Soil legacy effects on aboveground plant-insect interactions

Robin Heinen

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Contents

Chapter 1

General Introduction

Robin Heinen

Soil organisms are of vital importance in natural ecosystems (Bardgett & Van der Putten, 2014; Bardgett & Wardle, 2010). They regulate many important processes in the soil which are vital to sustain plant life, such as decomposition and mineralization (Bardgett & Wardle, 2010). Moreover, many soil organisms live in close symbiosis with plants, which can be beneficial to the soil organisms and the plant. For instance, arbuscular mycorrhizal fungi are abundant symbiotic organisms that infect plants and acquire carbon from the plant for their own benefit. In turn, they benefit the plants by enhancing the uptake of soil nutrients, such as phosphates, as well as water (Harrison, 1997; Parniske, 2008). Furthermore, soil is also home to many organisms that feed on plant material, or act as pathogens, and as such can be detrimental to plant performance (Van der Putten et al., 2001; Soler et al., 2005; Johnson et al., 2013; Mendes et al., 2013). Examples of this are well-documented, for instance in agricultural crops, which are under constant threat of specific fungal or bacterial pathogens, or root-feeding herbivores (Oerke, 2006). Soils are rich in biodiversity (Orgiazzi, Bardgett & Barrios., 2016). As such, individual species in the soil are part of vast communities of soil organisms. Therefore, it is likely that countless species of soil (micro)organisms of the same and different species will interact with an individual plant simultaneously (Kaplan, Pineda & Bezemer, 2018). The balance between the positive and negative effects that are the result of these soil-plant interactions determine how plants perform in a specific soil (Van der Putten et al., 2016).

Soil organisms alter plant-insect interactions

Plants, as primary producers, are vitally important to sustain the world's many herbivores, and the higher trophic levels that prey on them. However, plants vary greatly in their nutritional quality and chemical composition (Mithöfer and Boland, 2012). Plant quality differs between species, but may also vary strongly within species, for instance, due to differences in nutrient availability or soil health (Mendes, Garbeva & Raaijmakers, 2013). As discussed previously, soil organisms play an important role in determining plant growth, i.e., above- and belowground biomass (Berg, 2009), but also affect key physiological processes occurring in plant tissues, that determine plant quality (Pozo & Azcón-Aguilar, 2007; Mendes et al., 2013). Through this, they may affect those organisms that consume plant tissues (Bezemer & Van Dam, 2005; Pineda et al., 2010). Insect herbivores are among the most numerous herbivores on our planet. A vast body of research has revealed that taxa of soil organisms can have contrasting effects on different groups of insects. For instance, plant growth promoting rhizobacteria (Pineda et al.,

2010), arbuscular mycorrhizal fungi (Gehring & Bennett, 2009; Hartley & Gange, 2009; Koricheva, Gange & Jones, 2009), plant pathogenic nematodes (Wondafrash et al., 2013), and root feeding arthropods (Johnson et al 2012; Soler et al., 2012), four taxonomically and functionally very different groups of soil organisms, have been shown to, sometimes drastically, affect insect herbivores that feed aboveground on a shared host plant.

Soil organisms can influence plant insect interactions through various mechanisms. As many soil organisms are very important for nutrient cycling, one obvious mechanism can be that soil microbes determine nutrient availability in the soil, and through this, can alter plant growth and plant nutrient levels (Prudic, Oliver & Bowers, 2005; Schade et al., 2003; Kos et al., 2015a,b). However, soil organisms that actively interact with a plant, may also invoke physiological responses in the plant. For example, belowground interactions between plants and various abundant soil bacteria can induce systemic resistance in the plant (ISR). In this process, soil organisms prime the plant, so that its defense system responds faster or stronger when subsequently attacked, for instance by a fungal pathogen or an insect herbivore (Hammerschmidt Nuckles & Kuc, 1982; Van Loon, Bakker & Pieterse, 1998; Kloepper, Ryu & Zhang, 2004; Pieterse et al., 1998; 2014). Furthermore, several soil organisms cause systemic acquired resistance (SAR) in plants, a phenomenon through which attack by a pathogen, results in a local response that limits the proliferation of the pathogen as well as a systemic elevation of defenses throughout the plant, which can additionally protect the plant against aboveground insect herbivores (Ryals et al., 1996; Sticher, Mauch-Mani & Métraux, 1997; Durrant & Dong, 2004). Soil organisms may also alter the profile of the volatile blends that plants emit aboveground in response to herbivory (herbivore-induced plant volatiles, HIPV), that attract natural enemies of aboveground herbivores, such as predators or parasitoids (Pangesti et al., 2013; Pineda et al., 2013a). Moreover, soil organisms can interfere with the production of extrafloral nectar, which, in turn, attracts beneficial organisms aboveground, such as ants, that defend the plant against herbivore enemies (Wäckers & Bezemer, 2003; Godschalkx et al., 2015; Huang et al., 2015). The attraction of natural enemies may be beneficial to the plant by providing an external layer of indirect defense against insect herbivores.

As part of this PhD project, I conducted a literature review in which I searched the literature for evidence that soil organisms affect plant insect interactions, specifically under natural conditions (Heinen et al., 2018a). There is a considerable number of studies that have reported

effects of soil organisms on plant insect interactions. I observed that under natural conditions, effects of soil organisms are common, but appear to be more variable in terms of direction and strength, than those observed under laboratory conditions. The between-study variability under laboratory conditions is already quite high. Nonetheless, the work discussed in this review confirms that soil organisms do play an important role in shaping plant insect interactions in nature. Soil conditions in nature are highly variable, both in terms of abiotic and biotic conditions. It is therefore not surprising that many soil organisms can have contrasting effects under different conditions. Importantly, this literature review revealed that there is a knowledge gap concerning the effects of entire soil microbial communities on the interactions between plants and their associated insect herbivores. This gap provides a niche for future research and is the basis for the experimental work that has been performed and presented in this thesis.

Soil legacy effects I: Plant-soil feedbacks

Throughout their lifecycle, plants influence their surroundings. When a fresh seed arrives and germinates in a new location, often, the first thing that will happen after germination is that the young seedling will grow a root into the soil, which provides anchorage and a means to obtain water. As the seedling starts to grow, belowground, its taproot will fork many times, creating a network of fine roots. Through these fine roots, the seedling will obtain more water, as well as nutrients that are essential to its growth. Simultaneously, aboveground the plant shoot will develop and provide plants with sugars through photosynthesis. The sugars are distributed throughout the plant, and beyond, as plants also exudate a considerable portion of their photosynthates into the soil, via their root network, along with various other primary and secondary metabolites (Bais et al., 2006; Phillipot et al., 2013). During the course of its growth, bits and pieces of the root system and senescing aboveground plant parts from the aging plant may end up on or in the soil in the form of litter (De Long et al., 2019). Altogether, these plant-derived materials are the primary resources for soil biota. Indeed, plants are also the main primary producers belowground.

Considering the amount of resources that plants excrete into the soil, it is hardly surprising that plants also have a great influence on the organisms that surround their root systems. In response to plant input into the soil, be it in the form of exudates or litter, some soil organisms may be attracted to the roots, while others may be repelled. As a result, plant species often

develop highly specific communities of soil organisms around their roots. These communities may persist in the soil even after a plant disappears, as a soil legacy effect. Plant species-specific accumulation of soil organisms has been shown for various groups, e.g., bacteria, fungi, and nematodes (Bezemer et al., 2006a; Kos et al., 2015b; Heinen et al., 2018b). This accumulated community in the soil can affect the plants that grow simultaneously or later in the same soil, a concept known as *plant-soil feedback* (Van der Putten et al., 1993; Bever et al., 1994; Kulmatiski et al., 2008; Van der Putten et al., 2013). A common observation in plant-soil feedback studies is that plant species often have a negative effect (although neutral and positive effects also occur), via their soil, on plants of the same species (*conspecific* feedback) (Kulmatiski et al., 2008; Van der Putten et al., 2013). This has been hypothesized to be due to the accumulation of plant species-specific pathogens in the soil, which may limit the growth of other individuals of that same plant species. However, plants that belong to different species - and which may not be affected by the accumulation of species-specific soil pathogens - may respond very differently (*heterospecific* feedback) and much more variable, with effects ranging from positive to negative. Negative conspecific plant-soil feedback plays an important role in agricultural systems, and are one of the reasons why farmers use crop rotation schemes. In natural systems, plant-soil feedback has been pointed out as a driver of successional processes (Kardol et al., 2006; Morriën et al., 2017), species replacement (Bever, Westover & Antonovics 1997; Eppenga et al., 2018; Crawford et al., 2019) and species dominance or rarity in plant communities (Klironomos, 2002). Furthermore, plant-soil feedback may play an important role in plant invasions (Klironomos, 2002; Levine et al., 2006) or plants shifting their distributions in response to global change (Van Grunsven et al., 2007; 2010; Engelkes et al., 2008; McCarthy-Neumann & Ibáñez., 2012).

Soil legacy effects II: Traits and predictability of plant-soil feedbacks

Plant-soil feedback, both conspecific and heterospecific, can vary greatly between plant species in its strength and direction. What is the reason that plants accumulate different communities of soil organisms? What mechanisms can explain the differences that are observed between plant species? In the past decades, ecologists have tried to answer these questions based on the life history of their model plants. Plants differ profoundly, not only in how they look, but also in *when* they grow, *where* they grow, and *how* they grow and defend themselves. As a result, plants can have widely differing life history strategies. Different strategies require

specific characteristics. Functional traits describe morphological, physiological, phenological and other characteristics that define life history strategies (Cornelissen et al., 2003; Perez-Harguindeguy et al., 2016; Reich et al., 2003). Throughout the history of ecology, scientists have used traits to explain important ecological phenomena. Indeed, plant-soil ecologists have also tried trait-based and phylogenetic approaches to understand what explains differences in plant-soil feedbacks between different plant species (Klironomos, 2002; Lemmermeyer et al., 2014; Anacker et al., 2014; Mehrabi, Bell & Lewis, 2015; Mehrabi & Tuck, 2015; Bergmann et al., 2016; Cortois et al., 2016; Teste et al., 2017; Kutakova, Herben & Münzbergová., 2018).

Plants are unique in the fact that they have aboveground and belowground parts. Both parts may play a role in shaping soil communities and thus in creating soil legacy effects. Aboveground parts, for instance, may determine the quality of leaf litter input, and through this, influence organisms that live in the soil. However, the fact that roots are embedded in the soil, makes it more likely that root traits better explain how plants interact with their soil communities (Bardgett, Mommer & De Vries, 2014). Hence, several attempts have been made to explain plant soil feedbacks using root traits, such as specific root length, relative growth rate of roots, or nutrient acquisition strategies.

The role of growth rate in plant soil feedbacks

Ecological theory predicts that plants that grow fast, invest the majority of their resource budget on growth, and as a result, they have less to spend on other important functions, such as defense. Slow-growing plants, on the other hand, invest fewer resources into growth, which means that they can invest more resources into defense (Coley, Bryant & Chapin, 1985; Herms & Mattson, 1992). It has been hypothesized that plants that grow fast and are poorly defended, will accumulate more pathogens in the soil, leading to negative plant-soil feedbacks (Van der Putten et al., 2013). Following this hypothesis further, pathogens will accumulate far less with plants that grow slow and are better defended. These plants may invest some of their resources into mutualistic relationships, leading to increased densities of mutualists in the soil and neutral or even positive plant-soil feedbacks. Indeed, there has been some support for this hypothesis. For instance, a study that tested the plant growth-defense hypothesis, confirmed that plants that had higher relative growth rates, suffered more from negative feedbacks than those with lower relative growth rates (Lemmermeyer et al., 2014). In addition, studies have also shown that early successional plants, which are often fast growers, have more negative plant-soil

feedbacks than those that are later successional (Kardol et al., 2006). Other studies have found links between specific root length, which is often highly correlated with growth rate, and plant-soil feedbacks (Bergmann et al., 2016; Cortois et al., 2016). One key element that all these studies have in common, is that they look at conspecific plant soil feedbacks, i.e., the effects of a plant, via the soil, on other individuals of the same species. Much less is known about how plant growth rates affect heterospecific feedbacks, i.e., the soil legacy effects of a plant on other plant species.

The role of plant functional type in plant soil feedback

Plants can be categorized into phylogenetic groups, such as family, genus, species, and even subspecies. However, in ecology, plants are also often classified into coarser groups, also known as plant life-forms, plant functional groups, or *plant functional types*. There are good reasons to do so. As an example, consider the Rosaceae family. Some genera, such as the genus *Potentilla,* encompass small herbaceous plants that commonly occur in grasslands. Species from the *Prunus* genus are often large shrubs or trees. Small herbs and trees obviously have different impacts on their environment, in terms of competitive ability. Yet, they are phylogenetically quite close. Plants from very different phylogenetic backgrounds may evolve very similar appearances. As such various alternative classifications have been proposed that categorize plants by similarities in life history strategies and ecological functions, rather than by phylogenetic relatedness (e.g., Humboldt, 1806; Raunkiaer, 1934). Grouping plants by general appearance, or habit has become common and plants can be roughly divided into trees, shrubs and herbs. The latter are often further divided into forbs (leguminous and non-leguminous) and graminoids, or grasses. In grassland ecosystems, the herbaceous groups forbs and grasses are highly abundant. Grasses, being monocots, differ evolutionarily from forbs, which are eudicots. Moreover, the morphological differences between the two are evident, in roots, leaves and reproductive organs. As a result, the two functional types also vary in the way they interact with their biotic environment. An obvious example illustrates this well for the aboveground multitrophic interactions in plants of these two functional types. Many forbs display colorful flowers, in order to attract insect pollinators that are vital for reproduction, whereas grasses often have rather dull flowers that usually rely on wind rather than insect pollination.

Grass species are phylogenetically more closely related to each other than forbs, all grasses belong to the Poaceae. As such, different grasses may be more similar in their chemical defenses than different forbs. Studies on several cereals, such as wheat, rye and maize, reveal that grasses have rather conserved defences, using secondary metabolites abbreviated as DIMBOA-like compounds (Vicari & Bazely, 1993; Frey et al., 1997;2009; Hu et al., 2018), and silica-based defences (McNaughton et al., 1985; Massey, Ennos & Hartley, 2006). Forbs originate from a phylogenetically broad range of plant families, which, over the course of evolutionary history, have all developed very specific secondary defense mechanisms, and thus probably are more variable in their defenses than grasses. The following examples illustrate the variability in chemical defenses within forbs. Ribwort plantain, *Plantago lanceolata*, belonging to the Plantaginaceae family, has secondary chemical defenses that are characterized by iridoid glycosides (Darrow & Bowers, 1997 ;1999; Marak, Biere & Van Damme, 2002a;2002b). Black mustard, *Brassica nigra*, as well as other species belonging to the Brassicaceae, defend itself using glucosinolates (Heaney et al., 1987; Van Dam, Witjes & Svatoš, 2004). Tansy ragwort, *Jacobaea vulgaris*, belonging to the Asteraceae family, contains pyrrolizidine alkaloids (Hol et al., 2003; 2004; Joosten et al., 2009; Kostenko et al., 2012). These three different plant species apply very different secondary defenses. However, all three of them are forbs.

Belowground, grasses and forbs also differ (Roumet et al., 2008). Grasses root quite shallow, in the upper layers of the soil, whereas many forbs send taproots deeper into the soil. Root architecture also differs between the two. Forbs often have root structures that are characterized by thick anchoring roots, combined with more finely structured roots. Grasses, on the other hand, have very densely packed root systems that consist of numerous very fine roots. Root architecture also influences other soil properties. For example, both in field and glasshouse studies, we have observed in our group that in soils from grasses, or from communities where grasses dominate over forbs, soil moisture content is generally lower than in soils from forbs, or from communities where forbs dominate over grasses (Bezemer, unpublished data; Heinen et al., *in prep*aration a, b). Abiotic conditions such as soil moisture can be important drivers of microbial community composition in the soil (Ettema & Wardle, 2002; Fierer & Jackson, 2006). Grasses and forbs also have been shown to differ in the way they interact with soil microorganisms. For instance, it has been shown that grasses accumulate bacteria in their rhizosphere that produce antifungal compounds (Latz et al., 2012;2015;2016).

Via these compounds, these bacteria may help grasses in fending off fungal pathogens in the soil. Several studies have also shown that grasses and forbs accumulate different soil microbial communities, which, in turn, initiates plant-soil feedback effects (Kos et al., 2015; Heinen et al., 2018b). Although grasses generally exhibit negative conspecific feedbacks (Kulmatiski et al., 2008), several studies have shown that their soils positively affect other plant species, especially forbs (Wubs & Bezemer, 2016; Ma et al., 2017). Forbs also generally have negative conspecific feedbacks, but in contrast to grasses, their soils have more negative feedback effects on other plant species (Wubs & Bezemer, 2016; Ma et al., 2017).

For the work in this PhD project, I selected common grassland plant species, that differed in their growth rate and functional type. The selection of species allowed me to test the effects of both factors on heterospecific feedbacks, but also allowed me to investigate how they would affect soil plant-insect interactions aboveground.

Soil legacy effects III: Plant-soil feedbacks and aboveground plant-insect interactions

As briefly mentioned previously, an important gap in the field of soil-plant-insect interactions is the knowledge on how entire soil (microbial) communities may influence plant-insect interactions. Given their importance in determining plant growth in the form of plant-soil feedbacks, combined with the fact that examples of individual soil organisms affecting plant-insect interactions are plenty, it is likely that entire soil communities also shape how plants interact with their associated aboveground herbivores (Wurst & Ohgushi, 2015). Indeed, several studies that were published just before I started my own work, suggested just that (Kostenko et al., 2012; Badri et al., 2013; Kos et al., 2015).

Tansy ragwort, *Jacobaea vulgaris*, is a plant that is native to Europe and is common in the Netherlands. The species has long been studied in relation to plant soil feedback, as it is characterized by having very negative conspecific feedback effects. When grown on its own soil, ragwort suffers strong drawbacks in terms of growth. On soils conditioned by other plant species, ragwort shows a broad range of responses, with some soils limiting its growth and others seemingly boosting its growth (Van de Voorde et al., 2011). These characteristics made it an ideal first candidate to study the effects of soil legacies created by different plant species on the interactions between and their insect herbivores.

In a paper published in 2015, Martine Kos and colleagues did just that. For several weeks, they grew 10 common grassland plant species in live soil that was collected from a natural grassland area. Then, these soils with specific legacies were used in a subsequent experiment, in which ragwort was grown on each of the soils individually. After a period of establishment, the plants were exposed to one of two aphid species. These aphids started colonies that increased over time. However, in both aphid species, colony growth was strongly determined by the soil that its host plant grew in. Importantly, ragwort plants in different soils also strongly differed in the levels of secondary defense metabolites that were found in the phloem (Kos et al., 2015). As aphids strictly feed on plant phloem, the secondary defense metabolites, which in part determine phloem quality, may be the driving mechanism of the soil legacy effects on aphid colony growth.

A publication from the same group, this time led by Olga Kostenko (2012), had shown that chewing insect herbivores also could be affected by the soil community that its host plant was growing in. Specifically, in this study ragwort was grown with and without root and shoot herbivores, in a full factorial combination. Herbivory on ragwort changed the plant's interaction with other soil organisms, resulting in differences in fungal community composition in the soil. Then, a subsequent generation of ragwort plants was grown in these soils that had different legacies of plant-herbivore interactions. Similar to what was observed in the study on aphid colony growth, different soil legacies affected the levels of secondary defense metabolites in ragwort, which in turn affected the caterpillars feeding on the plants (Kostenko et al., 2012; Bezemer et al., 2013).

At the end of 2015, when I started my work on this PhD project, these two studies, to the best of my knowledge, were the only two to show that plant species-specific soil legacies, or plant-soil feedbacks, could affect plant-herbivore interactions (but see Badri et al., (2013), who reported effects of soil slurries from different soil management and cropping systems on interactions between *Arabidopsis thaliana* and *Trichoplusia ni*). These two studies used the same plant model system. Indeed, ragwort was highly responsive to different microbial soil conditions and this, in turn, affected insect herbivores feeding on it. What was unknown at the time, was whether this process also occurred in other plant species. Can soil legacy effects on plant-herbivore interactions be considered a general phenomenon? Or perhaps, is ragwort simply the odd one out? While I was conducting my own experiments with other plant species,

further evidence that microbial soil legacies can affect aboveground plant-herbivore interactions in other model systems has been accumulating (e.g. De la Peña et al., 2016; Hu et al., 2018; Lu et al., 2018).

Soil legacy effects IV: Plant-mediated soil legacy effects and direct soil legacy effects on aboveground plant-insect interactions

Effects of individual taxa of soil organisms on aboveground insect herbivores have been well-documented in the scientific literature (see Chapter 2). A common assumption that is made is that these effects are mediated by the shared host plant. Plants are often very well-defended, and these defenses require local and systemic regulation. For their defenses, plants use phytohormones that regulate complicated defense pathways. These pathways have been well-conserved across the plant kingdom and thus can be observed in many plant species. Although there are various hormones involved in these pathways at different levels, two important hormonal pathways stand out; the jasmonic acid (JA) and salicylic acid (SA) pathway (Pieterse et al., 2012; 2014). These two pathways are activated by distinct biotic interactions between plants and their attackers. Specifically, in response to chewing herbivores and biotrophic pathogens, plants activate the JA pathway in their tissues (Pieterse et al., 2012; 2014). Several proteins play a role in this cascade and have been used in (molecular) plant ecology to study plant defense responses. In Chapter 5, we used two marker genes in *Plantago lanceolata* that encode proteins associated with the JA pathway. Pl-LOX2 is a marker area for a gene coding for lipoxygenase, an enzyme that acts upstream of JA production (Chauvin et al., 2013). Furthermore, we used Pl-PPO7, a marker for a gene coding for polyphenol oxidase, which acts downstream of JA production (Mayer 2006; Bosch et al. 2014). On the other hand, the SA pathway is activated by phloem feeding insects and necrotrophic pathogens. Upon activation of the SA pathway, plants upregulate pathogenesis-related (PR) genes. In Chapter 5, we also used two markers (Pl-PR1 and Pl-PR2, respectively) coding for pathogenesis related proteins in (Van Loon et al., 2006). These four marker genes allowed us to assess whether plants would respond differently, e.g., to varying pathogen levels in different plant-mediated soil legacies. Furthermore, it allows us to investigate whether soil microbial legacies and aboveground herbivores would interactively shape plant defense responses.

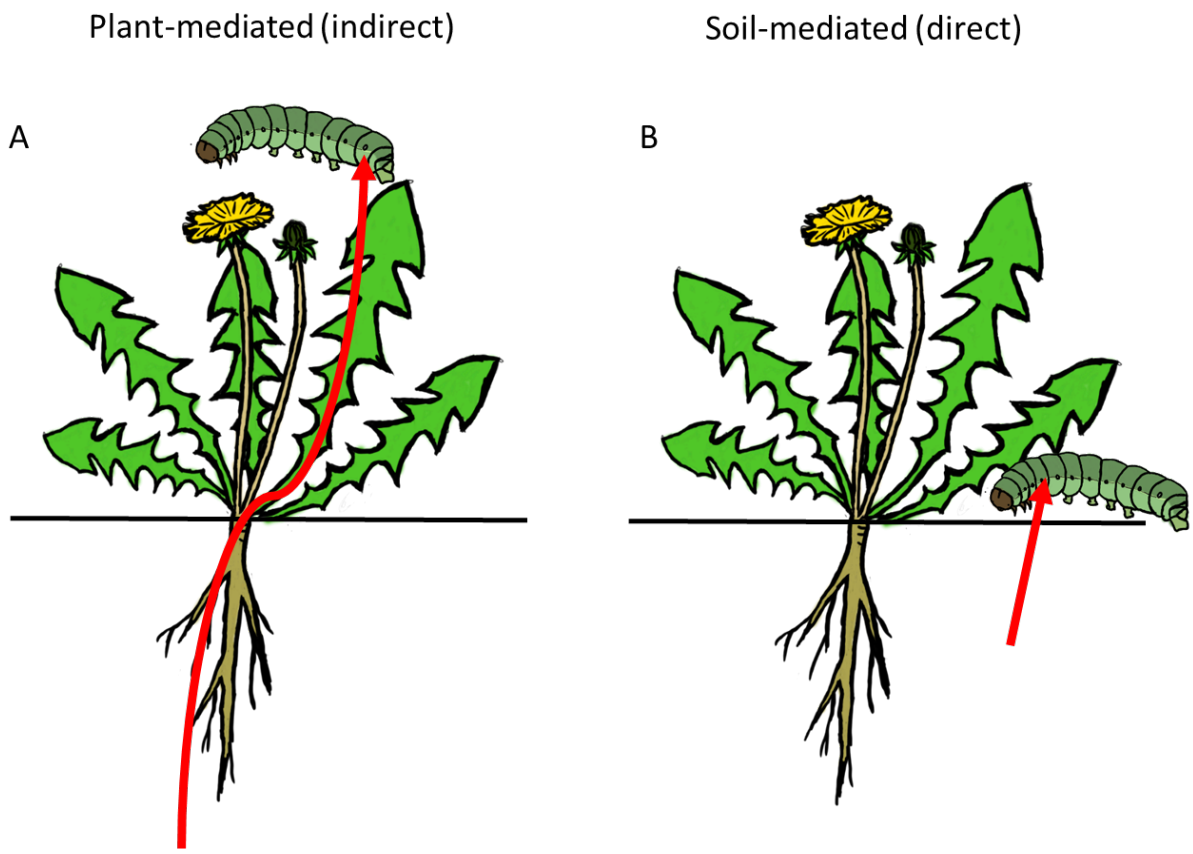

Figure 1.1: Schematic representation of two hypothetical pathways via which soil microbiomes may affect the caterpillar microbiome. A) In the plant-mediated pathway, soil microbes are transferred from the soil to the root to the shoot parts, where they are ingested by the caterpillar and end up inside their gut. B) In the direct pathway, soil microbiomes are affecting the caterpillar directly, either via passive or active soil-insect contact.

Thus far, the assumption in ecology has been that soil legacy effects on plant-insect interactions are mediated via plant phytohormonal pathways or plant chemistry. However, an exciting alternative possibility is that the microbes themselves may also play a role in altering plant-insect interactions. Microbes play an important role in many organisms, including humans. It has also been shown that microbes play important role in the gut of various insect species (Douglas, 2015). For instance, various bacterial species may aid caterpillars in detoxification of plant materials, digestion of food, or provide elevated defense against pathogens (Van Frankenhuyzen, Liu & Tonon, 2010; Chen et al., 2016). However, recent studies also suggest that insect microbiomes may be transient and change over time (Hammer et al., 2017). These findings further strengthen the idea that caterpillars pick up microbes throughout their life cycle. Recent studies indicate that plants take up their root and shoot microbiome as a subset from the soil (Chi et al., 2005; Lundberg et al., 2012; Bulgarelli et al., 2012; Bai et al., 2015). This raises the question whether these microorganisms can also influence aboveground insect

performance. In Chapter 6, we tested specifically whether soil microbial communities, shaped by different plant communities, are transferred to the caterpillars feeding on aboveground plant parts. We specifically investigated whether microbes would potentially be ingested via the plant during feeding, or, alternatively, whether they were taken up directly from the soil (see Figure 1.1).

Research questions

In this Phd thesis I explored soil-plant-insect interactions from many different angles. Below, Table 1.1 gives an overview of the questions asked in each of the chapters presented in this thesis. The specific questions and hypotheses are discussed in further detail in the introductions of the individual chapters.

Table 1.1: A brief overview of the main research questions that provided the basis for each of the chapters in this PhD thesis.

Chapter 2	Q1: Do soil organisms alter aboveground plant-insect interactions under natural conditions? Q2: How do soil-plant-insect interactions under natural conditions compare to results from controlled studies? Q3: How does methodology influence the effects of soil on aboveground plant-insect interactions under natural conditions?
Chapter 3	Q1: Do plants with contrasting growth rates and of different functional types have different soil legacy effects on aboveground plant-insect interactions in individual plants? Q2: How general are soil legacy effects on aboveground plant-insect interactions in individual plants
Chapter 4	Q1: Do plants with contrasting growth rates and of different functional types have different soil legacy effects on aboveground plant-insect interactions in plant communities? Q2: How do soil legacy effects affect aboveground caterpillar feeding behavior in plant communities?
Chapter 5	Q1: Do soil legacy effects alter herbivore-induced secondary plant shoot defenses? Q2: How does aboveground herbivory interact with soil legacies and how does this affect the jasmonic and salicylic acid pathways?
Chapter 6	Q1: Do soil microbial legacy effects influence aboveground insect microbiomes? Q2: Are microbial legacies transferred to aboveground insects via plant, or directly via the soil? Q3: Do microbial soil legacies alter performance of plants and aboveground herbivore?

Plant species selection

To answer the research questions in my PhD project, twelve plant species were selected that commonly occur in grasslands in Western Europe. Previous work on soil legacy effects on plant growth in terms of biomass production, i.e. plant soil feedbacks, has suggested an important role for root traits, as well as functional types of the plants as mediators of these soil legacy effects. Therefore, I selected species for my studies that had contrasting root growth traits and were members of two dominant functional types, grasses and forbs. This selection was made from a larger pool of 24 plant species native to the Netherlands. As we were interested in specific traits, we measured various above- and belowground traits in all 24 plant species. A subset of the replicates was used to measure qualitative traits, such as specific leaf area, carbon to nitrogen ratio, and traits related to root architecture. The remaining replicates were used to acquire important information regarding the growth rate of each species. For this, all 24 species were grown, with enough replicates for each species, under greenhouse conditions. Over the course of 10 weeks, three randomly selected individuals were harvested and roots and shoots dried and weighed separately. Then, growth curves were fitted through the data and from this, cumulative root, shoot and total biomass were estimated. For my studies, I then selected the three species with the smallest and the largest cumulative root biomass, within both functional types, totaling four different categories (i.e., fast-growing forbs, slow-growing forbs, fast-growing grasses, and slow-growing grasses). This selection allowed me to test the effects of plant growth rate and functional type on the legacies that they leave in the soil, as well as their responses to soil.

Insect herbivore selection

For my studies, I required foliar feeding insect herbivores which I could use to test my hypotheses. As I planned to work on a broad range of plant species, there were some important choices to be made. Different plant species generally harbor different fairly specialized insect herbivores. However, there are also insect herbivores that are less picky about their diet, which may readily accept whatever host plant they encounter as a food source. My choice fell on the latter. The reason for this is twofold; working with one species of polyphagous herbivore is practically much more feasible than working with 12 different herbivores. Polyphagous herbivores may be most relevant from an ecological viewpoint as well, as there are some key differences between polyphagous and more specialized insect herbivores. Specialist insect

herbivores, are closely associated with their host plant species and this association has established after a long history of coevolution. During this history, specialized insects may have developed specific mechanisms to deal with plant defenses, as natural selection will favor those individuals that survive best on a host plant. Polyphagous insect herbivores do not share such a long history with one specific plant species. Neonates will simply start feeding on a suitable plant that is close to where the female oviposited. This is not to say that polyphagous herbivores do not exert preferences, they certainly do. They are simply less tied to one host plant, and often lack the specific mechanisms to deal with specific plant chemical defences or have lower capacity to do so. It has been argued that fluctuations in chemical defenses may thus have less of an impact on specialized herbivores than on generalist herbivores (Ali & Agrawal, 2012).

The Noctuidae family, commonly known as the owlet moths, are an abundant group of insects. The caterpillars of many species in this family are highly polyphagous chewing herbivores. As several species, such as the beet army worm (*Spodoptera frugiperda*), the cotton bollworm (*Helicoverpa armigera*), and the cabbage moth (*Mamestra brassicae*) and others, can turn into agricultural pests (as their names suggest), they are also widely studied by agroecologists and entomologists. Their names are misleading in that these species are known to accept a much broader range of host plant species than just the crop species they were named after. *Mamestra brassicae* is a common moth species with a wide distribution. It occurs across the palearctic realm and it has been shown to feed on dozens of plant species in over 20 plant families, making it an ideal herbivore to use in our studies.

Thesis outline

In Chapter 2, as already briefly discussed in an earlier paragraph, I attempted to synthesize the scientific literature that is available on soil-plant-insect interactions under natural conditions. Specifically, I describe effects of four main groups of soil organisms, i.e., soil bacteria, soil fungi, soil nematodes, and soil arthropods on aboveground plant insect interactions. My findings in this chapter highlight that effects of soil organisms on aboveground plant-insect interactions are fairly common in nature. My findings also underpin the context dependency of many of these interactions; the outcome of any interaction is highly dependent on the species of soil organism, the plant species it interacts with, and strongly depends on the type of aboveground interaction that is studied. Furthermore, this work emphasises how little is known about the role of soil communities as a whole, in shaping aboveground plant-insect interactions.

In Chapter 3, I performed a large-scale greenhouse experiment, in which I grew twelve plant species on live field soil in a conditioning phase. Then, I grew all plant species on all twelve soils in a full factorial combination. I then introduced insect herbivores on each plant-soil combination. I aimed to investigate the effects of plant mediated microbial soil legacy effects on plants that grew later in the same soil, as well as the growth and leaf consumption of an insect herbivore that was kept on the plants. This set up allowed me to examine the impact of different soil microbiomes on future plant-insect interactions. In most plant species different soil microbiomes caused the plant growing in them, as well as the insect herbivores growing and feeding on the plant, to differ significantly from the average performance measured across all soils. This suggests that soil microbiomes may generally play a large role in shaping aboveground plant-insect interactions.

In Chapter 4, I used the same set of twelve plant species to create soils with different microbial legacies. This time, I did not grow individual plants, but plant communities on the different soils. I designed three plant communities that consisted of fast-growing plants, and three communities that consisted of slow-growing plants. In each plant community, I introduced the insect herbivore. The results from this study show that insect herbivore biomass significantly differed, depending on the soils the plant communities grew in. Moreover, I observed that, in some plant communities, feeding preferences of the insect herbivore for different plants within each plant community were altered by the legacy of the soil in which the plant community grew. These results suggest that insects may perceive soil mediated changes in plant quality, and can respond by switching host plants. However, the results also highlight that the effects of soil legacies, via plants, on insect herbivores, can strongly depend on the composition of the plant community that grows in soil with a certain legacy.

In Chapter 5, we used one of our selected plant species, *Plantago lanceolata*, to investigate how different microbial soil legacies would affect its interaction with insect herbivores. In the first experimental chapters, we could not provide mechanistic insights into the observed effects of microbial soil legacies on plant insect interactions. One of the reasons for this is that not much is known about physiology or biochemistry of many of the plant species that we work with. *Plantago* is one of the species of which the defense mechanisms have been well-described. Furthermore, we can follow the transcription of specific genes that are involved in the jasmonic and salicylic acid pathway, which play a vital role in plant defense against invaders

(chewing herbivores and biotrophic pathogens, respectively). Our results show that the levels of secondary defense metabolites differ depending on which soil the plant was growing in. Moreover, we show effects of soil and herbivory on the expression of two marker genes of the plant that are related to jasmonic acid defenses. Interestingly, two genes related to salicylic acid defenses were not affected by soil or herbivory treatments. Our results suggest that soil legacy effects alter the plant's ability to defend itself against herbivory.

In Chapter 6, we investigated the role of the microbial part of soil legacies on insect herbivore microbiomes. Specifically, we aimed to test whether a plant takes up subsets of the soil microbiome, first into the root, then into the shoot. Using two parallel assays, we explored whether the microbes that ended up in the shoot, would affect the microbes in the insect herbivore. In one assay, we reared caterpillars on caged dandelion plants in soils with different plant-induced legacies, allowing the insects to walk freely in their environment. In another assay, we fed caterpillars with clipped leaves from plants that had been growing in soils with the same legacies. Then, we characterized microbial communities in soils, roots, shoots, and caterpillars of both assays. We observed that caterpillar microbiomes that had been fed clipped leaves were fairly simple in microbial composition, as were the leaves. To our surprise, in the caged plant assay, caterpillar microbiomes were highly diverse, and closely matched the microbiome in the soil. Our results suggest that this herbivorous insect picks up most of its microbiome from the soil, and not from its (plant) diet. Interestingly, the specific legacies (changes in soil microbial community) that were left by the different plant communities in the soils were also detected in the insect. Although plant growth was equally affected by soil legacies in both parallel assays, the growth of the insect was only affected in the caged plant assay where the insects had access to the soil. These results suggest that in addition to the plant-mediated pathways through which soil organisms can affect aboveground plant-insect interactions, there may also be an alternative pathway via which soil organisms can affect insects, namely via their microbiome.

In Chapter 7, I discuss the results and implications of these studies and I place these findings in a broader context. I highlight the lessons learned from these experiments. More specifically, I discuss whether plant traits can be used to predict soil legacy effects on plants and aboveground insect herbivores. Furthermore, I will discuss how my findings relate to other recent scientific discoveries in the field.

Chapter 2

Effects of Soil Organisms on Aboveground Plant-Insect Interactions in the Field: Patterns, Mechanisms and the Role of Methodology

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Abstract

Soil biota-plant interactions play a dominant role in terrestrial ecosystems. Through nutrient mineralization and mutualistic or antagonistic interactions with plants soil biota can affect plant performance and physiology and via this affect plant-associated aboveground insects. There is a large body of work in this field that has already been synthesized in various review papers. However, most of the studies have been carried out under highly controlled laboratory or greenhouse conditions. Here, we review studies that manipulate soil organisms of four dominant taxa (i.e., bacteria, fungi, nematodes, and soil arthropods) in the field and assess the effects on the growth of plants and interactions with associated aboveground insects. We show that soil organisms play an important role in shaping plant-insect interactions in the field and that general patterns can be found for some taxa. Plant growth-promoting rhizobacteria generally have negative effects on herbivore performance or abundance, most likely through priming of defenses in the host plant. Addition of arbuscular mycorrhizal fungi (AMF) has positive effects on sap sucking herbivores, which is likely due to positive effects of AMF on nutrient levels in the phloem. The majority of AMF effects on chewers were neutral but when present, AMF effects were positive for specialist and negative for generalist chewing herbivores. AMF addition has negative effects on natural enemies in the field, suggesting that AMF may affect plant attractiveness for natural enemies, e.g., through volatile profiles. Alternatively, AMF may affect the quality of prey or host insects mediated by plant quality, which may in turn affect the performance and density of natural enemies. Nematodes negatively affect the performance of sap sucking herbivores (generally through phloem quality) but have no effect on chewing herbivores. For soil arthropods there are no clear patterns yet. We further show that the methodology used plays an important role in influencing the outcomes of field studies. Studies using potted plants in the field and studies that remove target soil taxa by means of pesticides are most likely to detect significant results. Lastly, we discuss suggestions for future research that could increase our understanding of soil biota-plant-insect interactions in the field.

Introduction

Soils are an important source of diversity of microbes worldwide (Ramirez et al., 2018), but soil is also home to various other higher taxa, such as nematodes, root feeding insects or even vertebrates (Bardgett and van der Putten, 2014). The role of soil biota in ecosystem functioning

is widely recognized and the study of soil biota-plant interactions has developed into a very active and large field in ecology. Soil organisms fulfill key processes in the soil, such as decomposition and nutrient mineralization. Many microorganisms engage in mutualistic interactions with plant hosts, aiding in the uptake of nutrients and water (e.g., arbuscular mycorrhizal fungi, AMF), in exchange for photosynthates or other plant metabolites. Other groups of soil micro- and macro-organisms have antagonistic effects on plant health, for example via pathogenicity (e.g., pathogenic fungi) or herbivory (e.g., root herbivorous insects). It has been shown previously in studies carried out under artificial/controlled conditions that mutualistic and antagonistic players in the soil not only impact the growth (i.e., biomass production) of plants, but also lead to the alteration of various physiological processes in plant tissues, resulting in changes in tissue quality or palatability of the plant (e.g., Bezemer and van Dam, 2005). Through such mechanisms, soil biota can mediate interactions between the host plant and aboveground organisms, such as insect herbivores and pollinators. Despite all the attention that this subject has received, the majority of published studies have been conducted under more controlled conditions (hereafter “controlled studies”), such as in greenhouses or growth chambers. Hence, an important question is whether the results are a realistic representation of ecological processes that occur in natural systems.

Mechanisms through which soil organisms can affect aboveground insects in the field are mostly plant-mediated (Figure 2.1). Various organisms, most notably plant growth promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF), can boost plant growth (e.g., Saravanakumar et al., 2008; Gadhave et al., 2016), which has been hypothesized to increase plant palatability (i.e., the plant vigor hypothesis; Price, 1991; Cornelissen et al., 2008). On the other hand, plants under

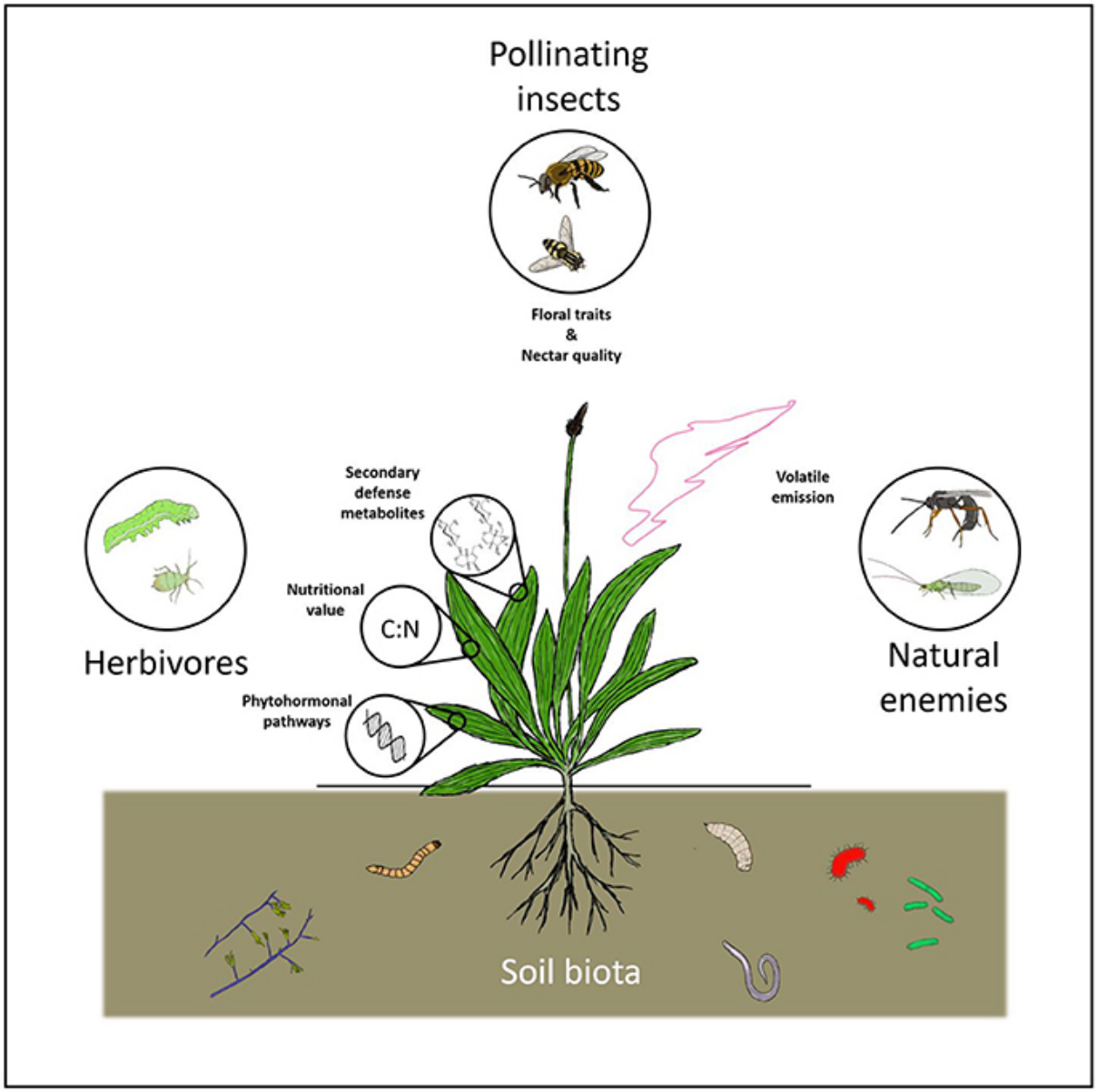

Figure 2.1. A schematic overview of mechanisms through which soil organisms can affect plant phenotype and associated aboveground insects. Soil organisms can affect a variety of host plant traits, including nutritional quality and palatability, size, morphology and floral traits, as well as the activation of defense pathways and the emission of plant volatile organic compounds. Through these mechanisms they can influence insect herbivores, pollinators and natural enemies.

biotic or abiotic stress can also be more vulnerable to attack by herbivores (i.e., the plant stress hypothesis; White, 1969). Evidence for the former has been reported from field studies (e.g., for some AMF species in Wolfe et al., 2005; Ueda et al., 2013). Several studies also find support for the plant stress hypothesis (e.g., for nematodes in Alston et al., 1991; Vockenhuber et al., 2013). However, many field studies report plant-mediated effects of soil organisms on aboveground insects, without reporting any effects on plant vigor or stress, which suggests that other factors related to plant performance (see Figure 2.1) could play an important role in mediating aboveground plant-herbivore interactions.

Plant nutritional value (most importantly, nitrogen and sugar content) in the field can be positively affected by soil organisms (Gange and West, 1994; Gange et al., 2005a,b; Younginger et al., 2009; Moon et al., 2013; Brunner et al., 2015; Godschalx et al., 2015; Ryalls et al., 2016). Moreover, plant secondary defense metabolites, that play a role in the palatability of host plants, can be affected by soil organisms in the field (Wurst et al., 2008; Megías and Müller, 2010). Interactions with soil organisms can also sensitize the immune system of plants so that they can respond faster or more strongly to subsequent attack by antagonists (e.g., Pieterse et al., 2014). This process, better known as induced systemic resistance (ISR), can play an important role in plant-insect interactions in the field (Saravanakumar et al., 2008; Prabhukarthikeyan et al., 2014). Soil organisms can also interfere with plant volatile emissions, which are important cues for herbivores (e.g., for oviposition), as well as for many natural enemies, to detect host plants (Megali et al., 2015). Finally, several studies have shown that, for instance AMF can affect plant functional traits, such as flower size and stamen number (Gange and Smith, 2005; Gange et al., 2005a; Varga and Kytöviita, 2010).

