# Insects on Individual Plants:

Plant quality, plant diversity and aboveground-belowground effects

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**Olga Kostenko**

**Thesis**

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*To my Mother*

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## *Abstract*

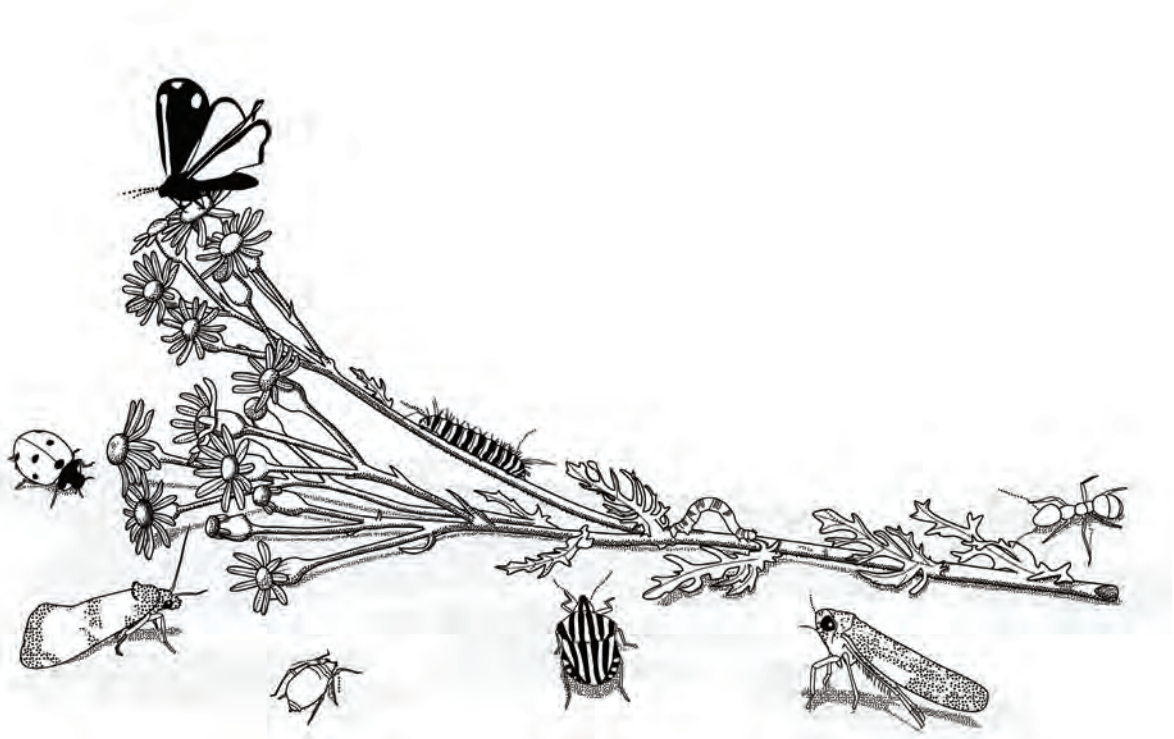
The density and composition of insects on a given plant species can vary greatly among individuals of that species. Understanding factors causing this variability can help us to predict the composition of insect communities on plants and their responses to environmental changes. The main aim of this thesis was to elucidate factors that structure the insect community associated to individual plants. Plant quality and diversity are increasingly recognized as important determinants of the composition and abundance of terrestrial insects. Hence, I specifically examined how individual variation in plant quality and local variation in the diversity of the plant community determine the performance and abundance of insects on individual plants. As a model system I used aboveground and belowground communities associated to ragwort (*Jacobaea vulgaris* Gaertner synonym *Senecio vulgaris*), an outbreak plant species native to the Netherlands.

In experiments under controlled greenhouse conditions I found that belowground herbivory caused a decrease in the concentration of the secondary plant compounds in the aboveground parts of *J. vulgaris* but did not affect the performance of aboveground insects that fed on the same plant. However, aboveground and belowground herbivores created unique soil legacy effects via herbivore-induced changes in the composition of the soil microbial community. These soil legacies affected the growth and secondary chemistry of plants that later grow in the same soil, as well as the aboveground multitrophic interactions occurring on those plants. It reveals that plant quality-mediated interactions between aboveground and belowground insects can also be important when they do not feed simultaneously on the same plant. Future studies should estimate the importance of these legacy effects in relation to other factors structuring insect communities on individual plants in the natural systems.

Using a field experiment, where plant species diversity was manipulated experimentally, I demonstrated that both the presence and the diversity of the surrounding vegetation affected the nutritional and chemical quality and size of focal *J. vulgaris* plants growing in that community. However, the abundance of an aboveground specialist herbivore that naturally colonised the focal *J. vulgaris* plants was influenced directly by the surrounding plant community and not via the effects of surrounding vegetation on the performance of the focal plants. Parasitoid foraging behaviour in the diversity plots was not affected by plant diversity, but by the structural complexity of the plant community surrounding the host-infested *J. vulgaris* plants. Belowground, increasing plant species diversity enhanced the level of predation of root herbivores indirectly by modifying the prey densities, but there were no effects of plant diversity on

predator abundance. Finally, in a chronosequence consisting of ten ex-arable fields that are restored to grasslands, the abundance and diversity of insects reared out from individual *J. vulgaris* plants differed among fields but did not correlate with the intraspecific changes in plant size or quality.

I conclude that the role of plant quality in structuring insect communities on individual plants in natural settings is subordinate to the effects of the surrounding plants on the aboveground and belowground communities associated to individual plants. Therefore, individual plant-insect interactions should be considered from the community perspective and future studies should aim at further disentangling the role of plant quality in structuring insect communities in natural settings.



# Chapter 1

## General introduction

Insects are the largest group of macroscopic organisms on Earth and they inhabit a range of trophic levels and functional groups. Insects are involved in a variety of ecological interactions with almost all other living organisms as they consume plants and dead organic matter, but also predate or parasite on other consumers including human being. The interactions between plants and insects have attracted scientific interest of ecologists for more than a century. This is not surprising, as plants harbour diverse multitrophic insect communities with myriad interactions that form the foundation of communities and ecosystems. Furthermore, these multitrophic insect communities are responsible for a variety of important functions within ecosystems, and can help us understand the relationship between plant diversity and ecosystem functioning (Price et al. 2011). Besides, insects are fascinating to study and many of them are still to be discovered.

Ecological communities can be defined as "groups of species that interact, or have the potential to interact, with each other" (Strong et al. 1984). Here, I use the term "insect community" to delineate a group of herbivorous insects and their natural enemies (predators and parasitoids) associated to an individual plant. During the past three decades, ecologists and entomologists have become increasingly aware that the density and composition of insects on a given plant species can vary greatly among individuals of that species. Understanding how species interactions contribute to community composition and ecosystem functioning constitutes a central topic in ecology. Therefore, the question what determines the composition of an insect community on a plant has received considerable attention (Strong et al. 1984; Lawton et al. 1993; Lewinsohn et al. 2005). Different approaches have been employed to study how host plants influence the composition of the insect community associated to those plants. One line of research has focussed on the effects of intraspecific differences in plant quality as a factor structuring insect communities. Plant quality is extremely important in every aspect of plant-insect interactions, including host-plant selection, growth, survivorship, and reproduction (reviewed in Awmack & Leather 2002). However, what the importance is of plant quality in structuring insect communities in natural habitats remains unknown.

Another line of research has focussed on how characteristics of the habitat or plant community can structure the insect assemblages on plants growing in that community. In nature, plants usually occur in mixed plant communities, where aboveground and belowground communities of insects and other biota associated to a plant are also influenced by interactions that occur on the neighbouring plants. The importance of the surrounding vegetation in determining how many insects are found on a particular plant was already recognized in the early seventies

(Tahvanainen & Root 1972; Feeny 1976; Atsatt & O'Dowd 1976). These studies reported that the probability that a plant is found by an insect often depends not only on its own inherent characteristics (such as plant quality), but also on the chemistry, morphology, distribution, and abundance of the neighbouring plants. Furthermore, interactions between insects within a community, such as between species that inhabit the same trophic level and that are potential or actual competitors, between root and shoot feeding insects, or between different trophic levels, such as herbivores and their natural enemies, can also greatly affect the composition of insect communities on individual plants.

In nature, when searching for their hosts insects are confronted with a complex of factors that will affect their behaviour, survival and performance. Therefore, the interplay between plant quality, plant diversity and insect diversity is a complex area of research that is essential for understanding the interactions between plants and their multitrophic insect communities. In this thesis, I link larger-scale patterns of plant diversity with insect diversity and plant quality at the scale of individual plants, to explore how the insect communities on individual plants can be affected by the quality of the host plant and by the characteristics of the surrounding plant community in which the host plant is embedded.

## Plant quality

The quality of food plants for individual herbivorous insects is usually described by three components: nutritional (primary compounds, water content), allelochemical (secondary compounds) and morphological quality.

*Nutritional plant quality*

Primary compounds such as nitrogen, carbon, and phosphorus are fundamental elements of all organisms, and maintaining the elemental balance in an organism is essential for its metabolism and cell functions. Inherently, the elemental composition of plants is greatly different from animals and, in particular, from insects. For example, the contents of nitrogen (N) and phosphorus (P) in leaves are roughly 2% and 0.05%, respectively, whereas the content of nitrogen in insect tissue varies from 10 to 12%, and that of phosphorus is about 0.5% (Strong et al. 1984; Fagan et al. 2002). Therefore, insect herbivores are inherently nutrient (N and P) limited. Furthermore, C-based nutrients also have a major impact on the performance of herbivorous insects. For example, high concentrations of soluble carbohydrates in plant tissues result in dilution of other nutrients, such as nitrogen that is already extremely limited, thereby forcing herbivores

to increase their consumption rates resulting in prolonged development times, decline in growth rates and fecundity (Awmack & Leather 2002). Similarly, in plants growing under elevated $CO_2$ conditions C:N ratios increased that adversely affect the performance of herbivorous insects (Bezemer & Jones 1998). Thus, for the majority of herbivorous insects, even for herbivores closely adapted to their host plant, the nutritional imbalance of the food is a major factor limiting their performance and development (Awmack & Leather 2002; Huberty & Denno 2006).

*Allelochemical plant quality*

Secondary compounds are organic metabolites that are not directly involved in the primary metabolic process, but derived from primary metabolic routes, for example, glucosinolates, terpenes, tannins, alkaloids, phenolics and others. Secondary plant compounds play a major role in plant-insect interactions, e.g., by protecting plants from being attacked by herbivorous insects (Rosenthal & Berenbaum 1991). Various secondary plant compounds also act as toxins to mammals or microorganisms such as bacteria and fungi, or inhibit the growth of competing plants by allelopathy; or provide other functions than defence such as protection from UV radiation, desiccation, or cold (Crawley 1997; Inderjit et al. 2011; Price et al. 2011). Secondary compounds can be constitutively expressed within a plant independent of whether the plant is exposed to herbivory or not. However, several secondary compounds can also be produced, or increased in content in response to herbivory. This is named induced response (Karban & Baldwin 1997; Agrawal et al. 1999). Moreover, some compounds that have been classified as constitutive can also be induced when the plant is damaged by herbivores (e.g., Van Dam et al. 1993). In addition to these quantitative differences in the concentration of secondary compounds, qualitative differences in the chemical composition of secondary plant compounds can also affect the performance and preference of individual herbivore species (Bukovinszky et al. 2008; Gols et al. 2008; Poelman et al. 2009).

Primary and secondary metabolites tend to interact and/or correlate with each other, which often makes it even more difficult for herbivorous insect to satisfy their nutrition requirements (Crawley 1997; Thamer et al. 2011). For example, low plant nitrogen concentrations are typically correlated with low water content and high amounts of lignin making the extraction and digestion of nitrogen even less efficient (Scriber & Slansky 1981). Plant water content can also affect the ability of sap-feeders to assess nitrogen by controlling the cell turgor pressure (Huberty & Denno 2004). Moreover, some plants store nitrogen in the form of non-protein N-based chemicals (e.g., alkaloids) that are toxic to insect herbivores (Crawley 1997).

*Morphological plant quality*

Plant morphological characteristics can directly affect interactions between plants and their antagonists. Visual characteristics of a plant, such as colour and shape of flowers, can guide herbivores to the host plant for oviposition or feeding. Morphological structures, such as spines, trichomes, or thorns on the plant surface can act as mechanical defences against herbivores, by preventing them to consume plant tissues, inhibit colonization, or hamper movement (Schoonhoven et al. 2005). Hardness and toughness help plants to withstand environmental pressures, such as damage by wind, but they also reduce the palatability of a plant to herbivorous insects. While such morphological characteristics clearly can play an important role in determining insect-plant interactions, these will not be addressed here. In this thesis, I use plant size (plant height) as a measure of morphological plant quality. Plant size has also been hypothesized to be an important determinant of the insect community associated to that plant, as large plants provide more resources to insects than small plants (Lawton 1983).

Finally, the quality of food plants that insect herbivores encounter is heterogeneous in space and time making it even harder for herbivores to locate optimal food resources for their survival and development. Variation in plant quality can occur within a single plant e.g., between different organs or tissues, or due to ontogenetic, diurnal or seasonal changes (Awmack & Leather 2002). There is also remarkable variation in plant nutrition, chemistry and morphology within different individuals of the same species. Plant primary and secondary chemistry, for example, can vary owing to heterogeneity in abiotic factors, such as light, nutrient and water availability, or biotic factors, such as feeding by invertebrate and vertebrate herbivores, pathogen infections, plant-plant interactions and allelopathy (reviewed in Crawley 1997). This intraspecific variation in plant quality places further constrains on insect performance and population dynamics (Awmack & Leather 2002; Huberty & Denno 2004; Gols et al. 2008; Poelman et al. 2009; Hakes & Cronin 2011; Kleine & Muller 2011).

*Plant quality and herbivorous insects*

Herbivores as primary consumers directly depend on quality of their host plants but *"...most species of plants are inedible and unavailable to most herbivorous insects most of the time"* (Strong et al 1984). Furthermore, the effects of plant quality on herbivorous insects are not universal and can vary greatly between species, or even between different instars of a single species. Insects of different feeding guilds (e.g., chewers, phloem/sap-suckers, leaf-miners, borers, gall formers etc) or feeding breadths may also be differently affected by plant quality even when they feed on the same plant (reviewed in Price et al. 2011). Secondary plant chemicals, for example, are known to have differential effects on generalist and specialist herbivores. Secondary

compounds can decrease the growth rates and fecundity of generalist feeders, or act as repellents, deterrents, or digestion inhibitors. In contrast, specialist herbivores have adapted to the chemicals of their host plant and often can even make use of these secondary plant compounds e.g., for host plant recognition or sequestration for their own defence (Schoonhoven et al. 2005).

*Plant quality and higher trophic level insects*

Nutritional and allelochemical plant quality, through its effects on herbivores, can also influence the performance of organisms inhabiting higher trophic levels (Harvey et al. 2005; Ode 2006). Because herbivores derive their nutrition from plants, natural enemies using herbivores as prey will obtain their nutrition indirectly from plants. If an herbivorous insect feeds on a well-defended host plant, it may have to invest resources in the detoxification of plant defence compounds. Therefore, herbivore fitness may be reduced and this may result in longer development times of parasitoids (Ode 2006). Non-metabolized plant defence compounds sequestered by specialist insect herbivores might have a detrimental effect on parasitoids developing inside the host (e.g., Bukovinszky et al. 2008). On the other hand, plants can also positively influence higher trophic level insects, e.g., by facilitating the location of their host via the emission of volatiles or production of sugar-type compounds such as (extra) floral nectar that serve as energy sources for adult parasitoids and predators (Vet & Dicke 1992; Kessler & Baldwin 2001). Several studies have shown that plant quality, via affecting herbivores and parasitoids, can even influence organisms inhabiting the fourth and higher trophic layers in the food chain (Ode 2006; Harvey et al. 2009; Poelman et al. 2012).

## Soil-plant-insect interactions

Plants critically depend on indirect and direct interactions with soil organisms for nutrient acquisition, pathogenesis, and herbivory (Wardle et al. 2004a). Soil organisms, in turn, depend on plants for basal resource inputs. Plants differ in the amount and quality of resources that they provide into the soil food web, and this influences the composition and functioning of the soil community surrounding the roots. This, in turn, can influence the survival and growth of a plant. For example, plant-soil feedback studies have shown that through their effects on soil biota and nutrient availability, plants can affect the biomass of other plants that later grow in the same soil (e.g., Bever et al. 1997; Ehrenfeld et al. 2005). The long-term effects of a plant on biotic and abiotic soil properties that influences the performance and dynamics of other plants that later grow in that soil is called the "soil legacy effect" (Kardol et al. 2007). Whether and how plant-soil feedback or soil legacies influence the nutritional or allelochemical quality of the next generation of plants remains largely unknown.

In nature, plant roots are often exposed to belowground insect herbivores. Despite being separated in space, belowground insect herbivores can significantly influence aboveground plant growth and the composition and concentration of primary and secondary plant compounds in aboveground plant parts (Van der Putten et al. 2001; Blossey & Hunt-Joshi 2003; Bezemer & Van Dam 2005; Erb et al. 2009; Johnson et al. 2012; Soler et al. 2012b). Through these changes in host plant quality, belowground herbivores can subsequently affect the survival and functioning of aboveground insect herbivores (e.g., Bezemer et al. 2003; Van Dam et al. 2005; Kaplan et al. 2008a; Erb et al. 2011a), and via changes in the herbivores, organisms inhabiting higher trophic levels aboveground, such as parasitoids (Soler et al. 2012b). Yet, our knowledge on the effects of root herbivory on higher trophic levels is scarce. Similarly, aboveground herbivory can influence root quality and root-associated multitrophic communities but these aboveground effects on belowground plant quality have been relatively less studied so far (Soler et al. 2007a). The vast majority of studies, that have examined interactions between aboveground and belowground herbivores, have used designs in which aboveground and belowground insects were feeding simultaneously on the same plant (Johnson et al. 2012). Therefore, the temporal dynamics of these aboveground-belowground interactions remain largely unexplored, although several studies have examined the effects of sequential feeding by aboveground and belowground insect herbivores on the same plant (Erb et al. 2011b; Barber et al. 2012).

Whether a plant is exposed to aboveground or belowground herbivores may also alter the composition of the microbial community in the soil in which the plant is growing (Wardle et al. 2004b; Bennett 2010). This is most evident for belowground herbivores that can directly interact with other soil organisms through their effects on the quality and quantity of root tissues, root exudates and organic matter content in the soil (Anderson et al. 1983; Bardgett et al. 1999; Gange 2007; Van Dam 2009). Although aboveground herbivores are physically separated from soil organisms, they can also influence soil microbial community composition and functioning. These effects can be indirect, by altering the allocation or production of biomass, nutrients or allelochemicals to root tissues, or by affecting the amount or quality of root exudates; or direct via deposition of frass or honeydew on the soil (Bardgett et al. 1998; Mikola et al. 2001; Soler et al. 2007a; Hamilton et al. 2008; Bennett 2010). Soil microorganisms, in turn, can affect aboveground plant quality and this can then influence aboveground herbivores and their antagonists (Bonkowski et al. 2001; Guerrieri et al. 2004; Bezemer et al. 2005; Bennett & Bever 2007; Gange 2007; Bonte et al. 2010; Eisenhauer et al. 2010b; Hol et al. 2010). Thus, via their effects on aboveground plant quality, soil organisms can influence the structure and functioning of aboveground communities associated with the plant.

## Associational effects

In natural communities, the interactions between insects and an individual plant can be strongly influenced by the specific associations of that plant with its surrounding community. The surrounding plant community can vary in species richness, composition, structure and density. These variations create physical and chemical heterogeneity, which can directly affect insect colonization on the focal plant both above- and belowground (Agrawal et al. 2006; Fig. 1.1 Pathway A). Moreover, surrounding plant communities can affect insects colonizing focal plants indirectly, through their effects on (1) the local pool of insects, changes in microclimate, host abundance, alternative resources (e.g., nectar, enemy-free space, alternative hosts) (Fig. 1.1 Pathway B); and/or (2) the quality and growth of the focal plant that, in turn, influence the interaction between a focal plant and the insect community associated to that plant (Fig. 1.1 Pathway C). Below, I discuss different characteristics of surrounding plant community and the mechanisms through which they can affect insect communities on focal plant.

*Diversity of the surrounding community*

The *associational resistance hypothesis* predicts that insects on a focal plant will be less abundant in complex and more diverse plant communities than in simple ones (Tahvanainen & Root 1972), because a focal plant is more difficult to detect in a diverse than in a homogeneous surrounding community due to physical and chemical obstruction. Moreover, diverse communities can provide alternative hosts; or can affect the microclimate that reduces the amount of the time spent by insects on a focal plant (Atsatt & O'Dowd 1976; Hambäck et al. 2000; Agrawal et al. 2006; Barbosa et al. 2009). Diverse plant communities typically produce more biomass and have denser plant structures per unit area than simple ones (e.g., Van Ruijven & Berendse 2005). This provides more resources for the local pool of insects. Therefore, through the effect on the size and composition of the local pool of insects that could subsequently "spill over" to the focal plant, the diversity of a plant community may directly influence the number of insects on a focal plant growing within that community (White & Whitham 2000). This is in line with the *associational susceptibility hypothesis* that predicts higher levels of herbivory on focal plants in diverse plant communities compared to monocultures (Barbosa et al. 2009). In contrast, the abundance and diversity of herbivore natural enemies, such as parasitoids, is predicted to be higher in more diverse plant communities (*Enemies hypothesis,* Root 1973). Increased parasitoid abundance can result in higher rates of parasitism of herbivores in more diverse communities thereby providing associational resistance to the focal plant (Stiling et al. 2003).

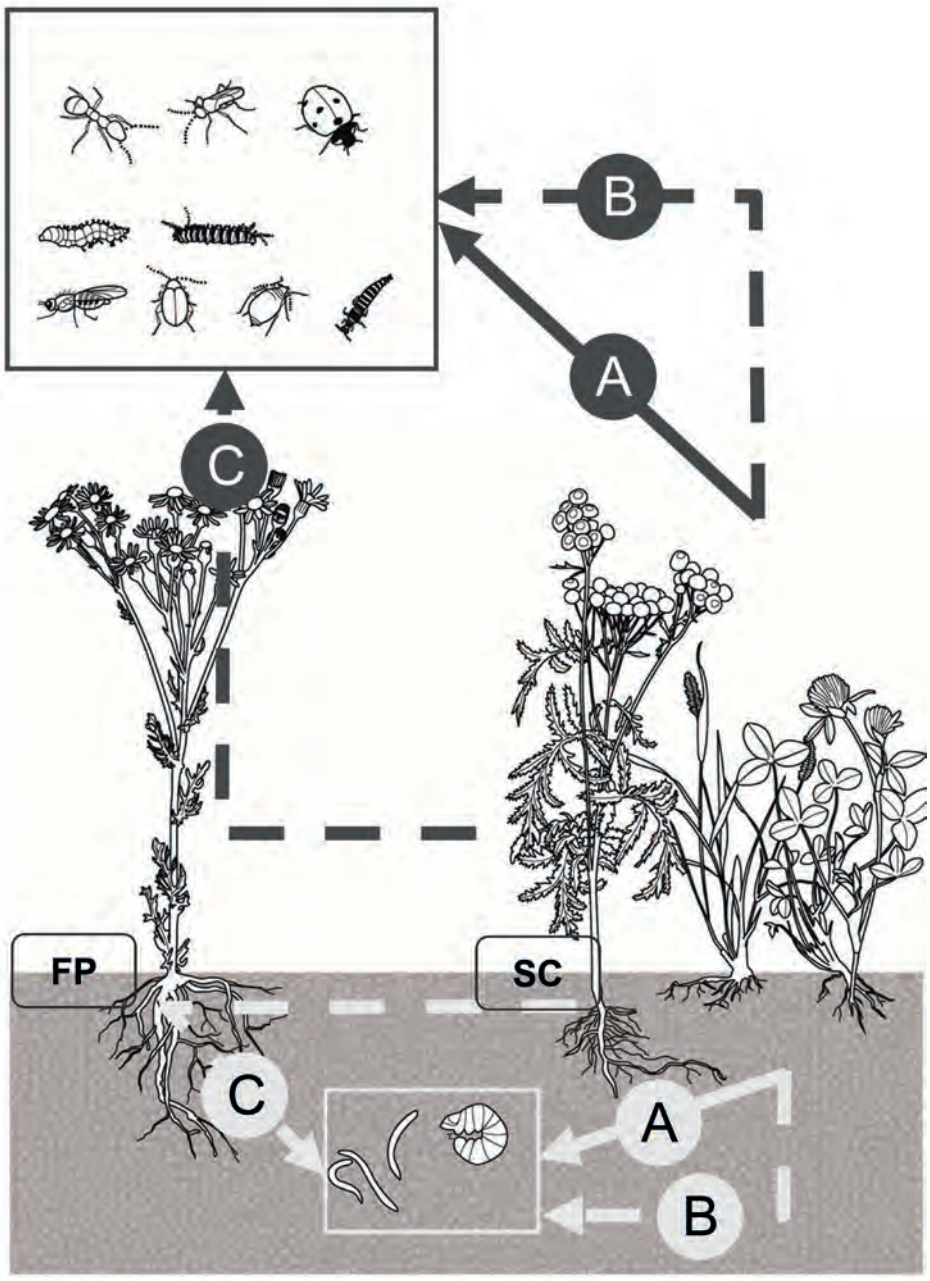



**Figure 1.1** Conceptual scheme illustrating the effects of surrounding plant community on the aboveground insect community associated to a focal plant (dark grey arrows) and the belowground insect community (light grey arrows). Solid arrows indicate direct effects and dashed lines indicate indirect effects. FP - focal plant; SC - surrounding plant community.

*Identity of the surrounding community*

Several studies have shown that the likelihood that a plant is detected by an insect or is vulnerable to herbivory also depends on the identity of the surrounding plants. This may also lead to associational resistance or susceptibility of plants to herbivore attack (Barbosa et al. 2009). For example, more palatable neighbours may attract more insects and subsequently increase the probability of a spill over of these insects from the neighbours to a focal plant (White & Whitham 2000). However, the likelihood of the spill over will probably strongly depend on the phylogenetic distance between focal and neighbouring plants, as phylogenetically close plants may be more similar in their chemical composition and morphology than phylogenetically distant ones. Therefore, if a focal plant is phylogenetically more similar to the neighbouring plants the probability of spill over will be higher (Feeny 1976; White & Andow 2006). The species and functional group identity of neighbouring plants can also be important in the belowground interactions between a focal plant and its neighbours (Dakora 2003; Bezemer et al. 2010a). For example, root exudates produced by a large number of legumes contain isoflavonoids, a group of allelochemicals that deter belowground insect larvae, therefore, plants that neighbour legumes can be released from belowground herbivore pressure (Dakora 2003). Furthermore, the damaged roots of young maize plants release secondary metabolites in the soil that attract entomopathogenic nematodes of the species *Heterorhabditis megidis* that could also potentially spill over on the insect larvae feeding on the roots of neighbouring plants (Rasmann & Turlings 2007). The associational effects between the focal plant and its surrounding community and consequences for insect communities belowground, however, have rarely been studied.

*Structure of the surrounding community*

Independent of the diversity and identity of the plant community, the physical structure or height of the surrounding vegetation can affect the insect abundance on a focal plant, for example via its effect on the apparency of the focal plant (Lawton 1983; Langellotto & Denno 2004). Plant apparency or "susceptibility to discovery" can be characterized by a variety of factors; one being the size of a focal plant (Feeny 1976). In the field, the size of plant individuals that belong to the same species can vary greatly (Kostenko & Bezemer 2013). Most plant individuals can support a large number of insects and the performance of an individual insect is often not limited by the quantity of the host plant (Strong et al. 1984). Yet, large plants are easier to detect and have therefore been proposed to be more readily colonized by insects than small plants (Lawton 1983). However, the presence of taller neighbours can physically conceal the host plants and may disrupt their location and colonization by insects (Castagneyrol et al. 2013). This

is particularly so for herbivorous insects. Many parasitoid species also use host plant cues, such as plant-emitted volatiles to locate their hosts. Large plants may emit larger quantities of volatiles than small plants, simply because they are large, or because they contain larger numbers of herbivores due to their higher apparency (Beyaert & Hilker 2013).

*The effect of surrounding community on the quality of the focal plants*

The surrounding plant community can also influence the insect community on a focal plant by modifying the size or quality of the focal plant (Box 1 Pathway C). The performance of a plant is greatly affected by whether it competes or not with other plants for nutrients or light (Crawley 1997). However, the identity of the competing plants, and hence the diversity or identity of the surrounding plants, can greatly affect the outcome of competition (McEvoy et al. 1993; Tilman 1997; Scherber et al. 2003; Agrawal 2004) and can also affect the expression of plant secondary metabolites (Barton & Bowers 2006; Broz et al. 2010; Mraja et al. 2011). Plant-plant interactions with leguminous plants, for example, may result in more nitrogen becoming available for growth of the focal plant relative to situations in which it competes with non-leguminous species. Alternatively, the increased availability of nitrogen can also lead to increased production of N-based allelochemicals (Bryant et al. 1983; Coley et al. 1985). Plants competing with non-leguminous neighbours will probably have fewer resources available for growth compared to plants growing without competitors. However, if light is not limited and photosynthesis is not impaired, carbon availability will increase relative to the plant's demand and this can result in increased production of C-based allelochemicals (e.g., phenolics; Bryant et al. 1983). Emission of volatiles by neighbouring plants may also influence the resistance of a focal plant by inducing the expression of defensive chemicals in the focal plant (reviewed in Heil & Karban 2010).

*Plant density*

A different mechanism by which the surrounding plant community can affect the insect community on a focal plant is through the relative abundance of focal and surrounding plants of the same species in that plant community. The *resource concentration hypothesis* (Root 1973) states that when the concentration of host plants is high, specialised insect herbivores will be abundant, as in those conditions, hosts will be easily located and food will be abundant. Moreover, generalist natural enemy pressure is often less compared to diverse plant communities (*Enemies hypothesis,* Root 1973). Insect densities in this context are expressed per unit area and not per plant individual. Therefore, it is possible that higher insect densities may simply be caused by increases in the number of host plants per unit area. An increase in plant density, therefore, will not necessarily lead to an increase in insect densities on all plants. Instead, it could lead to an increase in performance or fitness

of the insects that are locally present because the insects can choose between a greater variety of host plants and this will optimize food selection. Indeed, the few studies that have examined the effects of host plant density on insect numbers on individual plants in those communities typically report negative effects of host plant density on insect densities (Scherber et al. 2006; Lau et al. 2008).

*Temporal changes in environment*

The composition of the plant community surrounding a focal plant may change over time as plant communities often undergo successional changes. During the process of secondary succession plant communities typically become more diverse and complex resulting in increased plant competition (e.g., Connell & Slatyer 1977; Huston & Smith 1987; Tilman 1990). Along with the successional changes in the plant community, there will also be changes in the quality of individual plants growing in those communities (Reader & Southwood 1981; Bach 1990), for example, because soil nutrient availability declines during succession (Tilman 1990; Knops & Tilman 2000). Moreover, plant investment in chemical defences is related to plant apparency (plant life span) and plant apparency also changes during succession (Feeny 1976). More apparent plants (e.g., trees) are defended by quantitative defences, which typically reduce plant digestibility and are not easily overcome by specialist herbivores. Unapparent plants (e.g., herbs) are often defended by qualitative defences, typically toxins that limit feeding damage by a subset of specialized herbivores (Feeny 1976). Several studies have shown that aboveground insect communities also change during succession, both in terms of species composition and in the degree of host plant specialization (Tscharntke et al. 1998; Siemann et al. 1999; Brown & Gange 2002). When succession proceeds, the host specificity and diversity of the insects associated to the entire plant community often increase (Southwood et al. 1979; Brown & Gange 2002). While there are ample studies that have examined how insect communities change during succession, how individual plant quality and insect communities associated to individual plants change during succession and the factors that influence these changes are not well known.

## Model system

In this thesis I will use ragwort (*Jacobaea vulgaris* Gaertner ssp. *vulgaris)* synonym *Senecio jacobaea* and its associated aboveground and belowground communities as a model system to examine factors that structure the insect community associated to individual plants.

*Biology of* Jacobaea vulgaris

*Jacobaea vulgaris* is a biennial or short-lived perennial monocarpic plant in the family *Asteraceae* (Cameron 1935; Harper & Wood 1957). The seeds ripen and begin to set during mid-August. The seeds disperse by wind, but the majority drop within a few meters from the parent plant (McEvoy & Cox 1987). After emergence of the seedling, a rosette of leaves is formed and the plant overwinters in the rosette stage (Fig. 1.2A). During the next summer flowering stems are produced (Fig. 1.2B). Flowering may be delayed to later years when the plant has been damaged or when the size of the rosette is too small (Harper & Wood 1957; Van der Meijden & Van der Waals-Kooi 1979). Plants can also regenerate after flowering and persist for few more seasons (Islam & Crawley 1983). Most individual plants die after flowering but vegetative reproduction and polycarpy have also been observed (McEvoy 1984). In both rosette and flowering stages, *J. vulgaris* has a strong and fleshy taproot that contains large quantities of accumulated carbohydrates that are used by the plant for regrowth after complete defoliation (Van der Meijden et al. 2000).

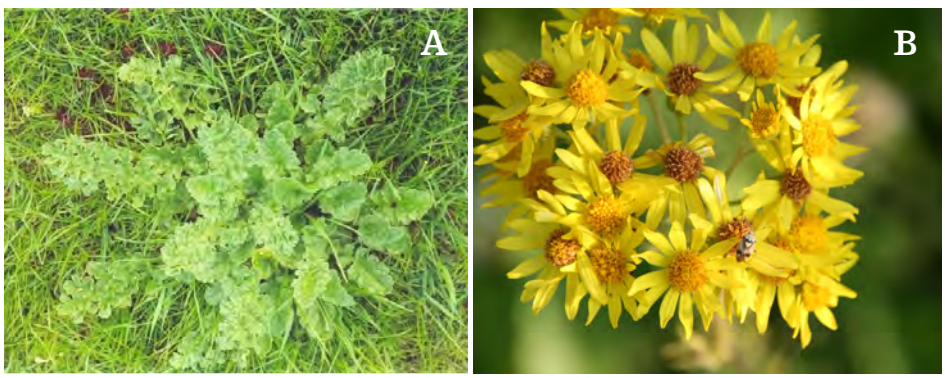



**Figure 1.2** *Jacobaea vulgaris* rosette of leaves (A) and inflorescence (B).

*Jacobaea vulgaris* is native to Europe and Asia where it is widely distributed. In the Netherlands, the species is found all over the country, but it is less abundant in the north-east (Van der Meijden et al. 1996). Ragwort is a typically ruderal species, which is able to grow fast and colonize recently disturbed areas. In the absence of environmental disturbance, self-replacement in the openings left in vegetation after the plant dies, may explain how ragwort can persist in the long-term (McEvoy 1984). The plant usually grows in patches (Dempster 1971; Van der Meijden E. & Van der Veen-Van Wijk 1997). However, in early successional habitats, especially in recently abandoned ex-arable fields on sandy soils in the centre and south of the Netherlands ragwort is highly abundant and dominant resulting in stands that resemble monocultures. The plants in those "monocultures" are large and produce large amounts of biomass. Subsequently,

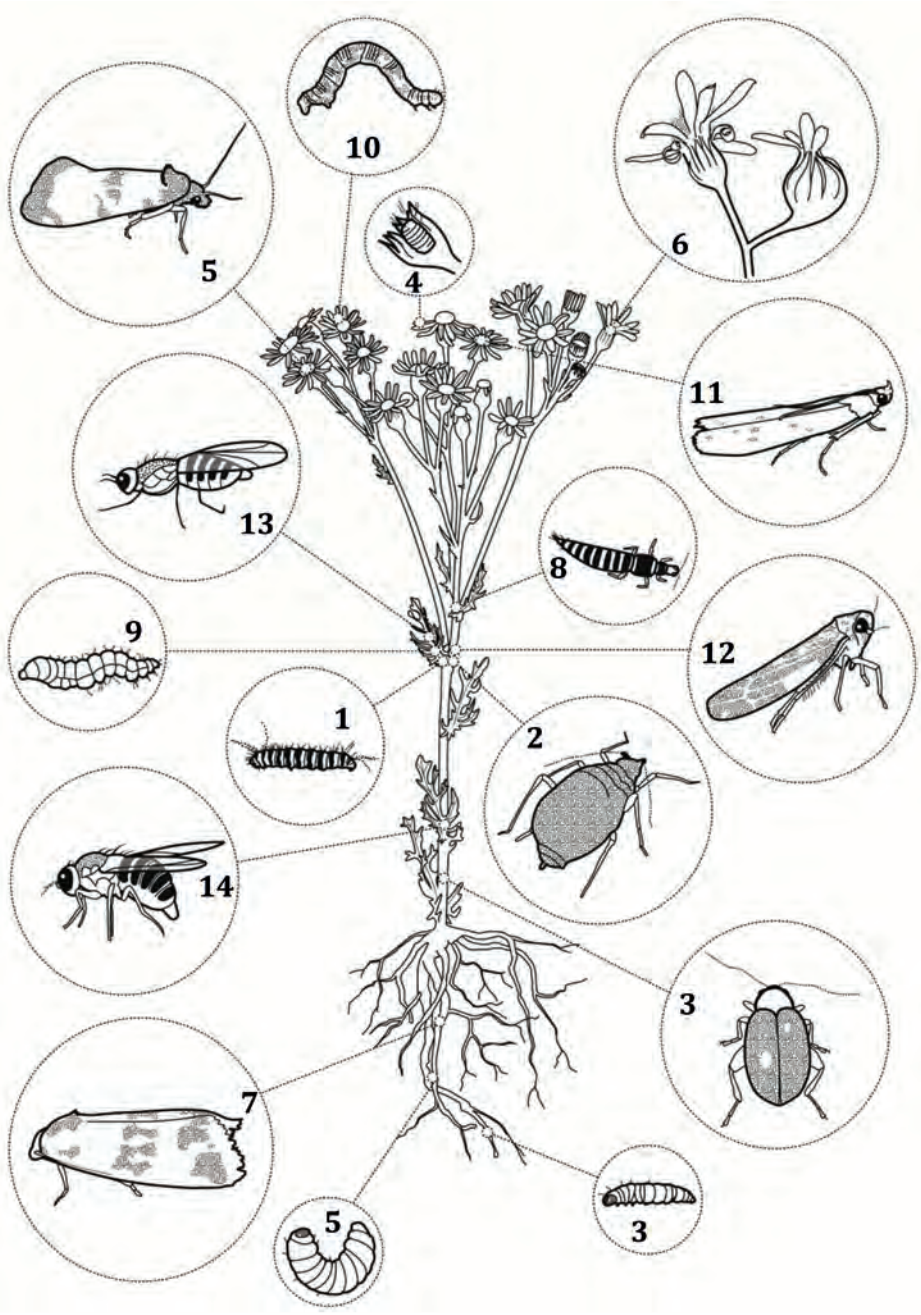
ragwort abundance, as well as the size of individual ragwort plants decline over time (Bezemer et al. 2006; Van de Voorde et al. 2012). *J. vulgaris* has been introduced in other parts of the world, such as Australia, New Zealand, America and South Africa where it has spread rapidly and became a serious invasive weed.

*Insects associated to* Jacobaea vulgaris

*Jacobaea vulgaris* harbours a rich insect fauna of more than 70 recorded species of herbivores (Cameron 1935; Harper & Wood 1957). The principal herbivores are illustrated in Box 1. One of the common herbivores of *J. vulgaris* in the Netherlands is the cinnabar moth, *Tyria jacobaeae* L. (Lepidoptera: Arctiidae; 1 in Box 1) although it is less abundant in the north-eastern part of the country where I performed my experiments. The larvae feed on leaves, flowers and top parts of the stems and the interactions between *T. jacobaeae* and its host-plant have been extensively studied (e.g., Dempster 1971; Myers 1980; Cox & McEvoy 1983; Crawley & Gillman 1989; Van der Meijden & Van Veen-Van Wijk 1997). Previous work has also shown that plants are attacked by other specialists herbivores, for example the specialist aphid *Aphis jacobaeae* Schrk. (Hemiptera: Aphididae; 2), flea beetle *Longitarsus jacobaeae* Wat. (3), *L. dorsalis* F., *L. flavicornis* Steph. (all Coleoptera: Chrysomelidae), ragwort seed fly *Pegohylemyia seneciella* Meade (4) and *P. jacobaeae* Hardy (both Diptera: Anthomyiidae), crown boring moth *Cochylis atricapitana* Steph. (Lepidoptera: Tortricidae; 5), flower galler *Contarinia jacobaeae* (Diptera: Cecidomyidae; 6) and root-feeding moth *Commophila aeneana* (Lepidoptera: Tortricidae; 7) and thrips *Haplothrips senecionis* Bagnall (Thysanoptera: Phlaeothripidae; 8). The plant is also attacked by a variety of generalists e.g., lepidopterans: *Autographa* spp. (9), *Arctia* spp., *Eupithecia* spp. (10), *Phycitodes* spp. (11); hemipterans: *Eupteryx* spp. (12), *Brachycaudus* spp.; more than 20 species of thrips and leaf-mining insects [e.g., *Chromatomyia syngenesiae* Hardy (13), *Liriomyza strigata* Meigen (all Diptera: Agromyzidae)] and stem-boring insects [e.g., *Melanagromyza* spp. (Diptera: Agromyzidae; 14)]. However, ragwort is also a valuable nectar and pollen supplier for more than 150 Dutch insect species.

Several studies have shown that ragwort abundance and plant size are important determinants of the insect communities in the field (Harrison & Thomas 1991; Kunin 1999; Bezemer et al. 2006). However, overall, insect communities on ragwort are not strongly affected by the spatial isolation of ragwort patches (Harrison et al. 1995; Kunin 1999; Brunzel et al. 2004; Esch et al. 2005). The composition of insects on ragwort plants varies among habitats (Macel & Klinkhamer 2010) and is affected by interspecific plant competition and disturbance (Crawley & Gillman 1989; Bonsall et al. 2003).

**Box 1. Herbivores of *Jacobaea vulgaris***




*The insect names, indicated by the numbers, are listed in the text.*

*Pyrrolizidine alkaloids*

*Jacobaea vulgaris* produces a variety of pyrrolizidine alkaloids (hereafter abbreviated PAs), which are a well-studied group of nitrogen-based constitutive defence compounds (Hartmann & Witte 1995). In the roots, the basic alkaloid structure senecionine *N*-oxide is produced, and this is converted by basic biotransformations into several structurally related senecionine-type PAs. These PAs are transported exclusively via the phloem path to the aboveground plant parts where additional diversification takes place, resulting in the formation of jacobine- and erucifoline-type PAs (Hartmann 1999; Cheng et al. 2011a). The process of diversification is highly plastic and depends on a number of physiological processes in the plant (reviewed in Hartmann 1999), however, the exact mechanism of PA diversification is still unclear. A simplified representation of the structural diversity of PAs and their biosynthetic pathways is illustrated in Fig. 1.3. PAs generally occur in plants in *N*-oxide form and in tertiary amine (free base) form. Both forms are interchangeable and can occur together within a plant (Boppre 2011). PA *N*-oxides are the specific molecular form for long-distance translocation, transport into the cell vacuole and for storage. Tertiary amines are regarded as degradation products of *N*-oxides (Hartman & Dierich 1998). PA synthesis in *J. vulgaris* is closely linked to root growth and negatively correlated with shoot-root ratio (Hol et al. 2003; Schaffner et al. 2003). In several studies nutrient, water or light availability have been shown to affect PA levels, whereas in others no such effect was found (Vrieling & Van Wijk 1994; Brown & Molyneux 1996; Hol et al. 2003). Several studies have argued that there are no fitness costs for the production of PAs (Vrieling & Van Wijk 1994; Vrieling et al. 1996) but that the plant suffers from ecological costs as PAs can attract specialist herbivores (Macel & Klinkhamer 2010).

There is great variability in amounts and patterns of PAs in natural populations of *J. vulgaris* and the concentration and composition of PAs is genetically determined (Vrieling et al. 1993; Macel et al. 2004). A number of studies demonstrated that PAs are not always constitutively present in the plant but that the concentration and composition of PAs can change in response to abiotic factors and to interactions of the plant with other aboveground and belowground organisms. For example, mechanical leaf damage (Van Dam et al. 1993), mechanical root damage and aboveground herbivory (Hol et al. 2004), and soil-borne microorganisms (Joosten et al. 2009; Carvalho et al. 2012) can all cause changes in PA concentration in *J. vulgaris*. Abiotic factors, such as soil or climate can also contribute to the variability in amounts and patterns of PAs in natural populations of *J. vulgaris* (e.g., Kirk et al. 2010; Macel & Klinkhamer 2010). However, whether these changes in PA concentration are caused by a reallocation of PAs within the plant or by changes in PA production is unclear (but see Hol et al. 2004).

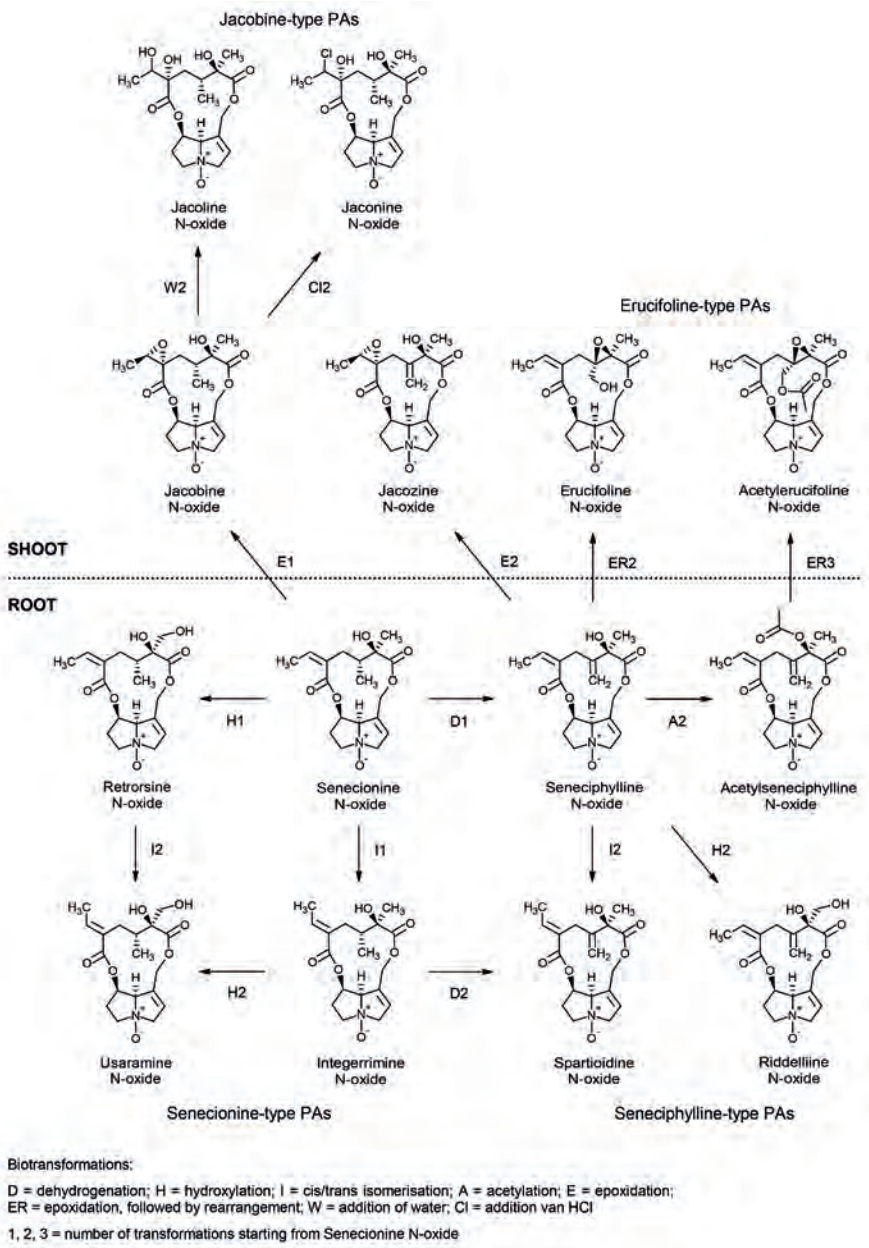



**Figure 1.3** Chemical structures of pyrrolizidine alkaloids found in *J. vulgaris* and their biosynthetic pathways (P.P.J. Mulder personal communication).

PAs play an important role in plant-insect interactions. PAs are toxic to a wide range of generalist insects and soil organisms (Hol & Van Veen 2002; Kowalchuk et al. 2006; Thoden et al. 2009; Macel 2011), whereas some generalist plant feeders are not negatively affected by PAs. These generalists either tolerate particular PAs or certain concentrations of PAs, degrade the PAs into other non-toxic chemicals, or excrete them (reviewed in Boppre 2011; Macel 2011). Certain specialized insects have evolved adaptations to sequester and utilize PAs for their own defence against predators and parasitoids. Thus, PAs can also affect the preference and performance of the third (and higher) trophic levels, although these effects are not well ascertained yet (Trigo 2011). The structure of PAs is important for their activity to insects as molecular structures differ in toxicity. A number of studies have shown that tertiary amines are more toxic for herbivorous insects than their corresponding *N*-oxides (Dreyer et al. 1985; Van Dam et al. 1995; Macel et al. 2005). At the same time, non-toxic *N*-oxides, can be converted into the potentially toxic forms in the gut of generalist herbivores and can have a negative effect on their performance and population growth (Hartman & Witte 1995). In addition, jacobine tertiary amines are more toxic to generalist herbivores than senecionine-type tertiary amines (Leiss et al. 2009; Macel & Klinkhamer 2010; Cheng et al. 2011b). The same PA compound can affect various generalist insect species in different ways (Macel et al. 2004), but the ecological functions of the majority of PAs in *J. vulgaris* remain unknown. Even though the importance of PAs in plant-insect interactions has been studied in great detail, little is known about the role of PAs in interactions between aboveground and belowground organisms (but see Hol et al. 2004; Joosten et al. 2009; Reidinger et al. 2011).

## Research objective and thesis outline

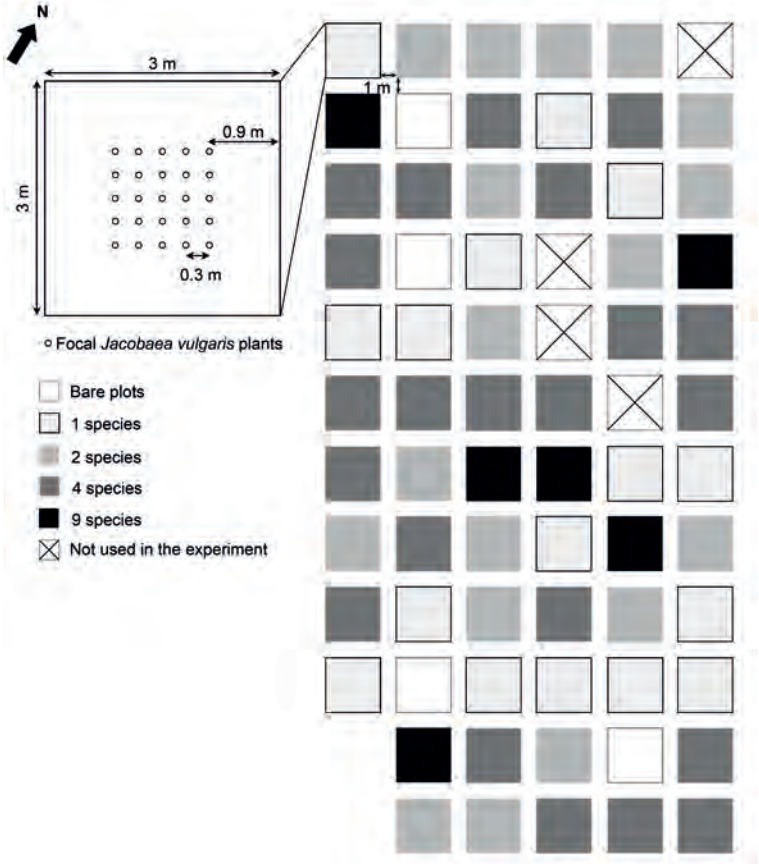
In this thesis I elucidated factors that structure insect communities associated to individual plants in a community context. Specifically, I examined the importance of the quality of the individual plant and of the plant community surrounding individual plants for the aboveground insect community associated to these plants.

First, I examined the importance of plant quality in mediating interactions between belowground and aboveground multitrophic communities associated to *J. vulgaris*. In *Chapter 2* I used a greenhouse experiment to determine the effects of root feeding insects on the performance of an aboveground insect herbivore and its parasitoid. I tested the hypothesis that root herbivory will affect aboveground plant quality, in particular the concentration of PAs, and thereby influence the

performance of aboveground insects. In addition, I examined how PA composition and allocation in roots and shoots of *J. vulgaris* is affected by root herbivory. Furthermore, in *Chapter 3* I tested the novel hypothesis that aboveground and belowground herbivory will cause legacy effects in the soil that will subsequently affect the growth and chemistry of plants growing later in the same soil, and that this, in turn, will influence interactions of the plant with aboveground herbivores and natural enemies. Further, I examined whether legacy effects caused by belowground herbivory differ from those arising from aboveground herbivory. Thus far, such soil legacy mediated interactions between aboveground and belowground insects feeding on plant individuals that grow after each other in the same soil have been ignored in insect-plant interaction studies.

In nature, individual plants are embedded in plant communities that may influence the quality of the focal plant and the aboveground and belowground insect communities on these focal plants. In *Chapters 4, 5, 6 and 7* I describe the results of a field experiment, in which individual *J. vulgaris* plants have been planted into experimental plant communities that differ in diversity and composition (Box 2). I examined how surrounding plant communities influence aboveground insect communities associated to focal plants, and tested to what extent the insect communities on a focal plant are driven by host plant quality and by the surrounding community. In *Chapter 4* I examined the effects of the diversity and identity of the surrounding plant community on the performance of focal *J. vulgaris* plants and the aboveground insect community associated to these plants. This chapter describes the results of the first season after the focal plants were planted into each plant community, and hence all plants were at the rosette phase. In *Chapter 5* I studied the longer-term effects of plant diversity on the growth and allelochemical quality of (vegetative and reproductive) plants. In *Chapter 6* I assessed how the diversity and complexity of the neighbouring plant community affects the behaviour of individual insects in these communities. I used a release-recapture experiment and trap plants to examine the effects of diversity and identity of the surrounding plant community on the host finding behaviour of parasitoids of a leaf-mining herbivore of *J. vulgaris*. Finally, in *Chapter 7* I address whether plant diversity and identity affect the abundance of predatory soil organisms and the predation level belowground. As predatory organisms, I used entomopathogenic (EPN) and carnivorous non-EPN nematodes that are important components of soil food webs. To get an estimation of the potential prey or food availability I also measured the abundance of soil insects and non-predatory nematodes and quantified root biomass production in the experimental biodiversity communities. I used structural equation modelling to investigate four possible pathways by which plant diversity may affect EPN infectivity and the abundance of carnivorous non-EPNs.

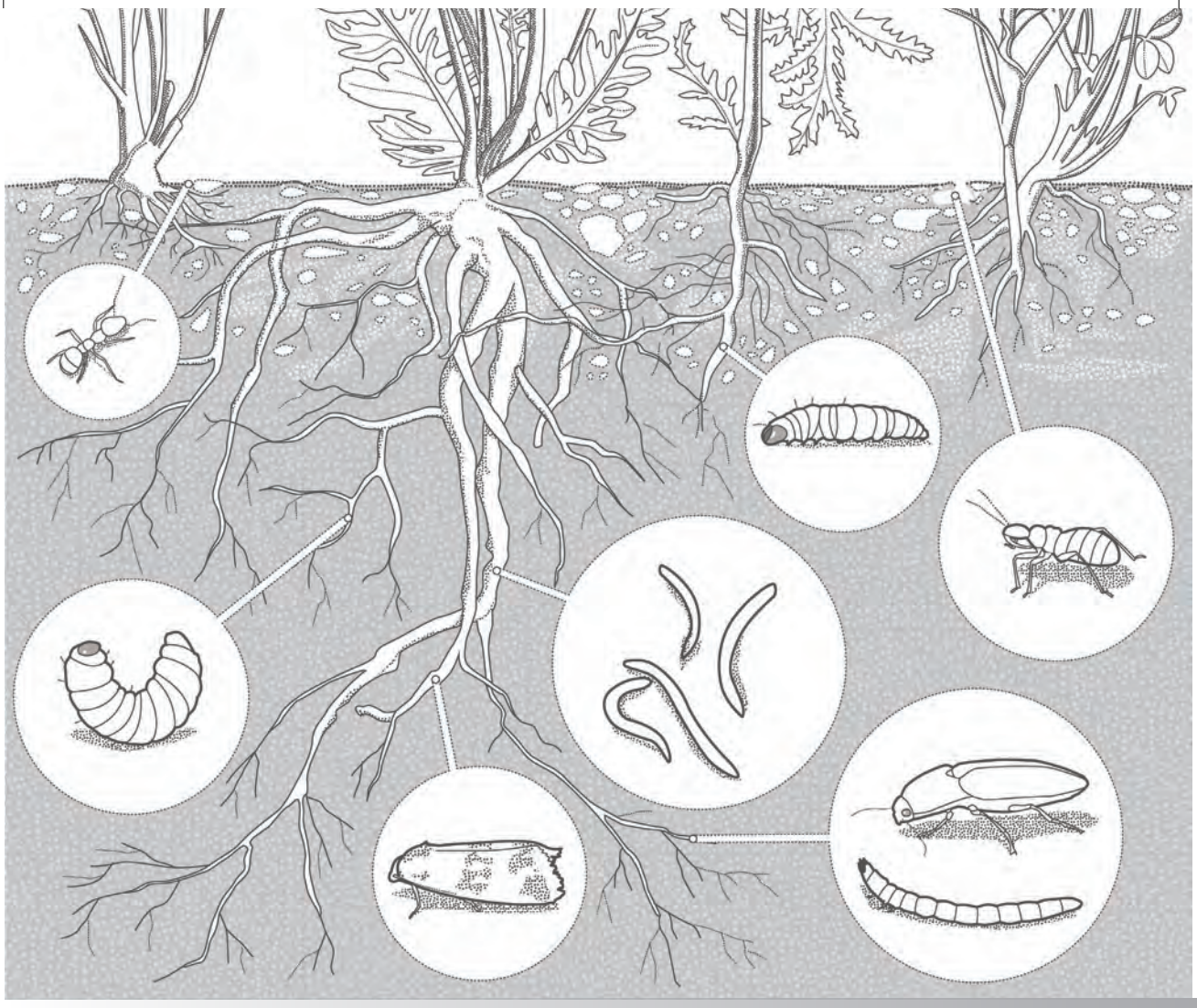
**Box 2. Schematic overview of the biodiversity field experiment**




The experimental field site that was set-up in the summer of 2008 on an ex-arable field at a nature restoration site Mossel (Ede, the Netherlands). The restoration started in the fall 1995 when the last crop was harvested (Van der Putten *et al.* 2000). The area of 25 × 50 m was cultivated and seventy plots of 3 × 3 m separated by 1-m-wide paths were laid out. In September 2008, the plots were sown with 1, 2, 4 or 9 grassland species that naturally co-occur with *J. vulgaris*. Plots with the same species composition were replicated twice using a complete randomized design. Initial sowing density was 4000 seeds per $m^2$. The sown species composition was maintained by hand weeding and paths between plots were regularly mown during the growing season. To avoid disturbance by vertebrate herbivores the experimental site was fenced. In August 2009, eight monocultural plots were poorly established. Four of them were kept free of vegetation and served as bare soil treatment, and the other four were excluded from analyses. Twenty five eight-week old *J. vulgaris* seedlings were planted in a regular grid (0.3 × 0.3 m) in the central 1.2 × 1.2 m square of each plot. The seedlings were grown from seeds collected from *J. vulgaris* plants growing in the direct vicinity of the experimental site. A detailed description of the experiment is presented in *Chapter 4*.