In this review, we aim to answer three main questions. (1) What is the role of whole soil communities and plant-soil feedbacks in mediating aboveground plant-insect interactions in the field? (2) What is the role of the individual taxa of soil organisms in mediating aboveground plant-insect interactions in the field and how do potential patterns compare to those that are observed in controlled studies? (3) How does the experimental methodology used in the field affect the outcome of above-belowground studies? Furthermore, we will discuss potential applications and suggest future directions to advance this scientific field.

Literature Search Methodology

The scientific literature was searched using Web of Science for combinations of "soil 'faunal group'" AND "insect" AND "field," in which "faunal group" was replaced by; bacteria, fung*, nematod*, arthropod* or insect*, respectively. Furthermore, the literature was searched for combinations of "plant-soil feedback" AND "insects" AND "field". Suitable studies were selected first based on title and subsequently on abstract or full manuscript. Additionally, reference lists from suitable papers, as well as from recent reviews (Gehring and Bennett, 2009; Hartley and Gange, 2009; Koricheva et al., 2009; Pineda et al., 2010; Johnson et al., 2012; Soler et al., 2012; Wondafrash et al., 2013) on soil biota-plant-insect interactions were examined to detect additional publications. Lastly, for all suitable publications, the studies that cited these publications were scanned to detect additional studies that were published later.

In total, the literature search yielded 50 field studies, covering a total of 185 individual soil biota-plant-insect interactions (Supplementary Tables 1–4).

Plant-Soil Feedback Effects on Plant-Insect Interactions in the Field

Plants are not only influenced by soil organisms, but they also play an active role in shaping the biome around their roots. Plant species typically manipulate the microbiome around their roots, e.g., via exudation of carbohydrates and other chemical substances (Bais et al., 2006), resulting in specific microbial rhizosphere profiles (Lakshmanan et al., 2014). Such species-specific microbial profiles can influence the performance of other plants that grow later in the same soil (Kostenko et al., 2012; Bezemer et al., 2013; Kos et al., 2015; Heinen et al., 2018). This process is known as plant-soil feedback (Van der Putten et al., 2013) and can be an important driver of plant community dynamics (Kardol et al., 2006). In recent years, it has become evident that such changes in soil microbial communities, via plant-mediated processes, can affect the performance of aboveground organisms that interact with these plants. For example, several greenhouse studies have shown that soil legacy effects, the effects of earlier plant growth on the microbial community in the soil, can have strong effects on aboveground herbivores feeding on later growing conspecific plants in those soils (Kostenko et al., 2012; Kos et al., 2015). A recent study, for example, revealed that soil legacies left by grasses and forbs have contrasting effects on a chewing herbivore that fed on plant communities growing on soils with these legacies (Heinen et al., 2018).

Although most studies on the impact of whole soil microbiomes on plant-insect interactions have been performed in greenhouses and climate chambers, several studies have explored such relationships in the field. For example, in a field experiment, the proportion of ragwort (*Jacobaea vulgaris*) plants attacked by stem borers, leaf miners and flower feeders was much lower (up to 50%) for plants that were grown in soils with a ragwort legacy compared with plants grown in soils without this legacy, probably because of a soil legacy-induced reduction in plant size (Bezemer et al., 2006). Negative plant-soil feedback is generally seen as a result of the accumulation of pathogenic organisms (Nijjer et al., 2007; Van der Putten et al., 2013), and the effects observed in ragwort and their associated aboveground insects are likely caused by belowground pathogens (e.g., Van de Voorde et al., 2012). Another field study with the same plant species, found a positive correlation between the occurrence of seed feeding insects and colonization of ragwort roots by mycorrhizal arbuscules (Reidinger et al., 2012). These results indicate that soil legacies, most likely driven by soil organisms, can play a role in shaping plant-insect interactions in the field. We have not been able to identify any manipulative studies that have, thus far, investigated plant-insect interactions in a plant-soil feedback framework. However, numerous studies have investigated the effects of the experimental manipulation of various groups of soil organisms on aboveground plant-insect interactions, and this area is discussed in more detail below.

Soil Biota-Plant-Insect Interactions in the Field

Bacteria

Bacteria are a dominant group of organisms in the soil that can have strong effects on plant growth and quality. For example, nitrogen-fixing rhizobia that associate with leguminous plant species fix atmospheric nitrogen and thereby often increase nitrogen content in the plant tissues. On the other hand, plant-growth promoting rhizobacteria (PGPR) are known to have yield enhancing effects on plants, but also are known to induce systemic resistance by priming plants for the activation of defense pathways, which often results in negative effects on insect herbivores in controlled studies (Pineda et al., 2010).

The Effect of Nitrogen-Fixing Rhizobia on Aboveground Herbivores

One would expect that the increased plant quality resulting from plant mutualisms with nitrogen fixing bacteria would benefit aboveground insects. However, this is not necessarily the

case, as rhizobia have been shown to also affect plant defense responses directly (e.g., Thamer et al., 2011) and indirectly (Godschalx et al., 2015). The latter is illustrated by a study with potted plants placed in the field that reported positive effects of the addition of *Rhizobium* sp. on plant protein levels in Lima bean, *Phaseolus lunatus*, but negative effects on extrafloral sugar content. This, in turn, led to 75% lower visitation numbers of the associated mutualist ant *Tetramorium caespitum*. Ants can act as natural enemies of herbivores and this study suggests that rhizobia can interfere with this indirect plant defense mechanism. In the presence of rhizobia, cyanogenesis (a chemical defense in legumes) is increased, and this may reduce the need for the plant to produce extrafloral nectar to attract ants (Godschalx et al., 2015).

The Effect of Plant Growth-Promoting Rhizobacteria on Aboveground Herbivores

Plant-mediated effects of the addition of PGPR on aboveground insects in the field are consistently negative in the studied systems. All interactions (n = 17) revealed from the literature search were negative for the aboveground herbivore, regardless of the insect feeding guild (Figure 2.2A, Supplementary Table 2.1, Zehnder et al., 1997; Commare et al., 2002; Saravanakumar et al., 2008; Gadhave et al., 2016). For instance, the addition of four different *Pseudomonas fluorescens* strains (individually, as well as in mixtures) to rice fields in India resulted in a ~3 fold reduction of leaf rolling by the rice leaf roller *Cnaphalocrocis medialis* (Commare et al., 2002; Saravanakumar et al., 2008). These effects are most likely driven by ISR, as plants generally express higher levels of defense gene transcription after exposure to herbivory in plants that received bacterial treatments (Saravanakumar et al., 2008; Prabhukarthikeyan et al., 2014).

The Effect of Plant Growth-Promoting Rhizobacteria on Aboveground Natural Enemies

Inoculation with PGPR can also influence the performance or attraction of insects at higher trophic levels, such as predatory insects or parasitoids (Saravanakumar et al., 2008; Gadhave et al., 2016). It is difficult to elucidate clear patterns as from all interactions (n = 18), 50% reported negative effects while 44% of the studies reported positive effects (Figure 2.2A, Supplementary Table 2.1). For example, a study investigating the effects of inoculation with *Bacillus* spp. on field-grown broccoli (*Brassica oleracea*) reported consistently reduced numbers of the ladybug (*Coccinella septempunctata*) and various unidentified syrphid flies on plants that received bacterial inoculations, compared to control plants that did not receive

additional bacteria (Gadhave et al., 2016). However, in the same study, the authors found that the percentage of cabbage aphids (*Brevicoryne brassicae*) parasitized by the parasitoid wasp *Diaraetiella rapae* was two to three times higher in plants grown on soils treated with *Bacillus cereus* and *B. subtilis*, but not in those treated with *B. amyloliquefasciens* or a mixture of the species (Gadhave et al., 2016).

Fungi

Soil fungi are a diverse group of organisms and their role in above-belowground interactions has been studied for many years. The most studied taxa are mycorrhizal fungi that associate with the majority of plant species. Ectomycorrhizal fungi (EMF) generally form mutualistic bonds with trees, whereas AMF form mutualisms with plants throughout the plant kingdom. EMF have been poorly studied within the soil biota-plant-insect framework and hence they are only briefly discussed. Relationships between AMF and aboveground insects, mediated by plants, are commonly reported in literature, and these effects have already been summarized in various other reviews (e.g., Pozo and Azcón-Aguilar, 2007; Gehring and Bennett, 2009; Hartley and Gange, 2009; Jung et al., 2012) and a meta-analysis (Koricheva et al., 2009).

The Effect of Ectomycorrhizal Fungi (EMF) on Aboveground Herbivores

Studies on the influence of EMF on plant-insect interactions are limited, but the published reports suggest that they can also affect insects in different directions. One study showed that numbers of the sap sucking poplar aphid *Chaitophorus populicola* were five times higher on poplar trees (*Populus angustifolia* x *P. fremontii*) that were treated with the EMF *Pisolithus tinctorius* than in controls that did not receive EMF. However, another study showed that various insects, even of the same feeding guild, respond differently to EMF in the same study and more importantly, results differ strongly between the various methodologies used (Gange et al., 2005b), as will be discussed in more detail further onwards in this review.

The Effect of Arbuscular Mycorrhizal Fungi (AMF) on Aboveground Herbivores

A general pattern that has emerged from controlled studies is that AMF negatively influence generalist chewers, while specialist chewers are positively affected by AMF (Hartley and Gange, 2009; Koricheva et al., 2009). From the interactions with generalist chewing herbivores

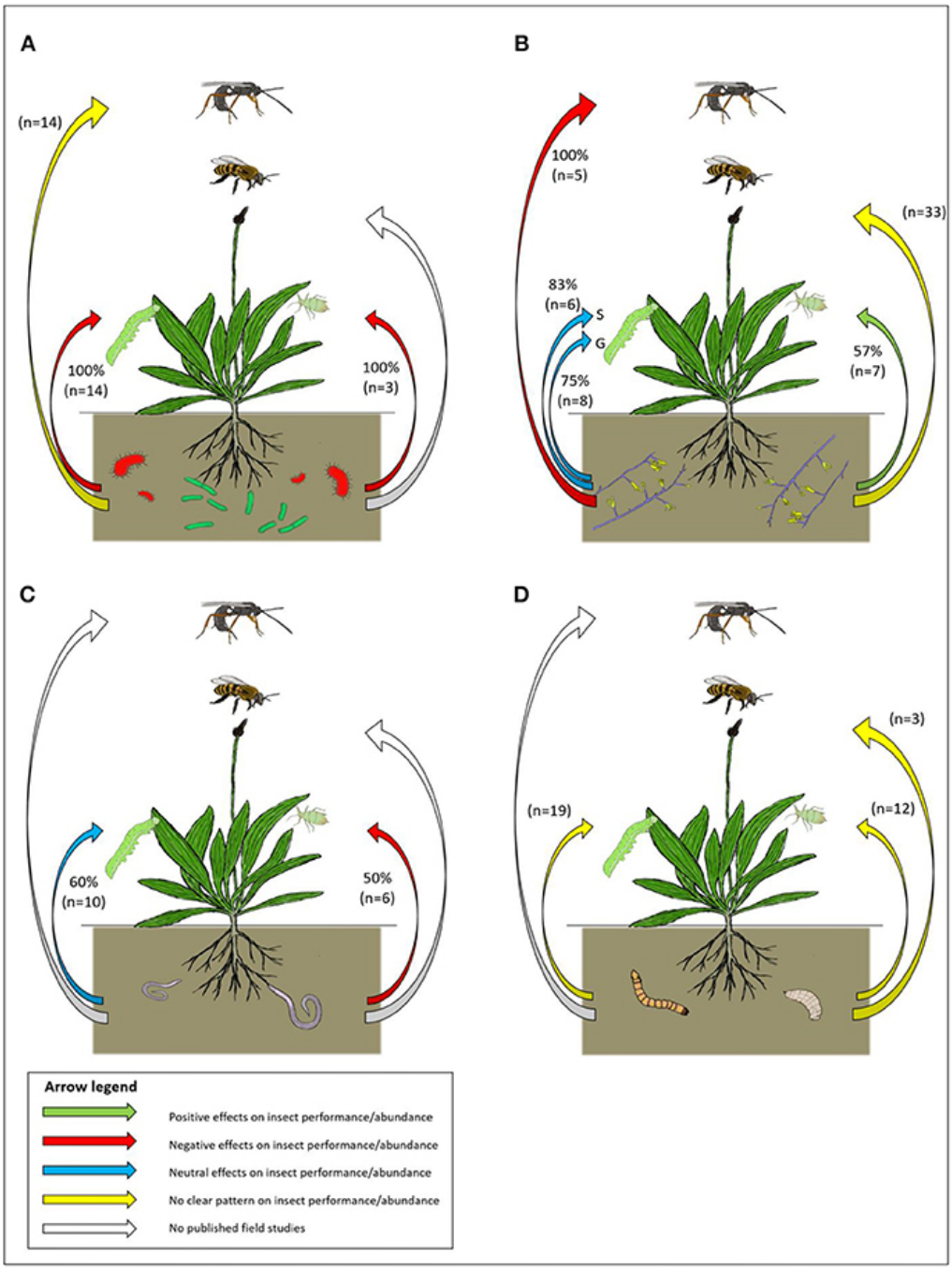

Figure 2.2. A schematic overview of the effects of (A) plant growth-promoting bacteria, (B) arbuscular mycorrhizal fungi, (C) plant-parasitic nematodes and (D) soil arthropods on the most frequently reported aboveground plant-insect interactions (interactions between plants and chewing and sap sucking herbivores, pollinators and natural enemies, respectively). In (B) S, Specialist; G, Generalist. Arrows indicate plant-mediated effects of soil organisms on aboveground insects. Green arrows represent generally positive indirect effects on aboveground insects, red arrows represent generally negative indirect effects on aboveground insects, blue arrows represent generally neutral effects on aboveground insects. Yellow arrows indicate that effects are observed, but no clear patterns emerged and white arrows indicate that interactions have not been reported in literature. Percentages with the green, red and blue arrows represent the percentage of the total reported interactions that followed the pattern (sample size between brackets).

revealed by our literature search (n = 8), 75% reported no effect and 25% reported negative effects of AMF on generalist chewers (Figure 2.2B, Supplementary Table 2.2, Gange and West, 1994; Vicari et al., 2002) or herbivore diversity (Guo et al., 2015) in the field. For example, in a field study on ribwort plantain, *Plantago lanceolata*, caterpillars of the highly polyphagous woolly bear moth, *Arctia caja*, were 25% smaller in plots with AMF than in plots with AMF removed (Gange and West, 1994). On the other hand, from the interactions with specialist chewers (n = 6) 83% report neutral (Younginger et al., 2009), and 17% reported a positive plant-mediated effect on specialist chewers (Figure 2.2B, Supplementary Table 2.2, Barber et al., 2013). Plant-mediated AMF effects on chewing herbivores also differ between different plant functional groups. A recent study showed that AMF presence increased total levels of herbivory in tallgrass prairie plots, but at the plant functional group level herbivory levels only differed between AMF and control plots for C3 grasses, but not for C4 grasses or forbs (Kula and Hartnett, 2015).

In controlled studies, sap sucking insects generally benefit from the presence of AMF and the degree of specialization of the sap sucking insects does not appear to influence the effects of AMF (Hartley and Gange, 2009; Koricheva et al., 2009). From the interactions revealed from our literature search (n = 7), 43% were neutral (Colella et al., 2014) and 57% reported positive plant-mediated effects of AMF on sap suckers (Figure 2.2B, Supplementary Table 2.2, Gange and West, 1994; Ueda et al., 2013). For example, a recent field study reports more than tenfold higher numbers of *Aulacorthum solani* on soybean (*Glycine max*) inoculated with *Gigaspora margarita*, than on untreated control plants (Ueda et al., 2013), which is in line with the commonly observed patterns in controlled studies. Only one study reports that treatment with AMF led to two- to three-fold lower numbers of the poplar aphid *C. populicola* on poplar trees, *P. angustifolia* x *P. fremontii* that were placed in pots in the field (Gehring and Whitham, 2002). Why aphids responded negatively in this study is hard to pinpoint. The authors report no significant effects of AMF on plant performance, but they did not investigate effects on plant chemistry, which may have changed in response to the AMF interaction. AMF effects on plant-insect interactions may also differ among plant functional groups. Most previous studies have been performed with herbaceous species, thus studies on woody shrubs and trees may give contrasting results.

As discussed in Koricheva et al. (2009), patterns in AMF-plant-insect effects on insects belonging to feeding guilds other than leaf chewers and sap suckers, such as cell content feeders and leaf miners, are not straightforward to interpret. However, addition of AMF to plants in the field had neutral (Gange et al., 2003, 2005b; Colella et al., 2014) to positive effects on cell-content feeders, leaf miners and gall makers in several studies (Gange et al., 2003; Younginger et al., 2009; Moon et al., 2013; Ueda et al., 2013). Within the same study system, results may even vary between generations of insects. For instance, when AMF levels were reduced using iprodione, this did not at first affect proportions of leaves mined by the leaf-mining fly *Chromoatomyia syngenesiae* in ox-eye daisy, *Leucanthemum vulgare* (Gange et al., 2003). However, in a follow-up study, the authors report AMF species-specific differences in the proportion of *Leucanthemum* leaves mined by *C. syngenesiae*, and a 50% increase in pupal biomass of the leafminer in plots with higher levels of AMF. These significant effects were only found for the second generation of flies in the year of study (Gange et al., 2005a).

The Effect of Arbuscular Mycorrhizal Fungi (AMF) on Aboveground Natural Enemies

Several studies have incorporated higher trophic levels in the study of AMF-plant-insect interactions and in all of the studied interactions (n = 5) AMF presence had a negative effect on the performance or density of predatory insects (Ueda et al., 2013) or parasitoids (Gange et al., 2003; Moon et al., 2013). In one study on Sea myrtle, *Baccharis halimifolia*, parasitism rates of two species of co-occurring leafminers (*Amauromyza maculosa* and *Liriomyza trifolii*, respectively) and a gall making fly (*Neolasioptera lathami*) by parasitoid wasps were all negatively affected by AMF application (Moon et al., 2013). AMF colonization resulted in more leaves per plant, which also had higher nitrogen levels, subsequently leading to healthier and potentially more strongly defended insect hosts, negatively affecting the respective parasitoids (Moon et al., 2013).

The Effect of Arbuscular Mycorrhizal Fungi (AMF) on Aboveground Pollinators

AMF-plant interactions can have contrasting effects on pollinating insects in the field. From the interactions revealed by our literature search (n = 35), 34% were positive, 17% were negative and 49% reported no effects on pollinators (Figure 2.2B, Supplementary Table 2.2). Several studies report higher pollinator visitation or flower probing on plants that received AMF treatment (Gange and Smith, 2005; Wolfe et al., 2005; Cahill et al., 2008; Barber et al., 2013),

whereas others report neutral or negative effects on pollinator visitation (Varga and Kytöviita, 2010). It is important to notice that effects of soil organisms on pollinating insects can vary between different levels of measurement (e.g., plot/community/species/pollinator taxa level). For example, in one study, levels of AMF were reduced by application of benomyl and the effects of AMF on six common forb species were investigated (Cahill et al., 2008). At plot level, plots with natural AMF levels showed an overall 67% higher number of pollinator visits per flowering stem, whereas the total number of visits per plot was not affected. AMF associations also led to a three-fold higher visitation by large-bodied bumblebees and a three-fold decrease in visitation by small-bodied pollinators such as bees and flies. At the plant species level, *Aster laevis* and *Solidago missouriensis* showed two to four times higher numbers of floral visits by pollinators in plots with higher AMF levels, whereas *Cerastium arvensis* showed a 80% decrease in total pollinator numbers in plots with higher AMF levels. Pollinator visitation of the herbs *Achillea millefolium*, *Campanula rotundifolia* and *Erigeron philadelphicus* was not affected by soil AMF levels (Cahill et al., 2008). More studies are needed to elucidate patterns for plant-mediated effects of AMF on pollinators in the field.

Nematodes

Nematodes are important soil dwelling organisms that belong to a range of trophic groups in the soil food web, and include bacterial feeders, fungal feeders, root feeders, and predators/carnivores. Their effect on host plants has been studied intensively, although fewer studies have focused on the indirect effects of nematodes on aboveground insects (reviewed in Wondafrash et al., 2013). As the literature search for field studies only revealed studies of plant-parasitic nematodes on aboveground insects, only this group will be discussed here. It should be noted that other nematodes (e.g., fungal feeders, bacterial feeders) may, however, also indirectly affect plant-insect interactions by interacting with other soil organisms. Plant-parasitic nematodes, by feeding on the roots of shared host plants, can influence the defense status and nutritional quality of host plants, potentially leading to effects on herbivores (Bezemer et al., 2003; Bezemer and van Dam, 2005; Wondafrash et al., 2013; Biere and Goverse, 2016). Results from laboratory studies of the effects of plant-parasitic nematodes on aboveground insects are often variable for chewing insects, but generally show negative effects on either the performance or preference of sap sucking insects (Johnson et al., 2012; Wondafrash et al., 2013). As the number of field studies on plant-parasitic nematodes that

describe effects on insect herbivores is rather low, we will treat plant-parasitic nematodes (PPNs) with different life styles (free-living, endoparasitic) as one group, and describe their effects on different types of insect herbivores. No studies that incorporated higher trophic levels or pollinating insects have been identified and therefore these are not discussed here.

The Effect of Plant-Parasitic Nematodes on Aboveground Herbivores

From the interactions revealed from our literature search (n = 10), 60% report neutral (e.g., Carter-Wientjes et al., 2004; Kaplan et al., 2009; Guo et al., 2016) and 40% report positive effects of PPNs on aboveground chewing herbivores (Figure 2.2C, Supplementary Table 2.3, Alston et al., 1991; Kaplan et al., 2009; Vockenhuber et al., 2013). For example, the addition of the root-knot nematode, *Meilodogyne incognita* to tobacco (*Nicotiana tabacum*) in field plots did not affect numbers of the specialist tobacco hornworn, *Manduca sexta*, or the growth of the generalist beet armyworm, *Spodoptera exigua*. In contrast, in the same experiment, nematode-treated plants had 30% higher numbers of chewing *Epitryx* flea beetles than untreated plants (Kaplan et al., 2009). Although correlative data should be interpreted with caution as they do not imply causation, numbers of free-living PPNs were also positively related to the levels of leaf consumption by chewing herbivores, although the observed correlations for PPNs were not significant for the three most abundant nematode genera *Tylenchorhynchus*, *Pratylenchus*, and *Xiphinema* (Kaplan et al., 2009).

From the interactions revealed from our literature search for nematode effects on sap suckers (n = 6), 50% reported no effects (e.g., Vandegehuchte et al., 2010; Heeren et al., 2012) and 50% reported negative effects (Figure 2.2C, Supplementary Table 2.3, Kaplan et al., 2009). In soy bean fields, *G. max*, the presence of the nematode *Heterodera glycines* did not correlate with total aphid abundance in one study (Heeren et al., 2012), but was negatively correlated with the number of alates of the soy bean aphid *Aphis glycines* at the onset of the peak season in another study (Hong et al., 2011). It is important to note that in the former study, plant yield was also not affected, whereas yield also negatively correlated with the number of nematode eggs in the latter (Hong et al., 2011; Heeren et al., 2012).

Soil Arthropods

A relatively large number of studies have examined the effect of soil arthropods on aboveground plant-insect interactions. Soil arthropods are an abundant group of macro-

invertebrates that can affect plants either directly, via root herbivory or indirectly, via decomposition of organic material. Although an increasing number of studies report on mechanisms through which root herbivory might impact aboveground plant-insect interactions (e.g., reviewed in Soler et al., 2012; Barber and Soper Gorden, 2014), most reviews remain inconclusive about the drivers behind the effects that are often observed. A meta-analysis showed that root herbivory by Diptera generally results in significantly negative effects on aboveground herbivores (Johnson et al., 2012), whereas herbivory by Coleoptera influences only aboveground Homoptera (positively) and herbivorous Hymenoptera (negatively), but has no significant effect on other groups.

The Effect of Root Herbivores on Aboveground Herbivores

From the interactions revealed by our literature search for root herbivore effects (regardless of taxa) on aboveground chewing herbivores (n = 20), 55% reported no effects, 10% reported positive effects and 35% reported negative effects.

Several studies in the 1990's investigated the effects of root herbivores on aboveground insects by means of reducing the total densities of soil arthropods with insecticides. In all of these studies, natural densities of soil arthropods had either no influence (Evans, 1991) or led to an increase (Evans, 1991; Masters et al., 1993, 2001; Masters, 1995) in aboveground herbivory. As there is little specificity in insecticide treatments, it is impossible to disentangle the effects of different soil arthropod taxa on plant-insect interactions from these older studies. Yet, they shed some light on the role of soil arthropods in shaping plant-aboveground insect interactions.

In field studies, plant-mediated effects of coleopteran root herbivores on aboveground chewing herbivores can be neutral (Hunt-Joshi et al., 2004; Barber et al., 2015; Borgström et al., 2017), positive (Wurst et al., 2008), or negative (White and Andow, 2006; Wurst et al., 2008; Megías and Müller, 2010, see Figure 2.2D, Supplementary Table 2.4). Interestingly, on ribwort plantain, *P. lanceolata* that were exposed to belowground herbivory by *Agriotes* spp., aboveground herbivory levels were three times lower on a high-iridoid glycoside (secondary defense metabolites in *Plantago*) producing lineage, compared to controls without root herbivores. In contrast, herbivory levels were nine times higher in response to the root herbivore on a low iridoid glycoside lineage (Wurst et al., 2008). This study illustrates that the genetic background of a plant can play an important role in determining plant-mediated effects

of root insect herbivores on aboveground chewing insect herbivores. Although a meta-analysis (Johnson et al., 2012) concluded that dipteran root herbivores generally have negative plant-mediated effects on aboveground herbivores, there is no consistent support from field studies for this (see Figure 2.2D, Supplementary Table 2.4). For example, Cabbage root fly, *Delia radicum* negatively affected numbers of chewing *Phyllotreta* sp. leaf beetles (this genus comprises mostly specialists and oligotrophs) in potted black mustard (*Brassica nigra*) in an experimental garden (Soler et al., 2009), but the addition of root flies had no plant-mediated effect on any lepidopteran chewers (Soler et al., 2009; Pierre et al., 2013).

There seems to be no pattern for the plant-mediated effects of coleopteran root herbivores on sap suckers in the field. From the interactions revealed by our literature search (n = 22), 54% reported no effects, compared to 23% that reported positive effects and 23% that reported negative effects (see Figure 2.2D, Supplementary Table 4). One study reports positive effects of root herbivory by coleopteran herbivory on aboveground sap suckers (Poveda et al., 2005). However, in other studies, the addition of coleopteran root herbivores had either no effect (Megías and Müller, 2010) or negative effects on sap suckers (Megías and Müller, 2010; Ryalls et al., 2016). For example, addition of larvae of a combination of the two beetle species *Morica hybrida* and *Cebrio gypsicola* on *Moricandia moricandioides* resulted in a more than three times lower number of aphids on the shared host plant, compared to controls. Similarly, in the same study, the addition of soil organisms resulted in a decrease in the total number of unidentified aphids on the plants, compared to controls, whereas the total number of planthoppers was not affected by the treatment with only *C. gypsicola*, but were 30% lower on plants that received only *M. hybrida* (Megías and Müller, 2010). This result could be driven by the fact that the latter is largely detritivorous and, thus, these two coleopteran soil arthropods may affect plant physiology in different ways. There is also no consistent effect of dipteran root herbivores on sap sucking herbivores in the field. Plants treated with root herbivores were found to have increased numbers of specialist aphid *B. brassicae* (Pierre et al., 2013) and decreased numbers of the same species in another study (Soler et al., 2009). Numbers of the generalist aphid *Myzus persicae* were not affected by the presence of root herbivores in either of the two studies (Soler et al., 2009; Pierre et al., 2013).

As we identified only one study that described the effect of root herbivores on other feeding guilds, it is not possible to elucidate patterns. In this study, the abundance of the leafminer

Stephensia brunnichella was 30% lower on Wild basil, *Clinopodium vulgare* plants that were infested with wireworms, *Agriotes* spp. than on controls without herbivores, whereas the size of the herbivores remained unaffected by the treatments (Staley et al., 2007).

The Effect of Root Herbivores on Aboveground Natural Enemies

The number of studies that have examined the effects of root-feeding insects on aboveground natural enemies in the field is limited. The available reports suggest that the presence of root feeding herbivores may have little effect on aboveground natural enemies in the field (e.g., Soler et al., 2009; Megías and Müller, 2010). Evans (1991) reported that soil arthropod reduction did not affect abundance of unspecified parasitic Hymenoptera, Arachnida and unspecified predatory and entomophagous insects in experimental field plots. In contrast, Megías and Müller (2010) found higher levels of parasitism by the braconid parasitoid *Cotesia kazak* in larvae of two pierid butterflies, *Euchloe crameri* and *Pontia daplidice*, when soil dwelling larvae of the tenebrionid beetle *M. hybrida* were present in potted *M. moricandioides* plants. It is important to note that this beetle species is largely detritivorous and therefore may not directly affect plants, but its presence may influence plant-insect interactions by making nutrients available in the soil that may affect physiological processes in the plant.

The Effect of Root Herbivores on Aboveground Pollinators

The literature is inconclusive on the plant-mediated effects of root herbivores on pollinators. Soil arthropods often cause association-specific effects on their host plants, ranging from changes in flower number to flower size and nectar quality, which all may influence different types of pollinating insects (Barber and Soper Gorden, 2014). Likewise, there is no evident pattern for field studies (Figure 2.2D, Supplementary Table 2.4). Three studies investigated the effects of addition of root herbivores on pollinator visits in the field. In all cases, the plants were in pots in the field and the treatment was an addition of coleopteran root herbivores. Addition of wireworms, *Agriotes* spp. to charlock mustard, *Sinapis arvensis* consistently resulted in an increase in total pollinator visits (Poveda et al., 2003, 2005). However, in another study using cucumber plants, *C. sativus*, addition of larvae of the striped cucumber beetle, *Acalymma vittatum* resulted in half the number of pollinator visits, compared to untreated controls and pollinator visits showed a negative relationship with root herbivore density (Barber et al., 2015).

Methodology Determines the Outcome of Field Experiments

Although similarities between controlled studies and field studies can be found for some soil taxa, the field literature also shows considerable variation in responses and neutral effects are commonly observed for soil biota-plant-insect interactions. This may be at least partly due to the experimental methodologies applied in the field. Three main methodologies are widely applied; (1) Addition of soil organisms to potted plants that are placed in experimental outdoor areas; (2) Addition of soil organisms to plants that are grown in field plots; (3) Removal of specific soil organism taxa by application of pesticides (see Figure 2.3). Direct comparisons between potted plants and field grown plants were made in two studies. For instance, in Marram grass, presence of a PPN of the genus *Heterodera* had a negative effect on the aboveground aphid *Schizaphis rufula* in pots, but in the field this correlation was not significant (Vandegehuchte et al., 2010). In another study, when *Eucalyptus* trees were grown in pots in the field, addition of EMF had a negative effect on feeding by larvae of the chafer *Anomala cupripes*, but for trees growing directly in the field, no effect on chafer feeding was observed. Damage by geometrid moths was significantly increased under EMF treatment in the potted plants, whereas it was decreased in the field-grown *Eucalyptus*. However, the EMF treatment led to a reduction in leaf folding by *Strepsicrates* sp. in both potted plants in the field and in field-grown plants (Gange et al., 2005b). These two studies clearly illustrate that choice of methodology used in field experiments can strongly influence the outcome, and suggests that studies using potted plants are more likely to show significant effects of belowground organisms on aboveground insects than studies that examine plants grown directly in the soil in the field. This also emphasizes the need for standardized methodologies, in order to make comparisons between different field studies more powerful.

Interestingly, there is a strong difference between effects reported for the different methodologies among the studies compiled in this literature review (see Table 2.1). In the published literature, only for the taxa soil fungi and soil arthropods were there reports on all three methodologies used in the field (see Figure 2.3). When we compare methodologies within these two taxa, potted plant studies and field removal studies more often reported significant results (in either direction) than studies where soil organisms were added to field plots. For example, in the studies with fungi, 63% of the interactions studied in pots showed a significant plant-mediated effect (in either direction) on aboveground insects. Field removal

studies also showed a significant plant-mediated impact in 73% of the studies, but only 25% of the field addition studies showed significant effects (see Table 1).

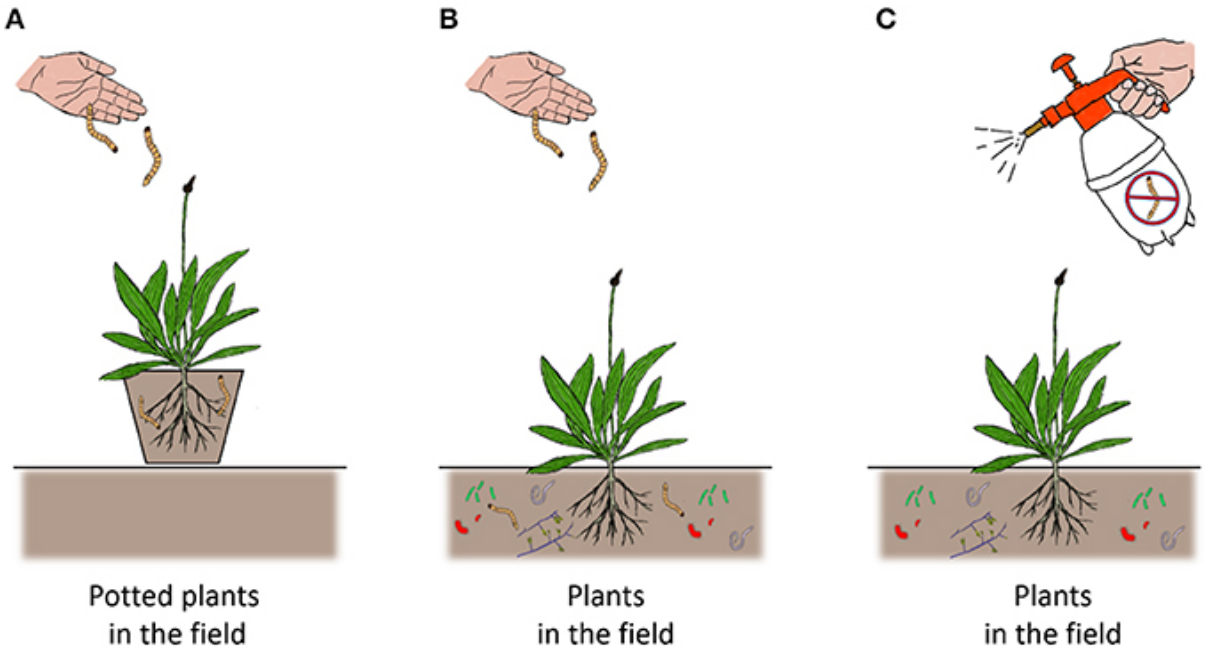

Figure 2.3. A schematic overview of the three most widely used methodologies to investigate soil biota-plant-insect interactions in the field. In this representation we used additions of wireworms, *Agriotes* spp. to Ribwort plantain *Plantago lanceolata* as an example. (A) Potted plants, which are often grown in a greenhouse for a number of weeks, are placed in experimental fields or gardens after being treated with soil organisms. Interactions between the potted plants and natural herbivores or pollinators are then tested in the field. (B) Plants are planted in the field under natural conditions, including a resident soil community. Soil organisms are added to plots and thus in the treated plots the numbers of added soil organisms are augmented, compared to untreated control plots. (C) Plants are planted in the field under natural conditions, including a resident soil community. However, in this method, the soil organisms under investigation are reduced by means of application of a pesticide. Hence, the treated plots have reduced levels of soil organisms, compared to the control plots, which have natural (but higher) levels of the soil organism.

A similar pattern emerges for the manipulation of soil insects. Here, 64% of the studied interactions resulted in significant plant-mediated effects on insect herbivores in pot experiments. Field removal studies showed significant plant-mediated effects in 70% of the studies, compared to only 33% in the field addition studies (see Table 2.1). These numbers suggest that there is a strong effect of methodology applied in the field, although it should be noted that publication bias may have also led to a bias toward studies that report significant results and in reality, the fraction of studies that report significant effects may be lower.

The use of pots comes with a range of disadvantages that may affect the study system, especially so in the field. First of all, studies often use sterilized soil or steamed potting soil, which excludes the interactions with resident soil organisms. Furthermore, pots not only impose a barrier to the root system, but also to the movement of the study organisms. Moreover, it prevents the influx of other soil organisms. Although pots may have the advantage of ensuring that the soil organisms are present at the root system, this methodology may be highly artificial compared to field plots. The barrier also inherently limits plant growth (i.e., pot limitation), leading to changes in plant growth and physiology (Poorter et al., 2012), which may either be beneficial or detrimental to insect performance. Lastly, abiotic conditions in pots can be quite different from conditions in soil. Placing pots (often of dark color, which absorbs more energy) on top of the soil, may increase soil temperature in the pot under warm conditions. Moreover, they may cool down more rapidly under cold conditions. We propose that pots can be extremely useful in studying soil organisms, both in laboratory and field conditions, but that they should be used with caution and that abiotic constraints should be countered as much as possible (for example by burying the pots, using large enough pots and including live soils into the design).

The use of pesticides in field experiments was a common approach in the early years of the development of this niche in ecology. However, this also comes with many obvious disadvantages. Several studies have shown that, although the pesticides are often rather specific and indeed reduce target organisms, there are also undesirable side-effects that influence many other soil processes (e.g., Wang et al., 2004). We propose that addition of soil organisms to field plots may be the best methodology, as this allows for interactions of both the added soil organisms and the plant with resident soil communities. From an applied perspective, results from soil organism addition studies are perhaps also the most useful as these scenarios are most comparable to application of soil organisms (e.g., in Integrated Pest Management). However, it is very hard to standardize both the abiotic and biotic conditions of live field soils, and this can lead to considerable variation between or even within study sites. Introduced soil organisms may encounter antagonists, or effects may be “diluted” as field plots often do not have barriers and organisms may move away.

Table 2.1 Comparison of the three most widely used field methodologies in studies investigating above-belowground interactions (potted plants placed in the field, inoculation of soil organisms in experimental plots, species removal by means of pesticides in experimental plots).

			Effect on herbivore		
Method	**Number of studies**	**Number of studied interactions**	**Percentage no effect**	**Percentage positive**	**Percentage negative**
FUNGI					
Pot	9	27	37.0	40.7	22.2
Field removal	4	11	36.4	45.5	18.1
Field inoculation	7	40	65.0	20.0	5.0
SOIL ARTHROPODS					
Pot	9	25	36.0	40.0	24.0
Field removal	5	10	30.0	60.0	10.0
Field inoculation	4	9	66.7	11.1	22.2

Shown are the total number of studies and the total number of organismal interactions for which relationships between soil organisms and aboveground herbivorous insects were investigated. The percentages were calculated for the studies that showed no significant effect on the herbivore, a significant positive effect on the herbivore or a significant negative effect on the herbivore. Only soil fungi and soil insect manipulation studies were included, since removal and pot studies were rare or non-existent in the other groups.

Discussion and Future Directions

In this review we have explored the scientific literature that discusses the effect of biotic manipulations of the soil on aboveground plant-insect interactions in the field. First, we asked if there is a role for soil organisms in shaping aboveground plant-insect interactions under field conditions. We searched the literature for studies that report on manipulations of the whole soil microbiome and how changes in soil community composition may affect aboveground insects in the field. It appears that there is ample evidence for effects of changes in whole soil communities on insect assemblages, but these findings are all correlative, not causative. This immediately highlights a first gap in the current scientific knowledge; how biotic "soil legacies" or plant-soil feedback (PSF) effects may influence aboveground insect communities in the field. To our knowledge, no studies thus far, have assessed these effects in a field setting. This is an important aspect of above-belowground ecology that deserves more attention in the future. We argue that introducing the PSF concept as a fourth applicable field method to shift soil communities in a certain direction would be less disruptive than the commonly used methodologies and would incorporate more ecological realism.

Our second question was whether the manipulation of specific taxa in the soil has the same effects on aboveground insects in the field as under more controlled conditions in greenhouses or growth chambers. Our survey indicates that this is true for most taxa except for soil arthropods. Bacterial inoculation in the field generally promotes plant growth and depresses abundance and performance of insects in the field, as they do in laboratory studies (e.g., Pineda et al., 2010). For AMF, the effects observed in laboratory settings have been thoroughly reviewed (Gehring and Bennett, 2009; Hartley and Gange, 2009; Koricheva et al., 2009) and the general patterns differ for insects from different feeding guilds and depend on the degree of specialization of the insects. Field studies, we show, report similar patterns; AMF negatively influences generalist chewers, but positively affect specialist chewing insects. AMF also generally benefit sap-sucking insects, regardless of their specialization. Under field conditions, nematodes affect chewing herbivores positively and sap suckers negatively and this is also in line with the general observations in laboratory studies (Wondafrash et al., 2013). Patterns in the effects of soil arthropods are less straightforward. In the current review of field literature, we have not been able to observe a clear pattern. One of the reasons for this could be the variation in abiotic and biotic conditions in the reported study systems. Furthermore, often only

very few interactions are studied for each combination of taxa (both below and aboveground). Therefore, there is currently a lack of relevant data and this makes it hard to compare the different results more thoroughly, e.g., in a meta-analysis. The same problem arises when we attempt to elucidate patterns for less abundant feeding guilds (such as leaf miners, gall makers or stem borers) or natural enemies and pollinators. Very few studies, so far, have investigated the effects of soil organism manipulations in the field on these less apparent aboveground feeding guilds and this is an area that requires further attention in order to better understand patterns in soil arthropod-plant-insect interactions.

Although we observed similarities between field and laboratory studies, in the field, it is also important to note that a relatively large fraction of the studies that we detected reported neutral effects. We suggest that field methodology can drastically affect the outcome of above-belowground studies and that ecologists should be aware of this when designing experiments. Although there is a current lack of studies that compare the different field methodologies directly, the pattern is rather clear. In the case of pot experiments and removal experiments in the field, the likelihood of observing a statistically significant effect of any kind, are twice as high as those in field addition experiments. However, we argue that the latter is, to date, by far the most realistic and useful methodology to understand ecological processes. Clearly, there are opportunities to explore alternative ways to manipulate soil organisms, or steer soil communities in specific directions. For example, through manipulation of soil via plant-soil feedback mechanisms where soils are manipulated in the field by plant species with specific effects on soil communities, or by inoculation of plots with soils that have been conditioned by specific plant species. Moreover, soil organisms can be manipulated via exclusion methods using variable mesh sizes that exclude certain soil taxa based on their sizes (e.g., Johnson et al., 2001, 2002), or via the addition of antagonistic organisms, that can impact specific groups of soil organisms.

Four aspects of the field of above-belowground ecology deserve further development. First, the response of insect species from less apparent feeding guilds (such as gall makers, stem borers, leaf miners and cell content feeders) has often been overlooked so far. In order to further elucidate patterns and more fully understand the ecological role of soil organisms in shaping plant-insect interactions, we need to use a more holistic approach that considers players from a broader range of guilds and trophic levels. Responses of natural enemies and

pollinators aboveground have been studied infrequently, and are completely missing for certain types of soil manipulations, or soil taxa. The life history of the various natural enemies is quite diverse and their responses to soil biota-plant interactions may vary. Parasitoids and other flying natural enemies may respond more quickly than wingless, cursorial predators like spiders. Furthermore, parasitoids are affected by changes in the quality of their herbivore hosts, as their life cycles intimately depend on host ecophysiology (e.g., MacKauer, 1996; Harvey, 2000; Harvey et al., 2004). Moreover, when we searched for studies in the scientific literature, we could not detect any that focused on the effect of soil organisms, via plants, on interactions between plants and non-arthropod taxa, such as slugs, snails, but also higher vertebrates, such as grazers. As plants are the primary producers that support food chains, it is likely that other organisms will also be affected by belowground organisms.

Second, to increase our ecological understanding, it is important to also include more ecologically realistic model systems, as the current systems are often based on crops, as well as on insect species that are either crop pests or chosen for convenience, rather than based on ecological relevance (Chen et al., 2015). This could be accomplished, for example, by using a range of wild plant species that vary in functional traits, which could give better insight into what traits may predict certain plant responses. Studying their natural associated insect communities may also increase our understanding of which traits are important in mediating soil biota-plant-insect interactions. Future work could fill in these important gaps in our current knowledge.

Third, more emphasis should be placed on the role of time and space in these aboveground-belowground interactions in the field. It is currently unknown whether performing manipulations with the same soil organisms at different locations (e.g., differing in altitude and latitude, as well as abiotic conditions) will lead to differential effects on aboveground insects or not. Future studies should also focus on the temporal aspects of above-belowground interactions in the field. As soil communities are dynamic and species-specific soil communities accumulate over time (Diez et al., 2010; Flory and Clay, 2013; Van der Putten et al., 2013; Heinen et al., 2018), it is likely that these temporal dynamics will strongly influence the performance of aboveground insect communities over time. Various controlled studies have shown that the sequence of arrival of aboveground and belowground herbivores on the plant can greatly alter the outcome of soil biota-plant-insect interactions (e.g., Erb et al., 2011; Wang

et al., 2014) and to some extent, this has also been shown in field studies (e.g., Gange et al., 2005a), although the link between temporally changing soil communities and temporal variation in aboveground insect communities has not been made. In the field, insect communities also change throughout the season. How soil treatments affect insects early compared to late in the season, and to what extent this is due to changes in plant-soil interactions or changes in plant-insect interactions is not known.

Fourth, most of the current research is focused on indirect effects that are mediated by shared host plants, but potential direct interactions should not be overlooked. There are various organisms, such as entomopathogens in the soil that can have direct impacts on aboveground insect performance. For instance, infection by entomopathogenic fungi, such as *Beauveria bassiana* and *Metarhizium anisoplae* can result in the quick death of many insect species (Meyling and Eilenberg, 2007; Vega et al., 2009, 2012), although its direct effects on aboveground insects in the field has been poorly documented. Interestingly, these fungi can also be endophytic in plants, and can influence both plant and herbivore performance (Meyling and Eilenberg, 2007; Vega et al., 2009, 2012; Senthilraja et al., 2010; Prabhukarthikeyan et al., 2014). Moreover, it has been shown for the fungus *Metarhizium* that it forms bridges between infected dead insects and plants, through which the fungus can provide the plant with extra nitrogen obtained from the insect bodies, which may also affect plant-insect interactions (Wang and St Leger, 2007; Behie et al., 2012; Sasan and Bidochka, 2012). Little is known about the extent to which aboveground insects pick up soil microorganisms and how this may affect their fitness, either through pathogenicity, or perhaps mutualistic interactions (e.g., in the gut microbiome), leaving an important gap in our current knowledge.

We conclude that there is strong support for a significant role of soil organisms in shaping plant-insect interactions in the field. With the exception of soil arthropods, we find that most field studies report effects that are similar to those of laboratory studies. We argue that future studies should be carefully planned, as the methodology applied in the field strongly affects the chance of finding robust results. Nonetheless, there are ample opportunities to develop this research field further, especially in terms of exploring alternative and more realistic methods to steer soil biomes into a targeted direction. It should be emphasized that there is a large gap in our knowledge when it comes to less apparent insect herbivore taxa such as leaf miners, stem borers and others. There is virtually nothing known about the effects of soil organisms on

a broad range of natural enemies (predators and parasitoids). However, as there are consistent reports of effects of soil organism addition in the field on aboveground insects, this opens up opportunities for the exploration of soil organism manipulation in agriculture or ecosystem restoration (e.g., Pineda et al., 2017). Some groups of soil organisms may be promising agents for crop yield enhancement and protection. Other groups of soil organisms may affect aboveground plant diversity at the community level and this gives rise to new opportunities to use soil organisms to "steer" the development of aboveground vegetation (Wubs et al., 2016), which may then subsequently affect aboveground insect communities. A challenge is to disentangle the drivers of soil organism manipulation effects on insects in the field. This will be an important step toward understanding how belowground organisms drive aboveground insect abundance, diversity and impacts in the field.

Author Contributions

TB and RH conceived the idea for the literature review. TB, AB, and RH designed the structural framework of the review. RH performed the literature study. TB and AB provided several additional references. RH wrote the first version of the manuscript. TB, AB, JH, and RH contributed critically to later versions of the manuscript.

Effects of Soil Organisms on Aboveground Plant-Insect Interactions in the Field: Patterns, Mechanisms and the Role of Methodology

Supplementary Information Chapter 2

Supplementary Table S2.1: An overview of the literature studies that were used for this literature review. For studies that investigated multiple interactions, these different interactions were detailed in separate rows. Detailed are the **soil bacterium** (species and strain), **soil organism type** (NF=nitrogen fixing; PGPR=Plant growth promoting bacteria), **method** (ADD= Field addition; REM= Field species removal; POT= Potted plants in the field), **Plant** (species), **Insect** (species), **Guild** (SH= Sucking herbivore; CH=Chewing herbivore; MT= Mutualist; PO=Pollinator; LM=Leafminer; CF=Cell-content feeder; GM=Gallmaker; SP=Seed predator; PI=Predatory insect), Enemy (species) and the **effects on plants, insects and enemies** (indicated by 0 (no effect on respective study organism), +(significant positive effect on respective study organism) or –(significant negative effect on respective study organism), or NA where the interactions were not assessed) and **Reference** (reference to original study).

Soil organism	Type	Method	Plant	Insect	Gld	Enemy	Plant effect	Insect effect	Enemy effect	Reference
Bradyrhizobium japonicum	NF	ADD	*Glycine max*	*Aphis glycines*	SH	NA	0	+	NA	Dean, Mescher & De Moraes, 2009
Bradyrhizobium japonicum	NF	ADD	*Glycine max*	*Aphis glycines*	SH	NA	0	-	NA	Brunner et al., 2015
Rhizobium DJB1033	NF	POT	*Phaseolus lunatus*	*Tetramorium caespitum*	MT	NA	+	-	NA	Godschalx et al., 2015
Pseudomonas fluorescens PF1	PGPR	ADD	*Oryza sativa*	*Cnaphalocrocis medinalis*	CH	various parasitoid and spiders	+	-	+	Radja Commare et al., 2002
Pseudomonas fluorescens FP7	PGPR	ADD	*Oryza sativa*	*Cnaphalocrocis medinalis*	CH	various parasitoid and spiders	+	-	+	Radja Commare et al., 2002
Bacillus subtilis EPC8	PGPR	ADD	*Solanum lycopersicum*	*Helicoverpa armigera*	CH	NA	+	-	NA	Prabhukarthikeyan, Saravanakumar & Raguchander, 2014

Pseudomonas putida 89B-61	PGPR	ADD	*Cucumis sativus*	*Acalymma vittatum*	CH	NA	+	-	NA	Zehnder et al, 1997
Pseudomonas putida 89B-61	PGPR	ADD	*Cucumis sativus*	*Diabrotica unidecimpunctata howardi*	CH	NA	+	-	NA	Zehnder et al, 1997
Serratia marcescens 90-166	PGPR	ADD	*Cucumis sativus*	*Acalymma vittatum*	CH	NA	+	-	NA	Zehnder et al, 1997
Serratia marcescens 90-166	PGPR	ADD	*Cucumis sativus*	*Diabrotica unidecimpunctata howardi*	CH	NA	+	-	NA	Zehnder et al, 1997
Flavomonas oryzihabitans INR-5	PGPR	ADD	*Cucumis sativus*	*Acalymma vittatum*	CH	NA	+	-	NA	Zehnder et al, 1997
Flavomonas oryzihabitans INR-5	PGPR	ADD	*Cucumis sativus*	*Diabrotica unidecimpunctata howardi*	CH	NA	+	-	NA	Zehnder et al, 1997
Bacillus pumillus INR-7	PGPR	ADD	*Cucumis sativus*	*Acalymma vittatum*	CH	NA	+	-	NA	Zehnder et al, 1997
Bacillus pumillus INR-7	PGPR	ADD	*Cucumis sativus*	*Diabrotica unidecimpunctata howardi*	CH	NA	+	-	NA	Zehnder et al, 1997
Pseudomonas fluorescens Pf1	PGPR	ADD	*Oryza sativa*	*Cnaphalocrocis medinalis*	CH	spiders	+	-	+	Saravanakumar et al., 2008
Pseudomonas fluorescens TDK1	PGPR	ADD	*Oryza sativa*	*Cnaphalocrocis medinalis*	CH	spiders	+	-	+	Saravanakumar et al., 2008

Pseudomonas fluorecens PY15	PGPR	ADD	*Oryza sativa*	*Cnaphalocrocis medinalis*	CH	spiders	+	-	+	Saravanakumar et al., 2008
Pseudomonas fluorescens Pf1	PGPR	ADD	*Oryza sativa*	*Cnaphalocrocis medinalis*	CH	damselflies	+	NA	-	Saravanakumar et al., 2008
Pseudomonas fluorescens TDK1	PGPR	ADD	*Oryza sativa*	*Cnaphalocrocis medinalis*	CH	damselflies	+	NA	-	Saravanakumar et al., 2008
Pseudomonas fluorecens PY15	PGPR	ADD	*Oryza sativa*	*Cnaphalocrocis medinalis*	CH	damselflies	+	NA	-	Saravanakumar et al., 2008
Pseudomonas fluorescens Pf1	PGPR	ADD	*Oryza sativa*	*Holochlora albida*	CH	NA	+	NA	-	Saravanakumar et al., 2008
Pseudomonas fluorescens TDK1	PGPR	ADD	*Oryza sativa*	*Holochlora albida*	CH	NA	+	NA	-	Saravanakumar et al., 2008
Pseudomonas fluorecens PY15	PGPR	ADD	*Oryza sativa*	*Holochlora albida*	CH	NA	+	NA	-	Saravanakumar et al., 2008
Bacillus cereus	PGPR	ADD	*Brassica oleracea*	*Brevicoryne brassicae*	SH	*Diaraetiella rapae, Cocinella septempunctata, syrphid flies*	0	-	-	Gadhave et al., 2016
Bacillus subtilis	PGPR	ADD	*Brassica oleracea*	*Brevicoryne brassicae*	SH	*Diaraetiella rapae, Cocinella septempunctata, syrphid flies*	0	-	-	Gadhave et al., 2016
Bacillus amyloliquefasciens	PGPR	ADD	*Brassica oleracea*	*Brevicoryne brassicae*	SH	*Diaraetiella rapae, Cocinella*	0	-	-	Gadhave et al., 2016

						septempunctata, syrphid flies				
Mixtures										
Pseudomonas fluorescens Pf1, *Pseudomonas fluorescens* TDK1, *Pseudomonas fluorecens* PY15	PGPR	ADD	*Oryza sativa*	*Cnaphalocrocis medinalis*	CH	spiders	+	-	+	Saravanakumar et al., 2008
Pseudomonas fluorescens Pf1, *Pseudomonas fluorescens* TDK1, *Pseudomonas fluorecens* PY15	PGPR	ADD	*Oryza sativa*	*Holochlora albida*	CH	NA	+	NA	+	Saravanakumar et al., 2008
Pseudomonas fluorescens Pf1, *Pseudomonas fluorescens* TDK1, *Pseudomonas fluorecens* PY15	PGPR	ADD	*Oryza sativa*	*NA*	NA	damselflies	+	NA	+	Saravanakumar et al., 2008
Bacillus cereus, Bacillus subtilis,	PGPR	ADD	*Brassica oleracea*	*Brevicoryne brassicae*	SH	*Diaraetiella rapae, Cocinella*	0	-	0	Gadhave et al., 2016

Bacillus amyloliquefasciens	*septempunctata,* *syrphid flies*

Supplementary Table S2.2: An overview of the literature studies that were used for this literature review. For studies that investigated multiple interactions, these different interactions were detailed in separate rows. Detailed are the **soil fungus** (species and strain), **soil organism type** (AMF=Arbuscular mycorrhizal fungi; EMF=Ectomycorrhizal fungi; EP=Entomopathogenic fungi), **method** (ADD= Field addition; REM= Field species removal; POT= Potted plants in the field), **Plant** (species), **Insect** (species), **Guild** (SH= Sucking herbivore; CH=Chewing herbivore; MT= Mutualist; PO=Pollinator; LM=Leafminer; CF=Cell-content feeder; GM=Gallmaker; SP=Seed predator; PI=Predatory insect), Enemy (species) and the **effects on plants, insects and enemies** (indicated by 0 (no effect on respective study organism), +(significant positive effect on respective study organism) or –(significant negative effect on respective study organism), or NA where the interactions were not assessed) and **Reference** (reference to original study).

Soil organism	Type	Method	Plant	Insect	Gld	Enemy	Plant effect	Insect effect	Enemy effect	Reference
Funneliformis caledonium (syn. *Glomus caledonium*)	AMF	ADD	*Eucalyptus urophylla*	*Anomala cupripes*	CH	NA	-	0	NA	Gange et al., 2005
Funneliformis caledonium	AMF	ADD	*Eucalyptus urophylla*	Unidentified geometrid	CH	NA	-	0	NA	Gange et al., 2005
Rhizoglomus clarum(syn *Glomus clarum*)	AMF	ADD	*Cucumis sativus*	*Acalymma vittatum*	CH	NA	0	0	NA	Barber et al., 2013
Rhizoglomus custos (syn *Glomus custos*)	AMF	ADD	*Cucumis sativus*	*Acalymma vittatum*	CH	NA	0	0	NA	Barber et al., 2013

Rhizophagus irregularis (syn. *Glomus intraradices*) 09	AMF	ADD	*Cucumis sativus*	*Acalymma vittatum*	CH	NA	0	0	NA	Barber et al., 2013
Rhizophagus irregularis DAOM 197198	AMF	ADD	*Cucumis sativus*	*Acalymma vittatum*	CH	NA	0	0	NA	Barber et al., 2013
Glomus caledonium	AMF	ADD	*Eucalyptus urophylla*	*Strepsicrates spp.*	LM	NA	-	0	NA	Gange et al., 2005
Rhizoglomus clarum	AMF	ADD	*Cucumis sativus*	Honeybees	PO	NA	0	-	NA	Barber et al., 2013
Rhizoglomus custos	AMF	ADD	*Cucumis sativus*	Honeybees	PO	NA	0	-	NA	Barber et al., 2013
R.irregularis 09	AMF	ADD	*Cucumis sativus*	Honeybees	PO	NA	0	-	NA	Barber et al., 2013
Rhizophagus irregularis DAOM 197198	AMF	ADD	*Cucumis sativus*	Honeybees	PO	NA	0	0	NA	Barber et al., 2013
Rhizoglomus clarum	AMF	ADD	*Cucumis sativus*	Bumblebees	PO	NA	0	0	NA	Barber et al., 2013
Rhizoglomus custos	AMF	ADD	*Cucumis sativus*	Bumblebees	PO	NA	0	0	NA	Barber et al., 2013
Rhizophagus irregularis 09	AMF	ADD	*Cucumis sativus*	Bumblebees	PO	NA	0	0	NA	Barber et al., 2013

Rhizophagus irregularis DAOM 197198	AMF	ADD	*Cucumis sativus*	Bumblebees	PO	NA	0	0	NA	Barber et al., 2013
Rhizoglomus clarum	AMF	ADD	*Cucumis sativus*	Lepidoptera	PO	NA	0	0	NA	Barber et al., 2013
Rhizoglomus custos	AMF	ADD	*Cucumis sativus*	Lepidoptera	PO	NA	0	0	NA	Barber et al., 2013
Rhizophagus irregularis 09	AMF	ADD	*Cucumis sativus*	Lepidoptera	PO	NA	0	0	NA	Barber et al., 2013
Rhizophagus irregularis DAOM 197198	AMF	ADD	*Cucumis sativus*	Lepidoptera	PO	NA	0	0	NA	Barber et al., 2013
Gigaspora margarita 'Central Glass'	AMF	ADD	*Glycine max*	*Thrips* spp.	CF	NA	+	-	NA	Ueda et al., 2013
Funneliformis mosseae	AMF	ADD	*Lolium perenne*	*Phlogophora meticulosa*	CH	NA	-	-	NA	Vicari et al., 2002
Funneliformis caledonium	AMF	ADD	*Eucalyptus urophylla*	*Anomala cupripes*	CH	NA	-	0	NA	Gange et al., 2005
Funneliformis caledonium	AMF	ADD	*Eucalyptus urophylla*	Unidentified herbivory	CH	NA	-	+	NA	Gange et al., 2005
Rhizophagus irregularis	AMF	ADD	*Chamerion angustifolium*	Unidentified herbivory	CH	NA	0	0	NA	Wolfe, Husband & Klironomos, 2005

Gigaspora gigantea	AMF	ADD	*Chamerion angustifolium*	Unidentified herbivory	CH	NA	+	0	NA	Wolfe, Husband & Klironomos, 2005
Gigaspora margarita 'Central Glass'	AMF	ADD	*Glycine max*	*Pleuroptya ruralis*	CH	NA	+	0	NA	Ueda et al., 2013
Gigaspora margarita 'Central Glass'	AMF	ADD	*Glycine max*	*Ascotis selenaria*	CH	NA	+	0	NA	Ueda et al., 2013
Funneliformis caledonium	AMF	ADD	*Eucalyptus urophylla*	*Strepsicrates spp.*	LM	NA	-	0	NA	Gange et al., 2005
Gigaspora margarita 'Central Glass'	AMF	ADD	*Glycine max*	NA	NA	*Orius sauteri*	+	NA	-	Ueda et al., 2013
Rhizophagus irregularis	AMF	ADD	*Chamerion angustifolium*	Pollinating Hymenoptera	PO	NA	0	+	NA	Wolfe, Husband & Klironomos, 2005
Gigaspora gigantea	AMF	ADD	*Chamerion angustifolium*	Pollinating Hymenoptera	PO	NA	+	+	NA	Wolfe, Husband & Klironomos, 2005
Clareideoglomus claroideum (syn. G*lomus claroideum*)	AMF	ADD	*Geranium sylvaticum*	Pollinating Hymenoptera	PO	NA	+ (flower quality), - (fitness)	0	NA	Varga & Kytöviita, 2010
Simiglomus hoi (syn. *Glomus hoi*)	AMF	ADD	*G. sylvaticum*	Pollinating Hymenoptera	PO	NA	+ (flower	-	NA	Varga & Kytöviita, 2010

							quality), 0 (fitness)			
Clareidoglomus claroideum	AMF	ADD	*G. sylvaticum*	Pollinating Diptera	PO	NA	+ (flower quality), - (fitness)	0	NA	Varga & Kytöviita, 2010
Siniglomus hoi	AMF	ADD	*G. sylvaticum*	Pollinating Diptera	PO	NA	+ (flower quality), 0 (fitness)	0	NA	Varga & Kytöviita, 2010
Gigaspora margarita 'Central Glass'	AMF	ADD	*Glycine max*	*Aulacorthum solani*	SH	NA	+	+	NA	Ueda et al., 2013
Glomus ssp. (CCS Aosta)	AMF	ADD (tunnel)	*Solanum lycopersicum*	*Frankliniella occidentalis*	CF	NA	0	0	NA	Colella et al., 2014
Laccaria laccata	EMF	ADD	*Eucalyptus urophylla*	*Anomala cupripes*	CH	NA	0	0	NA	Gange et al., 2005
Laccaria laccata	EMF	ADD	*Eucalyptus urophylla*	unidentified geometrid	CH	NA	0	-	NA	Gange et al., 2005
Laccaria laccata	EMF	ADD	*Eucalyptus urophylla*	*Strepsicrates* spp.	LM	NA	0	-	NA	Gange et al., 2005

Laccaria laccata	EMF	ADD	*Eucalyptus urophylla*	*Anomala cupripes*	CH	NA	0	-	NA	Gange et al., 2005
Laccaria laccata	EMF	ADD	*Eucalyptus urophylla*	Unidentified herbivory	CH	NA	0	+	NA	Gange et al., 2005
Laccaria laccata	EMF	ADD	*Eucalyptus urophylla*	*Strepsicrates* spp.	LM	NA	0	-	NA	Gange et al., 2005
Pisolithus tinctorius	EMF	ADD	*Populus angustifolia x Populus fremontii*	*Chaitophorus populicola*	SH	NA	NA	+	NA	Gehring & Whitham, 2002
Beauveria bassiana B2	EP	ADD	*Solanum lycopersicum*	*Helicoverpa armigera*	CH	NA	+	-	NA	Prabhukarthikeyan, Saravanakumar & Raguchander, 2014
Mixtures										
Fungi Perfecti; *Funneliformis mosseae*, *Rhizophagus irregularis, Clareidoglomus clarum, Funneliformis*	AMF	ADD	*Baccharis halimifolia*	*Trirhabda baccharidis*	CH	NA	NA	0	NA	Younginger, Barnouti & Moon, 2009

monosporus, Septoglomus. deserticola, Paraglomus brasilianum, Gigaspora margarita, Pisolithus tinctorus and four species of *Rhizopogon*											
Rhizoglomus clarum, Rhizoglomus custos, Rhizophagus irregularis	AMF	ADD	*Cucumis sativus*	*Acalymma vittatum*	CH	NA	0	+	NA	Barber et al., 2013	
Fungi Perfecti	AMF	ADD	*Baccharis halimifolia*	*Neolasioptera lathami*	GM	NA	NA	+	NA	Younginger, Barnouti & Moon, 2009	
Fungi Perfecti	AMF	ADD	*Baccharis halimifolia*	*Amauromyza maculosa*	LM	NA	NA	+	NA	Younginger, Barnouti & Moon, 2009	
Fungi Perfecti	AMF	ADD	*Baccharis halimifolia*	*Liriomyza trifolii*	LM	NA	NA	+	NA	Younginger, Barnouti & Moon, 2009	
Fungi Perfecti	AMF	ADD	*Baccharis halimifolia*	*Amauromyza maculosa*	LM	Unidentified parasitoid	+	+	-	Moon, Barnouti & Younginger, 2013	

Fungi Perfecti	AMF	ADD	*Baccharis halimifolia*	*Liriomyza trifolii*	LM	Unidentified parasitoid	+	+	-	Moon, Barnouti & Younginger, 2013
Fungi Perfecti	AMF	ADD	*Baccharis halimifolia*	*Neolasioptera lathami*	GM	Unidentified parasitoid	+	+	-	Moon, Barnouti & Younginger, 2013
INOQ; *Clareidoglomus etunicatum, Clareidoglomus claroideum*, *Rhizophagus irregularis*	AMF	ADD	*Trifolium pratense*	Overall herbivore diversity	NA	NA	+	0 (consumption), - (insect diversity)	NA	Guo et al., 2015
INOQ	AMF	ADD	*Lolium perenne*	Overall herbivore diversity	NA	NA	0	0	NA	Guo et al., 2015
Rhizoglomus clarum, Rhizoglomus custos, Rhizophagus irregularis	AMF	ADD	*Cucumis sativus*	Honeybees	PO	NA	0	0	NA	Barber et al., 2013
Rhizoglomus clarum, Rhizoglomus custos,	AMF	ADD	*Cucumis sativus*	Bumblebees	PO	NA	0	0	NA	Barber et al., 2013

Rhizophagus irregularis										
Rhizoglomus clarum, *Rhizoglomus custos*, *Rhizophagus irregularis*	AMF	ADD	*Cucumis sativus*	Lepidoptera	PO	NA	0	+	NA	Barber et al., 2013
Glomus ssp. (CCS Aosta)	AMF	ADD (tunnel)	*Solanum lycopersicum*	*Trialeurodes vaporariorum*	SH	NA	0	0	NA	Colella et al., 2014
Glomus ssp. (CCS Aosta)	AMF	ADD (tunnel)	*Solanum lycopersicum*	*Macrosiphom euphorbiae*	SH	NA	0	0	NA	Colella et al., 2014
Glomus ssp. (CCS Aosta)	AMF	ADD (tunnel)	*Solanum lycopersicum*	Unidentified leafhopper	SH	NA	0	0	NA	Colella et al., 2014
Natural mycorrhizal community	AMF	REM	*Plantago lanceolata*	*Arctia caja*	CH	NA	+	-	NA	Gange & West 1994
Natural mycorrhizal community	AMF	REM	*Leucanthemum vulgare*	*Chromatomyia syngenesiae*	LM	*Diglyphus isaea*	+	0	-	Gange, Brown & Aplin, 2003
Natural mycorrhizal community	AMF	REM	*Leucanthemum vulgare*	*Chromatomyia syngenesiae*	LM	NA	+	+	NA	Gange, Brown & Aplin, 2003

Natural mycorrhizal community	AMF	REM	Tallgrass prairie system of C3 and C4 grasses and forbs.	Herbivore consumption	NA	NA	0	+	NA	Kula & Hartnett, 2015
Natural mycorrhizal community	AMF	REM	*Achillea millefollium*	Total pollinator visits	PO	NA	0	0	NA	Cahill et al., 2008
Natural mycorrhizal community	AMF	REM	*Aster laevis*	Total pollinator visits	PO	NA	0	+	NA	Cahill et al., 2008
Natural mycorrhizal community	AMF	REM	*Campanula rotundifolia*	Total pollinator visits	PO	NA	0	0	NA	Cahill et al., 2008
Natural mycorrhizal community	AMF	REM	*Cerastium arvense*	Total pollinator visits	PO	NA	-	-	NA	Cahill et al., 2008
Natural mycorrhizal community	AMF	REM	*Erigeron philadelphicus*	Total pollinator visits	PO	NA	0	0	NA	Cahill et al., 2008
Natural mycorrhizal community	AMF	REM	*Solidago missouriensis*	Total pollinator visits	PO	NA	0	+	NA	Cahill et al., 2008

Natural mycorrhizal community	AMF	REM	*Plantago lanceolata*	*Myzus persicae*	SH	NA	+	+	NA	Gange & West 1994
Funneliformis mosseae & *Rhizophagus irregularis*	AMF	POT	*Tagetes patula*	Pollinating Hymenoptera	PO	NA	0	+	NA	Gange & Smith, 2005
Funneliformis mosseae & *Rhizophagus irregularis*	AMF	POT	*Tagetes erecta*	Pollinating Hymenoptera	PO	NA	+	+	NA	Gange & Smith, 2005
Funneliformis mosseae & *Rhizophagus irregularis*	AMF	POT	*Centaurea cyanus*	Pollinating Hymenoptera	PO	NA	+	+	NA	Gange & Smith, 2005
Funneliformis mosseae & *Rhizophagus irregularis*	AMF	POT	*Tagetes patula*	Pollinating Diptera	PO	NA	0	+	NA	Gange & Smith, 2005
Funneliformis mosseae & *Rhizophagus irregularis*	AMF	POT	*Tagetes erecta*	Pollinating Diptera	PO	NA	0	+	NA	Gange & Smith, 2005

Funneliformis mosseae & *Rhizophagus irregularis*	AMF	POT	*Centaurea cyanus*	Pollinating Diptera	PO	NA	+	+	NA	Gange & Smith, 2005
Glomus spp. & *Glomus etunicatum, Clareidoglomus clarum* and *Entrophospora columbiana*	AMF	POT	*Populus angustifolia* x *Populus fremontii*	*Chaitophorus populicola*	SH	NA	NA	-	NA	Gehring & Whitham, 2002

Supplementary Table S2.3: An overview of the literature studies that were used for this literature review. For studies that investigated multiple interactions, these different interactions were detailed in separate rows. Detailed are the **soil nematode** (species), Type (PPN= plant-parasitic nematode), **method** (ADD= Field addition; REM= Field species removal; POT= Potted plants in the field), **Plant** (species), **Insect** (species), **Guild** (SH= Sucking herbivore; CH=Chewing herbivore; MT= Mutualist; PO=Pollinator; LM=Leafminer; CF=Cell-content feeder; GM=Gallmaker; SP=Seed predator; PI=Predatory insect), Enemy (species) and the **effects on plants, insects and enemies** (indicated by 0 (no effect on respective study organism), +(significant positive effect on respective study organism) or –(significant negative effect on respective study organism), or NA where the interactions were not assessed) and **Reference** (reference to original study).

Soil organism	Type	Method	Plant	Insect	Gld	Enemy	Plant effect	Insect effect	Enemy effect	Reference
Total PPN community	PPN	CORR	*Nicotiana tabacum*	*Manduca sexta*	CH	NA	NA	+	NA	Kaplan, Sardanelli & Denno, 2009
Tylenchorhynchus sp.	PPN	CORR	*Nicotiana tabacum*	*Manduca sexta*	CH	NA	NA	0	NA	Kaplan, Sardanelli & Denno, 2009
Pratylenchus sp.	PPN	CORR	*Nicotiana tabacum*	*Manduca sexta*	CH	NA	NA	0	NA	Kaplan, Sardanelli & Denno, 2009
Xiphinema sp.	PPN	CORR	*Nicotiana tabacum*	*Manduca sexta*	CH	NA	NA	0	NA	Kaplan, Sardanelli & Denno, 2009
Heterodera sp.	PPN	CORR	*Ammophila arenaria*	*Schizaphis rufula*	SH	NA	-	0	NA	Vandegehuchte, De la Peña & Bonte, 2010
Heterodera glycines	PPN	CORR	*Glycine max*	*Aphis glycines*	SH	NA	-	-	NA	Hong, Macguidwin & Gratton, 2011
Heterodera glycines	PPN	CORR	*Glycine max*	*Aphis glycines*	SH	NA	0	0	NA	Heeren et al., 2012
Tylenchorhynchus sp.	PPN	CORR	*Nicotiana tabacum*	*Myzus persicae*	SH	NA	NA	-	NA	Kaplan, Sardanelli & Denno, 2009

Total PPN community	PPN	CORR	*Nicotiana tabacum*	*Myzus persicae*	SH	NA	NA	0	NA	Kaplan, Sardanelli & Denno, 2009
Heterodera glycines	PPN	CORR	*Glycine max*	*Helicoverpa zea*	CH	NA	-	+	NA	Alston et al., 1991
Meiloidogyne incognita	PPN	ADD	*Nicotiana tabacum*	*Manduca sexta*	CH	NA	NA	0		Kaplan, Sardanelli & Denno, 2009
Meiloidogyne incognita	PPN	ADD	*Nicotiana tabacum*	*Spodoptera exigua*	CH	NA	NA	0		Kaplan, Sardanelli & Denno, 2009
Meiloidogyne incognita	PPN	ADD	*Nicotiana tabacum*	*Epytrix spp.*	CH	NA	NA	+		Kaplan, Sardanelli & Denno, 2009
Heterodera schachtii	PPN	ADD	*Lathyrus vernus*	Unidentified herbivory	CH	NA	-	+	NA	Vockenhuber et al., 2013
Meiloidogyne incognita	PPN	ADD	*Nicotiana tabacum*	*Myzus persicae*	SH	NA	NA	-		Kaplan, Sardanelli & Denno, 2009
Meiloidogyne incognita	PPN	ADD (tunnel)	*Glycine max*	*Pseudoplusia includens*	CH	NA	0	0	NA	Carter-Wientjes et al., 2004

Supplementary Table S2.4: An overview of the literature studies that were used for this literature review. For studies that investigated multiple interactions, these different interactions were detailed in separate rows. Detailed are the **soil arthropod** (species), **soil organism taxon** (C= Coleopteran soil insect; D= Dipteran soil insect), **method** (ADD= Field addition; REM= Field species removal; POT= Potted plants in the field), **Plant** (species), **Insect** (species), **Guild** (SH= Sucking herbivore; CH=Chewing herbivore; MT= Mutualist; PO=Pollinator; LM=Leafminer; CF=Cell-content feeder; GM=Gallmaker; SP=Seed predator; PI=Predatory insect), Enemy (species) and the **effects on plants, insects and enemies** (indicated by 0 (no effect on respective study organism), +(significant positive effect on respective study organism) or –(significant negative effect on respective study organism), or NA where the interactions were not assessed) and **Reference** (reference to original study).

Soil organism	Type	Method	Plant	Insect	Gld	Enemy	Plant effect	Insect effect	Enemy effect	Reference
Hylobius transversovittatus	C	ADD	*Lythrum salicaria*	*Galerucella calmariensis*	CH	NA	0	0	NA	Hunt-Joshi & Blossey, 2004
Diabrotica virgifera virgifera	C	ADD	*Zea mays*	*Ostrinia nubilalis*	CH	*Macrocentrus grandii*	-	-	-	White & Andow, 2006
Agriotes spp.	C	ADD	*Clinopodium vulgare*	*Stephensia brunnichella*	LM	Unidentified Microgastrinae	NA	-	-	Staley et al., 2007
Hylobius transversovittatus	C	POT	*Lythrum salicaria*	*Galerucella calmariensis*	CH	NA	0	0	NA	Hunt-Joshi & Blossey, 2004
Cebrio gypsicola	C	POT	*Moricandia moricandioides*	*Total Chewing herbivores (Pontia daplidice; Euchloe crameri; Pieris rapae; Pieris brassicae)*	CH	NA	-	-	NA	Megías & Müller, 2010

Acalymma vittatum (larval)	C	POT	*Cucumis sativus*	Total herbivory	CH	NA	-	0	NA	Barber et al., 2015
Agriotes spp.	C	POT	*Plantago lanceolata* (Low IG)	Herbivore damage	CH	NA	+	+	NA	Wurst et al., 2008
Agriotes spp.	C	POT	*Plantago lanceolata* (High IG)	Herbivore damage	CH	NA	+	-	NA	Wurst et al., 2008
Morica hybrida	C	POT	*Moricandia moricandioides*	*Total Chewing herbivores (Pontia daplidice; Euchloe crameri; Pieris rapae; Pieris brassicae)*	CH	*Cotesia kazak*	0	-	+	Megías & Müller, 2010
Agriotes spp.	C	POT	*Sinapis arvensis*	Total pollinator visits	PO	NA	+	+	NA	Poveda et al., 2003
Agriotes spp.	C	POT	*Sinapis arvensis*	Total pollinator visits	PO	NA	0	+	NA	Poveda et al., 2005
Acalymma vittatum (larval)	C	POT	*Cucumis sativus*	Total pollinator visits	PO	NA	-	-	NA	Barber et al., 2015
Cebrio gypsicola	C	POT	*Moricandia moricandioides*	Total seed predators	SP	NA	-	-	NA	Megías & Müller, 2010
Morica hybrida	C	POT	*Moricandia moricandioides*	Total seed predators	SP	NA	0	-	NA	Megías & Müller, 2010

Agriotes spp.	C	POT	*Sinapis arvensis*	*Brevicoryne brassicae*	SH	NA	0	+	NA	Poveda et al., 2005
Cebrio gypsicola	C	POT	*Moricandia moricandioides*	Total planthoppers	SH	NA	-	0	NA	Megías & Müller, 2010
Cebrio gypsicola	C	POT	*Moricandia moricandioides*	Total aphids	SH	NA	-	0	NA	Megías & Müller, 2010
Sitona discoideus	C	POT	*Medicago sativa*	*Acyrthosiphon pisum*	SH	NA	+	-	NA	Ryalls et al., 2016
Morica hybrida	C	POT	*Moricandia moricandioides*	Total planthoppers	SH	NA	0	-	NA	Megías & Müller, 2010
Morica hybrida	C	POT	*Moricandia moricandioides*	Total aphids	SH	NA	0	0	NA	Megías & Müller, 2010
Agriotes spp.	C	POT	Community (*Achillea millefolium, Leucanthemum vulgare, Plantago lanceolata, Lotus corniculatus, Trifolium pratense, Agrostis capillaris, Dactylis*	*Chorthippus albomarginatus*	CH	NA	0	0	NA	Borgström et al., 2017

			glomerata, Lolium perenne, Festuca rubra							
Delia radicum	D	POT	*Brassica nigra*	*Phyllotreta ssp.*	CH	NA	NA	-	NA	Soler et al., 2009
Delia radicum	D	POT	*Brassica nigra*	*Pieris rapae*	CH	NA	NA	0	NA	Soler et al., 2009
Delia radicum	D	POT	*Brassica nigra*	NA	PI	*Chrysoperla carnea*	NA	NA	0	Soler et al., 2009
Delia radicum	D	POT	*Brassica nigra*	*Brevicoryne brassicae*	SH	NA	NA	-	NA	Soler et al., 2009
Delia radicum	D	POT	*Brassica nigra*	*Myzus persicae*	SH	NA	NA	0	NA	Soler et al., 2009
Delia radicum	D	ADD (tunnel)	*Brassica oleracea subsp. Italica (var. Monaco)*	*Pieris brassicae*	CH	NA	0	0	NA	Pierre et al., 2013
Delia radicum	D	ADD (tunnel)	*Brassica oleracea subsp. Italica (var. Monaco)*	*Pieris rapae*	CH	NA	0	0	NA	Pierre et al., 2013
Delia radicum	D	ADD (tunnel)	*Brassica oleracea subsp. Italica (var. Monaco)*	*Plutella xylostella*	CH	NA	0	0	NA	Pierre et al., 2013
Delia radicum	D	ADD (tunnel)	*Brassica oleracea subsp. Italica (var. Monaco)*	*Mamestra brassicae*	CH	NA	0	0	NA	Pierre et al., 2013

Delia radicum	D	ADD (tunnel)	*Brassica oleracea subsp. Italica (var. Monaco)*	*Brevicoryne brassicae*	SH	NA	0	+	NA	Pierre et al., 2013
Delia radicum	D	ADD (tunnel)	*Brassica oleracea subsp. Italica (var. Monaco)*	*Myzus persicae*	SH	NA	0	0	NA	Pierre et al., 2013
Mixtures										
Morica hybrida & *Cebrio gypsicola*	C	POT	*Moricandia moricandioides*	*Total Chewing herbivores (Pontia daplidice; Euchloe crameri; Pieris rapae; Pieris brassicae)*	CH	NA	0	-	NA	Megías & Müller, 2010
Morica hybrida & *Cebrio gypsicola*	C	POT	*Moricandia moricandioides*	Total planthoppers	SH	NA	0	0	NA	Megías & Müller, 2010
Morica hybrida & *Cebrio gypsicola*	C	POT	*Moricandia moricandioides*	Total aphids	SH	NA	0	-	NA	Megías & Müller, 2010
Total soil arthropods		REM	Tallgrass prairie	Unspecified	CH	NA	NA	+	NA	Evans, 1991
Total soil arthropods		REM	Tallgrass prairie	Unidentified Orthoptera	CH	NA	NA	0	NA	Evans, 1991
Total soil arthropods		REM	Tallgrass prairie	Unidentified Coleoptera	CH	NA	NA	0	NA	Evans, 1991

Total soil arthropods	REM	Tallgrass prairie	Ants	MT	NA	NA	-	NA	Evans, 1991
Total soil arthropods	REM	Grassland community	Total insect number	NA	NA	NA	+	NA	Masters, Brown & Gange, 1993
Total soil arthropods	REM	Tallgrass prairie	NA	PW	Unspecified Parasitica	NA	NA	0	Evans, 1991
Total soil arthropods	REM	Tallgrass prairie	NA	PI	Unidentified entomophagous	NA	NA	0	Evans, 1991
Total soil arthropods	REM	Tallgrass prairie	NA	PI	Unspecified Arachnida	NA	NA	0	Evans, 1991
Total soil arthropods	REM	Tallgrass prairie	NA	PI	Unidentified predatory insects	NA	NA	0	Evans, 1991
Total soil arthropods	REM	*Cirsium palustre*	*Terrelia ruficauda*	SP	*Pteromalus elevatus* and *Torymus chloromerus*	-	+	+	Masters, Jones & Rogers, 2001
Total soil arthropods	REM	Tallgrass prairie	Unspecified	SH	NA	NA	+	NA	Evans, 1991
Total soil arthropods	REM	Tallgrass prairie	Unidentified Auchenorrhyncha	SH	NA	NA	+	NA	Evans, 1991
Total soil arthropods	REM	Tallgrass prairie	Unidentified Sternorrhyncha	SH	NA	NA	0	NA	Evans, 1991
Total soil arthropods	REM	Grassland community	Aphids	SH	NA	NA	+	NA	Masters, 1995

Combinations										
Bacillus subtilis EPC8 + *Beauveria bassiana* B2	Mix	ADD	*Solanum lycopersicum*	*Helicoverpa armigera*	CH	NA	+	-	NA	Prabhukarthikeyan, Saravanakumar & Raguchander, 2014
Effective Microorganisms (EM); *Lactobacillus plantarum, Lactobacillus casei, Streptococcus lactis, Sacchermoyces* spp., *Rhodopseudomonas plastris, Rhodobacter sphacrodes* and *Streptomyces* spp.	Mix	ADD	*Zea mays*	Overall herbivore diversity	NA	Overall predator diversity	0	0	-	Megali et al., 2015
Bradyrhizobium japonicum + Delfia acidovorans	Mix	ADD	*Glycine max*	*Aphis glycines*	SH	NA	-	-	NA	Brunner et al., 2015
Bradyrhizobium japonicum + Azospirillum brasilense	Mix	ADD	*Glycine max*	*Aphis glycines*	SH	NA	0	0	NA	Brunner et al., 2015
Microsat F	Mix	ADD	*Solanum lycopersicum*	*Trialeurodes vaporariorum*	SH	NA	0	0	NA	Colella et al., 2014

Micosat F	Mix	FINOC	*Solanum lycopersicum*	*Macrosiphon euphorbiae*	SH	NA	0	0	NA	Colella et al., 2014
Micosat F	Mix	FINOC	*Solanum lycopersicum*	*Frankliniella occidentalis*	SH	NA	0	0	NA	Colella et al., 2014
Micosat F	Mix	FINOC	*Solanum lycopersicum*	Unidentified leafhopper	SH	NA	0	0	NA	Colella et al., 2014
Pseudomonas fluorescens TDK1, *Pseudomonas fluorescens* PF1, *Beauveria bassiana* B2, *Beauveria bassiana* B4	PGPR	FINOC	*Arachis hypogaea*	*Aproaerema modicella*	LM	NA	NA	-	NA	Senthilraja et al., 2010

Chapter 3

Plant functional group and growth rate interactively shape soil legacy effects on individual plant-insect interactions

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Abstract

Plant-mediated soil legacy effects can be important determinants of the performance of plants and their aboveground insect herbivores, but so far, such effects on plant-insect interactions have been tested for only a limited number of host plant species and soils. Here, we tested the performance of a polyphagous aboveground herbivore, *Mamestra brassicae* on twelve host plant species that were grown on a set of soils conditioned by each of these twelve species. We tested whether functional traits (growth rate: fast- versus slow-growing species, and functional type: grasses versus forbs) of the plant species that conditioned the soil and the test plant species growing in those soils affected the response of insect herbivores to conditioned soils. Our results show that plants and insect herbivores had lower biomass on soils that were conditioned by fast-growing forbs than on soils conditioned by slow-growing forbs. On soils conditioned by grasses, growth type of the conditioning plant had the opposite effect, i.e., plants and herbivores had higher biomass on soils conditioned by fast-growing grasses, than on soils conditioned by slow-growing grasses. The degree to which herbivores were affected by soil legacy effects also depended on the host plant species. On *Taraxacum officinale* and *Festuca ovina*, herbivory differed between host plants that were grown on conspecific and heterospecific soils. For two other plant species, *Holcus lanatus* and *Briza media*, herbivory was affected by the traits of the conditioning plant species. We provide evidence that soil communities can play an important role in shaping plant-insect interactions aboveground. Our results further emphasize the important, but differentiated role of traits in mediating soil-plant-insect interactions.

Introduction

Understanding what drives the performance of insect herbivores on their host plants has been an important area in the field of ecology. Many mechanistic explanations for variation in herbivore performance on different plants have been put forward, including individual plant vigour (e.g. Price 1991), plant tissue nutrient content (Awmack & Leather, 2002; Wetzel et al., 2016), levels of abiotic stress in plants (e.g. White, 1969; 1974), or levels of constitutive and inducible plant defences (Kessler & Baldwin, 2002). Plants that grow more vigorously, may present a higher-quality food source to herbivores than less vigorous plants (Price 1991). However, in most cases herbivore performance is determined by a combination of these factors, which makes understanding plant-insect relationships challenging (Agrawal & Fishbein, 2006). A recurring problem is that patterns observed in plant-insect ecology are often plant- or herbivore-specific, which adds another layer of complexity.

As primary producers, plants interact with a wide array of organisms, ranging from micro-organisms to grazing mammals. Plants are modular and possess different structures with different functions, such as roots, shoots and floral parts, which often simultaneously interact with different organisms. Roots, being embedded in the soil, encounter soil microorganisms and soil invertebrates, whereas aboveground structures, such as leaves or flowers, interact with insect herbivores or pollinators. It has been shown that interactions with the plant in one plant module, can influence interactions in other plant parts (e.g. Soler et al., 2005; 2007; Erb et al., 2011; Soler, Erb & Kaplan, 2013; Wang et al., 2015), which is regulated by complex phytochemical defence pathways (e.g. Bezemer & van Dam, 2005; Biere & Goverse, 2016; Erb & Reymond, 2019). For example, a vast body of work has revealed how individual soil dwelling species can influence the performance of aboveground foliar feeding herbivores, mediated by the shared host plant (e.g. reviewed in Pineda et al., 2010; Koricheva et al., 2009; Johnson et al., 2012; Wondafrash et al., 2013). However, soils are inhabited by a vast amount of different (micro) organisms, and how these soil communities as a whole can influence aboveground herbivore performance on different plant species is not well understood (Kostenko et al. 2012; Heinen et al., 2018a; 2018b; Pineda et al. 2017).

Soil communities and plants are intimately linked (Van der Putten et al., 2013; Bardgett & Van der Putten, 2014). First, plants can steer the soil communities around their roots through exudation of metabolites into the soil (Phillipot et al., 2013), and as a consequence, different

plant species leave very different microbial footprints in the soil (Bezemer et al., 2006; Kos et al., 2015; Heinen et al., 2018b). These specific soil communities, in turn, can have differential effects on the performance of plants that grow in the same soil, a process known as plant-soil feedback (Van der Putten et al., 2013). Recent studies have shown that such soil legacy effects also influence the performance of aboveground herbivores that feed on plants that grow in differently conditioned soils (Kostenko et al., 2012; Kos et al., 2015; Heinen et al., 2018b; Heinze et al., 2018). For instance, on ragwort, *Jacobaea vulgaris*, colony development of aphids highly depends on the microbial communities in the different soils that the host plant is growing in (Kos et al., 2015). Moreover, the performance of a polyphagous chewing herbivore is also strongly influenced by microbial soil legacy effects when feeding on ragwort (Kostenko et al., 2012, Bezemer et al., 2013), or on multispecies plant communities (Heinen et al., 2018b). However, whether insect herbivores are also affected by plant-soil feedback across a broader range of host plant species is not known.

Soil legacy effects are strongly influenced by plant traits. For instance, plants that have a higher growth rate, leave more negative soil legacy effects than those that have a lower growth rate (Cortois et al., 2016; Bergmann et al., 2016). Soil legacy effects on plants also differ between plant functional types (Kulmatiski et al., 2008). Grasses, for example, generally create more positive soil conditions for the growth of future plant species than forbs (Van de Voorde et al., 2011; Wubs et al., 2016; Ma et al., 2017). Moreover, grasses and forbs, may, via soil legacy effects, influence aboveground herbivores on plants that grow in these soils (Kos et al., 2015; Heinen et al., 2018b). For instance, *Mamestra brassicae* caterpillars that were reared on plant communities growing on soils that were previously conditioned by grass species, had a lower biomass than caterpillars reared on plant communities growing on forb soils (Heinen et al., 2018b).

In a full-factorial greenhouse experiment we reared a polyphagous chewing herbivore, *Mamestra brassicae*, on twelve common grassland host plant species that differed in growth rate (fast or slow) and functional type (grasses or forbs). Each host plant species was grown on soils that were previously conditioned by the same twelve plant species (conditioning plants) individually. We measured herbivore performance and consumption of the host plants, to test the generality of soil legacy effects on plant-herbivore interactions.

We hypothesize that (1) the performance of a polyphagous insect herbivore feeding on different host plants will be affected by the conditioning of the soils in which their host plants grow, and that the effect can be explained by the traits of the plants that conditioned the soil. We hypothesize that: (2) growth of host plants will differ on soils conditioned by different plant species and this can be explained by traits (i.e. functional type and growth rate) of the conditioning plant. More specifically, we expect forbs and fast-growing plant species to have a negative soil-mediated effect on plants growing in the conditioned soil, and grasses and slow-growing plants to have a positive effect on plants growing in their conditioned soils and that this would also affect insect herbivores feeding on the plants. We investigate in detail whether (3) individual host plant species and a polyphagous insect herbivore feeding on these plant species show host plant-specific responses to soil. Lastly, we hypothesize that (4) herbivore performance will follow the pattern of feedback responses observed for the host plants, i.e., soils that have accumulated pathogens are likely to have a negative impact on plant vigour, and via this also on their herbivorous insects, as these plants may be of lower quality and show stronger induced cross-resistance.