In *Chapter 8* I examined insect communities on *J. vulgaris* plants in a series of restoration grasslands on abandoned, former arable, fields in the Netherlands. The grasslands form a chronosequence of different stages of plant succession. I conducted a field survey to examine how apparency, nutritional quality and secondary chemistry of *J. vulgaris* and the associated insect communities change during succession. In this chapter, I also address the question whether the variability in insect communities on individual plants can be explained by changes in plant quality. The local variation in nutritional quality of wild plants growing in their natural habitat and the importance of this variation for insect-plant interactions occurring on these plants has been largely overlooked so far. I also describe a common garden experiment with *J. vulgaris* plants collected from different grassland fields that was designed to examine whether the performance of the specialist herbivore *T. jacobaeae* performance differed between plants from different successional stages in the absence of other environmental variables that may vary between sites.

In *Chapter 9*, I summarize the main findings of my thesis, and discuss their contributions to the field of plant-insect interactions. Finally, I consider the possibilities for application of my results for biological control of *J. vulgaris* and propose several directions for future research in the field.



# Chapter 2

## Effects of root herbivory on pyrrolizidine alkaloid content and aboveground plant-herbivore-parasitoid interactions in *Jacobaea vulgaris*

**Olga Kostenko, Patrick P. J. Mulder & T. Martijn Bezemer**

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## Abstract

The importance of root herbivory is increasingly recognized in ecological studies, and the effects of root herbivory on plant growth, chemistry, and performance of aboveground herbivores have been relatively well studied. However, how belowground herbivory by root feeding insects affects aboveground parasitoid development is largely unknown. In this study, we examined the effects of root herbivory by wireworms (*Agriotes lineatus*) on the expression of primary and secondary compounds in the leaves and roots of ragwort (*Jacobaea vulgaris*). We also studied the effects of root herbivory on the performance of a generalist aboveground herbivore, *Mamestra brassicae* and its parasitoid *Microplitis mediator*. In contrast to what most other studies have reported, root herbivory in *J. vulgaris* had a strong negative effect on the total concentration of pyrrolizidine alkaloids (PAs) in shoot tissues. The composition of PAs in the shoots also changed after root herbivory. In particular, the concentration of less toxic *N*-oxide PAs decreased. There was no significant effect of root herbivory on PA composition and concentration in the roots. Although the concentration of PA in the leaves decreased, *M. brassicae* tended to grow slower on the plants exposed to root herbivory. Parasitoid performance was not affected by root herbivory, but parasitoids developed faster when the concentration of jacobine-type PAs in the foliage was higher. These results point at a putative role of individual PAs in multitrophic interactions and emphasize that generalizations about aboveground-belowground effects should be made with great caution.

## Introduction

Root feeding insects can be very abundant in natural and agricultural systems and the importance of root herbivory is increasingly recognized in ecological studies (Blossey & Hunt-Joshi 2003; Whittaker 2003; Rasmann & Agrawal 2008; Van Dam 2009). Roots are essential for acquiring water and nutrients from the soil, and damage to the roots often results in decreased plant growth (Brown & Gange 1990). Besides the direct damage to the roots, belowground herbivory can also lead to changes in the concentration and composition of primary and secondary compounds in the roots. Due to root-shoot signalling, these changes frequently do not only occur in the roots, but also in the aboveground parts of a plant (Blossey & Hunt-Joshi 2003; Bezemer & Van Dam 2005; Johnson et al. 2008; Erb et al. 2009; Soler et al. 2012). Root herbivory can result in increases (e.g., Bezemer et al. 2003; Van Dam et al. 2005; Soler et al. 2005; Erb et al. 2008) or decreases in concentrations of aboveground secondary plant compounds (e.g., Kaplan et al. 2008a), although increased concentrations have been reported much more frequently than decreases (Kaplan et al. 2008b). As a result, this variation in plant responses to root herbivory may have important consequences for aboveground communities associated to the plant and interactions between aboveground and belowground herbivory.

Root herbivore-induced changes in aboveground plant chemistry can subsequently affect the performance of aboveground herbivores feeding on the plant (e.g., Bezemer et al. 2005; Van Dam et al. 2005; Soler et al. 2005; Erb et al. 2011b). Moreover, via these changes in the plant and in the herbivores, root herbivory can affect the performance and the behaviour of consumers of these herbivores such as parasitoids (Soler et al. 2012). A number of studies has shown that the level of parasitism or the host location behaviour of parasitoids is affected by whether or not the herbivorous host is feeding on a plant that is also exposed to root herbivory (Masters et al. 2001; Rasmann & Turlings 2007; Soler et al. 2007b; Staley et al. 2007; Olson et al. 2008). In contrast, the effects of belowground herbivory by root feeding insects on aboveground parasitoid development are less well studied. As far as we are aware, the impact of root feeding insects on aboveground parasitoid development have only been studied for *Cotesia glomerata*, a parasitoid of the specialist herbivore *Pieris brassicae*. In this system, root herbivory or even jasmonic acid application to the roots increases the glucosinolate contents in the leaves of Brassica plants and results in increased developmental times and reduced pupal weights of the parasitoid (Soler et al. 2005; Qiu et al. 2009). In the present study we examine the effects of root herbivory on aboveground multitrophic interactions for another plant-herbivore-parasitoid system. We exposed ragwort plants (*Jacobaea vulgaris*

Gaertn., Asteraceae) to root herbivory by wireworms (*Agriotes lineatus* L., Coleoptera: Elateridae), and examined the influence of root herbivory on the concentration and composition of pyrrolizidine alkaloids in roots and in foliar tissues, and on the performance of a generalist aboveground insect herbivore, *Mamestra brassicae* L. (Lepidoptera: Noctuidae) and its parasitoid *Microplitis mediator* Haliday (Hymenoptera: Braconidae).

Pyrrolizidine alkaloids (hereafter PAs) in *J. vulgaris* are root produced secondary metabolites (Hartmann 1999). PAs are a well-studied group of plant allelochemicals due to their important role in plant-insect interactions. They serve as feeding and oviposition stimulants to specialist herbivores and are known to deter generalist insect herbivores (reviewed in Macel 2011). In the roots, the basic alkaloid structure senecionine *N*-oxide is produced, and this is transformed into several related senecionine-type PAs. These PAs are transported exclusively via the phloem path to the aboveground plant parts where additional diversification takes place, resulting in the formation of jacobine- and erucifoline-type PAs (Hartmann 1999; Cheng et al. 2011a). PAs generally occur in plants in tertiary amine (free base) form and in *N*-oxide form. Tertiary amines are regarded as degradation products of *N*-oxides (Hartman & Dierich 1998). A number of studies have shown that tertiary amines are more toxic for herbivorous insects than their corresponding *N*-oxides (Van Dam et al. 1995; Macel et al. 2005). Even though the importance of PAs in plant-insect interactions has been studied in great detail, little is known about the role of PAs in interactions between aboveground and belowground organisms (e.g., Hol et al. 2004; Joosten et al. 2009; Kostenko et al. 2012b; Reidinger et al. 2012). Furthermore, the effects of PAs on parasitoid development and performance are not yet well ascertained (reviewed in Trigo 2011).

In a greenhouse experiment, we investigated the effects of root herbivory on the expression of primary and secondary compounds in the leaves and roots of ragwort. We further examined whether the survival and performance of the foliar feeding generalist herbivore and its parasitoid differed between plants exposed to root herbivory and control plants. Finally, we tested whether aboveground insect performance correlated with qualitative and quantitative characteristics of the chemistry of the leaves or roots. In line with what has been reported in other studies (e.g., Bezemer et al. 2003; Van Dam et al. 2005; Soler et al. 2005; Erb et al. 2008), we hypothesized that root herbivory (i) will increase total PA concentration in the shoots of *J. vulgaris*, and consequently (ii) will have a negative effect on aboveground herbivore and parasitoid performance.

## Materials and Methods

*Insects*

Wireworms are larvae of the click beetle *A. lineatus* and considered to be generalist root feeders. *A. lineatus* larvae were obtained commercially from Applied Plant Research (PPO-WUR), Lelystad, the Netherlands. Larvae of *M. brassicae* are generalist leaf-chewing insects that feed on a wide variety of food-plants, including *J. vulgaris* (De Boer 1999; Hol et al. 2004). *Microplitis mediator* is a solitary larval endoparasitoid of *M. brassicae* (Harvey & Gols 2011). This parasitoid develops in first to fourth instar larvae of its host. Larvae of *M. mediator* feed solely on host hemolymph, and thus can be directly exposed to the plant allelochemicals ingested into hemolymph by host. *Mamestra brassicae* and *M. mediator* were obtained from an insect culture at the Laboratory of Entomology of Wageningen University, the Netherlands. Cultures of *M. brassicae* and *M. mediator* were maintained on Brussels sprouts cv. Cyrus in climate rooms at 22 ± 2 °C, with a light regime of 16:8 L/D.

*Experimental set-up*

Seeds of *J. vulgaris* were collected from a single population at a semi-natural grassland in the Mossel nature restoration area (Ede, the Netherlands, 52°03'38''N, 5°45'04''E) where cropping ceased in 1995. Seeds were surface sterilized (1 min in a 0.1% sodium chloride solution and rinsed with water) and germinated on glass beads. Three *J. vulgaris* seedlings were planted in each of 80 one-litre pots filled with a mixture of sterilized and non-sterilized field soil (1:1 ratio). The sandy-loam soil (particle size distribution: < 2 $\mu$m, 3%; 2-63 $\mu$m, 17%; > 63 $\mu$m, 80%) was collected from the same area as the seeds and contained 4.5% organic matter. In the laboratory, the soil was sieved through a 0.5 cm mesh to remove stones and large arthropods and was subsequently homogenized. Half of the soil was sterilized using gamma irradiation (> 25 KGray gamma irradiation, Isotron, Ede, the Netherlands). The plants were grown in a greenhouse (21/16 °C day/night, 16 hours photoperiod). Natural daylight was supplemented by 400 W metal halide lamps (1 lamp per 1.5 $m^2$). Plants were watered three times per week and randomly redistributed within the greenhouse once a week. After one week, the seedlings were randomly thinned to two seedlings per pot.

Six weeks after transplantation, two late-instar wireworm larvae were introduced into each of 40 randomly chosen pots assigned to the root herbivory treatment. Wireworms were placed into a small hole (1 cm deep) made in the soil. The larvae immediately burrowed into the soil. Similar holes were also made in the soil of the remaining 40 control pots. Prior to their introduction,

wireworm larvae were starved for three days in moist soil at room temperature. Two weeks later, all pots were placed individually into a fine meshed cylindrical cage (70 cm height, 25 cm diameter). Two second-instar larvae of *M. brassicae* were then introduced to 20 control and 20 root herbivory pots. The remaining pots received two parasitized *M. brassicae* larvae. Larvae were introduced onto the plant by carefully placing them with a small brush on the youngest fully mature leaf of the plant. Parasitized larvae were parasitized individually using freshly mated *M. mediator* female parasitoids and then immediately introduced on the plant. The two larvae could move freely on the plants within each cage. Insects were kept on the plant for four weeks. Once a week, starting two weeks after introducing them on the plant, all larvae were collected from the plants, weighed on the microbalance, and returned to the same cage. Unparasitized larvae remained in the larval stage throughout the entire experiment. Cages with parasitized *M. brassicae* larvae were checked daily for egression of cocoons. Parasitoid cocoons were carefully collected from the plant and placed individually in Petri dishes until adult emergence. To record adult parasitoid emergence cocoons were checked twice a day. At emergence, the date of eclosion was recorded and parasitoids were sexed. Hind tibia length was recorded as a measure of adult size (Godfray 1994), using a calibrated slide and a stereomicroscope. Development time was calculated as days between parasitism and adult emergence. At harvest, shoots were clipped and roots were carefully removed from the soil and rinsed. Shoot and root biomass of each pot was oven-dried at 70 °C for three days and weighed. All wireworm larvae were recovered alive from the soil.

*Chemical analysis*

Eight weeks after germination, just prior to the introduction of the unparasitized and parasitized *M. brassicae* larvae, the fifth youngest leaf of 20 control plants and 20 plants with root herbivory was removed with a razor blade, immediately freeze-dried and finely ground. The root samples were taken from the oven-dried root material for the same plants and pulverized. For both treatments there were 10 plants allocated for unparasitized and 10 for parasitized larvae. Carbon (C) and Nitrogen (N) content were determined only for leaf samples using a Flash EA1112 CN analyzer (Interscience, Breda, the Netherlands). PA composition and content was determined using a Waters Acquity ultra performance liquid chromatographic system coupled to a Waters Quattro Premier XE tandem mass spectrometer (Waters, Milford, MS, USA); see also Cheng et al. (2011a, b). For each sample, 10 mg of ground plant material was mixed with 1.0 ml 2% formic acid solution. Heliotrine was added to the extraction solvent as an internal standard at a concentration of 1 $\mu g \cdot ml^{-1}$. The mixture was centrifuged and filtered through a 0.2 $\mu$m nylon membrane filter (Acrodisc, Pall Life Sciences, MI, USA).

An aliquot of 25 $\mu$l of the extracted filtrate was diluted with 975 $\mu$l of 10 mM ammonium hydroxide solution and injected in the LC-MS/MS system. PAs were separated on a Waters BEH C18 UPLC column (150 $\times$ 2.1 mm, 1.7 $\mu$m particles) applying 5 mM ammonium hydroxide as mobile phase and using acetonitrile as organic modifier (0-50%) in a 12-min linear gradient. The mass spectrometer was operated in positive electrospray mode and the samples were screened for a total of 37 PAs. Details on the mass spectrometric settings are described in Cheng et al. (2011b). PAs were quantified against a calibrant of PA standards added to *Tanacetum vulgare* plant extract (which itself is free of PAs) to minimize matrix effects that otherwise could play a role when using standards in solvent only. The calibrant solution was injected every 20 samples to monitor for variations in detector response. Samples were injected in a randomized order. Data were processed using Masslynx 4.1 software (Waters, Milford, MA, USA).

*Statistics*

The impact of root herbivory on plant biomass, chemistry, herbivore and parasitoid performance was assessed using a Welch's robust t-test which does not require homogeneity of variances. In the robust Welch t-test the degrees of freedom are corrected with the Welch-Satterthwaite modification (Welch 1947). The percentage difference in individual PA concentrations was calculated as: (mean PA concentration of plants subjected to root herbivory treatment – mean PA concentration of control plants)/ mean PA concentration of control plants. The overall difference in the concentration of *N*-oxides and tertiary amines was compared using a paired t-test. The relative concentration of *N*-oxides was calculated as: % *N*-oxide = *N*-oxide concentration/(*N*-oxide concentration + the corresponding tertiary amine concentration)$\times$100. Percentage data were arcsine square-root transformed prior to statistical analysis. For graphical representation we calculated the natural logarithm of the ratio between *N*-oxides and tertiary amines that is symmetrical around the 1:1 ratio point. The relationship between plant characteristics and herbivore and parasitoid performance were analyzed using Pearson's product-moment correlation. As the number of replicates was relatively low, significance in multiple statistical tests was not corrected (Moran 2003). To examine whether root herbivory influenced the PA composition aboveground or belowground we used multivariate principal component (PCA) and redundancy (RDA) analyses. The choice of linear methods was justified by the short length of gradients (less than 2.0). RDA was also used to test the relationship between the shoot PA composition and herbivore or parasitoid performance. Significances in multivariate analyses were tested using a Monte Carlo permutation test with 999 permutations. Univariate analyses were performed in R statistical language, ver. 2.15.0 (R Development Core Team 2012) and multivariate analyses in CANOCO version 4.5 (Ter Braak & Šmilauer 2002).

## Results

*Plant responses*

Plant shoot and root biomass did not differ significantly between treatments (Table 2.1). Root herbivory did also not influence leaf nitrogen concentrations and leaf C:N ratios. The total PA concentration in shoots of plants exposed to root herbivory was significantly lower (38%) than in control plants (Table 2.1). The total PA concentration in roots was slightly higher (12%) in plants exposed to root herbivory than in control plants but this was not statistically significant (Table 2.1). Twenty-nine PAs were detected in shoots and 33 PAs in roots of *J. vulgaris* (Table 2.2). The detected PAs belonged to four structural groups: erucifoline-type, jacobine-type, senecionine-type and otosenine-type (Table 2.2). Otosenine-type PAs were only identified in roots. In shoots, dehydrojaconine was detected in trace amounts and only occurred as tertiary amine. All other PAs were found in *N*-oxide and in tertiary amine form. In roots, senecivernine, senkirkine, otosenine, onetine and desacetyldoronine were only present as tertiary amines. The concentration of tertiary amines in shoots was not affected by root herbivory ($t_{35.5} = 0.61$, $P = 0.54$), whereas the overall levels of *N*-oxides in shoots decreased by 52% in the plants exposed to root herbivory ($t_{29.5} = 3.24$, $P = 0.003$; Fig. 2.1). In roots, there was no significant difference in the concentration of tertiary amines ($t_{35.2} = 0.81$, $P = 0.42$) and *N*-oxides ($t_{37.5} = -1.28$, $P = 0.21$; Fig. 2.1) between treatments, although the levels of *N*-oxides were 14% higher in roots exposed to root herbivory. The contribution of tertiary amines increased from 34% to 48% in the total shoot PA concentration while in the total root PA concentration it decreased from 9% to 7% (Fig. 2.1).

In shoots, independent of root herbivory, jacobine and jacobine *N*-oxide were present in the highest concentrations in all plants (35% and 33% respectively of the total PA concentration) and the total concentration of jacobine-type PAs decreased after root herbivory (Table 2.2). The total concentration of senecionine-type PAs in shoots was lower in plants exposed to root herbivores, but the total concentration of erucifoline-type PAs did not differ between the treatments, although levels of acetylerucifoline (+1024%) and acetylerucifoline *N*-oxide (+337%) responded most strongly to root herbivory (Table 2.2). In roots, the total concentrations of none of the four groups of PAs was affected by root herbivory (Table 2.2).

**Table 2.1** Effects of root herbivory by wireworms on plant, herbivore and parasitoid performance parameters. Means (±SE) are shown for control plants (-RH) and plants exposed to the root herbivory by wireworms (+RH) and results of a statistical test.

| | -RH | +RH | N | | $P^a$ |
|---|---|---|---|---|---|
| Shoot biomass (g dw) | 0.42 ± 0.01 | 0.42 ± 0.01 | 80 | $t_{77.5}$=0.40 | 0.69 |
| Root biomass (g dw) | 1.30 ± 0.05 | 1.24 ± 0.04 | 80 | $t_{69.3}$=0.89 | 0.38 |
| Leaf nitrogen concentration (%) | 1.26 ± 0.05 | 1.30 ± 0.03 | 40 | $t_{31.0}$=-0.76 | 0.45 |
| C:N ratio | 34.04 ± 1.43 | 32.25 ± 0.86 | 40 | $t_{31.0}$=1.07 | 0.29 |
| Total shoot PA concentration (mg·g$^{-1}$ dw) | 1.44 ± 0.14 | 0.89 ± 0.07 | 40 | $t_{35.4}$=3.27 | 0.0024 |
| Total root PA concentration (mg·g$^{-1}$ dw) | 3.49 ± 0.24 | 3.91 ± 0.28 | 40 | $t_{37.3}$=-1.26 | 0.22 |
| Herbivore RGR (mg·day$^{-1}$) | 0.10 ± 0.009 | 0.07 ± 0.008 | 31 | $t_{28.9}$=2.01 | 0.054 |
| Herbivore mortality (%) | 43.0 ± 7.5 | 38.0 ± 9.0 | 40 | $t_{37.1}$=0.43 | 0.67 |
| Parasitoid tibia length (mm) | 0.87 ± 0.02 | 0.82 ± 0.08 | 10 | $t_{2.1}$=0.59 | 0.62 |
| Successful pupation (%) | 15.0 ± 5.26 | 12.5 ± 6.15 | 40 | $t_{37.1}$=0.31 | 0.76 |
| Adult emergence (%) | 12.5 ± 4.97 | 10.0 ± 5.85 | 40 | $t_{37.0}$=0.33 | 0.75 |
| Parasitoid development time (days) | 31.20 ± 1.02 | 34.33 ± 1.45 | 10 | $t_{1.4}$=-1.27 | 0.38 |

[a]Differences between the two treatments were tested using a Welch robust t-test (t) which does not require homogeneity of variances.

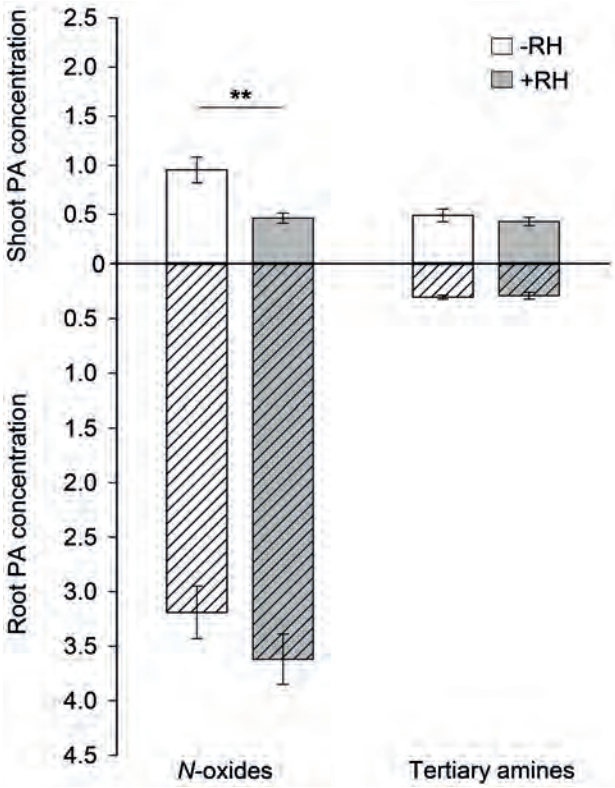


**Figure 2.1** Mean *N*-oxide and tertiary PAs concentration (N = 40; ± SE, mg·g$^{-1}$ dw) of *J. vulgaris* shoots (bars without pattern) and roots (hatched bars) in plants kept without root herbivory (-RH, white bars) and plants exposed to root herbivory by *A. lineatus* (+RH, grey bars). Asterisks indicate a significant difference based on a Welch's robust t-test ** $P < 0.01$.

**Table 2.2** Mean concentration (±SE, $\mu g \cdot g^{-1}$ dw) of individual PAs detected in shoots and roots of control (-RH) plants and plants exposed to belowground herbivory (+RH). The % difference in mean concentration between –RH and +RH is also presented. The "-" sign indicates that the concentration of a specific PA decreased when plants were exposed to root herbivory compared to control plants. AcEr – Acetylerucifoline, AcSp – Acetylseneciphylline, DADn – Desacetyldoronine, DHJn – Dehydrojaconine, Er – Erucifoline, Ir – Integerrimine, Jb – Jacobine, Jl – Jacoline, Jn – Jaconine, Jz – Jacozine, On – Onetine, Ot – Otosenine, Rd – Riddelliine, Rt – Retrorsine, Sk – Senkirkine, Sn – Senecionine, Sp – Seneciphylline, St – Spartioidine, Sv – Senecivernine, Us – Usaramine, -ox – *N*-oxide form of the corresponding PA.

| PA | Shoot | | | | | Root | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | -RH | +RH | % difference | t | $P^a$ | -RH | +RH | % difference | t | $P^a$ |
| **Erucifoline-type** | | | | | | | | | | |
| AcEr | 0.2 ± 0.1 | 2.3 ± 0.7 | 1024 | -3.20 | ** | 0.4 ± 0.1 | 0.2 ± 0.1 | -41 | 1.48 | ns |
| AcEr-ox | 8.0 ± 4.7 | 34.9 ± 11.4 | 337 | -2.18 | * | 26.2 ± 7.3 | 18.7 ± 2.7 | -28 | 0.96 | ns |
| Er | 6.8 ± 1.6 | 6.2 ± 1.2 | -8 | 0.28 | ns | 7.0 ± 1.2 | 8.0 ± 2.0 | 15 | -0.45 | ns |
| Er-ox | 114.2 ± 18.3 | 49.9 ± 7.9 | -56 | 3.23 | ** | 45.2 ± 10.9 | 36.1 ± 4.7 | -20 | 0.77 | ns |
| Total | 129.2 ± 20.9 | 93.3 ± 11.5 | -28 | 1.1 | ns | 78.7 ± 18.4 | 63.0 ± 7.9 | -20 | 0.05 | ns |
| **Jacobine-type** | | | | | | | | | | |
| DHJn | 0.04 ± 0.01 | 0.08 ± 0.02 | 114 | -1.79 | ns | - | - | - | - | ns |
| Jb | 439.5 ± 58.6 | 382.3 ± 37.8 | -13 | 0.82 | ns | 21.7 ± 2.1 | 22.6 ± 2.1 | 4 | -0.32 | ns |
| Jb-ox | 522.6 ± 101.6 | 239.5 ± 38.9 | -54 | 2.60 | * | 202.7 ± 24.0 | 205.2 ± 28.0 | 1 | -0.07 | ns |
| Jl | 32.8 ± 4.7 | 26.9 ± 2.9 | -18 | 1.06 | ns | 37.2 ± 3.7 | 33.0 ± 2.3 | -11 | 0.97 | ns |
| Jl-ox | 14.8 ± 2.4 | 8.0 ± 1.1 | -46 | 2.59 | * | 48.0 ± 4.7 | 50.0 ± 6.0 | 4 | -0.27 | ns |
| Jn | 1.2 ± 0.2 | 1.6 ± 0.2 | 35 | -1.23 | ns | 16.5 ± 1.7 | 13.0 ± 1.2 | -21 | 1.7 | ns |
| Jn-ox | 0.08 ± 0.03 | 0.20 ± 0.1 | 156 | -2.02 | ns | 8.7 ± 1.0 | 6.0 ± 0.6 | -31 | 2.32 | * |
| Jz | 2.7 ± 0.4 | 2.5 ± 0.4 | -9 | 0.40 | ns | 0.3 ± 0.1 | 0.4 ± 0.1 | 40 | -1.07 | ns |
| Jz-ox | 2.8 ± 0.6 | 1.7 ± 0.3 | -40 | 1.59 | ns | 3.0 ± 0.5 | 3.8 ± 0.6 | 27 | -0.99 | ns |
| Total | 1016.4 ± 112.9 | 662.7 ± 67.4 | -35 | 2.73 | ** | 337.9 ± 33.0 | 333.8 ± 37.1 | -1 | 0.07 | ns |

Table 2.2 continued

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Senecionine-type** | | | | | | | | | | |
| AcSp | 0.06 ± 0.02 | 0.12 ± 0.1 | 84 | -0.94 | ns | 17.6 ± 4.1 | 9.1 ± 1.4 | -48 | 1.98 | ns |
| AcSp-ox | 0.5 ± 0.1 | 0.4 ± 0.1 | -33 | 1.37 | ns | 274.8 ± 32.2 | 378.1 ± 47.5 | 38 | -1.80 | ns |
| Ir | 0.4 ± 0.2 | 0.3 ± 0.1 | -17 | 0.36 | ns | 17.2 ± 1.7 | 14.7 ± 1.8 | -14 | 1.02 | ns |
| Ir-ox | 40.7 ± 7.1 | 17.7 ± 2.7 | -57 | 3.01 | ** | 357.8 ± 31.0 | 380.6 ± 22.8 | 6 | -0.59 | ns |
| Rd | 0.06 ± 0.02 | 0.03 ± 0.01 | -38 | 0.93 | ns | 1.6 ± 0.4 | 1.4 ± 0.3 | -14 | 0.45 | ns |
| Rd-ox | 2.7 ± 0.5 | 2.2 ± 0.3 | -21 | 0.95 | ns | 65.6 ± 10.7 | 79.6 ± 8.2 | 21 | -1.04 | ns |
| Rt | 0.3 ± 0.1 | 0.2 ± 0.04 | -27 | 1.07 | ns | 13.5 ± 2.0 | 13.0 ± 1.8 | -4 | 0.19 | ns |
| Rt-ox | 12.1 ± 2.2 | 6.2 ± 0.8 | -49 | 2.48 | * | 238.0 ± 30.9 | 270.8 ± 40.9 | 14 | -0.64 | ns |
| Sn | 2.9 ± 0.7 | 2.9 ± 0.5 | -1 | 0.02 | ns | 120.9 ± 10.0 | 113.4 ± 11.1 | -6 | 0.51 | ns |
| Sn-ox | 164.1 ± 34.3 | 66.6 ± 12.6 | -59 | 2.67 | * | 1471.1 ± 123.0 | 1616.0 ± 121.6 | 10 | -0.84 | ns |
| Sp | 1.8 ± 0.5 | 1.6 ± 0.3 | -10 | 0.33 | ns | 27.5 ± 2.4 | 34.4 ± 5.5 | 25 | -1.14 | ns |
| Sp-ox | 64.8 ± 12.2 | 31.2 ± 4.6 | -52 | 2.59 | * | 418.0 ± 56.6 | 543.3 ± 62.6 | 30 | -1.49 | ns |
| Sv | - | - | - | - | - | 4.7 ± 0.5 | 4.4 ± 0.8 | -5 | 0.25 | ns |
| St | 0.05 ± 0.01 | 0.06 ± 0.01 | 17 | -0.54 | ns | 0.7 ± 0.1 | 0.7 ± 0.1 | 3 | -0.20 | ns |
| St-ox | 2.3 ± 0.4 | 1.8 ± 0.2 | -23 | 1.19 | ns | 9.2 ± 1.3 | 9.4 ± 0.8 | 1 | -0.08 | ns |
| Us | 0.15 ± 0.03 | 0.08 ± 0.02 | -45 | 1.89 | ns | 1.8 ± 0.2 | 1.5 ± 0.2 | -17 | 1.13 | ns |
| Us-ox | 2.8 ± 0.5 | 1.1 ± 0.2 | -61 | 3.13 | ** | 22.7 ± 3.4 | 22.9 ± 4.8 | 1 | -0.03 | ns |
| Total | 295.8 ± 55.2 | 132.4 ± 19.0 | -55 | 2.51 | * | 3062.7 ± 215.9 | 3493.1 ± 218.4 | 14 | -1.34 | ns |
| **Otosenine-type** | | | | | | | | | | |
| Sk | - | - | - | - | | 7.3 ± 5.0 | 11.2 ± 10.0 | 53 | -0.35 | ns |
| Ot | - | - | - | - | | 4.2 ± 1.8 | 5.3 ± 3.3 | 26 | -0.28 | ns |
| On | - | - | - | - | | 2.3 ± 1.0 | 2.7 ± 1.6 | 13 | -0.17 | ns |
| DADn | - | - | - | - | | 1.0 ± 0.5 | 1.3 ± 0.8 | 27 | -0.30 | ns |
| Total | - | - | - | - | | 14.9 ± 8.1 | 20.5 ± 15.6 | 37 | -0.04 | ns |

[a]Asterisks indicate significant differences analyzed by t-test *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ns – not significant.

Overall, the relative concentration of *N*-oxides was higher than that of tertiary amines (shoots: $t_{39}$ = -2.58, $P$ = 0.014; roots: $t_{39}$ = -32.15, $P$ < 0.001; Fig. 2.1). In shoots, for erucifoline- and senecionine-type PAs the relative concentration of *N*-oxides was much higher than the concentration of tertiary amines ($P$ < 0.01 in all cases), while for jacobine-type PAs concentrations of *N*-oxides were equal or lower than concentrations of tertiary amines (Fig. 2.2). In roots, the relative concentration of *N*-oxides was much higher for all compounds except for jaconine (Fig. 2.2). Root herbivory significantly decreased the relative concentration of *N*-oxides for senecionine ($t_{28.4}$ = -2.63, $P$ = 0.014), erucifoline ($t_{28.5}$ = 2.73, $P$ = 0.011) and integerrimine ($t_{33.2}$ = 2.98, $P$ = 0.005) in shoots, and increased the relative concentration of *N*-oxides for acetylseneciphylline in roots ($t_{38.0}$ = -3.05, $P$ = 0.005; Fig. 2.2).

Principle component analysis of the shoot PA composition showed that most of the variation in PA profiles could be explained by three principle component axes (74.3% cumulative explained variation). Shoot PA profiles differed significantly between plants exposed to root herbivory and control plants (RDA: F = 4.50, $P$ = 0.002; 10.6% explained variation). Shoot PA profiles of plants exposed to root herbivory and control plants clearly separated in an unconstrained analysis (PCA; Fig. 2.3). In the PCA, the levels of acetylerucifoline, acetylerucifoline *N*-oxide, jaconine *N*-oxide were higher in plants with root herbivory, whereas levels of jacobine *N*-oxide, jacoline *N*-oxide, erucifoline *N*-oxide, senecionine *N*-oxide, integerrimine *N*-oxide, usaramine *N*-oxide, seneciphylline *N*-oxide and retrorsine *N*-oxide were higher in control plants (Fig. 2.3). The PA composition in roots was not affected by root herbivory (RDA: F = 0.62, $P$ = 0.74; data not shown).

*Herbivore and parasitoid performance*

The relative growth rates of unparasitized *M. brassicae* larvae tended to be lower on plants with root herbivory, but this was only marginally significant ($P$ = 0.054; Table 2.1). Mortality of *M. brassicae* did not differ significantly between the two treatments (Table 2.1). Herbivore growth rate and survival were not significantly related to leaf nitrogen concentration, C:N ratio, total shoot PA concentration, levels of individual PAs in the shoots, or shoot PA composition ($P$ > 0.05 in all cases). Parasitoid performance, measured as hind tibia length, % successful cocoon egression, % adult emergence, and development time, also did not differ between the two treatments (Table 2.1). However, there was a negative relationship between parasitoid development time and total shoot *N*-oxide concentration (r = -0.79, $P$ = 0.033). Analyses of individual shoot PA compounds revealed that parasitoid development time negatively correlated with concentrations of jacoline *N*-oxide (r = -0.90, $P$ = 0.006), jacobine *N*-oxide (r = -0.85, $P$ = 0.016), and usaramine (r = -0.77, $P$ = 0.046).

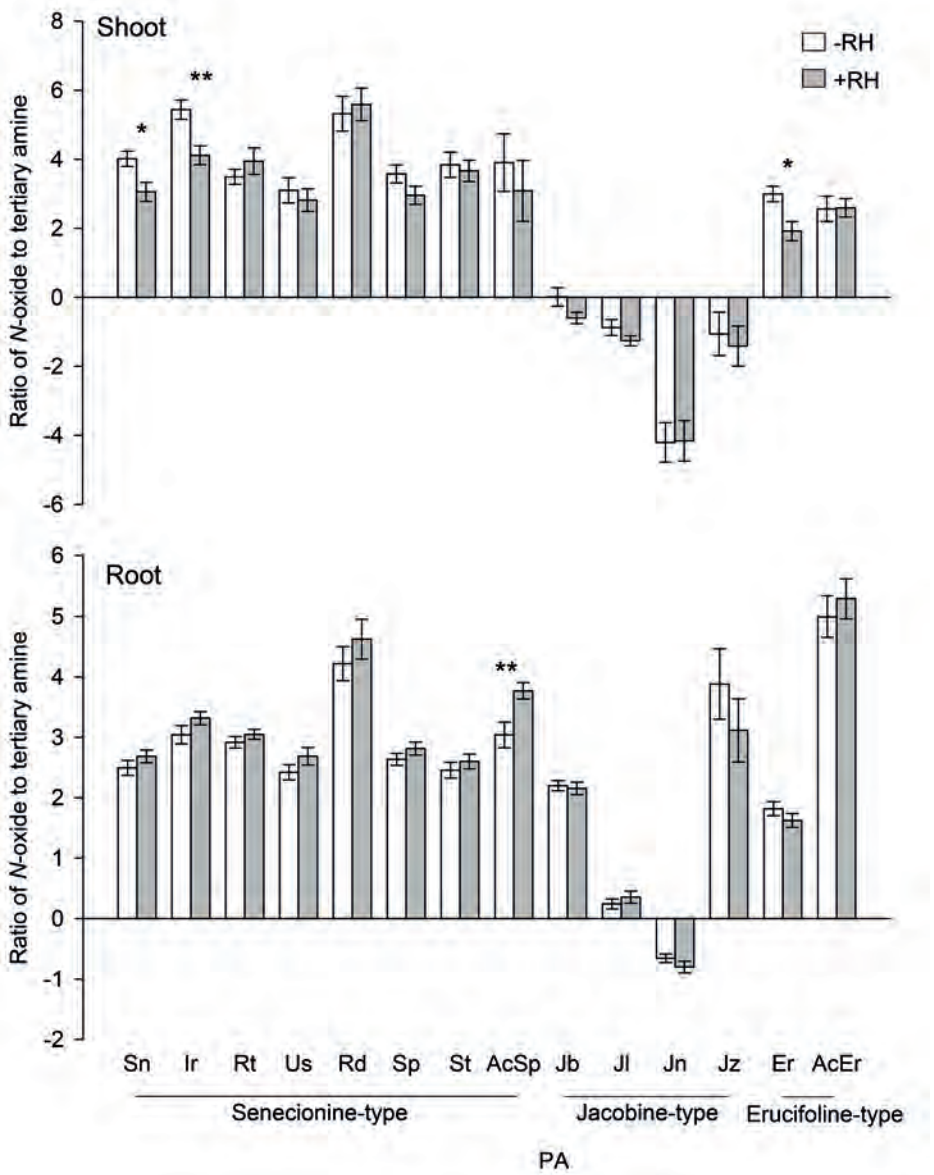


**Figure 2.2** Ratio of *N*-oxides to tertiary amines (± SE) of individual pyrrolizidine alkaloids of *J. vulgaris* shoots and roots in plants kept without root herbivory (-RH, white bars) and plants exposed to root herbivory by *A. lineatus* (+RH, grey bars). The ratio was calculated as ln ([total *N*-oxide concentration]/[total tertiary amine concentration]). Values larger than 0 indicate that the concentration of the *N*-oxide form of a PA is higher than that of the tertiary amine form, and values less than 0 indicate that the concentration of the *N*-oxide form is lower than that of the tertiary amine form. Dehydrojaconine and otosenine-type PAs occurred only as tertiary amine and therefore were not included in the figure. For the legend of PA names see Table 2.2.

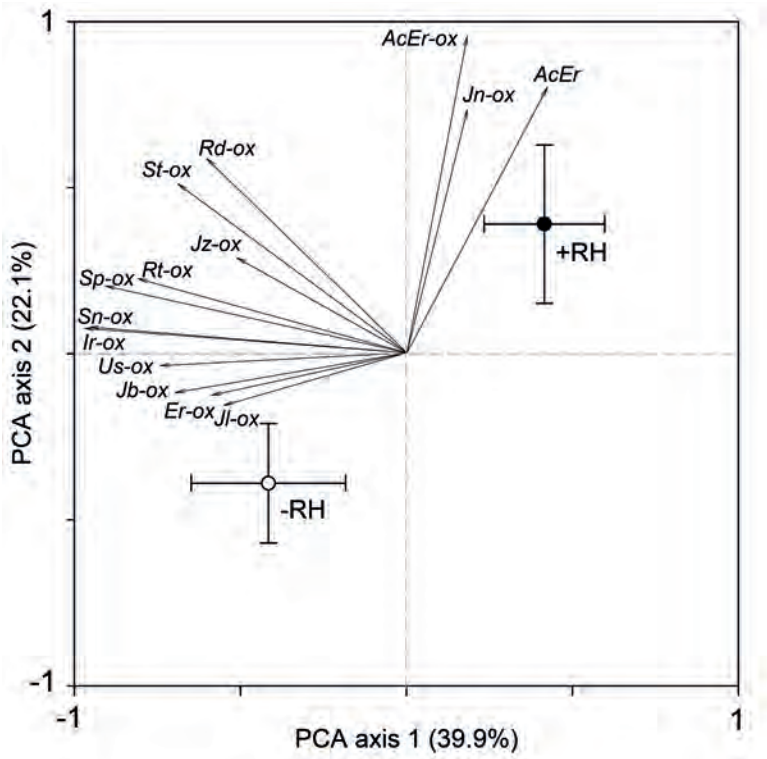


**Figure 2.3** Biplot showing the first and second axis of a principal component analysis (PCA) of the shoot pyrrolizidine alkaloid profiles. The mean (± SE) sample scores of undamaged control plants (-RH, open circles) and plants exposed to root herbivory by *A. lineatus* (+RH, filled circles) are shown, and all PAs with more than 30% fit. The numbers between brackets show the amount of variation explained by each axis. For the legend of PA names see Table 2.2.

## Discussion

In our study, root herbivory greatly affected the concentration and composition of PAs in the leaves of *J. vulgaris*. However, in contrast with our hypothesis, total PA concentration in the shoots of *J. vulgaris* decreased strongly (38%) when plant roots were exposed to herbivory by *A. lineatus*. In a previous study, Hol et al. (2004) found that mechanical root damage caused an increase in PA concentrations in the roots of *J. vulgaris* but that mechanical damage to roots had only weak and inconsistent effects on shoot PA concentrations. Clearly, mechanical tissue damage may not elicit the same effect on the expression of allelochemicals as actual herbivory (Bezemer et al. 2004b; Kaplan et al. 2008a). The majority of studies that have examined the effects of root damage by real herbivores on concentrations of aboveground secondary plant compounds for

other plant species report increases in the amount of secondary metabolites following root herbivory (e.g., Bezemer et al. 2003, 2004b; Van Dam et al. 2005; Soler et al. 2005; Erb et al. 2008; Kaplan et al. 2008b; Wurst et al. 2008). One of the reasons for the discrepancy between the results of these studies and ours may be that PAs are synthesized in the roots whereas many of the secondary compounds included in the other studies can be produced in the shoots. Similar to our results, root herbivory by the nematode *Meloidogyne incognita* in tobacco plants causes a decline in the concentrations of the alkaloid nicotine in the foliage, and nicotine is also synthesized in the roots (Hanounik & Osborne 1977; Kaplan et al. 2008a). However, Hanounik and Osborne (1977) also showed that root herbivory by *M. incognita* caused an increase in nicotine in leaves of a resistant tobacco cultivar showing that the effects of root herbivory can greatly vary even within a single plant species. In the study of Kaplan et al. (2008a), even though root herbivory caused a decline in the concentrations of nicotine aboveground, concentrations of other secondary plant compounds that are not exclusively produced in the roots increased in the foliage. In another study, terpenoid aldehydes in cotton (*Gossypium* spp.), which are also synthesized in roots, increased in the foliage of cotton following root herbivory by wireworms (Bezemer et al. 2004b). Synthesis of gossypol is also known to occur in the foliage of cotton plants but in lower concentrations (Bezemer et al. 2004b). Therefore, it is plausible that the synthesis of gossypol was enhanced in the shoots rather than in the roots by belowground herbivory. However, a more likely explanation for the different responses observed among the different plant species is that there are various mechanisms by which belowground herbivory can lead to changes in aboveground plant chemistry (reviewed in Soler et al. 2012b). These results therefore emphasize that generalizations about aboveground-belowground effects should be made with great caution.

An important question that requires further study is whether root herbivory in ragwort negatively interferes with PA synthesis in the roots, or whether the negative effects of root herbivory on aboveground PA concentrations result from a difference in allocation of PAs to aboveground tissues. PA production in *J. vulgaris* is closely linked to root growth (Frischknecht et al. 2001). Interestingly, in our study, root biomass was not significantly affected by the belowground herbivory. Such a lack of a response in root biomass to root herbivory has also been observed in other experiments in which *J. vulgaris* was exposed to root herbivory (M. Bezemer, unpublished data) and can be the result of compensatory growth or a reallocation of resources from shoots to roots. For *J. vulgaris* roots are more essential organs than shoots, because roots accumulate resources that are used by plant for regrowth after complete defoliation (Van der Meijden et al. 2000). As root biomass did not change after root herbivory this

suggests that the production of PAs in the roots could be maintained at the same level. Indeed in our study, total root PA concentration and composition were not significantly affected by belowground herbivory. Although, the effect of root herbivory on total root PA concentration was not significant, the total amount of PAs in the roots tended to increase (12%) in presence of root herbivory whereas the total amount of PAs in shoots decreased significantly (38%). This suggests that root herbivory caused a reallocation of PAs from the shoots to roots, or that less PAs were transported from the roots to the shoots in plants exposed to root herbivory. Overall, concentrations of PAs were much higher in roots than in leaves. These results, in line with other studies (Van der Meijden et al. 2000; Hol et al. 2004) suggest that roots are more important to *J. vulgaris* than shoot tissues. However, it is important to note that, in our study, the root samples were collected later than the leaf samples, and after a period of aboveground herbivore feeding.

The use of the LC-MS/MS procedure allowed us to detect both the tertiary amine and *N*-oxide forms of PAs, as well as PAs that are present only in extremely low concentrations in the plant (Joosten et al. 2009). Earlier studies were restricted to the major PAs that are present in plants and in these studies the authors were not able to discriminate between the two forms of PAs (e.g., Hol et al. 2004; Macel & Klinkhamer 2010). Our results in line with other more recent studies (e.g., Joosten et al. 2011) show that the concentration of tertiary amine forms in jacobine-type PAs is higher than in other PA groups (for a discussion on the selective formation of jacobine tertiary amines see Joosten et al. 2011). Interestingly, in our study most of the individual PAs in plant shoots that responded to the root herbivory treatment were *N*-oxides. As a result, the ratio of *N*-oxides to tertiary amines in the shoots changed from 2:1 in control plants to 1:1 in plants exposed to root herbivores. At the same time, there was a slight increase in the *N*-oxide concentration in the roots, mostly due to an increase in the concentration of senecionine *N*-oxide, while the total tertiary amine concentration in the roots remained constant. The concentration of *N*-oxides of major PAs such as jacobine *N*-oxide, jacoline *N*-oxide and erucifoline *N*-oxide did not increase in the roots in response to root herbivory, suggesting that it is unlikely that *N*-oxides are actively back-transported from shoots to roots when the plant is exposed to root herbivory. Therefore, our data suggest that plants, when they are exposed to root herbivory, alter PA concentrations in shoots and roots via restrictions in the flow of *N*-oxides from root to shoot tissues. As a result, if PA transport from roots to shoots is restricted, over time the PA concentration in the shoots will decrease, because the plant continues to grow (dilution effect). At the same time the conversion from *N*-oxides to tertiary amines continues to take place in the shoots. This conversion further reduces the concentration of *N*-oxides in the shoots, but stabilizes the tertiary amines concentrations.

Apart from affecting the total PA concentration in the plant, root herbivory also caused a change in the relative composition of PAs in the leaves. Traditionally, it was assumed that PAs are produced in the root as senecionine *N*-oxide only, and that diversification of this compound then occurs in the foliage (Hartmann & Dierich 1998). Recent studies, however, have shown that PA diversification may already start in the roots, where besides senecionine *N*-oxide, considerable amounts of compounds that are closely related to senecionine *N*-oxide, such as seneciphylline *N*-oxide, acetylseneciphylline *N*-oxide and integerrimine *N*-oxide have been detected (Joosten et al. 2009; Cheng et al. 2011a). Further conversion of PAs takes place in the leaves and this process is highly plastic and depends on a number of physiological processes in the plant (reviewed in Hartmann 1999). The exact mechanism of PA diversification remains unclear. Interestingly, in our study the concentration of acetylerucifoline and acetylerucifoline *N*-oxide in shoots increased greatly in plants exposed to root herbivory, while the concentration of erucifoline *N*-oxide significantly decreased. At the same time, the overall concentration of erucifoline-type PAs remained constant between the treatments. Acetylerucifoline *N*-oxide can be formed by acetylation of erucifoline *N*-oxide or by conversion of acetylseneciphylline *N*-oxide to acetylerucifoline *N*-oxide. Acetylseneciphylline *N*-oxide was not found in significant amounts in the shoots indicating that this compound is not transported well from roots to shoots perhaps due to its chemical properties. Therefore, we hypothesize that root herbivory causes an increase in the acetylation of erucifoline *N*-oxide in aboveground plant parts. Similarly, acetylseneciphylline *N*-oxide is synthesized by introducing an acetyl functional group to seneciphylline *N*-oxide in the root system (Cheng et al. 2011a). Acetylseneciphylline *N*-oxide also slightly increased in the roots of plants exposed to belowground herbivory. The ecological functions of acetylerucifoline and acetylseneciphylline are not yet known. Studies are needed that further explore how environmental stresses such as root herbivory affect the diversification of PAs and what the ecological consequences are of changes in plant PA composition for other organisms in natural communities.

The performance of the aboveground generalist herbivore *M. brassicae* was not significantly affected by root herbivory although unparasitized larvae tended to grow faster on undamaged plants containing higher concentrations of PAs in the shoots. This is a rather unexpected result that may be explained by the differences in the ratios between *N*-oxides and tertiary amines. *N*-oxide and tertiary amine forms of PAs are known to differently affect herbivorous insects. Several studies have shown, for example, that PAs in the form of *N*-oxides have less deterrent or toxic effects on generalist insect herbivores than tertiary PAs (Dreyer et al. 1985; Van Dam et al. 1995; Macel et al. 2005).

In addition, individual PAs differ in their effects on herbivores. For example, jacobine tertiary amine has been shown to adversely affect the performance of non-specialized herbivorous insects (Leiss et al. 2009; Macel & Klinkhamer 2010; Cheng et al. 2011b). In our study, jacobine was one of the major PAs present in leaves and the ratio of *N*-oxide to tertiary amine of this compound changed from 1.19 in control plants to 0.63 in plants exposed to root herbivory. Therefore, *M. brassicae* caterpillars feeding from root damaged plants may have suffered from the higher concentration of more toxic compounds that were present in the leaves even though the total PA concentration decreased. Furthermore, in our study larval mortality was high, and none of the unparasitized caterpillars pupated, even though they were kept on the plants for four weeks. The caterpillars performed much worse on *J. vulgaris* plants than on artificial diet (Kostenko, unpublished data), and this suggests that PA levels may already have been too high for this herbivore, independent of whether the plant was exposed to root herbivory or not. However, in a choice experiment where the individual and combined effects of six PAs were tested in an artificial diet, Macel et al. (2005) did not find a deterrent effect of PAs on *M. brassicae*. These authors concluded that *M. brassicae* is a generalist herbivore that is relatively insensitive to various secondary metabolites in its diet. Alternatively, root herbivory may have caused an increase in other defensive compounds in *J. vulgaris* such as phenolics or may have induced changes in morphological characteristics such as trichomes that can increase physical resistance of the plant to herbivory.

Clearly, besides plant defences, other plant characteristics may also have affected the performance of *M. brassicae* on *J. vulgaris* plants. In line with the plant-stress hypothesis (White 1984), Masters et al. (1993) proposed that stress induced by root herbivory will cause an increase in the concentrations of nitrogen and carbohydrates in foliar tissues of a plant. For the majority of herbivorous insects, the amount of nitrogen in the diet is the major limiting nutritional factor determining insect growth (Awmack & Leather 2002) and root herbivory would therefore lead to increased performance of aboveground herbivores. However, in our study, feeding by *A. lineatus* did not affect leaf nitrogen concentrations or C:N ratios in *J. vulgaris* plants.

The diet of an herbivorous host may also affect parasitoids that develop in this host by exposing them to unmetabolized defensive chemicals (Ode 2006). In our study, root herbivory did not affect the performance of the parasitoid *M. mediator*. Interestingly, although we did not detect a relationship between PA concentrations and *M. brassicae* performance, in our study parasitoids developed faster when the concentration of jacobine-type PAs, such as jacobine *N*-oxide

and jacoline *N*-oxide in the plant was higher. This suggests that *N*-oxides indeed could have less adverse effects on the performance of insects than tertiary amines. Future studies should examine whether there is a true causal positive relationship between jacobine-type *N*-oxides and parasitoid performance, or whether this is merely a coincidental correlation, and what the mechanisms are that underlie these interactions.

In summary, this study shows that root herbivory by wireworms has a strong negative effect on the concentration of PAs in the leaves of *J. vulgaris* possibly via the mechanism of restricted transport of PA *N*-oxides from roots to leaves. However, this does not result in a positive effect on the performance of the generalist insect herbivore *M. brassicae* or its parasitoid *M. mediator*. In contrast, *M. brassicae* tends to grow slower on plants exposed to root herbivory. This decline in herbivore performance can be explained by changes in foliar PA composition in plants exposed to root herbivory whereby the relative concentration of less toxic PAs decreases. Moreover, in our study the performance of parasitoids was also positively correlated with the concentration of less toxic PAs. Further research should aim at elucidating the putative role of individual PAs in aboveground-belowground multitrophic interactions.

## Acknowledgements

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# Chapter 3

## Legacy effects of aboveground-belowground interactions

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## Abstract

Root herbivory can greatly affect the performance of aboveground insects via changes in plant chemistry. These interactions have been studied extensively in experiments where aboveground and belowground insects were feeding on the same plant. However, little is known about how aboveground and belowground organisms interact when they feed on plant individuals that grow after each other in the same soil. We show that feeding by aboveground and belowground insect herbivores on ragwort (*Jacobaea vulgaris*) plants exert unique soil legacy effects, via herbivore-induced changes in the composition of soil fungi. These changes in the soil biota induced by aboveground and belowground herbivores of preceding plants greatly influenced the pyrrolizidine alkaloid content, biomass and aboveground multitrophic interactions of succeeding plants. We conclude that plant-mediated interactions between aboveground and belowground insects are also important when they do not feed simultaneously on the same plant.

## Introduction

A rapidly increasing number of studies is showing that belowground herbivory can influence plant growth and the composition and concentration of aboveground primary and secondary plant compounds (reviewed in Blossey & Hunt-Joshi 2003; Johnson et al. 2008; Van Dam 2009). Through these changes in host plant quantity and quality, root herbivores can affect the growth and survival of foliar feeding herbivores (e.g., Bezemer et al. 2003; Kaplan et al. 2008; Erb et al. 2011a; Van Dam & Heil 2011), as well as the enemies of these herbivores (Bezemer et al. 2005; Soler et al. 2005). These studies have focused on interactions that occur simultaneously and on a shared host plant. Little is known about the temporal dynamics of belowground-aboveground interactions and their feedback effects (Bardgett et al. 2005; Van der Putten et al. 2009). Here we show how aboveground and belowground herbivores can create soil legacy effects that affect the growth and nutritional quality of subsequent plants, as well as the aboveground multitrophic interactions occurring on those plants. These transferrable aboveground-belowground interaction effects due to induced legacy effects in the soil community have received little, if any, attention in ecology.

Ecological soil legacies can arise from effects on soil biota that subsequently affect the growth of plants colonizing the soil at a later stage (Kardol et al. 2007; Van de Voorde et al. 2011). Both root and foliar herbivores can alter the composition of the soil microbial community (Bardgett & Wardle 2010; Bennett 2010). Belowground herbivores can directly interact with soil microorganisms through competition, facilitation or predation, and indirectly through their effects on the quality and quantity of root tissues, root exudates, and organic matter content in the soil (Anderson et al. 1983; Bardgett et al. 1999; Van Dam 2009). Aboveground herbivores are physically separated from soil organisms. Nevertheless, they can influence soil microbial communities, for example by affecting the amount or quality of root exudates, or by altering the allocation or production of biomass, nutrients or allelochemicals to root tissues (Bardgett et al. 1998; Mikola et al. 2001; Soler et al. 2007a; Hamilton et al. 2008).