Materials and methods

Plants

Twelve plant species were selected based on functional type (6 grasses and 6 forbs). Within each functional group, three of the species had high growth rates while the other three were slow growers (Supplementary Table S3.1). Briefly, thirty replicates of 24 common grassland plant species (12 grasses, 12 forbs) were grown in pots with field soil for 10 weeks. For ten weeks, each week, three replicates of each species were harvested (above- and belowground biomass), dried, and weighed. Based on these data, growth curves were fitted through the root and shoot biomass data according to Paine et al. (2012). Cumulative root and total biomass were derived from the models and the three highest and lowest ranking species within each functional type were selected (see Supplementary Table S3.1). Supplementary Figures S3.1c-d confirm that the growth rate selection was valid in the current study (indicated by significant main effects of growth rate on shoot and root biomass; Table 3.1).

Seeds of all species were surface-sterilized using 2% bleach solution and then rinsed with water. For germination, seeds were placed on sterile glass beads in a climate cabinet (light regime

16:8, L:D, day temperature 21°C, night temperature 16°C). After germination, the seedlings were stored at 4°C under the same light regime, for later use in experiments. Seeds were obtained from Cruydt-Hoeck (Nijberkoop, The Netherlands).

Insects

Eggs of the cabbage moth, *Mamestra brassicae* (Lepidoptera: Noctuidae) were obtained from the Department of Entomology at Wageningen University. The cabbage moth had been reared for many years on Brussel's Sprout, *Brassica oleracea* var. *gemmifera* cv. Cyrus. The larvae were originally collected from cabbage fields near the university.

Soil

Field soil was collected from a restoration grassland area 'De Mossel' (Natuurmonumenten, Ede, The Netherlands). Live soil was taken from the top 10 cm, the well-rooted layer containing most of the rhizosphere biota. For sterile soil, the 5-20 cm layer just below the dense root layer was collected and sterilized by γ-irradiation (Synergy Health, Ede, The Netherlands). Both soil types were first sieved to remove roots, stones and most macro-invertebrates (sieve mesh Ø 1.0 cm).

Soil Conditioning Phase

Sixty square one-Litre pots (11x11 cm) were filled with 1050 gram live field soil, for each species (12x60=720 pots total). One individual seedling was grown for 10 weeks in each pot. The first four days seedlings were covered with shade cloth to aid in their establishment. Germination of seeds in the soil and egg deposition by fungus gnats were prevented by adding a layer of coarse sand to the surface of the pots. Germinating seeds originating from the seedbank were weeded daily. Plants were watered three times per week. After 10 weeks, the plants and their roots were removed from the soil and the soil was used in the feedback phase.

Feedback Phase

Sixty individually conditioned pots per species were divided over five separate replicates. Each replicate thus contained all soil from twelve independently conditioned pots. The replicate soils were homogenized, and then mixed with sterilized field soil (one volume conditioned soil to

two volumes sterilized soil) to obtain a sufficient amount of soil and to minimize abiotic differences among the conditioned soils. This resulted in 60 mixed conditioned soils (5 replicates x 12 conditioning species). Each of the 60 soil mixes was divided over 12 pots (9x9 cm, 650 g soil), each receiving an individual seedling from one of the 12 host plant species (12 conditioning species x 12 host plant species x 5 replicates = 720 pots).

After 4 weeks of growth, all 720 pots were caged with a plastic tube made of transparent plastic with insect mesh fitted on top (9 cm diameter, 30 cm height). In each cage, a freshly hatched *M. brassicae* caterpillar was introduced. After 7 days of feeding the caterpillars were collected and weighed to measure their performance. Moreover, for each plant the total area of leaf consumption by caterpillars was assessed using a reference area of 5 mm x 5 mm and counting the number of times the reference area fitted within the consumed area on the plant (as in Heinen et al., 2018b). All plants were then clipped and fresh shoot biomass was recorded. All shoot samples were flash-frozen in liquid nitrogen. Roots from each pot were washed and belowground biomass was oven-dried at 70°C and weighed.

Data analysis

In this experiment, we measured herbivore and plant responses to soils by four response variables; caterpillar biomass, leaf consumption by caterpillars, host plant shoot biomass and host plant root biomass. Plant and insect responses were analysed in two separate ways: via overall analyses across plant species and via species-specific analyses.

Effects of conditioning and host plant traits across plant species

In the overall analysis, we tested (1) whether traits of conditioning plants and host plants (functional group, growth type) affect caterpillar biomass, caterpillar consumption, shoot and root biomass, using linear mixed models with 'Conditioning plant functional type' (C_f, grass/forb), 'Conditioning plant growth rate' (C_g, fast/slow), 'Host plant functional type' (H_f, grass/forb), 'Host plant growth rate' (H_g, fast/slow) and all interactions as fixed effects, and using 'Conditioning plant' and 'Host plant' as random effects.

Host species-specific effects of soil conditioning, soil origin and traits

In the species-specific analyses all plant and insect responses were analysed per host plant species, using subsets of the total dataset. In separate one-way ANOVAs, we tested (2) the

effect of 'Soil id' (12 species) and (3) 'Soil origin' (conspecific or heterospecific soil) on all response variables; leaf consumption by caterpillars, caterpillar biomass, shoot and root biomass. Additionally, in linear mixed models, we analysed (4) the effects of 'Conditioning plant functional group', 'Conditioning plant growth type' and their interaction as fixed effects, with 'Conditioning plant species' (12 plant species) as a random effect, on all response variables. Post-hoc Tukey tests were performed when the effects of 'Soil id' were significant.

Soil feedbacks on plants and insects

We determined feedback responses for each individual sample, relative to the average of that species. For instance, using shoot biomass we calculate feedbacks by:

$$\text{Soil effect}_{\text{individual}} = (\text{observed shoot biomass}_{\text{species X}} - \text{mean shoot biomass}_{\text{species X}})/\text{mean shoot biomass}_{\text{species X}}$$

This calculated feedback tells us whether the shoot biomass of an individual plant of species 'X' responds positively or negatively to a soil, relative to the overall mean of that species 'X'. We calculated this effect for leaf consumption by caterpillars, caterpillar biomass, and for shoot biomass. Then (5), using oneway-ANOVAs, we analysed whether 'Soil id' (i.e. the conditioning plant species) affected the feedback responses for each parameter. Post-hoc Tukey tests were performed when the effects of 'Soil id' were significant.

Relationships between plant-soil feedbacks and insect performance

Lastly (6), we explored relationships between individual and averaged species soil legacy effects on the leaf consumption by the associated caterpillar, caterpillar biomass and plant shoot biomass, using linear regression.

All analyses were performed in R Studio version 1.1.419 (RStudio, Inc., Boston, USA) using R version 3.3.1 (R Development Core team, 2008). General Linear Mixed Models were performed using the R package 'nlme' (Pinheiro et al., 2018). Post-hoc Tukey tests were performed using the R package 'emmeans' (Lenth et al., 2019).

Results

Effects of conditioning plant and host plant traits on herbivory

Leaf consumption by caterpillars was higher on plants that were grown in soils conditioned by slow-growing forbs, than on plants grown in soils conditioned by fast-growing forbs, whereas for soils conditioned by grasses, the effect of growth type was opposite, i.e., leaf consumption tended to be lower on plants growing in soils conditioned by fast-growing than on soils conditioned by slow-growing grasses (significant C_f x C_g interaction, Table 3.1, Figure 3.1a). Leaf consumption across host plant species did not differ significantly between host plant categories (Supplementary Figure S3.1a, Table 3.1).

Caterpillar biomass responded to the conditioning plant treatments in a pattern that was similar to the one observed for standardized leaf consumption (significant C_f x C_g interaction, Table 3.1, Figure 3.1b). Caterpillar biomass differed between host plant functional types, with caterpillar biomass, on average, being slightly higher on grass hosts than on forb hosts (Supplementary Figure S3.1b, Table 3.1). Caterpillar biomass also depended on host plant growth rate, and was higher on slow-growing host plants than on fast-growing host plants (Supplementary Figure S3.1b, Table 3.1).

Shoot and root biomass of the host plants were affected by conditioning plant functional type and growth rate. Shoot and root biomass were higher on soils conditioned by grasses and slow-growing plants, than on soils conditioned by forbs or fast-growing plants, respectively (Table 3.1). However, various interactions were observed. Firstly, both shoot and root biomass were higher when plants were grown on soils conditioned by slow-growing forbs, than when they were grown on soils conditioned by fast-growing forbs, whereas on soils conditioned by grasses, the effect of growth rate was weaker, but opposite (Figure 3.2a (shoot) and 3.2d (root) (significant C_f x C_g interactions, Table 3.1)). Moreover, the effects of conditioning plant functional type and growth rate on shoot and root biomass

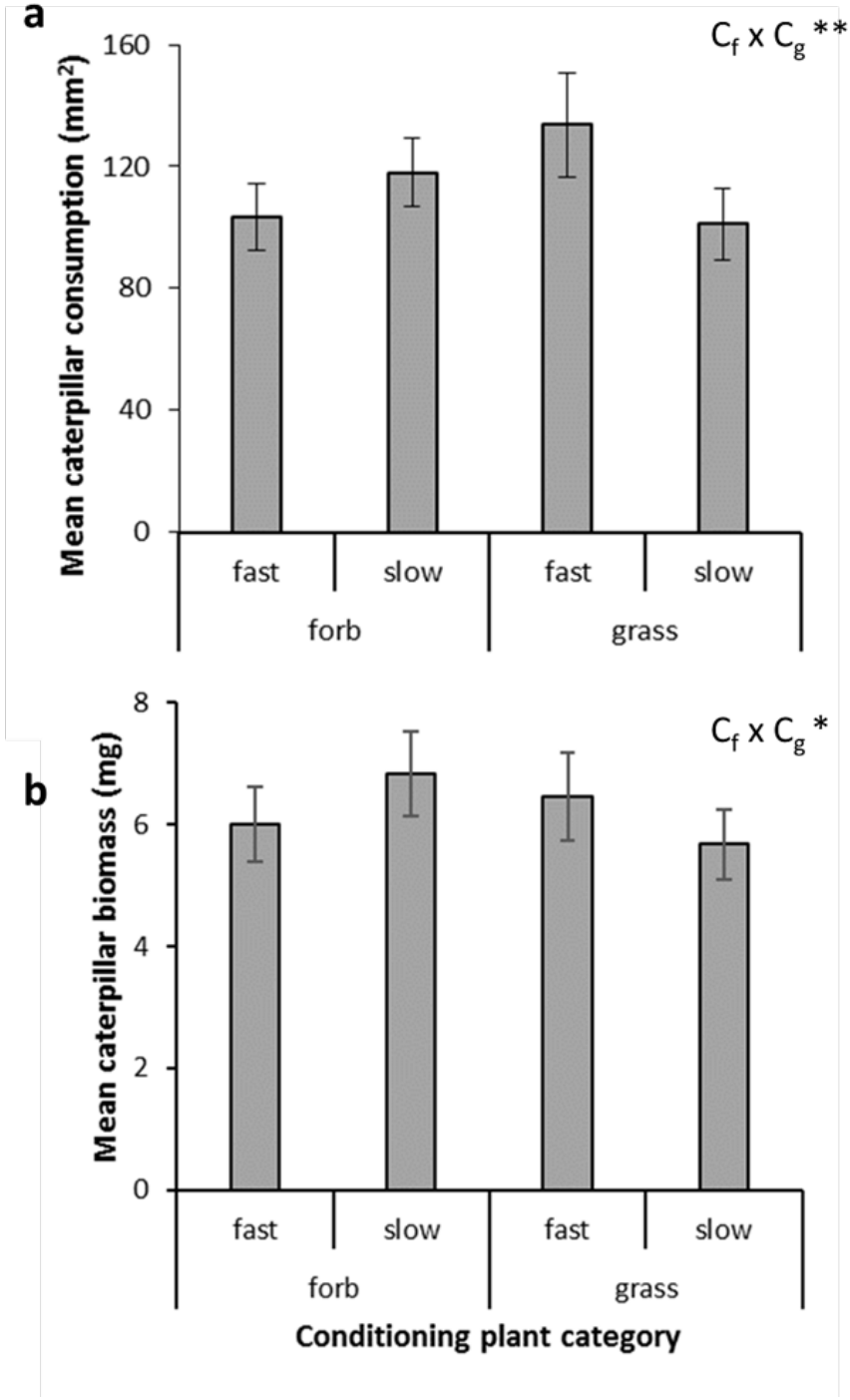


Figure 3.1: The effects of traits of the conditioning plants on a) consumption by caterpillars, and b) caterpillar biomass. The four categories on the x-axis represent conditioning plant categories, consisting of combinations of fast- and slow-growing forbs and grasses. Error bars represent standard errors, which were calculated on values that were averaged across 5 replicates (12 x 12 species = 144 combinations), leading to n=36 per bar. Asterisks represent significant results. Statistical output of the full linear mixed model is presented in Table 1.

Table 3.1: Statistical output a general linear mixed model testing the effects of 'Conditioning plant functional type' (grass or forb), 'Conditioning plant growth rate' (fast or slow growth), 'Host plant functional type' (grass or forb), 'Host plant growth rate' (fast or slow growth) and all interactions on leaf consumption by caterpillars, caterpillar biomass, shoot and root biomass. Linear mixed models were performed on the full dataset, including all plant species (and conditioning and host plant species included as random effects). Presented are degrees of freedom, F-statistics and p-values. Significant effects ($p<0.05$) are highlighted in bold.

	Leaf consumption [1]			Caterpillar biomass			Shoot biomass			Root biomass		
	df1,df2	F	p	df1,df2	F	p	df1,df2	F	p	df1,df2	F	P
Conditioning plant functional type (C_f)	1,630	0.4	0.546	1,619	0.1	0.702	**1,689**	**41.5**	**<0.001**	**1,688**	**27.7**	**<0.001**
Conditioning plant growth rate (C_g)	1,630	1.0	0.328	1,619	3.7	0.054	**1,689**	**15.0**	**<0.001**	**1,688**	**7.5**	**0.006**
Host plant functional type (H_f)	1,8	0.0	0.846	**1,8**	**5.4**	**0.048**	**1,8**	**10.2**	**0.013**	1,8	0.0	0.865
Host plant growth rate (H_g)	1,8	0.2	0.690	**1,8**	**45.2**	**<0.001**	**1,8**	**19.3**	**0.002**	**1,8**	**13.6**	**0.006**
C_f x C_g	**1,630**	**5.8**	**0.016**	**1,619**	**4.5**	**0.034**	**1,689**	**14.4**	**<0.001**	**1,688**	**19.5**	**<0.001**
C_f x H_f	1,630	1.4	0.231	1,619	0.6	0.458	**1,689**	**24.3**	**<0.001**	**1,688**	**16.2**	**<0.001**
C_g x H_f	1,630	0.1	0.707	1,619	1.4	0.238	**1,689**	**7.4**	**0.006**	**1,688**	**8.1**	**0.005**
C_f x H_g	1,630	0.0	0.871	1,619	0.1	0.790	1,689	3.3	0.072	1,688	0.6	0.442
C_g x H_g	1,630	0.0	0.941	1,619	1.4	0.239	1,689	2.5	0.111	1,688	0.1	0.806
H_f x H_g	1,8	1.8	0.216	1,8	5.1	0.054	1,8	0.0	0.974	1,8	0.6	0.447
C_f x C_g x H_f	1,630	0.1	0.808	1,619	1.5	0.222	1,689	1.7	0.198	1,688	2.6	0.110
C_f x C_g x H_g	1,630	0.4	0.541	1,619	0.2	0.647	1,689	1.2	0.276	1,688	0.4	0.544
C_f x H_f x H_g	1,630	0.0	0.894	1,619	1.4	0.237	1,689	0.3	0.594	1,688	1.0	0.317
C_g x H_f x H_g	1,630	3.5	0.063	1,619	1.15	0.284	1,689	0.1	0.797	1,688	0.6	0.449
C_f x C_g x H_f x H_g	1,630	0.6	0.423	1,619	0.3	0.577	1,689	0.0	0.853	1,688	2.0	0.156

1) Data were sqrt(x+1) transformed to obtain normality of residuals

were both dependent of host plant functional group (significant C_f x H_f and C_g x H_f interactions, Table 3.1). Specifically, host forbs grew smaller on forb soils than on grass soils, whereas for host grasses, growth was not dependent on functional type of the conditioning plant (Figures 3.2b (shoot) and 3.2e (root)). Similarly, growth rate of the conditioning plant strongly affected host forbs, but not grasses. Specifically, forb shoot and root biomass, on average, were lower on soils conditioned by fast-growing plants than on soils conditioned by slow-growing plants, whereas for host plants that were grasses, this was not the case (Figures 3.2c (shoot) and 3.2f (root)).

Host species-specific effects of soil conditioning, soil origin and conditioning plant traits

For three out of twelve host plant species, leaf consumption by caterpillars was significantly affected by soil conditioning. Leaf consumption on *Taraxacum officinale* was significantly affected by the species that conditioned the soil. Specifically, leaf consumption was low when host plants grew in conspecific soil compared to heterospecific soils (Supplementary Table S3.2, Figure 3.3a). Further, both for *Holcus lanatus* and *Briza media*, leaf consumption was higher on soils that were conditioned by slow-growing than fast-growing forbs, whereas on grass soils, the effect of growth type was opposite (significant C_f x C_g interaction, Supplementary Table S3.2, Figures 3.3b,c). All host plant species-specific responses to soils are visualized in Supplementary Figures S3.2a-l and summary statistics presented in Supplementary Table S3.2.

For two out of twelve host plant species, *H. lanatus* and *B. media*, both grasses, caterpillar biomass was also affected by traits of the conditioning plants. On these host plants, caterpillars grew larger when the plants were grown on soils that were conditioned by slow-growing forbs, than on soils conditioned by fast-growing forbs, whereas on grass soils, the effect of growth type was opposite (significant C_f x C_g interaction, Supplementary Table S3.2, Figures 3.4a-b). Further, on *Festuca ovina*, caterpillar biomass was affected by soil origin. Specifically, caterpillars had lower biomass on these host plants when they were grown on conspecific soils than on heterospecific soils (Supplementary Table S2, Figure 4c). All host plant

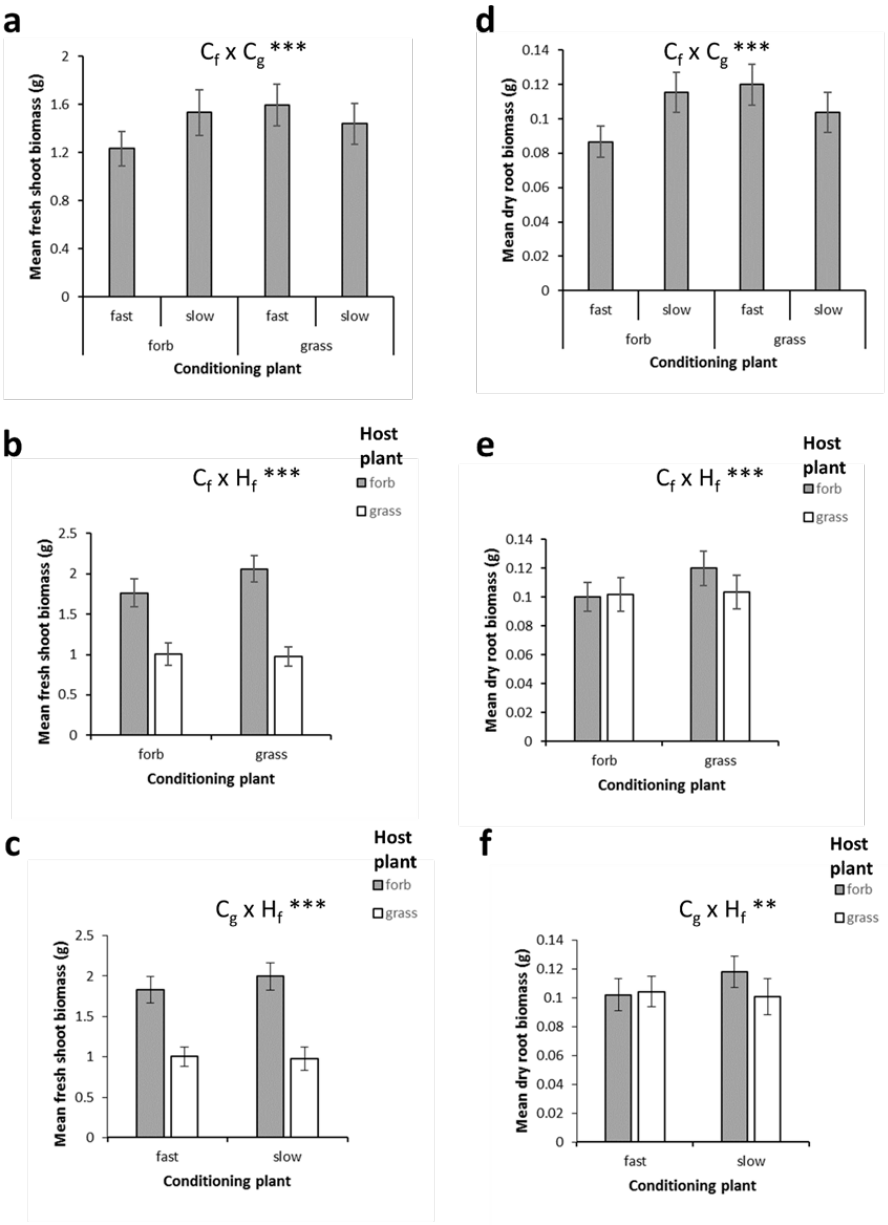


Figure 3.2: The effects of traits of the conditioning plants on plant biomass. The three panels on the left (a-c) represent shoot biomass, and the three panels on the right (d-f) represent root biomass. Panels represent the three significant two-way interactions that were observed for shoot and root biomass. Note that all panels are based on the same dataset and that the different panels are shown to clearly visualize the interactive effects. The full model is presented in Supplementary Figure 1c (shoot) and 1d (root). Error bars represent standard errors, which were calculated on values that were averaged across 5 replicates (12 x 12 species = 144 combinations), leading to n=36 per bar. Asterisks represent significant results. Statistical output of the full linear mixed model is presented in Table 1.

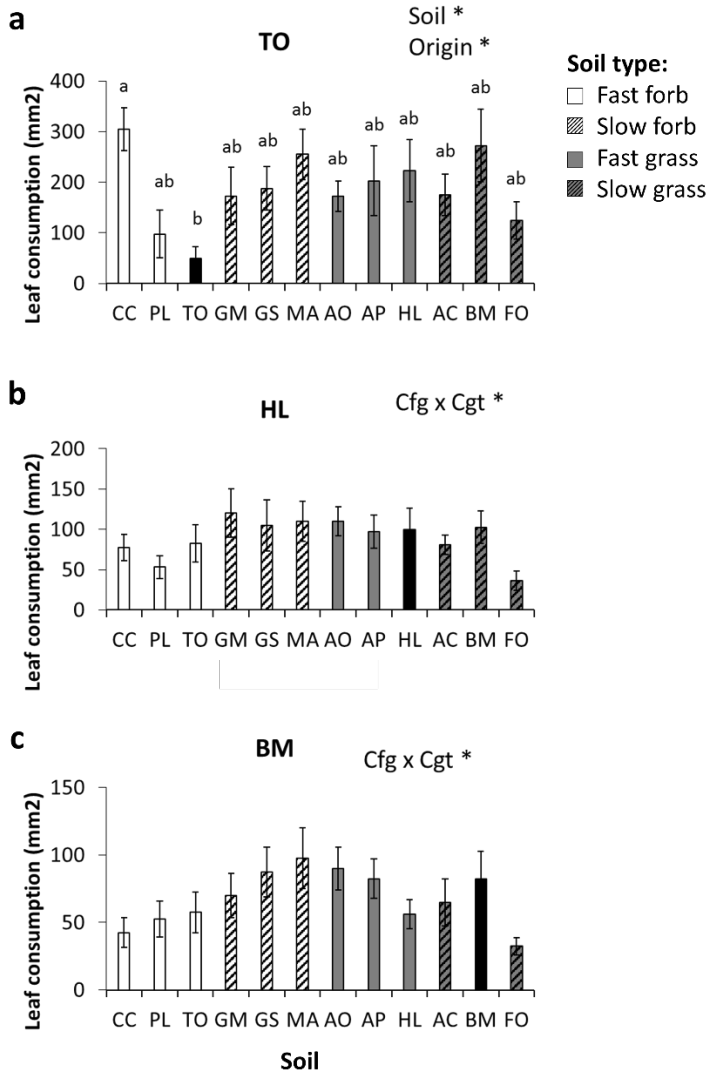


Figure 3.3: The effect of soil on average leaf consumption by *Mamestra brassicae* on a) *Taraxacum officinale*, b) *Holcus lanatus*, and c) *Briza media*. White bars represent soils conditioned by forbs, grey bars represent soils conditioned by grasses. Open bars represent soils conditioned by fast-growing species and dashed bars represent soils conditioned by slow-growing species. Black bars represent conspecific soils. Error bars represent standard errors calculated across 5 replicates. Statistically significant effects are indicated by asterisks. Presented are only significant responses and visualization for all species is presented in Supplementary Figure S2. Full summary statistics are presented in Supplementary Table S2. Different letters above the bars indicate significantly different means, as tested with post-hoc Tukey tests. Abbreviations are as follows for fast-growing forbs CC= *Crepis capillaris*, PL= *Plantago lanceolata*, TO= *Taraxacum officinale*, for slow-growing forbs GM= *Geranium molle*, GS= *Gnaphalium sylvaticum*, MA= *Myosotis arvensis*, for fast-growing grasses AO= *Anthoxanthum odoratum*, AP= *Alopecurus pratensis*, HL= *Holcus lanatus*, and for slow-growing grasses AC= *Agrostis capillaris*, BM= *Briza media* and FO= *Festuca ovina*.

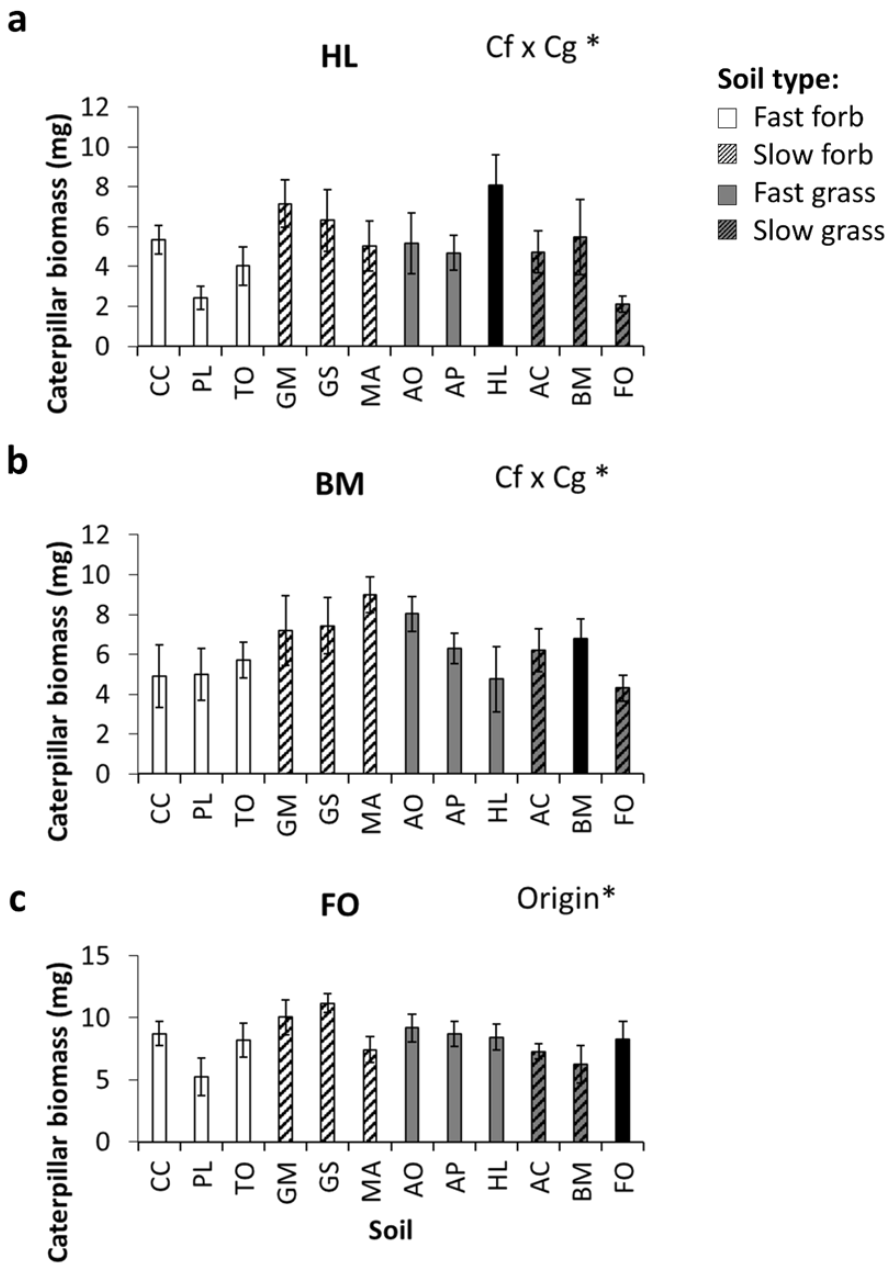


Figure 3.4: The effect of soil on biomass of *Mamestra brassicae* on a) *Holcus lanatus*, b) *Briza media*, and c) *Festuca ovina*. White bars represent soils conditioned by forbs, grey bars represent soils conditioned by grasses. Open bars represent soils conditioned by fast-growing species and dashed bars represent soils conditioned by slow-growing species. Black bars represent conspecific soils. Error bars represent standard errors, calculated across 5 replicates. Statistically significant effects are indicated by asterisks. Presented are only significant responses and visualization for all species is presented in Supplementary Figure S3. Full summary statistics are presented in Supplementary Table S2. Different letters above the bars indicate significantly different means, as tested with post-hoc Tukey tests. Abbreviations are as follows for fast-growing forbs CC= *Crepis capillaris*, PL= *Plantago lanceolata*, TO= *Taraxacum officinale*, for slow-growing forbs GM= *Geranium molle*, GS= *Gnaphalium sylvaticum*, MA= *Myosotis arvensis*, for fast-growing grasses AO= *Anthoxanthum odoratum*, AP= *Alopecurus pratensis*, HL= *Holcus lanatus*, and for slow-growing grasses AC= *Agrostis capillaris*, BM= *Briza media* and FO= *Festuca ovina*.

species-specific responses to soils are visualized in Supplementary Figure S3.3a-l and summary statistics presented in Supplementary Table S3.2.

Soil conditioning affected shoot biomass of four out of twelve host plant species, three of which were forbs. Specifically, shoot biomass of *T. officinale* was affected by the identity of the species that conditioned the soil and was larger on grass soils than on forb soils (Supplementary Table S3.2, Supplementary Figure S3.4c). Shoot biomass of *Gnaphalium sylvaticum* was also affected by the identity of the species that conditioned the soil, and by soil origin. Shoot biomass was much larger on heterospecific soils than on conspecific soil and also larger on grass soils than on forb soils (Supplementary Table S3.2, Supplementary Figure S3.4e). Shoot biomass of *Myosotis arvensis* was affected by soil identity and larger on grass than on forb soils (Supplementary Table S3.2, Supplementary Figure S3.4f). Shoot biomass of *F. ovina* was larger on soils conditioned by slow-growing forbs, compared to fast-growing forbs, whereas on grass soils, the effect was opposite (significant C_f x C_g interaction, Supplementary Table S3.2, Supplementary Figure S3.4l). All host plant species-specific responses to soils are visualized in Supplementary Figures S3.4a-l and summary statistics presented in Supplementary Table 3.2.

Soil conditioning affected root biomass of nine out of twelve host plant species. Root biomass of *Crepis capillaris* (Supplementary Figure S3.5a), *T. officinale* (Supplementary Figure S3.5c), *Geranium molle* (Supplementary Figure S5d), *G. sylvaticum* (Supplementary Figure S3.5e), *M. arvensis* (Supplementary Figure S3.5f), and *Anthoxanthum odoratum* (Supplementary Figure S3.5g), was affected by soil identity (Supplementary Table S3.2). Root biomass of *Plantago lanceolata* (Supplementary Figure S3.5b) and *M. arvensis* (Supplementary Figure S3.5f) was larger on grass- than on forb-conditioned soils (Cf, Supplementary Table S3.2). Root biomass of *B. media* (Supplementary Figure S3.5k) was larger on soils conditioned by slow-growing forbs than by fast-growing forbs, whereas on grass soils, the effect was opposite (significant C_f x C_g interaction, Supplementary Table S3.2). Root biomass of *G. sylvaticum* (Supplementary Figure S3.5e) was smaller on conspecific soils than on heterospecific soils, whereas roots of *Alopecurus pratensis* (Supplementary Figure S3.5h) were larger on conspecific than on heterospecific soils (Supplementary Table S3.2). All species-specific responses to soils are visualized in Supplementary Figures S3.5a-l and summary statistics presented in Supplementary Table S3.2.

Soil feedbacks on plants and insects

Conditioning plant species did not significantly differ in their soil-feedback effects on leaf consumption by caterpillars on the twelve host plant species (Soil id: $F_{11,631}$= 1.5; p=0.119, Figure 3.5a).

Conditioning plants significantly differed in their soil-feedback effects on the biomass of caterpillars feeding on the twelve different responding host plant species, and on average, conditioning negatively affected caterpillar biomass (Soil id: $F_{11,620}$= 1.9; p=0.034, Figure 3.5b). Soils of *P. lanceolata*, *T. officinale* and *F. ovina* caused caterpillars to be much smaller than average across host plant species, whereas soils of *A. pratensis*, *G. molle* and *M. arvensis* had rather positive effects on caterpillar biomass across host plant species, although post-hoc testing did not indicate significant differences between soils (Figure 3.5b).

Conditioning plants also significantly differed in their soil-feedback effects on the shoot biomass across twelve host plant species (Soil id: $F_{11,690}$= 9.1; p<0.001, Figure 3.5c). Soils conditioned by *P. lanceolata*, *G. sylvaticum* and *T. officinale* caused plants to have a lower than average shoot biomass than other conditioning plants, whereas on soils conditioned by *H. lanatus* host plants tended to have a higher than average shoot biomass (Figure 3.5c).

The individual soil feedback effects on leaf consumption by caterpillars, caterpillar biomass and shoot biomass - on which the averaged values that are reported above are based - are presented separately for each responding host plant species as background information in Supplementary Figure S3.6. These will not be discussed in detail, but provide an indicator of the importance of soils in plant-insect interactions across different species.

Briefly, on all host plant species except *Agrostis capillaris* and *A. odoratum*, soil feedback effects on leaf consumption significantly differed from zero on at least one soil, indicated by the 95% confidence intervals not crossing the zero line (Supplementary Figure S3.6; left column).

On all twelve tested host plant species, soil feedback effects on caterpillar biomass significantly differed from zero on at least one soil, indicated by the 95% confidence interval not crossing the zero line (Supplementary Figure S3.6; middle column).

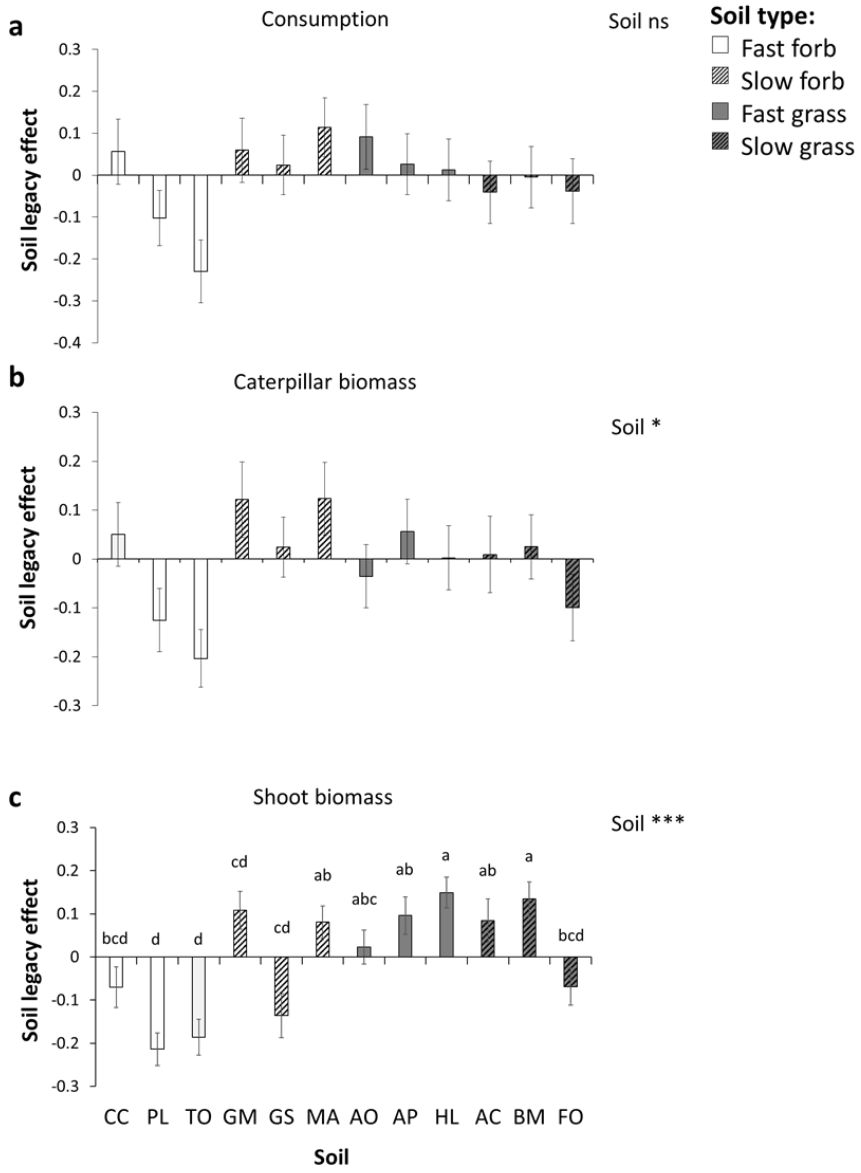


Figure 3.5: Averaged soil-feedback effects of conditioning plants on a) leaf consumption by caterpillars, b) caterpillar biomass and c) host plant shoot biomass. Individual soil effects were calculated for each sample by: *(observed shoot biomass$_{species\ X}$ - mean shoot biomass$_{species\ X}$)/ mean shoot biomass$_{species\ X.}$* Positive values represent positive soil effects and negative values represent negative effects, standardized per host plant species. Presented are the averaged soil effects of a conditioning plant species on twelve plant species or their associated herbivore. White bars represent forbs, grey bars represent grasses. Open bars represent fast-growing species and dashed bars represent slow-growing species. Black bars represent conspecific soils. Error bars represent standard errors (calculated across12 plant species with 5 replicates, n=60 per bar). Statistically significant effects of soils are indicated in individual graphs. Different letters above the bars indicate significantly different means, as tested with post-hoc Tukey tests. Abbreviations are as follows for fast-growing forbs CC= *Crepis capillaris*, PL= *Plantago lanceolata*, TO= *Taraxacum officinale*, for slow-growing forbs GM= *Geranium molle*, GS= *Gnaphalium sylvaticum*, MA= *Myosotis arvensis*, for fast-growing grasses AO= *Anthoxanthum odoratum*, AP= *Alopecurus pratensis*, HL= *Holcus lanatus*, and for slow-growing grasses AC= *Agrostis capillaris*, BM= *Briza media* and FO= *Festuca ovina*.

In all host plant species except *P. lanceolata*, soil legacy effects on shoot biomass significantly differed from zero on at least one soil, indicated by the 95% confidence interval not crossing the zero line (Supplementary Figure S3.6; right column).

Relationships between plant-soil feedbacks and insect performance

There was a positive relationship between effects of individual conditioning plants, via the soil, on the shoot biomass of host plants growing in their conditioned soil, and their effects on the biomass and leaf consumption of the caterpillars feeding on these host plants (leaf consumption: R^2= 0.018; $F_{1,652}$=12.2; p<0.001; Figure 3.6a, caterpillar biomass: R^2= 0.031; $F_{1,641}$=20.8; p<0.001; Figure 3.6b). The individual soil-mediated effects on caterpillar biomass were also positively correlated with the individual soil-mediated effects on leaf consumption (R^2= 0.40; $F_{1,640}$=421.1; p<0.001, Figure 3.6c). Moreover, there was a strong positive correlation between the average effect of a conditioning plant species, via the soil, on the shoot biomass of all other species and the effect of that same conditioning plant species on the biomass of caterpillars and their leaf consumption on these plants (leaf consumption: R^2= 0.35; $F_{1,10}$=5.3; p=0.044; Figure 3.6d, caterpillar biomass: R^2= 0.49; $F_{1,10}$=9.5; p=0.012; Figure 3.6e). Finally, the average soil-mediated effects of a conditioning plant species on caterpillar biomass and on leaf consumption by caterpillars were strongly positively correlated (R^2= 0.73; $F_{1,10}$=27.1; p<0.001, Figure 3.6f).

Discussion

The importance of soil legacy effects on plants for associated aboveground herbivores have recently been discussed (e.g. Wurst & Ohgushi, 2015; Kaplan, Pineda & Bezemer, 2018). In this study, we set out to test whether plant-mediated soil legacy effects on insect and plant performance are a general phenomenon across a range of plant species and can be linked. Our study with twelve host plant species growing in twelve conditioned soils shows that there are strong patterns in how plant-mediated soil legacies affect consumption by and biomass of insect herbivores feeding on host plants, as well as how these soil legacies affect those host plants. This pattern is to a large extent determined by an interaction between the functional group and growth type of the conditioning plant species. Despite these overall patterns, there is also considerable variation in how different host plant species respond to the different conditioned soils, and this is also true for the aboveground insect herbivore feeding on them.

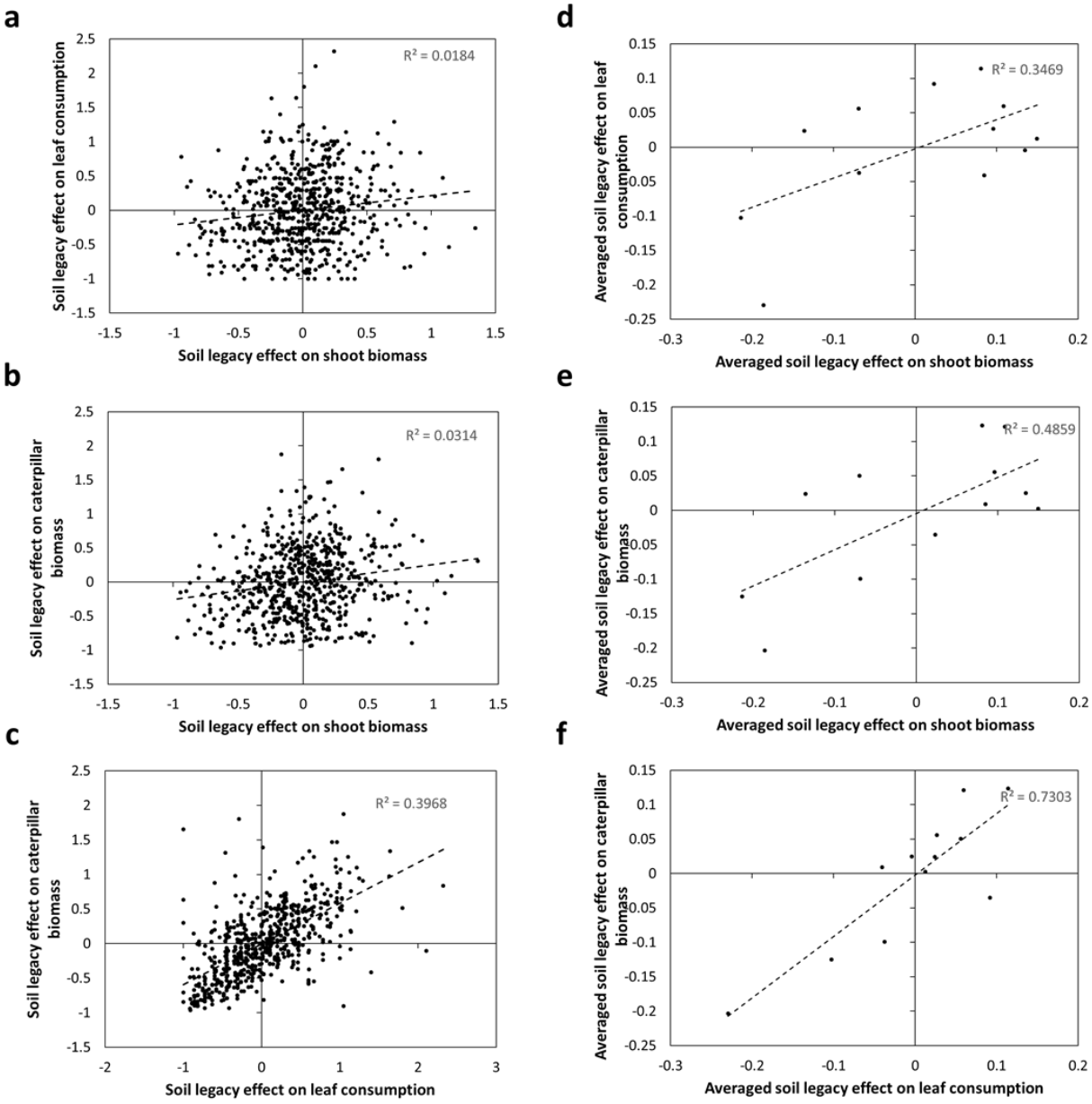


Figure 3.6: Relationships between a) the *individual* soil effect on shoot biomass of host plants and the *individual* soil effect on leaf consumption by caterpillars on those host plants, b) the *individual* soil effect on shoot biomass of host plants and the *individual* soil effect on biomass of caterpillars on those host plants, c) the *individual* soil effect on caterpillars on host plants and the *individual* soil effect on leaf consumption by caterpillars on host plants, d) the *averaged* conditioning plant soil effect on shoot biomass of host plants and the *averaged* conditioning plant soil effect on leaf consumption by caterpillars on those host plants, e) the *averaged* conditioning plant soil effect on shoot biomass of host plants and the *averaged* conditioning plant soil effect on growth of caterpillars on those host plants, and f) the *averaged* conditioning plant soil effect on caterpillars on host plants and the *averaged* conditioning plant soil effect on leaf consumption by caterpillars on host plants. In addition, we find that there is a strong similarity between plants and insects in their overall response to conditioned soils.

For insect biomass and leaf consumption, we find that responses to conditioned soils are dependent on functional type and growth rate of the conditioning plant species. Specifically, on plants growing in soils conditioned by fast-growing forbs insect biomass and leaf consumption are lower than on plants growing in soils conditioned by slow-growing forbs. On soils conditioned by grasses, insect growth rates had a weaker, but opposite effect. Interestingly, the effects of soil do not depend on the host plant, as is indicated by the absence of interactive effects between host and conditioning plants. This is an important finding, as it suggests that soil legacy effects on insects are rather consistent across our host plant categories. Previous studies have also shown that the functional group which the conditioning plant belongs to can be important for insect performance (Kos, et al., 2015; Heinen et al., 2018b). However, in a previous study with communities of response plants rather than individual plants but with the same set of plant species and the same insect herbivore, interactions between conditioning plant functional type and growth rate were not observed (Heinen et al., 2018b), suggesting that soil legacy effects may affect insects on individually grown plants differently than those that feed on plant communities, e.g. due to selective feeding from different plant species.

Similar to what was observed for insect herbivores, we also observed that fast-growing forbs created soil legacies that negatively affected later growing plants, whereas slow-growing forbs create soils that have a more positive effect. On soils conditioned by grasses, the effect of growth type was also opposite. On grass soils, plants tend to accumulate higher than average biomass. This finding is largely in line with previous studies that showed effects of plant functional group of the conditioning plants, via the soil, on plant growth (Petermann et al., 2008; De Kroon et al., 2012; Wubs et al., 2016). However, we also observed that these effects are mostly driven by host plants that are forbs, which respond quite strongly to both growth rate and functional types of conditioning plants, whereas host plants that are grasses did not show such a response. This is also consistent with earlier observations in our group using the same model species and soils (Heinen et al., 2018; Zhu et al., 2018; Heinen et al., in preparation a). This indicates that plant traits are important drivers of soil legacy effects such as plant-soil feedbacks in forbs, but that we cannot extrapolate these effects to plants that belong to other functional types.

Generally, plants and associated insects followed similar patterns in their response to soils, and plant-insect interactions could thus be predicted by the soil effects on host plants (i.e. plant-soil feedback). Indeed, we did find a positive relationship between the soil effect on individual plants and the effect on their associated herbivore in this study. If plants grew more vigorously in a specific soil, the insects feeding on plants growing in that specific soil also showed a positive growth response. These findings are in line with the vigour hypothesis (Price, 1991). Obviously, there may be other aspects than plant vigour alone that may explain our findings. Negative plant-soil feedbacks are often hypothesized to be due to accumulation of pathogens. Pathogens can negatively affect plant biomass (Berendsen, Pieterse & Bakker, 2012), but plant biomass was not a limiting factor for insect performance in this study. It has also been shown that soil microbiomes can alter plant chemistry (Kostenko et al., 2012; Badri et al., 2013; Zhu et al., 2018). Moreover, pathogens, as well as other plant-associated organisms are known to invoke resistance to aboveground herbivores, via priming of defences or inducing systemic resistance (Pieterse et al., 1998; Pozo & Azcon-Aguilar, 2007). Hence, a likely explanation for the observed soil legacy effects on insect herbivores is that soils affected plant chemistry and that these changes may have affected insect feeding behaviour and performance. Future work on the effects of soil legacies on plant chemistry should focus on the mechanisms of these above-belowground interactions (Zhu et al., 2018).

It remains difficult to explain mechanistically in what ways drivers (i.e. soil conditioning by plants that differ in traits) differ in how they condition the soil. Soil legacy effects reflect the effects of previous plant growth on soil biota and abiotic soil parameters. Each plant species interacts with different organisms in the soil, exuding different metabolites and thus creating different conditions (Phillipot et al., 2013). As such, each species leaves a distinct biotic pattern in the soil. We have previously reported that the composition of soil bacteria and fungi was strongly affected by conditioning plant species, as well as the functional type that they belong to, for the same set of plant species as used here, grown under very similar conditions and in similar soils (Heinen et al., 2018b). Thus, we have a broad idea of what microorganisms are present in the soil. However, for a large part, we do not have species names or the functional roles for many of the operational taxonomic units. Understanding the role of thousands of individual species of soil microorganisms that collectively shape plant-insect interactions is an immense challenge and requires further attention in ecology. As our understanding of functions

of belowground organisms is rapidly expanding with advancement of high throughput sequencing technologies, the 'black box' of soil is gradually opened.

There is an abundance of ecological theories on the role of individual soil biota on aboveground plant-insect interactions (e.g. arbuscular mycorrhizal fungi or plant-growth-promoting rhizobacteria), which has been reviewed in various reviews (e.g. Bezemer and Van Dam 2005; Pineda et al., 2010; 2017; Koricheva et al., 2009; Johnson et al., 2012; Wondafrash et al., 2013; Heinen et al., 2018a). However, a common theme in these reviews is the difficulty of testing the role of individual soil organisms in natural (soil) communities in shaping plant-insect interactions. Future studies should take selective approaches to create different soil communities with different functions, for instance through sieving approaches (Johnson et al., 2001; 2002; Wagg et al., 2011; 2014; Wang et al., 2019), or via assembly of simplified artificial communities (Bai et al., 2015). When the presence of mutualists, pathogens or decomposers (Van der Putten et al., 2016), as well as their relative abundance, can be experimentally manipulated, this will allow us to empirically test standing hypotheses in more natural communities on a range of plant and insect species.

Lastly, in our study, insect biomass was not only affected by the functional group and growth type of the plant species that conditioned the soils on which their host plants were growing, but it was also strongly affected by the functional group and growth type of the host plants themselves. This is hardly surprising, as plant traits usually have a large impact on caterpillar growth and feeding. However, despite the fact that the biomass strongly differed between caterpillars feeding on different host plant species, the amount of leaf area that they consumed from these different host species was quite similar, indicating that these host plants were of different quality. What is even more surprising is that insect biomass was twice as high on slow-growing plant species than on fast-growing plant species, and this pattern was true in grasses and forbs. This is not what ecological theory predicts, as fast-growing plants are assumed to be less well-defended hosts than slow-growing plants that adopt a more conservative nutrient use strategy coinciding with better protection of produced plant tissue (Coley et al., 1985; Herms & Mattson, 1992). Perhaps slow-growing host plant species invest in higher quality tissues that for instance have higher concentrations of leaf nitrogen, which could have driven this effect. Moreover, we observed that insect biomass was 23% higher on grass host plants than on forb hosts. *Mamestra brassicae*, as the name implies, is mostly studied on brassicaceous host plants,

but has also been recorded on a range of other forb species (e.g. Rojas et al., 2000). We show here that they may alternatively accept grasses as hosts and perform well on them.

In conclusion, our study shows that plant-mediated soil legacies can play an important role in shaping plant-insect interactions. Soil legacy effects are mediated by growth rate of the conditioning plants, but also strongly depend on plant functional type of the conditioning plants. Our results also show that the effects of soils on plant growth and insect performance are positively linked. We argue that when studying insect performance, especially in natural soils, the role that soil communities can play in shaping plant-insect interactions should not be overlooked.

Plant functional group and growth rate interactively shape soil legacy effects on individual plant-insect interactions

Supplementary information Chapter 3

Supplementary Table S3.1: Overview of the species used in the experiment and their functional type (grass or forb) and growth rate (fast or slow-growing). Selection of species was based on cumulative total biomass and cumulative root biomass (over ten weeks), which were measured in soil from the same area, in the same greenhouse conditions as the current study. Parameters presented here were derived from growth curve models that were fit in the growth data (see Methods).

Plant species	Label	Functional type	Growth rate	Cumulative total biomass (g)	Cumulative root biomass (g)
Crepis capillaris	CC	Forb	Fast	125.53	71.26
Plantago lanceolata	PL	Forb	Fast	120.75	60.89
Taraxacum officinale	TO	Forb	Fast	115.62	84.26
Geranium molle	GM	Forb	Slow	101.57	39.59
Gnaphalium sylvaticum	GS	Forb	Slow	58.58	19.76
Myosotis arvensis	MA	Forb	Slow	82.60	35.53
Anthoxanthum odoratum	AO	Grass	Fast	96.54	49.78
Alopecurus pratense	AP	Grass	Fast	139.84	71.43
Holcus lanatus	HL	Grass	Fast	122.96	71.67
Agrostis capillaris	AC	Grass	Slow	62.55	29.87
Briza media	BM	Grass	Slow	57.59	29.26
Festuca ovina	FO	Grass	Slow	60.64	27.18

Supplementary Table S3.2: Statistical output of two one-way ANOVAs testing the effects of 1) soil identity ('soil id' 12 conditioning plant species), 2) Origin' (conspecific or heterospecific soil), and 3) a general linear mixed model testing the effects of 'Conditioning plant functional group'(grass or forb) and 'Conditioning plant growth type' (fast or slow growth) and their interaction on absolute values of leaf consumption by caterpillars, caterpillar biomass, shoot and root biomass. Linear models were performed for each host plant species individually and are presented as such. Presented are degrees of freedom, F-statistics and p-values. Significant effects ($p<0.05$) are highlighted in **bold.**

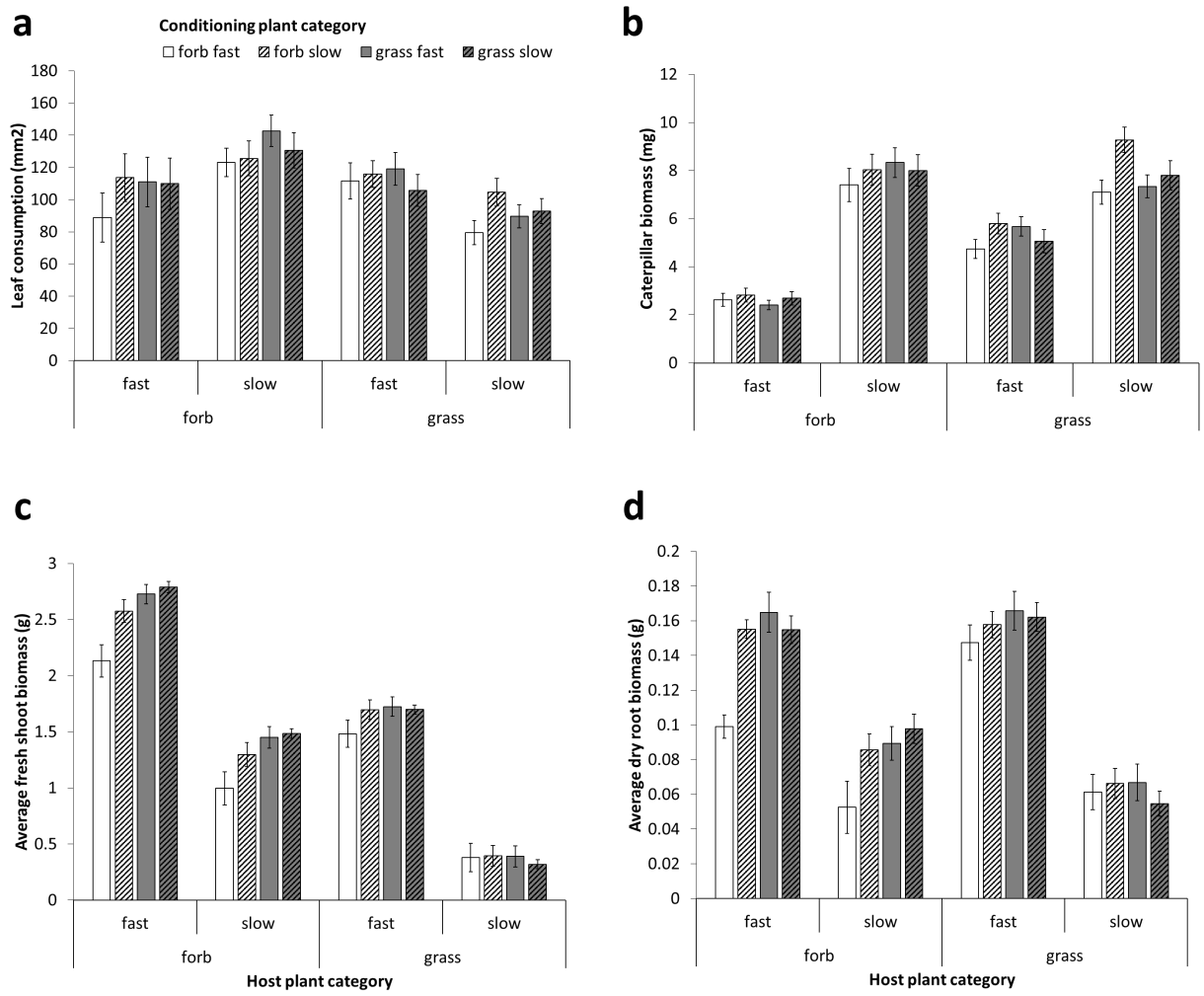
Plant functional group and growth rate interactively shape soil legacy effects on individual plant-insect interactions

			Leaf consumption		Caterpillar biomass		Shoot biomass		Root biomass
Fast forbs	Factor	df	F (p-value)	Df	F (p-value)	df	F (p-value)	df	F (p-value)
Crepis capillaris (CC)	Soil id	11,38	1.0 (0.506)	11,38	1.3 (0.278)	11,48	1.8 (0.075)	**11,48**	**2.6 (0.012)**
	Origin	1,10	0.8 (0.385)	1,10	0.4 (0.518)	1,10	0.4 (0.547)	1,10	1.5 (0.247)
	Cfg	1,8	0.7 (0.433)	1,8	1.2 (0.302)	1,8	2.5 (0.155)	1,8	1.4 (0.264)
	Cgt	1,8	0.1 (0.773)	1,8	4.2 (0.076)	1,8	1.0 (0.349)	1,8	0.0 (0.879)
	Cfg x Cgt	1,8	2.3 (0.165)	1,8	0.2 (0.685)	1,8	4.5 (0.066)	1,8	4.2 (0.074)
Plantago lanceolata (PL)	Soil id	11,42	1.3 (0.278)	11,37	0.6 (0.842)	11,48	0.5 (0.919)	11,48	1.9 (0.071)
	Origin	1,10	0.0 (0.984)	1,10	0.4 (0.564)	1,10	0.9 (0.361)	1,10	0.3 (0.586)
	Cfg	1,8	1.5 (0.261)	1,8	0.0 (0.974)	1,8	1.5 (0.250)	**1,8**	**7.6 (0.025)**
	Cgt	1,8	0.1 (0.717)	1,8	0.1 (0.826)	1,8	0.9 (0.382)	1,8	0.3 (0.607)
	Cfg x Cgt	1,8	0.0 (0.848)	1,8	0.2 (0.674)	1,8	2.1 (0.187)	1,8	5.0 (0.055)
Taraxacum officinale (TO)	Soil id	**11,41**	**2.2 (0.034)**	11,48	1.8 (0.087)	**11,48**	**6.4 (<0.001)**	**11,48**	**4.9 (<0.001)**
	Origin	**1,10**	**6.2 (0.032)**	1,10	0.1 (0.727)	1,10	4.2 (0.066)	1,10	2.3 (0.164)
	Cfg	1,8	0.2 (0.679)	1,8	2.8 (0.133)	**1,8**	**13.8 (0.006)**	1,8	2.2 (0.175)
	Cgt	1,8	0.3 (0.580)	1,8	0.2 (0.702)	1,8	4.9 (0.058)	1,8	6.4 (0.035)
	Cfg x Cgt	1,8	0.6 (0.455)	1,8	0.0 (0.919)	1,8	0.0 (0.884)	1,8	2.0 (0.197)
Slow forbs									
Geranium molle (GM)	Soil id	11,35	1.5 (0.175)	11,35	1.4 (0.200)	11,48	1.1 (0.405)	**11,48**	**2.8 (0.007)**
	Origin	1,10	0.7 (0.413)	1,10	0.2 (0.640)	1,10	0.1 (0.754)	1,10	3.8 (0.080)

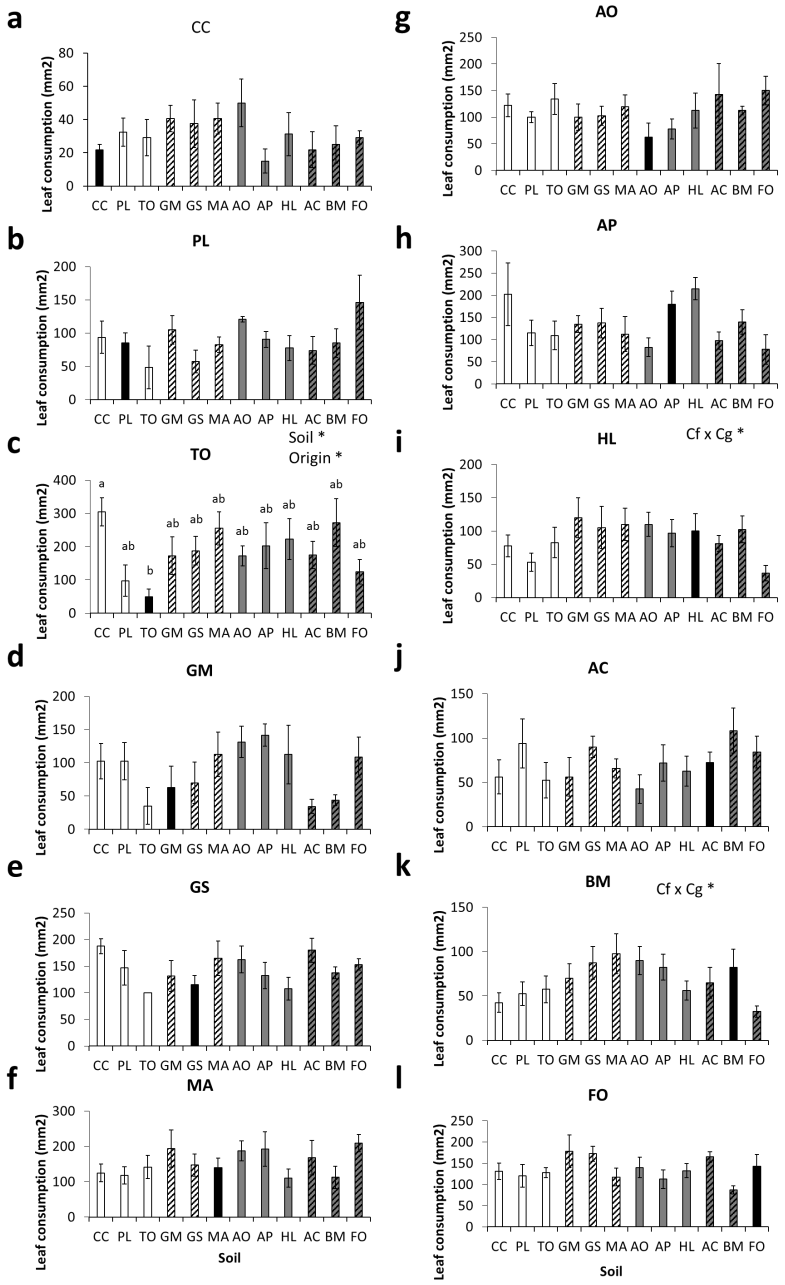
	Cfg	1,8	0.1 (0.780)	1,8	0.1 (0.744)	1,8	3.1 (0.117)	1,8	3.8 (0.087)
	Cgt	1,8	4.7 (0.061)	1,8	1.6 (0.246)	1,8	1.3 (0.279)	1,8	4.8 (0.060)
	Cfg x Cgt	1,8	3.5 (0.099)	1,8	4.6 (0.065)	1,8	2.6 (0.143)	1,8	3.2 (0.110)
Gnaphalium sylvaticum (GS)	Soil id	11,43	1.5 (0.182)	11,44	1.7 (0.104)	**11,48**	**5.2 (<0.001)**	**11,48**	**3.9 (<0.001)**
	Origin	1,10	1.7 (0.227)	1,10	0.0 (0.915)	**1,10**	**6.8 (0.026)**	**1,10**	**6.0 (0.034)**
	Cfg	1,8	0.0 (0.928)	1,8	0.2 (0.690)	**1,8**	**8.7 (0.018)**	1,8	5.2 (0.052)
	Cgt	1,8	0.1 (0.719)	1,8	0.6 (0.447)	1,8	1.2 (0.298)	1,8	2.9 (0.129)
	Cfg x Cgt	1,8	1.6 (0.244)	1,8	1.7 (0.230)	1,8	0.5 (0.497)	1,8	0.4 (0.559)
Myosotis arvensis (MA)	Soil id	11,41	1.0 (0.472)	11,41	1.4 (0.198)	**11,46**	**3.1 (0.003)**	**11,46**	**2.5 (0.013)**
	Origin	1,10	0.2 (0.702)	1,10	0.4 (0.534)	1,10	0.3 (0.569)	1,10	2.8 (0.125)
	Cfg	1,8	1.0 (0.356)	1,8	1.0 (0.345)	**1,8**	**12.2 (0.008)**	**1,8**	**7.3 (0.027)**
	Cgt	1,8	0.7 (0.434)	1,8	0.7 (0.442)	1,8	3.7 (0.092)	1,8	1.5 (0.252)
	Cfg x Cgt	1,8	0.6 (0.476)	1,8	0.9 (0.360)	1,8	1.0 (0.338)	1,8	0.3 (0.606)
Fast grasses									
Anthoxanthum odoratum (AO)	Soil id	11,45	0.7 (0.693)	11,45	0.5 (0.881)	11,48	2.2 (0.310)	**11,48**	**2.8 (0.006)**
	Origin	1,10	2.4 (0.156)	1,10	0.9 (0.368)	1,10	0.4 (0.531)	1,10	1.1 (0.310)
	Cfg	1,8	0.0 (0.978)	1,8	0.9 (0.383)	1,8	0.2 (0.649)	1,8	0.0 (0.979)

	Cgt	1,8	1.4 (0.276)	1,8	0.3 (0.620)	1,8	1.3 (0.294)	1,8	0.1 (0.747)
	Cfg x Cgt	1,8	3.5 (0.097)	1,8	0.3 (0.580)	1,8	2.1 (0.188)	1,8	1.7 (0.231)
Alopecurus pratensis (AP)	Soil id	11,45	1.7 (0.100)	11,45	0.9 (0.585)	11,48	1.5 (0.182)	11,48	1.5 (0.160)
	Origin	1,10	1.3 (0.277)	1,10	1.8 (0.204)	1,10	1.5 (0.242)	**1,10**	**9.8 (0.011)**
	Cfg	1,8	0.0 (0.900)	1,8	0.1 (0.815)	1,8	3.8 (0.086)	1,8	3.8 (0.089)
	Cgt	1,8	2.1 (0.182)	1,8	0.0 (0.912)	1,8	0.5 (0.485)	1,8	0.2 (0.666)
	Cfg x Cgt	1,8	0.7 (0.443)	1,8	2.2 (0.175)	1,8	0.9 (0.372)	1,8	0.8 (0.400)
Holcus lanatus (HL)	Soil id	11,43	1.3 (0.263)	11,43	1.8 (0.089)	11,48	0.8 (0.594)	11,48	1.4 (0.218)
	Origin	1,10	0.1 (0.732)	1,10	3.5 (0.091)	1,10	0.4 (0.529)	1,10	0.1 (0.739)
	Cfg	1,8	0.2 (0.656)	1,8	0.2 (0.686)	1,8	0.7 (0.417)	1,8	0.0 (0.892)
	Cgt	1,8	0.3 (0.620)	1,8	0.2 (0.663)	1,8	1.1 (0.333)	1,8	0.0 (0.856)
	Cfg x Cgt	**1,8**	**7.6 (0.025)**	**1,8**	**6.6 (0.033)**	1,8	1.6 (0.241)	1,8	0.7 (0.428)
Slow grasses									
Agrostis capillaris (AC)	Soil id	11,39	1.1 (0.418)	11,39	1.6 (0.133)	11,43	0.9 (0.537)	11,43	0.8 (0.635)
	Origin	1,10	0.0 (0.886)	1,10	2.3 (0.163)	1,10	0.3 (0.620)	1,10	0.0 (0.907)
	Cfg	1,8	0.0 (0.872)	1,8	0.8 (0.401)	1,8	2.1 (0.188)	1,8	0.4 (0.568)

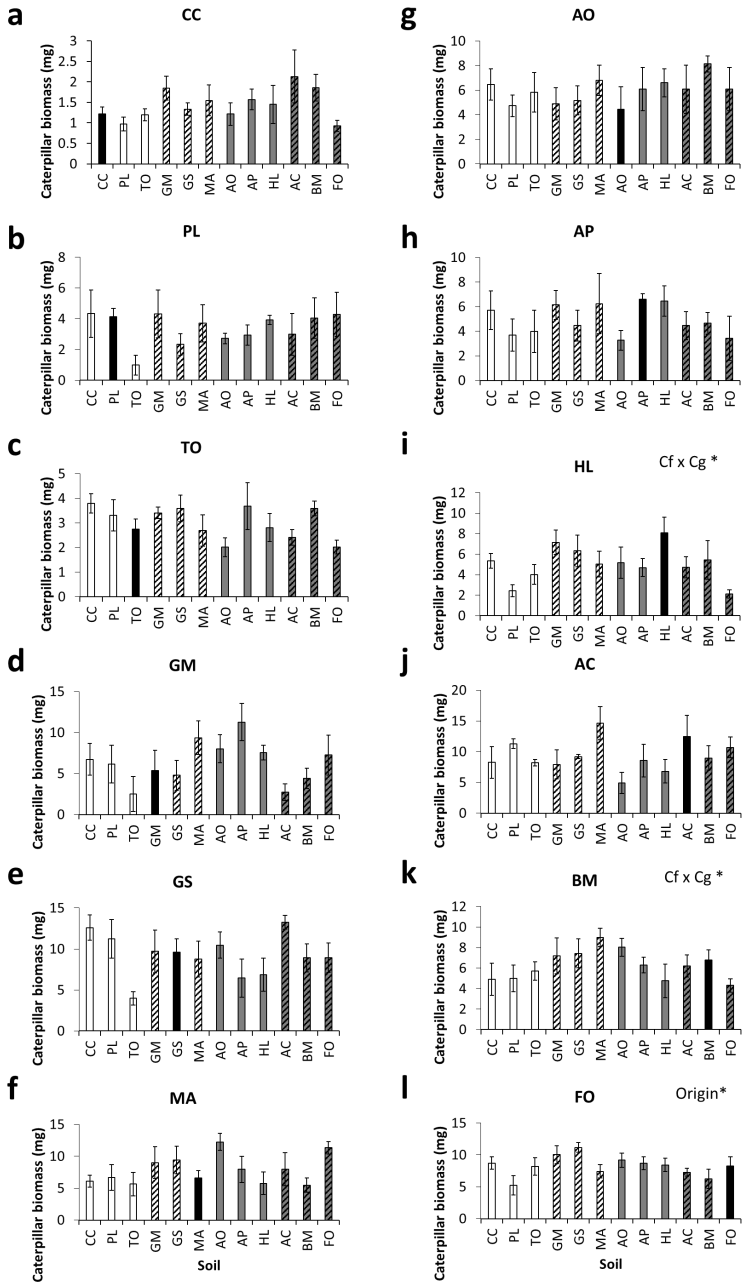
	Cgt	1,8	2.4 (0.158)	1,8	5.3 (0.051)	1,8	0.6 (0.479)	1,8	0.0 (0.953)
	Cfg x Cgt	1,8	1.1 (0.331)	1,8	1.6 (0.248)	1,8	0.0 (0.897)	1,8	0.3 (0.590)
Briza media (BM)	Soil id	11,46	1.7 (0.111)	11,46	1.5 (0.168)	11,48	1.3 (0.245)	11,47	1.5 (0.147)
	Origin	1,10	0.6 (0.447)	1,10	0.1 (0.717)	1,10	0.1 (0.758)	1,10	0.3 (0.597)
	Cfg	1,8	0.0 (0.893)	1,8	0.3 (0.573)	1,8	0.1 (0.801)	1,8	0.5 (0.507)
	Cgt	1,8	0.8 (0.406)	1,8	2.1 (0.181)	1,8	0.0 (0.841)	1,8	0.8 (0.384)
	Cfg x Cgt	**1,8**	**7.7 (0.024)**	**1,8**	**6.2 (0.037)**	1,8	2.4 (0.159)	**1,8**	**10.0 (0.014)**
Festuca ovina (FO)	Soil id	11,45	1.5 (0.173)	11,45	1.8 (0.074)	11,48	1.6 (0.134)	11,48	1.5 (0.152)
	Origin	1,10	0.1 (0.767)	1,10	0.0 (0.974)	1,10	2.5 (0.148)	1,10	0.2 (0.655)
	Cfg	1,8	0.6 (0.453)	1,8	0.3 (0.598)	1,8	0.3 (0.606)	1,8	0.2 (0.675)
	Cgt	1,8	1.3 (0.294)	1,8	0.2 (0.691)	1,8	0.2 (0.650)	1,8	1.3 (0.290)
	Cfg x Cgt	1,8	0.9 (0.383)	**1,8**	**7.0 (0.030)**	**1,8**	**7.6 (0.025)**	1,8	3.7 (0.090)



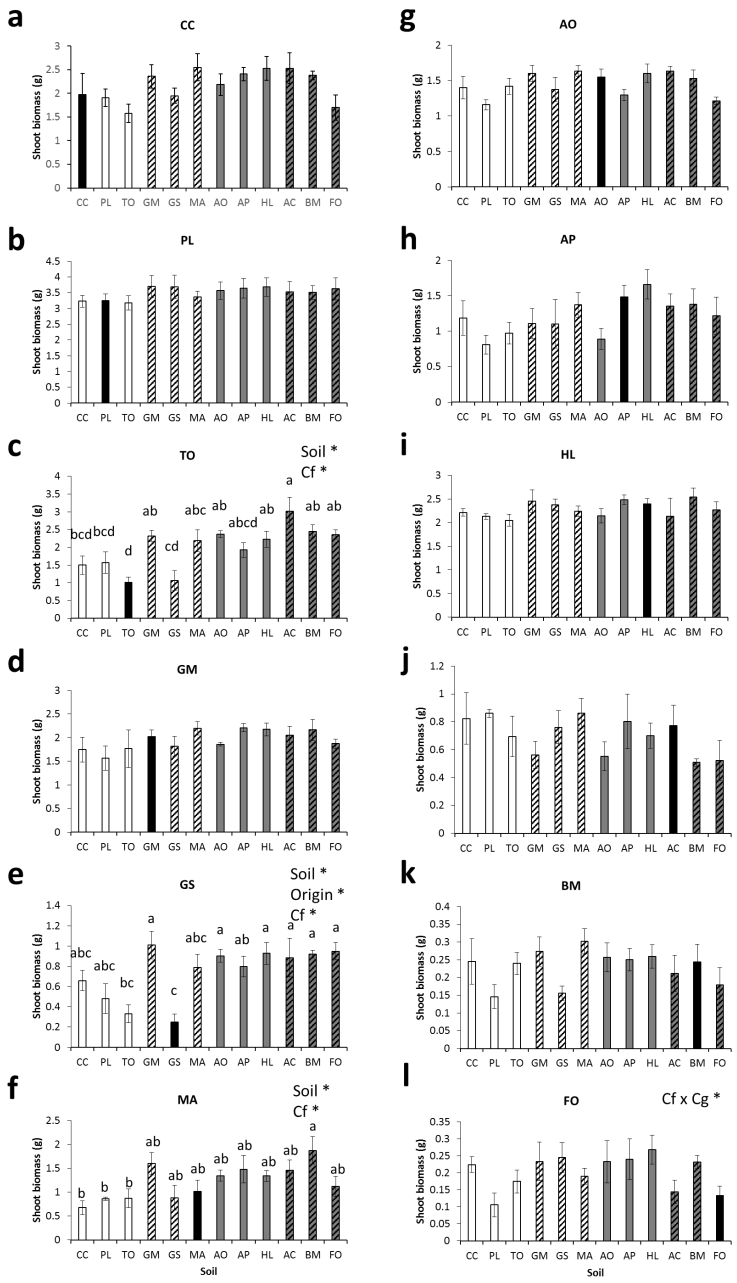
Supplementary Figure S3.1: Overview of the effects of growth rate and functional type of the conditioning and responding host plants on a) average leaf consumption by caterpillars, b) average caterpillar biomass, c) fresh shoot biomass and d) dry root biomass. The four categories on the x-axis represent host plant trait categories. Bars represent conditioning plant categories. White bars represent forbs, grey bars represent grasses. Open bars represent fast-growing species and dashed bars represent slow-growing species. Error bars represent standard errors. Each bar represents three conditioning plant species crossed with three host plant species, replicated five times (total per bar n=45). Statistical output of the full linear mixed model is presented in Table 1.



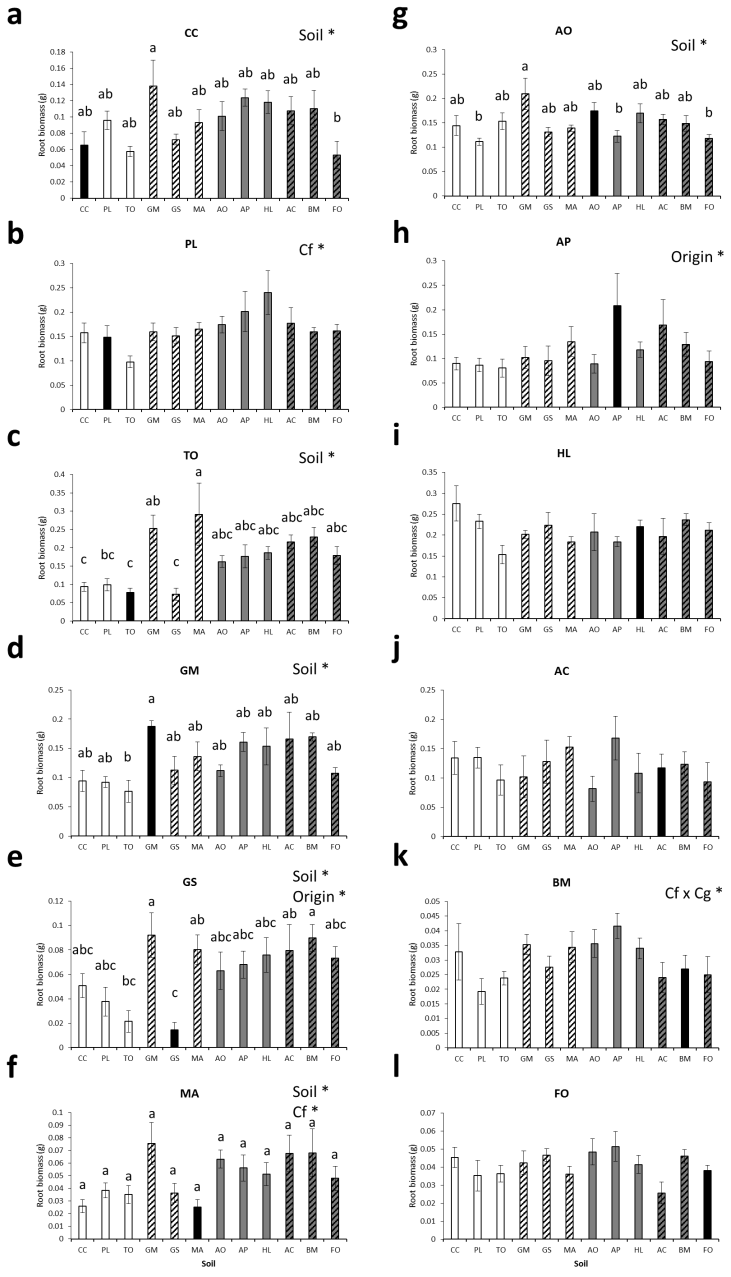
Supplementary Figure S3.2: Overview of the effect of soil on average leaf consumption by *M. brassicae* on twelve individual host plant species. White bars represent soils conditioned by forbs, grey bars represent soils conditioned by grasses. Open bars represent soils conditioned by fast-growing species and dashed bars represent soils conditioned by slow-growing species. Black bars represent conspecific soils. Error bars represent standard errors. Each bar represents five replicates. Statistically significant effects are indicated in individual graphs and full statistical output is presented in Supplementary Table 2. Different letters above the bars indicate significantly different means, as tested with post-hoc Tukey tests. Abbreviations are as follows for fast-growing forbs CC= *Crepis capillaris*, PL= *Plantago lanceolata*, TO= *Taraxacum officinale*, for slow-growing forbs GM= *Geranium molle*, GS= *Gnaphalium sylvaticum*, MA= *Myosotis arvensis*, for fast-growing grasses AO= *Anthoxanthum odoratum*, AP= *Alopecurus pratensis*, HL= *Holcus lanatus*, and for slow-growing grasses AC= *Agrostis capillaris*, BM= *Briza media* and FO= *Festuca ovina*.



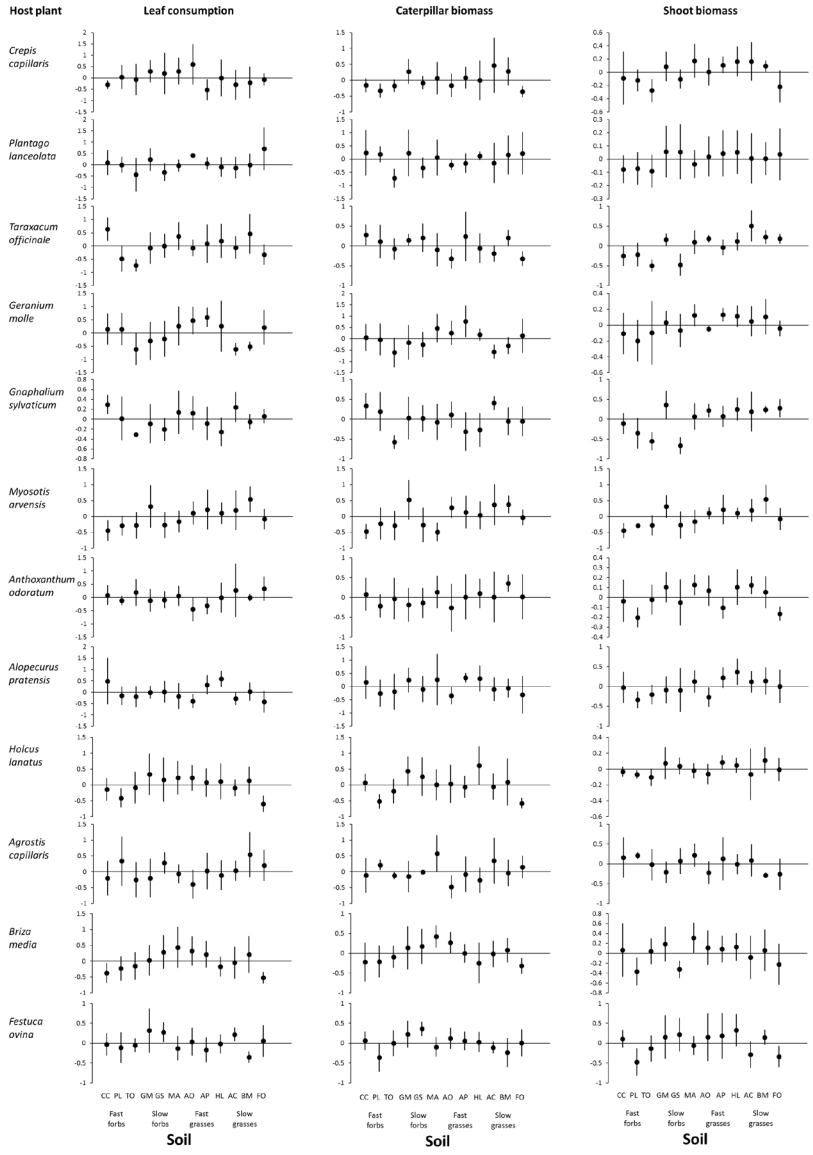
Supplementary Figure S3.3: Overview of the effect of soil on biomass of *M. brassicae* on twelve individual host plant species. White bars represent soils conditioned by forbs, grey bars represent soils conditioned by grasses. Open bars represent soils conditioned by fast-growing species and dashed bars represent soils conditioned by slow-growing species. Black bars represent conspecific soils. Error bars represent standard errors. Each bar represents five replicates. Statistically significant effects are indicated in individual graphs and full statistical output is presented in Supplementary Table 2. Different letters above the bars indicate significantly different means, as tested with post-hoc Tukey tests. Abbreviations are as follows for fast-growing forbs CC= *Crepis capillaris*, PL= *Plantago lanceolata*, TO= *Taraxacum officinale*, for slow-growing forbs GM= *Geranium molle*, GS= *Gnaphalium sylvaticum*, MA= *Myosotis arvensis*, for fast-growing grasses AO= *Anthoxanthum odoratum*, AP= *Alopecurus pratensis*, HL= *Holcus lanatus*, and for slow-growing grasses AC= *Agrostis capillaris*, BM= *Briza media* and FO= *Festuca ovina*.



Supplementary Figure S3.4: Overview of the effect of soil on average shoot biomass of twelve individual host plant species. White bars represent soils conditioned by forbs, grey bars represent soils conditioned by grasses. Open bars represent soils conditioned by fast-growing species and dashed bars represent soils conditioned by slow-growing species. Black bars represent conspecific soils. Error bars represent standard errors. Each bar represents five replicates. Statistically significant effects are indicated in individual graphs and full statistical output is presented Supplementary Table 2. Different letters above the bars indicate significantly different means, as tested with post-hoc Tukey tests. Abbreviations are as follows for fast-growing forbs CC= *Crepis capillaris*, PL= *Plantago lanceolata*, TO= *Taraxacum officinale*, for slow-growing forbs GM= *Geranium molle*, GS= *Gnaphalium sylvaticum*, MA= *Myosotis arvensis*, for fast-growing grasses AO= *Anthoxanthum odoratum*, AP= *Alopecurus pratensis*, HL= *Holcus lanatus*, and for slow-growing grasses AC= *Agrostis capillaris*, BM= *Briza media* and FO= *Festuca ovina*.



Supplementary Figure S3.5: Overview of the effect of soil on average root biomass of twelve individual host plant species. White bars represent soils conditioned by forbs, grey bars represent soils conditioned by grasses. Open bars represent soils conditioned by fast-growing species and dashed bars represent soils conditioned by slow-growing species. Black bars represent conspecific soils. Error bars represent standard errors. Each bar represents five replicates. Statistically significant effects are indicated in individual graphs and full statistical output is presented in Supplementary Table 2. Different letters above the bars indicate significantly different means, as tested with post-hoc Tukey tests. Abbreviations are as follows for fast-growing forbs CC= *Crepis capillaris*, PL= *Plantago lanceolata*, TO= *Taraxacum officinale*, for slow-growing forbs GM= *Geranium molle*, GS= *Gnaphalium sylvaticum*, MA= *Myosotis arvensis*, for fast-growing grasses AO= *Anthoxanthum odoratum*, AP= *Alopecurus pratensis*, HL= *Holcus lanatus*, and for slow-growing grasses AC= *Agrostis capillaris*, BM= *Briza media* and FO= *Festuca ovina*.



Supplementary Figure S3.6: Calculated averaged soil-feedback effects on individual host plant species and herbivores feeding on them. The left row of panels represents soil-feedbacks on leaf consumption by the caterpillar on that host plant. The middle row of panels represents soil-feedbacks on biomass of the caterpillar feeding on that host plant. The right row of panels represents soil-feedbacks on host plant shoot biomass. Soil-feedbacks were calculated for individual samples as the soil effect relative to the mean by:

$$Soil\ effect_{individual} = (observed\ shoot\ biomass_{species\ X} - mean\ shoot\ biomass_{species\ X}) / mean\ shoot\ biomass_{species\ X}$$

Shown feedbacks are the averages of replicates of each soil-plant-insect combination, with error bars representing 95% confidence intervals calculated across five replicates. When confidence intervals do not cross the zero-line, that soil invokes an effect that is significantly different from the average for that host plant species or the herbivore feeding on it. Abbreviations are as follows for fast-growing forbs CC= *Crepis capillaris*, PL= *Plantago lanceolata*, TO= *Taraxacum officinale*, for slow-growing forbs GM= *Geranium molle*, GS= *Gnaphalium sylvaticum*, MA= *Myosotis arvensis*, for fast-growing grasses AO= *Anthoxanthum odoratum*, AP= *Alopecurus pratensis*, HL= *Holcus lanatus*, and for slow-growing grasses AC= *Agrostis capillaris*, BM= *Briza media* and FO= *Festuca ovina*.

Plant functional group and growth rate interactively shape soil legacy effects on individual plant-insect interactions

Chapter 4

Plant community composition but not plant traits determine the outcome of soil legacy effects on plants and insects

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Abstract

Plant community composition but not plant traits determine the outcome of soil legacy effects on plants and insects

Plants leave species-specific legacies in the soil they grow in that can represent changes in abiotic or biotic soil properties. It has been shown that such legacies can affect future plants that grow in the same soil (plant–soil feedback, PSF). Such processes have been studied in detail, but mostly on individual plants. Here, we study PSF effects at the community level and use a trait-based approach both in the conditioning phase and in the feedback phase to study how 12 individual soil legacies influence six plant communities that differ in root size. We tested if (1) grassland perennial species with large root systems would leave a stronger legacy than those with small root systems, (2) grass species would leave a more positive soil legacy than forbs, and (3) communities with large root systems would be more responsive than small-rooted communities. We also tested (4) whether a leaf-chewing herbivore and a phloem feeder were affected by soil legacy effects in a community framework. Our study shows that the six different plant communities that we used respond differently to soil legacies of 12 different plant species and their functional groups. Species with large root systems did not leave stronger legacies than species with small root systems, nor were communities with large root systems more responsive than communities with root systems. Moreover, we show that when communities are affected by soil legacies, these effects carry over to the chewing herbivore *Mamestra brassicae* (Lepidoptera: Noctuidae) through induced behavioural changes resulting in better performance of a chewing herbivore on forb-conditioned soils than on grass-conditioned soils, whereas performance of the phloem feeder *Rhopalosiphum padi* (Hemiptera: Aphididae) remained unaffected. The results of this study shed light on the variability of soil effects found in previous work on feedbacks in communities. Our study suggests that the composition of plant communities determines to a large part the response to soil legacies. Furthermore, the responses to soil legacies of herbivores feeding on the plant communities that we observed, suggests that in natural ecosystems, the vegetation history may also have an influence on contemporary herbivore assemblages. This opens up exciting new areas in plant–insect research and can have important implications for insect pest management.