A number of studies have shown that soil microorganisms can influence not only plant growth, but also aboveground plant nutritional quality (Bonkowski et al. 2001; Bezemer et al. 2005; Gange 2007; Hol et al. 2010; Eisenhauer et al. 2010b). This can subsequently affect aboveground herbivores and their antagonists (Van Dam & Heil 2011). Thus, alteration of the soil microbial community by aboveground and belowground insect herbivory could potentially lead to soil legacy effects that impact the growth or nutritional quality of another plant

individual, and its interactions with herbivores and carnivores. The aim of this study was to test whether such aboveground-belowground multitrophic interactions can occur.

We tested the hypothesis that aboveground and belowground herbivory will lead to legacy effects in soil that will subsequently affect the growth and chemistry of plants growing later in the same soil, and their interactions with aboveground herbivores and natural enemies. We further studied if legacy effects of belowground herbivory differ from those arising from aboveground herbivory. To test our hypothesis we performed a greenhouse experiment in two phases. In the first phase, plants were exposed to aboveground and belowground herbivory in a full factorial design. At the end of this phase the composition of the soil microbial community was determined. In the second phase, new plants were grown in the conditioned soils in order to assess whether the legacy effects of aboveground and belowground herbivory influenced plant growth, and primary and secondary plant compounds in the foliage. We then exposed these plants to aboveground insect herbivores and parasitoids, in order to determine the soil legacy effects on aboveground multitrophic interactions.

## Materials and Methods

*Plant and insects*

The study system consisted of ragwort plants, *Jacobaea vulgaris* Gaertn. ssp. *vulgaris* (synonym *Senecio jacobaea L.,* Asteraceae), wireworms as belowground herbivores (*Agriotes lineatus* L. Coleoptera: Elateridae), *Mamestra brassicae* L. (Lepidoptera: Noctuidae) caterpillars as aboveground herbivores, and *Microplitis mediator* Haliday (Hymenoptera: Braconidae) as parasitoid of the aboveground herbivore. Ragwort is a biennial monocarpic plant native to Europe and Asia, where it is widely distributed (Bezemer et al. 2006). It produces a variety of pyrrolizidine alkaloids (PAs), a group of plant defence compounds that are toxic to a wide range of generalist insects and soil organisms (Hol & Van Veen 2002; Thoden & Boppre 2010; Macel 2011). PAs are constitutively biosynthesized in roots as senecionine *N*-oxide, which is transformed into several related senecionine-type PAs. These PAs are transported to aboveground plant parts where additional diversification takes place (Hartmann 1999; Cheng et al. 2011a). The concentration and composition of PAs can alter in response to abiotic factors, and to interactions of the plant with other aboveground and belowground organisms (Hol et al. 2004; Joosten et al. 2009).

*Agriotes lineatus* is the generalist root-feeding larva of a click beetle, usually called wireworm. Wireworms are pests of many cultivated crops. They are also common in semi-natural grasslands where *J. vulgaris* occurs (T. M. Bezemer, personal observation). Wireworms were obtained commercially from Applied Plant Research Lelystad (WageningenUR), the Netherlands. *Mamestra brassicae* is a generalist leaf chewer that has been reported to feed on *J. vulgaris* (Hol et al. 2004). *Microplitis mediator* is a solitary larval endoparasitoid that attacks first to fourth instar larvae of *M. brassicae* and a few closely related hosts of the family Noctuidae (Gols et al. 2008). *M. brassicae* and *M. mediator* were obtained from an insect culture maintained at the Laboratory of Entomology of Wageningen University, the Netherlands.

*Experimental setup*

*Phase 1: J. vulgaris* seeds were collected from a single population from a restoration grassland at Planken Wambuis (Ede, the Netherlands). The seeds were surface sterilized (1 min in 0.1% sodium chloride solution and rinsed with water) and germinated on glass beads. Forty pots of 2 l (15 cm diameter) were filled with 2.2 kg field soil (based on dry weight) collected from the restoration grassland at 5-20 cm below the soil surface. The soil was a sandy loam with particle size distribution: 3% $< 2$ $\mu$m, 17% 2-63 $\mu$m, 80% $> 63$ $\mu$m, with 4.5% organic matter. In the laboratory the soil was sieved through a 0.5 cm mesh and homogenized. During sieving, all insects were manually removed from the soil. Into each pot five seedlings were transplanted. Seedlings that died during the first week of the experiment were replaced. Pots were randomly located within a greenhouse (21 / 16 °C day / night, 16 hours photoperiod). Natural daylight was supplemented by 400 W metal halide lamps (225 $\mu$mol $m^{-2}$ $s^{-1}$ PAR). Plants were watered three times per week and randomly rearranged within the greenhouse once a week.

Seven weeks after transplanting, the pots were randomly allocated to one of the following treatments: belowground herbivory (B), aboveground herbivory (A), belowground and aboveground herbivory (AB) and undamaged control (C). All treatments were replicated 10 times. Four late-instar *A. lineatus* individuals were introduced into each pot assigned to one of the two root herbivory treatments. The larvae were placed into 1 cm deep holes and covered by soil. Similar holes were made for pots without root herbivory. Two third-instar larva of *M. brassicae* were placed individually in clip-on cages of 1.5 cm diameter on the youngest fully mature leaf of two plants in all A and AB treatment pots. Empty clip-on cages were attached to the same leaf of plants in pots that were not allocated to aboveground herbivory. The clip-on cages were kept on the leaf for a period of two days. During this period the larva had consumed the

entire leaf area available within the cage. Hereafter, the clip-on cages were moved to a similar-aged leaf of another plant within the same pot. Frass of *M. brassicae* larvae was removed from the clip cages every two days and it did not enter the soil. Plants were exposed to this treatment for three weeks. During this period, each plant within every pot was exposed to two bouts of herbivory. All herbivory treatments were initiated during the same day. Three weeks after initiating the herbivory treatments, shoots were clipped; roots were carefully removed from the soil and rinsed. Shoot and root biomass of each pot was oven-dried (70 °C for three days) and weighed. All wireworm larvae were recovered from the soil. A soil sample of 10 g was collected from each pot for molecular analysis (see below). The rest of the soil in each pot was homogenized, divided in five equal parts and used as inoculum in the second phase. Soil from each individual pot was kept separately during the entire following process.

*Phase 2*: Soil from each pot of the first phase was mixed with sterilized field soil (1:1 ratio) and used to fill five 1 l pots. This resulted in a total of 200 pots for Phase 2. The mixing of conditioned soil with sterilized soil minimized potential nutrient deficiencies after the first phase. Soil was sterilized by gamma irradiation (> 25 KGray) at Isotron, Ede, the Netherlands. Two *J. vulgaris* seedlings were planted into each pot. Plants were grown under the same conditions as during the first phase. Seven weeks after planting, the fifth youngest leaf of each plant was removed with a razor blade, immediately frozen at −20 °C, freeze-dried for three days under vacuum (−55 °C collector temperature, Labconco Free Zone 12 l Freeze Dry System, USA), and ground for chemical analysis (see below). The next day, all pots were caged individually using fine meshed cylindrical cages (70 cm height, 25 cm diameter). Two weighed second-instar larvae of *M. brassicae* were introduced to 160 pots. Eighty pots from each treatment received two non-parasitized (20 pots × 4 herbivory treatments) and the other eighty pots received two larvae parasitized by *M. mediator*. Larvae were introduced by carefully placing them with a small brush on the youngest fully mature leaf. The larvae had been parasitized individually in plastic vials using freshly mated female parasitoids. Parasitism was performed immediately prior to introducing the larvae into the cages. Within each cage, larvae could move freely on the plants. Insects were kept on the plants for four weeks. The remaining 40 pots (one replicate from each Phase 1 pot) were kept without insects and were used to measure plant growth (see Fig. S3.1 in Supporting Information for a scheme of the experimental design). The weight of non-parasitized and parasitized larvae was recorded once a week for four weeks starting 14 days after introduction. Mean relative growth rates and mortality were calculated. Cages with parasitized larvae were checked daily for egression of cocoons. Parasitoid cocoons were carefully collected from the

plant and kept individually in Petri dishes until adult emergence. Cocoons were checked twice a day for adult parasitoid emergence. At emergence, the date of eclosion was recorded and parasitoids were sexed, and tibia length was recorded as a measure of adult size (Godfray 1994). Tibia length was measured using a calibrated slide under a stereomicroscope. Development time was calculated as days between parasitism and adult emergence. The percentage of parasitized larvae that emerged as adults was also calculated. Twelve weeks after planting, for each of the 40 pots that was not exposed to herbivory, all aboveground and belowground biomass was harvested, oven dried at 70 °C for three days, and weighed.

*Chemical analysis of plants from Phase 2*

Chemical analyses were carried out on 20 pots of each treatment (10 of these pots were assigned to receive non-parasitized larvae and 10 to receive parasitized larvae). Carbon (C) and Nitrogen (N) content were determined using a Flash EA1112 CN analyzer (Interscience, Breda, the Netherlands). PA analysis was carried out using liquid chromatography-tandem mass spectrometry (LC-MS/MS) following the procedure outlined by Cheng et al. (2011a). In brief, 10 mg of freeze-dried ground plant material was extracted with 1.0 ml 2% formic acid solution containing heliotrine (1 $\mu g \cdot ml^{-1}$) as internal standard. After centrifugation and filtration, 25 $\mu$l of the extracted filtrate was diluted with 975 $\mu$l of 10 mM ammonium hydroxide solution and 10 $\mu$l was injected in a Waters Acquity ultra-performance chromatographic system coupled to a Waters Quattro Premier tandem mass spectrometer (Waters, Milford, MA, USA). Data were processed using Masslynx 4.1 software.

*Molecular analysis of the soil fungal community at the end of Phase 1*

The composition of the soil fungal community in each of the 40 pots at the end of Phase 1 was determined by T-RFLP (Terminal restriction fragment length polymorphisms analyses). Total DNA was extracted from 0.5 g frozen soil (−20 °C) with a Power Soil DNA isolation kit (MOBIO laboratories, Inc.) using a bead beating system. DNA quantity was checked using 1.5% agarose gel electrophoresis. The ITS region of the fungal rDNA was amplified by PCR using the primers ITS1F (White et al. 1990) and ITS4 (Gardes & Bruns 1993), which were labelled with FAM and NED respectively. The PCR reaction contained 13.8 $\mu$l Milli-Q, 2.5 $\mu$l 10× Fast Start High Fidelity Reaction Buffer (Roche Diagnostics), 2.5 $\mu$l DNTP Mix (2mM each), 2.5 $\mu$l ITS1F-6FAM primer (10 $\mu$M), 2.5 $\mu$l ITS4-NED primer (0.2 $\mu$M), 0.2 $\mu$l Fast Start High Fidelity Enzym Blend (5 $U \cdot \mu l^{-1}$) (Roche Diagnostics) and 1 $\mu$l template DNA. PCR programme conditions were 5 min at 95 °C, 35 cycles of 30 s at 95 °C, 40 s at 55 °C and 1 min at 72 °C, followed by 10 min at 72 °C before cooling. PCR product presence and

quality were verified on 1.5% agarose gels prior to restriction digestion. Two restriction enzymes, HhaI and TaqαI (New England Biolabs, Ipswich, MA, USA), were used to digest dual end-labelled DNA amplicons. A mixture containing 3.5 $\mu$l $ddH_2O$, 1 $\mu$l buffer, 0.1 $\mu$l Bovine Serume Albumin, 5 $\mu$l PCR product and 0.4 $\mu$l restriction enzyme was incubated at 37 °C (HhaI) or at 65 °C (TaqαI) for three hrs, and inactivation at 80 °C for 20 min. Restriction products were purified using ethanol precipitation. Fragment length polymorphism analysis was performed on an automated 3130 Genetic Analyzer sequencer (Applied Biosystems) with GeneScan™-500 LIZ, Applied Biosystems as a size standard. Samples which were over- (highest peak > 80000 rfu) or under-loaded (highest peak < 1000 rfu) were re-run with an adjusted concentration. Peaks were aligned to TRFs among the samples by applying a clustering threshold of 0.5 bp. Only peaks higher than 0.3% of the sum of all peaks in a sample were included.

*Data analysis*

All univariate analyses were performed using the R statistical language, version 2.10.1 (R Development Core Team 2010), and multivariate analysis using CANOCO version 4.55 (Ter Braak & Šmilauer 2002). Plant biomass data from both phases were analysed using two-way ANOVA. Other data from Phase 2 were analyzed using two-way mixed effects ANOVA (restricted maximum likelihood method, Pinheiro & Bates 2000), with legacy effects of above- and belowground herbivory and their interaction as fixed factors. Individual pot identity during Phase 1 was used as a random factor. In all analyses, the interaction term was never significant, so that it was removed from the model. Sex was included as a fixed factor when differences in parasitoid performance were compared. Prior to analyses, plant biomass, foliar nitrogen concentration and herbivore relative growth rate were log transformed; C:N ratio and parasitoid tibia length were square-root transformed to fulfil assumptions of normality. Percentage data on herbivore mortality and parasitoid adult emergence were analysed using a logit model with aboveground and belowground herbivory as fixed factors. The Wald z-statistic was used to test the statistical significance of each coefficient in the logit model.

Differences in PA composition among treatments in Phase 2 were analyzed using linear multivariate analyses (principle component analysis [PCA] and redundancy analysis [RDA]) as the longest gradient resulted from detrended correspondence analysis was < 3 (Lepš & Šmilauer 2003). Pot identities from Phase 1 were analyzed as "whole plots" and replicates pots in Phase 2 as "split plots". Whole plots were permuted freely and split plots were not permuted. The presence/absence matrix of T-RFLP fingerprints was analysed using distance-based redundancy analyses (db-RDA, Legendre & Anderson 1999), using the Jaccard coefficient of

similarity. The calculation of distance matrix and principal coordinates analyses (PCoA) were carried out in PrCoord 1.0 (CANOCO). All eigenvalues were positive. Significances in multivariate analyses were inferred by Monte Carlo permutation tests (999 permutations). Nonmetric multidimensional scaling (nMDS) was used to display variation in fungal communities among the four treatments. nMDS was performed in PAST (Hammer et al. 2001).

## Results

*Phase 1*

In soil with plants exposed to the different herbivore treatments 754 different fungal TRFs were detected; on average 59 TRFs per enzyme/dye combination per sample. Both belowground and aboveground herbivory significantly affected the composition of the soil fungal community (B: $F = 1.41$, $P = 0.023$; A: $F = 1.902$, $P = 0.003$). Soil fungal communities belonging to the root herbivory treatment separated most distinctly from the other treatments (Fig. 3.1). Plant root biomass, at the end of Phase 1, did not significantly differ between herbivore treatments (B: $F_{1,38} = 0.54$, $P = 0.47$; A: $F_{1,38} = 0.015$; $P = 0.90$). Shoot biomass tended to decrease when plants were exposed to belowground herbivore but the effect was not significant (B: $F_{1,38} = 3.76$, $P = 0.060$; A: $F_{1,38} = 0.69$; $P = 0.41$).

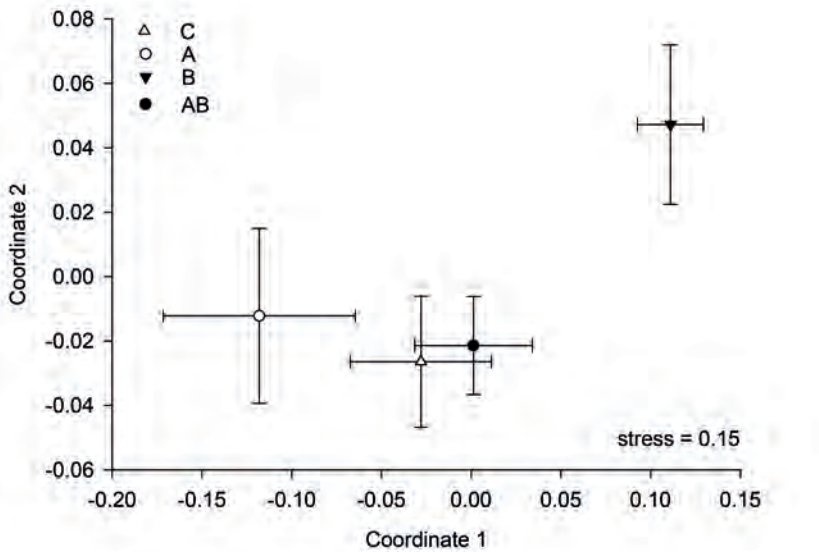


**Figure 3.1** Nonmetric multidimensional scaling (nMDS) plot. Shown are mean sample scores (±SE) of the T-RFLP community composition in soil with undamaged plants (C), or in soil, in which plants had grown that were exposed to aboveground (A), belowground (B), or aboveground and belowground herbivory (AB).

*Plant responses in Phase 2*

In Phase 2, there was a significant negative legacy effect of aboveground herbivory on root biomass ($F_{1,38}$ = 4.87, $P$ = 0.034; Fig. 3.2a). The legacy effect of belowground herbivory on root biomass was not significant ($F_{1,38}$ = 2.83, $P$ = 0.10; Fig. 3.2a). Shoot biomass was not affected by herbivore legacy effects in the soil (B: $F_{1,38}$ = 0.65, $P$ = 0.42; A: $F_{1,38}$ = 0.90, $P$ = 0.35). The legacy effect of root herbivory tended to cause an increase in foliar nitrogen concentration and a decrease in C:N ratio. However, this effect was not significant (%N: $F_{1,38}$ = 2.63, $P$ = 0.11 and C:N ratio: $F_{1,38}$ = 3.14, $P$ = 0.085).

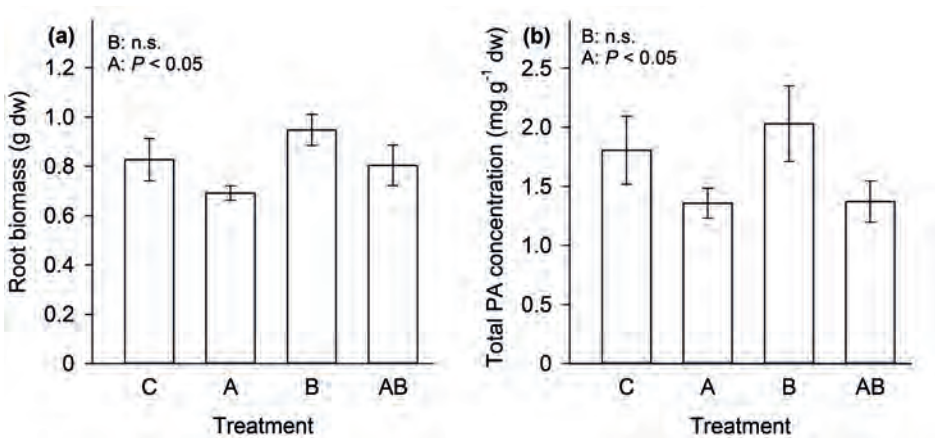


**Figure 3.2** Means (±SE) root biomass (a) and total shoot PA concentration (b) of *J. vulgaris* plants growing in soil with a legacy of undamaged plants (C), or with a legacy of aboveground (A), belowground (B), or aboveground and belowground herbivory (AB). Significance of the main effects of belowground and aboveground herbivory are based on linear mixed model analyses; n.s. is non-significant. The interaction between A and B was never significant and therefore was excluded from the model.

A total of 29 PAs were detected in the leaves of *J. vulgaris* (Table 3.1). Total PA concentration decreased in plants growing in soil with a legacy of aboveground herbivory ($F_{1,38}$ = 5.22, $P$ = 0.028; Fig. 3.2b), but not in soil with a legacy of belowground herbivory ($F_{1,38}$ = 0.24, $P$ = 0.63; Fig. 3.2b). However, the composition of PAs was significantly affected by a legacy effect of belowground herbivory (RDA: F = 9.29, $P$ = 0.001, 19% explained variation). PCA analysis revealed that the unconstrained variation in PA composition on the second axis could be very well explained by whether plants were growing in soil with a legacy of root herbivory (Fig. 3.3). Acetylerucifoline, acetylerucifoline *N*-oxide, erucifoline, erucifoline *N*-oxide and jaconine *N*-oxide, contributed most to the separation of plants between treatments (Table 3.1).

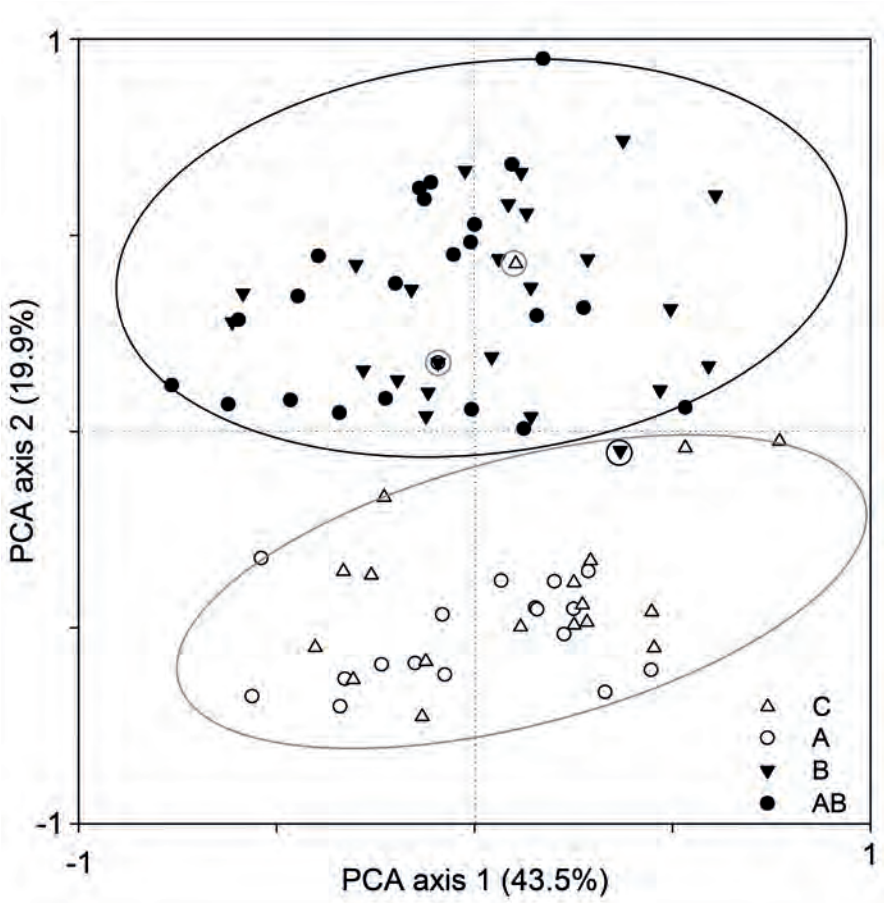


**Figure 3.3** Ordination diagram of principal component analysis (PCA) of the shoot PA composition of *J. vulgaris*. Plants were grown in soil with a legacy of undamaged plants (C), or with a legacy of aboveground (A), belowground (B), or aboveground and belowground herbivory (AB). Percentages of total explained variation by PCA axes are given in parentheses.

*Herbivore performance in Phase 2*

Mean relative growth rate (RGR) of *M. brassicae* larvae was significantly reduced on plants growing in soil with a legacy effect of root herbivory, and significantly increased on plants growing in soil with a legacy effect of aboveground herbivory (B: $F_{1,36}$ = 6.47, $P$ = 0.015; A: $F_{1,36}$ = 5.47, $P$ = 0.025; Fig. 3.4a). Larval mortality did not differ between treatments (B: z = 0.53, $P$ = 0.60; A: z = -0.60, $P$ = 0.55). RGR of *M. brassicae* was positively related to foliar nitrogen concentration ($R^2$ = 0.19, $P$ = 0.0087). There was also a significant relationship between RGR and PA composition (RDA: F = 3.02, $P$ = 0.022, 8.6% explained variation).

**Table 3.1** Mean concentration (±SE, $\mu g \cdot g^{-1}$ dw) and scores on the first two PCA axes of individual PAs of *J. vulgaris* plants growing in Phase 2 in soil with a legacy of undamaged plants (C), or with a legacy of aboveground (A), belowground (B), or aboveground and belowground herbivory (AB). Differences in concentrations of individual PAs between treatments are analyzed using linear mixed model with aboveground (A) and belowground (B) herbivory as fixed factors and individual pot identity in Phase 1 as random factor. The interaction between A and B was never significant and therefore was excluded from the model; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, the absence of asterisks denote no significant effects.

| PA† | C | A | B | AB | $F_{1,38}$ | | PCA | |
|---|---|---|---|---|---|---|---|---|
| | | | | | A | B | Score 1 | Score 2 |
| AcEr | 3.8 ± 1.0 | 2.9 ± 0.6 | 0.24 ± 0.07 | 0.13 ± 0.04 | 0.55 | 38.53*** | 0.284 | -1.671 |
| AcEr-ox | 71.3 ± 13.3 | 78.9 ± 12.4 | 7.3 ± 1.9 | 3.5 ± 1.6 | 0.11 | 71.53*** | 0.601 | -2.003 |
| AcSp | 0.2 ± 0.1 | 0.18 ± 0.05 | 0.16 ± 0.05 | 0.07 ± 0.03 | 1.94 | 4.80* | 0.105 | -0.595 |
| AcSp-ox | 3.2 ± 1.0 | 2.5 ± 0.6 | 4.7 ± 3.8 | 3.2 ± 1.9 | 0.24 | 0.20 | 0.568 | -0.662 |
| DHJn | 0.19 ± 0.03 | 0.23 ± 0.06 | 0.04 ± 0.01 | 0.04 ± 0.01 | 0.42 | 33.65*** | 0.149 | -1.057 |
| Er | 6.2 ± 3.4 | 2.7 ± 0.6 | 8.5 ± 1.4 | 9.4 ± 3.1 | 0.57 | 8.08** | 0.380 | 0.947 |
| Er-ox | 30.3 ± 5.1 | 29.5 ± 5.1 | 164 ± 26 | 108 ± 19 | 3.07 | 30.11*** | 0.802 | 0.944 |
| Ir | 2.0 ± 1.1 | 0.7 ± 0.2 | 1.1 ± 0.3 | 1.4 ± 0.6 | 0.46 | 0.03 | 0.845 | 0.740 |
| Ir-ox | 93.8 ± 21.4 | 64.6 ± 10.2 | 119 ± 28 | 53.7 ± 11.1 | 5.91* | 0.13 | 1.432 | 0.255 |
| Jb | 352 ± 39 | 348 ± 32 | 366 ± 48 | 358 ± 37 | 0.02 | 0.08 | 0.087 | 0.527 |
| Jb-ox | 446 ± 84 | 352 ± 70 | 467 ± 82 | 402 ± 68 | 1.08 | 0.21 | 0.780 | 0.126 |
| Jl | 23.5 ± 2.7 | 20.7 ± 1.7 | 23.2 ± 3.1 | 21.8 ± 2.2 | 0.59 | 0.02 | 0.084 | 0.414 |
| Jl-ox | 9.6 ± 1.5 | 7.6 ± 1.3 | 11.3 ± 1.9 | 10.9 ± 1.7 | 0.45 | 2.20 | 0.570 | 0.272 |
| Jn | 3.4 ± 0.8 | 3.0 ± 0.6 | 1.0 ± 0.2 | 1.3 ± 0.2 | 0.01 | 20.95*** | 0.401 | -0.686 |
| Jn-ox | 1.0 ± 0.2 | 0.9 ± 0.2 | 0.08 ± 0.03 | 0.05 ± 0.01 | 0.17 | 63.85*** | 0.520 | -1.540 |
| Jz | 2.3 ± 0.4 | 2.8 ± 0.3 | 2.3 ± 0.4 | 2.3 ± 0.2 | 0.53 | 0.50 | 0.188 | 0.210 |
| Jz-ox | 5.1 ± 1.3 | 3.9 ± 0.9 | 4.4 ± 0.8 | 3.3 ± 0.7 | 1.62 | 0.52 | 1.185 | 0.055 |
| Rd | 0.2 ± 0.1 | 0.06 ± 0.02 | 0.04 ± 0.01 | 0.04 ± 0.01 | 1.06 | 2.36 | 0.460 | -0.157 |

Table 3.1 continued

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Rd-ox | 7.5 ± 1.1 | 6.7 ± 1.1 | 5.5 ± 1.1 | 3.2 ± 0.7 | 2.09 | 6.00* | 1.296 | -0.630 |
| Rt | 1.6 ± 1.1 | 0.4 ± 0.1 | 0.7 ± 0.2 | 0.7 ± 0.3 | 1.17 | 0.40 | 0.849 | 0.524 |
| Rt-ox | 30.0 ± 6.5 | 15.3 ± 2.0 | 31.5 ± 6.7 | 17.8 ± 3.7 | 7.42* | 0.15 | 1.371 | 0.434 |
| Sn | 31.1 ± 19.7 | 7.8 ± 2.3 | 18.3 ± 6.1 | 16.9 ± 10.9 | 0.99 | 0.03 | 1.038 | 0.593 |
| Sn-ox | 413 ± 104 | 261 ± 43 | 585 ± 139 | 240 ± 62 | 6.67* | 0.58 | 1.419 | 0.343 |
| Sp | 16.5 ± 8.4 | 4.4 ± 1.4 | 8.0 ± 2.6 | 7.6 ± 4.3 | 1.36 | 0.35 | 1.030 | 0.520 |
| Sp-ox | 239 ± 75 | 132 ± 24 | 193 ± 42 | 101 ± 21 | 5.17* | 0.80 | 1.440 | 0.054 |
| St | 0.2 ± 0.1 | 0.08 ± 0.02 | 0.09 ± 0.02 | 0.14 ± 0.08 | 0.25 | 0.30 | 0.560 | 0.482 |
| St-ox | 7.5 ± 1.7 | 4.6 ± 0.8 | 3.7 ± 0.6 | 2.3 ± 0.5 | 4.69* | 10.87** | 1.315 | -0.659 |
| Us | 0.2 ± 0.1 | 0.12 ± 0.02 | 0.19 ± 0.03 | 0.16 ± 0.03 | 1.37 | 0.64 | 0.734 | 0.500 |
| Us-ox | 4.9 ± 1.2 | 3.1 ± 0.6 | 4.2 ± 1.0 | 3.5 ± 0.7 | 1.89 | 0.02 | 1.179 | 0.242 |

*†AcEr – Acetylerucifoline, AcSp – Acetylseneciphylline, DHJn – Dehydrojaconine; Er – Erucifoline, Ir – Integerrimine, Jb – Jacobine, Jl – Jacoline, Jn – Jaconine, Jz – Jacozine, Rd – Riddelliine, Rt – Retrorsine, Sn – Senecionine, Sp – Seneciphylline, St – Spartioidine, Us – Usaramine, -ox – N-oxide form of the corresponding PA*

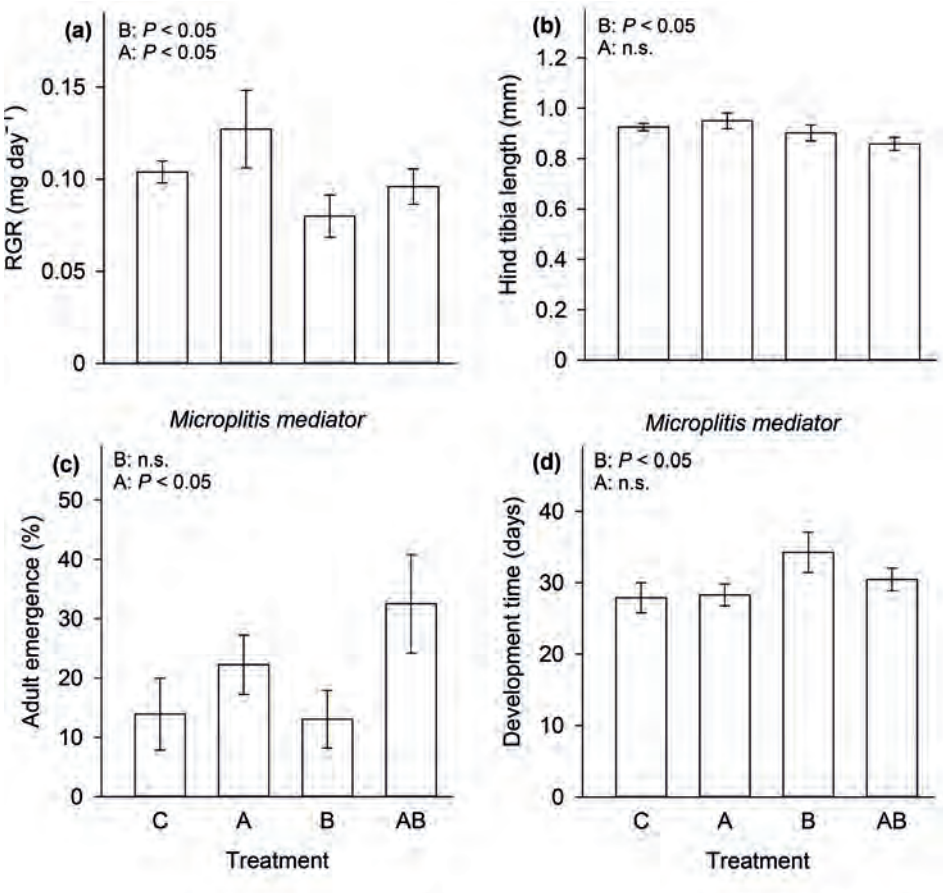


**Figure 3.4** Performance of the herbivore *M. brassicae* and the parasitoid *M. mediator* on *J. vulgaris* plants in Phase 2, growing in soil with a legacy of undamaged plants (C), or with a legacy of aboveground (A), belowground (B), or aboveground and belowground herbivory (AB). Means (±SE) of (a) larval relative growth rate of the herbivore, and (b) hind tibia length, (c) adult emergence and (d) development time of the parasitoid. Significance of the main effects of below- and aboveground herbivory are based on linear mixed model analyses; n.s. − non-significant. The interaction between A and B was never significant and therefore was excluded from the model.

*Parasitoid performance in Phase 2*

Parasitoid adult size was reduced on plants growing in soil with a legacy effect of root herbivory but not when grown in soil with a legacy of aboveground herbivory (B: $F_{1,21} = 4.48$, $P = 0.046$; A: $F_{1,21} = 0.11$, $P = 0.74$; Fig. 3.4b). In contrast, more adults of *M. mediator* emerged on plants growing in soil with a legacy effect of aboveground herbivory (B: $z = 0.716$, $P = 0.47$; A: $z = 2.208$, $P = 0.027$; Fig. 3.4c). Development time of *M. mediator* was significantly

longer for females than for males ($F_{1,7}$ = 11.12, $P$ = 0.013), and increased on plants growing in soil with a legacy of belowground herbivory (B: $F_{1,20}$ = 4.72, $P$ = 0.045; A: $F_{1,20}$ = 0.27, $P$ = 0.61; Fig. 3.4d).

## Discussion

This study demonstrates that aboveground multitrophic interactions can be affected by soil legacy effects created by aboveground and belowground herbivory on preceding plants. So far studies on aboveground-belowground interactions have mainly focused on interactions occurring simultaneously and on the same host plant (e.g., Bezemer et al. 2003; Soler et al. 2005; Kaplan et al. 2008a; Erb et al. 2011a). In this study, aboveground and belowground herbivory elicited specific effects on the soil fungal community, and, probably mediated by these effects on soil fungi, affected the growth and nutritional quality of plants growing later in the soil. These soil legacy effects also had multitrophic consequences aboveground, as they influenced the performance of aboveground herbivores and parasitoids on those plants. Other studies have already shown that herbivory can influence soil microbial communities (Bardgett et al. 1999; Mikola et al. 2001; Hamilton et al. 2008; Van Dam 2009), and that soil microbial communities can influence plant secondary chemistry aboveground (e.g., Joosten et al. 2009, Hol et al. 2010). However, these interactions were studied on the same plant and not on plant individuals that were growing in sequence. Therefore, the novelty of our results is that aboveground and belowground herbivore effects on microbial communities in the soil can be induced in one generation of plants and still influence multitrophic interactions on a subsequent generation of plants.

This study also adds a novel dimension to plant-soil feedback research. A large number of studies have shown that plants, through soil legacy or plant-soil feedback effects can affect the performance of plants that grow subsequently in the conditioned soil (Bever et al. 1997; Young et al. 2001; Van de Voorde et al. 2011). Our findings show that (i) both aboveground and belowground herbivory during the conditioning phase can affect soil conditioning; and (ii) that plant-soil feedback effects go beyond affecting plant biomass, as they can also affect plant chemistry and aboveground multitrophic interactions.

In this study, soil legacy effects of aboveground and belowground herbivory influenced both the concentration and the composition of PAs in foliage. Remarkably, the effects differed greatly between the two types of herbivory. Aboveground herbivory caused a soil legacy effect that resulted in a reduction of the total shoot PA concentration. The overall PA composition was not affected, although the concentration of a number of individual PAs was significantly

reduced in plants growing in soil with a legacy of aboveground herbivory. Legacy effects arising from root herbivory, on the other hand, resulted in changes in the composition of PAs but not in the total shoot PA concentration. These effects might depend on the species, or feeding guild acting as aboveground or belowground herbivore, but solving that question would require additional studies. Although this study does not provide a conclusive mechanism by which aboveground and belowground herbivory caused these specific soil legacies that affected plant growth and defence compounds of subsequent plants, we propose that pathogenic fungi caused the observed effects. Aboveground and belowground herbivory can differentially affect the concentration of PAs in the roots (Hol et al. 2004). We suggest that these changes in PA concentrations influenced the abundance and composition of (pathogenic) fungi as we observed in this study. This, in turn, influenced the growth and chemical composition of plants that grew subsequently in the soil, and these changes in the plant then affected aboveground insect performance. Alternatively, it could have been possible that the observed effects in Phase 2 were the result of differences in nutrient availability. However, as plants were grown in conditioned soil mixed with 50% sterilized soil, this is unlikely. The short time span of the experiment makes it also unlikely that the effects were caused by differences in decomposition by fungi.

Data from other studies support our proposed mechanism. First, root pathogenic fungi are important antagonists of *J. vulgaris* and certain pathogens are sensitive to PAs (Hol & Van Veen 2002). In general, plant pathogens are often suppressed by plant defence compounds (Kowalchuk et al. 2006; Van Dam 2009). Second, *J. vulgaris* exhibits a strong negative plant-soil feedback caused by soil (pathogenic) fungi (Bezemer et al. 2006; Van de Voorde et al. 2011). Third, soil-borne microorganisms such as soil fungi can greatly affect the composition of PAs in *J. vulgaris* leaves (Joosten et al. 2009). Finally, aboveground herbivory by *M. brassicae* on *J. vulgaris* has been shown to cause a reduction in the concentration of PAs in root tissues (Hol et al. 2004). The results from this study therefore suggest that aboveground herbivory caused a decrease in PA concentration in the roots that led to an increase in soil fungal pathogens, which decreased root biomass of plants growing subsequently in that soil. Root herbivory could have caused a leakage of plant defence compounds from the roots into the rhizosphere, which may have negatively affected soil pathogens and changed the composition of other soil microorganisms. Inoculation trials with fungi that respond to the herbivory treatments are needed to verify whether changes in the abundance of these fungi indeed have caused the observed legacy effects.

We observed a significant relationship between herbivore performance and the concentration of primary and secondary compounds in the plant tissues during the second phase. As less than five percent of the foliage was consumed in all cages (O. Kostenko, personal observation), we can assume that the legacy effects on herbivore performance were driven by changes in host plant quality and not by food quantity. The development and larval performance of parasitoids are strongly influenced by the size and the quality of their herbivorous hosts (Godfray 1994). Therefore, it appears that legacy effects, via changes in plant quality influenced herbivore and parasitoid performance in this study. Similar direct effects have been shown for root herbivory on aboveground herbivore-parasitoid interactions (Soler et al. 2005). Interestingly, in this study root herbivory exerted a negative soil legacy effect on herbivore and parasitoid performance while a soil legacy of foliar herbivory resulted in increased performance of herbivore. These results underline suggestions made in other studies that specific interactions between plants and insects can have far stretching consequences for other multitrophic interactions (Kaplan & Denno 2007; Soler et al. 2012a).

An important question is whether our experiment, which was performed under controlled conditions in a greenhouse, represents a process that also plays a role under natural conditions where interactions are more complex. We argue that these soil legacy effects can significantly affect plant population dynamics and insect communities in systems that comply with three rules. First, the plant species should grow sequentially at the same location. In Europe, semi-natural grasslands can be severely dominated by *J. vulgaris* for a number of years, during which the plant goes through several plant generations and new rosettes often appear close to flowering plants. Second, in the field, there should be effects of soil biota on plant growth and aboveground insect performance and abundance. A recent study by Reidinger et al. (2012) showed that interactions with arbuscular mycorrhizal fungi can affect aboveground insects in *J. vulgaris* plants grown in such semi-natural grasslands, and that these effects may have been mediated by changes in PA composition. Third, there must be differences among plants in aboveground and belowground herbivory. It is well documented that in the field insect abundances vary greatly among individual plants, and recently we showed that this is also the case for *J. vulgaris* (Kostenko et al. 2012a). Therefore, we conclude that these soil legacy effects can influence the growth of individual ragwort plants as well as the abundance and performance of insects on those plants in nature. Field studies are needed to understand how long this legacy effects would last, how widespread they are in natural communities, and how important they are for insect performance relative to abiotic effects such as changes in temperature or rainfall. Interestingly, we observed that aboveground herbivory had a negative effect on subsequent plant

growth and quality but positively affected aboveground insect performance. This positive aboveground-belowground feedback effect could be an alternative mechanism that can explain the decline of *J. vulgaris* in natural populations.

We conclude that herbivore-induced soil legacy effects can mediate interactions between spatially and temporally separated organisms. Our study shows that specific interactions between plants and insects can even extend beyond a single growth period of a plant, emphasizing the complexity of ways by which plants and insects interact, and that the insect community present at any stage of ecosystem development may reflect insect-plant interactions from the past. The observed soil legacies of aboveground and belowground insect herbivores can play a role in the field, but tests under more complex conditions are required. Isolation and inoculation studies to determine which soil organisms are involved are also needed. The implication of this study is that there are connections between the concepts of plant-soil feedback and aboveground-belowground multitrophic interactions, and this opens up new avenues for research in these areas.

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## Supporting Information




**Figure S3.1.** Scheme of the experimental setup. In Phase 1, plants were kept without insects (C), or exposed to aboveground (A), belowground (B), or aboveground and belowground herbivory (AB). In Phase 2, five identical experimental pots were created from each single pot from Phase 1. Two of them were used to assess the performance of the herbivore *Mamestra brassicae*, two to examine the performance of the parasitoid *Microplitis mediator*, and one pot was used to measure plant growth.

# Chapter 4

## Effects of diversity and identity of the neighbouring plant community on the abundance of arthropods on individual ragwort (*Jacobaea vulgaris*) plants

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## Abstract

The diversity of plant community can greatly affect the abundance and diversity of arthropods associated to that community, but can also influence the composition or abundance of arthropods on individual plants growing in that community. We sampled arthropods and recorded plant size of individual ragwort, *Jacobaea vulgaris ssp. vulgaris* (synonym *Senecio jacobaea* L., Asteraceae), plants transplanted into 70 experimental grassland plots that differed in plant diversity (1-9 species) or that were kept without vegetation. The arthropod fauna was dominated by the specialist aphid *Aphis jacobaeae* Schrank (Hemiptera: Aphididae). The abundance of aphids on ragwort plants decreased significantly with increasing plant diversity. The abundance of other arthropod species was not affected by the diversity of the surrounding plant community. Plant size was also not affected by the diversity of the surrounding plant community, but varied significantly among monocultures. Ragwort plants were largest in monocultures of legumes. Aphid abundance on ragwort plants, however, was not related to the size of the individual ragwort plants, but was high in monocultures consisting of *Tanacetum vulgare* L. (Asteraceae) plants. This plant is morphologically similar to ragwort. Even though we observed significant effects of plant diversity, ragwort plants were considerably larger – and the abundance of aphids and other arthropods on ragwort plants substantially higher – in plots without vegetation than in vegetated plots. Our results show that the presence and the diversity of neighbouring plants can provide associational resistance to focal plants growing in that community. We conclude that the surrounding plant community directly affects the abundance of arthropods on focal ragwort plants, and not via the effects of neighbouring plants on the performance of the focal plants.

## Introduction

The abundance and diversity of insects is often positively related to the diversity of the plant community (Haddad et al. 2009; Scherber et al. 2010). This has been explained by the greater diversity of resources that is present in more diverse plant communities (Hutchinson 1959; Strong et al. 1984). Other theory predicts that specialized insect herbivores will be more abundant in monocultures or simple plant communities compared to diverse plant communities, because the preferred host plant is more, and natural enemies are less abundant in those communities (Elton 1958; Root 1973). Furthermore, diverse plant communities may be structurally more complex than simple ones, providing a wider range of niches and refuges for insects (Bukovinszky 2004; Randlkofer et al. 2010b). However, structural complexity of the vegetation may also negatively influence insects such as parasitoids and predators by lowering their foraging efficiency (Sheehan 1986; Andow 1991; Bezemer et al. 2010b).

Most studies that examined the effects of plant diversity on insect abundance and diversity in natural systems have focused on the insect community associated to the entire plant community (e.g., Siemann et al. 1998; Koricheva et al. 2000; Haddad et al. 2001; Otway et al. 2005). How the composition or abundance of insects on a focal plant is affected by the diversity or identity of the surrounding plants has been widely investigated in agricultural systems, where insect abundances were compared between monocultures and mixed cropping systems, or between cultivated crops and weedy communities (reviewed in Kareiva 1983). In agricultural systems, absence of competing vegetation often results in changes in the size or quality of individual plants, which in itself can influence the arthropod community (Kareiva 1983). Fewer studies have examined responses of insects on individual plants in natural systems or in manipulated long-term semi-natural plant communities (e.g., Smith & Whittaker 1980; Raucher 1981; Bezemer et al. 2004a; Schreber et al. 2006; Unsicker et al. 2006; Lau et al. 2008). In natural systems, the diversity of a plant community may directly influence the number of insects on a focal plant growing within that community through its effect on the size and composition of the local pool of insects that could subsequently "spill over" to the focal plant (Andow 1991; White & Whitham 2000). However, diverse plant communities also often produce more biomass and have denser plant structures than simple ones and an increase in arthropod abundance in high diverse plant communities may be caused by the increase in plant biomass per unit area. This does not necessarily have to lead to an increase in insects on a particular plant individual growing in that community. The associational resistance hypothesis predicts that insects on a focal plant will be less abundant in complex than in simple plant communities (Tahvanainen &

Root 1972). Independent of the diversity of the surrounding plant community, the structure or height of the vegetation may affect the insect abundance on a focal plant, for example via its effect on the apparency of the focal plant (Kareiva 1983; Lawton 1983; Langellotto & Denno 2004). Other studies have shown that the likelihood that a plant is detected by an insect, or is vulnerable to herbivory, also depends on specific plant-plant associations, which can lead to associational resistance or susceptibility to certain plant species (reviewed in Barbosa et al. 2009). This implies that the identity of the neighbouring plants may be more important than the diversity of the plant community in affecting insects on a focal plant.

Neighbouring plants can also affect insect abundance on a focal plant via their impact on the size of the focal plant (Kareiva 1983). The performance of a plant is greatly affected by whether it competes with other plants for nutrients or light (Tilman 1988; Crawley 1997). However, the identity of the competing plants can greatly affect the outcome of competition (McEvoy et al. 1993; Agrawal 2004; Schädler et al. 2007). The impact of a competing plant on the size of a focal plant will depend on the aboveground and belowground architecture of the competing plants, but also on characteristics such as its ability to fix nitrogen. Plant-plant interactions with leguminous plants, for example, may result in more nitrogen being available for growth of the focal plant relative to competition with non-leguminous species. The size of a plant is directly related to its apparency and insect herbivores preferentially oviposit and feed on large plants (Randlkofer et al. 2009).

In this study, we examine how the diversity and identity of the surrounding plant community affects the arthropod assemblages on individual ragwort, *Jacobaea vulgaris ssp. vulgaris* (synonym *Senecio jacobaea* L., Asteraceae) plants in a field experiment where plant diversity has been manipulated experimentally. We collected insects on 1750 *J. vulgaris* plants transplanted into plots that were sown and maintained at different plant diversity levels (1-9 species) or kept without plants (i.e. bare soil). For each *J. vulgaris* plant, we also recorded its size and examined the relationships between plant diversity, focal plant size, and insect abundance.

## Materials and Methods

*Plant species*

Ragwort is a biennial or short-lived perennial monocarpic plant. A rosette of leaves is formed during the first year and flowering stems are produced in the second year. Flowering may be delayed to later years when the plant has been damaged or if the rosette is too small (Harper & Wood 1957; Van der Meijden & Van der Waals-Kooi 1979). *Jacobaea vulgaris* is a suitable model system to study the effects of plant community diversity on arthropod assemblages because it supports a wide variety of specialist and generalist insects (Harper & Wood 1957). The composition of insects on this plant is influenced by characteristics of the surrounding habitat (Macel & Klinkhamer 2010). In addition, *J. vulgaris* is a dominant species in recently restored semi-natural grasslands in the Netherlands and its performance can vary greatly within and among habitats (Bezemer et al. 2006; Van de Voorde et al. 2011).

*Field experiment*

In the summer 2008, a biodiversity field experiment was set-up within a nature restoration site on former arable land at Mossel, Ede, the Netherlands. The total area was approximately 55 ha and abandoned after harvesting the last crop in the fall of 1995 (detailed characteristics of the area are reported in Van der Putten et al. 2000). The experimental site was fenced to exclude large vertebrate herbivores and consisted of 70 plots (3 × 3 m), separated by 1-m-wide paths. Plots were sown in September 2008 with a single species or with species mixtures randomly chosen from a pool of 12 local grassland species (treatments: 1, 2, 4, or 9 species mixtures). Three grasses [*Anthoxanthum odoratum* L., *Agrostis capillaris* L., and *Festuca rubra* L. (all Poaceae)], three legumes [*Lotus corniculatus* L., *Trifolium arvense* L., and *Trifolium repens* L. (all Fabaceae)], and six forbs [*Achillea millefolium* L., *Hypochaeris radicata* L., *Leucanthemum vulgare* Lamk., *Tanacetum vulgare* L., *Tripleurospermum maritimum* (L.) W.D.J. Koch (all Asteraceae), and *Plantago lanceolata* L. (Plantaginaceae)] were used in the experiment. There were 12 monocultures (one for each plant species), nine combinations of two species (2 × 2 grasses, 2 × 2 legumes, 5 × 2 forbs), 11 combinations of four species (3 × 4 forbs, 3 × 2 forbs + 2 legumes, 3 × 2 forbs + 2 grasses, 2 × 2 grasses + 2 legumes), and three combinations of nine species (each with 5 forbs + 2 legumes + 2 grasses; Table 4.1). The seed density was 4000 seeds per $m^2$. Each of the sown plant species mixtures and monocultures was replicated twice using a complete randomized design. Four plots were kept free of all vegetation and served as bare soil treatment. Plant community composition was maintained by hand weeding from the beginning (late April) until the end (late August) of the growing season in 2009 and 2010. Paths were mown regularly

during the growing season but the experimental plots were not mown. One legume (*T. arvense*), one forb (*T. maritimum*), and two grass species (*A. capillaris* and *A. odoratum*) established poorly in monocultures, although these species were present in mixed communities. The monocultures of these four species were therefore excluded from analyses.

**Table 4.1** Plant species sown in the experimental plots. There were two plots for each species combination, and plots were sown with grasses (G), legumes (L), and other forbs (F), and with 1, 2, 4 or 9 plant species.

| | G | G | G | L | L | L | F | F | F | F | F | F |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species richness | *Anthoxanthum odoratum* | *Agrostis capillaris* | *Festuca rubra* | *Lotus corniculatus* | *Trifolium arvense* | *Trifolium repens* | *Achillea millefolium* | *Hypochaeris radicata* | *Leucanthemum vulgare* | *Plantago lanceolata* | *Tanacetum vulgare* | *Tripleurospermum maritimum* |
| 1 | | | + | + | | + | + | + | + | + | + | |
| 2A | | | | | | | | | + | | + | |
| 2B | | | | | | | + | + | | | | |
| 2C | | | | | | | + | | | + | | |
| 2D | | | | | | | | | + | | | + |
| 2E | | | | | | | | + | | | + | |
| 2F | | | | + | | + | | | | | | |
| 2G | | | | + | + | | | | | | | |
| 2H | + | + | | | | | | | | | | |
| 2I | + | | + | | | | | | | | | |
| 4A | | | | | | | + | + | + | | + | |
| 4B | | | | | | | + | + | | + | | + |
| 4C | | | | | | | | | + | + | + | + |
| 4D | | | | + | + | | | | + | | + | |
| 4E | | | | + | | + | + | + | | | | |
| 4F | | | | + | + | | + | | | + | | |
| 4G | + | | + | | | | | | + | | + | |
| 4H | | + | + | | | | + | + | | | | |
| 4I | + | + | | | | | + | | | | + | |
| 4J | | + | + | + | | + | | | | | | |
| 4K | + | | + | + | + | | | | | | | |
| 9A | + | + | | + | | + | | + | + | + | + | + |
| 9B | + | | + | + | + | | + | + | + | + | + | |
| 9C | | + | + | + | | + | + | + | | + | + | + |

Capital letters in the first column specify different species combinations that have the same species richness. + indicates that the species was sown in a plot. The monocultures of *Trifolium arvense*, *Tripleurospermum maritimum*, *Agrostis capillaris*, and *Anthoxanthum odoratum* were excluded from analyses because plants established poorly in these plots.

*Jacobaea vulgaris* seeds were collected from plants growing in the direct vicinity of the experimental field site. After germination on glass beads, individual seedlings were transplanted into seedling trays filled with sterilized potting compost. Plants were grown in a greenhouse at L16 (21 ± 2 °C): D8 (16 ± 2 °C) photoperiod. Natural daylight was supplemented by 400-W metal halide lamps (one lamp per 1.5 $m^2$). Plants were watered three times per week. In August 2009, when the plants were eight weeks old, 25 *J. vulgaris* plants were transplanted into the central 1.2 × 1.2 m square of each plot. The distance between plants was 30 cm. In total 1750 ragwort plants were used in the experiment.

*Arthropod abundance*

During the summer of 2010, two years after sowing the plots, and the first season following transplantation of ragwort plants into the plots, arthropods on each ragwort plant were collected. Insects were collected three times (June, July, August). During each collection, all plants were carefully inspected and all arthropods that were observed on a plant were collected using an aspirator, by three simultaneous collectors distributed evenly over the field. Each collector inspected all 1750 plants, spending an approximately equal amount of time at each plant. All arthropods were stored individually in 70% ethanol in labelled Eppendorf tubes. Most insects were identified to species or family level and other arthropods to order level. Data from the three collection periods were pooled for each plant because the number of arthropods collected at each date was low, and for some species and families it varied greatly between different collection dates. The arthropod composition data were analyzed at the order level.

*Plant measurements*

In 2010, all ragwort plants were at the rosette stage. In August 2010, immediately after the third collection of arthropods, we counted the leaves and measured the length of the longest leaf of each plant. To determine the relationship between the longest leaf, number of leaves, and aboveground biomass, we harvested 140 vegetative plants (two from each plot). For each plant the aboveground dry weight was determined. Various regression models were fitted to describe the relationship between plant biomass and the number of leaves, length of the longest leaf, or the product of these two. From the fitted models we selected the relationship that described plant biomass with the highest coefficient of determination ($R^2$ = 87%):

$$ln(\text{aboveground biomass}) = 1.220 \times ln(\text{longest leaf size} \times \text{number of leaves}) - 9.305.$$

We used this formula to predict aboveground plant biomass (g) for each ragwort plant.

*Statistical analysis*

Because there were multiple ragwort plants growing within a single plot, data were analyzed using mixed models with plot identity as random factor [nlme package; R statistical language, version 2.10.1 (R Development Core Team 2010)]. To fulfil the requirements of normality and homogeneity of variances, arthropod counts were square root transformed, plant biomass data were log-transformed, and proportions data were arcsine square root transformed. Because the arthropod community was dominated by one monophagous aphid species (>90% of all collected arthropods), separate analyses were carried out for this aphid and the other arthropods. To determine how the abundance of aphids or other arthropods on individual plants, or ragwort plant size, was affected by plant community diversity, we performed a mixed model ANOVA with plant species richness (0, 1, 2, 4, or 9 species) as fixed factor. We also used a linear mixed model with plant species richness as a continuous variable to incorporate the continuity of plant diversity in our analysis. We repeated both analyses by excluding the bare plots. We also examined whether the occurrence of aphids or other arthropods was affected by the plant community diversity using one-way ANOVA. Occurrences were calculated as the proportion of plants within a plot with aphids or other arthropods.

To test whether the identity of the surrounding plant species affected the abundance of aphids or other arthropods on *J. vulgaris* plants or plant size, we analysed the data from the monocultures using mixed model one-way ANOVA with surrounding plant identity as fixed factor. We also tested whether the proportion of plants with aphids or other arthropods differed among monocultures using one-way ANOVA. To determine whether aphid abundance or the abundance of other arthropods was related to plant size we used a linear mixed model with plant biomass as fixed factor. To asses whether the effect of plant diversity on aphid abundance or the abundance of other arthropods was due to changes in plant size, we first regressed the arthropod abundance on plant biomass, and then used the residuals from this regression in a linear mixed model with plant species richness as a continuous variable. To test whether arthropod community composition differed between diversity treatments or was related to plant size, we used canonical correspondence analysis (CCA) in CANOCO (Lepš & Šmilauer 2003). CCAs were conducted with total number of arthropods per plot (summed over the 25 plants). Significances in multivariate analyses were inferred by Monte Carlo permutation tests (unrestricted 999 permutations).

## Results

In total, 3673 arthropod individuals were collected on the transplanted ragwort plants (Table 4.2). The arthropod fauna was dominated by the specialist aphid *Aphis jacobaeae* Schrank (Hemiptera: Aphididae). The cinnabar moth, *Tyria jacobaeae* L. (Lepidoptera: Arctiidae), a specialist herbivore of *J. vulgaris*, was not found on the rosettes. Only six individuals of the other specialist herbivore, the flea beetle *Longitarsus jacobaeae* Waterhouse (Coleoptera: Chrysomelidae), were collected.