Introduction

Soil biota critically depend on plants, because they provide the primary resources for the soil food web (Bardgett & Wardle, 2010; Wardle et al., 2004). Plant growth, in turn, also depends on the composition of the soil biotic community, as soil biota recycle and provide nutrients to the plant or influence plant health (Berendsen, Pieterse, & Bakker, 2012; Van Der Heijden, Bardgett, & Van Straalen, 2008). Plant species can differ greatly in how they influence soil biota as well as soil abiotic conditions such as pH, or the concentration of allelochemicals in the soil (Bais, Vepachedu, Gilroy, Callaway, & Vivanco, 2003; Bais, Weir, Perry, Gilroy, & Vivanco, 2006). Furthermore, via their effect on the soil, plants can also influence other plants that grow later in the soil, a process known as plant–soil feedback (PSF; Bever, 1994).

Plants differ in how they influence the soil, but species also vary greatly in how they respond to differences in soil conditions. An important question is whether these effects on soils and responses to soils can be predicted by plant traits, such as those related to defence (Bardgett, Mommer, & de Vries, 2014; Kulmatiski, Beard, Stevens, & Cobbold, 2008; Van der Putten et al., 2013). Several studies have shown that the strength and direction of the PSF effect induced by a species differs between plant functional groups, and that grasses induce overall more positive effects than forbs (Kos, Tuijl, de Roo, Mulder, & Bezemer, 2015; Van de Voorde, van der Putten, & Bezemer, 2011; Wubs & Bezemer, 2016). Plant roots directly interact with the soil and soil biota and hence plants with large root systems may have a larger zone of influence per unit soil, or a larger contact area for interacting with soil organisms than plants with small roots. It is well known for many plant species that there is a positive relationship between the root size and root growth rate of a plant and the amount of exudates that the roots deposit in the soil (De Deyn, Cornelissen, & Bardgett, 2008; Dennis, Miller, & Hirsch, 2010; der Krift, Kuikman, Möller, & Berendse, 2001). Larger root systems also provide more habitat for root-associated (micro)-organisms such as bacteria or nematodes, for example, by having a larger surface area (Latz, Eisenhauer, Scheu, & Jousset, 2015). The surface area of the roots could also affect the response to soil. Roots with a larger size and surface area may, by chance, encounter more soil organisms. The size of a root system at any particular point in time will be influenced by growth rate, since a plant that grows fast, will accumulate more biomass in a fixed time frame than a plant that grows more slowly. Another determinant of root size could be the relative investment of plant species in their root biomass. Several studies have shown that fast growing,

early successional plant species typically create negative PSF effects, while slow growing, later-successional plants tend to leave a more positive legacy (Cortois, Schröder-Georgi, Weigelt, van der Putten, & De Deyn, 2016; Heinze, Bergmann, Rillig, & Joshi, 2015; Jing, Bezemer, & van der Putten, 2015; Kardol, Bezemer, & van der Putten, 2006). Previous studies suggest that fast growers may accumulate more pathogens in their rhizosphere than slow growers (Bever, Westover, & Antonovics, 1997; Van der Putten, Van Dijk, & Peters, 1993; Van der Putten et al., 2013). Fast growing plants may invest less in plant defence such as allelochemicals than slow growing ones (Coley, Bryant, & Chapin, 1985; Herms & Mattson, 1992). Hence, root traits related to growth and defence may also play a vital role in a plant's response to soil legacy effects.

Most PSF studies focus on plant growth effects, but several recent studies have shown that PSF effects can also influence above-ground herbivorous insects and their natural enemies (Kos et al., 2015; Kostenko, van de Voorde, Mulder, van der Putten, & Martijn Bezemer, 2012; Wurst, 2013). Soil biota can influence above-ground insect herbivores via influencing the size and ontogeny of the host plant, or via changing the nutritional quality of above-ground plant parts (Wardle et al., 2004). How different feeding guilds of above-ground insect herbivores respond to PSF is poorly understood. Insects of different feeding guilds vary greatly in how they respond to qualitative or quantitative changes in their host plants (Awmack & Leather, 2002; Bezemer & Jones, 1998). Furthermore, many studies have shown that the magnitude and even direction of effects of soil biota such as root herbivores, mycorrhizal fungi or even non-pathogenic bacteria on above-ground insects can differ between feeding guilds (Biere & Goverse, 2016; Johnson et al., 2012; Pangesti, Pineda, Pieterse, Dicke, & Van Loon, 2013; Soler et al., 2012). Root damage, for example, often increases the performance of above-ground sap suckers while it reduces the performance of leaf chewers (Bezemer & Jones, 1998; Johnson, Mitchell, McNicol, Thompson, & Karley, 2013; Johnson et al., 2012).

Plant–insect interactions are likely to differ between individual plants, monocultures and mixed communities. Moving from single species to mixed cultures increases biological diversity, chemical diversity and phylogenetic diversity of the study system (Andow, 1991; Salazar, Jaramillo, & Marquis, 2016). Studies show that performance of generalists increases in more diverse systems, as a result of higher productivity in diverse plant communities (Loranger et al., 2014; Marquard et al., 2009; Roscher et al., 2005; Scherber et al., 2006). Most likely, the

increased performance of generalists in such systems can be explained by increased plant diversity, as they can digest a wider range of host plants (Andow, 1991; Root, 1973). It should be noted that herbivores differ in their tolerance to different chemical compounds (Ali & Agrawal, 2012; Lankau, 2007), which may play an important role in the performance of different generalists on a range of different communities. In mixed plant communities, PSF effects may also influence above-ground insect herbivores by altering the relative abundance of host plants within the community (Jing et al., 2015; Kardol et al., 2006). However, how PSF influences above-ground insects in mixed plant communities remains largely unknown (Wurst & Ohgushi, 2015).

In this study, we examine the effects of soil legacies on a selection of large- and small-rooted grasses and forbs (based on their accumulation of root biomass over 7 weeks) and in turn how this affects the performance of two generalist herbivores from different feeding guilds. The cabbage moth (*Mamestra brassicae* L., Lepidoptera: Noctuidae) is a polyphagous chewing herbivore with a wide range of host plants and occurs all over the Palearctic (Metspalu, Jõgar, Hiiesaar, & Grishakova, 2004; Turnock & Carl, 1995). The bird cherry-oat aphid (*Rhopalosiphum padi* L., Hemiptera: Aphididae) is a phloem feeder that has a world-wide distribution and feeds on a wide range of grasses during its vegetative (summer) cycle (Dixon, 1971). We conditioned the soil by growing monocultures of each species for 10 weeks. We then planted mixed plant communities consisting of either large- or small-rooted plants on the conditioned soils and introduced M. brassicae and R. padi to each plant community. We predicted that (1) large-rooted plants will create more negative soil legacies than small-rooted plants, and this will, in turn, affect above-ground herbivores; (2) legacies left by grasses will be more positive than legacies left by forbs; (3) large-rooted plant communities will be more responsive to soil legacies than small-rooted communities. (4) Lastly, we expected that the two insect species will be differentially affected by soil legacies.

Materials and methods

Field soil and soil sterilization

Field soil used in this experiment was collected from a restoration grassland field site, “De Mossel” (Natuurmonumenten, Ede, The Netherlands) that has been abandoned from agriculture in 1996. This site has sandy loam soils (83% sand, 10% silt, 4% clay, 3% organic

matter, for chemistry see Table S1); the area is known to be poor in nutrients, except for phosphorus (a legacy of decades of heavy fertilization with manure). The live field soil originated from the top 5–10 cm of soil. For sterile soils, the soil layer of 10–30 cm depth was sterilized by γ-irradiation (Synergy Health, Ede, The Netherlands). Soil was sieved to remove roots, stones and most macro-invertebrates (sieve mesh Ø1.0 cm).

Plants

Growth of roots and shoots of 24 common grassland species was followed under standard greenhouse conditions over the course of 6 weeks, simultaneous with the conditioning phase of present study. A selection of 12 species was made based on root biomass; large root (R+) or small root (R−) and functional group; grass (G) or forb (F) (see Table S2).

Seeds were surface-sterilized using 2.5% bleach solution and then rinsed with water. For germination, seeds were placed on sterile glass beads in a climate cabinet (light regime 16:8, L:D, day temperature 21°C, night temperature 16°C). Because plants differ in their germination time, as soon as a species had germinated, the seedlings were stored at 4°C under the same light regime, until all species had sufficiently germinated. Seeds were obtained from Cruydt-Hoeck (Nijberkoop, The Netherlands).

Insects

Eggs of the Cabbage moth, *M. brassicae* were provided by the Department of Entomology at Wageningen University. The colony has been in production for many years on Brussel's Sprout, *Brassica oleracea* var. *gemmifera* cv. Cyrus. The larvae were originally collected from cabbage fields near Wageningen University.

A starter colony of the bird cherry-oat aphid, *R. padi*, was provided by Plant Research International at Wageningen University. The colony has been in rearing for more than 25 years. The original specimens were caught in Wageningen and have since been reared on Oat, *Avena sativa*, in a climate chamber with long day light (16:8, L:D) at 19°C.

Soil conditioning phase

To condition the soils by each of the 12 conditioning plant species, six round 2-L pots per plant species were filled with 1,800 g of homogenized live field soil. In each of the pots, five seedlings were grown to condition the soil. In addition, 10 smaller square pots (11 × 11 cm) were filled with 1,050 g homogenized live field soil in which only one seedling was planted, resulting in a total of 2,850 g of conditioned soil per plant species. The smaller pots were planted for an experiment that was performed simultaneously with the same live soils and seed batches in the same greenhouse compartment. These pots were also used to determine the root and shoot productivity for the 12 species used in this experiment. The soils were carefully homogenized per replicate. After planting, the seedlings were covered with shade cloth for 4 days to acclimatize. Pots were topped off with a 1 cm layer of fine sand against weeds and fungus gnats. Weeds that emerged from the soil were removed daily. The used plant species differed in their water use and soil moisture was kept at 17%. After a conditioning phase of 10 weeks, soils were harvested by removing all root pieces. For each of the conditioning species, soils of the individual and community conditionings were mixed by volume in a 1:1 ratio and divided over five independent replicates (each consisting of soil from one of the large and two of the smaller pots) to avoid pseudo-replication in the feedback phase. Soil from the sixth large pot per conditioning species was equally divided over the five replicates. The resulting conditioned soils were mixed with sterilized (by γ-irradiation) field soil (1:1 v:v). A subsample of each replicate soil was frozen at −80°C and the composition of soil bacteria and fungi was determined using Illumina Miseq sequencing. Results and details about the methods and analysis are presented in the Supporting Information.

Six different plant communities were composed before the start of the feedback phase of the experiment. Three communities contained plants that invest in quick root biomass addition (large-rooted communities; C+) and the other three communities contained plants that remain small rooted (small-rooted communities; C−). Each community consisted of four individuals: two forbs and two grasses (see also Table S3a). The experiment had a fractional factorial design (see Table S3b). Each of the six communities was grown on eight of the 12 conditioned soils (two R+ grasses; two R+ forbs, two R− grasses; two R− forbs) and thus, on every soil, four out of six communities were grown (see Table S3b for experimental combinations). Every

combination was replicated five times, using soil from one of the independent pools from the conditioning phase.

Feedback phase

Four round 2-L pots were filled per independent replicate pool. Each round pot was filled with a fixed volume (1.3 L) of conditioned soil. Soils were then topped off with a 1–2 cm layer of fine filter sand. All pots were watered and left to acclimatize for 2 days. Four germinated seedlings were planted in a square shape with roughly 5 cm distance between individuals to form the distinct communities. Plants were watered as needed three times per week. On day 41, the plants were placed in Bugdorm rearing bags (66 × 100 cm, MegaView Science, Taiwan) that were modified into hanging cylindrical cages for the insect assays (33 cm wide × 90 cm high). After the insect assay ended, on day 66 of the experiment, all above-ground parts were harvested for each plant species individually. Roots were harvested per community, as they could not be separated by species. Root parts were washed on a sieve to remove sand, stones and foreign organic material. Plant material was weighed after oven-drying for at least 72 hr at 70°C.

Caterpillar assay

On day 43 of the feedback phase, two *M. brassicae* were placed in each cage. Caterpillar damage was scored for each individual plant in each community on days 9, 16 and 23 of the insect assays. The larger of the two caterpillars was left on the plant after the first weighing for continuation of the assay. On days 10, 17 and 24, caterpillars were weighed and damage was measured as the estimated number of 25 mm2 squares that were eaten per plant. After the third measurement, the caterpillars were taken off the plants.

Aphid assay

On day 15 of the caterpillar assay, five *R. padi* individuals of nymphal instar 4 were placed in each cage. The aphids were left to reproduce asexually for 19 days, after which the above-ground biomass of the plants was harvested and the number of aphids was counted on each plant species.

Statistical analyses

Multivariate analyses of individual plant biomass and individual consumption

Unconstrained, principal component analyses were performed separately for each community for the response variables "individual plant biomass" and "consumed leaf area per individual plant" in each pot. Furthermore, constrained, redundancy analyses (RDA) were performed separately for each community for the same response variables, with root size (R+/R−) of the conditioning species, functional group (G/F) of the conditioning species and identity of the species (eight soil species per community) that conditioned the soils, as explanatory variables. All multivariate analyses were conducted in Canoco 5.03 (Microcomputer Power, Ithaca NY, USA).

Across-community effects

General linear mixed-effect models were used to analyse community root and shoot biomass, as well as caterpillar consumption, caterpillar biomass and aphid colony size. The raw data were z-transformed (as follows: $z = (x - \mu)/\sigma$, in which x = the observed value, μ = the community mean and σ = the community standard deviation) in order to allow assessing effects of soil conditioning on plant community types (C+/C−) while taking into account the differences in community composition. We analysed the main effects and interactions between root size of the conditioning plant species (R+/R−), functional group of the conditioning plant species (G/F) and community type (C+/C−) as fixed effects, with soil identity (conditioning plant species) nested in community identity (composition 1–6) as random effect. Analyses were performed in r version 3.0.3 (R Development Core Team, 2008) using the nlme package (Pinheiro, Bates, DebRoy, Sarkar, & R Core Team, 2017).

Within-community effects on plant and insect biomass

We analysed (1) the main effects and interaction between root size (R+/R−) and functional group (G/F) as factors as well as (2) the effect of soil identity (conditioning plant species) as factor on total shoot biomass, total root biomass, caterpillar biomass, caterpillar consumption and aphid colony size by ANOVAs. Analyses were performed for each community separately, using the raw data (log-transformed for root and shoot biomass, and square root-transformed for caterpillar biomass and aphid colony size) because we wanted to compare communities of

the same composition on different soils, not different communities, as was the case in the z-score analyses. Analyses were performed in r version 3.0.3 (R Development Core Team, 2008).

Growth of individual plants and leaf consumption of individual plants across six communities

The biomasses of individual species within each community are not independent samples and therefore should not be treated as such. Hence, the main body of this paper contains only the multivariate analyses of these data. However, because how the plant species grow and compete in different communities on different soils contains valuable information, these results are presented in the Supporting Information, accompanied by the respective ANOVAs (see Figure S4.1, Table S4.4).

Likewise, the data of the individual consumption gives valuable insights into the behaviour and preferences of the caterpillars in different communities and therefore are also supplied along with the accompanying ANOVAs (see Figure S4.2, Table S4.4).

Results

Multivariate analyses

The relative distribution of above-ground biomass across plant species within a community was affected by the soils the communities were grown on. There was a significant effect of the identity of the species that conditioned the soils on the composition of the biomass in communities II, IV and VI (RDA: community II: $F = 2.1$, $p < .001$; IV: $F = 1.8$, $p = .05$; VI: $F = 2.1$, $p = .01$, respectively, see Figure 4.1). In community I, II and V there was a significant effect of the functional group of the conditioning species (I: $F = 6.1$, $p < .01$; II: $F = 6.7$, $p < .01$; V: $F = 3.1$, $p = .02$, resp., see Figure 4.1). Only in community VI, was there an effect of root size of the species that conditioned the soil ($F = 4.2$, $p = .01$, see Figure 4.1).

The relative consumption of the different plant species by M. brassicae, was significantly affected only by functional group of the species that conditioned the soils. This effect was found in communities I, II and V (I: $F= 3.7$, $p = .01$; II: $F = 2.9$, $p = .05$; V: $F = 3.7$, $p = .01$, resp., see Figure 4.2).

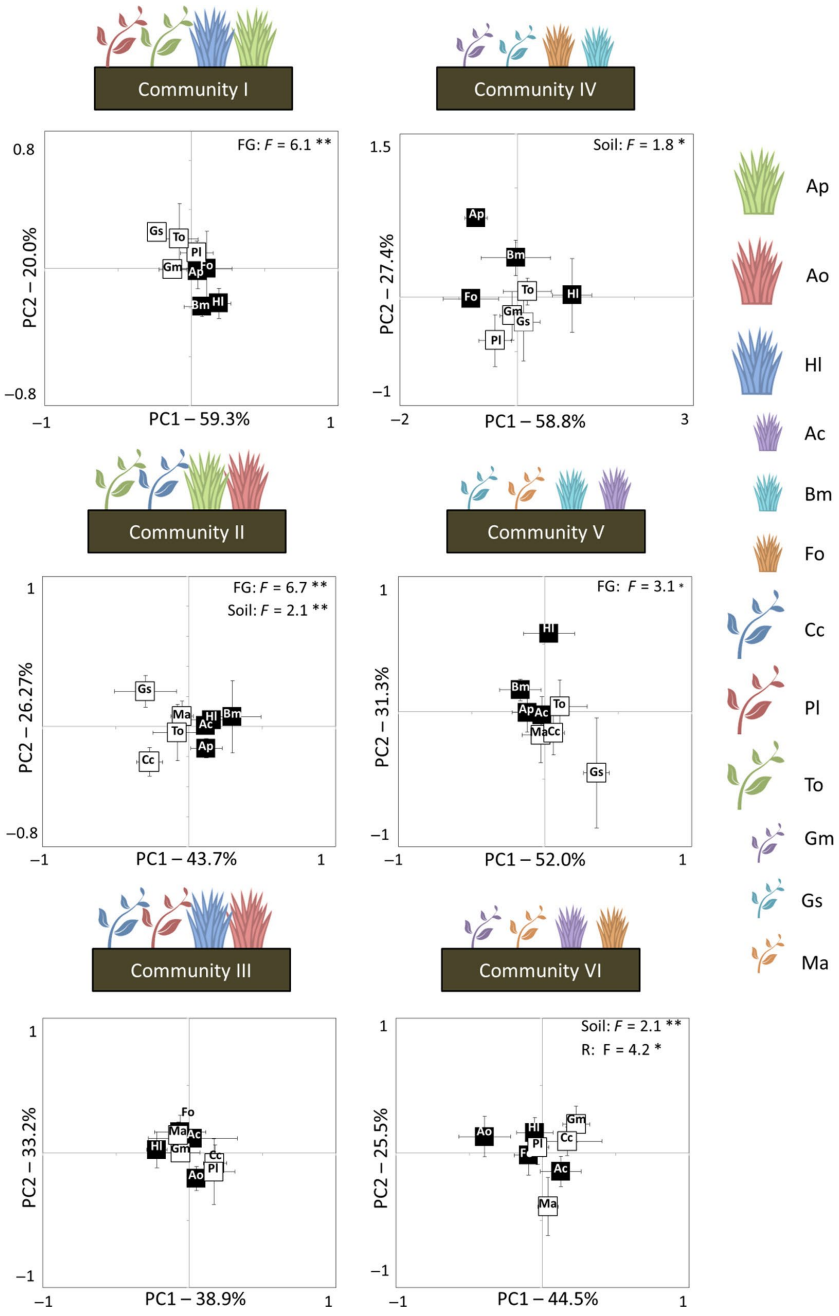

Figure 4.1: Principal component analysis (unconstrained PCA) plots showing effects of soil conditioning by 12 plant species on the distribution of shoot biomass over the four individual plant species in six different plant communities (I–VI). Each plant community was grown on 8 of 12 soils (fractional factorial design). Squares represent mean sample scores for the different conditioned soils (n = 5 for each square). Error bars represent SEs of the mean PCA scores for both axes. White squares represent forb soils and black squares represent grass soils. The composition of each of the six communities is also presented above each panel. Ac = *Agrostis capillaris*, Ao = *Anthoxanthum odoratum*, Ap = *Alopecurus pratensis*, Bm = *Briza media*, Cc = *Crepis capillaris*, Fo = *Festuca ovina*, Gm = *Geranium molle*, Gs = *Gnaphalium sylvaticum*, Hl = *Holcus lanatus*, Ma = *Myosotis arvensis*, Pl = *Plantago lanceolata*, To = *Taraxacum officinale*. Statistics shown in the panels are the F-statistic of constrained redundancy analysis (RDA) on functional group (FG), root size (R) and soil identity (Soil). Asterisks represent significance: *p < .05; **p < .01; ***p < .001

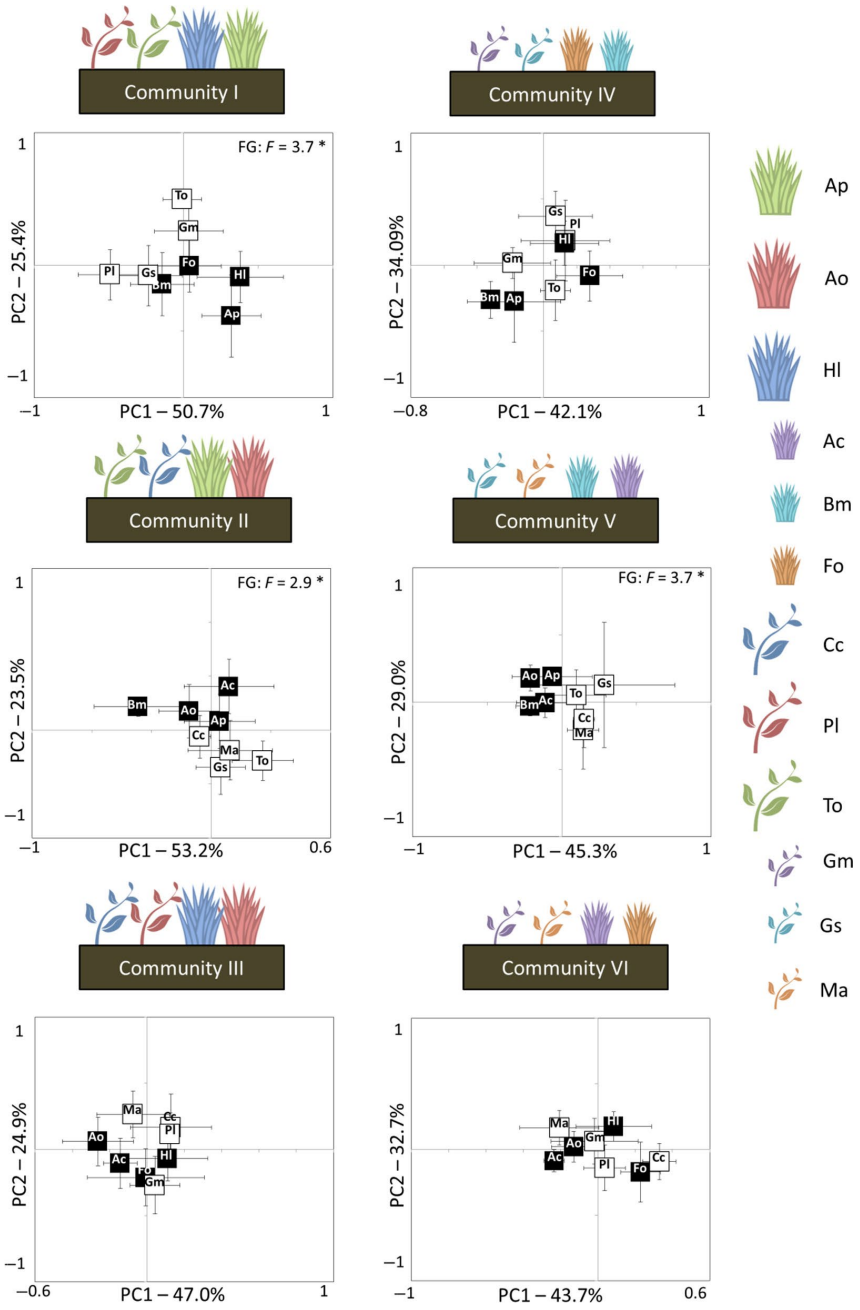

Figure 4.2: Principal component analysis (unconstrained PCA) plots showing effects of soil conditioning by 12 plant species on the distribution of herbivory (measured as consumed area) over the four individual plant species in six different plant communities (I–VI). Each plant community was grown on 8 of 12 soils (fractional factorial design). Squares represent mean sample scores for the different conditioned soils ($n = 5$ for each square). Error bars represent *SE*s of the mean PCA scores for both axes. White squares represent forb soils and black squares represent grass soils. The composition of each of the six communities is also presented above each panel. Ac = *Agrostis capillaris*, Ao = *Anthoxanthum odoratum*, Ap = *Alopecurus pratensis*, Bm = *Briza media*, Cc = *Crepis capillaris*, Fo = *Festuca ovina*, Gm = *Geranium molle*, Gs = *Gnaphalium sylvaticum*, Hl = *Holcus lanatus*, Ma = *Myosotis arvensis*, Pl = *Plantago lanceolata*, To = *Taraxacum officinale*. Statistics shown in the panels are the *F*-statistic of constrained redundancy analysis (RDA) on functional group (FG) and soil identity (Soil). Asterisks represent significance: *$p < .05$; **$p < .01$; ***$p < .001$

Across-community effects

Total above-ground biomass was not affected by main effects of root type (R+/R−) or functional group (G/F) of the conditioned soils, or the type of community (C+/C−). However, a marginally significant interaction was found between community type and functional group of the species that conditioned the soil. As shown in Figure 3a, on forb-conditioned soils large-rooted communities tended to have a higher above-ground biomass than small-rooted communities, whereas on grass-conditioned soils, the small-rooted communities tended to have a higher biomass than the large-rooted communities (non-significant: $F_{1,36} = 3.95$, p = .055, see Figure 4.3a).

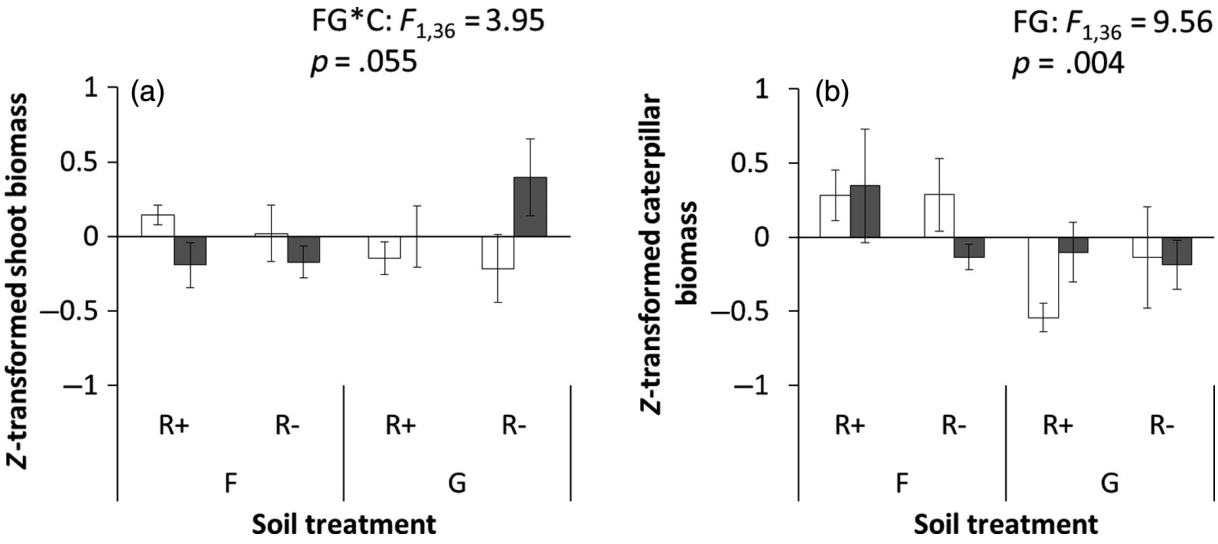

Figure 4.3: Effects of soil conditioning by large-rooted (R+) or small-rooted (R−) grasses (G) and forbs (F) on (a) community shoot biomass and (b) caterpillar biomass after 24 days of feeding. As different plant communities inherently differ in their community shoot biomass, as well as the suitability as food source for herbivores, data for shoot biomass and caterpillar biomass were *z*-transformed (*M* = 0 and *SE* = 1, See methods) for each of the six (different) plant communities. In this way, the mean and *SD*s were centralized, which makes it possible to compare the effects of soil conditioning between communities and test for general treatment effects across the data. Error bars represent *SEs*. White bars represent large-rooted communities (C+) and grey bars represent small-rooted communities (C−). Statistics shown are main effects and interactions of community type (C), functional group (FG) and root size (R) derived from mixed models

The identity of the functional group of the species that conditioned the soil had a significant effect on caterpillar biomass after 3 weeks of feeding. Caterpillars were significantly larger on food plants grown on forb-conditioned soils than on grass-conditioned soils ($F_{1,36} = 9.56$, p <

.01, see Figure 4.3b). Neither root size of the conditioning species nor community type significantly affected caterpillar biomass.

No effects of functional group or root type of the conditioning species were found on aphid numbers (data not shown). Since only one plant species (*Alopecurus pratensis*) supported formation of aphid colonies and this species only occurred in two of six communities, no further analyses were performed.

Within-community effects on plant and insect biomass

Conditioning species identity had a significant effect on total above-ground biomass in three of six communities (I: $F_{7,31} = 7.95$, $p < .001$; V: $F_{7,26} = 4.38$, $p < .001$; VI: $F_{7,30} = 3.08$, $p = .01$ resp., see Figure 4.4). Community I accumulated most biomass on Gnaphalium soil, whereas biomass was approximately one-third lower on *Briza* and *Holcus* soils. Community V had highest biomass on *Taraxacum, Alopecurus* and *Agrostis* soils and lowest biomass on *Crepis* soils. Similarly, community VI grew best on *Agrostis* soil and worst on *Crepis* and *Festuca* soils.

The functional group identity of the conditioning species only affected total above-ground biomass in community I ($F_{1,35} = 13.1$; $p < .001$). Communities grown on forb soils (*Plantago*, *Taraxacum*, *Geranium*, *Gnaphalium*) on average accumulated more biomass than those grown on grass-conditioned soils (*Alopecurus*, *Holcus*, *Briza*, *Festuca*). Root size of the conditioning plant species did not affect total above-ground biomass of any of the communities.

Functional group or identity of the conditioning species did not have any effects on total root biomass in any community. However, in community I we observed a significant effect of root size on the total root biomass of that community ($F_{1,35} = 6.8$; $p < .001$, see Figure S4.4). This community had significantly larger root systems when grown on soils that were conditioned by large-rooted grass or forb species, than when they were grown on those of small-rooted species.

Functional group of conditioning species had an effect on caterpillar biomass, but only in those feeding on community I and II (I: $F_{1,33} = 6.7$, $p = .01$; II: $F_{1,22} = 12.1$, $p < .01$, resp. see Figure 4.5). In both communities, the caterpillars grew larger on plants grown on soils conditioned by forbs.

Conditioning led to significant differences in the composition of bacteria and fungi. These effects were significant when all species were compared and when comparing grasses and forbs. However, the latter effect was much stronger for fungi than for bacteria (Figure S4.4a,b).

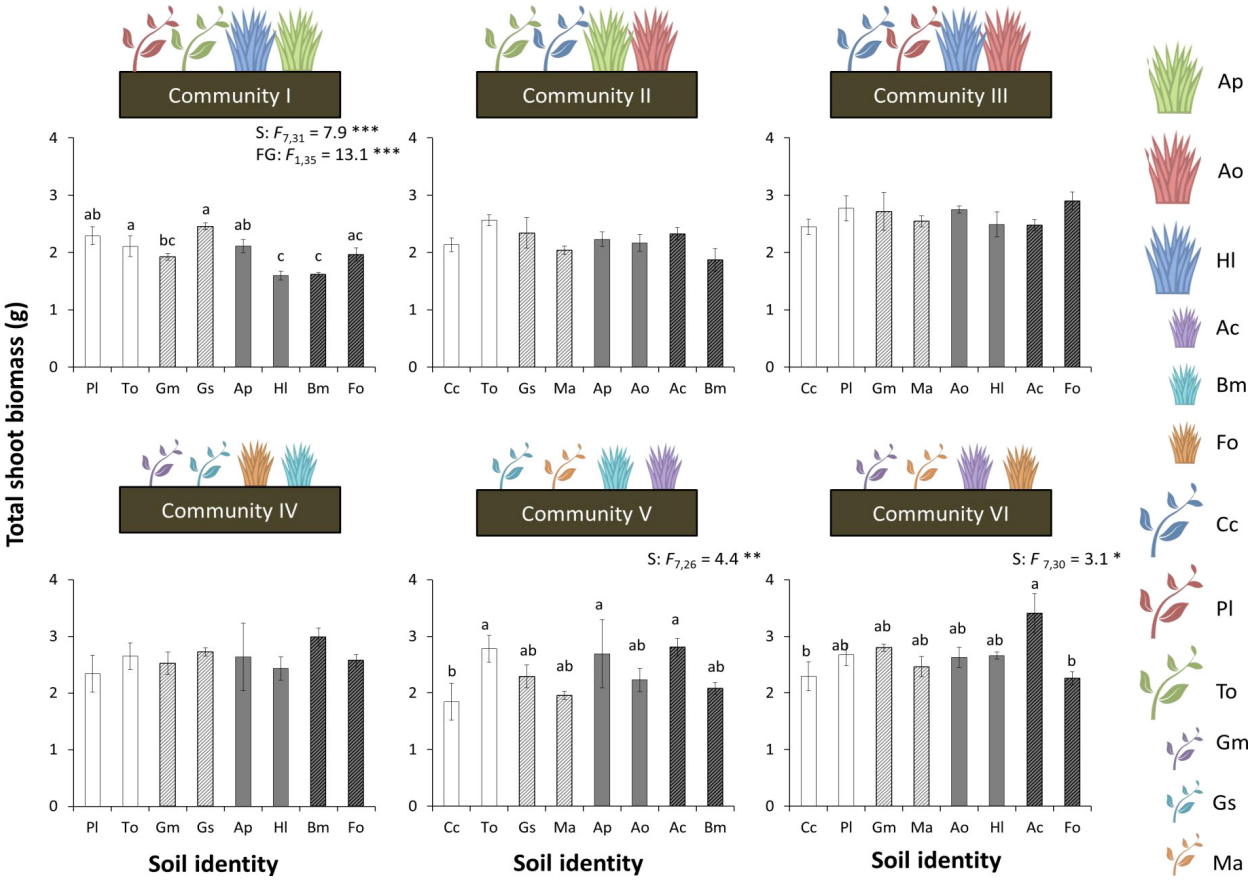

Figure 4.4: Effects of soil conditioning of species of grasses and forbs on community shoot biomass. White bars represent large-rooted forbs, striated white bars represent small-rooted forbs; grey bars represent large-rooted grasses, striated grey bars represent small-rooted grasses. Error bars represent *SEs*. The composition of each of the six communities is also presented above each panel. Ac = *Agrostis capillaris*, Ao = *Anthoxanthum odoratum*, Ap = *Alopecurus pratensis*, Bm = *Briza media*, Cc = *Crepis capillaris*, Fo = *Festuca ovina*, Gm = *Geranium molle*, Gs = *Gnaphalium sylvaticum*, Hl = *Holcus lanatus*, Ma = *Myosotis arvensis*, Pl = *Plantago lanceolata*, To = *Taraxacum officinale*. Statistics in the panels represent main effects of soil identity (S), root size (R) and soil functional group (FG) derived from one-way ANOVAs. Asterisks represent significance: *$p < .05$; **$p < .01$; ***$p < .001$

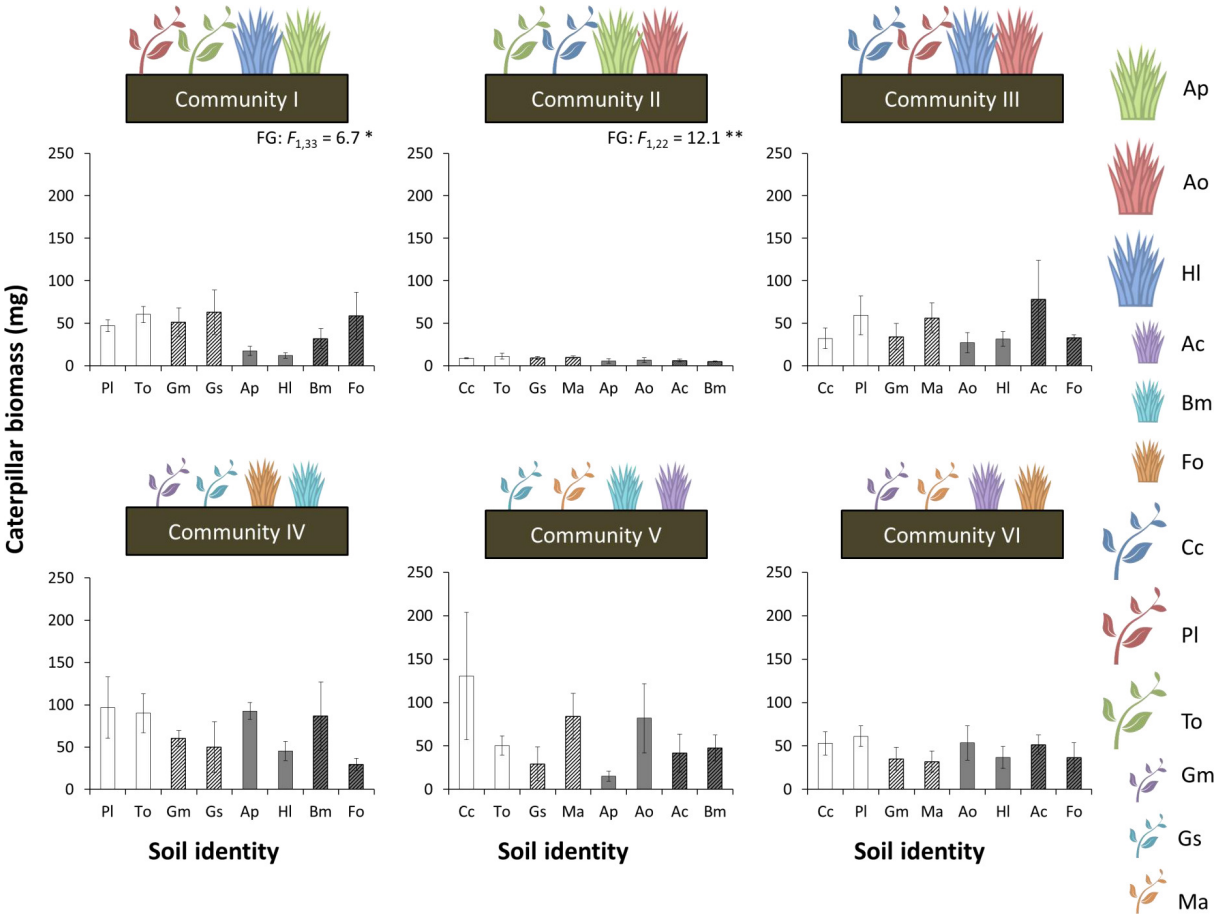

Figure 4.5: Effects of soil conditioning of species of grasses and forbs on *Mamestra brassicae* biomass after 24 days. White bars represent large-rooted forbs, striated white bars represent small-rooted forbs; grey bars represent large-rooted grasses, striated grey bars represent small-rooted grasses. Error bars represent SEs. The composition of each of the six communities is also presented above each panel. Ac = *Agrostis capillaris*, Ao = *Anthoxanthum odoratum*, Ap = *Alopecurus pratensis*, Bm = *Briza media*, Cc = *Crepis capillaris*, Fo = *Festuca ovina*, Gm = *Geranium molle*, Gs = *Gnaphalium sylvaticum*, Hl = *Holcus lanatus*, Ma = *Myosotis arvensis*, Pl = *Plantago lanceolata*, To = *Taraxacum officinale*. Statistics in the panels represent main effects of soil identity (S), root size (R) and soil functional group (FG) derived from one-way ANOVAs. Asterisks represent significance: *p < .05; **p < .01; ***p < .001

Discussion

Plant species differ in the way they influence the soil and via these changes they can affect plants that grow later in the same soil, as well as the insects that develop on them. In this study, we tested if such effects are still apparent if whole plant communities are grown on the soils in a feedback phase and whether insects would be affected by soil legacies in plant communities with several host plant species. Furthermore, we tested whether grassland plants that differ in root traits and functional group create different legacy effects.

We show here that 12 test plant species left specific soil legacies that differed in soil microbial composition, and that these legacies affected the relative performance of plant species in plant communities that grew later on the conditioned soils. In turn, this led to altered performance in an associated chewing herbivore, whereas a phloem feeder was not affected. Remarkably, while we found a clear effect of functional group on composition of soil communities and on plant community performance, root size of the conditioning plant species had very little influence on composition of soil communities and on plant community performance. The rooting type (large or small rooted; C+/C−) of the response community also did not affect the response to legacy effects.

The functional group the conditioning plant species belonged to, grass or forb, significantly explained the distribution of plant biomass over the plant species during the feedback phase in three out of six communities. This in itself is an interesting finding, as many studies incorporate just one focal plant or one focal community in the feedback phase and show the effects of different soils on this single plant species or plant community (e.g. Kardol, Cornips, van Kempen, Bakx-Schotman, & van der Putten, 2007). We did find plant species-specific (as well as functional group-specific) microbial profiles in the soil. This is in line with other studies using the same study system that show that plants leave species-specific microbial profiles in the soils, and that changes in soil biota differ significantly between the species and functional group the conditioning plants belong to (Kos et al., 2015). Our findings suggest that biotic legacies indeed are generally present in the soils, but that it is very much dependent on the composition of the community that grows later on these soils whether and how a community responds to these changes in soils. In our experiment we used 50% of conditioned soil and mixed this with 50% sterilized soil. Hence, potential differences in soil nutrients among the conditioned soils were diluted, but we cannot exclude that they may have played a role in the observed effects

on plants and herbivores, in addition to the effects incurred by plant-induced changes in microbial communities.

Several studies have shown that grasses leave different biotic profiles in the soil than forbs (e.g. Kos et al., 2015; Latz et al., 2012, 2015). Grass-conditioned soils have been shown in previous studies to be rich in plant growth-promoting rhizobacteria (Latz et al., 2012), which may prime plant defences in some plant species (Pangesti et al., 2015; Van Oosten et al., 2008). It has been proposed that these rhizobacteria may aid the grasses in fighting off (fungal) pathogens (Hol, Bezemer, & Biere, 2013; Latz et al., 2012, 2015). Alternatively, conditioning by different functional groups (as well as species) may lead to different endophyte communities in the plants of the feedback community, which in turn may also affect herbivores (Cripps, Edwards, & McKenzie, 2013; Zhang, Li, Nan, & Matthew, 2012). A lowered level of pathogens in grass soils as opposed to forb soils could result in different defence patterns in future plants growing on their soils, thus explaining our findings in this study. Unfortunately, interactions between the plant species used in this study and soil pathogens are poorly understood, making it difficult to test such hypotheses and draw definite conclusions.

We found significant effects of functional group of the conditioning species on productivity (total above-ground biomass) in only one community. Furthermore, we found significant effects of soil conditioning species on productivity in three of six communities. The other three communities were remarkably stable in their efficiency to convert the available resources into biomass, regardless of the soil legacy they grew on. As we observed effects of soil conditioning on individual species in all communities, this exemplifies that in plant communities where a species is negatively affected by a soil legacy, other species may exploit the resources that this species would otherwise have utilized. It is difficult to pinpoint what exactly caused three communities to respond to soil legacies, whereas three others remained unchanged. Communities without a significant overall response to soil conditioning could have consisted of species that all did not respond to the changes in the soil. However, in this study, we find that in all communities, at least one plant species in the communities responded differently to the different conditioned soils (see also Figure S4.1), regardless of whether the community as a whole was responsive. Furthermore, several studies have shown that conspecific PSF is generally negative and often is stronger when plants are grown in competition with other plants

than when they are alone in a pot (Jing et al., 2015; Petermann, Fergus, Turnbull, & Schmid, 2008).

Because our design allowed us to test for differences in response to soils by communities differing in root productivity, we can thus conclude that the root productivity (C+/C−) of a community does not influence its response to soil legacies. Interestingly, the species composition of communities that were responsive to soils conditioned by different functional groups partly overlapped with the species composition of communities that were non-responsive. This suggests that there is not just one species that explains the observed functional group effect, as each species always occurred in two of three communities of that type. More likely, it is the competitive interplay between the four species in each community that determines the outcome of its response to soil legacies. How balances between different plant species may influence the interactions between soil organisms and plants in a community, is a largely unexplored area that requires further study.

In the three communities where biomass distribution was affected by functional group of the conditioning species, we also found that herbivore behaviour was affected by the functional group to which the conditioning plant belonged. Studies have shown effects of functional group of conditioning species on insect performance (e.g. Kos et al., 2015), but, to our knowledge, this study is the first one to show altered feeding preferences in plant communities due to soil legacies and suggests that *M. brassicae* is able to detect soil legacy-mediated changes in host plant quality. Perhaps the herbivore switched between host plants in an attempt to escape host plants in which soil legacies had affected nutritional quality too negatively. Alternatively, herbivores may forage for those plants that are poorly defended above-ground, but these hypotheses require further study. This is especially relevant in the context of soil legacy studies, since legacy effects are often attributed to either pathogens (negative feedback) or growth promotors (positive feedback) (Van der Putten et al., 2013). If allocation of defences to local attack by root pathogens is traded off with defence against attack by above-ground herbivores, then interactions with soil pathogens, that is, negative soil legacies, may render above-ground plant parts less defended and more prone to attack by herbivores (Bezemer & van Dam, 2005).

Not only did the functional group of the conditioning plant species affect behavioural aspects of plant–herbivore interactions (as discussed above) but we also found a strong overall effect of functional group of the soil conditioning plant species on the performance (biomass) of the

herbivore. That is, herbivores grew bigger on plant communities growing on soils that were conditioned by forbs than on soils that were conditioned by grasses. Conditioning by plants of different functional groups may result in differences in resource uptake and use, leading to a nutritional legacy effect, which may not always be evident in the biomass of a community. However, such effects could be reflected in individual plant nutritional values and in turn affect herbivore performance. However, biomass (both of the community as a whole and individual plants) was not limiting to the herbivore, we cannot exclude that a difference in nutritional value may have played a role, as this was not measured.

Although we found a strong effect of functional group of the conditioning species on the generalist chewing herbivore, we found no effect of soil identity or functional group on performance of a generalist grass-feeding aphid (*R. padi*). Recent work has demonstrated that performance of the specialist aphid *Aphis jacobaeae* on *Jacobaea vulgaris* was affected by the functional group of the plant species that conditioned the soil. Grass-conditioning showed positive effects on aphid colony size, whereas performance of the generalist *Brachycaudus cardui* was not affected by functional group (Kos et al., 2015). The aphid used in our study has a broad host range of monocots (Dixon, 1971). Likely, the degree of specialism plays an important role in an herbivore's capability to cope with variation in host plant quality (Ali & Agrawal, 2012; Lankau, 2007). It is important to note that different feeding guilds often show different responses to changes in plant quality, due to differences in feeding strategies, as well as in the defence pathways invoked by plants (Awmack & Leather, 2002; Pangesti et al., 2013; Pineda, Zheng, van Loon, Pieterse, & Dicke, 2010). In plant cells, secondary (defence) chemicals and the hydrolytic enzymes that activate them are often stored in different intracellular compartments. Phloem feeders, using their stylets to penetrate individual cells during feeding, often leave these compartments largely intact. Leaf chewers damage cells and intracellular compartments and bring defence chemicals and hydrolytic enzymes into contact, leading to stronger defence responses (Gehring & Bennett, 2009; Koricheva, Gange, & Jones, 2009; Pangesti et al., 2013; Pineda et al., 2010). Therefore, possible changes in defence chemistry in response to soil legacy effects may affect different feeding guilds in different ways. However, to test this would require additional studies using multiple species from each feeding guild.

Conclusions

Our study shows that 12 common grassland species created species-specific soil legacies, which, in the feedback phase, influenced the composition of the plant communities. There was no effect of root size of the conditioning plants on the response of plants or insects. Instead, the soil effects were partly explained by the functional group the plant species that conditioned the soil belonged to. Soil legacies also affected the feeding behaviour of a chewing herbivore. The chewing herbivore performed significantly better on communities growing on forb-conditioned soils than on grass-conditioned soils. To our knowledge, this is the first time that this has been shown in a community context. This finding may have implications in natural communities and it may explain why insects are often found on certain individuals of a host species in a particular area, but not on other individuals of the same species in the same area (or other areas). Future studies should focus on unravelling mechanisms that underlie these soil legacy effects, first of all, through more thorough analysis of the soil communities and interactions and directional changes therein under different conditioning scenarios. Secondly, there is a need for better understanding of processes (such as defence chemistry and gene expression) that may occur in response to shifts in microbial communities, within a wider range of plants. Other studies are needed that examine the broader generalities of these plant–soil insect interactions also in real communities in the field. Such soil legacy effects could then potentially be used to improve the abundance of beneficial or "target" insects in natural communities, or instead repel or deter those that are unwanted or causing problems, such as pests, for example, in agricultural systems (Pineda, Kaplan, & Bezemer, 2017).

Acknowledgements

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Author contributions

RH, AB, JAH and TMB conceived the ideas and designed methodology; RH and MS collected the data; RH analysed the data and led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data accessibility

Data available from the Dryad Digital Repository:

https://doi.org/10.5061/dryad.39f10 (Heinen, van der Sluijs, Biere, Harvey, & Martijn Bezemer, 2017).

Plant community composition but not plant traits determine the outcome of soil legacy effects on plants and insects

Supplementary information Chapter 4

Supplementary methods

Beta-diversity soil fungi and bacteria:

Soil DNA was extracted from the soils using the PowerSoil® DNA Isolation Kit (MoBio, Carlsbad, CA, USA) and the library preparation for bacteria was done using tagged primers for 16S as described in detail in Dassen et al., 2017. For fungi, library preparation was done using tagged primers for ITS as described in Gweonn et al., 2015. Soil DNA was sequenced on the Illumina MiSeq platform (250 bp paired-end) by Beijing Genomics Institute (www.bgi.com; Shenzhen, China).

Statistical analysis soil beta diversity:

Sequences for bacteria and fungi were analyzed separately. Sequences were placed in operational taxonomic units (OTUs) and to account for differences in read numbers each OTU was standardized as a percentage of the total number of OTUs as described in Hannula et al., 2017. Unconstrained, principal coordinate analyses (PCoA/metric multi-dimensional scaling) were performed on dissimilarity matrices that were calculated from the OTU data. Furthermore, constrained, ANOSIM (Analysis of similarity, 999 permutations, using Bray-Curtis distance) were performed separately for bacteria and fungi, with root size (R+/R-) of the conditioning species, functional group (G/F) of the conditioning species and identity of the species (12 soil conditioning plant species) that conditioned the soils, as explanatory variables. All multivariate analyses on soil communities were conducted in R version 3.0.3 (R Core Team 2014), using the vegan package (Oksanen et al., 2017).

Supplementary Results

Growth of individual plants and leaf consumption of individual plants across six communities:
In community I, functional group effects on plant biomass were found for three out of the four plant species. *Holcus lanatus* tended to grow larger on forb-conditioned soils, compared to grass-conditioned soils ($F_{1,37}$ = 4.44; p=0.04, see Supplementary Figure S4.1). Similarly, *Plantago lanceolata* grew larger on forb-conditioned soils than on grass-conditioned soils ($F_{1,37}$= 6.63; p=0.01, see Supplementary Figure S4.1). Contrastingly, *Taraxacum officinale* grew larger on grass-conditioned soils than on forb-conditioned soils and was significantly affected by soil conditioning species as well (FG: $F_{1,37}$ = 15,81; p<0.01 S: $F_{7,31}$ = 6.90; p=0.01, see Supplementary Figure S4.1). Leaf consumption of *P. lanceolata* was significantly affected by identity and functional group of the species that conditioned the soil (FG: $F_{1,37}$ = 19.25; p<0.01, S: $F_{7,31}$ = 2.77; p=0.02, see Supplementary Figure S4.2). *T. officinale* consumption was affected by functional group of the soil-conditioning species. Caterpillars fed more on *Taraxacum* growing on grass-conditioned than on forb-conditioned soils ($F_{1,37}$ = 6.90; p=0.01, see Supplementary Figure S4.2).

In community II, *Anthoxanthum odoratum* grew significantly larger on soil conditioned by forbs than on grass-conditioned soils ($F_{1,35}$= 6.74; p=0.01, see Supplementary Figure S4.1). By contrast, *T. officinale* grew larger on grass-conditioned soils than on forb-conditioned soils and was significantly affected by soil conditioning species as well (FG: $F_{1,38}$ = 22.63; p<0.01 S: $F_{7,32}$ = 4.65; p<0.01, see Supplementary Figure S4.1). *Crepis capillaris* was significantly affected by soil conditioning species ($F_{7,32}$= 2.8; p=0.02, see Supplementary Figure S4.1).

In community III, *Crepis capillaris* produced more biomass on grass soils ($F_{1,35}$ = 6,28; p<0.01, see Supplementary Figure S4.1).

In community IV, *Briza media* grew larger on forb-conditioned soils than on grass-conditioned soils ($F_{1,29}$ = 5,43; p=0.03, see Supplementary Figure S4.1). *Gnaphalium sylvaticum* growth was significantly affected by identity of the soil conditioning species ($F_{7,23}$ = 2.55; p=0.04, see Supplementary Figure S4.1).

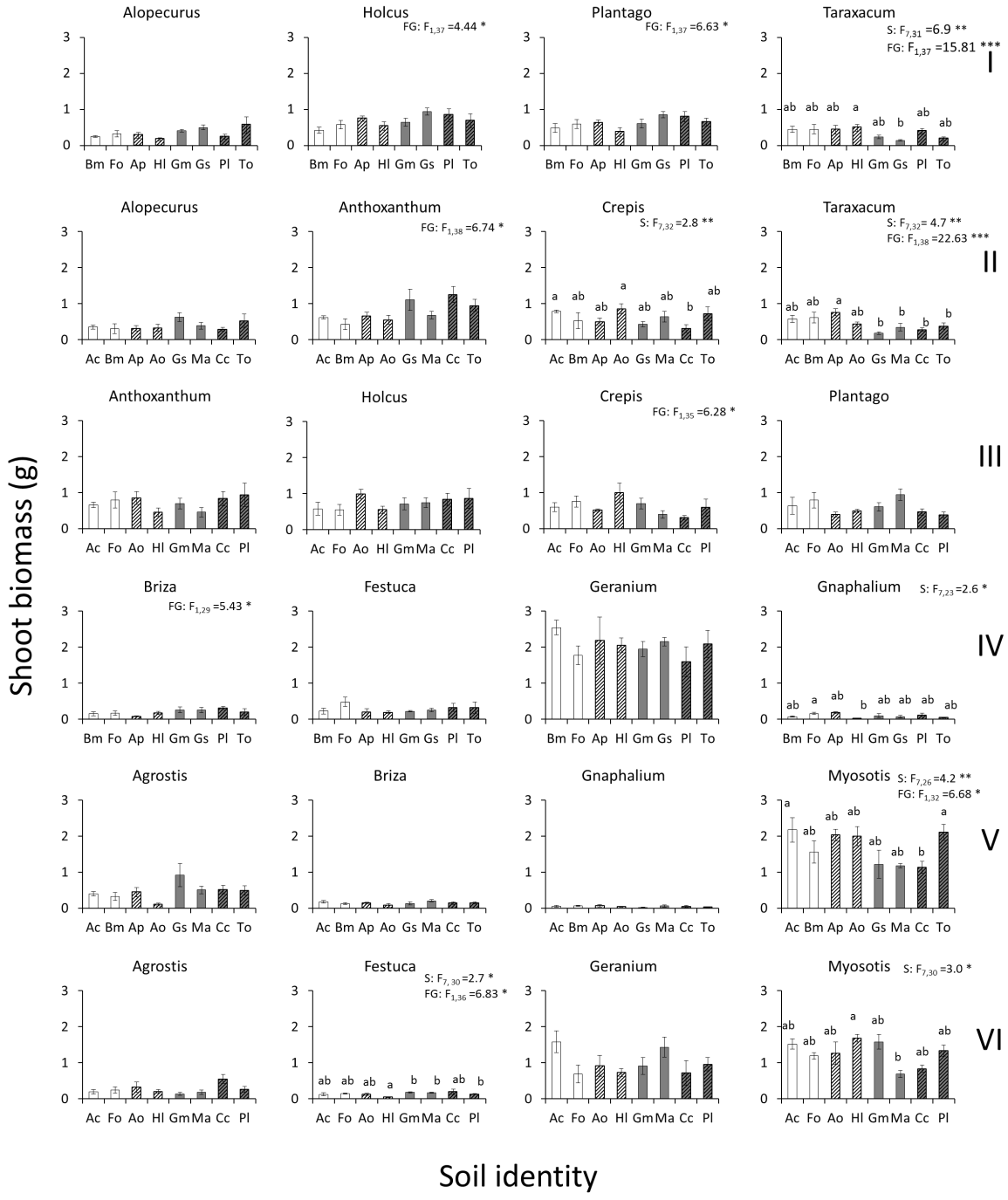
In community V, only *Myosotis arvensis* biomass was affected by identity and functional group of the species that conditioned the soil; they grew larger on grass-conditioned soils than on forb-conditioned soils (FG: $F_{1,32}$ =6.68; p=0.01, S: $F_{7,26}$ = 4.2; p=0.00, see Supplementary Figure S4.1). Consumption on *G. sylvaticum* leaves was significantly higher on *Gnaphalium* that grew on grass soils than on forb soils ($F_{1,32}$ = 5.93; p=0.02, see Supplementary Figure S4.2).

In community VI, *Festuca ovina* biomass was significantly affected by identity and functional group of the species that conditioned the soil; *Festuca* grew larger on soil conditioned by forbs (FG: $F_{1,36}$=6.83; p=0.01, S: $F_{7,30}$ =2.68; p=0.03, see Supplementary Figure S4.1). *Myosotis arvensis* was significantly affected by identity of the species that conditioned the soil (S: $F_{7,30}$ =2.99; p=0.02, see Supplementary Figure S4.1).

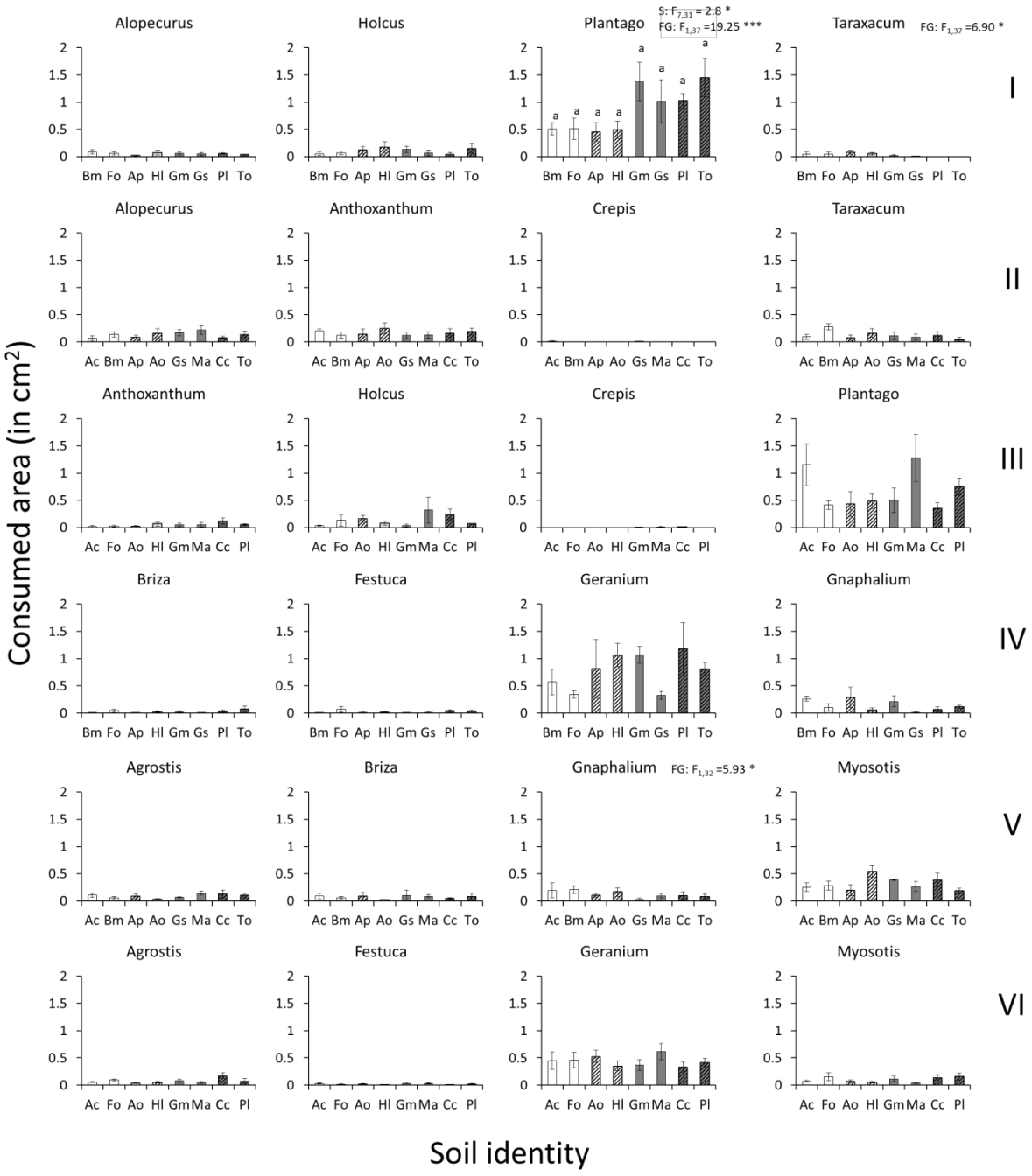
The majority of the consumption (depending on the communities) was on *Plantago, Geranium* and *Myosotis*, but it should be noted that grasses were not left untouched. Interestingly, consumption was not observed on *Crepis*, a forb. In all cages, the caterpillar fed on more than one host plant, and in the majority of the cages, caterpillars fed on all four plant species in the community. Because of the difference in species composition, the six communities differed in quality as a food source, leading to differences in caterpillar growth, as can be seen especially in community II. The low (but consistent on all soils) biomass of the caterpillars, as well as the low consumption of each individual plant species on *Crepis, Taraxacum, Alopecurus* and *Anthoxanthum* suggests that these species are not optimal food plants for herbivore growth (Fig 4B and Supplementary Figure S4.2, community II).

Beta-diversity soil fungi and bacteria:

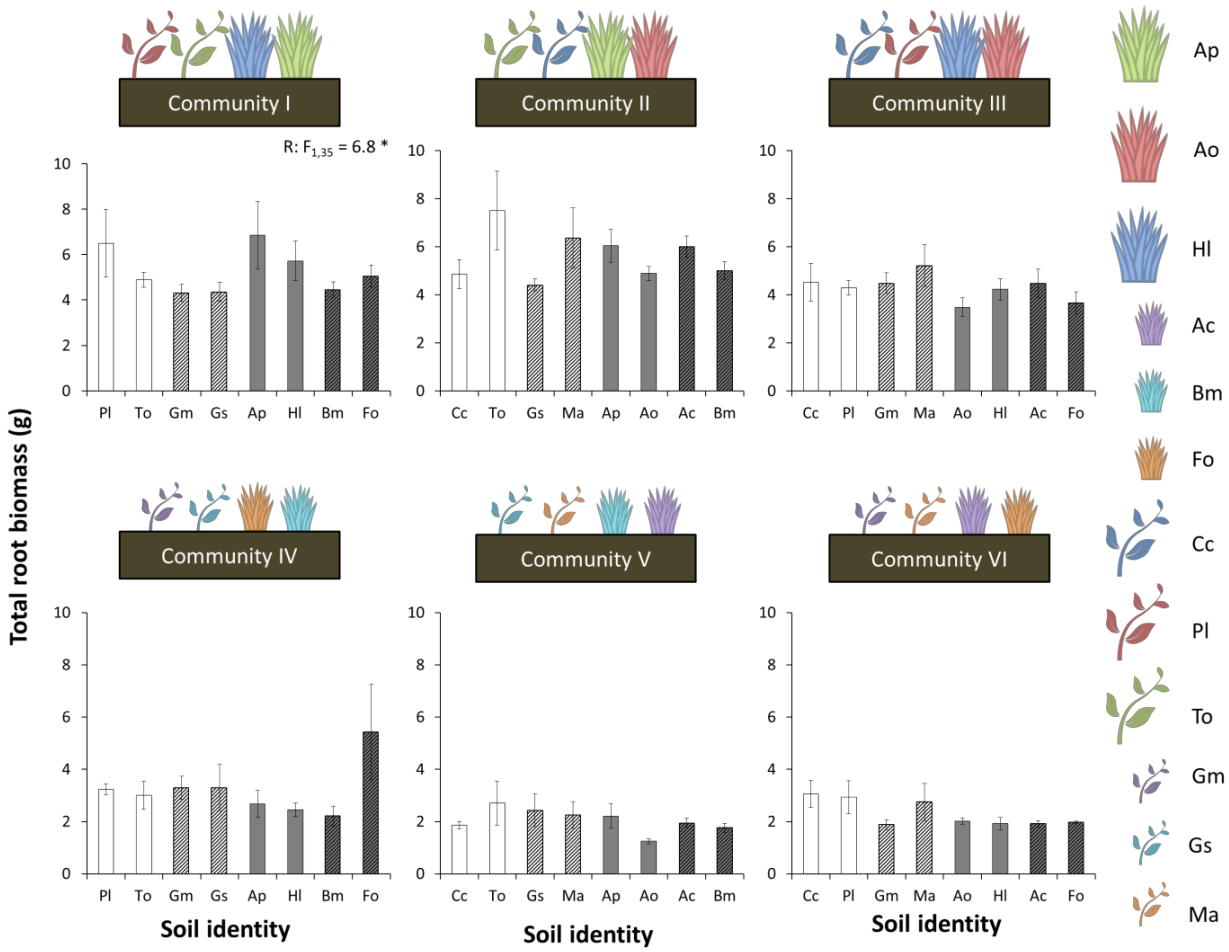
Beta diversity was significantly affected by soil conditioning plant species, as well as functional group for both the soil fungi (Soil: R=0.49; p<0.001; Functional group: R=0.19; p<0.001, see Supplementary Figure S4.4A) and soil bacteria (Soil: R=0.36; p<0.001; Functional group: R=0.19; p<0.05, see Supplementary Figure S4.4B). However, beta diversity was not affected in either group by the root size of plant species (Fungi: R= 0.009; p=0.23; Bacteria: R=0.008; p=0.26).



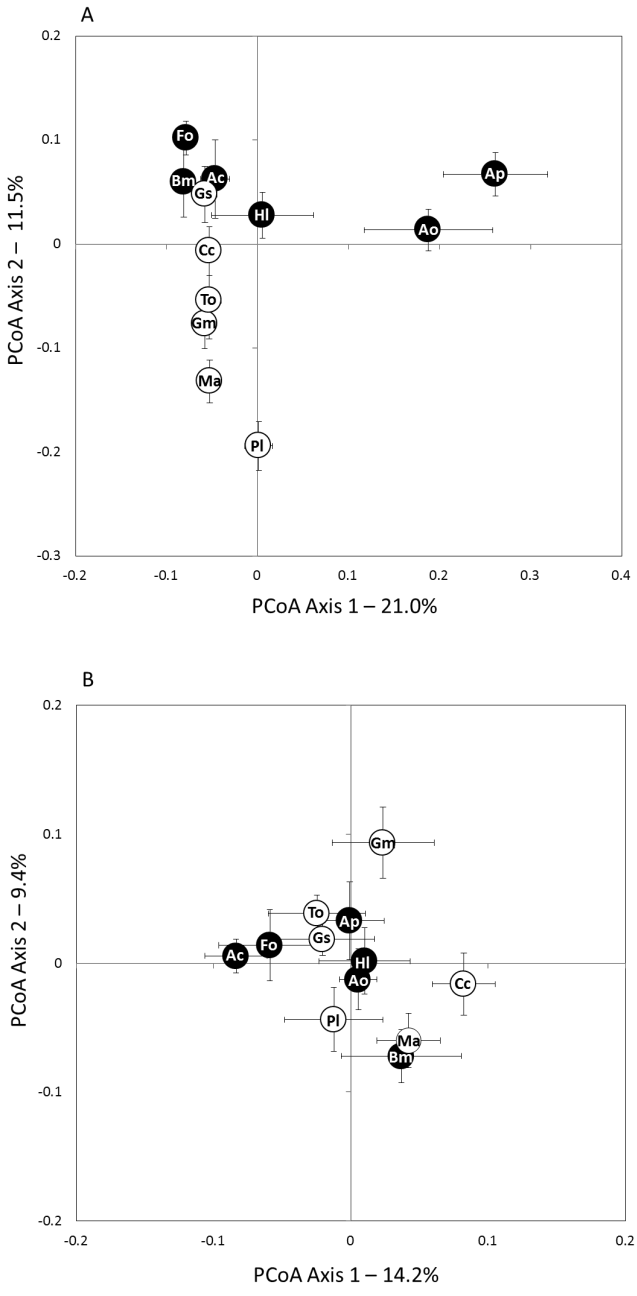
Supplementary Figure S4.1: Effects of soil conditioning of species of grasses and forbs on individual shoot biomass of all plant species (four total) per community (six communities). White bars represent large-rooted forbs, hatched white bars represent small-rooted forbs; grey bars represent large-rooted grasses, hatched grey bars represent small-rooted grasses. Error bars represent standard errors. Ac= *Agrostis capillaris*, Ao= *Anthoxanthum odoratum*, Ap= *Alopecurus pratensis*, Bm= *Briza media*, Cc= *Crepis capillaris*, Fo= *Festuca ovina*, Gm= *Geranium molle*, Gs= *Gnaphalium sylvaticum*, Hl= *Holcus lanatus*, Ma= *Myosotis arvensis*, Pl= *Plantago lanceolata*, To= *Taraxacum officinale*. Statistics in the panels represent main effects of soil identity (S) and soil functional group (FG) derived from one-way ANOVAs. Asterisks represent significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



Supplementary Figure S4.2: Effects of soil conditioning of species of grasses and forbs on areas consumed by herbivores on all individual plant species (four total) per community (six communities). White bars represent large-rooted forbs, hatched white bars represent small-rooted forbs; grey bars represent large-rooted grasses, hatched grey bars represent small-rooted grasses. Error bars represent standard errors. Ac= *Agrostis capillaris*, Ao= *Anthoxanthum odoratum*, Ap= *Alopecurus pratensis*, Bm= *Briza media*, Cc= *Crepis capillaris*, Fo= *Festuca ovina*, Gm= *Geranium molle*, Gs= *Gnaphalium sylvaticum*, Hl= *Holcus lanatus*, Ma= *Myosotis arvensis*, Pl= *Plantago lanceolata*, To= *Taraxacum officinale*. Statistics in the panels represent main effects of soil identity (S) and soil functional group (FG) derived from one-way ANOVAs. Asterisks represent significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



Supplementary Figure S4.3: Effects of soil conditioning of species of grasses and forbs on community root biomass. White bars represent large-rooted forbs, striated white bars represent small-rooted forbs; grey bars represent large-rooted grasses, striated grey bars represent small-rooted grasses. Error bars represent standard errors. Community composition for each community is as follows; Community I: Ap, Hl, Pl, To; Community II: Ap, Ao, Cc, To; Community III: Ao, Hl, Pl, Cc; Community IV: Gm, Gs, Bm, Fo; Community V: Gs, Ma, Ac, Bm; Community VI: Gm, Ma, Ac, Fo. Community composition is also represented by differently colored grass or forb symbols above each panel. Ac= *Agrostis capillaris*, Ao= *Anthoxanthum odoratum*, Ap= *Alopecurus pratensis*, Bm= *Briza media*, Cc= *Crepis capillaris*, Fo= *Festuca ovina*, Gm= *Geranium molle*, Gs= *Gnaphalium sylvaticum*, Hl= *Holcus lanatus*, Ma= *Myosotis arvensis*, Pl= *Plantago lanceolata*, To= *Taraxacum officinale*. Statistics in the panels represent main effects of soil identity (S), root size (R) and soil functional group (FG) derived from one-way ANOVAs. Asterisks represent significance: * = p<0,05; **= p<0,01; ***= p<0,001.



Supplementary Figure S4.4: Effects of soil conditioning on beta diversity of A) soil fungi and B) soil bacteria. Plots shown are the first two axes of PCoA analyses performed on sequence data for ITS (fungi) and 16S (bacteria) markers. Dots represent means of the replicates for each soil (n=5 for each soil, error bars represent standard errors for the means). Black dots represent grasses, white dots represent forbs. Species names are abbreviated as follows; Ac= *Agrostis capillaris*, Ao= *Anthoxanthum odoratum*, Ap= *Alopecurus pratensis*, Bm= *Briza media*, Cc= *Crepis capillaris*, Fo= *Festuca ovina*, Gm= *Geranium molle*, Gs= *Gnaphalium sylvaticum*, Hl= *Holcus lanatus*, Ma= *Myosotis arvensis*, Pl= *Plantago lanceolata*, To= *Taraxacum officinale*. Statistics in the panels represent main effects of soil identity (S) and soil functional group (FG) derived from permutational multivariate ANOVAs. Asterisks represent significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Supplementary Table S4.1: Soil characteristics of live and sterilized field soil. Means are shown with standard errors. For live soils n=8, for sterilized soils n=2.

	Live field soil		Sterile field soil			
	mean	se	mean	se	Method	Standard
pH	4.85	0.06	5.05	0.15	pH in 1:10 v:v KCl2; 1:10 v:v Ca2Cl2; 1:10 v:v H2O, potentiometry	(NEN-ISO 10390)
Organic matter (%)	3.29	0.14	3.00	0.00	Loss on ignition at 550 °C, C measured at 600 °C IR-spectrophotometry	(ISO 10694)
C/N ratio	15.00	0.46	16.00	0.00	Derived value	
N-total mg N/kg	1226.00	173.00	1085.00	25.00	N after burning with thermal resistance	(ISO 13878)
P-total mg P2O5/100g	69.00	7.67	75.00	1.00	P soluble in Ammonium lactate-acetic acid, DA spectrophotometry	(NEN 5793); (NEN-ISO 15923-1)
K-total mmol+/kg	1.48	0.05	1.80	0.10	K exchange with 0.0166M Cobalthexamine trichloride solution (Cohex)	ICP AES (ISO 23470)
S-total mg S/kg	195.00	8.66	180.00	10.00	Total S after sample preparation	(NEN 15587-2); ICP AES (NEN 6966)
N-available kg N/ha	112.13	1.72	108.00	1.00	Derived value	
P-available mg P/kg	4.33	0.49	5.70	0.70	P soluble in 0.01M Ca2Cl2 1:10 m/V DA spectrophotometry	(NEN 5704); (NEN-ISO 15923-1
K-available mg K/kg	49.86	12.88	35.00	1.00	K soluble in 0.01M Ca2Cl2 1:10 m/V DA spectrophotometry	NEN 5704; ICP-AES (NEN 6966)
S-available kg S/ha	6.13	0.30	6.00	1.00	Derived value	

Supplementary Table S4.2: The twelve plant species that were used in this study with functional group and root size. R- represents small rooted species and R+ represents large rooted species. F represents forbs, G grasses. Root biomass, shoot biomass and Root:Shoot Ratios were measured at six weeks and the presented data are mean values (with standard errors between brackets; n=3 for all species).

Symbol	Plant species	Root size	Functional group	Root biomass (g)	Shoot biomass (g)	Root:Shoot Ratio (g)
	*Briza media*	R-	G	0.19 (0.05)	0.35 (0.02)	0.54 (0.11)
	*Festuca ovina*	R-	G	0.67 (0.07)	0.83 (0.03)	0.81 (0.06)
	*Agrostis capillaris*	R-	G	0.69 (0.07)	0.89 (0.16)	0.80 (0.07)
	*Alopecurus pratensis*	R+	G	1.00 (0.15)	1.36 (0.1)	0.76 (0.18)
	*Anthoxanthum odoratum*	R+	G	1.40 (0.18)	1.48 (0.12)	0.95 (0.12)
	*Holcus lanatus*	R+	G	1.82 (0.35)	1.24 (0.11)	1.46 (0.22)
	*Gnaphalium sylvaticum*	R-	F	0.41 (0.03)	0.87 (0.01)	0.47 (0.04)
	*Myosotis arvensis*	R-	F	0.91 (0.02)	1.10 (0.06)	0.83 (0.02)
	*Geranium molle*	R-	F	1.00 (0.09)	1.65 (0.19)	0.61 (0.02)
	*Plantago lanceolata*	R+	F	1.67 (0.13)	1.82 (0.14)	0.92 (0.05)
	*Crepis capillaris*	R+	F	1.99 (0.13)	1.08 (0.06)	1.84 (0.16)
	*Taraxacum officinale*	R+	F	2.12 (0.05)	0.78 (0.02)	2.72 (0.05)

Supplementary Table S4.3: Overview of a) the selected small-rooted and large-rooted grasses and forb that occur in each individual community, b) the fractional factorial combinations of communities and conditioned soils.

a)

	Large-rooted Forbs			Small-rooted Forbs			Large-rooted Grasses			Small-rooted Grasses		
Plant Species	*Plantago*	*Taraxacum*	*Crepis*	*Geranium*	*Gnaphalium*	*Myosotis*	*Holcus*	*Alopecurus*	*Anthoxanthum*	*Festuca*	*Briza*	*Agrostis*
Com I	X	X					X	X				
Com II		X	X					X	X			
Com III	X		X				X		X			
Com IV				X	X					X	X	
Com V					X	X					X	X
Com VI				X		X				X		X

b)

	Soils conditioned by:											
	Large-rooted Forbs			Small-rooted Forbs			Large-rooted Grasses			Small-rooted Grasses		
	Plantago	*Taraxacum*	*Crepis*	*Geranium*	*Gnaphalium*	*Myosotis*	*Holcus*	*Alopecurus*	*Anthoxanthum*	*Festuca*	*Briza*	*Agrostis*
Com I	X	X		X	X		X	X		X	X	
Com II		X	X		X	X		X	X		X	X
Com III	X		X	X		X	X		X	X		X
Com IV	X	X		X	X		X	X		X	X	
Com V		X	X		X	X		X	X		X	X
Com VI	X		X	X		X	X		X	X		X

Supplementary Table S4.4: Output of one-way ANOVAs performed on raw data of each individual plant species within every community, with conditioning species (S) as factor. Statistically significant differences ($p < 0.05$) are presented in **bold** and values in *italics* indicate trends ($0.05 < p < 0.10$).

			Plant		Herbivore			
			Shoot biomass		Consumed area		Aphid number	
	Factors	df1,df2	F	p	F	P	F	P
Community I								
Alopecurus	S	7, 31	0.73	0.65	0.68	0.69	1.10	0.39
	FG	1, 37	*2.91*	*0.10*	0.00	0.95	1.58	0.22
Holcus	S	7, 31	1.87	0.11	0.94	0.49	1.26	0.30
	FG	1, 37	**4.44**	**0.04**	0.14	0.71	0.44	0.51
Plantago	S	7, 31	*1.99*	*0.09*	**2.77**	**0.02**		
	FG	1, 37	**6.63**	**0.01**	**19.25**	**0.00**		
Taraxacum	S	7, 31	**3.52**	**0.01**	1.28	0.29		
	FG	1, 37	**15.81**	**0.00**	**6.90**	**0.01**		
Community II								
Anthoxanthum	S	7, 32	*1.90*	*0.10*	0.62	0.74	0.22	0.98
	FG	1, 38	**6.74**	**0.01**	0.37	0.55	0.89	0.35
Alopecurus	S	7, 32	0.74	0.64	0.66	0.70	1.19	0.33
	FG	1, 38	1.49	0.23	1.87	0.18	1.27	0.27
Crepis	S	7, 32	**2.76**	**0.02**	0.87	0.54		
	FG	1, 38	2.13	0.15	0.06	0.81		
Taraxacum	S	7, 32	**4.65**	**0.00**	1.42	0.23		
	FG	1, 38	**22.63**	**0.00**	*3.21*	*0.08*		
Community III								
Anthoxanthum	S	7, 29	0.91	0.51	0.58	0.77	1.23	0.32
	FG	1, 35	0.00	0.99	1.41	0.24	1.58	0.22
Holcus	S	7, 29	0.91	0.52	1.21	0.33	1.15	0.36
	FG	1, 35	**6.28**	**0.02**	*3.09*	*0.09*	2.37	0.13
Crepis	S	7, 29	1.77	0.13	0.70	0.67		
	FG	1, 35	1.59	0.22	0.64	0.43		
Plantago	S	7, 29	1.27	0.30	1.59	0.18		
	FG	1, 35	0.06	0.80	0.12	0.74		

Community IV								
Briza	S	7, 23	1.21	0.34	0.70	0.67	0.53	0.80
	FG	1, 29	**5.43**	**0.03**	0.38	0.54	0.26	0.61
Festuca	S	7, 23	0.81	0.59	0.62	0.74	1.46	0.23
	FG	1, 29	0.11	0.75	0.20	0.66	1.41	0.25
Geranium	S	7, 23	1.02	0.44	*2.09*	*0.09*		
	FG	1, 29	1.26	0.27	0.83	0.37		
Gnaphalium	S	7, 23	**2.55**	**0.04**	*2.14*	*0.08*		
	FG	1, 29	0.05	0.82	0.92	0.35		
Community V								
Agrostis	S	7, 26	*1.99*	*0.10*	0.73	0.65	0.47	0.85
	FG	1, 32	*3.28*	*0.08*	2.14	0.15	0.07	0.79
Briza	S	7, 26	1.73	0.15	0.18	0.99	0.52	0.81
	FG	1, 32	1.31	0.26	0.10	0.76	0.42	0.52
Gnaphalium	S	7, 26	0.82	0.58	0.91	0.51		
	FG	1, 32	*3.24*	*0.08*	**5.93**	**0.02**		
Myosotis	S	7, 26	**4.15**	**0.00**	0.97	0.48		
	FG	1, 32	**6.68**	**0.01**	0.01	0.91		
Community VI								
Agrostis	*S*	7, 30	1.70	0.15	1.81	0.12	1.27	0.30
	FG	1, 36	0.01	0.95	1.22	0.28	1.80	0.19
Festuca	S	7, 30	**2.68**	**0.03**	1.43	0.23	1.89	0.11
	FG	1, 36	**6.83**	**0.01**	0.12	0.73	1.37	0.25
Geranium	S	7, 30	1.79	0.12	0.65	0.71		
	FG	1, 36	0.05	0.83	0.01	0.91		
Myosotis	S	7, 30	**2.99**	**0.02**	1.13	0.37		
	FG	1, 36	2.81	0.10	0.49	0.49		

Plant community composition but not plant traits determine the outcome of soil legacy effects on plants and insects

Chapter 5

Species-specific plant–soil feedbacks alter herbivore-induced gene expression and defense chemistry in *Plantago lanceolata*

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Species-specific plant–soil feedbacks alter herbivore-induced gene expression and defense chemistry in Plantago lanceolata

Abstract

Plants actively interact with antagonists and beneficial organisms occurring in the above- and belowground domains of terrestrial ecosystems. In the past decade, studies have focused on the role of plant–soil feedbacks (PSF) in a broad range of ecological processes. However, PSF and its legacy effects on plant defense traits, such as induction of defense-related genes and production of defensive secondary metabolites, have not received much attention. Here, we study soil legacy effects created by twelve common grassland plant species on the induction of four defense-related genes, involved in jasmonic acid signaling, related to chewing herbivore defense (LOX2, PPO7), and in salicylic acid signaling, related to pathogen defense (PR1 and PR2) in *Plantago lanceolata* in response to aboveground herbivory by *Mamestra brassicae*. We also assessed soil legacy and herbivory effects on the production of terpenoid defense compounds (the iridoid glycosides aucubin and catalpol) *in P. lanceolata*. Our results show that both soil legacy and herbivory influence phenotypes of *P. lanceolata* in terms of induction of Pl PPO7 and Pl LOX2, whereas the expression of Pl PR1 and Pl PR2-1 is not affected by soil legacies, nor by herbivory. We also find species-specific soil legacy effects on the production of aucubin. Moreover, *P. lanceolata* accumulates more catalpol when they are grown in soils conditioned by grass species. Our study highlights that PSF can influence aboveground plant–insect interactions through the impacts on plant defense traits and suggests that aboveground plant defense responses can be determined, at least partly, by plant-specific legacy effects induced by belowground organisms

Introduction

As plants are members of complex communities, they simultaneously interact with both antagonists and beneficial organisms occurring both above and below the ground (Pieterse et al. 2013; Biere and Goverse 2016). To cope with challenges by harmful pathogens and insect herbivores, plants have evolved a complex immune system that modulates plant defensive responses, from recognition of alien molecules or signals from damaged plant cells to activation of effective immune responses against the attackers (Jones and Dangl 2006; Howe and Jander 2008). The phytohormones jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) act as major players in coordinating the signaling pathways involved in multi-trophic species interactions among plants, microbes, and insects (Anand et al. 2008; Erb et al. 2012; Pieterse et al. 2012). In addition, beneficial relationships between plants and soil microbes are common in nature as well, improving plant growth or enhancing the plant's ability to cope with biotic or abiotic stress (Pineda et al. 2010; Pieterse et al. 2014). Benefits of the associations with microbes to the plants are often based on the growth-promoting effects of beneficial microbes, as well as on the activation of induced systemic resistance (ISR) resulting in sensitization of the plant immune system (priming) for a more efficient activation of plant defenses upon a future attack (Zamioudis and Pieterse 2012). Beneficial rhizosphere microbes can prime the plant for enhanced defense against a broad range of insect herbivores (Van Oosten et al. 2008; Van Wees et al. 2008; Jung et al. 2012; van de Mortel et al. 2012; Pangesti et al. 2013).

The fitness and performance of a plant can depend greatly on the conditions of the soil it grows in (Bardgett and Wardle 2010). The soil is where plants get their water and nutrients from, but it is also the center stage for interactions with a wide range of soil biota. Soil biota profoundly contribute to plant growth and productivity, and their effects range from positive to negative via respectively mutualistic or antagonistic interactions (Berendsen et al. 2012; van der Putten et al. 2013). Plants, in turn, influence the composition of the soil community around their roots via the excretion of root exudates or sheathing of dead root cells. Plant species can differ greatly in the composition and amount of these deposits, and this can lead to plant species-specific soil communities (Philippot et al. 2013; Shahzad et al. 2015). These specific soil communities can influence the performance of other plants that grow later in the same soil, a process called plant–soil feedback (PSF) (Bever 1994; van der Putten et al. 2013). PSFs can be conspecific, when the plant that grew previously in the soil affects future growth of plants of the same

species, or heterospecific, when the plant species that grew previously in the soil affects future growth of other plant species. During the past decade, PSF and its legacy effects have been extensively studied in the context of plant community dynamics, such as environmental change-related range shifts, ecological succession, biological invasion and biodiversity (van der Putten et al. 2013). Recent studies revealed that induced changes in the composition of soil biota by plants could also affect aboveground multitrophic plant–insect interactions (Kostenko et al. 2012; Kos et al. 2015a; Heinen et al. 2018). Moreover, aboveground herbivory in turn can affect the outcome of PSF effects (Heinze and Joshi 2018). The functional group that a plant belongs to may also explain the way in which it influences its soil. Several studies have observed that grasses induce more positive PSF effects than forbs (van de Voorde et al. 2011; Kos et al. 2015b), and that aboveground insect herbivores perform differently on plants growing in forb-conditioned and grass-conditioned soil (Heinen et al. 2018). So far, the mechanistic understanding of how PSFs influence aboveground plant–insect interactions through affecting induced defensive responses in the plant, and how this interacts with aboveground insect herbivory on the plant, remains poorly studied.

To date, a recurring problem in insect–plant research is that most of the knowledge on defense mechanisms, especially defense gene expression, is based on model species (Heidel and Baldwin 2004; de Vos et al. 2006), or on a selected group of economically important plants such as tomato, pepper or maize (Chen et al. 2015). However, some ecologically relevant wild plant species, such as *Jacobaea vulgaris*, *Plantago lanceolata* and various species in the Brassicaceae family, have been used to study chemical defenses in response to soil biota, which has led to a better understanding of above–belowground ecology (Bezemer et al. 2006a; Soler et al. 2007; Kostenko et al. 2012; Wang et al. 2014, 2015; Kos et al. 2015a). Ribwort Plantain, *P. lanceolata* has a worldwide distribution and has been used as model species addressing plant-mediated above–belowground interactions (e.g., Gange and West 1994; Wurst et al. 2008; Bennett and Bever 2009; Wang et al. 2015). A group of plant secondary defense metabolites that has been well-characterized and well-studied for its ecological role in *P. lanceolata* are iridoid glycosides (IGs). In response to aboveground herbivory and soil biota, such as mycorrhizae or root herbivorous insects, the production of IGs often increases in the plant (Gange and West 1994; Wurst et al. 2008; Bennett and Bever 2009; Schweiger et al. 2014; Wang et al. 2014, 2015). These compounds act as feeding deterrents against generalist herbivores (Puttick and Bowers

1988; Biere et al. 2004; Harvey et al. 2005; Reudler et al. 2011), but can also be used as feeding and oviposition stimulants by specialist herbivores (Bowers and Puttick 1989; Nieminen et al. 2003). Previous studies have examined the effects of addition of single soil organisms on secondary defense responses, but how 'whole community' PSF processes influence plant defense has thus far not been studied in detail.

To investigate whether PSF and insect herbivory affect *P. lanceolata* defense responses, we selected four orthologs of genes that are involved in the interactions between plant and biotic agents both above- and belowground. These included a polyphenol oxidase (Pl PPO7), a lipoxygenase (Pl LOX2-2), and two pathogenesis-related proteins (Pl PR1 and Pl PR2-1). Previous studies have shown that Pl LOX2-2 and Pl PPO7 are strongly induced in *P. lanceolata* after the application of JA, whereas Pl PR1 and Pl PR2-2 are induced by SA (Supplementary Figure S5.1). First, *Arabidopsis* LOX2 is a key enzyme in the JA biosynthesis pathway induced by (generalist) chewing insect herbivores. LOX2 orthologs are commonly used as markers of JA-mediated defense responses (Chauvin et al. 2013). Second, in several plant species foliar JA-inducible PPOs play a key role in defense against a number of leaf chewing herbivores (Mayer 2006; Bosch et al. 2014). Third, the pathogenesis-related protein PR1 is often used as a marker for SA-mediated disease resistance. It is among the most abundantly produced proteins in plants following infection by biotrophic pathogens (Breen et al. 2017). Finally, PR2 also serves as an SA-marker. Orthologs encode a ß-1,3-glucanase that has been proposed to degrade the cell walls of invading fungal pathogens. Possibly PR-proteins like PR-2 have enzymatic activities that generate elicitors of defense responses (van Loon et al. 2006).

In this study, to obtain species-specific conditioned soils, we grew twelve different co-occurring grassland plant species (including the current focal plant *P. lanceolata*) individually in live field collected soil. We then grew *P. lanceolata* in all twelve soils during a feedback phase and exposed a subset of these plants to aboveground herbivory by the chewing insect herbivore *Mamestra brassicae* (Lepidoptera: Noctuidae). We quantified the expression levels of *P. lanceolata* homologues of LOX2, PPO, PR1 and PR2. We also measured concentrations of the defense chemicals aucubin and catalpol (the two major IGs in *P. lanceolata*) in shoots. We address three main questions: (1) Do PSFs of the twelve plant species differ in how they influence the expression of above- and belowground defense-related genes in *P. lanceolata*, and does this interact with the response of the plant to aboveground herbivory? (2) Do PSFs

affect chemical defense in *P. lanceolata* leaves? (3) Do PSFs of grasses and forbs differ in how they influence IG levels and defense gene expression in *P. lanceolata* and interact with aboveground herbivory?

Materials and methods

Field soil

Field soil was collected from a natural grassland site 'De Mossel' (N52°3′, E5°44′, Natuurmonumenten, Ede, The Netherlands). This field has been in use as an experimental field site since 1996 and the soil has been used in numerous plant–soil studies (e.g., Bezemer et al. 2006a, b; Heinen et al. 2018). Live soil was taken from the top 10 cm, the well-rooted layer containing most of the rhizosphere biota. Soil was sieved to remove roots, stones and most macro-invertebrates (sieve mesh Ø1.0 cm).