**Table 4.2** Total number of arthropod individuals collected on individual *J. vulgaris* plants growing in the experimental plots.

| Taxonomic group | Individuals |
|---|---|
| Coleoptera | 22 |
| Diptera | 37 |
| Hemiptera | 3438 |
| Hymenoptera | 129 |
| Lepidoptera | 17 |
| Thysanoptera | 9 |
| Araneae | 21 |
| Total | 3673 |

The abundance of aphids per plant decreased significantly with increasing plant community diversity ($F_{4,61} = 23.09$, $P = 0.0001$; Fig. 4.1A). This effect remained significant when bare soil plots were excluded from the analysis ($F_{3,58} = 4.10$, $P = 0.010$). When plant community diversity was treated as a continuous variable, the effect was even stronger (including bare plots: $F_{1,64} = 33.69$, $P = 0.0001$; without bare plots: $F_{1,60} = 10.20$, $P = 0.0022$). The abundance of other arthropods on *J. vulgaris* also declined significantly with increasing plant community diversity ($F_{4,61} = 32.37$, $P = 0.0001$; continuous: $F_{1,64} = 19.93$, $P = 0.0001$; Fig. 4.1B). However, when bare plots were excluded from the analysis, the effect of plant diversity on arthropod abundance was no longer significant ($F_{3,58} = 0.33$, $P = 0.81$; continuous: $F_{1,60} = 0.70$, $P = 0.41$). The highest numbers of aphids and other arthropods were found on ragwort plants growing in bare plots. The proportion of plants with aphids also decreased significantly with increasing diversity of surrounding plant community (including bare soil plots: $F_{4,61} = 15.58$, $P = 0.0001$; without bare plots: $F_{3,58} = 4.87$, $P = 0.0043$; Fig. 4.1C).

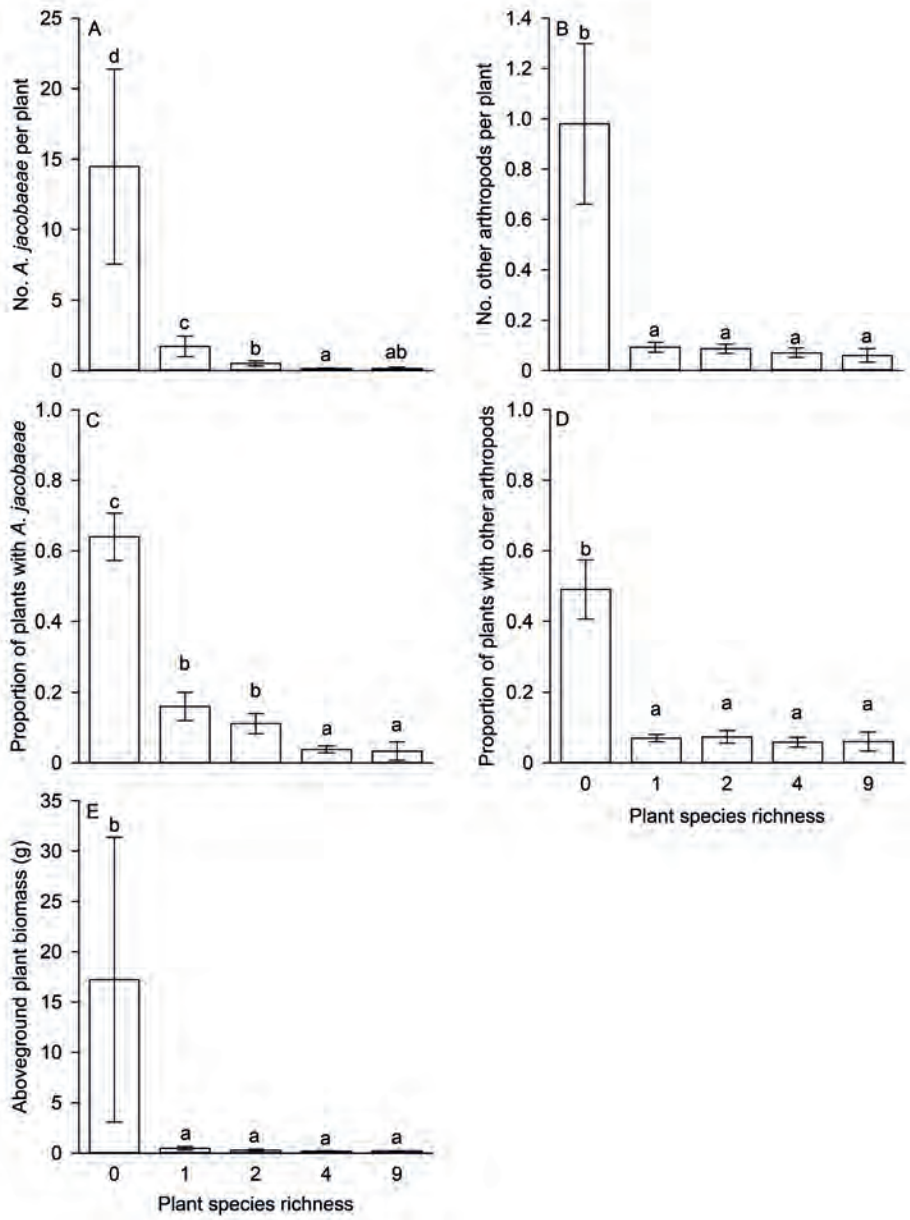


**Figure 4.1** Effect of plant community diversity on (A) aphid (*A. jacobaeae*) and (B) other arthropods abundance on individual ragwort plants, (C) proportion of ragwort plants with aphids, and (D) with other arthropods, and (E) ragwort aboveground plant biomass. Means are shown (calculated based on average values per plot ± between-plot SE). Significant differences are indicated by different letters based on mixed-model ANOVA for the abundance of aphids and other arthropods and plant biomass, and one-way ANOVA for the proportion of plants with aphids and other arthropods.

The occurrence of other arthropods, however, was only significantly affected by plant diversity when bare plots were included in the analysis (including bare plots: $F_{4,61} = 12.51$, $P = 0.0001$; without bare plots: $F_{3,58} = 0.48$, $P = 0.70$; Fig. 4.1D). When plant community diversity was treated in the analysis as a continuous variable, its effect on aphids and other arthropods occurrence remained the same. The composition of arthropods on ragwort plants did not differ between plant diversity treatments (CCA: $F = 0.86$, $P = 0.62$) and was not affected by plant size ($F = 0.36$, $P = 0.84$).

Ragwort plants were substantially larger in bare soil plots than in plots with surrounding vegetation (bare soil: 17.2 ± 14.1 g; vegetated plots: 0.29 ± 0.06 g) resulting in a significant effect of plant species richness on plant size ($F_{4,61} = 14.30$, $P = 0.0001$; continuous: $F_{1,64} = 17.90$, $P = 0.0001$; Fig. 4.1E). When bare plots were excluded from the analysis, ragwort plant biomass was no longer affected by plant diversity ($P > 0.05$). There was a trend for a positive relationship between the number of aphids or other arthropods per plant and the aboveground biomass of that plant, but this was not significant (aphids: $F_{1,1583} = 3.41$, $P = 0.065$; other arthropods: $F_{1,1583} = 3.51$, $P = 0.061$) and the amount of explained variation was low ($R^2 = 2.3$ and 0.9%, respectively). When aboveground ragwort plant biomass was used as covariate in the analysis, the effect of plant species richness on aphids and other arthropods abundance remained highly significant (aphids: $F_{1,64} = 14.06$, $P = 0.0004$; other arthropods: $F_{1,64} = 14.35$, $P = 0.0003$), indicating that the decrease in arthropod abundance on *J. vulgaris* in high diversity plots was not due to a negative effect of plant community diversity on ragwort biomass.

Aphid abundance on ragwort plants varied among monocultures, but this was not significant ($F_{7,8} = 2.85$, $P = 0.083$; Fig. 4.2A). The proportion of plants within a plot with aphids, however, differed significantly among monocultures ($F_{7,8} = 11.89$, $P = 0.0012$) and was highest in monocultures of *T. vulgare* and *T. repens* (Fig. 4.2C). The abundance of other arthropods and the proportion of plants with other arthropods did not significantly differ among monocultures (Fig. 4.2B, D). Ragwort biomass differed significantly among monocultures ($F_{7,8} = 14.93$, $P = 0.0005$) and was highest in monocultures of the legumes *T. repens* and *L. corniculatus* (Fig. 4.2E).

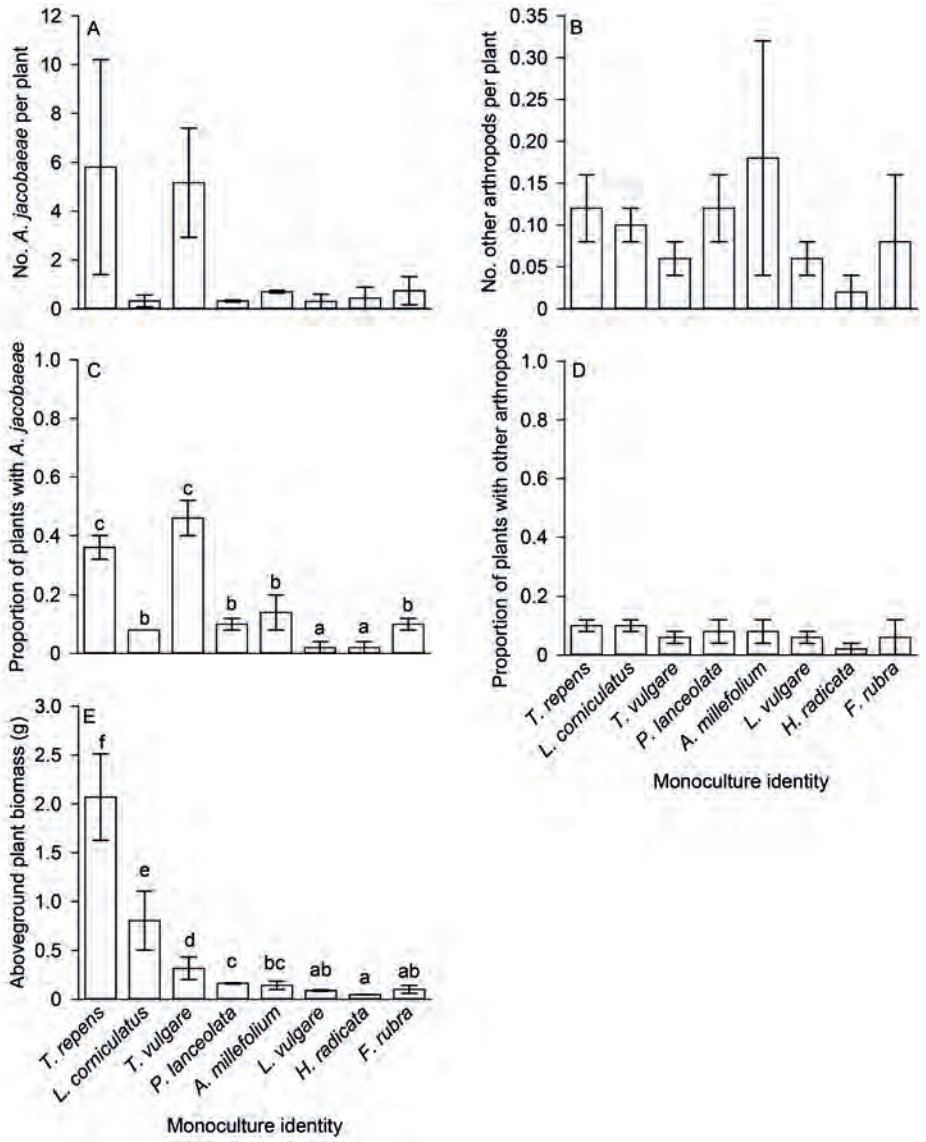


**Figure 4.2** Effect of the plant species identity of the monocultures on (A) the abundance of aphids and (B) other arthropods on individual ragwort plants, (C) the proportion of ragwort plants with aphids and (D) with other arthropods, and (E) ragwort aboveground plant biomass. Means are shown (calculated based on average values per plot ± between-plot SE). Significant differences are indicated by different letters based on mixed-model ANOVA for the aphids and other arthropods abundance and plant biomass, and one way ANOVA for the proportion of plants with aphids and other arthropods. Full names of the plant species are presented in Table 4.1.

## Discussion

In this study, we examined the effects of plant community diversity and identity on arthropod abundance on ragwort individuals that were transplanted into these communities. More than 90% of the arthropods that were collected from the ragwort plants belonged to *A. jacobaeae*, a species that is a specialized herbivore of ragwort.

Root & Cappuccino (1992) also reported for another plant species, *Solidago altissima* L., that the abundance in the associated arthropod community was dominated by a few species. It is important to note that in our study the number of arthropods collected on individual ragwort plants was relatively low. This might be because the arthropods were collected from plants that were at the rosette stage. Hence, flower- and stem-feeding arthropods were entirely absent. Moreover, due to competition with other plants, the rosettes in the intact plant communities remained relatively small throughout the season. Rosettes may be less attractive to arthropods than flowering plants because they are less apparent, less structurally complex, and do not provide nectar or other floral resources.

The abundance of *A. jacobaeae* on individual ragwort plants clearly declined when the diversity of the surrounding plant community increased. This result is in line with the prediction of Root (1973) that herbivore loads will be higher in simple than in diverse plant communities. There is ample evidence from agricultural systems that there is less herbivory in mixed cropping systems than in monocultures (Andow 1991). However, fewer studies have examined how plant diversity affects the abundance of insects, or the level of herbivory on plant individuals growing in natural plant communities. The studies that have investigated these effects show mixed results. For example, Scherber et al. (2006) examined the effects of plant species diversity on herbivory on individuals of four grassland species and reported that insect and mollusc herbivory levels were generally not related to plant species richness. Similarly, Bezemer et al. (2004a) reported that the density of aphids on *Cirsium arvense* (L.) Scop. plants was not affected by the diversity of the surrounding vegetation, although more species of aphids were found on plants growing in highly diverse plant communities. In contrast, Lau et al. (2008) showed for the prairie legume, *Lespedeza capitata* Michx., that plants growing in polycultures experienced much more damage from generalist herbivores, but less damage from specialist herbivores than plants growing in monocultures. In line with the findings of Lau et al. (2008), in our study there were also more specialised aphids on plants growing in monocultures. Importantly, our study shows that this result is not

only true when polycultures and monocultures are compared, but that there is an overall negative relationship between the diversity of the surrounding plant community and the abundance of specialized herbivores on plants growing in that community. Moreover, in our study, each plot contained exactly 25 ragwort individuals, planted in a fixed spatial design. Therefore, in contrast to many other biodiversity studies, there were no confounding effects of variation in plant density or patch size that might have affected longer distance attraction or plant preference of arthropods (Bezemer et al. 2006). This allows us to conclude that the abundance patterns of the specialized aphid herbivore on ragwort plants in our study indeed were due to differences in diversity of the neighbouring plant community among plots.

Although we observed a negative effect of plant community diversity on the abundance of a specialized herbivore, there was no significant effect of plant community diversity on ragwort performance. However, ragwort plant size varied significantly between the monocultures. These results emphasize that the species identity rather than the diversity per se of the surrounding plant community is important for growth of focal plants. Similar results have been shown in other biodiversity experiments (e.g., Scherber et al. 2006). These effects could be due to morphological differences between plant species that affect their ability to compete aboveground with ragwort for light or space. However, the species-specific effects could also be driven by plant-soil interactions. For example, in a study with 30 plant species that naturally co-occur with ragwort, Van de Voorde et al. (2011) recently showed that the performance of ragwort plants is greatly affected by the legacy effects of these other species on the soil biotic community, and that these effects differ between species, even directionally. Other work has shown that the identity or diversity of the surrounding plant community can influence interactions between ragwort and soil (mycorrhizal) fungi (Bezemer et al. 2006; Van de Voorde et al. 2010). In the present study, ragwort biomass was highest in the monocultures with leguminous plants. Legumes can fix nitrogen via the symbiosis with *Rhizobium* bacteria in the roots, and may directly or indirectly stimulate the growth of co-occurring plants via increased nitrogen availability in the soil (Temperton et al. 2007). Nutrients and especially nitrogen are known to positively affect the growth of ragwort in nutrient-poor soils (Hol et al. 2003).

The identity of the monoculture also significantly affected the proportion of plants with aphids within a plot. However, aphid abundance was not related to ragwort plant size, although aphid abundance was high on plants growing in monocultures of the legume *T. repens*, where plant biomass was also high. Interestingly, arthropod occurrence was also high on ragwort plants growing

in monocultures of the forb species common tansy, *T. vulgare*. The morphology of tansy is more similar to ragwort than other plant species used in the present experiment and this plant is sometimes even confused with *J. vulgaris*. Therefore, these results might be explained by associational susceptibility, and exemplify that when a plant is grown in association with a morphologically similar plant species, it may suffer from higher levels of herbivory (Barbosa et al. 2009). Similar results could also arise from chemical similarity between plants (Randlkofer et al. 2010). Besides, in monocultures of *T. vulgare*, we observed higher abundance and activity of ants (O. Kostenko, personal observation). *T. vulgare* is known to support numerous species of aphids, among which many species that are associated with ants (Stadler et al. 2002). *A. jacobaeae* is also tended by ants and there is some evidence that ants may transfer aphids between plants (Vrieling et al. 1991). Thus, the increased aphid abundance in monocultures of *T. vulgare* may have been the result of higher ant abundance.

The abundance of other arthropods was not affected by the diversity or identity of the surrounding plant community. It is possible that the effect was masked by the relatively low number of other arthropods that were collected in our study. It is also important to note that not only the diversity or identity of the neighbouring plant community can affect the arthropods colonizing a focal plant, but also the interactions among different arthropods co-occurring on that plant or on neighbouring plants (Kareiva 1983; Meyer 1993; Barbosa et al. 2009). Furthermore, the associations between co-occurring arthropods may increase or decrease the chance of being discovered by their natural enemies and this, in turn, can affect the abundance of arthropods on focal plants. More studies are needed that aim to disentangle the mechanisms by which neighbouring plants can affect the arthropod community on a focal plant.

Although plant community diversity affected the abundance of a monophagous herbivore on individual ragwort plants, in our study both aphid densities and abundance of other arthropods differed greatest when bare and vegetated plots were compared. Aphids and other arthropods were more abundant on ragwort plants growing in bare plots, and plants were 98% larger in those plots than in vegetated plots. Previous work on *J. vulgaris* has shown that this ruderal species is negatively affected by interspecific plant competition (McEvoy et al. 1993). The increased number of aphids and arthropods in open plots could be directly related to the higher biomass of plants in bare plots. However, we did not find a significant relationship between plant biomass and arthropod abundance. Ragwort plants were more apparent in open plots due to the absence of surrounding vegetation. This increase in plant apparency could also have resulted in the higher arthropod abundances in open plots (Kareiva 1983; Andow

1991). We cannot distinguish between these two mechanisms, but our results clearly show that the presence of vegetation per se can result in associational resistance for focal plants to their natural enemies. This finding is in line with the results of a meta-analysis conducted by Barbosa et al. (2009) who showed that the presence of surrounding vegetation resulted in decreased numbers of herbivores on focal plants. Also Tahvanainen & Root (1972) demonstrated that *Brassica* plants experienced associational resistance to their specialist herbivore *Phyllotreta cruciferae* (Goeze) when surrounded by other plant species. In conclusion, our results show that the abundance of arthropods on plant individuals depends on the presence, identity, and diversity of the neighbouring plants. Therefore, changes in the diversity of the plant community may not only lead to changes in the functioning or stability of that system (Tilman et al. 2006), or the diversity of the entire insect community associated to that plant community (Siemann et al. 1998; Haddad et al. 2009; Scherber et al. 2010), but it can also affect individual plant-insect interactions.

## Acknowledgments

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# Chapter 5

## Surrounding plants influence the nutritional quality of focal plants in a field experiment

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*Submitted in a slightly different form*

## Abstract

Surrounding plants may affect the size, nutritional quality and secondary chemistry of a focal plant individual via e.g., competitive interactions for nutrients or space that can result in intraspecific variation in plant quality. This variation in plant quality can affect the interactions between the focal plant and its multitrophic community. However, field studies examining whether and how surrounding plants can affect the growth and quality of focal plants are limited. Here, we investigated how the presence, diversity and identity of the surrounding plant community affected the development, growth and primary and secondary chemistry of the foliage of focal ragwort (*Jacobaea vulgaris* Gaertn.) plants in a field experiment. We planted focal *J. vulgaris* plants in experimental plant communities that differed in diversity and composition. Two years after planting, the focal plants that were either still in the rosette (vegetative) stage and focal plants that were flowering were harvested and their biomass and primary and secondary chemistry were measured. Additionally, we measured the soil mineral N content and the density of the surrounding plant community to test whether the effects on the diversity of the surrounding community are mediated by space or nutrient availability. The presence and diversity of the surrounding plant community strongly affected the development and growth of the focal *J. vulgaris* plants. However, the presence of the surrounding community was more important for the nutritional and chemical quality of the focal plants than effects of plant diversity per se. The effects also differed significantly between the two developmental stages. For vegetative plants, the foliar N and total pyrrolizidine alkaloid concentration differed significantly among the monocultures and were the highest in the monocultures of leguminous species. For flowering plants, the nutritional and chemical quality was not affected by monoculture identity. Regression analyses indicated that the effects of plant diversity on the secondary chemistry of the focal plants were positively related to plant size and N concentration of the focal plants but also by the density of the surrounding community. Our data suggest that the chemistry of a focal plant in the field is partly determined by the presence, diversity and identity of the surrounding plants.

## Introduction

Associational interactions between focal and neighbouring plants have been well documented in terrestrial and aquatic ecosystems (reviewed in Barbosa et al. 2009). Many studies have shown for example, that characteristics of surrounding plants can influence the level of herbivory on a focal plant (e.g., Hambäck et al. 2000; Cipollini & Bergelson 2002; Agrawal 2004; White & Andow 2006; Karban et al. 2007; Kostenko et al. 2012; Castagneyrol et al. 2013). Surrounding plants may also affect characteristics of the focal plant, such as plant size, nutritional quality and secondary chemistry. These effects on a focal plant likely result from competition between the focal plant and its neighbours that alters the availability of nutrients, light and space (Crawley 1997). However, field studies that examine whether and how surrounding plants can affect the growth, nutritional quality and secondary chemistry of focal plants are scarce (but see Barton & Bowers 2006; Mraja et al. 2011; Abbas et al. 2013).

Many empirical studies have shown in field experiments that increasing plant diversity leads to an increase in plant productivity (reviewed in Reich et al. 2012). More productive plant communities are usually denser, which can lead to increased competition for space and light, as well as soil nutrient depletion (Spehn et al. 2000b; Oelmann 2007; Lorentzen et al. 2008). Therefore, the diversity of the surrounding plant community, via these competitive interactions, can potentially affect the performance and quality of a focal plant. Indeed, in a large-scale biodiversity experiment, Eisenhauer and co-workers (2009) demonstrated that belowground plant competition increased with increasing plant species diversity and that this negatively affected the growth of the phytometer plant species *Centaurea jacea*. Recently, in the same experiment, it was also shown that an increase in plant species richness affects the expression of carbon-based secondary metabolites (i.e. iridoid glycosides) in *Plantago lanceolata* plants (Mraja et al. 2011). However, the effects of plant diversity differed between different types of iridoid glycosides. Whether and how the expression of other secondary compounds (e.g., alkaloids) in a focal plant is modified by manipulations in the diversity of the surrounding plant community is unknown.

Changes in the nutritional quality of a focal plant can also depend on the identity of the competing plants as this can greatly influence the outcome of plant-plant competition (Grace 1990; McEvoy et al. 1993; Scherber et al. 2003; Agrawal 2004). For example, if a focal plant competes with a leguminous plant species, it may result in reduced competition for soil nitrate. This, in turn, can lead to increased nitrogen availability for the focal plant (Temperton et al. 2007) and to increased production of nitrogen-based secondary compounds by the focal plant

(Bryant et al. 1983; Coley et al. 1985). However, if the production of nitrogen-based secondary metabolites in the focal plant cannot keep up with the increase in plant growth, their concentration can be diluted (Koricheva 1999). Plants competing with neighbours will probably have fewer resources available for growth than plants growing without competitors. However, if light is not limited and photosynthesis is not impaired, carbon availability will increase relative to the plant's demand in conditions with competition, and this can result in increased production of carbon-based compounds in the focal plant (Bryant et al. 1983). Finally, if the focal plant is surrounded by conspecifics that require exactly the same resources, this can result in stronger competition, reduced plant growth and plant quality. Indeed, in greenhouse and field studies, the levels of carbon-based plant defence compounds in focal plants were higher when they were grown with conspecific neighbours than in plants growing with heterospecific neighbours (Barton & Bowers 2005; Broz et al. 2010).

The concentration of secondary plant compounds of a focal plant can also be greatly affected by insect herbivory, and this, in turn can also be influenced by neighbouring plants (Karban & Baldwin 1997; Barbosa et al. 2009). For example, herbivore attack by specialist herbivores is considered to be higher in close proximity of intraspecific neighbours (Root 1973). However, heterospecific neighbours can also be highly palatable and attract certain generalist herbivores that subsequently can move to the focal plants. In a field study where plant diversity was manipulated, individual *Lespedeza capitata* plants grown in the monospecific stands experienced greater herbivory by specialist insects, but the total amount of herbivore damage (from specialists and generalists) on individual plants was lower in monospecific than in more diverse plots (Lau et al. 2008).

The primary and secondary chemistry of a plant also often changes considerably with plant development. Usually young seedlings and leaves contain high concentrations of nitrogen and secondary compounds (Rhoades & Cates 1976). A recent meta-analysis of the developmental changes in plant secondary chemistry revealed that levels of secondary plant chemicals significantly increase during the ontogenetic development of a plant (Barton & Koricheva 2010). Whether the effects of surrounding plants on the quality of a focal plant differ at various stages of plant development is currently unknown.

In the field, we investigated the effects of the presence, diversity and identity of the surrounding plant community on the development, growth and primary and secondary chemistry of the foliage of individual ragwort (*Jacobaea vulgaris* Gaertn.) plants. We also recorded the levels of soil nutrients and the density of the surrounding plants to test whether the effects on the surrounding community are

mediated by space or nutrient availability. Specifically, we predicted that (i) an increase in plant diversity will increase interspecific competition, impair plant survival, development and growth and lead to a decline in plant nitrogen and secondary metabolite concentrations. Similarly, (ii) *J. vulgaris* plants growing in monocultures with species that form dense plant stands, such as the grass species: *Festuca rubra* and the forb species: *Hypochaeris radicata* and *Leucanthemum vulgare*, will be negatively affected via interspecific competition for space and this will lead to a decrease in the growth and concentration of primary and secondary metabolites. In contrast, (iii) plant survival and performance will be higher in bare plots and in monocultures of legumes, and *J. vulgaris* plants will contain higher nitrogen and secondary compound concentrations in these plant communities.

## Materials and Methods

*Focal species*

Ragwort is a biennial or short-lived perennial monocarpic plant of the family *Asteraceae*. In the first year, a rosette of leaves is formed and flowering stems are produced in the second year. However, flowering may be delayed to later years when the plant has been damaged or when the size of the rosette is too small (Harper & Wood 1957; Van der Meijden & Van der Waals-Kooi 1979). *Jacobaea vulgaris* produces pyrrolizidine alkaloids (PAs), a well-studied group of nitrogen-based secondary compounds that are toxic to a wide range of generalist insects, microorganisms, mammals and humans (reviewed in Boppre 2011; Macel 2011). Specialist insects, in contrast, are not deterred by PAs but utilize them to locate hosts or for their own defence (Ehmke et al. 1990; Hartmann & Witte 1995; Naberhaus et al. 2004). PAs are produced in the roots as senecionine *N*-oxide and transported to the aboveground parts via phloem pathways. Diversification of PAs takes place in both roots and shoots. PAs occur in plants in two forms, tertiary amine and *N*-oxide. Tertiary amine PAs are considered to be more toxic to non-adapted insects (e.g., Macel et al. 2005; Cheng et al. 2011b). Recently, around 40 PAs were recorded from *J. vulgaris* belonging to four major types: jacobine, erucifoline, senecionine and seneciphylline (Cheng et al. 2011a). During all stages of ontogenetic development the plant contains PAs and PA production is closely linked to the root biomass (Hol et al. 2003; Schaffner et al. 2003). PAs are regarded as constitutive defence compounds, however, increases in the levels of PA production were also observed after root herbivory (Hol et al. 2004). In addition, there is high phenotypic plasticity in the concentration and composition of PAs, and PAs vary depending on abiotic and biotic factors

(Vrieling & De Boer 1999; Macel et al. 2004; Joosten et al. 2009; Kirk et al. 2010). For example, nutrient addition negatively affects the PA concentration in *J. vulgaris* shoots and roots (Hol et al. 2003).

*Experimental design*

The associational effects of the surrounding community on the quality of the focal plant were studied in the biodiversity field experiment used in *Chapter 4*. A detailed description of the experiment and the set-up scheme is presented in *Chapters 1 and 4*. Briefly, in the summer of 2008, seventy plots (3 × 3 m), separated by paths (1 m wide) were established on an ex-arable field at a nature restoration site Mossel (Ede, the Netherlands). In September 2008, the plots were sown with a single plant species (monocultures) or with mixtures of 2, 4, or 9 species randomly chosen from a pool of 12 local grassland species that naturally co-occur with *J. vulgaris* in the studied area (*Anthoxanthum odoratum* L., *Agrostis capillaris* L., *Festuca rubra* L., *Lotus corniculatus* L., *Trifolium arvense* L., *Trifolium repens* L., *Achillea millefolium* L., *Hypochaeris radicata* L., *Leucanthemum vulgare* Lamk., *Plantago lanceolata* L., *Tanacetum vulgare* L., *Tripleurospermum maritimum* (L.) W.D.J. Koch). The focal species *J. vulgaris* was not sown. Plots with the same species composition were replicated twice using a complete randomized design. Four plots were kept free of all vegetation and served as bare soil treatment. Initial sowing density was 4000 seeds per $m^2$. The sown species composition was maintained by hand weeding from the beginning of the growing season (late April) until the end of the growing season (late August) throughout the years 2009 – 2011 and paths between plots were regularly mown during the growing season. The monocultures of *T. arvense, T. maritimum*, *A. capillaris* and *A. odoratum* were excluded from the experiment because of the poor establishment but these species were present in mixed communities. To exclude large vertebrate herbivores the experimental site was fenced.

In August 2009, when the sown plant communities were established, 25 eight-week old *J. vulgaris* rosettes were out-planted in a regular grid of 0.3 × 0.3 m in the central 1.2 × 1.2 m square of each plot. The resident plant community around the focal rosettes was not removed in order to test the effects of the surrounding community on the establishment of *J. vulgaris* seedlings (Fig. 5.1A). In bare plots, no other plants than the 25 focal *J. vulgaris* were present (Fig. 5.1B). The rosettes were grown from the seeds collected from *J. vulgaris* plants growing in the direct vicinity of the experimental site. After germination, individual seedlings were transplanted into seedling trays filled with sterilized potting compost. Plants were grown in a greenhouse (21/16 °C day/night, 16 h photoperiod) and watered three times per week. Natural daylight was supplemented by 400 W metal halide lamps (1 lamp per 1.5 $m^2$).

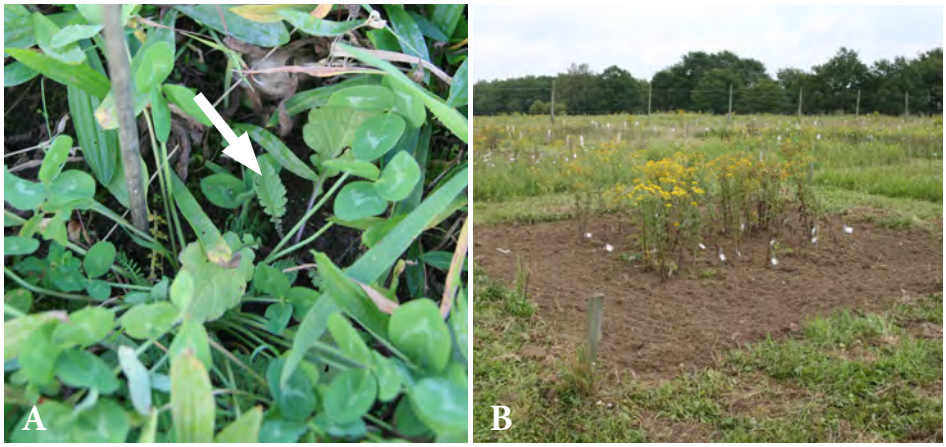


**Figure 5.1** (A) Rosette of *J. vulgaris* (indicated by the white arrow) in one of the biodiversity plots one month after the transplantation in September 2009; (B) Bare plot with the 25 focal *J. vulgaris* plants in August 2011.

*Focal plant and community sampling*

In August 2011, a total of 1402 (out of 1750 planted) focal plants were recovered in the experimental plots. We intended to collect four reproductive and four vegetative plants in each plot. However, only 424 of the *J. vulgaris* plants produced flowering stems (reproductive stage) two years after the transplantation and flowering was not evenly distributed among the plots (see Results). Therefore, in 17 plots less than four and in 18 plots no reproductive plants could be collected. The aboveground plant parts (rosettes of leaves or flowering stems) were clipped off and placed in a labelled paper bag. To collect the roots we placed a standard plastic circle (diameter 20 cm) around each plant and excavated a soil core of 20 cm deep within the plastic circle. The core was placed in the labelled plastic bag and together with the aboveground sample transported in a cool box to the laboratory for further processing. In the laboratory, the samples were stored at 4 °C till processing. The day after the plants had been collected, the fifth youngest fully expanded leaf from each rosette and flowering plant was removed with a razor blade, freeze-dried and finally ground for chemical analysis. The roots were carefully removed from the soil and rinsed. For each plant, the aboveground and belowground dry weight was determined after drying for 48 h at 70 °C. One week after plant sampling, five soil cores of 15 cm depth and 2.5 cm diameter were collected from each experimental plot at five random positions. The samples were pooled per plot and used for chemical analysis. As a proxy of light and space availability in the experimental communities the percentage plant cover was recorded in two 1 $m^2$ quadrants along a diagonal transect within each plot.

*Chemical analysis of plant and soil samples*

Leaf carbon (C) and nitrogen (N) concentration were determined using a Flash EA1112 CN analyzer (Interscience, Breda, the Netherlands). PA analysis was carried out using liquid chromatography-tandem mass spectrometry (LC-MS/MS) following the procedure outlined in *Chapter 2*. In brief, 5 mg of freeze-dried ground plant material was extracted with 0.5 ml 2% formic acid solution containing heliotrine (1 $\mu g \cdot ml^{-1}$) as internal standard. After centrifugation and filtration, 25 $\mu$l of the extracted filtrate was diluted with 975 $\mu$l of 10 mM ammonium hydroxide solution and 10 $\mu$l was injected in a Waters Acquity ultra-performance chromatographic system coupled to a Waters Quattro Premier tandem mass spectrometer (Waters, Milford, MA, USA). The information about the separation and mass spectrometric detection of the PAs is described in Cheng et al. (2011b). Data were processed using Masslynx 4.1 software. Mineral N content ($NH_4^+$ and $NO_3^-$) in soil samples was determined colorimetrically in the $CaCl_2$ extraction using a Traacs 800 autoanalyzer (TechniCon Systems Inc, USA).

*Data analyses*

To fulfil the requirements of normality and homogeneity of variances data were log- or square-root transformed. To examine the effect of plant species richness on the growth and chemistry of the focal plants we used mixed-effects models with plant species richness (0-9 species) as continuous log-linear factor and plot identity as random factor. We included plot as a random factor to incorporate that plants collected from the same plot are pseudoreplicates. Plant developmental stage (vegetative or reproductive) was also included in the analysis as fixed factor to test whether the effects of plant species richness differed between vegetative and reproductive stages. We repeated both analyses by excluding the bare plots. To test whether the identity of the monoculture surrounding the focal plants affected the growth or chemistry of *J. vulgaris* plants, we analysed the data from the monocultures only using mixed model one-way ANOVA with monoculture identity as fixed factor and plot identity as random factor. This was done for vegetative and reproductive plants separately as flowering plants were not present in some plots. To test whether the diversity of the surrounding community affected total PA concentration directly or by altering root biomass or leaf N concentration we fitted plant root biomass and leaf N as covariates in the mixed model. Finally, the data were averaged at the plot level to test whether the effects of plant species richness on PA concentrations were indirectly mediated by the associated changes in the focal plant (such as plant root biomass, leaf N concentration) or changes in the characteristics of the community (such as soil mineral N or total plant cover in the community). For this we used multiple linear regressions where the covariates were fitted prior to plant species richness. Data were analysed using R statistical language, version 2.15.1 (R Development Core Team, 2012).

## Results

Only 30% of the focal *J. vulgaris* produced flowering stems two years after the plants had been transplanted into the experimental plots. Focal plant survival ($F_{1,64}$ = 12.05, $P$ = 0.0009) and number of flowering plants decreased ($F_{1,64}$ = 5.84, $P$ = 0.019) with an increase in plant species richness. When bare plots were excluded from the model, the negative effect of plant species richness on focal plant survival was less strong but remained significant ($F_{1,60}$ = 4.99, $P$ = 0.029). Plant species richness had a negative effect on plant shoot ($F_{1,64}$ = 5.23, $P$ = 0.026) and root biomass ($F_{1,64}$ = 5.23, $P$ = 0.026) and reproductive plants produced always more biomass than vegetative plants (shoot and root, both $P < 0.0001$, data not shown). When the analysis was limited to plots with a background vegetation (excluding bare plots), plant biomass did not differ between the diversity levels (shoot and root $P > 0.05$) but reproductive plants were on average larger than vegetative (shoot and root $P < 0.0001$). The leaf N concentration of both reproductive and vegetative plants decreased with plant species richness (Fig. 5.2), but a significant interaction between richness and growth stage indicated that variation in leaf N concentration in response to the diversity of the surrounding community also depended on the developmental stage ($F_{1,348}$ = 19.46, $P < 0.0001$). The interaction effect was still significant when bare plots were excluded from the model ($F_{1,323}$ = 5.81, $P$ = 0.017). Interestingly, the leaf N concentration of vegetative plants growing in bare plots was almost 50% higher than that of the vegetative *J. vulgaris* plants growing in plant communities independent of plant species richness (Fig. 5.2). There was also a significant interaction between the effect of plant species richness and plant developmental stage on leaf C:N ratio when bare plots were kept in the model, suggesting that the increase in C:N ratio with plant species richness depended on plant developmental stage ($F_{1,348}$ = 4.92, $P$ = 0.027; Fig. 5.2).

Forty six different PAs were recorded from the leaves of the focal plants growing in the experimental plots. In vegetative plants, jacobine- (43.1% of the total PA concentration) and erucifoline-type (44.4%) PAs were the major PA groups, whereas in reproductive plants, only jacobine-type PAs were the dominant PA group with a relative abundance of 79.0% (Fig. 5.3). In general, leaves of the reproductive plants contained higher concentrations of total PA and of the different types of PAs than the leaves of vegetative plants, except for senecionine-type PAs ($P > 0.05$; Fig. 5.3). Overall, the PA concentration of the focal plants tended to decrease with increasing plant species richness of the surrounding community (Fig. 5.3). However, the significant decrease was only observed for jacobine- and senecionine-type PAs ($F_{1,64}$ = 7.24, $P$ = 0.0091; $F_{1,64}$ = 9.17, $P$ = 0.0036; respectively).

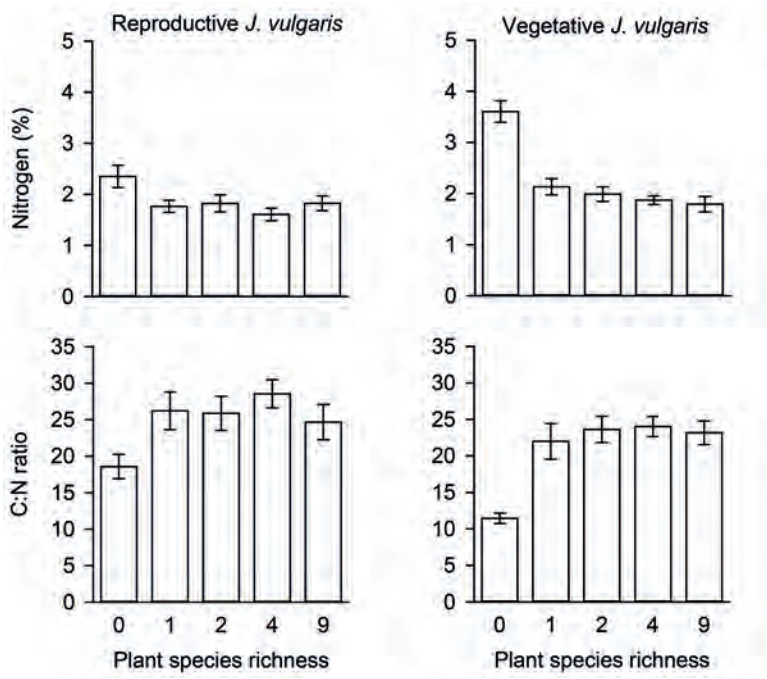


**Figure 5.2** Effect of the diversity of the surrounding community on the leaf N concentration and C:N ratio of the vegetative and reproductive focal *J. vulgaris* plants. Means ± between-plot SE are shown.

The effect of plant species richness on the total PA concentration was only marginally significant ($F_{1,64}$ = 3.76, $P$ = 0.057; Fig. 5.3). Both vegetative and reproductive plants contained higher concentrations of *N*-oxides than tertiary amines with an average *N*-oxide to tertiary amine ratio 2.0 ± 0.21 for reproductive and 2.5 ± 0.22 for vegetative plants. Interestingly, total *N*-oxide concentration decreased with an increase in plant diversity ($F_{1,64}$ = 5.15, $P$ = 0.027) whereas the concentration of tertiary amines did not change ($F_{1,64}$ = 0.54, $P$ = 0.71). Of all PA types, only jacobine PAs had higher total tertiary amine concentrations than total *N*-oxide concentrations and this was only true for vegetative plants (data not shown). In reproductive plants, the ratio of jacobine-type *N*-oxides to tertiary amines was 3.10 ± 0.70 for plants growing in bare plots and 1.20 ± 0.12 for plants growing in the vegetated communities. When bare plots were not included in the model there was no significant effect of plant species richness on the PA concentration ($P > 0.05$ in all cases). However, separate analysis of vegetative and reproductive plants showed that senecionine-type PAs concentration in vegetative plants decreased with an increase in plant diversity when bare plots were excluded from the model ($F_{1,60}$ = 6.74, $P$ = 0.012).

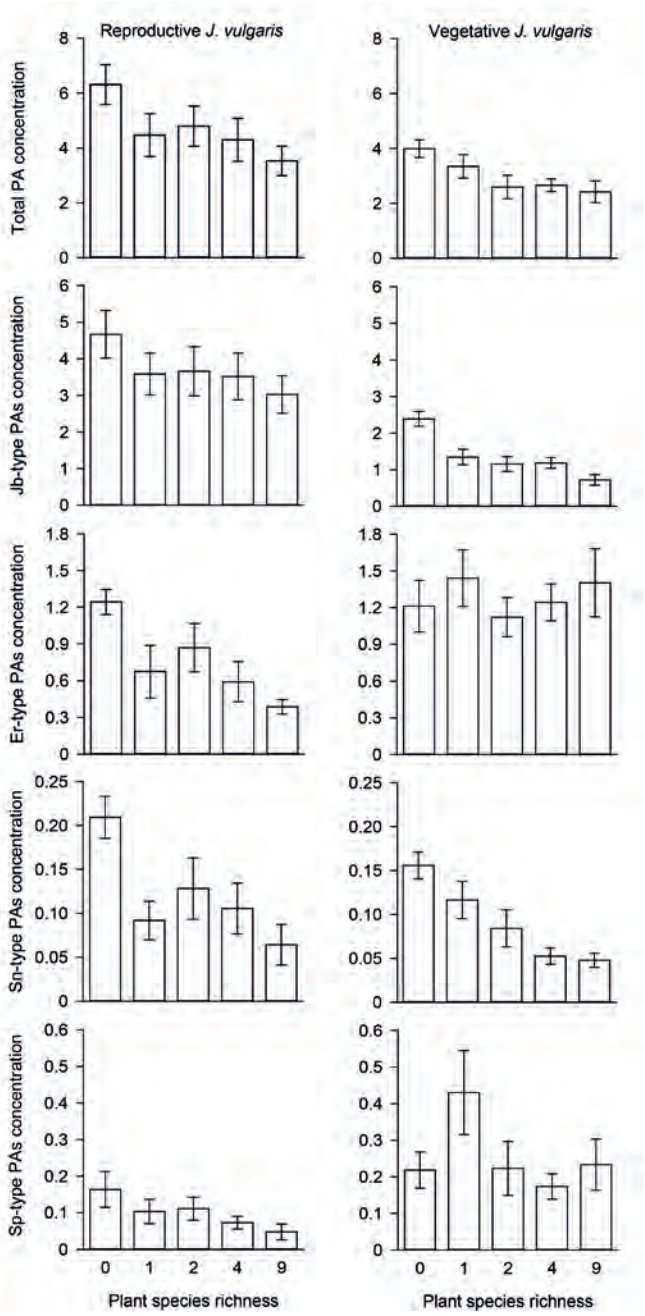


**Figure 5.3** Effect of the diversity of the surrounding community on the total PA concentration and the concentration of Jacobine-type (Jb), Erucifoline-type (Er), Senecionine-type (Sn), and Seneciphylline-type (Sp) PAs ($mg \cdot g^{-1}$ dw) in the leaves of the vegetative and reproductive focal *J. vulgaris* plants. Means ± between-plot SE are shown.

The biomass, C:N ratio, N and PA concentrations of reproductive focal plants did not significantly vary among monocultures ($P > 0.5$ in all cases, data not shown). For vegetative focal plants, leaf N concentration ($F_{7,8} = 4.43$, $P = 0.028$), C:N ratio ($F_{7,8} = 4.68$, $P = 0.023$), total PA concentration ($F_{7,8} = 6.55$, $P = 0.008$) and erucifoline-type PAs ($F_{7,8} = 3.50$, $P = 0.050$) significantly differed among monocultures (Fig. 5.4). Plants contained the highest concentration of N and total PAs when grown in the legume monocultures (*L. corniculatus* and *T. repens*) and the lowest in monocultures of the forbs *L. vulgare* and *H. radicata* (Fig. 5.4).

Total plant cover increased significantly with an increase in plant species richness when bare plots were not included in the model (Table 5.1). The mineral N content in the soil was not related to the diversity of the plant community (Table 5.1). Mixed-effects models accounting for within-plot variation revealed that plant root biomass and leaf N concentration positively affected total PA concentration of vegetative plants independent of whether bare plots were included in the model (Table 5.2). For reproductive plants, leaf N concentration was positively related to total PA concentration but only when bare plots were excluded from the model, whereas root biomass was positively related to total PA levels in both cases (Table 5.2). The analysis based on averaged values per plot showed that total PA concentration of vegetative plants was negatively related to the total plant cover even after accounting for changes in plant root biomass and leaf N concentration (Table 5.2). However, when the analyses were limited to plots with background vegetation, the effect of total plant cover was no longer significant (Table 5.2). Total PA concentration of reproductive plants was positively related to root biomass independent of including or excluding bare plots (Table 5.2). In addition, when bare plots were not included in the analysis the total PA concentration of focal plants was negatively, but marginally significantly, related to total plant cover (Table 5.2). Negative effects of plant species richness on total PA concentration did not remain statistically significant after accounting for covariates (Table 5.2) suggesting that plant diversity effects were entirely mediated by associated changes in the focal plants and in changes of total cover of the plant community. The relationships between total PA concentration and the root biomass, N concentration and total plant cover in the community are illustrated in Fig. S5.1 of the Supporting Information.

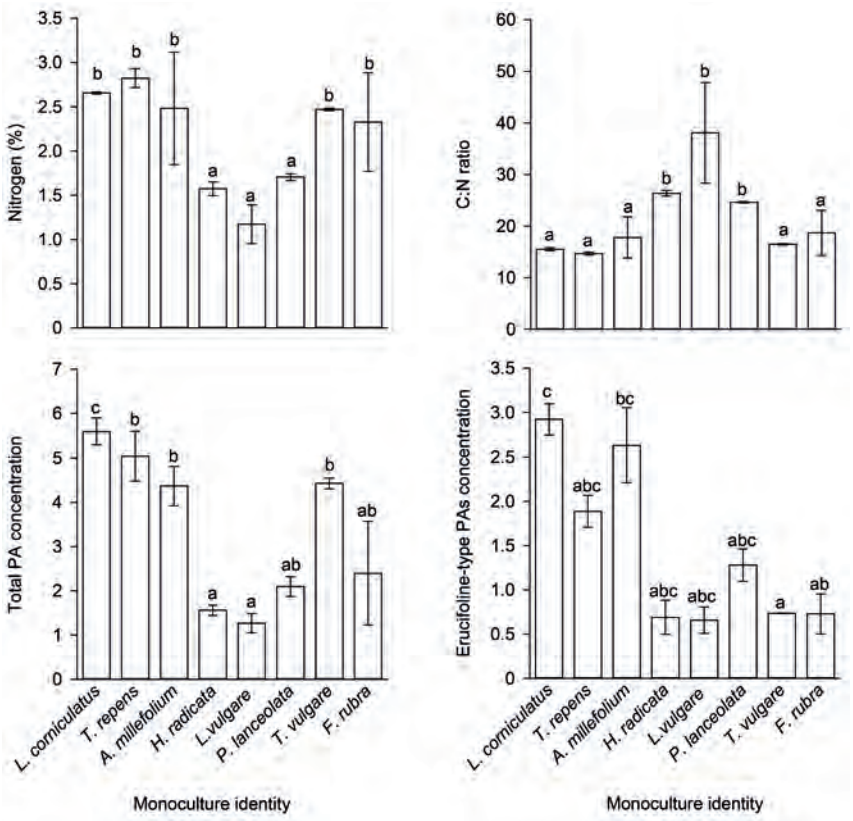


**Figure 5.4** Effect of monoculture identity on leaf N concentration, C:N ratio, total PA concentration (mg·g$^{-1}$ dw) and erucifoline-type PAs (mg·g$^{-1}$ dw) of vegetative focal *J. vulgaris* plants. Means ± between-plot SE are shown.

**Table 5.1** Mean (±SE) available mineral nitrogen in the soil and total plant cover in experimental plots that were sown with 1, 2, 4 or 9 species or kept without vegetation (0). Asterisks indicate significant effects based on a general linear model with plant species richness as fixed log-linear factor and bare plots included or excluded from the model. ** $P < 0.01$; *** $P < 0.001$; the absence of asterisks indicates that the effect is not significant.

| Plant species richness | Soil mineral N ($NH_4^+ + NO_3^-$) (mg·kg$^{-1}$) | Total plant cover (%) |
|---|---|---|
| 0 | 1.86 ± 1.02 | 0 ± 0 |
| 1 | 1.91 ± 0.37 | 132 ± 7.0 |
| 2 | 2.15 ± 0.47 | 152 ± 7.0 |
| 4 | 3.95 ± 0.94 | 160 ± 6.3 |
| 9 | 3.71 ± 1.66 | 166 ± 8.2 |
| Bare plots included | $F_{1,64} = 2.29$ | $F_{1,64} = 34.25$*** |
| Bare plots excluded | $F_{1,60} = 1.82$ | $F_{1,60} = 9.52$** |

**Table 5.2** Effects of characteristics of the focal plant and of the surrounding plant community on total PA concentration of vegetative and reproductive focal plants. The variables were fitted in the order as reported in the table. The analyses were run with and without bare plots. F-values are shown of mixed-effects models (for individual values per plant) and general linear models (for values averaged per plot). Asterisks indicate significant effects at *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; the brackets indicate marginally significant effect at $P < 0.06$; the absence of asterisks indicates that the effects were not significant. ↑ denotes positive and ↓ denotes negative effects.

| | Reproductive plants | | Vegetative plants | |
|---|---|---|---|---|
| | With bare | Without bare | With bare | Without bare |
| **Mixed-effects model** | | | | |
| Root biomass | ↑39.90*** | ↑53.82*** | ↑46.53*** | ↑58.24*** |
| Leaf nitrogen | 3.45 | ↑9.10** | ↑71.51*** | ↑76.03*** |
| Plant species richness | 0.0002 | 0.15 | 1.88 | 0.0039 |
| **General linear model** | | | | |
| Root biomass | ↑38.76*** | ↑56.18*** | ↑50.45*** | ↑50.35*** |
| Leaf nitrogen | 1.88 | 2.83 | ↑15.79*** | ↑22.12*** |
| Total plant cover | 1.26 | (3.70)↓ | 7.18**↓ | 1.50 |
| Soil mineral N | 0.68 | 0.44 | 0.25 | 0.74 |
| Plant species richness | 0.31 | 0.17 | 0.15 | 0.54 |

## Discussion

In this study, we demonstrate that characteristics of the plant community surrounding a focal plant can significantly affect the performance, nutritional quality and secondary chemistry of that focal plant both during vegetative and reproductive stages. However, our results also show that the effects differed significantly between the two developmental stages of the focal plant species. Moreover, our study shows that the presence of the surrounding community had a stronger effect on the growth, nutritional and chemical quality of the focal plants than the diversity of that plant community. The foliar N and total PA concentration of vegetative plants differed significantly among the monocultures and were highest in the monocultures of leguminous species. Independent of the diversity of the surrounding community and the developmental stage of the focal plant, the total PA concentration in the focal plant increased with an increase in plant root biomass of the focal plant and with a decrease in the percentage cover of the surrounding plant community. These results suggest that the surrounding plant community via associated changes in plant biomass and interspecific competition for space influenced the levels of defence compounds in the focal plant.

The presence and diversity of the surrounding plant community, in our study, strongly affected the development and growth of the focal *J. vulgaris* plants. In agreement with our prediction, plant survival and the number of plants that produced flowering stems significantly decreased with an increase in species richness of the surrounding community. *Jacobaea vulgaris* is a poor competitor, and there is a minimum size for the rosettes of the vegetative plants that is required before the plant can produce flowering stems (Van der Meijden & Van der Waals-Kooi 1979; Crawley & Gillman 1989; McEcoy et al. 1993). As the total plant cover in the community increased significantly with increases in plant species richness, this suggests that there was an increase in interspecific competition in more diverse plots. However, in our study there was no effect of plant species richness on the size of the focal *J. vulgaris* plants. Plant size differed only significantly between plants growing in the bare plots and vegetated plots indicating that the presence of the surrounding vegetation is more important for *J. vulgaris* growth than plant diversity per se. Therefore, not plant biomass but other factors, such as size of the rosette at the moment of vernalization, may explain the differences that we observed in plant flowering (Wesselingh & Klinkhamer 1996). Importantly, these findings are also consistent with the results from the first year of the study where plants grown in bare plots were significantly larger than the plants grown in plots with the surrounding vegetation (Kostenko et al. 2012a).

In the last decades, the importance of plant traits for understanding the relationship between ecosystem functions and species richness has been widely recognized in biodiversity studies (Lavorel & Garnier 2002; Diaz et al. 2004). It is well recognized that the plant traits such as plant nitrogen content and defence compound levels can greatly influence the interaction between individual plants and their multitrophic communities. Nevertheless, as far as we are aware, the question how plant diversity affects the levels of plant defence compounds has been only addressed in one study so far (Mraja et al. 2011). These authors showed that plant species richness positively influenced aucubin concentrations and negatively affected catalpol concentrations resulting in no net effect of plant diversity on total iridoid glycoside levels in *P. lanceolata*. Moreover, in the study of Mraja et al. (2011) the responses to plant species diversity were mediated by increases in specific leaf area that reduced the investment into these carbon-based secondary compounds. In our study, where we measured the effects of plant diversity on the levels of pyrrolizidine alkaloids (nitrogen-based secondary compounds), the diversity of the surrounding community significantly decreased the levels of PAs in the focal plants. Interestingly, the levels of almost all PA groups and the total PA concentration of the focal plants were lowest in the plots with highest species diversity (nine plant species) and highest in the bare

plots. The absence of the surrounding vegetation, indeed, had a strong effect on the levels of PAs in the focal *J. vulgaris* plants. PA production takes place in the roots and total PA concentration of the plant is closely linked to the root biomass (Hol et al. 2003; Schaffner et al. 2003). The total PA concentration also positively correlated with the plant root biomass in our experimental grassland plots. As the focal plants growing in bare plots produced significantly larger root biomass than the focal plants in the plots with the surrounding vegetation, this may explain why the absence of the background vegetation strongly affected PA concentration of *J. vulgaris*. Furthermore, the total PA concentration of the focal plants in our study decreased with an increase in the total plant cover of the surrounding community also when bare plots were not included in the analysis. It shows that the availability of open spaces in the vegetation or that plant density is mediating the effects of the surrounding plant community on the levels of PAs of the focal *J. vulgaris* plants. More studies are needed that examine how the manipulations of plant diversity alter the level of plant defence compounds and the mechanisms that are responsible for these changes.

The identity of monocultural stands significantly affected primary and secondary chemistry of vegetative plants. In line with our prediction, the foliar N and total PA concentrations increased in the monocultures of legumes. This is in line with previous studies reporting positive effects of the leguminous neighbours on the performance of focal plants (Temperton et al. 2007, but see Lagerström et al. 2011). In our study, this result was not mediated by the positive effects of legumes on the mineral soil N content as the total PA concentration was not affected by the soil mineral N content. Moreover, the soil mineral N content was lower in legume plots than in the other monocultures such as *H. radicata* that negatively affected PA concentration of the focal plants (data not shown). Therefore, other mechanisms, for example allelopathy, or the availability of other soil nutrients or microelements, might be responsible for the observed changes in PA concentration but this needs further testing.

Intraspecific variation in the expression of plant defence compounds can have a genetic basis (Iason et al. 2012). The PA composition in *J. vulgaris* plants is also partially genetically determined (Vrieling et al. 1993; Macel et al. 2004). We did not measure genetic variation among the focal plants. However, as we used seeds collected from one *J. vulgaris* population to grow focal plants we assume that the genetic variation was relatively low. Additionally, the levels of plant defence compounds can increase in response to herbivores, fungal pathogens or volatile emissions of damaged neighbouring plants (Agrawal et al. 1999; Heil & Karban 2010). In our study, we did not disentangle whether the observed changes in plant chemistry are influenced by differences in herbivory that the focal

plants may experience in the different plant diversity treatments. In a previous study we measured the densities of insects colonizing these focal plants in the experimental plots and we found that the number of specialist aphids, the most abundant herbivore species colonizing *J. vulgaris* rosettes in the first year after transplantation, decreased with an increase in plant species richness (Kostenko et al. 2012a). Therefore, it is also possible that the PA concentration decreased with a decrease in the herbivore pressure on the focal plants in more diverse plots. It is important to note though, that previous experiments with PAs demonstrated that PA expression decreases after artificial damage to the leaves (Van Dam et al. 1993) and is not induced in shoots in response to shoot herbivory (Hol et al. 2004).

In summary, our data show that the presence, diversity and identity of the surrounding community can affect the development, size, nutritional quality and secondary chemistry of focal plants growing in that community. However, the presence and the identity of the surrounding community are more important for growth, nutritional and chemical quality of the focal plants than plant diversity per se. Competition for open spaces was partly responsible, but did not fully mediate the effects of the surrounding plants community on the focal plants. Such intraspecific variation in plant quality at small spatial scales can have important consequences for interactions of focal plants with their multitrophic communities in the field.

## Acknowledgments

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## Supporting Information

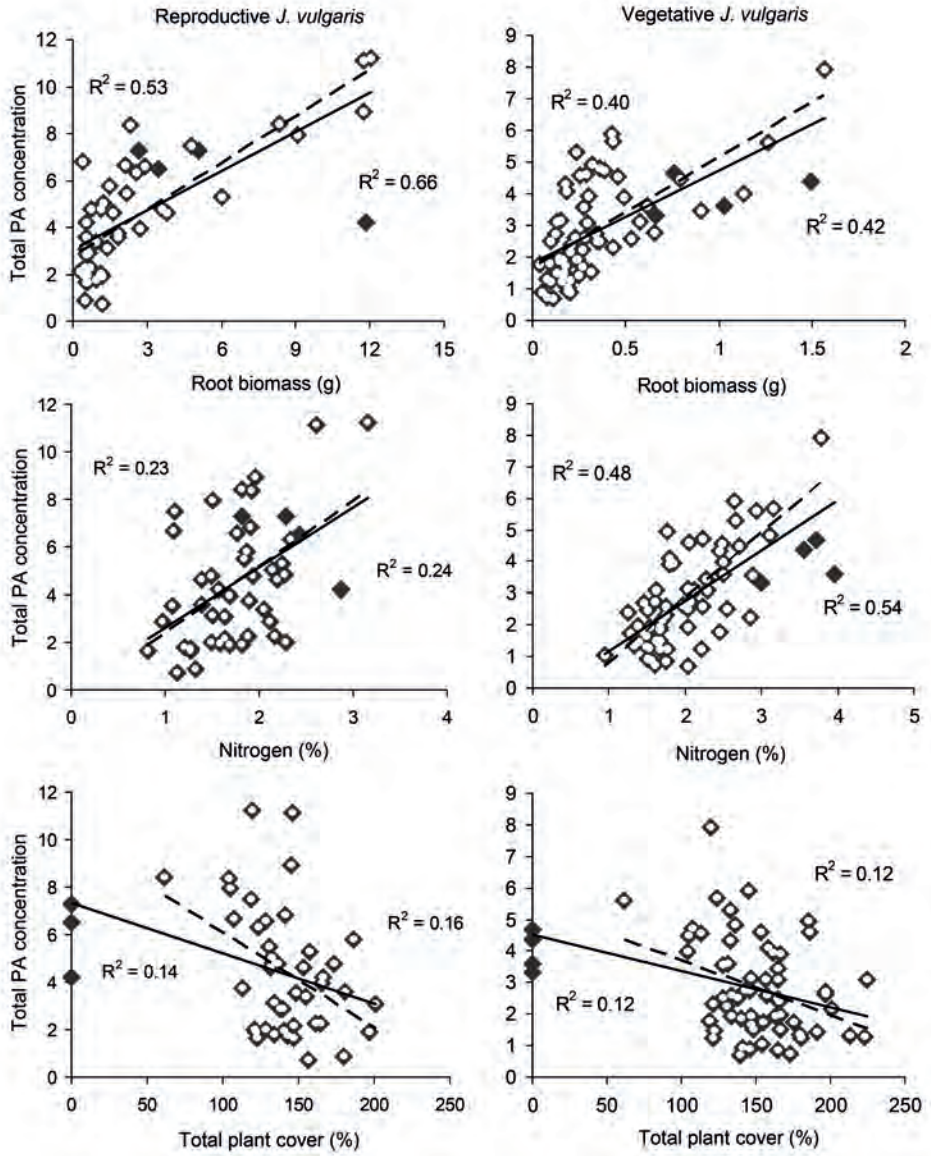


**Figure S5.1** The effects of plant root biomass, foliar N concentration and total plant cover in the community on the total PA concentration (mg·g$^{-1}$ dw) of focal *J. vulgaris* plants. Black dots indicate data from the bare plots, black and white dots – data from the plots with background vegetation. Solid line and $R^2$ value on the left represent the regression line of the model where bare plots were included; dashed line and $R^2$ value on the right represent the regression line of the model where bare plots were excluded.

# Chapter 6

## Behaviour of parasitoids in the field: effects of diversity and complexity of the surrounding plant community

**Olga Kostenko, T. Martijn Bezemer**

*Manuscript*

## Abstract

In natural ecosystems, host-infested plants are often embedded within heterogeneous plant communities. The plant community surrounding host-infested plants may influence the host-finding behaviour of parasitoids in those communities. In this study, we used a release-recapture approach to examine whether the diversity and structural complexity of the community surrounding a host plant influences the host-finding ability of the leaf-miner parasitoid *Dacnusa sibirica*. Potted *Jacobaea vulgaris* plants infested with the generalist leaf-miner *Chromatomyia syngenesiae* were placed in communities that differed in plant diversity (1 to 9 species) and habitat complexity (bare ground, mown vegetation, tall vegetation). Then, *D. sibirica* parasitoids were released in those communities and recaptured. Locally present leaf-miner parasitoids found on the experimental mines were also collected. After the recapture day, the potted plants were left in the field so that the mines could be parasitized by remaining *D. sibirica* and by naturally occurring parasitoids. One week later all plants were brought to a greenhouse. In the greenhouse all plants were caged individually and all emerging insects were collected. The impact of the diversity of the surrounding plant community on the locally present parasitoid community was then assessed. No *D. sibirica* parasitoids were recaptured in the bare ground plots. Plant diversity did not influence mean recapture rates of *D. sibirica* or captures of other locally present parasitoids. Mean recapture rate of *D. sibirica* generally increased with an increase in structural complexity of plant community and the capture rate of locally present parasitoids increased with an increase in the percentage bare ground in the community. There was a hump-shaped relationship between the number of reared local parasitoids from the trap plants and diversity of the surrounding vegetation with the highest density of emerged parasitoids at intermediate diversity levels. Our study adds to the scarce body of literature examining the foraging behaviour of parasitoids in the field and suggests that the preference of parasitoids to forage in complex versus simple stands might depend on the characteristics of the plant community they are searching in, but also on species specific behavioural traits of the parasitoids.

## Introduction

Understanding parasitoid foraging behaviour in complex natural systems is important from a fundamental ecological viewpoint, but can also be used to enhance pest control in agricultural systems. Parasitoid foraging or host-finding ability is often described as a three-step hierarchical process of habitat location, host plant location and host location (reviewed in Godfray 1994). Laboratory studies performed in simple controlled settings have shown that parasitoids can exploit a series of physical and chemical cues to locate their host (Dicke & Grostal 2001; Vet & Godfray 2007). In natural habitats, however, insect parasitoids have to search for their sparsely distributed hosts in spatially and temporally heterogeneous environments where host-infested plants are surrounded by neighbouring plant species that often are non-host plants. The detection of the host in this microhabitat is a crucial step for a parasitoid female and it can have a major influence on her fitness. The surrounding plant community may influence the way cues are perceived by the foraging wasp (reviewed in Andow 1991). For example, surrounding plants can decrease the reliability of the chemical cues from the host or the host-infested plant that are used by the parasitoid to locate its host (Sheehan 1986; Bukovinszky et al. 2007; Randlkofer et al. 2007). Additionally, surrounding plants can visually mask the host plant or hamper parasitoid movements (Gols et al. 2005; Obermaier et al. 2008). Yet, empirical studies on the effects of the surrounding vegetation on parasitoid foraging behaviour in the field are scarce.

Two important characteristics of a plant community that can affect parasitoid foraging behaviour are the diversity of that community and the density of host plants within that community. A high concentration of host-infested plants in monocultural stands may attract parasitoids that use plant cues to locate their hosts (Sheehan 1986). Similarly, the tendency to leave a highly diverse community may increase if a parasitoid repeatedly encounters non-host plants in a plant community. High levels of plant species and plant odour diversity might also hinder parasitoids from detecting the host cues in diverse plant communities (Price et al. 1980; Sheehan 1986; Randlkofer et al. 2007). Moreover, diverse communities may support higher abundances of generalist predators which might, in turn, suppress the abundance of parasitoids (Root 1973). Alternatively, more diverse plant communities may provide a higher abundance of nutritional resources, such as nectar, that can positively influence the longevity and fecundity of many parasitoids and influence the amount of time they spent in the community thereby increasing the probability of host encounters (Root 1973; Sheehan 1986; Russel 1989). A number of laboratory studies have shown that increasing the diversity of the surrounding plants negatively affects the

efficiency of parasitoids to find hosts on focal plants (e.g., Coll & Bottrell 1996; Gols et al. 2005; Bukovinszky et al. 2007). However, whether the same pattern will occur in natural communities remains largely unknown.

Diverse plant communities may be also structurally more complex than simple ones, in terms of, for example, size, abundance and diversity of plant parts, arrangement of biomass in space, or openness of the vegetation (Randlkofer et al. 2010b). Structurally more complex habitats may provide a wider variety of niches and resources and more favourable microclimate conditions to which searching parasitoids may be attracted (Strong et al. 1984; Andow 1991). On the other hand, increasing plant structural complexity may represent a constraint to small organisms such as parasitoids and may reduce host encounter rates. Previous studies that have examined the effect of plant structure on the foraging behaviour of parasitoids have shown that the host-finding ability of parasitoids is generally constrained in structurally more complex habitats than in simple ones, both in the laboratory and in the field (e.g., Andow & Prokrym 1990; Cronin 2003; Gingras et al. 2003; Perfecto & Vet 2003; Meiners & Obermaier 2004; Gols et al. 2005; Bezemer et al. 2010b; Randlkofer et al. 2010b). However, until now relatively few studies have examined the effects of habitat complexity on parasitoid searching behaviour in the field (e.g., Bezemer et al. 2010b).

To examine the effects of surrounding plant community diversity and structural complexity on host-searching behaviour of parasitoids in the field we carried out a release-recapture experiment with the leaf-miner parasitoid *Dacnusa sibirica* Telenga (Hymenoptera: Alysiinae). We placed potted *Jacobaea vulgaris* Gaertn. (Asteraceae) trap plants, which we a priori infested with the generalist leaf-miner *Chromatomyia syngenesiae* Hardy (Diptera: Agromyzidae), in field plots with tall vegetation that varied in plant diversity and structural complexity. In the diversity plots we also measured vegetation height, vertical distribution of plant parts, plant biomass and cover of bare ground. To determine the effect of habitat complexity on host-searching behaviour of parasitoids we also placed trap plants in plots without vegetation and in plots where vegetation was regularly mown. We released *D. sibirica* parasitoids and recaptured them on the trap plants. We also recorded the time when *D. sibirica* were collected and we expected that immediately after the release more parasitoids will be recaptured in bare plots or monospecific plots but that over time, parasitoids will become attracted and remain in more diverse plots where they will be able to find more food resources. Based on the results of previous studies we formulated the following hypotheses: (i) in more diverse plant communities fewer parasitoids will be able to find host-infested plants; (ii) the number of recaptured parasitoids will decrease with increasing structural complexity of

the surrounding community; (iii) recapture rates of the parasitoids will be lower in plots with tall vegetation than in mown or bare plots;. and (iv) there will be a significant interaction between time since release and the diversity of the surrounding community. Finally, we also captured locally present leaf-miner parasitoids that were exploring mines on the trap plants. After the recapture day, plants were left in the field allowing parasitism by remaining *D. sibirica* and locally present parasitoid community and later brought to the laboratory. All emerging parasitoids were collected and the effect of diversity of the surrounding plant community on the locally present parasitoid community was assessed. We hypothesized that (v) the number of captured and reared-out local parasitoids will increase with increasing plant diversity and complexity as parasitoids are more abundant in diverse plant communities.

## Materials and Methods

*Study system*

*Plants*. *Jacobaea vulgaris* is a biannual or short-lived perennial plant widely distributed in natural and semi-natural areas throughout Europe and Asia. Experimental *J. vulgaris* plants were grown from seeds collected from a single natural plant population at a restoration grassland at Planken Wambuis (Ede, the Netherlands), where agriculture was ceased 14 years ago at the time of seed collection.

*Leaf miners*. In its native range *J. vulgaris* leaves are frequently mined by agromyzids of the genera *Liriomyza*, *Chromatomyia, Ophiomyia* and *Phytomyza* (Pitkin et al. 2013). *Chromatomyia syngenesiae* is a highly polyphagous leaf-mining species that is considered to be a serious pest of many agricultural crops (Cornelius & Godfray 1984). It forms linear white mines both on the upper and lower surface of the leaf, avoiding the major veins. It is widely distributed in Europe and present in natural populations throughout the summer. *C. syngenesiae* pupa were commercially obtained from BCP Certis (United Kingdom) and reared on *Sonchus oleraceus* L. (Asteraceae). Adults were kept in a climate chamber (10 °C) until needed in the experiment.

*Parasitoids*. *Dacnusa sibirica* is widely used as a biological control agent against miner flies of vegetable crops (e.g., tomato, sweet pepper, lettuce) and ornamental plants (e.g., rose, gerbera, and chrysanthemum). *D. sibirica* is a solitary koinobiont and is able to parasitize all larval stages of *C. syngenesiae* (Croft & Copland 1994). The parasitoid larva develops within the host, feeding on non-vital tissues until the host pupates. Adult *D. sibirica* emerge from the pupa of

the leaf-miner. *D. sibirica* can hibernate in leaf-miner pupae thereby allowing it to occur simultaneously with its host already early in the season. Adult parasitic wasps do not host-feed. Natural parasitism of *C. syngenesiae* by *D. sibirica* has not been reported in the literature but *D. sibirica* has been widely used as a biological control agent for this leaf-miner species (e.g., Cornelius & Godfray 1984; Minkenberg 1990; Croft & Copland 1995). *D. sibirica* were commercially obtained from Koppert B.V. (the Netherlands). The sex ratio of the parasitoids was 50% males. Parasitoids were kept in 100 ml plastic containers (250 adults in each container) with a droplet of diluted honey and water in a climate chamber (10 °C) for one day.

*Plant and insect rearing*

Seeds of *J. vulgaris* were surface sterilized (1 min in 0.1% sodium hypochloride solution and rinsed with water) and germinated on glass beads. Two hundred eighty pots of 1 l were filled with 800 mg sterilised potting compost mixed with sterilized field soil in 50:50 ratio. Sterilized field soil was collected from the same location as the seeds of *J. vulgaris* and sterilized using gamma irradiation (> 25 KGray, Isotron, Ede, the Netherlands). One ragwort seedling was transplanted into each pot. Pots were placed in outdoor fine-meshed (0.1 cm) tents of 2.5 × 2.0 × 2.0 m in a common garden of the Netherlands Institute of Ecology at Heteren, the Netherlands. The weather conditions inside the cages closely resembled field conditions. Plants were watered three times per week and randomly rearranged within the cage once a week. Three months later, when all plants had rosettes with 10 or more fully expanded leaves, they were placed in small meshed cages of 0.4 × 0.4 × 0.6 m. There were nine plants per cage and 35 adult *C. syngenesiae* females were released into each cage. All adult leaf-miners were carefully removed from each cage 24 h later and plants were placed back in the large cages. Ten days later, the first mines appeared, and three days later all plants were transported to the field.

*Experimental set-up*

The effect of plant diversity on the searching behaviour of the parasitoid was studied in the existing biodiversity field experiment that was running at the same location as where the seeds of ragwort and the soil were collected. The biodiversity field experiment was created in 2008 by sowing 70 plots (3 × 3 m) with 1, 2, 4 or 9 plant species randomly selected from a pool of 12 local grasslands species belonging to three functional groups (forbs, legumes, grasses). Four plots were designated as bare soil treatment and kept free of the vegetation but only two bare plots were used in this experiment. Plots were weeded manually during the growing seasons of 2009 and 2010 to maintain the initial sown species composition. Plots were not mown but 1 m-wide paths between plots were mown regularly during

the growing season. Details of the experimental design have been presented in Kostenko et al. (2012a). In each plot, 25 small (10.0 ± 0.23 cm diameter) *J. vulgaris* plants were growing in the inner square (1.2 × 1.2 m) but these were not included in this experiment. We call these plants "focal plants". The potted plants used in the release experiment are called "trap plants". The leaves of the trap plants were visually inspected and plants with approximately the same number of mines (roughly 40 per plant) were selected. Four pots with leaf-miner-infested plants were placed in each plot at fixed positions 50 cm from the edge and 2 m from each other (see Fig. S6.1 in the Supporting Information for the experimental set-up). The height of the experimental pots was 11 cm and the experimental trap plants were approximately 10 cm tall (total height 21 cm). We also placed plants in two "bare ground" plots and on a mown grassland area of 50 × 5 m next to the experimental plots (Fig. S6.1). This type of habitat complexity was named "mown vegetation". In the mown area, plants were placed in four rows with four plants per row at a distance of 1 m between plants. The distance between the rows was 7 m. To test the effect of habitat complexity on host location of the parasitoids we compared recapture rates in the sown plots (tall vegetation), the bare plots and the mown plots. Prior and after the release experiment all focal plants were checked for naturally occurring leaf mines. Mines were found only on 42 of the 1750 focal plants (2%) and these plants only had one or two mines.

*Release-recapture experiment*

A release-recapture experiment was carried out on a sunny day during mid August of 2010. The wind speed was less than 0.3 $m \cdot s^{-1}$. One day before the parasitoids were released in the field, pots with trap plants were placed in the biodiversity field experiment. At the recapture day, at 10:00 a.m., 35 tubes with 250 *D. sibirica* adults (8750 adults in total) were released at regular positions within the experimental field (Fig. S6.1). All but 133 parasitoids left the release tubes. Starting immediately after the release and for seven consecutive hours all trap plants were checked thoroughly by eight collectors, and all parasitoids encountered on a plant were collected. Recaptures were made during three rounds and each collector visited each plant at least once during each collection round. All collectors spent an equal amount of time at each plant. Each recaptured parasitoid was kept separately in an Eppendorf tube and the identity of the plant, on which the insect was captured, and time of recapture were recorded. Parasitoids were stored in alcohol and *D. sibirica* were identified under microscope using the specimens received from the supplier. Other leaf-miner parasitoid species that were also collected from the trap plants were identified at family level (Table S6.1). One week after the plants had been brought to the field, all potted plants were collected, transported to the greenhouse and caged individually using fine-meshed cylindrical cages of 70 cm height and 25 cm

diameter. During a period of five weeks, for each plant all emerging insects were collected and stored individually in Eppendorf tubes in 70% ethanol. The total number of emerged parasitoids was calculated per plant.

*Plant community measurements*

One week after the field recapture event, plant community measurements were made in each plot, excluding the 25 focal plants, to estimate the structural complexity of the community. For each plant community we measured the height of the vegetation, the vertical diversity, the aboveground biomass and the total percentage of plant cover (Table 6.1). The height of the vegetation was measured using the vertical drop disc method (Stewart et al. 2001). The disc weighed 200 g, had a diameter of 300 mm, and was released from 1.5 m height. The height was measured at 10 random locations within each plot. At the same locations where height was measured, a wooden pin (10 mm diameter and 1.5 m height) with length markings was placed within the community and the height at which different plant parts (leaves, stems and flowers) hit the pin was recorded. Then, using the Shannon diversity formula we calculated the vertical diversity of the community as described in Woodcock et al. (2009). The percentage cover of bare soil was recorded in three 1 $m^2$ quadrants along a diagonal transect within each plot. Two weeks later the aboveground biomass of the vegetation was determined in each plot by clipping all plants in four randomly selected subplots of $0.25 \times 0.25$ m at 1 cm above the soil surface. The aboveground biomass of each plot was weighted after drying for three days at 70 °C. Biomass was calculated as gram dry weight per square meter.

*Data analysis*

All analyses were performed in R statistical language, ver. 2.15.0 (R Development Core Team 2012). The recapture data of *D. sibirica* and *Eulophidae* parasitoids and the counts of reared parasitoids were analyzed using a generalised linear mixed model with Poisson distribution and logarithmic link function. Plot identity was used as a random factor as multiple plants were sampled within each experimental plot. The mown area was subdivided into four plots (Fig. S6.1) and the four plants within each of these plots were also considered pseudoreplicates. Plant diversity was included as linear factor. The sum of the parasitoids recaptured or reared out from plants in bare, mown and tall vegetation plots were compared using a $\chi^2$ test. The relationship between the mean number of recaptures of *D. sibirica* or *Eulophidae*, or reared parasitoids and the characteristics of the surrounding vegetation (height, index of vertical diversity, biomass and percentage bare ground) were analysed using multiple linear regression based on averaged values per plot. Bare plots were excluded from this analysis. The order in which community variables were fitted in the

model was based on the Pearson correlation coefficients between parasitoid data and individual variables. The model simplification was performed by using "step" function in R. To test whether the mean recapture rate of *D. sibirica* from the sown (diversity) plots changed with time, we calculated the mean number of recaptured parasitoids for each one-hour time-period per plot and analysed the data using two-way ANOVA with plant species richness (1-9) and time as main factors. The effects of plant species richness on the characteristics of the surrounding vegetation were analysed using general linear models with averaged values per plot. The data were log- or square-root transformed to fulfil the requirements of normality and homogeneity of variances.

**Table 6.1** Mean (±SE) height, index of vertical diversity, standing biomass and percentage bare ground of the surrounding plant community in the experimental biodiversity plots; and the results of a general linear model with plant species richness as linear factor.

| Species richness | Height (cm) | Index of vertical diversity | Standing biomass ($g \cdot m^{-2}$) | Bare ground (%) |
|---|---|---|---|---|
| 1 | 17.9 ± 3.3 | 62.3 ± 9.5 | 203.1 ± 34.8 | 14.3 ± 3.3 |
| 2 | 17.7 ± 2.4 | 64.1 ± 8.6 | 133.3 ± 15.2 | 6.7 ± 1.1 |
| 4 | 17.5 ± 1.5 | 83.8 ± 7.0 | 216.1 ± 13.9 | 5.9 ± 1.1 |
| 9 | 16.2 ± 2.0 | 79.9 ± 20.5 | 246.9 ± 29.9 | 7.6 ± 3.0 |
| $F_{1,60}$ | 0.57 | 3.24 | 1.52 | 6.31 |
| *P*-value | 0.45 | 0.077 | 0.22 | 0.015 |

## Results

A total of 207 *D. sibirica* individuals and 172 other hymenopteran parasitoids were captured from the trap plants. Two Ichneumonoidae individuals were captured on the trap plants but these are not leaf-miner parasitoids and were excluded from further analysis. The non-released (local) parasitoid community were parasitoids of leaf-mining dipterans and the majority of them belonged to the family *Eulophidae* (Chalcidoidae, Table S6.1). Only seven individuals of *Pteromalidae* were collected. Therefore, we based our further analysis of the local parasitoids captured on the trap plants only on the *Eulophidae* family. Parasitoids were captured from 181 of the 280 plants. The spatial distribution of the captured parasitoids per plant is shown in Fig. S6.2. There was no effect of the diversity of the surrounding plant community on the number of recaptured *D. sibirica* ($z = 0.22$, $P = 0.83$; Fig. 6.1A) and of other collected *Eulophidae* ($z = -0.26$, $P = 0.80$). From the eight trap plants placed in the bare

plots no *D. sibirica* parasitoids were recaptured (Fig. 6.1B). The recapture rates of *D. sibirica* were significantly higher in plots with tall vegetation than in plots that were mown ($\chi^2_2 = 13.90$, $P = 0.0010$; Fig. 6.1B). In contrast, the number of captured *Eulophidae* parasitoids did not significantly differ among the studied microhabitats ($\chi^2_2 = 0.70$; $P = 0.71$). There was no significant interaction effect between plant diversity and time since release on mean recapture rate of *D. sibirica* ($F_{1,346} = 1.125$, $P = 0.29$; Fig. 6.2). The recapture rates decreased with the time since release ($F_{1,346} = 6.71$, $P = 0.010$).

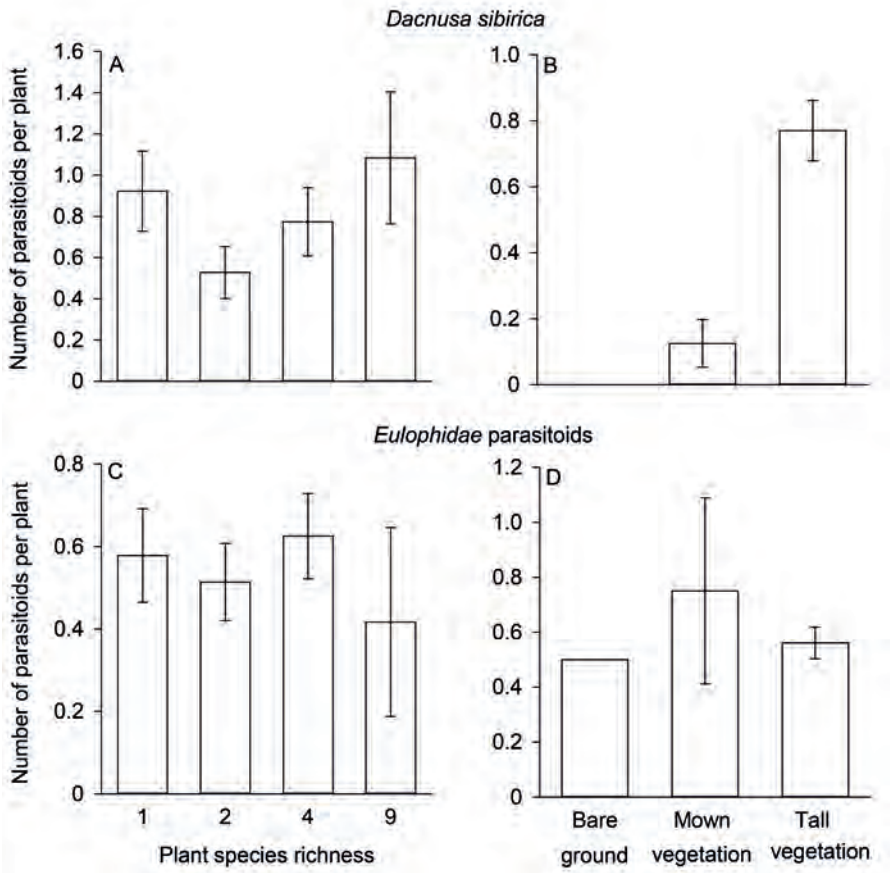


**Figure 6.1** Mean number (± SE) of recaptured *Dacnusa sibirica* and locally present *Eulophidae* leaf-miner parasitoids on each trap plant. Trap plants were placed in experimental plots differing in plant species richness (tall vegetation), in plots without surrounding vegetation (bare ground), and in plots with mown vegetation. Shown are (A, C) the effects of plant diversity (1, 2, 4 or 9 species) and (B, D) the effects of habitat complexity.

The mean number of recaptured *D. sibirica* parasitoids per plant increased with an increase in the vertical diversity of the surrounding vegetation ($F_{1,59} = 4.44$, $P = 0.039$). The biomass of the surrounding community was also kept in the model but it did not significantly affect the mean recapture rate of *D. sibirica* parasitoids. The mean number of collected *Eulophidae* parasitoids positively correlated with the percentage of bare ground in experimental plots ($F_{1,60} = 4.02$, $P = 0.049$). There were no significant correlations between densities of *D. sibirica* and other collected *Eulophidae* parasitoids per plant ($P > 0.05$, data not shown).

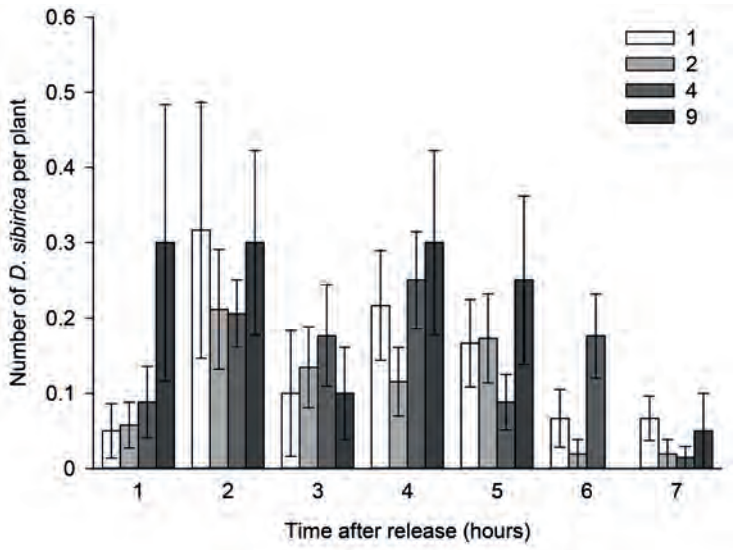


**Figure 6.2** Mean number (± SE) of recaptured *Dacnusa sibirica* on trap plants placed in experimental plots differing in plant species richness (1, 2, 4 or 9 species). Shown number of recaptures for each one-hour period since the parasitoids were released.

Approximately 5000 parasitoids were reared from the trap plants. The majority of them belonged to the family *Eulophidae* (data not shown). There was no linear relationship between the number of reared parasitoids and the diversity of the surrounding vegetation ($z = 1.06$, $P = 0.29$). However, there was a polynomial quadratic relationship between the mean number of reared parasitoids per plot and diversity of the surrounding vegetation ($F_{2,61} = 4.17$, $P = 0.020$; Fig. 6.3A) with the highest number of reared parasitoids in plots with four plant species. The number of reared parasitoids was higher in plots with tall vegetation, but the difference was not statistically significant ($\chi^2_2 = 1.26$, $P = 0.53$; Fig. 6.3B), and was not affected by the characteristics of the communities with tall vegetation ($P > 0.05$ in all cases, data not shown). The mean number of reared parasitoids from trap plants placed in plots with tall vegetation significantly correlated with the number of captured *Eulophidae* ($r = 0.26$, $P = 0.040$).

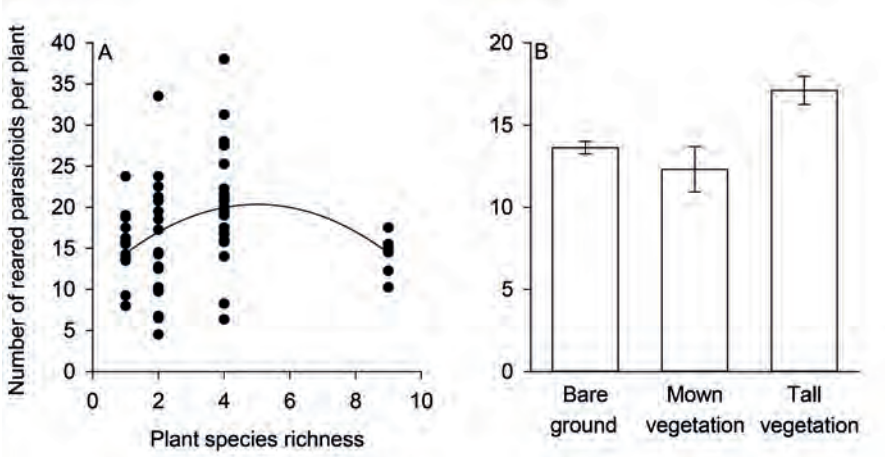


**Figure 6.3** (A) Number of parasitoids reared from the trap plants placed in experimental plots differing in plant species richness with a polynomial quadratic curve fitted to the data. Each circle represents the mean number of parasitoids reared out from plant averaged per plot. (B) Effects of habitat complexity on the mean number (± SE) of parasitoids reared from the trap plants used in the release-recapture experiment.

## Discussion

Overall, the diversity of the surrounding community did not affect the recapture rates of the generalist leaf-miner parasitoid *D. sibirica* and captures of the locally present *Eulophidae* parasitoids of leaf-mining *C. syngenesiae*. However, there was a hump-shaped relationship between the diversity of the surrounding plant community and number of reared leaf-miner parasitoids from the trap plants that were predominantly parasitized by the locally present parasitoid community. The number of reared leaf-miner parasitoids from the trap plants was highest at intermediate (four species) diversity level. Moreover, our study demonstrates that the host-finding behaviour of *D. sibirica* is strongly influenced by the complexity of the surrounding vegetation and that this parasitoid species prefers to forage in more complex environments. In contrast, the locally present *Eulophidae* parasitoids were more often captured in more open plots.

In an earlier field experiment, carried out at the same location, Bezemer and colleagues (2010b) examined the role of complexity of the surrounding vegetation on the host or host plant location behaviour of the parasitoid wasp *Cotesia glomerata* L. (Hymenoptera: Braconidae). In that study, very low numbers of parasitoids were recaptured in plots with tall vegetation, independent of the composition of the vegetation, whereas high numbers of parasitoids were

recaptured from plants placed on bare soil or in mown grassland (Bezemer et al. 2010b). On bare soil, *C. glomerata* was even recaptured on plants without hosts. Similarly, in another field experiment, the egg parasitism rate by *Oomyzus galerucivorus* was negatively affected by the complexity of the plant community in which the host plants were growing (Meiners & Obermaier 2004). The results of these studies sharply contrast with our experiment where no released parasitoids were recaptured from the plants placed in bare ground plots and most in the plots with tall vegetation. One explanation for the discrepancy in the results of these studies can be the difference in the feeding breadth of the studied parasitoid species. *C. glomerata* and *O. galerucivorus* are both specialist parasitoids whereas *D. sibirica* is a generalist parasitoid attacking a range of leaf-mining Agromyzidae. Generalist parasitoids are predicted to be more attracted to diverse and complex habitats because of the greater variety of the hosts and food resources available in those habitats (Root 1973). However, the specialist parasitoid *Macrocentrus grandii* (Hymenoptera: Braconidae) that parasitizes the European corn borer also preferred to forage in dense versus open habitats (White & Andow 2006). Another possible explanation for the opposite responses of *D. sibirica* and *C. glomerata* in habitats with short and tall vegetation could be a difference in innate searching preferences between these two parasitoid species. *C. glomerata* prefers to search for hosts in open areas and avoids dense vegetation (Benson et al. 2003; Bezemer et al. 2010b). This preference coincides with the habitats in which its host plants are most typically found. Biological control studies have shown that *D. sibirica* searches mainly low in the crop and is able to locate mines at very low densities (e.g., Minkenberg 1990). This is also supported by the positive correlation between the vertical diversity of the vegetation and the average recapture rates of *D. sibirica* in our study. The lack of consistent responses among parasitoid species in field studies indicates that generalizations about parasitoid searching behaviour should be made with the great caution.

One of the aims of our study was to obtain an estimate of the levels of parasitism of the local community of leaf-miner parasitoids, and to asses the effects of the diversity and complexity of the surrounding vegetation on locally occurring leaf-miner parasitoids. The majority of collected parasitoids that were not released belonged to the family *Eulophidae* (probably *Diglyphus* spp. and *Chrysocharis* spp., O. Kostenko pers. observation). In line with the results from the released and recaptured parasitoids there was no significant effect of the diversity of the surrounding community on the number of collected locally occurring parasitoids. However, in contrast to our hypothesis, fewer (although not statistically significant) parasitoids were found on and fewer parasitoids were reared out of the plants placed in the most diverse plant communities

indicating that these parasitoids may prefer less diverse habitats for foraging. The capture rates of the locally present *Eulophidae* parasitoids also positively correlated with the percentage cover of bare ground in the community. The preference of local parasitoids for more open and structurally simple vegetations in our study suggests that plant diversity could negatively affect the level of parasitism in these communities. This is in contrast to other studies that show a positive effect of plant diversity on the abundance of parasitoids and parasitism rates (e.g., Tscharntke et al. 1998; Haddad et al. 2009; Scherber et al. 2010). This difference can be explained by the innate searching preferences of parasitoids to forage in certain types of habitats. Biological control studies, for example, report that eulophid parasitoids, such as *Diglyphus* spp. prefer to forage in environments that are warmer and have more daylight, such as more open habitats (e.g., Cheah 1987; Bazzocchi et al. 2003). Parasitoids are often sensitive to environmental conditions, and hence, through the alteration of abiotic conditions, modifications in vegetation diversity can have a widespread effect on many natural enemies (Bezemer et al. 1998; Hance et al. 2007). Finally, several studies have also illustrated that a parasitoid's foraging success is linked to its movement ability and that complex vegetation structures exert a negative influence on the foraging efficiency of these natural enemies (e.g., Coll & Bottrell 1996; Randlkofer et al. 2010a).

Apart from the diversity and structural complexity of the surrounding vegetation, the structure of the host-infested plant, the density and distribution of hosts on the plant, and the spatial distribution of the host-infested plants, have all been shown to affect the foraging behaviour of parasitoids (e.g., Casas 1989; Cronin 2003; White & Andow 2005; Bezemer et al. 2010b). In our study, the potted *J. vulgaris* plants placed in the experimental plots were similar in size and distributed in a regular grid over the experimental biodiversity field. We did not measure the density of the leaf mines on the trap plants but we selected plants with approximately the same number of mines based on the visual estimation of the leaves. Moreover, in a study examining natural parasitism of leaf-miners belonging to the genus *Calycomyza*, the density of leaf mines per plant and per leaf did not affect the rate of parasitism of the leaf-miner (De Queiroz & Garcia 2009). Other studies have shown that habitat characteristics such as patch size and habitat heterogeneity can significantly affect the rate of parasitism of leaf-miners (e.g., De Queiroz & Garcia 2009). Therefore, we argue that it is unlikely that local differences in the trap plants or in host density interfered with the host-locating decisions of the parasitoids in our study. However, during the release-recapture experiment we did not release the parasitoids as close to the mown plots as to the rest of the plots. This may offer an alternative explanation for low number of recaptured *D. sibirica* in those plots.

Recently, Randlkofer and colleagues (2010b) proposed that high plant complexity and high odour diversity may have an interactive effect on host location behaviour of parasitoids in natural plant communities. In an earlier laboratory bioassay, Randlkofer et al. (2007) demonstrated that parasitoids respond only to pure host plant odours but not to complex odour blends that contained host odours. Other studies have shown that the emission of volatiles of a plant can be affected by the presence and identity of neighbouring plants (Kigathi et al. 2013) and that neighbouring plants via this mechanism can confuse parasitoid host location (Gols et al. 2005). Unfortunately, little is known about the importance of olfactory cues for host searching of parasitoids in natural environments (Casas et al. 2004; Heimpel & Casas 2007; Poelman et al. 2009a). We did not measure volatile profiles of the trap plants in the different plots. However, in a wind tunnel study, the parasitoid *D. sibirica* was attracted to volatile infochemicals emitted from tomato leaves infested by the agromyzid leaf-miner *Liriomyza bryoniae* (Dicke & Minkenberg 1991). Whether such olfactory cues emitted by the host plant also influence the foraging behaviour of *D. sibirica* in the field remains to be tested.

The spatial scale at which the behavioural experiments are performed and the characteristics of the matrix (the unsuitable intervening habitat that surrounds patches of suitable habitat) can greatly affect parasitoid behaviour and hence the outcome of the study (reviewed in Bommarco & Banks 2003; Cronin 2003). Our experimental plots were relatively small in size (9 $m^2$) and separated by narrow lanes of 1 m wide that consisted of mown grassland vegetation. Therefore, it may be incorrect to consider each plot as a separate habitat that parasitoids can distinguish and our set-up is not suitable to examine landscape dynamics of parasitoids (sensu Tscharntke & Brandl 2004; Thies et al. 2005; Kaartinen & Roslin 2011). Instead, in our study we focus on the microhabitat properties and assume that the immediate surrounding of each host-infested trap plant affected host location success of the parasitoids.

In conclusion, our study shows that host-finding success of the parasitoid *D. sibirica* in natural vegetation is not affected by the diversity of the surrounding plant community but rather by the structural complexity of the vegetation in which the host plant is embedded. The generalist leaf-miner parasitoid preferred to forage in structurally more complex plant communities that are characterized by tall vegetation and vertical diversity. In contrast, the locally present parasitoid species preferred to forage in more open and structurally less complex plots. Our study also suggests that the preference of parasitoids for complex or simple vegetation stands might depend on their behavioural traits. Further experiments that examine parasitoid host-finding behaviour in natural systems are needed to unravel how parasitoid, host, and vegetation characteristics interact in influencing parasitoid foraging decisions.

## Acknowledgements

Natuurmonumenten (Planken Wambuis) are acknowledged for permission to perform this study on their properties; Gregor Disveld for assistance in the greenhouse; Saskia Grootemaat, Thomas Kroon, Constanza Vidal, Jeff Harvey, Moniek Van Geem, Johan Veendrick for help in collecting parasitoids; Alex Gumovsky for help with parasitoid identification. Helpful suggestions of Wim Van der Putten greatly improved an earlier version of this chapter.

## Supporting Information

**Table S6.1** Parasitoids collected from *J. vulgaris* trap plants in the field.

| Superfamily | Family | Subfamily | Number of recaptured individuals |
|---|---|---|---|
| Chalcidoidea | *Eulophidae* | | 163 |
| | *Pteromalidae* | | 7 |
| Ichneumonoidea | *Braconidae* | *Microgastrinae* spp. | 1 |
| | *Ichneumonidae* | | 1 |
| Total | | | 172 |

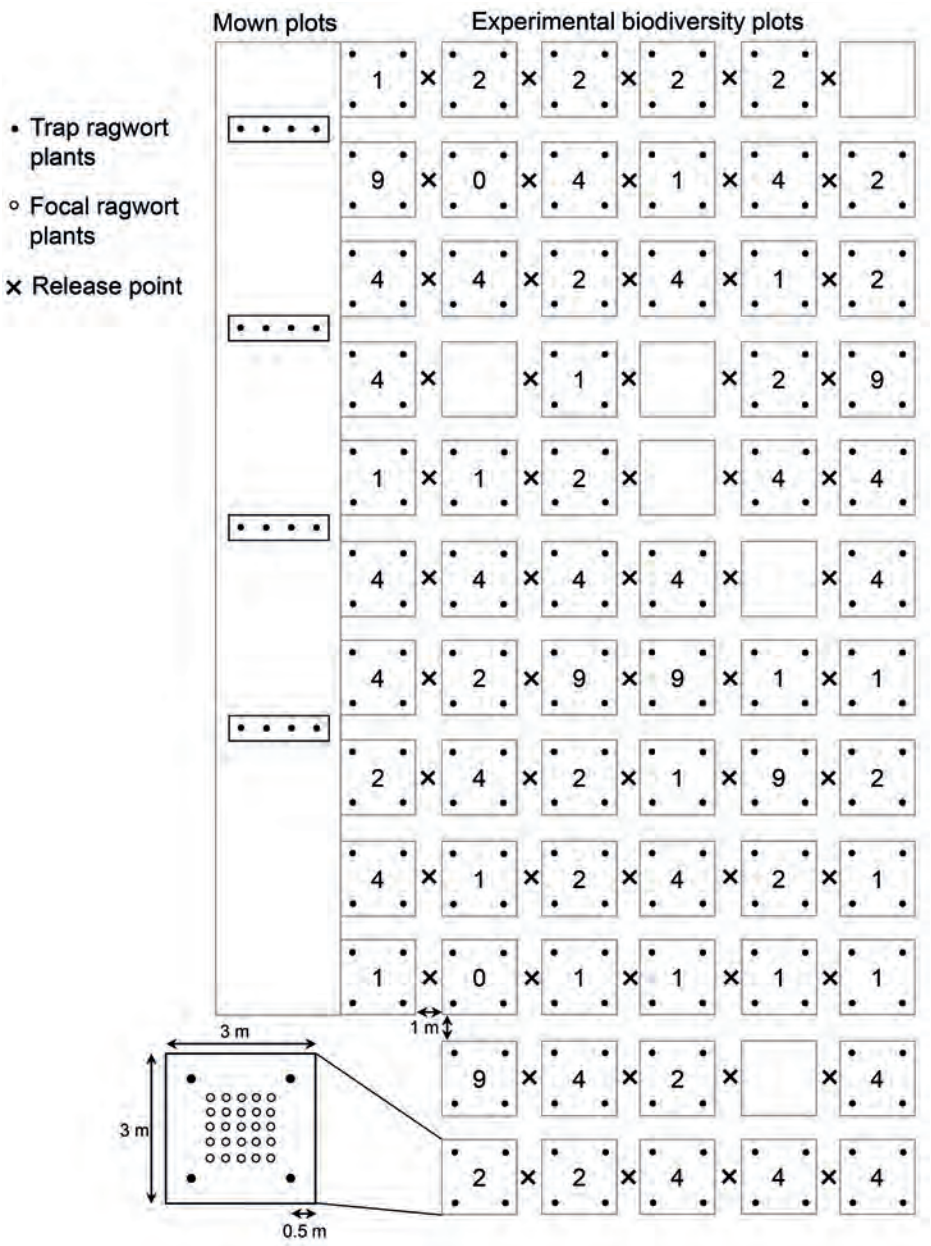


**Figure S6.1** Set-up of the release-recapture experiment. The × indicates locations where 250 *Dacnusa sibirica* adults were released. Closed circles indicate locations of the trap plants and open circles indicate locations of the focal plants (marked for one plot only). The number (1-9) in each square indicates the species richness of the surrounding vegetation; 0 indicates bare ground plots without the surrounding vegetation. Plots without numbers were not included in this study.

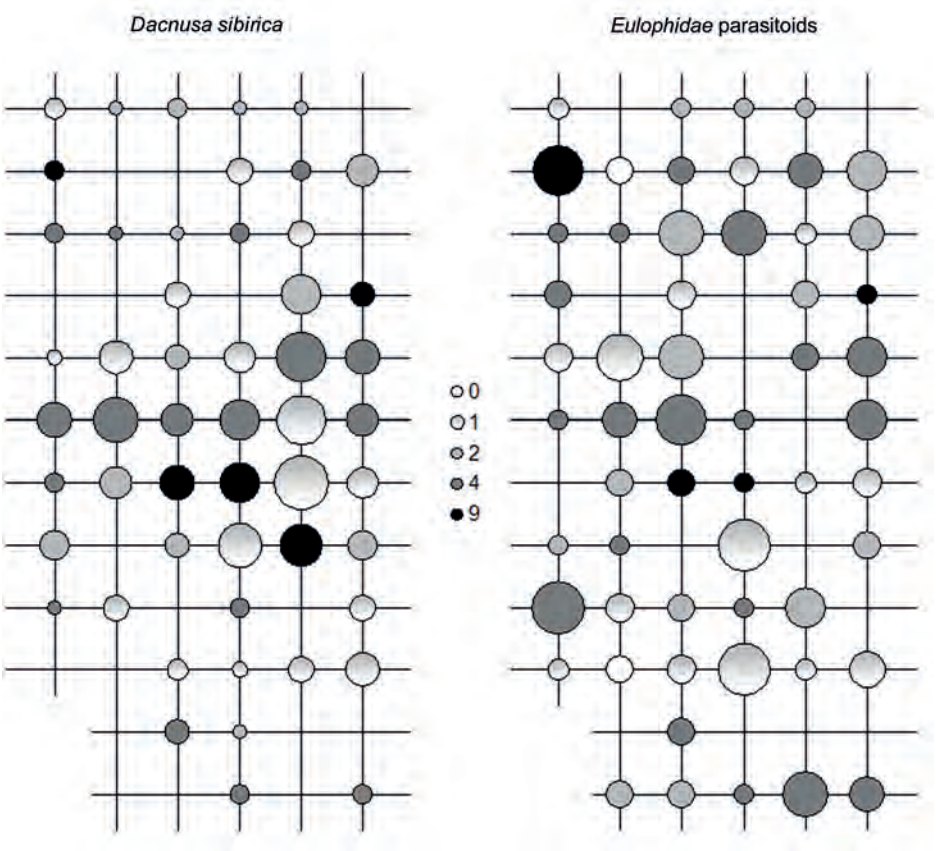


**Figure S6.2** Spatial distribution of the mean number of *Dacnusa sibirica* and *Eulophidae* parasitoids captured from the host-infested plants in experimental plots that differed in species richness of the surrounding plant community (from 1 to 9 species) and from bare ground plots. Each cross of vertical and horizontal lines designates an experimental plot. The size of the circle corresponds to the mean number of captured parasitoids per plant.

# Chapter 7

## Plant diversity and identity effects on entomopathogenic nematode infection and predatory nematode abundance in the soil

**Olga Kostenko, Henk Duyts, Saskia Grootemaat, Gerlinde B. De Deyn & T. Martijn Bezemer**

*Submitted in a slightly different form*

## Abstract

There is considerable evidence that plant diversity and identity influence the abundance of aboveground parasites and predators and the level of predation. However, how the abundance of predatory soil fauna, and the level of predation in the soil is related to plant diversity and identity is less well understood. Nematodes are important components of soil food webs comprising all major trophic levels, including predators, such as entomopathogenic nematodes (EPNs) which are natural enemies of soil insects, and carnivorous non-EPNs which feed mostly on other soil-dwelling nematodes. In a biodiversity field experiment where plant diversity and composition were manipulated, we examined the effects of plant diversity and identity on the EPN infectivity and abundance of carnivorous non-EPNs. In addition, we measured the abundance of soil insects and non-predatory nematodes and quantified root biomass production in the experimental plots to get a comprehensive view of the potential prey or food availability. We used structural equation modelling (SEM) to investigate three possible pathways by which plant diversity may affect EPN infectivity and the abundance of carnivorous non-EPNs. EPN infectivity and the abundance of carnivorous non-EPNs were not directly related to plant diversity or the proportion of legumes, grasses and forbs in the community, however, the infectivity of the EPN *Steinernema* was higher in monocultures of *Festuca rubra* and *Trifolium pratense* than in monocultures of the other six species. SEM revealed that plant diversity indirectly affected the infectivity of the EPN *Heterorhabditis* via effects on the abundance of soil insects. No significant link was found between plant diversity and infectivity of *Steinernema* or the abundance of carnivorous non-EPNs in SEM. The abundance of nematodes inhabiting lower trophic levels were positively affected by the proportion of legumes in the community whereas insect densities increased with higher plant diversity. Our results show that there is no direct effect of plant diversity on the number of carnivorous nematodes in the soil but that the plant diversity effects on EPN infectivity can be mediated indirectly via changes in prey densities. Our study also shows that these indirect plant diversity effects on belowground biota can differ between organisms that belong to the same feeding guild, such as the EPNs *Steinernema* and *Heterorhabditis*.

## Introduction

Biodiversity is rapidly declining worldwide, and many studies have shown that this can result in significant negative effects on ecosystem processes, including economically important ecosystem services such as control of pest insects (Cardinale et al. 2003; Hooper et al. 2005; Brussaard 2012). Most studies investigating the effects of species loss on ecosystem services and processes have focused on the aboveground effects of plant species diversity. There is increasing evidence that a decline in plant species diversity negatively affects the abundance or diversity of predators and parasitoids of foliar feeding herbivores (Andow 1991; Thies & Tscharntke 1999; Landis et al. 2000; Haddad et al. 2009; Scherber et al. 2010; Borer et al. 2012). However, how the abundance of predatory soil organisms and the level of predation in the soil are related to the diversity and identity of the plant community is less well understood (but see Wardle & Yeates 1993; Wardle et al. 2003; Scherber et al. 2010). Here, we investigate the effects of plant diversity and identity on the infectivity of entomopathogenic nematodes (EPNs) and the abundance of carnivorous non-entomopathogenic nematodes.

Entomopathogenic nematodes are natural enemies of insects or other arthropods that live in the soil or close to the soil surface (Kaya & Gaugler 1993; Gaugler 2002). EPNs are present in the soil of most terrestrial ecosystems and used in pest management programs worldwide. Studies that have estimated the effects of intercropping on the presence and infectivity of EPNs show that heterogeneous vegetation in agricultural systems can serve as a refuge for EPNs (Lawrence et al. 2006; Jabbour & Barberchek 2008). However, less is known about the role of EPNs in natural plant communities and how the infectivity and natural occurrence of EPNs is related to the diversity or composition of the plant community.

Carnivorous non-entomopathogenic nematodes feed predominantly on other nematodes and have evolved special features for ingesting nematode prey, such as root-feeding, bacterivorous, fungivorous and omnivorous nematodes (Yeates et al. 1993). Previous studies on effects of plant diversity on non-entomopathogenic nematodes mainly focused on functional shifts in nematode composition and have reported weak or non-existing effects of plant diversity on carnivorous non-entomopathogenic nematodes (e.g., De Deyn et al. 2004b; Viketoft et al. 2009; Eisenhauer et al. 2011a,b). However, the mechanisms of these weak responses have remained largely unclear, and, as far as we are aware, the effects of plant diversity on the infectivity of EPNs, i.e. their functioning rather than the effects on carnivore abundance, has not been examined yet.

Root-feeding arthropods and nematodes use plant roots as a food source and can be directly affected by changes in root diversity or biomass production (De Deyn et al. 2004a). Increases in root biomass can also indirectly cause an increase in the abundance of organisms that are part of the decomposer subsystem of the soil food web, such as bacterivorous and fungivorous nematodes, via increased amounts of litter or root exudates that serve as the basal resource for decomposition (Eisenhauer et al. 2011a,b). According to the diversity-trophic structure hypothesis (Hutchinson 1959), such increases in lower trophic level soil organisms may then positively affect predatory soil organisms, as their prey density increases. Alternatively, increases in plant diversity and biomass production may affect the abundance of soil predatory organisms directly, for example, by providing habitat or refuge in the case of abiotic extremes or competition (Lawrence et al. 2006). In turn, increases in the abundance of predators in the soil can potentially lead to increased predation rates and as a result lower prey abundance (Siemann 1998; Preisser 2003). Therefore, the relationship between plant diversity, productivity and higher trophic levels comprises a complex network of direct and indirect links and it is not known how the interactions in these multitrophic networks operate. Here we use structural equation modelling (SEM) to examine plant diversity effects on belowground multitrophic networks with a particular focus on entomopathogenic and other carnivorous nematodes. SEM is a multivariate method that can be used to examine how alternative pathways with direct and indirect relationships in networks may contribute to the observed species responses to experimental treatments (Grace 2006).

Many studies have argued that the effects of plant diversity on other organisms are not directly due to the number of plant species per se, but rather due to the abundance of certain plant species or functional groups in the plant community (e.g., Spehn et al. 2000a; Gastine et al. 2003; Wardle et al. 2003; De Deyn et al. 2004b; Viketoft et al. 2005; Viketoft et al. 2009). For example, aboveground invertebrate densities are often higher in plant communities that contain leguminous species, most likely because the nutritional quality of plant tissues is often higher in communities that contain nitrogen-fixing plant species (Mulder et al. 1999; Koricheva et al. 2000; Haddad et al. 2001). Belowground, the number of fungivorous nematodes in a long-term grassland experiment was enhanced in the presence of forbs, whereas bacterivorous nematodes were positively related to the presence of legumes (Viketoft et al. 2009). Whether the infectivity of EPNs in the soil is affected by the functional composition or plant species identity remains largely unknown.

In this study, we use a grassland biodiversity experiment, in which the diversity of plant community was manipulated and maintained, to examine the effects of plant diversity and identity on the abundance of free-living nematodes and soil insects. We determined the number of root-feeding, fungivorous, bacterivorous, omnivorous and carnivorous non-EPNs in soil samples and measured the EPN infectivity using a bioassay with wax moth (*Galleria mellonella* L.) larvae. Based on the findings of previous studies, we hypothesized that (i) increased plant diversity will enhance EPN infectivity, the abundance of carnivorous non-EPN and other nematodes, abundance of soil insects, and root biomass and that (ii) the effects on soil organisms will differ between monocultures of different plant species. Further, we hypothesized that (iii) the plant functional groups will strongly affect the densities of belowground organisms, in particular, the abundances of soil organisms will be positively related to the cover of legumes in the plant community. Finally, we examined whether the relationship between plant species richness and predation in the soil could be explained by changes in root biomass and/or prey abundances.

## Materials and Methods

*Entomopathogenic nematodes*

EPNs of the genera *Steinernema* and *Heterorhabditis* (Rhabditida: Steinernematidae and Heterorhabditidae) are roundworms that spend part of their life cycle in soil as free-living non-feeding infective juveniles (Kaya & Gaugler 1993). After they have entered an arthropod host, the developing juveniles release bacterial symbionts (Enterobacteriaceae) that kill the host and convert host tissues to a suitable nutrient substrate for EPNs. The nematodes feed on the bacteria and on partly decomposed insect tissues. Within the host, EPNs go through several reproduction cycles and multiply rapidly. When the resources inside the insect cadaver become depleted, juvenile EPNs emerge from the host and these juveniles can remain in the soil for several months (Kaya & Gaugler 1993). *Heterorhabditis* and *Steinernema* EPNs differ in their host-finding strategy (Gaugler et al. 1997; Lewis et al. 2006). Several *Steinernema* species are located close to the soil surface and use an ambusher foraging strategy, whereas members of *Heterorhabditis* genus employ a so-called "cruising foraging" strategy and are able to move within the soil profile (Gaugler et al. 1997). EPNs are sensitive to abiotic factors, such as temperature and moisture, and biotic factors such as competition and natural enemies, that can affect the survival of EPNs (Gaugler 2002). For long-term persistence, EPNs rely on high population densities of suitable hosts, as well as on plants that create favourable abiotic conditions in the soil (Kaya & Gaugler 1993).

*Field site and sampling*

A detailed description of the design of the field experiment has been presented elsewhere (Kostenko et al. 2012a). In brief, in 2008, seventy experimental plots of 3 × 3 m separated by 1-m-wide lanes were set-up in a nature restoration grassland area that had been agricultural land until 1996 (De Mossel, Ede, the Netherlands). The experimental area was fenced to exclude large vertebrate herbivores. The plots were sown with 1, 2, 4, or 9 plant species drawn from a pool of 12 grassland species including three grasses (*Anthoxanthum odoratum* L., *Agrostis capillaris* L., and *Festuca rubra* L.), three legumes (*Lotus corniculatus* L., *Trifolium arvense* L., and *Trifolium repens* L.), and six forbs (*Achillea millefolium* L., *Hypochaeris radicata* L., *Leucanthemum vulgare* Lamk., *Tanacetum vulgare* L., *Tripleurospermum maritimum* L. W.D.J. Koch and *Plantago lanceolata* L.). Each diversity level was replicated with several different mixtures in order to avoid confounding effects of species identity and species richness. Each of the sown plant species mixtures and monocultures was replicated twice using a complete randomized design. Due to poor establishment in monospecific plots, there were no monocultures of *A. odoratum*, *A. capillaris*, *T. arvense* and *T. maritimum*, but these species were present in the mixtures. There were 16 plots with monocultures, 18 plots with two species, 22 plots with four, and nine plots with nine species. In addition, four plots were kept free of all vegetation and served as "bare soil" treatment. Experimental plots were not mown, but hand-weeded during the growing season in 2009 and 2010 (from the end April until end August) to maintain the sown species composition. All soil samples were collected in September 2010.

*Infection Bioassay*

Twenty five soil cores of 15 cm depth and 5 cm diameter were collected from the inner 2.5 × 2.5 m square of each experimental plot in a regular 0.5 × 0.5 m grid. The samples were pooled per plot. To assess the EPN infectivity in the experimental plots we exposed the final instar of the greater wax moth's larvae (*G. mellonella*) to soil collected from the experimental plots (Bedding & Akhurst 1975). The insect larvae were obtained from Kreca V. O. F. (Ermelo, the Netherlands). Plastic containers of 10 × 10 × 5 cm were filled with 250 g soil from each plot and adjusted to field capacity (15%) by adding de-mineralized water. There were four containers per plot. Into each container, four *G. mellonella* were placed on the soil surface, the containers were closed and flipped over so that the larvae were covered by soil. The containers were kept in a dark climate chamber under controlled conditions at 22 °C, 50-60% humidity. After one week, all the larvae were retrieved from the soil and incubated individually in the labelled plastic vials (3 cm diameter, 5 cm height) in the climate chamber. Seven days later, all larvae were dissected and examined using a stereo microscope in

order to assess infection by *Heterorhabditis* or *Steinernema* EPNs. Assessments were based on the colour of the cadaver and the morphology of adult nematodes found in the dissected larvae (Stock & Hunt 2005). Because EPNs typically kill their hosts within 48 h (Kaya & Gaugler 1993), the two weeks scoring period virtually assured that we observed all nematode-imposed mortality. We also recorded whether larvae died from fungal or bacterial infection or were healthy. We then calculated the percentage of EPN-infected larvae and the percentage of larvae that died of other causes.

*Soil nematode extraction and identification*

The soil was subsampled from the pooled soil collected for EPN infectivity bioassay. Before the nematode extraction, soil moisture content was determined on a soil subsample of each plot by drying 50 g of fresh soil during three days at 120 °C. Nematodes were extracted from 100 ml (approximate 100 to 110 g, the exact weight was recorded) fresh soil using Oostenbrink elutriators (Oostenbrink 1960). The suspensions with nematodes were run through a series of 75 $\mu$m and 45 $\mu$m mesh-sized sieves and a double cotton filter on a sieve in a dish with a 100 ml layer of tap water. The nematodes were allowed to migrate through the filter into the water for 24 h at room temperature. The nematodes were collected in 100 ml jars and concentrated into 10 ml vials and subsequently into 2 ml of water by letting the nematodes settle to the bottom of the jars/vial and careful removal of the top layer of water. The concentrated nematode samples were then fixated by adding 4 ml of hot and 4 ml of cold 4% formalin. The total number of nematodes were then determined for each soil sample using a reversed-light microscope. Number of nematodes was calculated per 100 g fresh soil. Nematodes were categorized into feeding guilds according to Yeates et al. (1993), Andrassy (2005) and personal communication with Tom Bongers (Table S7.1, Supporting Information). We considered nematodes as being carnivorous if there is evidence that they consume other nematodes, although some of the listed carnivores might also feed on other organisms, e.g., bacteria (see Table S7.1 for details).

*Root biomass*

To determine community standing root biomass in each plot, three soil cores of 10 cm depth and 2.5 cm diameter were taken 1 m apart along a diagonal transect within each plot that started 50 cm from the edge of the plot. In the laboratory, the weight of the soil in each core was determined, and all root material was washed, oven-dried at 70 °C and weighed. Total root biomass was calculated as root dry weight per 100 g soil.

*Soil insects*

To estimate the abundance of soil-dwelling insects, four soil cores of 12.5 cm diameter and 15 cm deep were collected from four randomly selected locations within the inner 2.5 m × 2.5 m square of each plot. In the laboratory, each soil sample was weighed and then hand-sorted. All visible arthropods were collected and stored in 70% ethanol in labelled Eppendorf tubes. The arthropods were categorized as white grub larvae (scarab beetle larvae), wireworms (Elateridae beetle larvae), other insect larvae (Lepidoptera, Diptera, and other Coleoptera) and adult beetles (Coleoptera). The abundance of soil insects was expressed per 100 g fresh soil.

*Statistical analyses*

All statistical analyses were performed using R statistical language, version 2.15.1 (R Development Core Team 2012). Percentage data were arcsine square root-transformed, biomass and nematode data were log-transformed and insect data were square-root transformed prior to analysis to meet the requirements of normality and homoscedasticity of errors. If the assumptions were still violated, non-parametric tests were used to analyze the data (for these analyses $\chi^2$ are reported). Because there were four containers per plot, the effects of plant species diversity, monoculture identity and proportion of legumes, grasses or forbs in the vegetation on percentage EPN infectivity were analyzed using linear mixed models with plot identity as random factor. General linear models were used to test the effects of plant species diversity and proportion of legumes, grasses or other forbs in the vegetation on nematode and insect abundances, root biomass and soil moisture content. Plant species richness was included as continuous variable to test for linear effects. We also repeated the analyses by excluding the bare plots. Individual comparisons were based on a Tukey HSD test. Due to the low number of insects recovered from monocultures, the effects of monoculture identity on the soil insect abundance were not tested.

*Structural equation modelling*

We tested three alternative hypotheses linking plant species richness to EPN infectivity or predatory nematode abundance via changes in prey abundance (model A, Fig. 7.1); via changes in root biomass (model B, Fig. 7.1); and via changes in root biomass that subsequently controls prey abundance (model C, Fig. 7.1). There was no significant effect of plant species richness on the % EPN infectivity or predatory nematode abundance in our study (see results). This supported results from other studies (De Deyn et al. 2004b; Viketoft et al. 2009; Scherber et al. 2010). Therefore, we excluded a direct path from plant species richness to the % EPN infectivity or predatory nematode abundance from the initial model (Fig. 7.1).

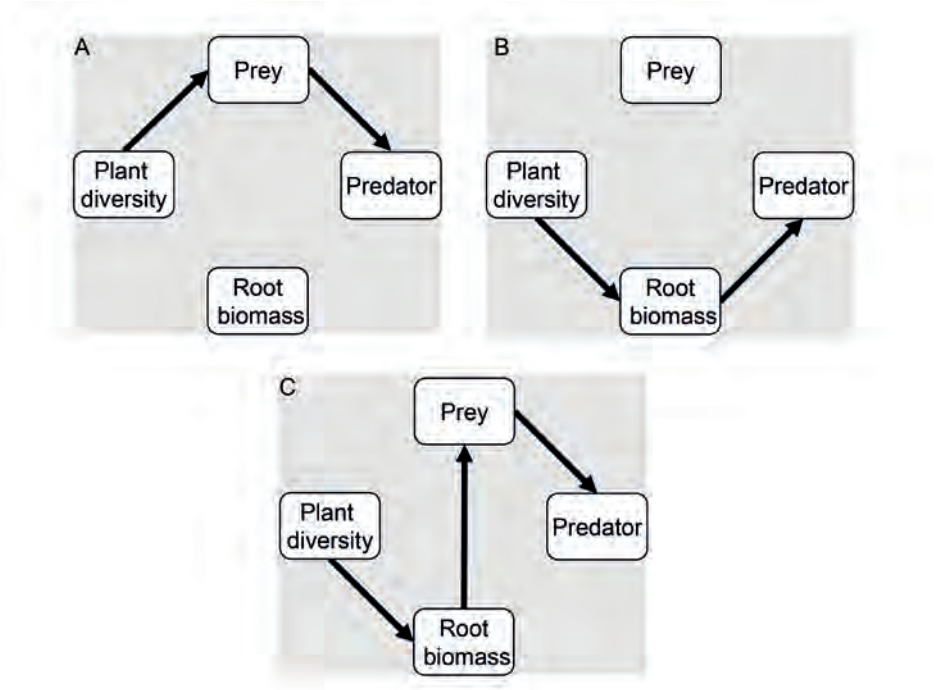


**Figure 7.1** Three alternative hypothetical pathways between plant species richness, root biomass, prey and predator abundances that were tested by structural equation modelling. The hypothesis A, B and C are explained in the text.

The reciprocal effects of soil insects or root-feeding nematodes on plant root biomass and higher trophic level organisms on their prey were also excluded from the initial model because plant diversity was experimentally manipulated in our study. Separate models were developed for *Heterorhabditis* infectivity, *Steinernema* infectivity, and abundance of non-entomopathogenic carnivorous nematodes. For EPN infectivity models, we included soil insects as prey; and for non-entomopathogenic nematodes models, we included the total of root-feeding, bacterivorous and fungivorous nematodes as prey. Omnivorous nematodes were not included in the model as they also can feed on other food sources, such as bacteria or fungi. All plots were used in the analysis. We used the log-transformed data for plant species richness and root biomass, the square-root transformed insect and nematode abundances and arcsine square-root transformed % EPN infectivity. We used likelihood ratios and chi-squared tests to determine if the model-implied variance-covariance matrix differed from the observed variance-covariance matrix and to perform model simplification. We removed non-significant terms from the initial model and selected the model that best fitted our data. This model was used to determine which of the proposed

hypothesis best explained the relationship between plant species richness and EPN infectivity or carnivorous non-EPN abundance. SEM was performed using *sem* package for R.

## Results

*Plant diversity and functional group effects*

Average total mortality of *Galleria* larvae in the bioassay was 78%, of which 21% were infected by *Heterorhabditis* and 12% by *Steinernema* while the other 43% died of unknown causes. Neither plant species richness nor the proportion of plant functional groups in the mixtures significantly affected infectivity by *Heterorhabditis* spp. (Table 7.1). However, the infectivity by EPNs was on average three times lower in the bare compare to vegetated plots (0.11 ± 0.03% and 0.27 ± 0.03% respectively). When the bare plots were excluded from the analysis, the effect of plant species richness remained non-significant (all $P > 0.05$; Table 7.1). There was also no significant effect of plant species richness on the infectivity of *Steinernema* spp. (Table 7.1). However, the infectivity of *Steinernema* spp. was less in plots where forbs were highly abundant; this effect was significant only when bare plots were excluded from the analysis (Table 7.1). The percentage of the larvae that died due to other causes was not affected by plant species richness or by the abundance of different functional groups of plants (Table 7.1).

The abundance of all but carnivorous non-entomopathogenic nematodes increased significantly with plant diversity but the effect became non-significant when the bare plots were excluded from the analysis (Table 7.1, Fig. 7.2). Nematodes of the family *Mononchidae* were the most dominant carnivorous non-entomopathogenic nematodes in our study (Table S7.1). The abundance of *Mononchidae* was highest in bare plots (227 ± 56 nematodes per 100 g soil) and lowest in nine species plots (81 ± 21 nematodes per 100 g soil), however, there was no significant effect of plant species richness on the *Mononchidae* abundance ($F_{1,61} = 0.56$, $P = 0.46$). Carnivorous nematodes of genus *Aporcelaimus* were also abundant in the biodiversity plots but their abundance did not differ between the plots with different diversity levels (data not shown). Carnivorous nematodes of genera *Nygolaimus*, *Paraxonchium* and *Sectonema* were not found in the bare plots (data not shown). There was a positive relationship between the proportion of legumes in a plant community and abundance of root-feeding, bacterivorous and fungivorous nematodes. This was also true when bare plots were not included in the analysis (Table 7.1). The proportion of grasses negatively affected fungivorous nematode abundance (Table 7.1). The abundance of root-feeding nematodes decreased with increasing proportion of forbs and increased with increasing proportion of grasses (Table 7.1).

**Table 7.1** Effects of plant species richness, proportion of legumes, grasses and forbs on the infectivity of entomopathogens, abundance of other nematodes, soil insect abundance and root community biomass. F-values are shown of linear mixed models for infectivity of entomopathogenic nematodes and other mortality causes and general linear models for other response variables. The respective response variable in those models was fitted first.

| | Plant species richness | Legumes | Grasses | Forbs |
|---|---|---|---|---|
| **Bare plots included** | | | | |
| Infectivity | | | | |
| *Heterorhabditis* | 1.15 | 0.003 | 1.85 | 0.09 |
| *Steinernema* | 1.25 | 3.26 | 1.32 | 3.27 |
| Other mortality | 0.46 | 0.22 | 0.19 | 0.013 |
| Soil biota abundance | | | | |
| Root-feeding nematodes | **↑5.47*** | **↑16.39***** | **↑5.79*** | **10.39**↓** |
| Bacterivorous nematodes | **↑7.90**** | **↑9.44**** | 2.03 | 0.0006 |
| Fungivorous nematodes | **↑7.20**** | **↑9.51**** | **4.84*↓** | 2.30 |
| Omnivorous nematodes | **↑5.36*** | 1.89 | 0.90 | 2.23 |
| Carnivorous nematodes | 0.91 | 0.41 | 0.24 | 2.80 |
| Insect abundance | **↑5.89*** | 0.68 | 1.75 | 0.14 |
| Root biomass | 1.74 | 0.0004 | **↑4.51*** | 0.0003 |
| **Bare plots not included** | | | | |
| Infectivity | | | | |
| *Heterorhabditis* | 0.16 | 0.051 | 1.32 | 0.58 |
| *Steinernema* | 0.60 | 2.90 | 1.08 | **4.88*↓** |
| Other mortality | 0.01 | 0.10 | 0.08 | 0.23 |
| Soil biota abundance | | | | |
| Root-feeding nematodes | 0.61 | **↑13.65***** | **↑4.05*** | **20.55***↓** |
| Bacterivorous nematodes | 3.59 | **↑7.82**** | 3.07 | 0.65 |
| Fungivorous nematodes | 0.43 | **↑6.39*** | **7.32**↓** | 0.06 |
| Omnivorous nematodes | 0.33 | 0.90 | 2.12 | 0.17 |
| Carnivorous nematodes | 1.17 | 0.71 | 0.48 | 1.63 |
| Insect abundance | **↑(3.69)*** | 0.99 | 1.22 | 0.01 |
| Root biomass | 0.22 | 0.19 | 2.81 | 1.13 |

Asterisks indicate significant effect at *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; the brackets indicate marginally significant effect at $P < 0.06$; the absence of asterisks indicates no significant effect. ↑ indicates positive effect and ↓ indicates negative effect.

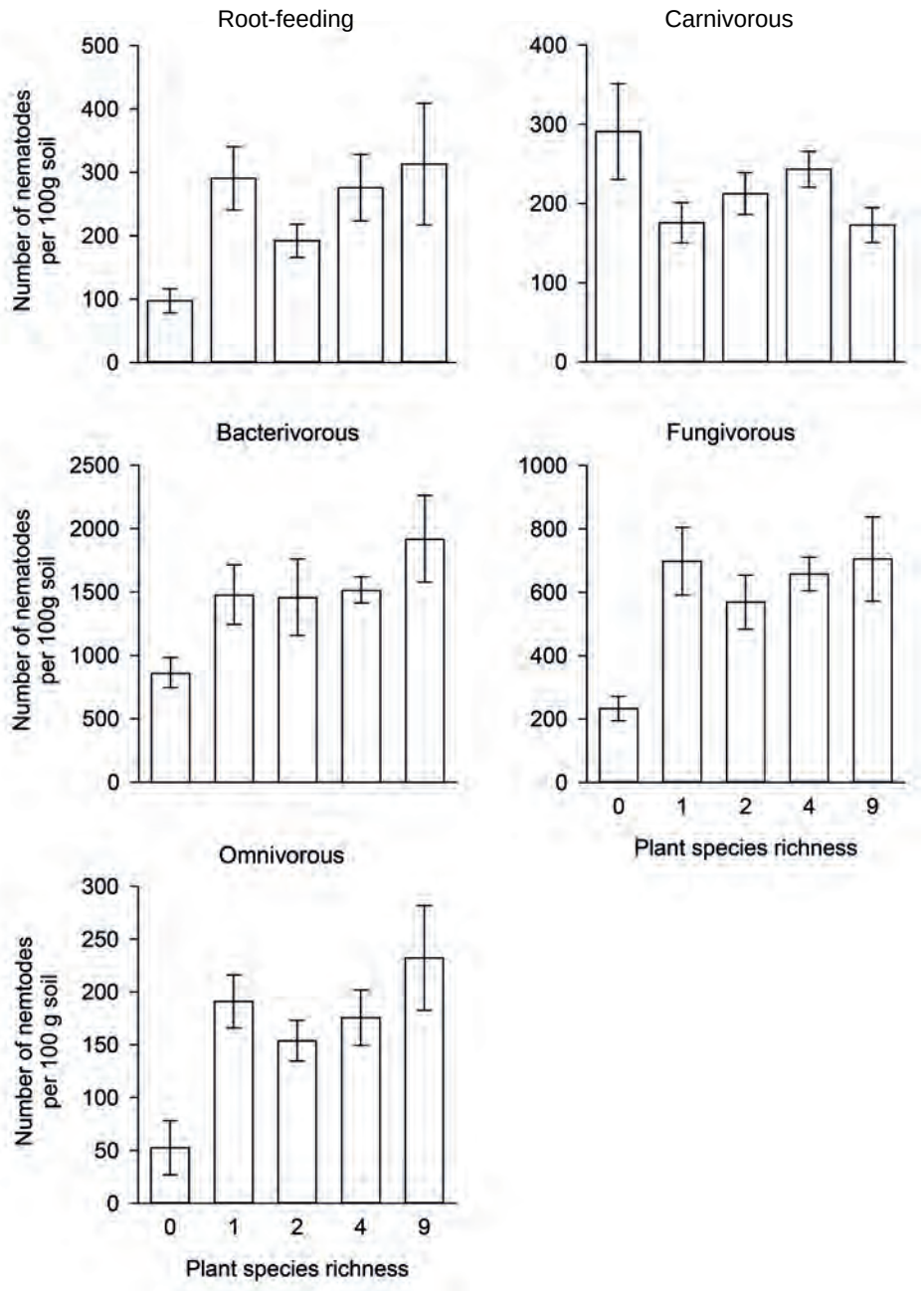


**Figure 7.2** Effect of plant species richness on the abundance of root-feeding, bacterivorous, fungivorous, omnivorous and carnivorous non-entomopathogenic nematodes. Means ± SE are shown. The number of nematodes was calculated per 100 g fresh soil. Results of statistical tests are presented in Table 7.1.

The majority of insects that were recovered from the soil were white grubs. No insects were recovered from the soil collected from bare plots (Fig. 7.3). There was a positive relationship between insect abundance and plant species richness when bare plots were included in the analysis (Table 7.1, Fig. 7.3). This relationship was marginally significant when bare plots were excluded from the model ($P = 0.059$). The density of soil insects was not affected by the abundance of any of the three plant functional groups in the plant community (Table 7.1).

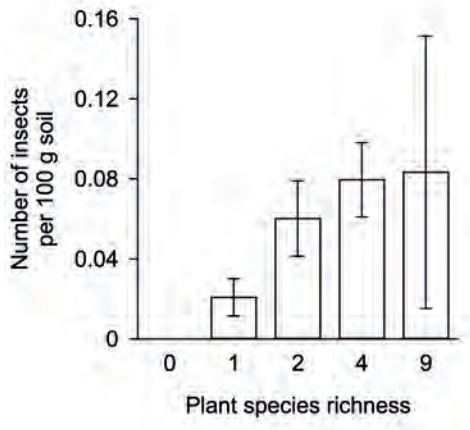


**Figure 7.3** Effect of plant species richness on abundance of soil insects. Means ± SE are shown. Results of statistical tests are presented in Table 7.1.

There was no relationship between plant species richness and root biomass (Table 7.1, Fig. 7.4A). However, root biomass positively correlated with the proportion of grasses in the community (Table 7.1). Soil moisture content was not related to the diversity or identity of plant community (all $P > 0.05$, data not shown).

*Monoculture identity effects*

Infectivity of *Heterorhabditis* spp. did not differ among monocultures ($F_{7,8} = 0.31$, $P = 0.93$), but the infection rate by *Steinernema* varied significantly among monocultures ($F_{7,8} = 3.67$, $P = 0.044$; Fig. 7.5). Infectivity of *Steinernema* spp. was highest in the monocultures of *F. rubra* and *T. repens*. Mortality of the wax moths larvae due to other causes did not differ among monocultures ($F_{7,8} = 1.27$, $P = 0.37$). Root-feeding ($\chi^2_7 = 10.42$, $P = 0.17$), bacterivorous ($\chi^2_7 = 10.63$, $P = 0.16$), fungivorous ($\chi^2_7 = 10.35$, $P = 0.17$), omnivorous ($\chi^2_7 = 5.91$, $P = 0.55$), and carnivorous non-EPN ($\chi^2_7 = 3.00$, $P = 0.89$) densities did not differ between monocultures. Root biomass differed significantly between monocultures ($F_{7,8} = 5.48$, $P = 0.014$; Fig. 7.4B) and was highest in monocultures of *H. radicata* and *P. lanceolata*.

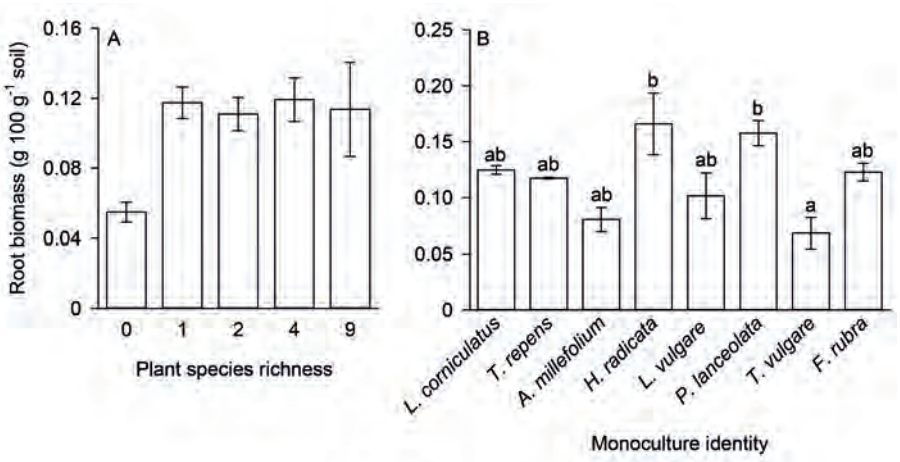


**Figure 7.4** Effect of (A) plant species richness and (B) monoculture identity on root biomass. Means ± SE are shown. Different letters denote significant differences between monocultures ($P < 0.05$) based on a Tukey HSD test.

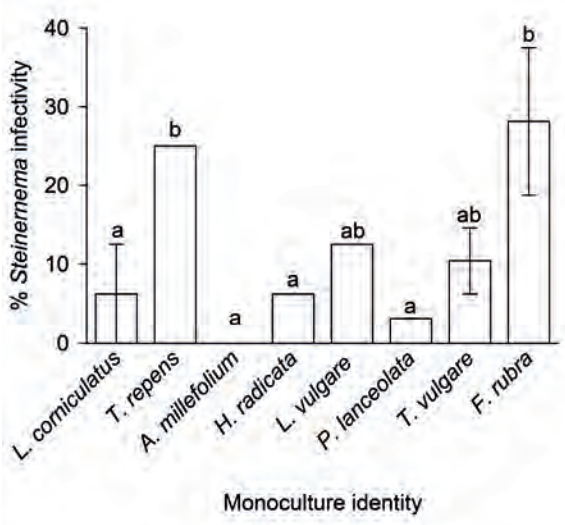


**Figure 7.5** Effect of monoculture identity on mortality of *G. mellonella* larvae caused by *Steinernema* spp. Means are calculated based on average values per plot ± between plot SE (if SE is not displayed the mortality of *G. mellonella* larvae was equal in both plots). Different letters denote significant differences between monocultures ($P < 0.05$) based on linear mixed model with plot identity as random factor.

*Structural equation modelling*

In the final SEM ($\chi^2_3 = 1.15$, $P = 0.77$) for *Heterorhabditis* spp., 10% of the variation in percentage EPN infectivity could be explained by plant species richness and soil insect abundances (Fig. 7.6A). For *Steinernema* spp. ($\chi^2_3 = 3.37$, $P = 0.50$), there was no significant pathway associated with percentage EPN infectivity in the final model (Fig. 7.6B). The only significant pathway that remained in this model was between plant species richness and soil insect abundance ($P = 0.013$) that explained 9% of the variation in the soil insect abundance. The final SEM for carnivorous non-EPN ($\chi^2_3 = 1.08$, $P = 0.78$) also did not reveal a significant pathway associated with carnivorous non-EPN nematode abundance (Fig. 7.6C), but there was a direct significant link between plant species richness and the abundance of non-carnivorous nematodes ($P = 0.0050$; Fig. 7.6C) that explained 10.8% of the variation in their abundance.

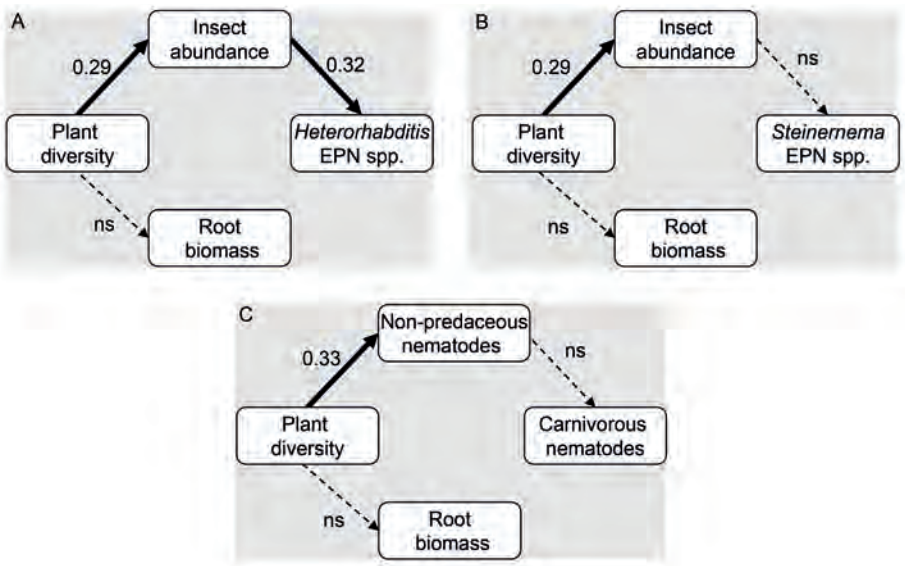


**Figure 7.6** Final structural equation models for infectivity of (A) *Heterorhabditis* spp., (B) *Steinernema* spp., (C) abundance of carnivorous non-EPN nematodes. Solid arrows indicate significant effects (at $P < 0.05$); dashed arrows represent non-significant effects kept in the model (ns) and the absence of arrows represents non-significant effects that were removed from the model. Values associated with solid arrows denote standardized path coefficients.

## Discussion

In our study, the infectivity of EPN spp. and abundance of carnivorous non-entomopathogenic nematodes were not directly affected by plant species richness. Other studies also found no or weak effects of plant species richness on higher trophic level nematodes (e.g., Wardle et al. 2003; De Deyn et al. 2004b; Viketoft et al. 2009 but see Spehn et al. 2000; Eisenhauer et al. 2011a). However, in our experiment plant diversity positively affected the abundance of soil insects and nematode prey. Although there was no direct effect of plant species richness on the infectivity of EPN spp., the structural equation models revealed a significant indirect effect of plant diversity on *Heterorhabditis* infectivity via changes in the abundance of soil insects. These effects of plant diversity on EPNs are in line with the diversity-trophic structure hypothesis, which states that a greater number of resources support a greater number of consumers (Hutchinson 1959). Plant diversity effects, neither directly nor indirectly, affected infectivity by *Steinernema*, so that plant diversity effects might be EPN-genus, or even species-specific.

The infection rates of wax moth larvae by *Heterorhabditis* spp. were higher than by *Steinernema* spp. but in general the infection rates for both genera were low. Although EPNs are widely distributed in soils of all sorts of ecosystems, there is considerable variability in EPN distribution across seasons and habitats (Stuart & Gaugler 1994; Spiridonov et al. 2007). Therefore, the low abundance and inconsistent results for the two EPN genera in our study may be the result of differences in local densities and patchy distributions of EPN populations. Alternatively, the different responses of EPNs could be due to differences in abiotic conditions or prey availability in the field. Soil moisture is one of the most important abiotic parameters for EPN survival (Gaugler 2002). However, there was no difference in the soil moisture content between different plots in our study. Therefore, we cannot attribute the variation in the EPN abundances to variation in soil moisture unless that operated at finer spatial and temporal scales than we could measure. The majority of insect prey found in our study was scarab beetle larvae that are feeding on plant roots and typical hosts of cruise-foraging nematodes, such as *Heterorhabditis*. Therefore, the difference in host suitability and life histories between the two EPN genera might explain differences in EPN responses in our study with *Heterorhabditis* responding more strongly to general insect host abundance than *Steinernema*.

In contrast to our hypothesis and in line with several other studies (e.g., Spehn et al. 2000; Gastine et al. 2003), belowground biomass did not increase with higher plant species richness at the time scale of our experiment. Moreover, while

we did not find significant effects of diversity on root biomass, SEM also did not reveal a significant relationship between abundance of nematodes and soil insects and root biomass. Therefore, these results suggest that soil nematodes and insects are not restricted by the quantity of primary resources and that belowground plant diversity effects are generally not mediated through root biomass (Verschoor et al. 2001; Bezemer et al. 2010). In contrast, in aboveground communities the effects of plant diversity on consumer diversity and abundance occur primarily via changes in plant production (Borer et al. 2012). It has been argued that root exudates or root quality may be more important for soil biota than root biomass per se (Verschoor et al. 2001; Viketoft et al. 2005; Eisenhauer et al. 2010a). Alternatively, to maintain the initial plant species composition the experimental communities were regularly hand-weeded and it is almost inevitable that part of the roots of the removed plants remained in the soil after weeding, even though the aboveground parts of these plants were removed entirely. This can also explain why there was some root biomass present in the bare plots in our experiment. These results emphasize that hand-weeding can cause perturbations in belowground systems that can obscure the "pure effect"of plant diversity in synthetic biodiversity experiments (Bezemer & Van der Putten 2007; Roscher et al. 2013). This will be the case in both seed addition and plant removal experiments.

Our study shows that the presence of particular plant functional groups often was more important for non-entomopathogenic nematodes than plant diversity per se. Prey abundance (root-feeding, bacterivorous and fungivorous nematodes) was positively influenced by the presence of legumes in the community. Prey abundance was also always higher in monocultures of one of the legume species *L. corniculatus* although in contrast to previous findings (De Deyn et al. 2004b; Viketoft et al. 2005; Viketoft et al. 2009) there was no significant difference in the abundance of non-carnivorous nematodes among monocultures in our study. Positive effect of legumes might be explained by the higher tissue nitrogen contents of the plant roots or litter that can lead to increased performance of root feeders and decomposers. Similarly, the infectivity by *Steinernema* spp. was relatively high in the monocultures of one of the leguminous species *T. repens*. However, we did not observe an overall positive effect of legumes on the infectivity of EPNs, probably due to inconsistent effects between individual legume species. Surprisingly, we did not observe an overall positive effect of legumes on the soil insect abundance. This can possibly be explained by the fact that root exudates of a large number of legumes contain isoflavonoids, which deter belowground insect larvae (Dakora 2003). Alternatively, this effect might also be obscured by the low number of soil insects retrieved from the field plots in our study. Finally, neither the abundance of higher trophic levels nematodes

was affected by the presence of certain functional group in the community or monoculture identity. Together these results suggest that site-specific differences such as pool of plant species, nematode species present and the history of the site are important for soil biota.

In our study the effect of monoculture identity was not consistent between the two genera of EPNs. The infectivity of EPNs of the genus *Steinernema* in contrast to the genus *Heterorhabditis*, differed significantly among different monocultures. The infectivity by *Steinernema* spp. was relatively high in the two monocultures of the grass species *F. rubra.* This might be explained by large amounts of fine roots produced by grass species altering soil structure and microclimate (but not soil moisture content) that potentially serves as refuges for EPNs (Eisenhauer et al. 2011a). In line with previous studies non-entomopathogenic nematodes also positively responded to the presence of grass species in the community (Verschoor et al. 2001; Wardle et al. 2003). Nematodes of genera *Paratylenchus* were the most dominant plant feeders in our study (Table S7.1) and the ectoparasitic *Paratylenchus* preferably feeds on grass roots (Korthals et al. 2001). However, we could not discriminate the effects of functional group from species identity for grasses as only the monoculture of *F. rubra* was used in our study. Interestingly, no infection of wax moth larvae by *Steinernema* occurred in the monocultures of *A. millefolium*, whereas other studies have shown that *A. millefolium* has a positive effect on free-living nematodes (Viketoft et al. 2005).

EPN and high trophic level nematodes are broadly used in biological control programmes to suppress pests of agricultural crops in soil and enhance crop yields (Peters 1996; Denno et al. 2008). In our study, where plant communities were manually manipulated we could not estimate the effect of predation on plant survival and productivity but our findings suggest that increasing plant diversity will indirectly positively affect EPN infectivity (in particular by *Heterorhabditis* spp.). Studies in which the abundance of EPNs or other nematodes was experimentally manipulated have demonstrated that the increased level of predation can strongly positively impact plant survival, productivity and diversity (Van der Putten & Van der Stoel 1998; Brussaard et al. 2001; Preisser et al. 2003; Khan & Kim 2007; Ram et al. 2008). It should also be emphasized that carnivorous non-EPN nematodes and EPNs are only part of the predatory soil community. Other groups of soil predators not estimated in our study (e.g., microarthropods), can also be affected directly or indirectly by plant diversity (Sabais et al. 2011). Ultimately, understanding the relationships between plant diversity and natural populations of predatory organisms in the soil may provide new insights in the functioning of soil communities and their use as biological control agents in managed and natural systems.

In conclusion, our results show that plant diversity effects on belowground communities are generally not mediated through root biomass. Plant functional group and the presence of vegetation are more important for belowground communities than plant diversity per se. Finally, increasing plant species diversity enhances the level of predation by *Heterorhabditis* EPNs in the soil indirectly by modifying the interactions with their prey, but does not affect predator abundance. However, the responses of belowground organisms to manipulation in plant diversity can be specific and may differ even between organisms that belong to different species but the same feeding guild, such as entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis*.

## Acknowledgements

We are grateful to Natuurmonumenten (Planken Wambuis) for permission to perform this study on their properties. We thank Ciska Raaijmakers, Chamila Darshanee and Sebastien Roumegous for technical assistance.

## Supporting Information

**Table S7.1** Number of nematodes per 100 g fresh soil (mean ± SE between plots), feeding type and taxa composition according to Yeates et al. (1993), Bongers (1994) and Andrássy (2005). The soil cores (15 cm depth and 5 cm diameter) were collected from the inner 2.5 × 2.5 m square of each experimental plot (3 × 3 m) in a regular 0.5 × 0.5 m grid.

| Order | Family | Genus | Abundance ± SE |
|---|---|---|---|
| **Root-feeding** | | | |
| Diphtherophorida | Trichodoridae | *Trichodorus* | 6.2 ± 1.6 |
| Dorylaimida | Nordiidae | *Pungentus* | 21.7 ± 2.9 |
| Tylenchida | Anguinidae | *Ditylenchus* | 7.0 ± 1.3 |
| Tylenchida | Hoplolaimidae | *Helicotylenchus* | 2.6 ± 1.6 |
| Tylenchida | Meloidogynidae | *Meloidogyne* | 36.8 ± 6.0 |
| Tylenchida | Paratylenchidae | *Paratylenchus* | 93.1 ± 18.8 |
| Tylenchida | Pratylenchidae | *Pratylenchus* | 33.7 ± 7.6 |
| Tylenchida | Tylenchidae | *Filenchus* | 14.1 ± 2.4 |
| Tylenchida | Tylenchidae | *Malenchus* | 0.2 ± 0.2 |
| Tylenchina | Telotylenchidae[a] | *Tylenchorhynchus* | 15.8 ± 4.0 |
| Tylenchida | Tylenchidae | Other Tylenchidae | 12.3 ± 3.0 |
| **Bacterivorous** | | | |
| Alaimida | Alaimidae | *Alaimus* | 13.3 ± 1.9 |
| Alaimida | Amphidelidae[e] | *Paramphidelus* | 0.6 ± 0.4 |
| Araeolaimida | Bastianiidae | *Bastiania* | 1.9 ± 1.0 |
| Araeolaimida | Cylindrolaimidae | *Cylindrolaimus* | 4.2 ± 1.4 |
| Araeolaimida | Metateratocephalidae[d] | *Metateratocephalus* | 0.3 ± 0.3 |
| Araeolaimida | Plectidae | *Anaplectus* | 58.0 ± 8.5 |
| Araeolaimida | Plectidae | *Plectus* | 167.5 ± 19.6 |
| Araeolaimida | Plectidae | *Tylocephalus* | 46.1 ± 10.2 |
| Araeolaimida | Plectidae | *Wilsonema* | 26.8 ± 3.1 |
| Enoplida | Prismatolaimidae | *Prismatolaimus* | 50.1 ± 5.7 |
| Monhysterida | Monhysteridae | *Eumonhystera* | 4.9 ± 1.3 |
| Monhysterida | Monhysteridae | *Monhystera* | 0.8 ± 0.5 |
| Rhabditida | Cephalobidae | *Acrobeles* | 413.7 ± 24.1 |
| Rhabditida | Cephalobidae | *Acrobeloides* | 300.0 ± 21.3 |
| Rhabditida | Cephalobidae | *Acrolobus* | 2.1 ± 0.9 |
| Rhabditida | Bunonematidae | *Bunonema* | 3.1 ± 1.3 |
| Rhabditida | Cephalobidae | *Cervidellus* | 17.4 ± 3.3 |
| Rhabditida | Cephalobidae | *Chiloplacus* | 6.3 ± 1.8 |
| Rhabditida | Cephalobidae[b] | *Eucephalobus* | 116.4 ± 18.5 |
| Rhabditida | Mesorhabditidae[c] | *Mesorhabditis* | 1.7 ± 0.9 |
| Rhabditida | Panagrolaimidae | *Panagrolaimus* | 83.5 ± 16.4 |
| Rhabditida | Rhabditidae | | 139.7 ± 29.4 |
| Rhabditida | Teratocephalidae | *Teratocephalus* | 6.3 ± 3.1 |

Table S7.1 continued

| | | | |
|---|---|---|---|
| **Fungivorous** | | | |
| Aphelenchida | Aphelenchidae | *Aphelenchus* | 341.7 ± 26.8 |
| Aphelenchida | Aphelenchoididae | *Aphelenchoides* | 230.1 ± 25.5 |
| Diphtherophorida | Diphtherophoridae | *Diphterophora* | 22.8 ± 2.9 |
| Dorylaimida | Tylencholaimidae[f] | *Tylencholaimus* | 5.2 ± 1.3 |
| **Omnivorous** | | | |
| Dorylaimida | Dorylaimidae[i] | *Mesodorylaimus* | 12.3 ± 3.2 |
| Dorylaimida | Qudsianematidae | *Crassolabium*[j] | 111.0 ± 9.0 |
| Dorylaimida | Qudsianematidae | *Dorydorella* | 9.8 ± 2.2 |
| Dorylaimida | Qudsianematidae[h] | *Ecumenicus* | 20.2 ± 3.6 |
| Dorylaimida | Qudsianematidae | *Epidorylaimus* | 1.2 ± 0.7 |
| Dorylaimida | Qudsianematidae | *Eudorylaimus* | 2.8 ± 0.8 |
| Dorylaimida | Qudsianematidae | *Microdorylaimus* | 14.6 ± 3.2 |
| **Carnivorous** | | | |
| Dorylaimida | Aporcelaimidae | *Aporcelaimus* | 55.6 ± 4.2 |
| Dorylaimida | Mydonomidae[g] | *Dorylaimoides* | 15.9 ± 4.2 |
| Dorylaimida | Nygolaimidae | *Nygolaimus* | 0.3 ± 0.3 |
| Dorylaimida | Nygolaimidae | *Sectonema* | 0.4 ± 0.3 |
| Dorylaimida | Paraxonchiidae[k] | *Paraxonchium* | 4.7 ± 1.3 |
| Mononchida | Mononchidae | | 137.4 ± 12.6 |
| Rhabditida | Neodiplogastridae | | 7.3 ± 1.8 |

According to Bongers (1994): [a]Dolichodoridae; [b]Diplopeltidae; [c]Rhabditidae; [d]Teratocephalidae; [e]Alaimidae; [f,g]Leptonchidae; [h,i]Thornenematidae; [j]*Thonus*; [k]Aporcelaimidae.

# Chapter 8

## Intraspecific variation in plant size, secondary plant compounds, herbivory and parasitoid assemblages during secondary succession

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## Abstract

During secondary succession on abandoned agricultural fields the diversity and abundance of insect communities often increases, whereas the performance and nutritional quality of early successional plants often declines. As the diversity and abundance of insects on a single plant are determined by characteristics of the environment as well as of the host plant, it is difficult to predict how insects associated with a single plant species will change during succession. We examined how plant characteristics of the early successional plant species ragwort (*Jacobaea vulgaris*), and the herbivores and parasitoids associated with these plants change during secondary succession. In ten grasslands that differed in time since abandonment (3-26 years), we measured the size and primary and secondary chemistry of individual ragwort plants. For each plant we also recorded the presence of herbivores in flowers, leaves and stems, and reared parasitoids from these plant parts. Ragwort plants were significantly larger but had lower nitrogen concentrations in recently abandoned sites than in older sites. Pyrrolizidine alkaloid (PA) composition varied among plants within sites but also differed significantly among sites. However, there was no relationship between the age of a site and PA composition. Even though plant size decreased with time since abandonment, the abundance of stem-boring insects and parasitoids emerging from stems significantly increased with site age. The proportion of plants with flower and leaf herbivory and the number of parasitoids emerging from flowers and leaves was not related to site age. Parasitoid diversity significantly increased with site age. The results of our study show that ragwort and insect characteristics both change during secondary succession, but that insect herbivore and parasitoid abundances are not directly related to plant size or nutritional quality.

## Introduction

The process of secondary succession on abandoned agricultural fields, also named "old field succession", has long been used as a model to study how plant communities change over time and the mechanisms behind these changes (e.g., Corbet 1995; Knops & Tilman 2000). Plant communities often become more complex and diverse during succession (e.g., Corbet 1995). The performance of individual plants of a single species can also change greatly during secondary succession (Bach 1990; Van de Voorde et al. 2012). Plant competition often increases and soil nutrient availability often declines during secondary succession (Knops & Tilman 2000) and this leads to increased competition and reduced plant growth.

Changes in plant species composition, competition and nutrient availability during succession can also affect the nutritional quality of individual plants growing in those communities. Several studies have shown, for example, that plant nitrogen content decreases during succession (e.g., Bach 1990). Although not well studied, changes in plant community or soil characteristics, as well as in levels of insect herbivory during succession can also affect the composition and concentration of plant secondary compounds (Hakes & Cronin 2011). For example, the resource availability hypothesis (Coley et al. 1985) states that concentrations of N-based allelochemicals are supposed to be high in conditions where nitrogen concentrations are high relative to the amount required by the plant for growth. Likewise, concentrations of C-based allelochemicals will typically be high when carbon availability is high relative to its demand. However, Hakes and Cronin (2011) recently showed for the perennial *Solidago altissima* that the concentration of phenolic compounds was lower in plants growing in late (high carbon availability) than in early successional communities. In late successional communities, greater environmental heterogeneity may also increase intraspecific variation in the amount or composition of secondary compounds (Agrawal et al. 2006). However, how much intraspecific variation there is in nutritional quality and secondary chemistry among individual plants that grow in natural areas and how this changes during succession remains largely unknown.

Insect communities also change during secondary succession and insect abundance and diversity often increases during succession (Steffan-Dewenter & Tscharntke 1997; Tscharntke et al. 1998; Siemann 1999; Brown & Gange 2002). This is true for herbivores but also for insects at higher trophic levels such as parasitoids or predators. However, the response of herbivorous insects to succession also varies between feeding guilds. For example, sap-feeding insects

often dominate during early successional stages, while stem-boring insects are often more abundant in late successional communities (Tscharntke & Greiler 1995). So far, most studies examining successional changes in insect communities have focused on insects associated with entire plant communities. How the insects associated with a single plant species will change during succession is less well known (Bach 1990).

Successional changes in insect densities on plants of a single species can be driven by habitat or plant community characteristics that change during succession (Kostenko et al. 2012). Alternatively, these effects can arise from changes in host plant characteristics such as plant nutritional quality or plant size (Awmack & Leather 2002). The concentration of secondary plant compounds can also affect insect herbivores, but also the performance and abundance of other groups of insects such as parasitoids that develop in or on other (herbivorous) insects (Ode 2006). Large plants are easier to detect and are therefore hypothesized to be more readily colonized by herbivorous insects than smaller plants (Feeny 1976). This may also lead to larger numbers of parasitoids on large plants, because they contain larger numbers of herbivores or simply due to their higher apparency (Andow 1991).

In this study we examine how characteristics of the surrounding habitat and concentrations of primary and secondary plant compounds, and insect abundances on individual ragwort (*Jacobaea vulgaris*) plants change during succession. Ragwort is a dominant species in semi-natural grasslands in the Netherlands, where nature is restored on former agricultural fields (Bezemer et al. 2006; Van de Voorde et al. 2012). We hypothesized (i) that due to cessation of fertilization after land abandonment, the availability of the soil nutrients will decrease and that this will negatively affect the growth of the plants and their nutritional quality, as well as the total concentration of secondary plant compounds. Next, we hypothesized that (ii) the similarity in the composition of secondary plant compounds between individual plants will decrease with time since abandonment as environmental heterogeneity will increase with succession. The population of insects associated with the entire plant community often builds-up with successional age, and this may potentially lead to "spill over" onto individual plants growing in those communities (e.g., Andow 1991). Therefore, we hypothesized (iii) that the proportion of plants with herbivore damage will increase, and that parasitoid communities will become more abundant and diverse during succession. Alternatively, if the plant quality is an important determinant of herbivore performance in the field, we may expect to find higher proportions of damaged plants in younger sites because of the predicted decline in plant nutritional quality with succession. To examine

whether successional changes in insect abundances were driven by plant quality we tested whether insect abundance was correlated with plant characteristics.

In the field, both plant and environmental characteristics vary. To determine whether insect performance varies on plants from different successional stages, we conducted a common garden experiment using *Tyria jacobaeae* L. larvae, a common specialist herbivore of *J. vulgaris* in the Netherlands (Van der Meijden & Van der Veen-Van Wijk 1997).

## Materials and Methods

*The study system*

*Jacobaea vulgaris* Gaertner ssp. *vulgaris* is a biennial or short-lived perennial plant in the family *Asteraceae.* The seeds disperse by wind but the majority drops within a few meters from their parent plant (McEvoy & Cox 1987). The plant contains pyrrolizidine alkaloids (PAs), a well studied group of secondary plant compounds that play an important role in plant-insect interactions (Macel 2011). PAs are feeding deterrents to a wide range of generalist insects, whereas certain specialized insects have evolved adaptations to sequester and utilize PAs for their own defence. The concentration and composition of PAs can vary greatly among different populations of ragwort plants (Macel et al. 2004). *J. vulgaris* harbours a rich insect community of 75 recorded species. The specialist herbivore *T. jacobaeae* feeds on leaves, flowers and top parts of the stems and exploits PAs to recognise host plants (Macel 2011). Insect communities on ragwort respond differently to ragwort abundance and plant size; and often are not strongly affected by spatial isolation of ragwort patches, but previous studies report different dispersal abilities (Harrison et al. 1995; Kunin 1999; Brunzel et al. 2004; Esch et al. 2005).

*Field sampling*

In July 2008, 600 individual ragwort plants were sampled from ten former arable sites that are being restored to species-rich grasslands. The sites were each at least 2 ha in size, and located in the Veluwe region in the central part of the Netherlands within a 25 × 25 km area (Table 8.1). All sites had similar physical soil properties, agricultural history, and were grazed mainly by wild cows and horses. We selected sites that were abandoned between three and 26 years ago at the time of sampling. Details about each site are presented in Table 8.1. Ragwort was present at all sites but its abundance varied among sites (Table S8.1 Supporting Information). Plants from all sites were sampled within two consecutive days. At each site, three transects were laid out of 40 m each.

Each transect started from the middle of the site ("tripod approach"). Along each transect at distances of 2 m we collected the nearest ragwort plant that was in full bloom (20 plants per transect, 60 per site). All plants were clipped at ground level, kept individually in plastic bags and transported to the laboratory. For each site, the percentage cover of ragwort was also estimated by eye in five 1 × 1 m quadrates along the transects and the average ragwort cover was calculated per site. Later, soil samples (5 cm diameter, 15 cm depth) were collected at nine locations within each site. The samples were homogenized, sieved (4 mm diameter), dried at 40 °C for one week and used for chemical analysis (see below).

**Table 8.1** Site descriptions. Data for soil characteristics are mean ± SE. Available P and K were extracted using 0.01M $CaCl_2$. The relationship between site age and ragwort cover is tested using linear regression, and soil characteristics using linear mixed models with site identity as a random factor. Pearson correlation coefficients are calculated based on the averaged values per site.

| Site | Age (yrs) | Ragwort cover (%) | Soil characteristics | | | | |
|---|---|---|---|---|---|---|---|
| | | | % Soil organic matter | $NH_4^+ + NO_3^-$ ($mg \cdot kg^{-1}$) | P ($mg \cdot kg^{-1}$) | K ($mg \cdot kg^{-1}$) | pH |
| 1 | 3 | 34 | 4.6 ± 0.2 | 4.5 ± 0.2 | 1.7 ± 0.3 | 82.9 ± 4.4 | 5.2 ± 0.04 |
| 2 | 3 | 40 | 5.3 ± 0.2 | 4.9 ± 0.4 | 1.1 ± 0.1 | 52.9 ± 5.4 | 5.2 ± 0.1 |
| 3 | 3 | 40 | 5.2 ± 0.1 | 3.0 ± 0.1 | 0.7 ± 0.1 | 41.2 ± 10.3 | 5.4 ± 0.05 |
| 4 | 6 | 40 | 5.2 ± 0.2 | 6.2 ± 0.4 | 4.8 ± 0.4 | 74.2 ± 11.4 | 4.9 ± 0.09 |
| 5 | 13 | 26 | 4.1 ± 0.2 | 5.3 ± 0.4 | 5.4 ± 0.8 | 54.7 ± 9.8 | 5.0 ± 0.06 |
| 6 | 14 | 14 | 3.9 ± 0.1 | 4.4 ± 0.6 | 3.0 ± 0.2 | 54.1 ± 2.1 | 4.7 ± 0.07 |
| 7 | 18 | 8 | 4.2 ± 0.1 | 6.7 ± 0.6 | 3.6 ± 0.2 | 48.5 ± 4.1 | 4.3 ± 0.03 |
| 8 | 20 | 8 | 5.2 ± 0.3 | 5.4 ± 0.4 | 3.4 ± 0.5 | 23.4 ± 1.7 | 4.5 ± 0.03 |
| 9 | 23 | 26 | 4.9 ± 0.2 | 9.6 ± 0.8 | 2.0 ± 0.3 | 47.0 ± 5.7 | 4.7 ±0.03 |
| 10 | 26 | 22 | 5.4 ± 0.3 | 8.0 ± 1.1 | 0.3 ± 0.1 | 33.0 ± 3.6 | 4.9 ±0.03 |
| Age | | $F_{1,8} = 9.35$<br>$P = 0.016$<br>r = -0.73 | $F_{1,8} = 0.0038$<br>$P = 0.95$<br>r = -0.02 | $F_{1,8} = 9.60$<br>$P = 0.015$<br>r = 0.75 | $F_{1,8} = 0.08$<br>$P = 0.79$<br>r = 0.08 | $F_{1,8} = 5.90$<br>$P = 0.041$<br>r = -0.62 | $F_{1,8} = 7.66$<br>$P = 0.024$<br>r = -0.70 |

*Insects*

In the laboratory, the height of each clipped plant was measured and visual herbivore damage of leaves, flowers and stems was estimated by eye. The presence of aphids on each plant was recorded as a binary measurement (present/absent). Each plant was subsequently separated into inflorescences, leaves and stems, and the biomass (fresh weight) of each plant part was recorded. A subsample of 1 g was oven-dried at 40 °C, ground and stored at −20 °C for chemical analyses (see below). The subsample consisted of flower, leaf and stem biomass, proportional to the contribution of each part to the total plant biomass. The remaining fresh plant material of each plant part was placed in plastic containers and kept in an insect

rearing chamber (22 °C, 16:8 h day: night regime, 60 ± 2% RH). Lids of the plastic containers were pierced to allow aeration. Each container was checked weekly for emerged insects. All insects (some herbivores but mainly parasitoids) were stored separately in 70% ethanol. Three months later, all plant material in each container was thoroughly checked for presence of insects, and all stems and flowers were dissected to detect cryptic insects or signs of herbivory. Within each stem the number of stem borer larvae and pupae were also recorded. All parasitoids were identified to family or genus level.

*Chemical analyses*

Nitrogen (N) content was determined for 300 plants (30 randomly selected plants per site) using a Flash EA1112 CN analyzer (Interscience). PAs were determined for a subsample of the plants analyzed for nitrogen (N = 150, 15 plants per site) using liquid chromatography-tandem mass spectrometry (LC-MS/MS) following the procedure described by Cheng et al. (2011a). Soil samples were analyzed for available P and K, using an 0.01M $CaCl_2$ extraction; N ($NH_4^+$and $NO_3^-$) was determined colorimetrically in the $CaCl_2$ extraction using a Traacs 800 autoanalyzer (TechniCon Systems Inc.). The percentage organic matter was determined following Nelson & Sommers (1982).

*Common garden experiment*

To determine whether the performance of the specialist herbivore *T. jacobaeae* differed between plants from different sites in the absence of other environmental variables that may differ between sites, we performed an experiment in a common garden (30 × 30 m) at the experimental field site of the NIOO Institute in Heteren in July 2010. Larvae were collected from a single *J. vulgaris* population (20 × 20 m area) at the Mossel (the Netherlands). Twenty randomly selected flowering plants were carefully dug out with an intact soil monolith of 20 × 20 × 20 cm from each of six sites (Site 1, 2, 3, 7, 9, 10). Each plant was placed in a large pot (20 × 20 × 20 cm), its height recorded and the plants were caged individually using cylindrical cages (25 cm diameter, 1 m height). The plants were placed randomly in two rows of 60 individuals. Distance between plants within each row was 30 cm and rows were 1 m apart. Into each cage two pre-weighed (22.1 mg; SE 1.01 mg) and similar-sized second instar *T. jacobaeae* larvae were then introduced. Larvae were introduced in a Petri dish (5.5 cm diameter, 1 cm height) that was placed on the soil surface next to the stem. Thirty minutes later, all larvae had moved onto the plant and the Petri dishes were removed. The larvae were kept on the plants for seven days and were then collected, weighed again and relative growth rate (RGR) was calculated. Hereafter, all larvae were dissected to check whether they were parasitized, since this can affect their feeding behaviour; none of the larvae was parasitized.

*Data analyses*

Data were analysed using the R statistical language, ver. 2.15.0 (R Development Core Team, 2012). To fulfil requirements of normality and homogeneity of variances, ragwort characteristics and soil chemistry data were log-transformed and proportion data were arcsine transformed prior to analysis. Linear relationships between site age and ragwort or soil characteristics were based on all measurements per site but analysed using linear mixed models with site age as fixed factor and site identity as random factor. By using site as random factor the analysis takes into account that multiple plants sampled within each site are pseudoreplicates. Relationships between site age and parasitoid abundances were analyzed using generalized linear mixed models with Poisson distribution and log link function. When only an average value per site was available (ragwort abundance, proportion of plants with herbivores, Shannon diversity per site, *T. jacobaeae* larval performance) data were analyzed using linear regressions. To examine differences in *T. jacobaeae* performance among sites we used ANOVA. The relationships between foliar and leaf feeders presence and ragwort characteristics within each site were analysed using generalized linear models with binomial distribution and logit link function. The relationships between stem borers or parasitoid abundance and ragwort characteristics within each site were analysed using multiple linear regressions. For this, stem borer and parasitoid abundance data were square root transformed prior to the analysis. Pearson correlation coefficients were calculated for all variables based on averaged values per site.

To determine whether there was a relationship between PA composition and site age we used multivariate redundancy analysis (RDA) in CANOCO version 4.55 (Ter Braak & Šmilauer 2002). The variation in PA profiles of the individual plants growing at each site was visualized using a linear discriminant function plot based on discriminant function analysis of the relative PA composition. The Bray-Curtis similarity in PA composition between plants that originated from the same site and between plants from different sites was also calculated. Differences in within and between-site similarities were analyzed using a paired t-test. Data were analyzed per plant. As there are more possible comparisons between individual plants from different sites, than between plants that originate from the same site, between-site comparisons were based on the same number of comparisons (random subset, but including plants from all sites) as within-site comparisons.

## Results

Plant height of individual ragwort plants declined with site age ($F_{1,8}$ = 25.47, $P$ < 0.001, r = -0.87; Fig. 8.1A; ranges of plant and soil characteristics are presented in Table S8.1). Flower and stem biomass also decreased with site age (flower: $F_{1,8}$ = 8.56, $P$ = 0.019, r = -0.70; stem: $F_{1,8}$ = 9.24, $P$ = 0.016, r = -0.73). Foliar biomass was not related to time since abandonment ($F_{1,8}$ = 3.29, $P$ = 0.11, r = -0.47). N-content of individual ragwort plants increased with site age ($F_{1,8}$ = 12.84, $P$ = 0.007, r = 0.79; Fig. 8.1B). When we included plant biomass as a covariate in the model the effect of site age was still significant ($F_{1,8}$ = 5.29, $P$ = 0.050). Soil mineral N also increased significantly with site age whereas soil K and pH decreased as succession proceeded (Table 8.1).

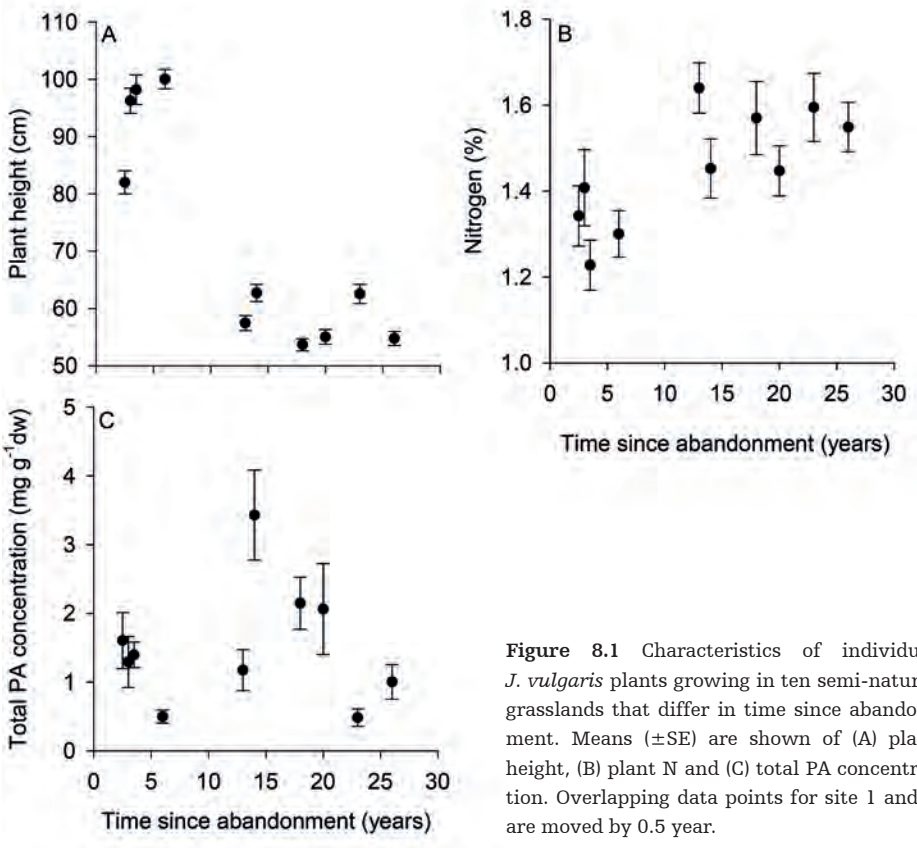



**Figure 8.1** Characteristics of individual *J. vulgaris* plants growing in ten semi-natural grasslands that differ in time since abandonment. Means (±SE) are shown of (A) plant height, (B) plant N and (C) total PA concentration. Overlapping data points for site 1 and 3 are moved by 0.5 year.

A total of 36 different PAs were detected in ragwort plants (Table S8.2). Total PA concentration did not significantly correlate with successional age ($F_{1,8}$ = 0.18, $P$ = 0.69, r = -0.041; Fig. 8.1C). PA composition varied greatly between plants within a site (Fig. S8.1) and was not significantly related to site age (RDA, F = 1.63, $P$ = 0.76). Overall, the PA compositions of two plants that originated from the same site were significantly more similar than the PA compositions of two plants from different sites (t = 8.34, $P$ < 0.0001). When this was analyzed for each site separately, this was significantly so for seven sites (Fig. 8.2). Similarity in PA composition within sites did not correlate with site age ($F_{1,8}$ = 1.71, $P$ = 0.23, r = 0.42).

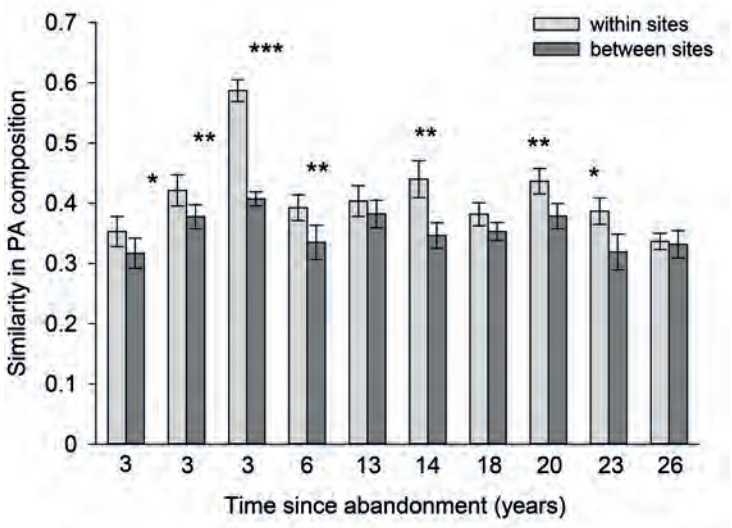



**Figure 8.2** Mean Bray-Curtis similarity (±SE) in PA composition of two plants that originated from the same site (light grey bars) and of two plants from different sites (dark grey bars). * $P$ < 0.05; ** $P$ < 0.01; *** $P$ < 0.001, based on a paired $t$-test.

There was no significant effect of site age on the proportion of plants with flower-feeding ($F_{1,8}$ = 1.48, $P$ = 0.26, r = 0.39; Fig. 8.3A) or leaf-feeding insects ($F_{1,8}$ = 0.22, $P$ = 0.65, r = -0.16; Fig. 8.3C). However, the proportion of plants with stem borers increased with successional age although the effect was marginally significant ($F_{1,8}$ = 5.03, $P$ = 0.055, r = 0.62; Fig. 8.3E). The number of stem borers per plant significantly increased with site age ($F_{1,8}$ = 9.61, $P$ = 0.014, r = 0.74; Fig. 8.3G). There were no significant relationships between plant characteristics and the presence of herbivores within each site ($P$ > 0.05 in all cases, data not shown). In the common garden experiment, RGR of *T. jacobaeae* did not differ between plants collected from different sites ($F_{5,108}$ = 1.21, $P$ = 0.31) and was not related to the age of the sites ($F_{1,112}$ = 0.26, $P$ = 0.62, r = 0.18).

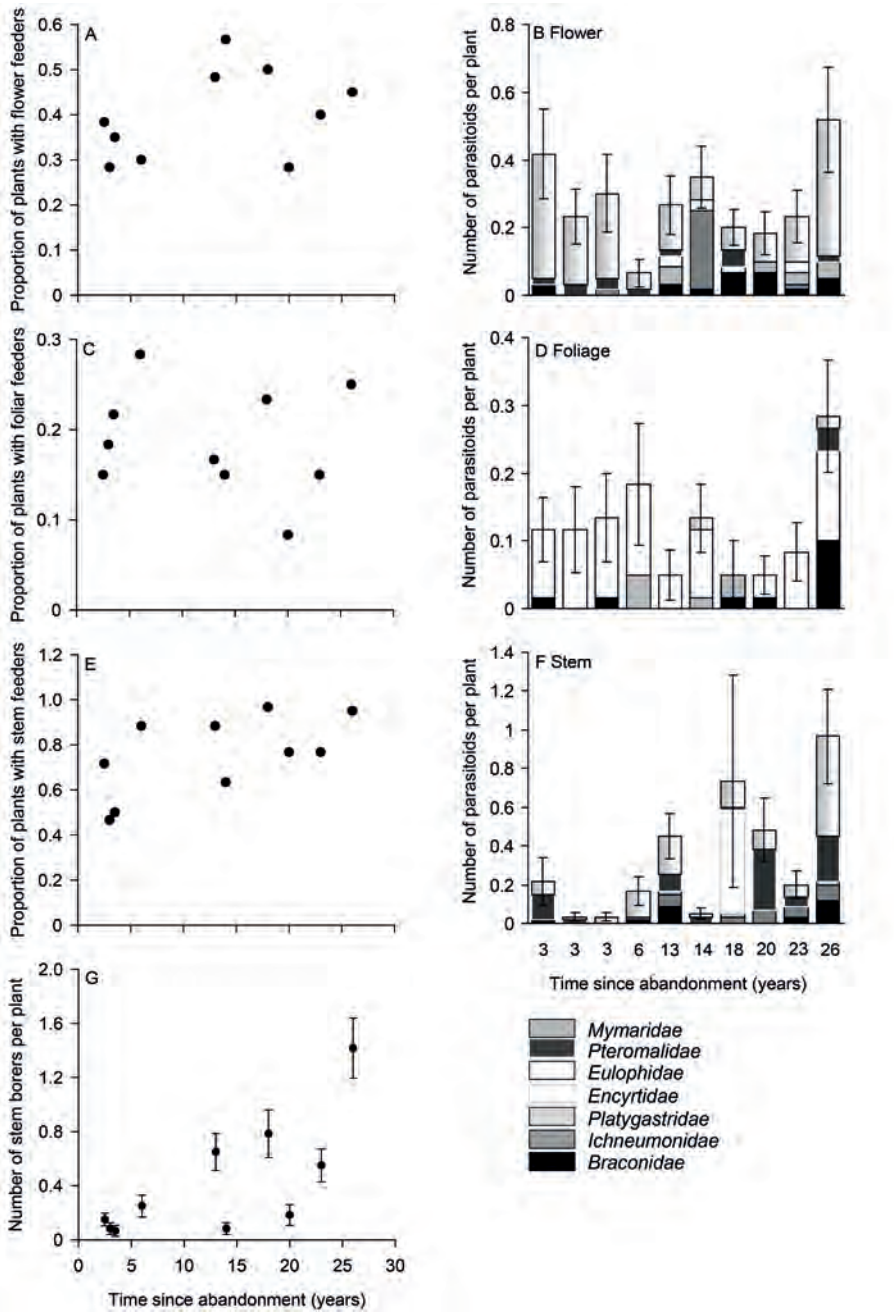



**Figure 8.3** Insect herbivory and parasitoid abundance on ragwort plants growing in ten semi-natural grasslands that differ in time since abandonment. Shown are proportions of plants with (A) flower and (C) leaf feeders, and (E) stem borers; (G) number of stem borers per plant; mean (±SE) number of parasitoids per family per plant that emerged from (B) flowers, (D) leaves, and (F) stems. Overlapping data points for site 1 and 3 are moved by 0.5 year.

A total of 438 parasitoids emerged and these belonged to seven Hymenoptera families (Table S8.3). The Shannon diversity of all parasitoids increased with increasing site age and this was true when analyzed per plant ($F_{1,8}$ = 8.52, $P$ = 0.019, r = 0.71; Fig. 8.4A) and per site ($F_{1,8}$ = 7.15, $P$ = 0.028, r = 0.68; Fig. 8.4B). There was no significant relationship between site age and the number of parasitoids that emerged from flowers ($F_{1,8}$ = 0.28, $P$ = 0.62, r = 0.20) or leaves of a plant ($F_{1,8}$ = 0.01, $P$ = 0.94, r = 0.074). However, the number of parasitoids that emerged from stems increased with site age ($F_{1,8}$ = 8.04, $P$ = 0.02, r = 0.71; Fig. 8.3B, D, F). Separate analyses of parasitoid groups/families revealed that *Braconidae*, *Ichneumonidae* and *Platygastridae* abundance significantly increased with site age (Table S8.3). Within each site, there were no significant relationships between plant size or plant chemistry characteristics and parasitoid abundances ($P > 0.05$ in all cases, data not shown).

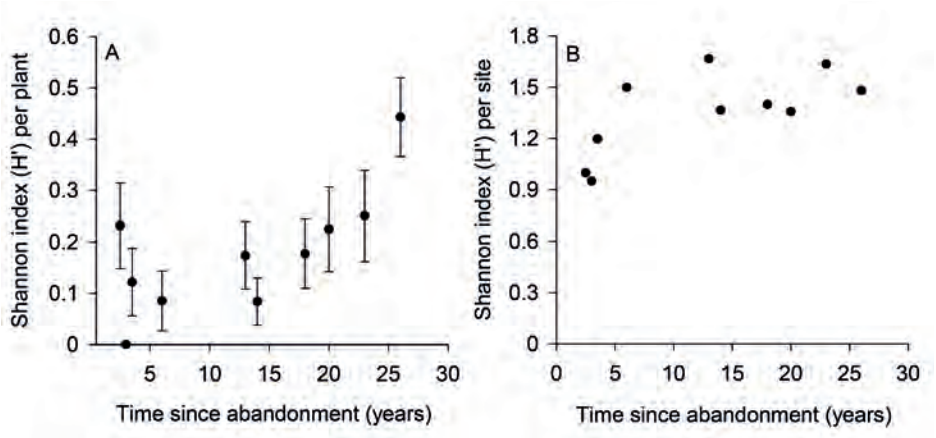



**Figure 8.4** Mean (±SE) Shannon diversity of parasitoids calculated (A) per plant and (B) per site in ten semi-natural grasslands that differ in time since abandonment. Overlapping data points for site 1 and 3 are moved by 0.5 year.

## Discussion

Our study shows that ragwort plant size and nutritional quality, and the presence and abundance of insects associated with these plants change during secondary succession. Plant size declined during succession while nitrogen content increased. In contrast, the total concentration or composition of PAs did not follow a successional pattern but differed between sites. Stem-borer densities and overall parasitoid diversity increased with successional age. Below we will first discuss the

successional effects on ragwort plants and subsequently discuss the successional changes we observed in insects.

Ragwort plants became less abundant and smaller as secondary succession proceeded. This has also been observed in other studies (Bezemer et al. 2006; Van de Voorde et al. 2012). The novel finding of our study is that we observed an increase in the plant N-content over time. This could simply be the result of a dilution effect as plants were bigger in younger sites. However, when we used plant biomass as a covariate in the model, the relationship between N-content and site age remained significant. Alternatively, higher levels of plant N could have resulted from higher availability of N in the soil of late successional sites. Indeed, N soil availability increased with time since abandonment (Table 8.1). During secondary succession on former arable land, the size and complexity of soil food webs increases and this can subsequently lead to an improved functioning of these food webs, including increased decomposition of organic matter which can lead to increased nitrogen mineralization (Holtkamp et al. 2011). Notably, our findings contradict the assumption made in many studies on secondary succession in grasslands that nutrient availability declines with time since abandonment due to cessation of fertilization (Kardol et al. 2005).

In our study, the PA composition varied considerably among individual plants originating from different sites but also among plant individuals growing within a single site. We did not find a successional pattern in total PA concentration or PA composition, but observed that the similarity in PA composition of ragwort plants was always higher within a site than among sites. The PA composition in *J. vulgaris* plants can be determined by genetic and by environmental factors (Vrieling et al. 1993; Joosten et al. 2009). We did not measure genetic variation. However, the ten successional sites were located within a confined area of roughly 25 $\times$ 25 km and ragwort was abundantly present in the entire area, therefore we may expect that there was genetic mixing among sites but this remains to be tested. Joosten and colleagues (2009) demonstrated that the composition of the soil microbial community can also strongly affect PA composition of *J. vulgaris*. During the process of succession there are spatio-temporal changes in soil biota (Van der Putten 2003; Kardol et al. 2005). Moreover, within a single plant community, the composition of the soil community can vary greatly locally, even among individual co-occurring plants (Bezemer et al. 2010a). Interactions with other plants or with insects can also change during succession and this can, in turn, influence the chemical composition of the plants (e.g., Macel 2011). Our study, in line with others (Kleine & Muller 2011; Hakes & Cronin 2011), emphasizes that secondary plant compounds in the field can vary greatly from plant to plant and may be the result of a suite of interactions between the plant and its biotic and abiotic environment.

In agreement with our hypothesis, we observed a positive relationship between site age and stem-borer densities as well as the number of parasitoids that emerged from stems. Other studies have also shown that parasitism of stem-boring insects increases with successional age (Tscharntke & Greiler 1995). In contrast, in our study, the proportion of plants with flower and leaf feeders as well as the abundance of parasitoids that emerged from flowers and leaves did not correlate with site age. It is possible that different groups of parasitoids respond differently to succession. Many chalcidoids that dominated the parasitoid communities from flowers and leaves are parasitoids of eggs or leaf-mining insects, which are highly abundant in both ruderal and later successional communities (Brown & Gange 2002). In contrast, Ichneumonidae, which often attack endophagous herbivores such as stem and flower-head borers, are known to colonize later successional communities (Tscharntke & Greiler 1995; Brown & Gange 2002). It is important to note that in our study, plants were surveyed only once during the growing season. Hence, our results may not represent the entire parasitoid community present in the field and this may explain why we did not observe significant differences in parasitoids from leaves and flowers. At the time of sampling, in recently abandoned sites, ragwort plants had only been present for a few years, whereas in the older sites, ragwort populations had been present for more than a decade. Therefore, it is also possible that insects had not yet fully colonized the recently abandoned sites and that the differences we observed in insect abundances are due to colonization dynamics. A similar argument was made by Kunin (1999), who showed that herbivore communities in newly created experimental ragwort populations differed from those in established populations. Colonization success of insects greatly decreases with increasing isolation, and the effects of isolation are often more pronounced for parasitoids than for herbivores (Kruess & Tscharntke 2000). In our study, three of the four younger sites were located within a distance of less than one kilometre of older sites and there are numerous ragwort populations in the areas in between the sites that could act as source populations. This makes it less likely that the differences among sites in our study were caused by dispersal limitation or isolation.

The plant apparency hypothesis states that more apparent plants are more readily colonized by herbivorous insects and, consequently, parasitoids are more likely to locate hosts on these plants and will be concentrated on them (Feeny 1976). We found that ragwort plants were larger and more abundant in newly abandoned sites. Therefore, according to the plant apparency hypothesis we would expect to find more insects on these plants. However, the proportion of plants with herbivore damage was lower, and parasitoids were less abundant and less diverse on plants in newly abandoned sites. Thus, our results do not

support this hypothesis. Individual plants can vary greatly in the amount of available nitrogen and secondary plant compounds and this can be an important determinant of the insect abundance. We found that plants that grew in older sites contained higher amounts of nitrogen, and parasitoid abundance and diversity on these plants was also higher. However, in our study plant size and nitrogen correlated with the successional age of the site. With most experimental designs, the effects of two co-varying factors cannot be disentangled. We sampled several plants within each site, and we did not merge samples. This enabled us to examine the "pure" effects of plant size and nutritional quality, by analyzing the effects for each site separately. This analysis showed that there were no significant effects of plant size and quality on insect abundances, even though there were considerable differences in plant and herbivory characteristics among individual plants within each site. We therefore suggest that the differences in herbivory and parasitoid abundance among sites are not primarily due to differences in plant quality. This is also in line with the results of the common garden experiment where the performance of the specialized herbivore did not differ between plants collected at different sites.

In conclusion, our results show that individual ragwort plant size and quality differs greatly among sites but also within sites. Herbivory and parasitoid communities associated with the individual plants also differ greatly among sites but this is not related to differences in plant quality between these sites.

## Acknowledgements

We thank Natuurmonumenten (Planken Wambuis) and Stichting het Utrechts Landschap for access to the study sites; André Kamp, Wiecher Smant, Saskia Grootemaat and Thomas Kroon for technical assistance; Alex Gumovsky for help in parasitoid identification; Patrick Mulder (RIKILT Institute of Food Safety, WageningenUR) for analysis of pyrrolizidine alkaloids; Wim Van der Putten and four anonymous referees for valuable comments on a previous version of this manuscript.

## Supporting Information

**Table S8.1** Minimum, maximum, and median of the measured variables in the studied sites.

| Variable | Minimum | Maximum | Median |
|---|---|---|---|
| **Ragwort characteristics** | | | |
| Height (cm) | 32.0 | 131.0 | 66.0 |
| Flower biomass (g) | 0.70 | 74.5 | 7.1 |
| Foliar biomass (g) | 0.05 | 30.0 | 2.7 |
| Stem biomass (g) | 0.90 | 93.9 | 11.9 |
| Nitrogen content (%) | 0.61 | 3.17 | 1.38 |
| Total PA concentration (mg $g^{-1}$ dw) | 0.04 | 10.8 | 1.03 |
| Ragwort cover (%) | 8.0 | 40.0 | 26.0 |
| **Soil characteristics** | | | |
| Organic matter (%) | 3.28 | 7.22 | 4.73 |
| Mineral N (mg·$kg^{-1}$) | 2.79 | 14.8 | 5.57 |
| P (mg·$kg^{-1}$) | 0.10 | 8.89 | 2.75 |
| K (mg·$kg^{-1}$) | 17.0 | 162.0 | 51.2 |
| pH | 4.19 | 5.63 | 4.83 |

**Table S8.2** Standardized canonical discriminant coefficients for the first three discriminant functions of the relative pyrrolizidine alkaloid composition of individual ragwort plants and the significance of the discriminant functions.

| PA | Function 1 | Function 2 | Function 3 |
|---|---|---|---|
| Acer | 0.390 | 0.740 | -0.159 |
| Acer-ox[a] | - | - | - |
| Acsp | 0.049 | -0.127 | -0.140 |
| Acsp-ox | -0.378 | -0.185 | -0.040 |
| DHEFox | 0.103 | -0.477 | 0.188 |
| DHJl | -0.194 | -0.484 | 0.050 |
| Ef-ox | 0.841 | 0.898 | -0.569 |
| Er | 0.494 | -0.330 | 0.038 |
| Er-ox | 2.289 | 2.772 | 0.052 |
| HOJb | 0.018 | -0.051 | -0.459 |
| HOJl | 0.149 | 0.398 | 0.807 |
| HOJn | 0.069 | 0.359 | 0.025 |
| Ir | 0.101 | -0.579 | 0.130 |
| Ir-ox | 0.043 | 0.097 | 0.193 |
| Jb | 0.256 | 0.884 | 0.931 |
| Jb-ox | 3.207 | 2.893 | 0.418 |
| Jl | 0.859 | -0.391 | -0.397 |
| Jl-ox | 1.374 | 0.329 | 0.028 |
| Jn | 0.675 | 0.038 | -0.378 |
| Jn-ox | -0.256 | -0.053 | 0.106 |
| Jz | 0.200 | 0.557 | -0.414 |
| Jz-ox | 0.165 | 0.467 | -0.098 |
| Rd | -0.140 | -0.305 | 0.867 |
| Rd-ox | 0.039 | 0.125 | -0.352 |
| Rt | 0.258 | 0.218 | 0.285 |
| Rt-ox | 1.033 | 0.413 | -0.240 |
| Sn | 0.415 | 0.216 | 0.203 |
| Sn-ox | 1.321 | 1.207 | -0.152 |
| Sp | -0.103 | 0.592 | -0.077 |
| Sp-ox | 0.715 | 0.979 | 0.252 |
| St | 0.145 | 0.194 | -0.051 |
| St-ox | 0.019 | -0.155 | 0.061 |
| Us | -0.071 | 0.429 | -0.216 |
| Us-ox | 0.368 | 0.463 | 0.030 |
| Un 4.98 | 0.145 | -0.101 | -0.429 |
| Un 5.03 | 0.182 | 0.188 | 0.353 |
| Wilks' lambda | 0.015 | 0.039 | 0.091 |
| *P* | <0.0001 | <0.0001 | 0.001 |

[a]this variable was automatically excluded from the analysis by the discriminant analysis procedure

AcEr – Acetylerucifoline, AcSp – Acetylseneciphylline, DHEf – Dehydroeruciflorine, DHJl – Dehydrojacoline, Ef – Eruciflorine, Er – Erucifoline, HOJb – Hydroxyjacobine, HOJl – Hydroyxjacoline, HOJn – Hydroxyjaconine, Ir – Integerrimine, Jb – Jacobine, Jl – Jacoline, Jn – Jaconine, Jz – Jacozine, Rd - Riddelliine, Rt – Retrorsine, Sn – Senecionine, Sp – Seneciphylline, St – Spartioidine, Us – Usuramine, Un – Unknown PA *N*-oxides (and their retention time in minutes), ox – the *N*-oxide form of corresponding PA.

**Table S8.3** Total number of parasitoids reared from *J. vulgaris* plants growing in ten semi -natural grasslands that differ in time since abandonment. Between brackets the total number of plants from which parasitoids emerged is presented. Asterisks indicate a significant relationship with site age based on a generalized linear mixed model with Poisson error distribution and log link function, including site as a random factor. **(*)** $P < 0.1$, * $P < 0.05$. The relationships were tested only at the family level and not tested for the *Encyrtidae* family (NA) because the parasitoids emerged from one plant.

| Taxon | Total number of individuals | | F | |
|---|---|---|---|---|
| Ichneumonoidea | | | | |
| *Braconidae* | 43 | (36) | 6.82 | * |
| *Aphidiinae* | 15 | (12) | | |
| *Microgastrinae* | 5 | (5) | | |
| Other *Braconidae* | 23 | (19) | | |
| *Ichneumonidae* | 35 | (28) | 4.58 | * |
| Platygastroidea | | | | |
| *Platygastridae* | 19 | (15) | 2.91 | **(*)** |
| Chalcidoidea | | | | |
| *Encyrtidae* | 32 | (1) | NA | |
| *Mymaridae* | 160 | (78) | 0.34 | |
| *Pteromalidae* | 90 | (75) | 2.52 | |
| *Eulophidae* | 59 | (43) | 0.37 | |
| *Diglyphus* | 36 | (25) | | |
| *Chrysocharis* | 12 | (10) | | |
| *Closterocerus* | 6 | (5) | | |
| *Diaulinopsis* | 2 | (1) | | |
| *Tetrastichinae* | 2 | (1) | | |
| *Euderus* | 1 | (1) | | |
| Total | 438 | (205) | 6.05 | * |

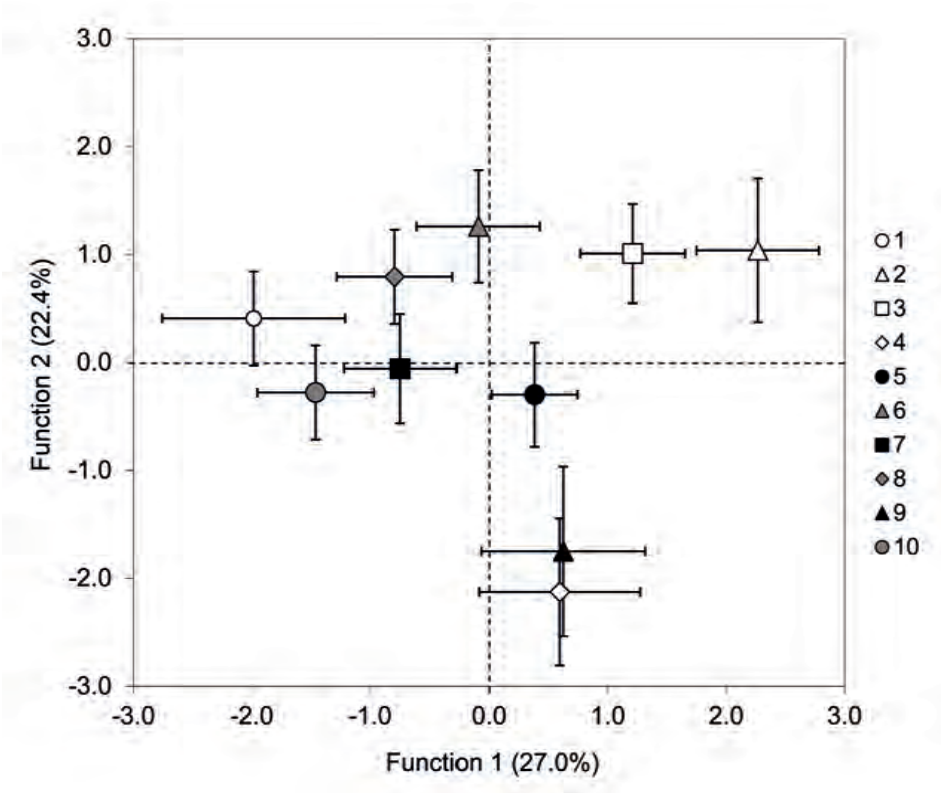
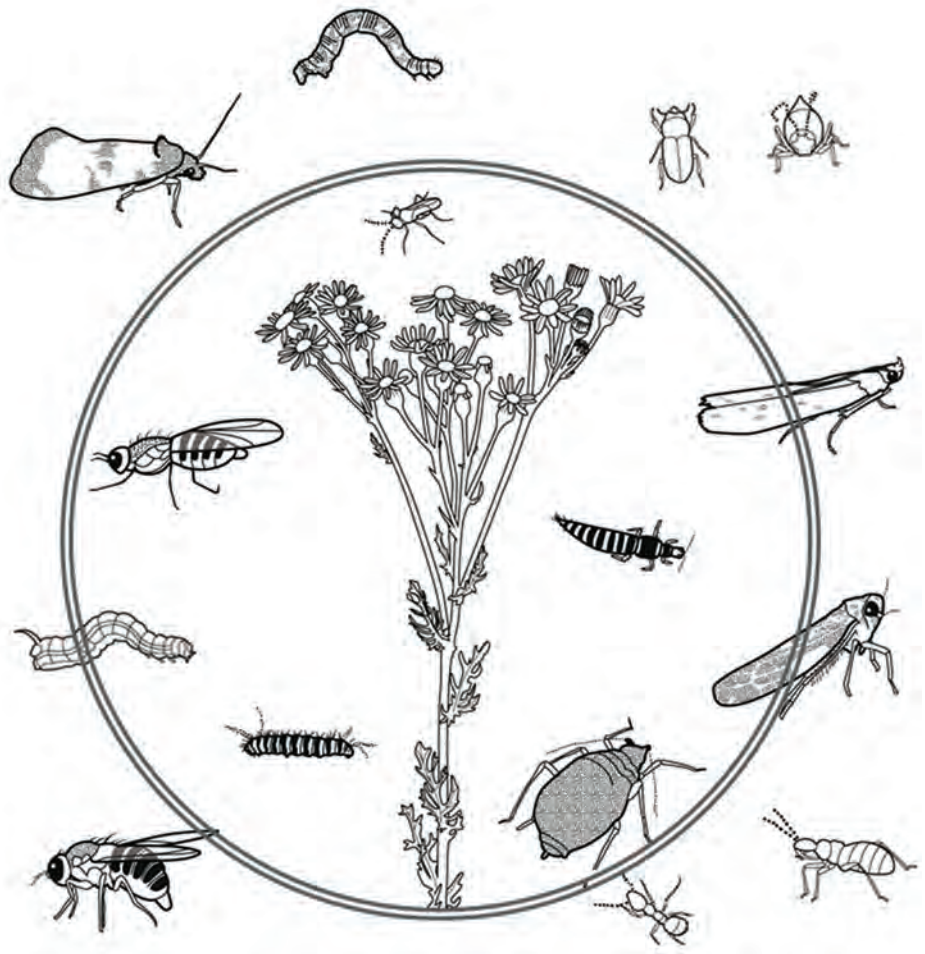



**Figure S8.1** The first and second discriminant functions of the relative PA composition of individual ragwort plants growing in ten semi-natural grasslands that differ in time since abandonment. The mean (±95% confidence interval) of discriminant sample scores are shown for each site. See Table 8.1 (main text) for description of the sites.



# Chapter 9

## General discussion

The main aim of my thesis was to elucidate, in a community context, the factors that structure the insect community associated to individual plants. I define community context as the aboveground and belowground multitrophic community surrounding an individual plant. In particular, I focused on the importance of the plant community surrounding individual plants as well as the nutritional quality of these plants for the aboveground insect community associated to these plants. Plant quality, often expressed as the content of nitrogen and secondary compounds in the plant, can directly influence insect communities by affecting the preference of an insect for the host plant, its development, survival and reproduction. However, plant quality varies in space and time and can be affected by a range of abiotic and biotic factors in a community context. In my thesis I also examined factors that can contribute to such intraspecific variation in plant quality and whether this variation in plant quality affects insect communities in the field. As a model system I used ragwort (*Jacobaea vulgaris* Gaertner ssp. *vulgaris)* and its associated aboveground and belowground communities. In this chapter I will discuss and synthesize the main results of my research, identify possibilities for application of these results for biological control of *J. vulgaris*, and propose several directions for future research.

## Intricate aboveground-belowground interactions

*Immediate belowground-aboveground interactions*

It is well established now that aboveground (hereafter AG) and belowground (hereafter BG) organisms and processes can interact with each other through plant-mediated mechanisms (Wardle et al. 2004a; Van der Putten et al. 2009). For instance, root herbivores can induce changes in the growth or chemistry of the shoot and this can have important consequences for AG herbivores. Changes in the diet of an insect herbivore can also affect the development and survival of higher trophic level organisms, such as parasitoids that are natural enemies of insect herbivores (Godfray 1994). These linkages between physically separated organisms, named as AG-BG interactions, can have major consequences for the dynamics of plant and herbivore communities in terrestrial systems (Bardgett & Wardle 2010). Although these AG-BG interactions have been intensively studied during past two decades, little is known about the effects of BG herbivory on the development of AG parasitoids (but see Soler et al. 2005; Qiu et al. 2009). Exploring the effects of AG-BG interactions on higher trophic level organisms may considerably contribute to the understanding of AG insect community dynamics and the ability to predict their responses to global changes. In *Chapter 2*, I studied whether root herbivores exert changes in the quality of the AG tissues and via these changes impact the development of AG herbivores and parasitoids.

Based on the results of the previous studies I expected that root herbivory by generalist root-feeding wireworms on *J. vulgaris* will increase the concentration of plant defence compounds, pyrrolizidine alkaloids (PAs), in the shoots (Bezemer et al. 2003; Soler et al. 2005; Van Dam et al. 2005; Erb et al. 2008); and consequently will negatively affect the development of a generalist AG herbivore and its parasitoid. In contrast to the majority of previous studies and our expectation, the results of *Chapter 2* showed that root feeding by wireworms had a strong negative effect on the total concentration of PAs in AG parts of *J. vulgaris*. Few earlier studies also observed a decline in the concentration of secondary compounds (alkaloid nicotine) in the foliage following root herbivory (Hanounik & Osborne 1977; Kaplan et al. 2008a). These defence compounds as well as PAs are produced in plant roots, suggesting that root herbivory may interfere with the production or translocation of these compounds, and thus indirectly benefit AG herbivores by reducing the concentration of plant secondary metabolites. Unexpectedly, in our study the decline in shoot PA concentration did not result in increased performance of the generalist insect herbivore or its parasitoid. On the contrary, there were no significant effects of root herbivory on AG insect performance. There was only a marginally significant negative effect of root herbivory on AG herbivore growth, but no effect on parasitoid development or survival. Interestingly, together with the quantitative changes there were qualitative changes in the composition of secondary compounds in the leaves of *J. vulgaris* upon root herbivory. Most of the compounds that decreased in the leaves after the root herbivory were compounds that are reported to be less toxic to herbivorous insects (Dreyer et al. 1985; Van Dam et al. 1995; Macel et al. 2005; Cheng et al. 2011b). The concentration of more toxic compounds did not change in the foliage after root herbivory and hence the performance of AG insects was not affected. These findings suggest that there might be various mechanisms by which BG herbivory can affect the chemistry of AG plant parts and AG multitrophic communities on the plants. Moreover, a reduction of root-produced defence compounds in the AG tissues after root herbivory implies that the response of a plant to BG herbivory may depend on whether the defence compounds are produced in the roots, or in the shoots or in both (Rasmann & Agrawal 2008; Erb et al. 2009; Van Dam 2009). However, more experimental support is needed to generalize these findings.

*Temporal dynamics of aboveground-belowground interactions*

AG-BG interactions have been studied extensively in experiments where AG and BG organisms were feeding on the same plant (*Chapter 2*; Bezemer et al. 2003; Soler et al. 2005; Van Dam et al. 2005; Kaplan et al. 2008b; Vandegehuchte et al. 2010; Erb et al. 2011a). The temporal dynamics of these interactions and their feedback effects remain poorly studied and are not well understood (Bardgett

& Wardle 2003; Bardgett et al. 2005; Van der Putten et al. 2009). Plant-soil feedback, on the other hand, is a temporal process in which plants, through their effects on soil biota and the abiotic environment, can affect the performance of other plants that later grow in the same soil (Bever et al. 1997; Ehrenfeld et al. 2005). Plant-soil feedbacks have also been recognised as important drivers of plant community dynamics in terrestrial ecosystems (reviewed in Van der Putten et al. 2013). In the long-term, plant-soil feedback in one generation can become a soil legacy for the next generation of plants (Kardol et al. 2007; Kardol et al. 2013). Since plants are continuously interacting with a number of different AG and BG insect herbivores in natural communities, understanding the interactions between the plant-soil feedbacks and AG-BG herbivores can be essential for predicting their impact on the structure and functioning of plant and insect communities. In *Chapter 3*, I combined these two concepts to test for the temporal component of AG-BG interactions.

In a greenhouse experiment, I exposed plants to either aboveground, belowground, or both herbivores or kept plants free of insects. I expected that the insect herbivory will have an effect on soil biota, particularly on soil fungi as they can be important determinants of *J. vulgaris* dynamics in the field (Bezemer et al. 2006). Then, I used this conditioned soil to grow new plants in order to test whether AG and BG herbivory could potentially lead to legacy effects in soil that will subsequently affect the growth and chemistry of the new plants. Furthermore, I exposed these new plants to the parasitized and unparasitized aboveground insect herbivores to examine whether these soil legacies affect multitrophic AG interactions on the new plants. In line with previous studies, the composition of soil fungi was significantly affected by aboveground and belowground herbivory (*Chapter 3*; Bardgett & Wardle 2010; Bennett 2010). Remarkably, these herbivore-induced changes in soil community composition also affected the growth and secondary chemistry of new plants, as well as the AG multitrophic interactions occurring on those plants (*Chapter 3*).

The legacy effects differed greatly between AG and BG herbivory. Belowground herbivory caused less negative plant-soil feedback allowing plants to grow bigger and to produce higher concentration of the defence compounds. Better defended plants negatively affected the performance of the AG herbivore and its parasitoid. Thus, belowground herbivory on one generation of plants, via positive plant-soil feedback, can negatively affect the performance of insects on the next generation of plants growing in the same soil, resulting in a negative indirect *belowground herbivore-soil feedback*. In contrast, the direct effects of BG herbivory on plant quality did not affect the same AG herbivore and parasitoid performance (*Chapter 2*) exemplifying how complex plant-

insect interactions are. AG herbivory, opposite to BG herbivory, increased the strength of the negative plant-soil feedback that led to reduced plant growth and production of defence compounds, thereby positively affecting the development of the AG herbivore and its parasitoid on these later growing plants. Hence, AG herbivory via negative-plant soil feedback can facilitate future AG herbivores, resulting in positive *aboveground herbivore-soil feedback*. There is increasing evidence that AG and BG herbivory can differentially affect the concentration and composition of defence compounds in plant roots (Kaplan et al. 2008b; Van Dam 2009). Recently, it has also been shown that variation in the concentration and composition of defence compounds in plant roots can exert changes in the composition of the soil community resulting in altered plant-soil feedbacks (Lankau et al. 2011). In addition, AG and BG herbivores can potentially affect the concentration of defence compounds in root exudates or BG herbivores can wound the roots causing increases in secretions of defence compounds to the rhizosphere. Therefore, I propose that these "herbivore-induced plant-soil feedbacks" were mediated through the effects of insect herbivores on the plant defence compounds (Fig. 9.1). These feedbacks can lead to spatial and temporal heterogeneity in soil biota and plant quality in the field.

As far as I am aware, the existence of "herbivore-induced plant-soil feedbacks" mediated by plant defence chemistry has never been reported before. This said it is important to mention that earlier studies examining the effects of vertebrate herbivory or artificial defoliation on plant-soil feedback responses also reported similar effect but mediated via changes in decomposer abundance and nutrient mineralization in the soil (Bardgett & Wardle 2003; Mikola et al. 2005; Sørensen et al. 2008; Veen 2011; Cease 2012). I propose that these indirect feedbacks will be important for the future research in both the "AG-BG interactions" and "plant-soil feedback" fields leading to "AG-BG feedback" (Box 3). Moreover, it shows that spatially and temporally separated organisms can interact in more complex ways than considered up to now by ecologists. To better understand the dynamics and structure of multitrophic insect communities, it will be important not only to focus on plant-insect interactions, but also on the feedback effects that occur between soil biota, plants and insects.

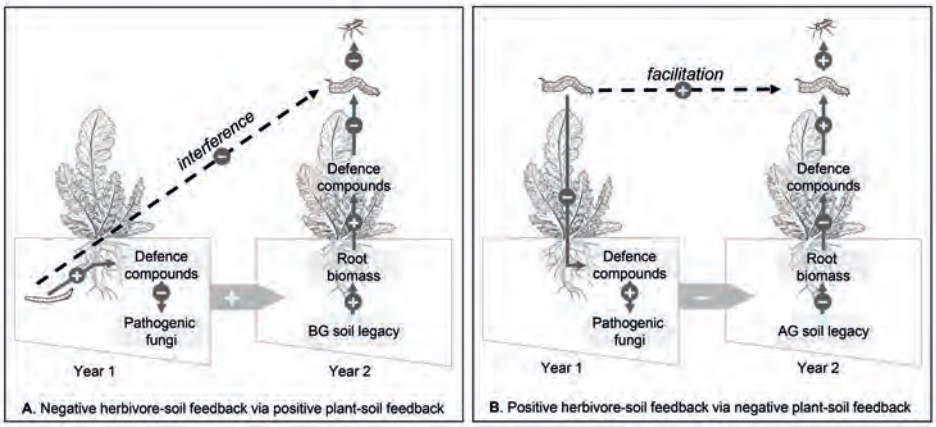
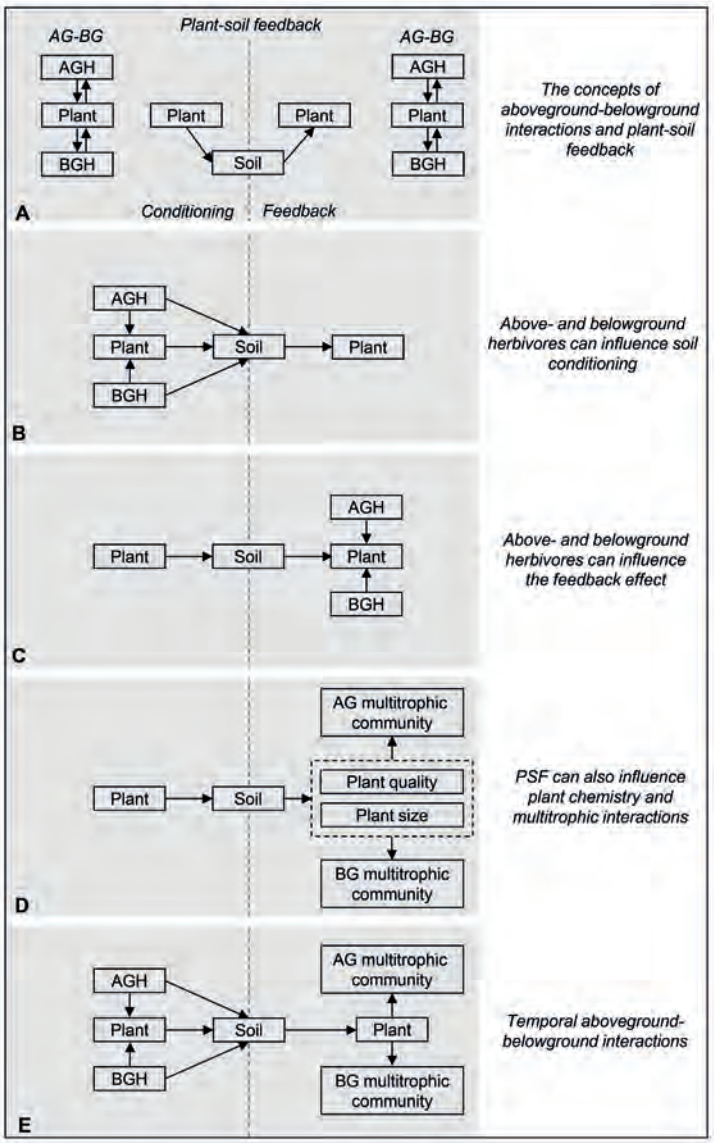



**Figure 9.1** Hypothetical interactions between temporally and spatially separated insect communities (based on the results of the *Chapter* 3). The "+" and "−" indicate the direction of the effects; the thick arrows show the interactions between temporally separated organisms. (A) Belowground herbivory damages the root tissues, which leads to the leakage of defence compounds into the soil. These compounds are toxic to soil pathogenic fungi and cause a decrease in the soil pathogen pressure (Hol & Van Veen 2003). The next generation of plants benefits from the pathogen release in the soil and produces more biomass resulting in positive plant-soil feedback. These plants also produce more defence compounds, which have a negative effect on the development of the aboveground generalist herbivores feeding on these plants and its parasitoids. Thus, belowground herbivory, via positive plant-soil feedback, negatively affects the aboveground multitrophic insect community on the next generation of plants, resulting in negative indirect belowground herbivore-soil feedback. (B) Aboveground herbivores weaken the plant but this does not lead to the release of defence compounds in the soil. As *J. vulgaris* exhibits strong negative plant-soil feedback through the increased abundance of soil pathogenic fungi (Bezemer et al. 2006; Van de Voorde et al. 2011), this leads to higher pathogen pressure in the soil. New plants grow less well in this soil and have lower concentrations of defence compounds that consequently result in a better performance of aboveground generalist herbivores and their natural enemies. Hence, aboveground herbivory via negative-plant soil feedback facilitates future aboveground herbivores, resulting in positive aboveground herbivore-soil feedback.

**Box 3. Conceptual schemes connecting the concepts of aboveground-belowground interactions (AG-BG) and plant-soil feedback (PSF)**




*AGH stands for aboveground herbivory; BGH – belowground herbivory; Soil – the biotic and abiotic components of the soil; Conditioning – the first phase of plant-soil feedback where plants or plant communities can induce changes in the soil biota or abiotic conditions; Feedback – the second phase of plant-soil feedback, where induced changes in soil can affect the growth and dynamics of plants colonizing this soil. Dashed rectangle outlines plant characteristics that can be affected by plant-soil feedback.*

(A) Plant soil-feedback is usually described as a two-phased process. During the soil conditioning phase, a plant or plant community can affect the biotic and abiotic components of the soil. In the feedback phase, the biotic and abiotic changes in the conditioned soil can feed back to the new generation of plants or plant communities. Plants can be damaged by herbivorous insects either during the conditioning or feedback phase, or both. (B) When a plant is exposed to AG or/and BG

herbivores during the conditioning phase it can modify the effect of a plant on soil biota and affect the outcome of PSF (Anderson et al. 1983; Bardgett et al. 1998; Mikola et al. 2005; Bezemer et al. 2013; *Chapter 3*). (C) Herbivory during the feedback phase is also likely to alter the plant's response to soil biota (Bezemer et al. 2013). For instance, negative PSF is often caused by the increased abundance of detrimental organisms such as pathogens in the soil. These pathogens, however, are sensitive to plant allelochemicals and feeding by an AG herbivore can induce the production of defence compounds in the roots (Soler et al. 2007). This may increase a plant's resistance against pathogens and, in turn, can result in less negative PSF. (D) PSF studies typically report effects on plant biomass (Kulmatiski et al. 2008 but see Mikola et al. 2005; Sørensen et al. 2008). However, soil microorganisms can also affect the nutritional quality or the concentration of plant defence compounds in both root and shoot tissues (Joosten et al. 2009; Hol et al. 2010; Badri et al. 2013; *Chapter 3*). These changes in the nutritional or allelochemical quality of the next generation of plants can have important consequences for multitrophic AG and BG communities on these plants. (E) Insects feeding on plants during the conditioning phase can indirectly interact with the insects colonizing plants during the feedback phase by modifying the outcome of PSF (*Chapter 3*).

## Interplay of plant quality and plant community effects on insect communities on individual plants

Understanding the factors influencing the dynamics and composition of insect communities in natural settings may considerably contribute to our ability to predict their responses to environmental changes. A large number of studies have shown that plant quality is an essential factor for preference and performance of herbivorous insects (reviewed in Awmack & Leather 2002). However, the vast majority of those studies have been performed in controlled environments because it is usually difficult to identify patterns and mechanisms of selection by herbivorous insects in natural communities due to the large phenotypic variation caused by environmental characteristics. The importance of plant quality for insect communities has also been demonstrated in semi-field conditions. For example, by using several cultivars of *Brassica oleracea* that considerably differed in nutritional and chemical quality Poelman and colleagues (2009b) showed that the composition of the herbivore community associated with plants was significantly affected by the intraspecific variation in plant quality. Furthermore, Bukovinszky and colleagues (2008) revealed that variation in plant quality of feral versus domesticated *B. oleracea* cascades up through the food web and can also affect insects inhabiting higher trophic levels. Whether plant quality is an important determinant of insect community in natural ecosystems remains unclear.

If we assume that insects use only plant quality cues to make their decisions regarding host plant location and acceptance it would not matter where plant grows as the number of insects that colonize a particular plant will be always

the same. The results of the survey carried-out in the chronosequence of ex-arable fields, revealed that stem-borer densities and overall parasitoid diversity associated to individual plants increased with the age of a field (time since cessation of agriculture) and that plant quality varied between and within fields. However, there was no significant correlation between the quality of the individual plants and insect occurrence or abundance in the chronosequence fields (*Chapter 8*). In line with our results, Macel & Klinkhamer (2010) reported that there was also no correlation between the total PA concentration and herbivore damage of the vegetatively propagated *J. vulgaris* plants that were transplanted in a field site close by to the chronosequence sites. The damage was limited and did not differ between different chemotypes or genotypes of *J. vulgaris*. Interestingly, when the same genotypes and chemotypes of *J. vulgaris* plants were planted at another site the amount of damage significantly correlated with the total PA concentration of the plant (Macel & Klinkhamer 2010). These findings together with the results of *Chapter 8* imply that the differences between sites and not plant quality affected the insect communities on individual *J. vulgaris* plants.

In natural communities, individual plants coexist and compete with neighbouring plants within diverse plant communities and these neighbouring plants can have a large effect on the ability of insects to find and accept their host plant and can also affect the quality of the host plant (reviewed in Barbosa et al. 2009). Indeed, in a controlled biodiversity experiment, individual *J. vulgaris* plants harboured higher abundance of insects in less diverse and more open communities than in more diverse communities (*Chapter 4*). This indicates that the presence and the diversity of surrounding community can provide associational resistance to focal plants growing in that community. The presence and diversity of the surrounding community also strongly affected the growth and quality of the focal *J. vulgaris* plants, but the associational effects were not mediated via the effects of the plant community on the performance of the focal plants (*Chapters 4* and *5*). In contrast, in another study investigating variation in secondary chemistry of wild *Tanacetum vulgare* plants the amount of damage and the performance of generalist insect herbivores were significantly different between various plant chemotypes in the field and under semi-field conditions (Kleine & Müller 2011). Unfortunately, this study does not report whether there are differences in the communities where the various chemotypes were grown. I argue that the plant quality can be an important factor affecting insect performance and preference for a focal plant but that in a natural context the surrounding plant community can overrule the effects of plant quality on the insect communities associated to a focal plant. However, future studies should examine whether these effects are common for other plant-insect systems or are species-specific. Finally, these results confirm that it is important to investigate plant-insect interactions

under the field conditions in which plants and insects interact in a community context. This is particularly important for studies that examine evolutionary relationships between plants and insects.

## Effects of the surrounding community on insect-plant interactions on individual plants

Several studies have shown that increased plant diversity leads to an increase in the diversity and abundance of insects (Haddad et al. 2009; Scherber et al. 2010). However, whether these effects are mediated through increases in the number of plant species in more diverse communities, where each plant harbours its own specific insect communities; or whether the combination of different plant species can lead to interactive effects influencing the insect communities of both or one of the plant species remains unsolved. To answer this question it will be important to study the effects of surrounding plants on insect communities associated to individual plants. Variable mechanisms have been proposed to explain how surrounding plants can affect insects colonizing individual plants. Insect host-finding processes consist of three consecutive steps of habitat location, host plant location and host acceptance, and surrounding plants can affect each of these steps via different mechanisms (Fig. 9.2). For example, if there are numerous high quality plants within the surrounding community ("high quality habitat") this can attract large numbers of generalist herbivores that subsequently spill-over on the focal plant. Similarly, natural enemies of herbivores, such as parasitic wasps, may accumulate on surrounding plants for a variety of reasons and this may either result in their moving to focal plants or to aggregation on the surrounding plants (Stilling et al. 2003). Moreover, surrounding plants can directly affect the colonization of individual plants by interfering with host-locating processes, for example, through physical or chemical masking (Hambäck et al. 2000; *Chapter 4* and 6) or impairment of insect movements (Kareiva 1983). Besides this, changes in microclimate, host plant volatile emissions, abundance of the alternative hosts and other resources (e.g., nectar, enemy-free space) caused by the surrounding community can indirectly influence the process of host location (Andow 1991; Heil & Karban 2010). The interactions between focal plants and their surrounding community (described in *Chapter 5*) may result in alteration of the quality of the focal plant that can have profound effects on host plant acceptance (*Chapter 4* and *5*). Finally, via the effects on the abundance of predators and parasitoids, the surrounding community can also affect the acceptance of the host plant for oviposition or feeding (Randlkofer et al. 2007; *Chapter 6*). Understanding these mechanisms

will enable us to predict when or where associational effects will be important in natural systems and can be used to improve biological control programmes in agroecosystems.

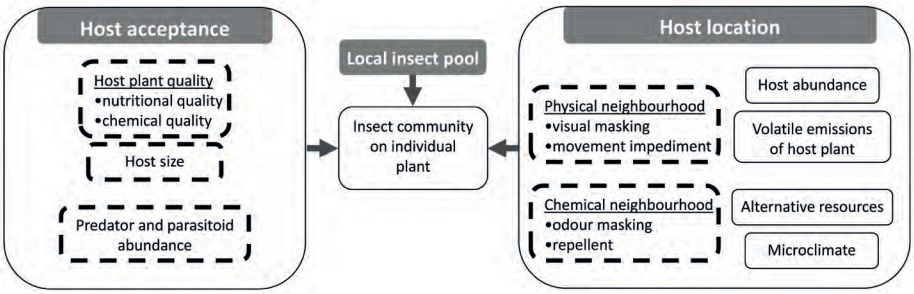



**Figure 9.2** The surrounding plant community can affect insects colonizing individual plants through their effects on the local pool of insects; the ability of insects to locate their host; and the host acceptance. The dashed lines indicate the mechanisms that have been addressed in this thesis.

## Belowground effects of plant diversity

One of the most significant consequences of current global changes is the decline of biodiversity in many ecosystems (Hooper et al. 2005; Cardinale et al. 2012). Soil biota are a significant component of all terrestrial ecosystems providing essential ecological functions, such as decomposition, nutrient cycling, and economically important functions, such as control of pest herbivores (Brussaard 2012). Most studies examining the effects of plant diversity on the control of pest insects have focused on aboveground communities (e.g., Andow 1991; Thies & Tscharntke 1999; Landis et al. 2000; Haddad et al. 2009; Scherber et al. 2010). These studies show that an increase in plant diversity often results in an increase in parasitoid and predator diversity and abundance leading to increased rates of parasitism (but see *Chapter 6*). However, less is known about the effects of plant diversity on the abundance of predatory soil organisms and the level of predation in the soil. Entomopathogenic nematodes (EPNs) are natural enemies of insects or other arthropods that live in the soil. EPNs play an important role in terrestrial ecosystems and used in biological control programmes in agricultural systems worldwide (Kaya & Gaugler 1993; Gaugler 2002). The question whether the infectivity of EPNs is related to the diversity or composition of the plant community has never been addressed before in grassland biodiversity experiments. Therefore, in my biodiversity field experiment, I tested the hypothesis that increased plant diversity will enhance

EPN infectivity in the soil (*Chapter 7*). I found that there was no direct effect of plant diversity on the infectivity of EPNs (*Chapter 7*). Instead, plant species diversity effects were indirectly mediated via changes in EPNs prey densities, and prey densities were not limited by root biomass. Similarly, there is also little support for increases in abundance of carnivorous non-EPN nematodes with increasing plant diversity, although the abundance of other nematodes is positively affected by plant diversity (De Deyn et al. 2004b; Viketoft et al. 2009; *Chapter* 7 but see Eisenhauer et al. 2011a). Several studies have highlighted that plant identity is more important than the plant diversity per se for the nematode community composition (Wardle et al. 2003; De Deyn et al. 2004b; Viketoft et al. 2005; *Chapter 7*). In my field experiment, plant identity exerted significant effects only on the infectivity of *Steinernema* EPNs suggesting that the responses of belowground organisms to manipulation in plant identity can be specific and differ even between organisms that belong to different species but the same feeding guild. Finally, my results suggest that plant diversity can exert bottom-up effects on multitrophic belowground communities, with predominantly strong effects on lower trophic levels organisms.

## PAs in aboveground-belowground interactions

In *Chapter 2*, I propose that a possible mechanism of changes in PA concentration and composition in plants exposed to root herbivores will be a restricted transport of PA *N*-oxides from the roots to the shoots (Fig. 9.3). If the transport of PA *N*-oxides from roots to shoots is constrained, it can result in a reduction in the concentration of PAs in shoot tissue over time. The PA concentration in the shoots will mostly decrease at the expense of PA *N*-oxides, as PA *N*-oxide concentrations in the shoots will be reduced due to shoot growth, which will result in a reduction to the corresponding tertiary amines. For the tertiary amines, dilution due to the shoot growth is compensated by the conversion of *N*-oxides to tertiary amines but reoxidation to the *N*-oxide form does not seem to take place in the shoots. Significant degradation of PAs is not expected on this time scale (Rosenthal & Berenbaum 1991). In line with our proposed mechanism, the PA concentration decreased at the expense of *N*-oxides when plants were directly damaged by root herbivores (*Chapter 2*), but also when plants were growing in the soil with a negative plant-soil feedback induced by AG herbivores (*Chapter 3*). This confirms the general hypothesis that PAs are distributed throughout the plants in the *N*-oxide form (Hartmann & Witte 1995). Moreover, the concentration of PAs in the roots and shoots of *J. vulgaris* is often related to the root to shoot ratio of the plant (Hol et al. 2003; Schaffner et al. 2003). The

bigger the shoot part becomes, the smaller the effect that roots can exert on the PA concentration in shoots. In other words, when the shoots are small most PAs are located in the roots. Therefore, restriction of PA transport will have a greater impact on the shoot PA content. When shoots are bigger, a larger proportion of PAs will be allocated to the shoot part. Thus, changes in transport will have a less dramatic effect on the shoot content. Finally, this restriction in PA transport most probably will not remain throughout the entire development of a plant as it would result in depletion of PAs in aboveground plant parts, which seems unlikely as all reproductive plants collected in the field contained PAs in AG parts (*Chapters 5* and *8*). Therefore, PA transport should be restored at certain point even if there is a constant pressure of root herbivores.

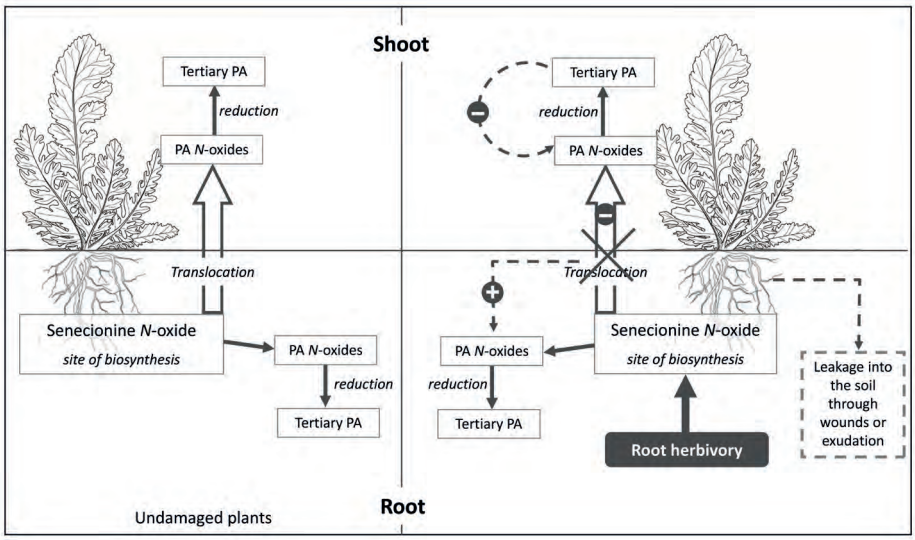



**Figure 9.3** Schematic overview of the potential mechanism of changes in pyrrolizidine alkaloid (PA) concentration and composition in vegetative *J. vulgaris* plants exposed to root herbivory (*Chapter 2*). The solid thin arrows represent the pathways of PA biosynthesis, the solid thick arrow – the root herbivory and the dashed arrows – the hypothetical changes in PA biosynthesis following root herbivory. "+" indicates processes leading to an increase and "–" a decrease in PA concentration. The left panel shows the production of PAs in undamaged plants. PAs are constitutively biosynthesised in roots as senecionine *N*-oxide. From the roots, senecionine *N*-oxide is exported to aboveground plant parts. *N*-oxides are constantly converted to the corresponding tertiary PAs both in root and shoot tissues ("reduction"). The right panel shows that upon root herbivory the transport of PA *N*-oxides from roots to shoots is restricted. This results in a reduction of PA concentration in the shoots over time at the expense of PA *N*-oxides. The decrease in PA *N*-oxide concentrations in the shoots is caused by two processes: dilution due to shoot growth and reduction to the corresponding tertiary amines. For the tertiary amines, dilution due to shoot growth is compensated by the conversion of *N*-oxides to tertiary amines. At the same time, root herbivory can result in the leakage of PAs from wounded root tissues to the soil; or increased exudation of PAs into the soil as an induced defence response against attackers (Van Dam 2009).

In the greenhouse experiment, I observed that upon root herbivory the concentration of erucifoline *N*-oxide (Er-ox) decreased whereas the concentration of acetylerucifoline *N*-oxide (AcEr-ox) increased in aboveground plant parts although the total concentration of the erucifoline-type PAs remained relatively constant (*Chapter 2*). Thus, root herbivory aside from quantitative changes also affected the composition of PAs in the shoots. Likewise, I found a shift of Er-ox towards AcEr-ox in plants growing in soils with a legacy of AG herbivory (*Chapter 3*). These plants were grown in soils with a negative plant-soil feedback possibly caused by an increase in the abundance of pathogenic fungi. I propose that this high pathogen pressure in the soil could exert a similar effect on plant roots as actual root herbivory resulting in comparable changes in Er-type PA profiles in aboveground plant parts. Similarly, but in the opposite direction, the undamaged (control) plants in *Chapter 2* and plants growing in soils with legacies of BG herbivory (*Chapter 3*) had higher levels of erucifoline and low levels of acetylerucifoline PAs. These plants were more robust because they were not damaged and were growing in soil with positive plant-soil feedback, perhaps resulting from a reduction in the abundance of pathogenic fungi.

Remarkable, almost all but three of the 150 plants collected from the chronosequence of ex-arable fields from the Veluwe region also contained high concentrations of erucifoline and low concentrations of acetylerucifoline PAs (*Chapter 8*). Because root and shoot herbivores are ubiquitous in natural communities I speculate that this shift in erucifoline-type PAs in plants collected from the chronosequence fields may be explained by legacy effects of AG-BG herbivory. Alternatively, it could indicate that the plants in those fields were not damaged belowground as their erucifoline profile was similar to control plants in *Chapter 2*. However, *J. vulgaris* plants (vegetative and reproductive) that were planted into the biodiversity plots contained relatively high concentrations of AcEr and AcEr-ox compare to Er and Er-ox, which is comparable with the erucifoline profile of the root-damaged plants (*Chapter 5,* data not shown). The biodiversity plots were set-up within one of the chronosequence fields. Therefore, root herbivory may be quite common in all these fields. These findings suggest that erucifoline-type PAs can be involved in plant-soil-insect interactions on *J. vulgaris* or are affected by some other processes or interactions that also occur in our experiments. Interestingly, several studies investigating *J. vulgaris* PA profiles also found changes in erucifoline-type PAs, though there are no records on the specific effects of erucifoline-type PAs on insect herbivores or other organisms in the literature (Hol et al. 2004; Joshi & Vrieling 2005; Macel & Klinkhamer 2010). For example, Hol et al. (2004) also reported that total erucifoline concentrations changed following aboveground herbivore attack and mechanical root damage while the other PA-types did

not change. In a field study of ten *J. vulgaris* genotypes that differed in the relative abundance of jacobine- and erucifoline-type PAs the total erucifoline concentration increased in all genotypes when they where planted in the sand dune area where the plant naturally occurs (Macel & Klinkhamer 2010). However, the increase in erucifoline concentration did not correlate with herbivore or pathogen damage. Future studies are needed to discover the causes and consequences of the changes of these intricate secondary metabolites.

## Potential applications

*Control of* J. vulgaris *by insect herbivores*

Because of the quick spread throughout several continents and due to numerous instances of poisoned domestic animals, *J. vulgaris* has become a prominent invasive weed. Also in its native range, due to the outbreaks in semi-natural grasslands and toxicity to livestock, especially cattle and horses, it is considered a noxious pest. Therefore, a range of management practices have been proposed in order to control *J. vulgaris* both in native and non-native ranges. For example, a biological control programme has been developed in which root and shoot feeding insect herbivores are released to suppress *J. vulgaris* densities (Leiss et al. 2011). The research in my thesis was not aimed at improving biological control of *J. vulgaris*. However, as I studied the insect community associated to *J. vulgaris*, my results and field observations may provide some insight into the factors that can impact the effectiveness of insect herbivores in controlling *J. vulgaris* in its natural range.

*Tyria jacobaeae* is considered the most important herbivore of *J. vulgaris* (Van der Meijden & Van der Veen-Van Wijk 1997). In the ex-arable fields at the natural area Veluwe (located in the central eastern part of the Netherlands), where I conducted my experiments (*Chapters 4-8*), the abundance of *J. vulgaris* can be very high (Van de Voorde et al. 2012; *Chapter 8*). Nevertheless, *T. jacobaeae* is rare or totally absent in some of those fields (Macel & Klinkhamer 2010; O. Kostenko, personal observation). The quality of the plants cannot explain the absence of the herbivore in the fields as in controlled conditions larvae of *T. jacobaeae* readily consumed plants that originated from different fields (*Chapter 8*). In contrast, in coastal open sand dune areas of the Netherlands, the density of *T. jacobaeae* is often high and larvae frequently completely defoliate *J. vulgaris* plants over large areas (Van der Meijden & Van der Veen-Van Wijk 1997). During four years of field research I have never observed complete defoliation of *J. vulgaris* plants by *T. jacobaeae* at the scale of more than 10 $m^2$

while patches of *J. vulgaris* can be multitudes larger and this plant can even cover entire fields of many hectares in size (Fig. 9.4). Also in the experimental biodiversity plots with 1750 *J. vulgaris* individuals no *T. jacobaeae* were found in the first year after plant transplantation (*Chapter 4*) and only about 150 larvae were collected during the second year (O. Kostenko, unpublished data). Similarly, Crawley & Gillman (1989) reported that *T. jacobaeae* defoliation had little or no impact on annual fluctuations in *J. vulgaris* abundance in densely vegetated areas in the United Kingdom. A study that compared the long-term herbivore-plant population dynamics in Dutch coastal dunes and UK habitats proposed that additional factors, such as interspecific plant competition and the natural enemies or competitors in habitats with dense background vegetation, may explain the differences between two study sites (Bonsall et al. 2003). Additionally, several studies measuring the impact of *T. jacobaeae* in invaded areas reported no or only local effects on the spread and density of *J. vulgaris* infestations (Wardle 1987; McLaren et al. 2000 but see Harris et al. 1975) altogether suggesting that *T. jacobaeae* may not be particularly effective in controlling *J. vulgaris* spread.

Roots are more essential organs for *J. vulgaris* than shoots and damage to the roots can be detrimental to the plant (Van der Meijden 1979; *Chapters 2* and *3*). Therefore, the herbivores attacking roots of *J. vulgaris*, such as flea beetles (*Longitarsus jacobaeae* and *L. flavicornis*) can potentially be successful biological control agents of *J. vulgaris*. Indeed, *L. jacobaeae* and *L. flavicornis* proved to be very effective in controlling *J. vulgaris* in invaded areas (about 90% reduction; Pemberton & Turner 1990; McEvoy et al. 1991; McLaren et al. 2000) and can reduce plant survival by 50% in the Dutch dunes (Windig 1991). The damage the beetles exert to the roots particularly negatively affects the plant during the vulnerable stage when it shifts from the rosette phase to the reproductive phase. In the biodiversity field experiment, I only collected six adults of *L. jacobaeae* on the plants during the first year (*Chapter 4*). However, the adults are difficult to catch due to their jumping behaviour and these results therefore might not represent the real densities in the field. Therefore, I also recorded leaf and stem damage on the experimental plants during the second year. These results indicate that *L. jacobaeae* can be a widespread herbivore on *J. vulgaris*. Recently, researchers of Dutch Louis Bolk institute have proposed to release *L. jacobaeae* as a biological control of *J. vulgaris* in the Netherlands (Bos 2010). However, previous studies suggest that its spread in the east of the Netherlands and the ability to control *J. vulgaris* can be hindered by less favourable soil abiotic factors and high levels of parasitism (Windig 1991; Bos 2010).

**Figure 9.4** *Jacobaea vulgaris* in an ex-arable field that was taken out of production three years earlier (Oud Reemst, Veluwe region, the Netherlands).

The most abundant specialist herbivore in my study area was the aphid species *Aphis jacobaeae* (*Chapter 4*, O. Kostenko personal observation). I could not estimate the effect of aphids on plant performance in the experimental plots because I removed aphids from the plants during each census but Vrieling and colleagues (1991) assumed that feeding by specialist aphids results in a reduction in seed output. Furthermore, *A. jacobaeae* is tended by ants that can effectively defend *J. vulgaris* plants infested with *A. jacobaeae* against larvae of *T. jacobaeae*, allowing plants to escape total defoliation and produce seeds although less than undamaged plants (Vrieling et al. 1991). This suggests that specialist aphids are not controlling *J. vulgaris* plants but instead may promote *J. vulgaris* persistence in natural communities.

To summarise, the characteristics of the habitat where plants grow, and the presence of predators can all influence the effectiveness of natural enemies in controlling *J. vulgaris* in their native range.

*Interspecific competition, soil legacies and* J. vulgaris *dynamics*

Recently, Van de Voorde and colleagues (2012) have shown that plant-soil feedbacks can be important drivers of *J. vulgaris* population dynamics and that these feedbacks can cause a decrease of *J. vulgaris* abundance over time in old fields. The field survey of 600 *J. vulgaris* plants that I carried out in the same fields as those used by Van de Voorde et al. (2012) revealed that the aboveground herbivores were present on *J. vulgaris* plants in all fields although their abundance (in particular stem-borers) increased with time since abandonment (*Chapter 8*). As I demonstrated in *Chapter 3*, feeding by aboveground herbivores can modify the outcome of plant-soil feedback and induce a legacy effect in the soil. This soil legacy negatively affects the growth and production of defence compounds in the plants growing later in the same soil and thereby positively affects generalist herbivores feeding on those plants (Fig. 9.1B). The increase in herbivore performance can lead to increased levels of herbivory that, in turn, weaken the plant and can cause the decline or disappearance of this plant species in the field. Thus, herbivore-induced soil legacies could be an alternative mechanism explaining why *J. vulgaris* declines over time in old fields. However, smaller plants, although they might be of a better quality for insects (*Chapters 3* and *8*), can also be physically obscured by the taller neighbouring plants or dense surrounding plant communities that can hinder insect herbivores in finding their host plant (*Chapter 4*). In line with this, Bezemer and colleagues (2006) previously showed that there are fewer insects on smaller *J. vulgaris* plants in the field. Consequently, these small plants could survive and reproduce leading to the persistence of *J. vulgaris* in later successional fields although at much lower densities.

It is important to mention that I used *Mamestra brassicae* as aboveground generalist herbivore in my greenhouse experiments and that *M. brassicae* does not control *J. vulgaris* populations in the field even though it has been recorded feeding on *J. vulgaris* (De Boer 1999; Hol et al. 2004; Macel et al. 2005). In the field and in common garden experiments I observed another aboveground generalist herbivore, larvae of the moth *Autographa gamma*, that readily consumed *J. vulgaris* rosettes. *J. vulgaris* is also frequently attacked by other generalist herbivores such as *Eupithecia* spp., *Phycitodes* spp., *Brachycaudus* spp., leaf-mining agromyzids and stem-boring agromyzids in the densely vegetated areas of the Veluwe region (Macel & Klinkhamer 2010; O. Kostenko personal observation). Therefore, generalist herbivores could play an important role in the feedbacks between *J. vulgaris* and soil biota in the densely vegetated areas.

*Jacobaea vulgaris* is a poor competitor and the ability of the plant to persist in the field will be largely determined by the presence of the open areas and by interspecific competition with the surrounding plants (Crawley & Gillman 1989; Bonsall et al. 2003). In accordance with this, in *Chapters 4* and *5*, I showed that *J. vulgaris* plants were significantly larger in bare soil plots than in any of the plots with the background vegetation. Moreover, approximately 20% of *J. vulgaris* plants in the vegetated plots and 80% in bare plots produced a flowering stem in the second year after *J. vulgaris* seedlings were transplanted in the experimental communities (*Chapter 5*). Similarly, the presence of open spaces in the vegetation caused by grazing activities of large herbivores (cows and horses) and local disturbance by wild boars in ex-arable fields at the Veluwe facilitates the germination of *J. vulgaris* plants. These local disturbances create open spaces in the vegetation and thereby allow new *J. vulgaris* plants to germinate. Therefore, I propose that the interplay between insect herbivory, soil legacy effects and the effects of the surrounding community determine the temporal pattern in *J. vulgaris* abundance in the field. Further studies are needed that examine the relative importance of these factors for *J. vulgaris* dynamics in natural areas.

Finally, it is important to note that management practices aiming at the control of *J. vulgaris* in its native range should take into account that *J. vulgaris* is part of the native ecosystem and that more than 150 insect species, several of which are endangered in the Netherlands (e.g., *Argynnis aglaja*, *Melitaea athalia, Satyrium ilicis, Hesperia comma, Issoria lathonia, Lycaena tityrus)* rely on *J. vulgaris* for food and survival.

## Conclusions

- Belowground herbivory, soil legacy effects of aboveground and belowground herbivores, surrounding plant communities, and temporal changes in the surrounding communities all contribute to the intraspecific variation in *J. vulgaris* quality aboveground.

- Belowground herbivory negatively affects the concentration of pyrrolizidine alkaloids in the leaves of *J. vulgaris* possibly via the mechanism of restricted transport of pyrrolizidine alkaloids *N*-oxides from roots to leaves, but it does not affect the development of aboveground parasitoid.

- Aboveground multitrophic communities are affected by soil legacies created by aboveground and belowground herbivory on preceding plants growing in the same soil.

- Insect herbivory can influence plant-soil feedback effects by affecting soil biota during the conditioning phase. Plant-soil feedback effects go beyond affecting plant biomass and can also affect plant quality and aboveground multitrophic interactions.

- The presence and the diversity of the surrounding community provide associational resistance against a specialist aboveground herbivore to focal *J. vulgaris* plants growing in that community. The surrounding plant community directly affects the abundance of the specialist herbivore on focal plants, and not via the effects of surrounding plants on the performance of the focal plant.

- Parasitoid host-finding abilities are not affected by the diversity of the surrounding plant community but rather by the structural complexity of the vegetation in which the host-infested plant is embedded.

- Belowground, increasing plant species diversity enhances the level of predation in the soil indirectly by modifying the interactions with their prey, but it does not affect predator abundance. However, the responses of belowground organisms to manipulation in plant diversity can be specific and may differ between organisms that belong to different species but the same feeding guild, such as entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis*.

- Characteristics of both *J. vulgaris* and its associated insect community change during secondary succession, but insect herbivore and parasitoid abundances are not directly related to host plant quality.

To summarise, my PhD research provides new insights into the role of individual plant quality and the surrounding plant community for insect communities associated to individual plants. One of the new insights/interactions I discovered is that herbivores can cause herbivore-induced soil legacies. These legacies potentially are an important mechanism for the spatiotemporal dynamics of plant-insect interactions in natural communities. My research shows that there is high intraspecific variation in plant quality among individual plants in the field. However, it remains difficult to disentangle the role of this intraspecific variation in plant quality in structuring insect communities in natural settings where the surrounding plant community may have a greater effect on the colonizing aboveground communities associated to individual plants than the host plant itself.

## Future challenges

Because belowground and aboveground herbivores are ubiquitous components of terrestrial ecosystems, herbivore-induced legacy effects may be an important process that links the dynamics of spatially and more importantly temporally separated aboveground and belowground communities. Since *Chapter 3* reports the first description of this phenomenon, it generates numerous questions that deserve further investigation. I will only list a few of them: it is not clear what mechanism is causing these legacies, or how widespread they are in nature, or how long they will last, and what their role is in structuring terrestrial communities compared to other important drivers.

In this thesis I demonstrated that there is a large amount of intraspecific variation in plant quality induced by herbivory, soil legacies, surrounding community and successional changes. Other studies have shown that this variation can affect herbivore performance and preference, and can even extend to higher trophic levels (Bukovinszky et al. 2008; Poelman et al. 2009b). However, less is known about the effects of individual variation in plant quality on the population dynamics and diversity of insects in natural settings, where also other factors independently or interactively can affect the composition of insect community. Therefore, it remains a challenge to further disentangle the effects of plant quality from e.g., effects of plant community, abiotic conditions, and environmental changes on structuring insect communities in terrestrial ecosystems.

Despite the large body of ecological work on associational resistance and susceptibility and aboveground-belowground interactions, less is known about belowground associational effects. There are numerous questions that will be important to address in the future studies of associational effects, for example, whether the presence of certain neighbours decreases/increases the likelihood of being attacked by both shoot and root herbivores; whether associational effects aboveground are independent of associational effects belowground; and whether the mechanisms by which plants gain associational resistance/susceptibility from neighbours differ above- and belowground.

A number of studies indicated that pathogenic fungi might be involved in the regulation of *J. vulgaris* dynamics in the natural communities (Hol & Van Veen 2002; Bezemer et al. 2006; Van de Voorde et al. 2012; Bezemer et al. 2013; *Chapter 3*). These soil pathogens could be used as potential biological agents to control *J. vulgaris* spread in its native and invasive ranges. However, identification and inoculation studies are needed to ascertain which fungal species is or are responsible for the decline in *J. vulgaris* performance and

whether this fungus does not affect the performance of other plants before it could be used in natural communities, especially in the invaded areas.

Finally, although the importance of PAs in plant-insect interactions has been increasingly recognised, the ecological functions of the majority of the PAs remain unknown. Also, the exact mechanism of the synthesis of PAs has not been described in detail hampering the interpretation of ecological effects. Further, the results of *Chapter 3* suggest that PAs can play an important role in the interactions between plants and soil biota. However, practically nothing is known about the abundance of PAs in the soil or in root exudates. From an applied perspective, PAs are harmful chemicals to human and livestock and given their wide occurrence in nature, knowledge on natural sources of PAs will be useful for quality control of medical herbs, livestock forage, milk, and honey products. This knowledge could also be used for generating an international regulation of PAs in food.

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# *Summary*

Plants harbour diverse multitrophic insect communities with myriad interactions that form the foundation of communities and ecosystems, and are responsible for a variety of important ecological functions. During the past three decades, ecologists and entomologists have become increasingly aware that the density and composition of insects on a given plant species can vary greatly among individuals of that species. Understanding factors causing this variability can help us to predict the composition of insect communities on plants and their responses to environmental changes. Therefore, the main aim of my thesis was to elucidate factors that structure the insect community associated to individual plants. Specifically, in a combination of greenhouse experiments and field studies I examined how the insect communities on individual plants can be affected by intraspecific variation in the quality of the host plant and by the characteristics of the surrounding plant community in which the host plant is embedded. I used ragwort (*Jacobaea vulgaris* Gaertner ssp. *vulgaris)* and its associated aboveground and belowground communities as a model system. *Jacobaea vulgaris* is a suitable model system to address this question because there is great variability in amounts and patterns of pyrrolizidine alkaloids (PAs, plant defence compounds characteristic for *J. vulgaris*) in natural populations and because the plant grows in communities ranging from almost monospecific stands to diverse grasslands. Moreover, *J. vulgaris* supports a wide variety of specialist and generalist insects.

Plant quality plays a major role in mediating interactions between belowground and aboveground insects. These belowground-aboveground interactions have been extensively studied over the past two decades, but how herbivory by root feeding insects affects the development of aboveground parasitoids and what the role is of PAs in these interactions remains largely unknown. In a greenhouse experiment, I tested the hypothesis that root herbivory will result in the increase of plant secondary compounds, in particular PAs, in aboveground plant tissues and thereby negatively influences the performance of an aboveground herbivore and its parasitoid (*Chapter 2*). Contrary to the majority of previous studies and my hypothesis, I found that root herbivory negatively affected the concentration of the secondary plant compounds in the shoots of *J. vulgaris*. Interestingly this reduction in plant defence compounds did not result in an increased performance of the aboveground generalist insect herbivore or its parasitoid. Aboveground herbivore and parasitoid performance were not significantly affected by root herbivory. Root herbivory also elicited changes in the composition of shoot PAs, but the concentration of more toxic PA compounds did not differ between control plants and plants exposed to root herbivory. This may explain why the performance of aboveground insects was not affected by the quantitative

changes in plant defence compounds caused by root herbivory. I propose a mechanism by which root herbivory can cause changes in PA concentration and composition in plants.

Although interactions between aboveground and belowground organisms have been extensively studied, the temporal dynamics of these interactions and their feedback effects remain poorly understood. I performed a two-phase greenhouse experiment (*Chapter 3*) to test a novel hypothesis that aboveground and belowground herbivory will cause legacy effects in the soil that will subsequently affect the growth and chemistry of plants growing later in that soil, and that this, in turn, will influence plant interactions with aboveground herbivores and natural enemies. I discovered that aboveground and belowground herbivores can create unique soil legacy effects through herbivore-induced changes in the composition of soil microbial community. Via these soil legacies, aboveground and belowground insect herbivores affected the chemical quality of the plants growing later in the same soil and the performance of insect herbivores and parasitoids feeding on these plants. A novel insight from this study was the suggestion that the insect community present at any stage of ecosystem development may reflect insect-plant interactions from the past. The results of this study also offer several important contributions to the research areas of aboveground-belowground interactions and plant-soil feedback, demonstrating that aboveground and belowground herbivory during the conditioning phase can affect plant-soil feedback responses; and that these effects go beyond affecting plant biomass, and can also affect plant chemistry and their multitrophic interactions. Therefore, to better understand the dynamics and structure of multitrophic insect communities it will be important not only to focus on plant-insect interactions, but also on the feedback effects that occur between soil biota, plants and insects.

To understand how the surrounding plant communities influence insects on individual plants I manipulated the diversity and composition of plant communities in a biodiversity field experiment (*Chapters 4, 5, 6 and 7*) and used a chronosequence of ten ex-arable fields where plant communities undergo successional changes (*Chapter 8*). Moreover, I also examined whether the surrounding plant communities affect the quality of the focal plants and to what extent the composition and abundance of the insect communities on these focal *J. vulgaris* plants are driven by changes in the host plant and by the surrounding community. In the biodiversity experiment, I planted individual *J. vulgaris* plants into experimental plant communities that were sown and maintained at different plant diversity levels (1–9 species) and composition, or that were kept without background vegetation and I studied natural colonization

of the local insect community on these plants. In the chronosequence, I sampled individual *J. vulgaris* that naturally colonized the ex-arable fields.

In the first year after the focal *J. vulgaris* plants were planted in the experimental grassland communities, the arthropod fauna was dominated by the specialist aphid *Aphis jacobaeae* (*Chapter 4*). Individual *J. vulgaris* plants harboured a higher abundance of the specialist aphids in less diverse and more open communities compared to the plants growing in more diverse (and more dense) communities indicating that the diversity of the surrounding community can provide associational resistance to focal plants growing in that community. The size of the individual plants was not affected by the diversity of the surrounding plant community, but in monocultures, the size of the focal *J. vulgaris* plants depended on the identity of the surrounding community. The occurrence of aphids was also significantly affected by the identity of the monospecific stand. Aphid densities were much higher and the focal plants were significantly larger in bare plots than in plots with the background vegetation. Aphid abundance on *J. vulgaris* plants, however, was not related to the size of the individual *J. vulgaris* plants, suggesting that the surrounding plant community directly affects the abundance of specialist herbivores on focal plants, and not via the effects of the surrounding vegetation on the performance of the focal plants.

The surrounding plant community also strongly affected the development of the focal *J. vulgaris* plants during the second growth period. Only 30% of the *J. vulgaris* produced flowering stems two years after the plants had been transplanted into the experimental plots (*Chapter 5*). Overall, increased diversity of the surrounding community tended to decrease the PA concentration of the focal plants. However, the presence and the identity of the surrounding community were more important for nutritional and chemical quality of the focal plants than plant diversity per se. In line with the previous results, I found that both reproductive and vegetative plants gained more biomass, contained higher nitrogen and PA concentrations when grown on bare soil than when grown in plots with surrounding vegetation. In general, reproductive plants contained higher foliar PA concentrations and lower nitrogen concentration than vegetative plants. The results of the second year show that the characteristics of the surrounding plants can greatly affect the intraspecific variation in plant nutritional and chemical quality.

Associational effects of the surrounding community on focal plants can also be mediated by higher trophic level organisms, such as parasitoids. For example, surrounding plant communities can physically obscure the herbivore-infested plants from herbivore natural enemies, such as parasitoids, thereby providing associational susceptibility to the plant. I used a release-recapture experiment to

examine whether the diversity and structural complexity of the plant community surrounding a host plant infested with the generalist leaf-miner *Chromatomyia syngenesiae* influences the host-finding ability of the parasitoid *Dacnusa sibirica* (*Chapter 6*). I also assessed the effect of the surrounding plant community on the locally present parasitoid community of the leaf-miner. In contrast to my hypothesis and the results of earlier studies, the generalist leaf-miner parasitoid *D. sibirica* preferred to forage in structurally more complex plant communities. The locally present parasitoid community preferred more open and structurally less complex plots. The preference of local parasitoids for less diverse communities in this study suggests that the rates of parasitism may decrease in high diverse plant communities resulting in associational susceptibility of plants to leaf-mining herbivores.

Increase in plant diversity often results in an increase in parasitoid and predator diversity and abundance leading to increased parasitism in aboveground communities. How plant diversity affects the abundance of predators and level of predation in belowground communities is less well understood. To study this, I measured the infectivity of entomopathogenic nematodes (EPNs) which are natural enemies of soil insects, and determined the abundance of other carnivorous nematodes in the experimental biodiversity plots. I used structural equation modelling (SEM) to analyse the possible pathways through which plant diversity influences predatory soil organisms (*Chapter 7*). To get a comprehensive view of the potential prey or food availability I measured the abundance of soil insects and non-predatory nematodes and quantified root biomass production in the experimental plots. SEM revealed that increasing plant species diversity enhanced the infectivity of EPNs in the soil indirectly by modifying the interactions with their prey, but plant diversity did not affect the abundance of carnivorous nematodes. The responses of belowground organisms to manipulation in plant diversity, however, were not consistent and differed between two genera of EPNs *Steinernema* and *Heterorhabditis* and other free-living nematodes.

During secondary succession the composition of plant community changes, but the size, abundance and other characteristics of individuals of a focal plant species also change over time. Both the changes in the characteristics of the surrounding plants and in the individuals of a focal species can influence the abundance and composition of the insects that are found on these focal plants. I investigated how size, nutritional quality, secondary chemistry and the insect communities associated to individual *J. vulgaris* plants change during succession in a chronosequence of ex-arable fields (*Chapter 8*). I also examined whether the variability in insect communities on these individual plants can be explained by

changes in plant nutritional quality. *Jacobaea vulgaris* plants were significantly larger but had lower nitrogen concentrations in recently abandoned sites than in older sites. The PA composition of these individual plants differed significantly among sites but there was no relationship between the age of a site and PA concentration or composition. The abundance of stem-boring insects, parasitoids emerging from stems and total parasitoid diversity significantly increased with site age. However, insect herbivore and parasitoid abundances were not directly related to the quality of the plant individual. This result was also supported by a common garden experiment, where I did not observe differences in performance of *Tyria jacobaeae* larvae, a common specialist herbivore on *J. vulgaris* plants originating from different successional sites.

In conclusion, my PhD research shows that herbivory by soil insects, soil legacies of aboveground and belowground herbivores, surrounding plant communities, and temporal changes in the surrounding communities all contribute to the intraspecific variation in *J. vulgaris* quality. These changes in plant quality affect the performance of aboveground herbivores and parasitoids on individual plants in controlled conditions. In natural ecosystems, however, plant communities surrounding individual plants may overrule the effects of plant quality on the insect communities on individual plants. Therefore, individual plant-insect interactions should be considered from the community perspective and future studies should aim at further disentangling the role of plant quality in structuring insect communities in natural settings.

# *Nederlandse samenvatting*

Planten en insecten vormen de basis van veel ecosystemen en ze zijn verantwoordelijk voor belangrijke ecologische functies. Gedurende de laatste drie decennia zijn ecologen en entomologen zich er steeds meer bewust van geworden dat het aantal insecten en de soortsamenstelling van die insecten op één plantensoort sterk kan variëren tussen individuen van die plantensoort. Het is echter nog steeds niet duidelijk welke factoren verantwoordelijk zijn voor deze variatie en op welke manier die variatie tot stand komt. Met meer kennis hierover kunnen we beter voorspellen hoe insectengemeenschappen er op een bepaalde plant uitzien en hoe ze zullen reageren op veranderingen in het milieu. Eerder onderzoek heeft aangetoond dat zowel de kwaliteit van de waardplant (zoals de hoeveelheid voedingsstoffen en plantverdedigingsstoffen) als haar omgeving insecten kunnen beïnvloeden. In dit promotieonderzoek heb ik met een combinatie van kas- en veldexperimenten onderzocht hoe intraspecifieke variatie in de waardplantkwaliteit en de omringende planten de insectengemeenschap op een individuele plant bepalen. Ik heb dit onderzoek gedaan met Jakobskruiskruid (*Jacobaea vulgaris* Gaertner ssp. *vulgaris)* en de bovengrondse en ondergrondse multitrofe gemeenschap waar deze plant mee geaffilieerd is. Populaties van Jakobskruiskruid vertonen veel variatie in concentraties pyrrolizidine alkaloïden (PAs), plantverdedigingsstoffen die karakteristiek zijn voor *J. vulgaris*. Bovendien komen natuurlijke populaties van *J. vulgaris* in verschillende types vegetaties voor, en komt de plant zowel in hoge dichtheden voor als in lage dichtheden in soortenrijke graslanden. Bovendien is *J. vulgaris* waardplant van een groot aantal gespecialiseerde en generalistische insecten.

In mijn onderzoek heb ik allereerst de rol van de voedingswaarde van de plant in de interacties tussen ondergrondse en bovengrondse insecten bestudeerd. Zulke ondergrondse-bovengrondse-interacties zijn de afgelopen twee decennia veelvuldig bestudeerd. Hoe ondergrondse, worteletende insecten de groei en ontwikkeling van bovengrondse sluipwespen (natuurlijke vijanden van de plantenetende insecten) beïnvloeden is echter nog praktisch niet onderzocht. Ook de rol van PA's in deze boven- en ondergrondse interacties is nog grotendeels onbekend. In een kasexperiment testte ik de hypothese dat wortelvraat resulteert in een toename van plantverdedigingsstoffen (zoals PA's) in bovengronds plantweefsel en daardoor een negatieve invloed heeft op de groei van bovengrondse plantenetende insecten en sluipwespen (***hoofdstuk 2***). In tegenstelling tot een grote meerderheid van eerder gepubliceerde studies en mijn hypothese ontdekte ik dat beschadiging van de wortels door ritnaalden resulteerde in een lagere

concentratie van plantverdedigingsstoffen in de bladeren van *J. vulgaris*. Interessant is dat deze vermindering in plantverdedigingsstoffen niet leidt tot een verhoogde groei van de bovengrondse generalistische plantetende insecten en van sluipwespen. De bovengrondse insecten werden nauwelijks beïnvloed door worteleters. Worteletende insecten beïnvloedden niet alleen de hoeveelheid, maar ook de samenstelling van PA's in het blad. Dit leidde echter niet tot een verschil in de concentratie van giftige PA's tussen controleplanten en planten die aangetast waren door worteletende insecten. Dit kan verklaren waarom de groei van bovengrondse insecten niet beïnvloed werd door worteleters terwijl de totale hoeveelheid plantverdedigingsstoffen in het blad verminderde. Op basis van deze resultaten beschrijf ik een mogelijk mechanisme dat kan verklaren hoe wortelvraat door insecten kan leiden tot veranderingen in PA-concentraties en de samenstelling van PAs in planten.

Hoewel er recentelijk veel onderzoek gepubliceerd is over de interacties tussen ondergrondse en bovengrondse organismen, is de temporele dynamiek van deze interacties nog vrij onbekend. Om deze temporele effecten te bestuderen voerde ik een kasexperiment uit dat bestond uit twee fasen (*hoofdstuk 3*). In dit experiment testte ik de hypothese dat bovengrondse en ondergrondse schade aan de plant door insecten kan leiden tot veranderingen in de dichtheid en soortensamenstelling van bodemmicro-organismen, die vervolgens de groei en chemie van een nieuwe generatie planten op diezelfde bodem kunnen beïnvloeden (ook wel "legacy of nalatenschapeffect" genoemd). Deze veranderingen in plantengroei en –chemie kunnen op hun beurt de interacties beïnvloeden tussen deze nieuwe planten en hun bovengrondse herbivoren en de natuurlijke vijanden van die herbivoren. De resultaten van het experiment lieten zien dat wortel en bladschade door insecten inderdaad kan leiden tot *legacy*-effecten in de bodem. Nieuwe planten die in grond opgroeiden met een legacy van boven- of ondergrondse schade, verschilden sterk in hun groei en chemische samenstelling. Opmerkelijk is dat deze veranderingen in de chemie van de plant ook de interacties tussen de nieuwe plant en plantenetende insecten en hun natuurlijke vijanden beïnvloedden. Dit impliceert dat de groei van een insect op een plant de interacties tussen planten en insecten uit het verleden kunnen weerspiegelen. De resultaten van dit onderzoek zijn belangrijk voor zowel het onderzoeksgebied van bovengrondse-ondergrondse-interacties en het onderzoek naar plant-bodem(organisme) interacties. Bovengrondse en ondergrondse vraat door insecten kan plant-bodemfeedback beïnvloeden en bodem micro-organismen kunnen behalve plantengroei ook de chemie van een plant en de multitrofe interacties op die plant beïnvloeden. Om de dynamiek en de structuur van multitrofe insectengemeenschappen beter te begrijpen, is het belangrijk

om plant-insect interacties te bestuderen in samenhang met feedback-effecten tussen bodemorganismen, planten en insecten.

Om te bestuderen hoe de planten die om een waardplant heen staan de insecten op die waardplant beïnvloeden heb ik een biodiversiteitsveldexperiment opgezet (*hoofdstukken 4, 5, 6 en 7*) en onderzoek gedaan op tien voormalige landbouwgronden die 3 tot 26 jaar geleden uit productie zijn genomen (een zogenaamde chronosequentie, *hoofdstuk 8*). Ik heb ook onderzocht of de omringende planten of de plantengemeenschap waarin de waardplant groeit de kwaliteit van de waardplant *J. vulgaris* kunnen beïnvloeden; en in welke mate het aantal insecten en de samenstelling van de insectengemeenschap op deze waardplanten verklaard kan worden door veranderingen in de waardplant of de omringende planten. In het biodiversiteitsexperiment heb ik veldjes aangelegd die verschilden in diversiteit van 1-9 plantensoorten en in soortensamenstelling. Ook waren er veldjes zonder vegetatie. In al die veldjes heb ik 25 individuele *J. vulgaris* planten geplant en ik heb de natuurlijke kolonisatie van de lokale insectengemeenschap op deze planten bestudeerd. In de chronosequentie bemonsterde ik individuele *J. vulgaris* planten die op natuurlijke wijze de voormalige landbouwvelden hebben gekoloniseerd.

In het eerste jaar nadat de *J. vulgaris* planten werden geplant in de experimentele veldjes, domineerde de specialistische bladluis *Aphis jacobaeae* op *J. vulgaris* (*hoofdstuk 4*). Het aantal bladluizen was hoger op individuele *J. vulgaris* planten die in minder diverse en meer open plantengemeenschappen groeiden, ten opzichte van *J. vulgaris* planten die in meer diverse (en compactere) plantengemeenschappen groeiden. Dit resultaat geeft aan dat de diversiteit van de omringende plantengemeenschappen ervoor kan zorgen dat individuele planten "associatieve afweer" tegen herbivoren kunnen ervaren. De groei van de *J. vulgaris* planten werd niet beïnvloed door de diversiteit van de omringende plantengemeenschappen. Echter, in veldjes waarin slechts één plantensoort groeide naast de *J. vulgaris* planten was er een significant effect van identiteit van de monocultuur op de groei van *J. vulgaris* en de aanwezigheid van bladluizen op die planten. Het aantal bladluizen was veel hoger en de *J. vulgaris* planten waren aanzienlijk groter in de veldjes zonder achtergrondvegetatie dan in de veldjes met achtergrond vegetatie. Het aantal bladluizen was niet gerelateerd aan de grootte van individuele *J. vulgaris* planten. Dit suggereert dat de omringende plantengemeenschap een direct effect had op het aantal specialistische herbivoren op *J. vulgaris*. Er was geen bewijs voor een indirect effect waarbij de omringende vegetatie de groei van de *J. vulgaris* beïnvloedt en waarbij de groei van de *J. vulgaris* plant vervolgens de bladluizen beïnvloedt.

Ook in het tweede jaar hadden de omringende planten een sterk effect op de ontwikkeling van de *J. vulgaris* planten. Slechts 30% van de *J. vulgaris* planten bloeide in het tweede jaar (*hoofdstuk 5*). De PA-concentratie van *J. vulgaris* planten was lager als deze planten in diverse plantengemeenschappen groeiden. De invloed van de aanwezigheid en de identiteit van de omringende planten op de voedings- en chemische kwaliteit van de *J. vulgaris* planten was echter belangrijker dan de diversiteit van de omringende planten. Zowel bloeiende als vegetatieve planten hadden meer biomassa en bevatten hogere stikstof- en PA-concentraties wanneer ze in veldjes zonder achtergrondvegetatie groeiden dan in veldjes met omringende planten. De bloeiende *J. vulgaris* planten hadden hogere PA-concentraties en lagere stikstofconcentraties dan de vegetatieve planten. De resultaten van het tweede jaar van dit veldexperiment laten zien dat omringende planten grote invloed kunnen hebben op de intraspecifieke variatie in de voedingsstoffen en verdedigingsstoffen van een plant

Zogenaamde "geassocieerde effecten" van omringende planten op een individuele plant kunnen ook worden bewerkstelligd door de invloed van die omringende vegetatie op natuurlijke vijanden van herbivoren, zoals sluipwespen. De omringende vegetatie kan bijvoorbeeld meer sluipwespen aantrekken door voedsel, zoals nectar, of schuilgelegenheden te bieden. Als dit leidt tot meer sluipwespen in een lokale plantengemeenschap dan kan dit ook leiden tot meer parasitering van herbivoren op een individuele plant. Om te onderzoeken hoe de diversiteit van de omringende vegetatie de parasitering van herbivoren op een individuele plant beïnvloedt, heb ik een *release-recapture*-studie (uitzet- en terugvangexperiment) uitgevoerd in het biodiversiteitsexperiment. Ik testte hiermee of de diversiteit en structuur van de plantengemeenschap waarin een waardplant groeit, invloed heeft op het vermogen van sluipwespen om gastheren (herbivoren) te vinden (*hoofdstuk 6*). Voor dit experiment gebruikte ik de generalistische bladmineerder *Chromatomyia syngenesiae* en de sluipwesp *Dacnusa sibirica*. In elk veldje van het biodiversiteitsexperiment plaatste ik *J. vulgaris* planten met mijnen van de bladmineerder. Vervolgens liet ik sluipwespen los en ving ze terug op deze planten. In tegenstelling tot de resultaten van eerdere studies was het aantal teruggevangen *D. sibirica* hoger in complexe plantengemeenschappen dan in plantengemeenschappen met weinig variatie in structuur. Ik bestudeerde ook hoe de diversiteit van de omringende plantengemeenschappen de lokaal aanwezige sluipwespen van de bladmineerder beïnvloedde. De lokale sluipwespsoorten werden vaker gevangen in meer open plantengemeenschappen met weinig variatie in structuur. De voorkeur van lokale sluipwespen voor mindercomplexe plantengemeenschappen

in deze studie suggereert dat de kans op parasitering van herbivoren (biologische bestrijding) lager is in hoog-diverse plantengemeenschappen. Dit kan leiden tot betere overleving van de bladminerende insecten en meer schade aan planten in plantengemeenschappen met veel soorten.

Een afname in plantensoorten leidt vaak tot een afname in het aantal en de diversiteit van bovengrondse predatoren en sluipwespen. Hoe de plantendiversiteit de predatie in ondergrondse gemeenschappen kan beïnvloeden, is minder goed bestudeerd. Om deze vraag te beantwoorden heb ik het infectievermogen van entomopathogene nematoden (EPN´s; de natuurlijke vijanden van bodeminsecten) bepaald in de verschillende biodiversiteitveldjes. Ook heb ik het totale aantal carnivore nematoden gemeten in het biodiversiteitsexperiment. Vervolgens heb ik een statistische techniek – *structural equation modelling (SEM)* – gebruikt om te bepalen via welke mogelijke route plantdiversiteit entomopathogene en carnivore nematoden kan beïnvloeden (*hoofdstuk 7*). Om een uitgebreid overzicht van de potentiële prooi- en voedselbeschikbaarheid in de grond te krijgen, heb ik het aantal bodeminsecten en niet-carnivore nematoden bepaald en de hoeveelheid wortelbiomassa in de experimentele veldjes gemeten. Uit de SEM-analyse bleek dat plantdiversiteit een indirect positief effect had op het infectievermogen van EPN's in de bodem. Plantendiversiteit beïnvloedde echter de dichtheid van de carnivore nematoden niet. De effecten van plantendiversiteit verschilden tussen de twee geslachten van EPN's (*Steinernema* en *Heterorhabditis*) en tussen EPNs en de andere vrijlevende carnivore nematoden.

Gedurende (secundaire) successie verandert niet alleen de samenstelling van de soorten in een plantengemeenschap, maar ook de kenmerken van individuele planten zoals hun grootte en dichtheid. Zowel veranderingen in de kenmerken van de omringende plantengemeenschap, als veranderingen in de eigenschappen van individuele waardplanten kunnen in potentie het aantal en de samenstelling van de insectengemeenschap op individuele waardplanten beïnvloeden. Daarom heb ik onderzocht hoe de concentraties van stikstof, koolstof. en plantverdedigingsstoffen, de grootte en de insectengemeenschappen op individuele *J. vulgaris* planten veranderen tijdens secundaire successie. Hiervoor heb ik een chronosequentie van tien voormalige landbouwvelden gebruikt (*hoofdstuk 8*). Daarnaast onderzocht ik of de variatie in de insectengemeenschappen op deze planten in elk veld kan worden verklaard door veranderingen in plantkwaliteit. *Jacobaea vulgaris* planten waren aanzienlijk groter maar hadden lagere stikstof concentraties in velden die recentelijk uit productie waren genomen dan in oudere velden. De PA-samenstelling van de planten verschilde aanzienlijk tussen de velden, maar er was geen relatie tussen

de leeftijd van een veld en de PA-concentratie of PA-samenstelling van *J. vulgaris* planten. Het aantal stengelborende insecten en hun sluipwespen alsmede de totale diversiteit aan sluipwespen was aanzienlijk hoger in de oudere velden. Er was echter geen significante relatie tussen het aantal herbivoren en sluipwespen en de chemische samenstelling of grootte van de plant. In een tuinexperiment met *J. vulgaris* planten verzameld uit verschillende jonge en oude velden was er geen relatie tussen de groei van *Tyria jacobaeae*-rupsen (de meest voorkomende specialistische herbivoor op *J. vulgaris*) en de leeftijd van het veld waaruit de plant kwam.

Samenvattend toont mijn promotieonderzoek aan dat vraat aan plantenwortels door bodeminsecten, nalatenschapeffecten in de bodem van zowel bovengrondse als ondergrondse vraat aan de plant, omringende planten en tijdelijke veranderingen in de omringende plantengemeenschap allemaal bijdragen aan de intraspecifieke variatie in de kwaliteit van *J. vulgaris* planten. Deze variatie in plantkwaliteit beïnvloedt bovengrondse herbivoren en hun natuurlijke vijanden in gecontroleerde omstandigheden. Echter in natuurlijke ecosystemen overstemt de invloed van de omringende planten de effecten van plantkwaliteit op de insectengemeenschap van *J. vulgaris* planten. Daarom moeten individuele plant-insect interacties bekeken worden binnen het perspectief van de omringende gemeenschap.

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Now my thesis is complete!

*Olga*, 15 December 2013

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# *Curriculum vitae*

Olga Kostenko was born on August 21, 1983 in Zhytomyr, Ukraine. After finishing high school specialized in Biology and Chemistry (graduated *Cum Laude* in 2000), she obtained a Bachelor degree (2004, *Cum Laude*) and an Engineer degree (2005, *Cum Laude*) in Ecology and Environmental Protection at the State University of Agriculture and Ecology in Zhytomyr, Ukraine. For her final thesis she studied ecology and allelopathy of crops that were introduced in Ukraine to intensify agricultural production. In 2005 she started a Master program in Ecology and Environmental Protection at the National Agricultural University (Kyiv, Ukraine) where she became also interested in Environmental Management and Impact Assessment. After one year she received a personal MATRA Fellowship (from the Dutch Ministry of Foreign Affairs) and switched to the Master Program of Environmental Sciences at Wageningen University (Wageningen, the Netherlands). Within her master she carried out a joined project, where she investigated the effects of local weather variability on behaviour and dispersal of butterflies, supervised by Dr J. Verboom, Drs A. Cormont and Prof Dr R. Leemans. During her internship she investigated the influence of weather parameters on the population dynamics of Dutch marshland birds during non-breeding season with the help of trend and trait analyses under the supervision of Dr C. Vos, Drs A. Cormont and Prof Dr R. Leemans. Her master and internship projects were part of the Dutch national research program "Climate Changes Spatial Planning (Adaptation of the Dutch Ecological Network)" and were performed at ALTERRA Landscape Centre. She graduated in 2008 with the major in Environmental Systems Analysis and minor in Environmental Policy. Right away, she started her PhD project with Dr Martijn Bezemer and Prof Dr Wim Van der Putten at the department of Terrestrial Ecology at the Netherlands Institute of Ecology (NIOO-KNAW) which resulted in this thesis. From May 1st 2013 Olga continued her scientific career as a postdoctoral researcher at the NIOO-KNAW, investigating the community assembly and re-assembly of range-expanding plant species with Wim Van der Putten.

## Awards

2013 The 1st prize of the National Ecological Research Network (NERN) for best paper published by a PhD-candidate.

2012 The 2nd prize of the Annual Publication Award by C.T. de Wit Graduate School for Production Ecology and Resource Conservation.

2006-2008 Personal Matra Training for European Cooperation Student Fellowship (MTEC), Netherlands Ministry of Foreign Affairs (covering study fee and living expenses).

2006 "The Best Student Thesis Award" from the Ministry of Agriculture of Ukraine.

2003, 2004 The Scholarship of the President of Ukraine for highly distinct students.

2003 The 1st prize in the "Student of the year" competition in the nomination "Science".

2002 The 1st prize at the National Ecological Olympiad among students.

## Publications

Kostenko O & Bezemer TM (2013) Intraspecific variation in plant size, secondary plant compounds, herbivory and parasitoid assemblages during secondary succession. *Basic & Applied Ecology*, 14, 337-346.

Bezemer* TM, Van der Putten, WH, Martens H, Van de Voorde TFJ, Mulder PPJ & Kostenko* O (2013). The effects of aboveground and belowground herbivory on belowground plant-fungus interactions and plant-soil feedback responses. *Journal of Ecology*, 10, 325-333. (*these authors contributed equally to the manuscript)

Kostenko O, Mulder PPJ & Bezemer TM (2013). Effects of root herbivory on pyrrolizidine alkaloid content and aboveground plant-herbivore-parasitoid interactions in *Jacobaea vulgaris*. *Journal of Chemical Ecology*, 39, 109-119.

Kostenko O, Van de Voorde TFJ, Mulder PPJ, Van der Putten WH & Bezemer TM (2012) Legacy effects of aboveground-belowground interactions. *Ecology Letters*, 15, 813-821.

**Featured in** *Vroege Vogels*; "*Hoe?Zo!*" Radio 5; *Delware's News at Noon with Allan Loudell* (USA); "*Mooney goes wild*" Irish national RTE Radio; BBC Mundo; Science News.eu; NWO; CORDIS and other media sources.

Kostenko O, Grootemaat S, Van der Putten WH & Bezemer TM (2012) Effects of diversity and identity of the neighbouring plant community on the abundance of arthropods on individual ragwort (*Jacobaea vulgaris*) plants. *Entomologia Experimentalis et Applicata*, 144, 27-36.

Cormont A, Malinowska AH, Kostenko O, Radchuk V, Hemerik L, WallisDeVries MF & Verboom J (2011) Effect of local weather on butterfly flight behaviour, movement, and colonization: Significance for dispersal under climate change. *Biodiversity & Conservation*, 20, 483-503.

Bezemer TM, Harvey JA, Kamp AFD, Wagenaar R, Gols R, Kostenko O, Fortuna T, Engelkes T, Vet LEM, Van der Putten WH & Soler R (2010) Behaviour of male and female parasitoids in the field: Influence of host density, patch size and habitat complexity. *Ecological Entomology*, 35, 341-351.

**PE&RC PhD Training Certificate**


With the educational activities listed below the PhD candidate has complied with the educational 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)**

- Multitrophic aboveground interactions on ragwort (*Jacobaea vulgaris*)

**Writing of project proposal**

- Placing individual interaction in a community context: multitophic aboveground interactions on ragwort (*Jacobaea vulgaris*)

**Post-graduate courses (7.5 ECTS)**

- Chemical approaches to parasitoid behavioural ecology; University of Nottingham & Lancaster, Environmental Centre, UK (2008)
- Spatiotemporal methods in ecology: an introduction to integro-differential equations; PE&RC (2009)
- Multivariate analysis of ecological data; University of South Bohemia, Czech Republic (2010)
- Ecological modelling with R; TU Dresden, Germany (2010)
- Parasitoid taxonomy and biology; NIOO, PE&RC (2010)
- Generalized linear models; PE&RC (2012)

**Laboratory training and working visits (2.1 ECTS)**

- Hymenoptera: identification and collection techniques; Laboratory of Insect Taxonomy, Bavarian State Collection of Zoology, Munich (2009)

**Invited review of (unpublished) journal manuscript (2 ECTS)**

- OIKOS: aboveground-belowground multitrophic interactions (2012)
- Insect Conservation and Diversity: responses of insect assemblages to environmental factors (2012)
- Oecologia: effects of plant fertilization on plant-insect interactions (2013)

**Competence strengthening / skills courses (2.8 ECTS)**

- Presentation skills; WGS (2008)
- PhD Competence assessment; WGS (2009)

- Techniques for writing and presenting a scientific paper; WGS (2009)
- PCDI PostDoc retreat; PCDI (2012)

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

- PE&RC Weekend (2008)
- PE&RC Days (2009 and 2012)
- Current themes in ecology (2008 and 2012)

**Discussion groups / local seminars / other scientific meetings (7.5 ECTS)**

- Workshop Plant-Insect Interactions: chemical defense; Leiden University (2008)
- NIOO Days (2008 and 2009)
- PhD Monthly discussion group; NIOO, TE (2008-2011)
- 5th Workshop Plant-Insect Interactions for young scientists; WUR (2010)
- Netherlands Annual Ecology Meeting; poster (2011) and oral (2012) presentation; Lunteren, the Netherlands (2011 and 2012)
- EPS Graduate School yearly meeting: interactions between plants and biotic agents; oral presentation (2012)
- Invited presentation Plant Ecology group; Leiden University (2012)

**International symposia, workshops and conferences (8.2 ECTS)**

- Annual Conferences of the Plant Population Biology Section of the Ecological Society of Germany, Switzerland and Austria; oral presentation; Plant PopBio, Nijmegen, the Netherlands (2010)
- 14th Symposium on Insect-Plant Interactions; oral presentation; Wageningen (2011)
- 2nd Entomophagous Insects Conference; oral presentation; Antibes, France (2011)
- Multitrophic Interactions workshop; oral presentation; Gottingen, Germany (2012)

**Supervision of a MSc student**

- Insect assemblages on ragwort

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