Plants and insects

Ribwort Plantain (*P. lanceolata*) was used as a focal species. In previous studies, this species has been shown to be responsive to soil legacies and various biotic players in the soil (Bezemer et al. 2006b; Wurst et al. 2008; Wang et al. 2014, 2015), and its secondary chemistry has been well characterized (Duff et al. 1965; Bowers et al. 1992). RNA transcriptional data (RNAseq) were available for primer design from previous work at the Netherlands Institute of Ecology (A. Biere, unpublished data).

Seeds of *P. lanceolata* were surface-sterilized using 2.5% bleach solution and then rinsed with demineralized water. For germination, seeds were placed on sterile glass beads in a climate cabinet (light regime 16:8, L:D, day temperature 21 °C, night temperature 16 °C). After germination, the seedlings were stored at 4 °C under the same light regime, for later use in experiments. Seeds were obtained from Cruydt-Hoeck (Nijberkoop, The Netherlands).

Eggs of the Cabbage moth, *M. brassicae* were obtained from the Department of Entomology at Wageningen University, The Netherlands. The cabbage moth had been reared for several years on Brussels Sprouts, *Brassica oleracea* var. *gemmifera* cv. Cyrus. The larvae were originally collected from cabbage fields near the university. *M. brassicae* is a generalist chewing herbivore native to the Palearctic. It is known to feed on many species of grasses and forbs, including *P. lanceolata* (Heinen et al. 2018).

Soil conditioning phase

Twelve common grassland plant species were chosen for soil conditioning, including six forbs: *P. lanceolata* (Plantaginaceae; PL), *Crepis capillaris* (Asteraceae; CC), *Taraxacum officinale* (Asteraceae; TO), *Myosotis arvensis* (Boraginaceae; MA), *Geranium molle* (Geraniaceae; GEM), and *Gnaphalium sylvaticum* (Asteraceae; GS); and six grasses (all Poaceae): *Anthoxanthum odoratum* (AO), *Alopecurus pratensis* (AP), *Holcus lanatus* (HL), *Agrostis capillaris* (AC), *Briza media* (BM), and *Festuca ovina* (FO). Per plant species, five replicate pots were used to condition the soil. Square pots (11×11 cm) were filled with 1050 g live field soil topped off with a 0.5 cm layer of fine white sand to prevent oviposition by fungus gnats. In each pot, one seedling was grown for 10 weeks. Plants were kept at 17% soil moisture. After 10 weeks, the plants and their roots were removed from each pot, and the conditioned soil was mixed with sterilized field soil (1:2 conditioned:sterile v/v) to reduce variation in soil nutrient availability, keeping the five replicates separate. Sterile soil was obtained by γ-irradiation (>25 Kgray, Synergy Health, Ede, The Netherlands), using the live soil that was collected from the field site.

Feedback phase

New 11×11 cm square pots were filled with 1050 g of the mixtures. Two pots were filled with the same soil for each of the replicates in this experiment, one was assigned to the aboveground herbivory treatment and the other one was kept without herbivory (12 conditioned soils, two treatments (herbivore/control), five independent replicates, totaling 120 pots). Each individual pot was planted with a P. lanceolata seedling and covered by shade cloth for 3 days. After the seedlings established, the shade cloth was removed. The individual plants were grown for 4 weeks.

Insect treatment

Plants from both the undamaged control and herbivory treatment were caged using a transparent plastic tube (8 cm Ø; 25 cm high) with a 5-cm mesh covering the top of the cage. Plants allocated to the insect herbivory treatment received one newly hatched L1 *M. brassicae* caterpillar just prior to placing the cage over the plant. The insects were left to feed for 7 days, after which they were removed and the plants were harvested. The removed caterpillars were weighed, and for each plant we measured the absolute leaf area that was consumed by the caterpillar. This was assessed using a visual reference square of 25 mm2 (5×5 mm) and then

estimating the number of times that this visual reference would fit in the total consumed area. The number of squares was multiplied by 25 to get the consumed area per plant in mm2.

Sampling

Immediately after removing the caterpillars, the plants were harvested by clipping the aboveground plant parts with sharp surgical scissors just above soil level. The scissors were cleaned between all clippings with 10% SDS (Biorad, The Netherlands). All leaves of each plant were then folded in aluminum foil and placed in liquid nitrogen before storage in −80 °C until subsequent sample preparations. Prior to analysis, samples were homogenized per plant in liquid nitrogen and a subsample was taken (fresh) for transcriptome analysis. A second subsample was taken and freeze-dried for use in the chemical analyses.

Quantitative real-time PCR

Total RNA was isolated and purified from finely ground and homogenized leaf material originating from individual replicate plants with the ISOLATE II RNA Plant Kit (Bioline). Subsequently cDNA was synthesized from RNA (adjusted to 1 µg/µl) using SensiFAST™ cDNA Synthesis Kit (Bioline). To investigate whether PSF and insect herbivory affect P. lanceolata defense responses, we selected four genes that are involved in the interactions between plant and biotic agents, including a polyphenol oxidase (Pl PPO7), a lipoxygenase (Pl LOX2-2), and two pathogenesis-related proteins (Pl PR1 and Pl PR2-1). Pl LOX2-2 and Pl PPO7 are induced by JA, involved in signaling of generalist chewing herbivores, whereas Pl PR1 and Pl PR2-2 are induced by SA, involved in signaling of biotrophic pathogens (Supplementary Figure S5.1). Gene specific primers were designed using Primer3Plus (http://www.bioinformatics.nl/primer3plus/) and were tested for specificity and efficiency before qPCR experiments. The primer sequences used in this study are listed in Table S1. Quantitative RT-PCR analysis was performed in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Each reaction was performed in a total volume of 20 µl containing 10 µl SensiFAST SYBR® No-ROX Mix (Bioline), 5 µl cDNA and 1 µl of 400 nM forward and reverse gene specific primer pair. For each reaction, two technical replicates were carried out and average values were used in the analyses. The following PCR program, including a melting curve analysis, was used for all PCR reactions: 3 min 95 °C, followed by 40 cycles of 5 s 95 °C, 10 s 60 °C, and 20 s 72 °C. The normalized expression level of each gene was calculated under the assumption of 100% primer efficiency by means of the

2−(ΔCt) method (formula 7 of Livak and Schmittgen 2001) using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (Pl GAPDH) as a reference. The ΔCt values were also used for statistics.

Iridoid glycosides

To determine iridoid glycoside levels in P. lanceolata, plant samples were freeze-dried for 3 days under vacuum (−55 °C collector temperature; Labconco Free Zone 12 L Freeze Dry System, USA), finely ground and weighed. Twenty-five mg of each sample was extracted overnight in 10 ml, at room temperature in 70% methanol (LichroSolv, VWR) using a horizontal shaker, then filtered and diluted ten times with ultrapure water. The concentrations of the IGs (aucubin and catalpol, Sigma-Aldrich) were analyzed using high-performance liquid chromatography (HPLC, Bioinert 1260 Infinity, Agilent) with electro chemical detection (ECD, Decade elite ECD, Antec). For HPLC quantification, five microliters of filtered extracts and standards was analyzed at 20 °C with a Dionex™ Guard column CarboPac PA1 2×50 mm, Main column CarboPac PA1 2×250 (Thermo Fisher Scientific). The isocratic mobile phase contained 100% 0.1 M NaOH at a flow rate of 0.25 ml/min, runtime 35 min. Retention time (RT) was 3 and 5 min for aucubin and catalpol, respectively. The standard concentration range was 0.125–2.5 ppm.

Statistical analyses

Main effects and interactions of 'soil' (12 conditioning species) and 'herbivory' (herbivory/control) on the relative expression levels (ΔCt) of the four selected P. lanceolata genes, as well as the concentrations of IGs (aucubin and catalpol) were analyzed by means of two-way ANOVAs. Post-hoc multiple comparisons were conducted using Tukey–Kramer tests to compare the differences among means if the models were significant.

As the conditioning species consisted of grasses and forbs, we subsequently analyzed the parameters with a general linear mixed model with 'functional group' as fixed factor, and 'soil identity' (12 conditioning species) as random factor.

The relationship between mean insect growth and consumption per soil treatment and mean levels of catalpol, aucubin, and four defense-related genes was determined using regression analysis.

All analyses were performed in R Studio, R version 3.0.3 (R Development Core Team 2008). Mixed models were performed using the 'nlme' package (Pinheiro et al. 2017).

Results

Effects on plant biomass

We found a marginally significant effect of soil on shoot fresh biomass ($F_{11,96}$=1.8, p=0.065, Figure 5.1a). Soil significantly affected *P. lanceolata* belowground dry biomass ($F_{11,96}$=3.1, p=0.001, Figure 5.1b). This effect was driven by the strongly negative effect of *T. officinale*, *C. capillaris* and *P. lanceolata* soils compared to other soils. There also was an almost significant interaction between functional group of the species that conditioned the soil and herbivore treatment ($F_{1,106}$=3.6, p=0.061). Plants grown on forb-conditioned soils tended to produce more root biomass when they experienced herbivory than control plants, whereas this was not observed for plants grown on grass-conditioned soils (see Figure 5.1b).

Effects on defense related gene expression

Among the four defense-related genes in *P. lanceolata*, the relative expression of Pl PPO7 was significantly affected by soil conditioning species and by herbivory (Soil: $F_{11,95}$=2.87; p=0.003; Herbivory: $F_{1,95}$=9.73; p=0.002). Pl PPO7 expression levels were higher under herbivory treatments, but the levels varied when plants were grown on different soils (Figure 5.2a). The expression level was highest when *P. lanceolata* was grown on soils that were previously conditioned by *G. sylvaticum* and lowest on soils conditioned by *M. arvensis*, *A. odoratum* and *A. pratensis*.

A significant interactive effect of herbivory treatment and soil conditioning species was found on the expression of Pl LOX2-2 (Herbivory×Soil: $F_{11,96}$=2.17; p=0.022). The expression was upregulated by herbivory treatment on some soils (i.e., *P. lanceolata*, *T. officinale*, *H. lanatus* and *F. ovina*), but downregulated (as compared to caged control plants on the same soils) on soils conditioned by some of the other species (most notably *A. capillaris*, *B. media*, *C. capillaris* and *G. sylvaticum*, Figure 5.2b).

Expression of Pl PR1 and Pl PR2-1 was not affected by herbivory treatments, although we found a marginally significant effect of soil on Pl PR1 expression (Soil: $F_{11,94}$=1.87; p=0.053, Figure

5.2c and 5.2d, Supplementary Table S5.2), most likely driven by the high levels found in *P. lanceolata* grown on soils conditioned by *C. capillaris*.

For Pl PPO7, the transcript levels were slightly higher in plants that had been grown in forb-conditioned soils compared to those that had been grown in grass-conditioned soils (Functional group: $F_{1,10}=4.53$; $p=0.059$).

Effects on plant chemistry

The plant species that conditioned the soil significantly differed in how they affected concentrations of aucubin in shoots of *P. lanceolata* ($F_{11,96}=2.40$; $p=0.011$; Figure 5.3a). Catalpol was not affected by soil conditioning (Supplementary Table S5.2). Aucubin levels of plants grown in soils conditioned by *T. officinale*, were relatively low, whereas levels in soils conditioned by *C. capillaris*, *M. arvensis* and *G. molle* were two to three times higher than those in soils conditioned by *T. officinale* (Figure 5.3a). Catalpol levels were significantly higher in *P. lanceolata* plants that were grown on grass-conditioned soils, than those that were grown on forb-conditioned soils ($F_{1,10}=5.76$; $p=0.037$, Figure 5.3b).

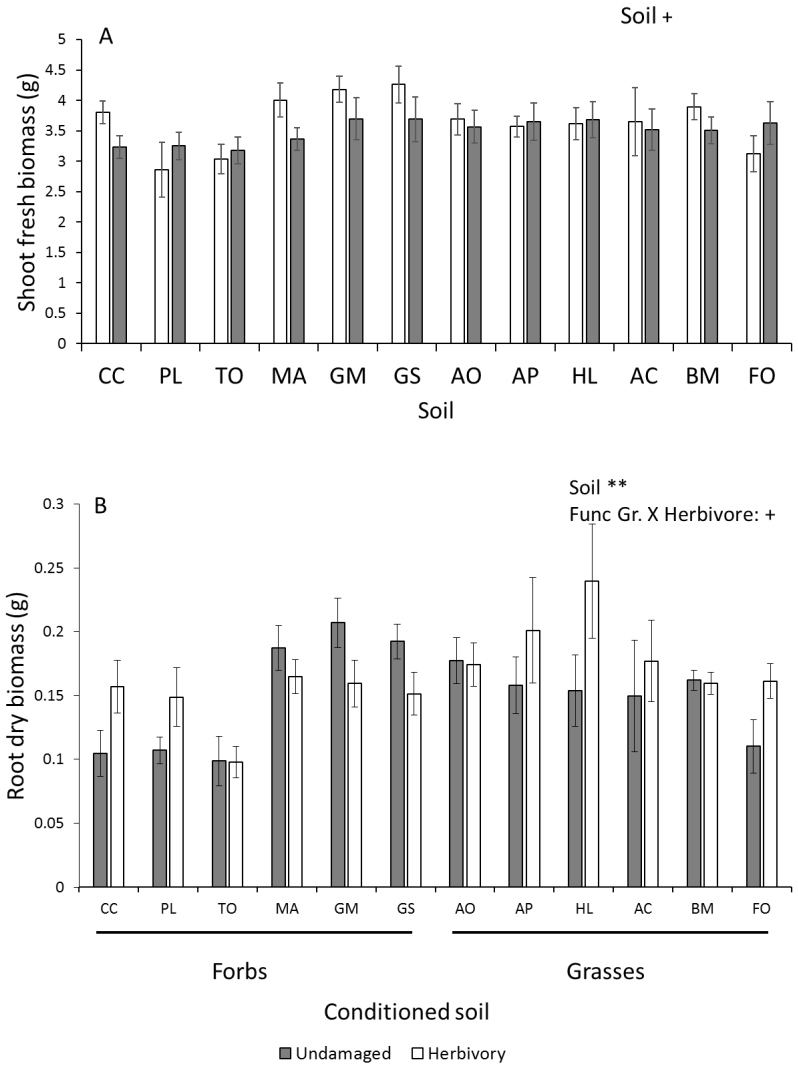

Figure 5.1: The effects of soil conditioning by twelve common grassland species and herbivory treatments on **a** shoot and **b** root biomass of *Plantago lanceolata.* Grey bars represent undamaged plants and white bars represent plants exposed to herbivory (*Mamestra brassicae*). Error bars represent standard errors. For each treatment combination, $n=5$. Asterisks represent significant effects; $+p<0.07$, $*p<0.05$, $**p<0.01$. Soils were conditioned by either forb or grass species. CC, *Crepis capillaris*; PL, *Plantago lanceolata*; TO, *Taraxacum officinale*; MA, *Myosotis arvensis*; GM, *Geranium molle*; GS, *Gnaphalium sylvaticum*; AO, *Anthoxanthum odoratum*; AP, *Alopecurus pratensis*; HL, *Holcus lanatus*; AC, *Agrostis capillaris*; BM, *Briza media*; FO, *Festuca ovina*

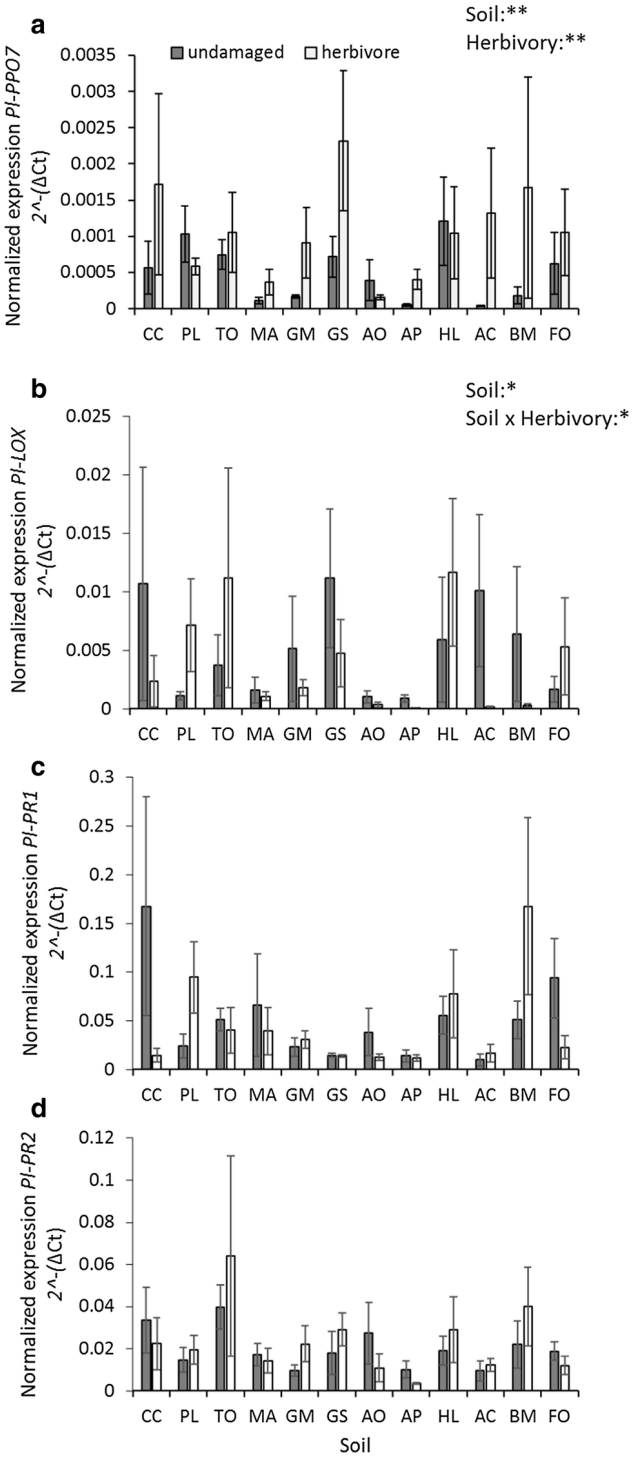

Figure 5.2: The effects of soil conditioning by twelve common grassland species and herbivory treatments on the relative gene expression levels of four genes in the shoots of Plantago lanceolata: Pl PPO7 (a), Pl LOX2-2 (b), Pl PR1 (c) and Pl PR2-1 (d). Values represent normalized gene expression levels [$2^{-(\Delta Ct)}$)] relative to GAPDH. Grey bars represent undamaged and white bars represent herbivory (Mamestra brassicae) treatments. Error bars represent standard errors. For each treatment combination, n=5. Asterisks represent significant effects; *p<0.05, **p<0.01. Soils were conditioned by either forb or grass species. CC, *Crepis capillaris*; PL, *Plantago lanceolata*; TO, *Taraxacum officinale*; MA, *Myosotis arvensis*; GEM, *Geranium molle*; GS, *Gnaphalium sylvaticum*; AO, *Anthoxanthum odoratum*; AP, *Alopecurus pratensis*; HL, *Holcus lanatus*; AC, *Agrostis capillaris*; BM, *Briza media*; FO, *Festuca ovina*.

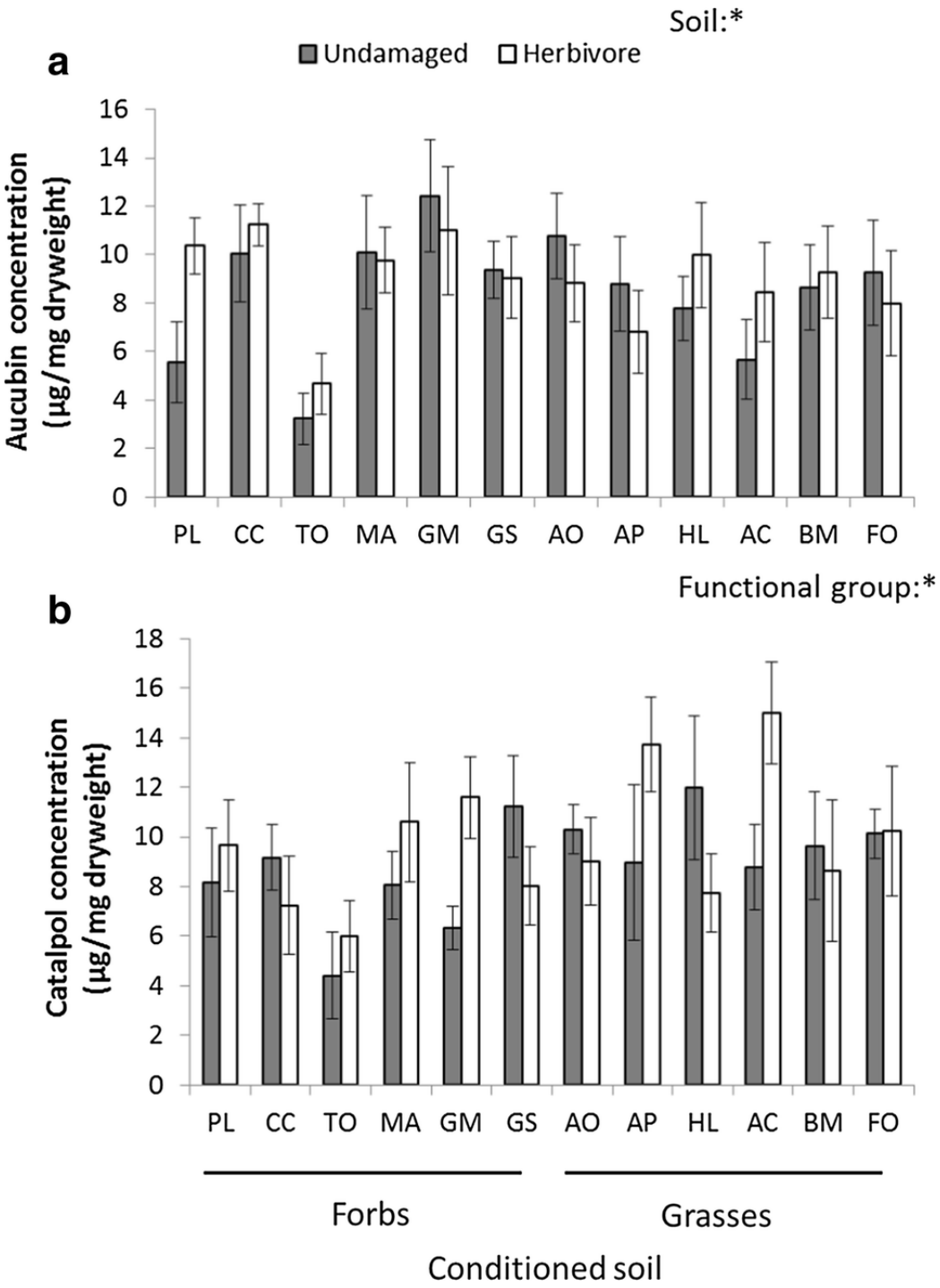

Figure 5.3 The effects of soil conditioning by twelve common grassland species and herbivory treatment on levels of aucubin (**a**) and catalpol (**b**), in the shoots of *Plantago lanceolata*. Grey bars represent undamaged and white bars represent herbivory (*Mamestra brassicae*) treatments. Error bars represent standard errors. For each treatment combination, $n=5$. Asterisks represent significant effects; *$p<0.05$, **$p<0.01$. Soils were conditioned by either forb or grass species. CC, *Crepis capillaris*; PL, *Plantago lanceolata*; TO, *Taraxacum officinale*; MA, *Myosotis arvensis*; GEM, *Geranium molle*; GS, *Gnaphalium sylvaticum*; AO, *Anthoxanthum odoratum*; AP, *Alopecurus pratensis*; HL, *Holcus lanatus*; AC, *Agrostis capillaris*; BM, *Briza media*; FO, *Festuca ovina*

Effects on caterpillar performance

Species-specific soil legacies did not influence biomass of *M. brassicae* larvae ($F_{11,37}$=0.57, p=0.84, Supplementary Figure S5.2a), nor leaf area consumption by the caterpillars ($F_{11,42}$=1.27; p=0.28, Supplementary Figure S5.2b).

Correlations between consumption and caterpillar biomass

Caterpillar biomass showed a marginally significant positive correlation with caterpillar consumption (R^2=0.33, p=0.052, Supplementary Figure S5.3).

Discussion

In this study, we examined how soil legacy effects and aboveground herbivory interact to influence growth and defense responses in the perennial forb *P. lanceolata*. We assessed treatment effects on the transcript levels of four defense-related genes, and measured the production of two secondary defense metabolites, catalpol and aucubin. Our results show that soil conditioning by plants can influence the response of the plant in terms of defense-related gene expression and the production of secondary defense metabolites.

Ribwort plantain, when exposed to *M. brassicae* infestation, showed an up-regulation in transcription of the defense-related gene Pl PPO7 that putatively codes for a polyphenol oxidase (PPO). PPOs are known to be induced by herbivory and confer resistance to a broad range of insect herbivores (War et al. 2012). Interestingly, we found that soil conditioning by different plant species also can influence transcript levels of Pl PPO7. Moreover, we found an interaction between herbivory and the plant species that conditioned the soil on the overall transcript levels of Pl LOX2-2, a gene that is involved in the biosynthesis of JA. Pl LOX2-2 was up-regulated by herbivory in some of the conditioned soils, most notably in soils conditioned by *P. lanceolata*, *T. officinale*, *H. lanatus* and *F. ovina*. However, on other soils, herbivory showed no effect on transcript levels of Pl LOX2-2, or the gene had a lower expression under the herbivory treatment, compared to control plants (most notably in soils conditioned by *C. capillaris*, *G. sylvaticum*, *A. capillaris* and *B. media*). These results suggest that, at the transcription level, the JA-mediated defensive responses against chewing herbivores may depend on the soil that *P. lanceolata* is growing in. In this study plant material was sampled when the caterpillars had fed on plants for 7 days, thus we were not able to detect the induction of Pl LOX2-2 at early stages of herbivory. As lipoxygenase genes are generally considered to

respond relatively fast to herbivore damage (Heitz et al. 1997), future studies should follow these induction patterns through a time series.

SA-regulated defense responses are often associated with piercing and sap-sucking insects and with biotrophic and hemibiotrophic phytopathogens (Anand et al. 2008; Pieterse et al. 2014). Soil pathogens are often considered to be important drivers of PSF effects (van der Putten et al. 2013). Therefore, we expected that specific PSFs would affect soil biotic conditions and thereby affect the activation of SA related genes in the plant. In our study, the transcript levels of Pl PR2-1, a marker related to the SA signaling pathway, was not strongly affected by the treatments although we found a marginally significant effect of soil conditioning on its homolog marker Pl PR1.

Besides harmful pathogens, soils also host microbes that have beneficial relationships with the host plants (Philippot et al. 2013). These beneficial soil microbes, such as mycorrhizae and plant-growth promoting rhizobacteria, have been shown to prime the plant for effective defense responses (Pozo and Azcon-Aguilar 2007; Jung et al. 2012). Soil conditioning likely also influences the compositions of other soil organisms that may alter a plant's phenotype. Although soil biotic composition was not specifically characterized in this study, in another experiment, performed with the same plant species as we used here, and carried out under similar experimental conditions, plants greatly impacted the structure of soil microbial communities (Heinen et al. 2018).

In the current study, chewing herbivores were used as the inducer of plant defenses. Since chewing herbivores generally invoke the JA pathway rather than the SA pathway (Ali and Agrawal 2012), the absence of an effect of herbivory on the expression of SA-related genes is in line with expectations. Future studies should be conducted to find out whether SA-related gene expression would respond more strongly to soil conditioning when plants are under attack by phloem-feeding herbivores that more commonly induce the SA signaling pathway.

Seeds of Ribwort plantain were not derived from the same genetic background, and plant material used for gene expression analysis was collected from individual *P. lanceolata* replicates. The relative expression values in our study exhibit large variation, indicating strong variability among individual plants in their response to the soils. Most studies on gene expression pool samples from multiple plants, and analyze these pooled samples, which can

greatly reduce the variation. We purposely did not pool samples in our study, since individual plants may not respond in the same way and this information cannot be inferred from pooled samples. It may well be that not all individual plants were induced to the same extent. This could be due to differences among individual plants in how they respond to a given set of soil microbes, but also due to differences in the composition of soil organisms among replicate soils. Certain microbes may be present or absent in replicates even though they originated from the same replicate pot with conditioned soil. Nevertheless, even without pooling, our study shows that *P. lanceolata* responded differently to combined soil legacy and herbivory effects with respect to the induction of defense-related genes.

The metabolites aucubin and catalpol have been well-studied in *P. lanceolata* and several studies have shown that both compounds can be induced by herbivory, and by soil organisms (Bowers and Stamp 1993; Marak et al. 2002; Biere et al. 2004; Harvey et al. 2005), such as soil pathogens or arbuscular mycorrhizal fungi (Gange and West 1994; Schweiger et al. 2014; Wang et al. 2015). In this study, *P. lanceolata* secondary defense metabolites were also affected by soil conditioning by twelve different plant species. We only found an effect of soil conditioning species on aucubin levels, which seems to be mainly driven by very low levels of aucubin in *P. lanceolata* growing in soils conditioned by *T. officinale*. In a previous study, *T. officinale* had a negative effect on microbial biomass in the soil (Wardle and Nicholson 1996). As IG levels are often elevated when the plant interacts with microorganisms and nematodes (Wurst et al. 2010), we speculate that differences in IG levels detected may be caused, at least partially, by variation in the activity or community composition of soil organisms. Previous studies have indicated that grasses and forbs differ in their microbial profile in the soil (Kos et al. 2015b; Latz et al. 2015, 2016) and that this can affect aboveground plant–insect interactions (van de Voorde et al. 2011; Kos et al. 2015b; Heinen et al. 2018). In our study, catalpol levels were significantly higher in *P. lanceolata* on soils that were conditioned by grasses, than on those that were conditioned by forbs, regardless of the herbivore treatment. It has also been shown that IG levels in *P. lanceolata* negatively correlate with nutrient levels available in the soil (Darrow and Bowers 1999; Marak et al. 2003), so a nutritional soil legacy effect cannot be ruled out. In this study, all soils were mixed with two volumes sterilized field soil, which was done to minimize the effect of soil nutritional differences in the feedback phase.

In conclusion, our results shed light on the effect of plant-induced variation in soil biotic and abiotic conditions on defense responses to aboveground herbivory in plants that grow later in these conditioned soils. Until now, mechanisms of how PSF may influence aboveground plant–insect interactions have been highly speculative. Further studies are required, but here we provide evidence that soil legacies can be important drivers of insect–plant interactions—via their influence on plant defense chemistry and the JA-pathway. We showed these effects in a relatively realistic ecological framework, using live soils and natural soil conditioning. Future studies should focus on disentangling the changes in the soil microbiome involved, and manipulating the different classes of soil organisms, such as decomposers, pathogens and beneficial organisms within this framework, to better understand what drives these changes in plant defense.

Acknowledgements

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Author contributions

FZ, RH and TMB conceived and designed the experiments. RH, MS, TMB and FZ performed the experiment, FZ performed transcriptome analyses. CR performed chemical analyses. FZ and RH analyzed the data. RH and FZ, AB and TMB wrote the manuscript. All authors approved final version for publication.

Species-specific plant–soil feedbacks alter herbivore-induced gene expression and defense chemistry in Plantago lanceolata

Supplementary information Chapter 5

Supplementary table S5.1. Specific primer sequences used for quantitative RT-PCR analyses.

Gene name	Forward primer	Reverse primer
Pl GAPDH	AGCAAGCTTCCCACCTTCTC	TGGGAATGTCACCCTTTCCG
Pl PPO7	TTTCCTGGAATCGGAGTTTG	GGTTGCGCGTCTATCTTAGC
Pl LOX2-2	CCTCAGTCCTCTCCAAACTCA	GGTTGGGAGCAAAGGCTTAT
Pl PR1	CGCAAGGAACTATGCACAAA	ACTCTCCTCCAACGCAAGAA
Pl PR2-1	CCCGGCTTATAGTTTCCACA	CTCCAGAGCCGGTGTAAGAG

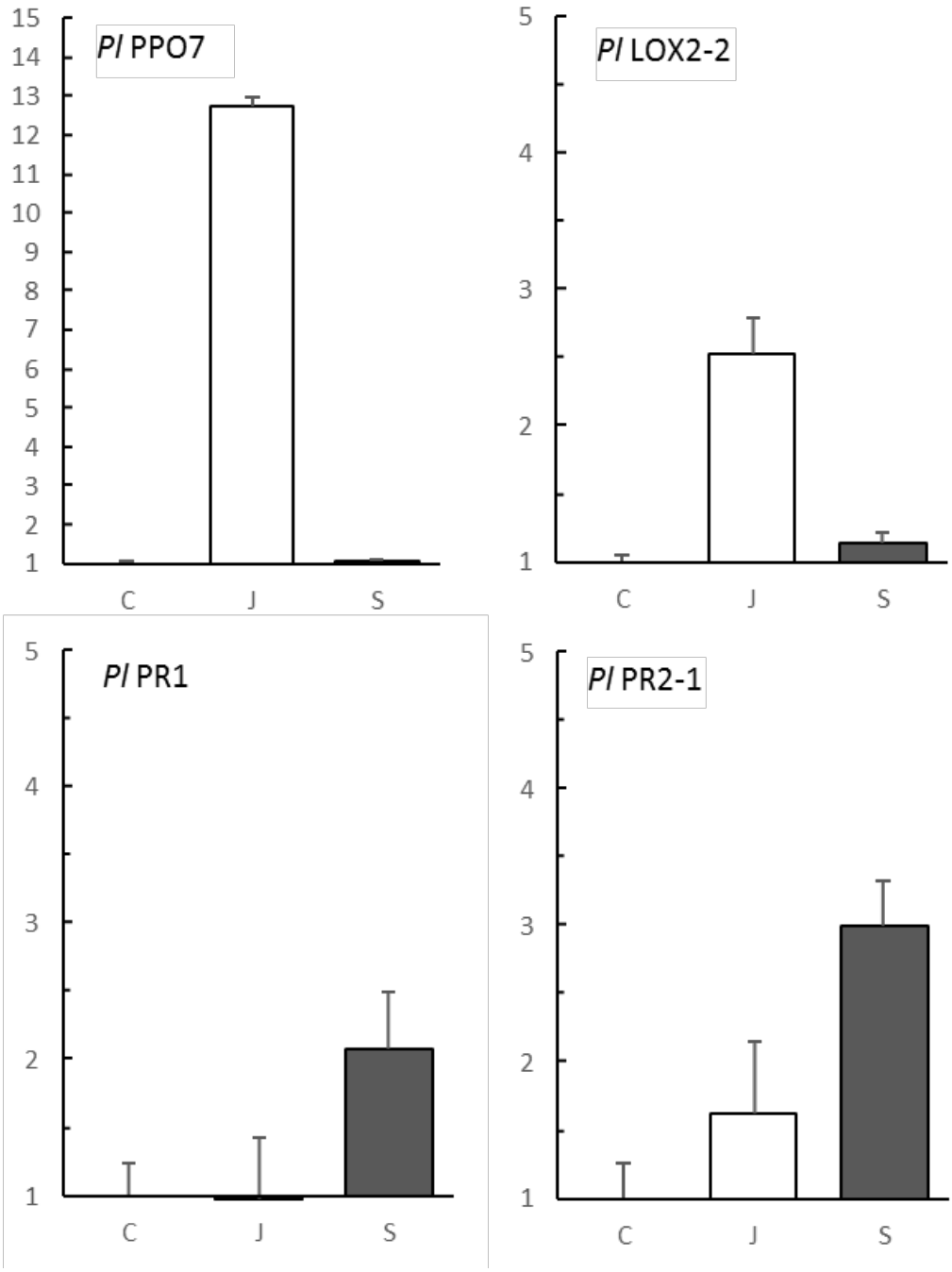
Supplementary table S5.2: Statistical results of the effect of herbivory, soil legacy and functional group of conditioning plant species on induction of defense-related genes in *Plantago lanceolata*. Shown are degrees of freedom, F-value and P-value of a two-way ANOVA with soil (conditioning species) and herbivory treatment (herbivory/control) as factors and the output of a general linear mixed model with functional group of the conditioning species (grass/forb) and herbivory treatment (herbivory/control) as fixed factors and soil as random factor.

Plantago gene	Model	Model factors	df1,df2	F-value	P-value
***P*PPO7**[a]	Two-way	herbivory	1, 95	9.73	**0.002**
	ANOVA	soil	11, 95	2.87	**0.003**
		herbivory x soil	11, 95	1.29	0.241
	GLMM	herbivory	1, 105	9.43	**0.003**
		grass-forb	1, 10	4.53	*0.059*
		herbivory x grass-forb	1, 105	0.53	0.468
***P*LOX2-2**[a]	Two-way	herbivory	1, 96	1.54	0.217
	ANOVA	soil	11, 96	1.91	**0.048**
		herbivory x soil	11, 96	2.17	**0.022**
	GLMM	herbivory	1, 106	1.38	0.242
		grass-forb	1, 10	3.82	*0.079*
		herbivory x grass-forb	1, 106	3.16	*0.079*
***P*PR1**[a]	Two-way	herbivory	1, 94	0.07	0.797
	ANOVA	soil	11, 94	1.87	*0.053*
		herbivory x soil	11, 94	1.30	0.235
	GLMM	herbivory	1, 104	0.06	0.811
		grass-forb	1, 10	0.36	0.564
		herbivory x grass-forb	1, 104	0.28	0.600
***P*PR2-1**[a]	Two-way	herbivory	1, 95	0.05	0.821
	ANOVA	soil	11, 95	1.61	0.108
		herbivory x soil	11, 95	0.91	0.536
	GLMM	herbivory	1, 105	0.05	0.822
		grass-forb	1, 10	1.82	0.207
		herbivory x grass-forb	1, 105	0.02	0.881

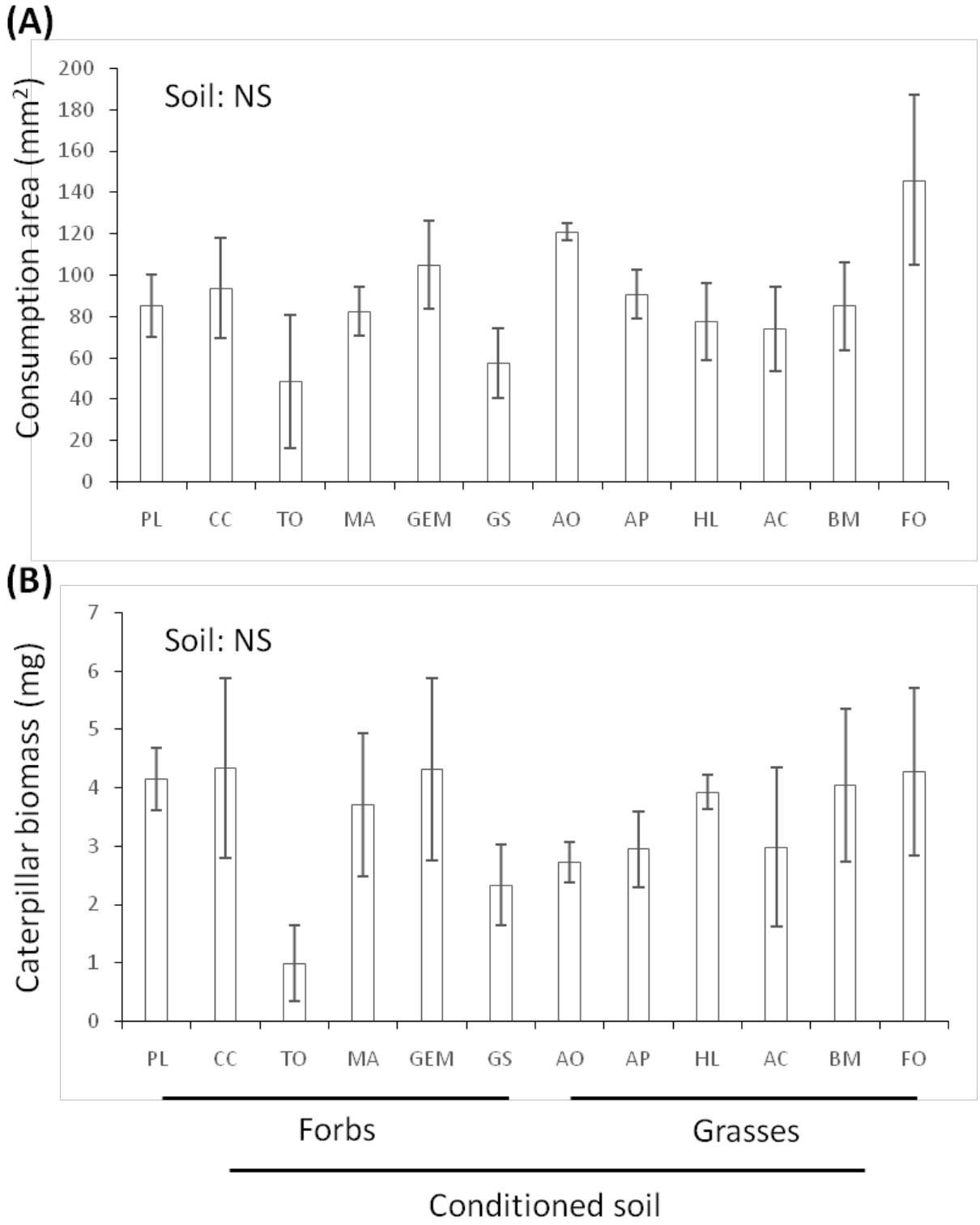
a) Values were log-transformed prior to statistical analysis.

Supplementary table S5.3: Statistical results of the effect of herbivory, soil legacy and functional group of conditioning plant species on production of iridoid glycosides (aucubin and catalpol) in *Plantago lanceolata*. Shown are degrees of freedom, F-value and P-value of a two-way ANOVA with soil (conditioning species) and herbivory treatment (herbivory/control) as factors and the output of a general linear mixed model with functional group of the conditioning species (grass/forb) and herbivory treatment (herbivory/control) as fixed factors and soil as random factor.

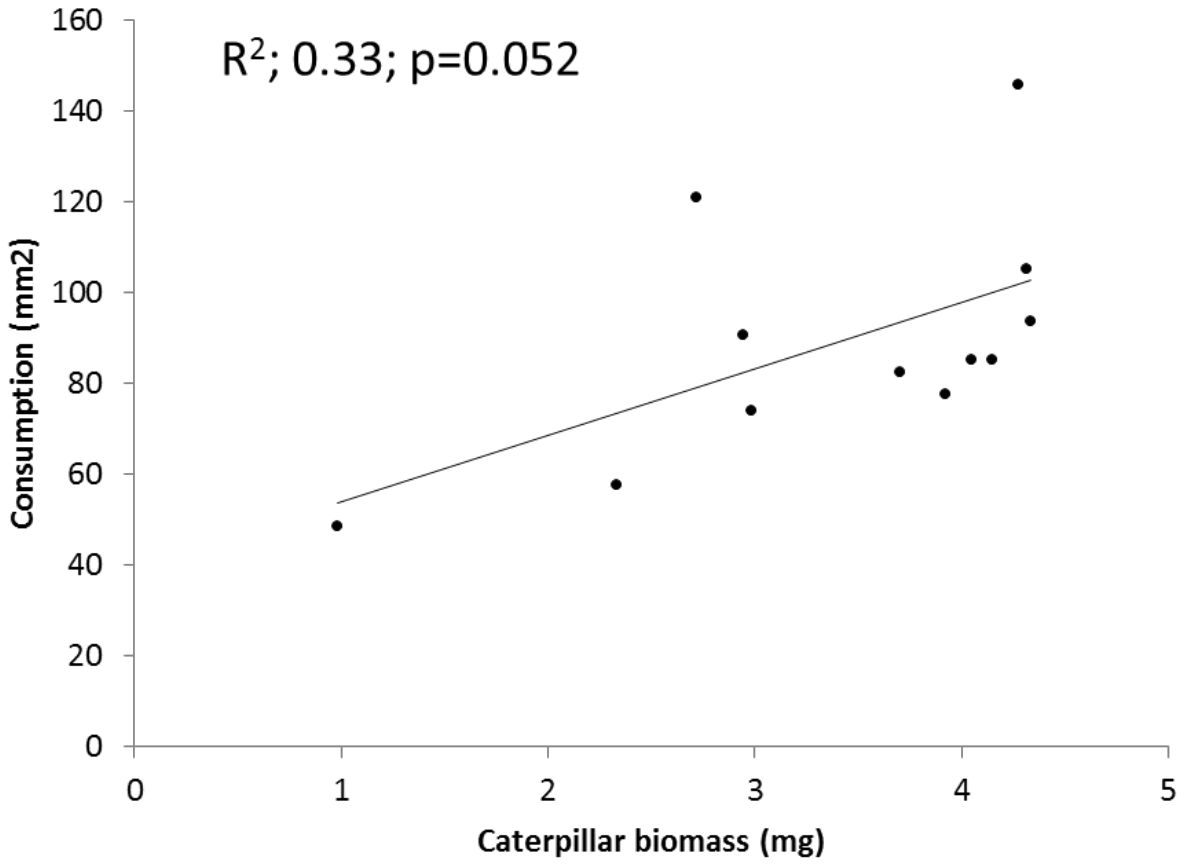
IGs	Model	Model factors	df1,df2	F-value	P-value
aucubin	Two-way	Herbivory	1, 96	0.43	0.513
	ANOVA	Soil	11, 96	2.40	**0.011**
		herbivory x soil	11, 96	0.68	0.752
	GLMM	Herbivory	1, 106	0.44	0.511
		grass-forb	1, 10	0.12	0.736
		herbivory x grass-forb	1, 106	0.32	0.576
catalpol	Two-way	Herbivory	1, 96	1.14	0.288
	ANOVA	Soil	11, 96	1.44	0.170
		herbivory x soil	11, 96	1.49	0.148
	GLMM	Herbivory	1, 106	1.08	0.300
		grass-forb	1, 10	5.76	**0.037**
		herbivory x grass-forb	1, 106	0.01	0.904



Supplementary Figure S5.1: Relative gene expression of *Plantago lanceolata* homologues of PPO, LOX2, PR-1, and PR-2 used in the experiment. Data from an unpublished RNA-seq experiment (Illumina Hi-seq100 paired end) in which the fourth-youngest fully expanded leaves of seven-week old plants were induced with 250 uL of jasmonic acid (10 mM; J), salicylic acid (5 mM; S), or mock treatment with acid water (C). Values are mean ± s.e. fold changes in expression of J and S plants compared to the control C, based on n=6 biological replicates of 9 pooled plants each (A. Biere, unpublished data). Stars indicate significant differences from the control (* P<0.05; ** P<0.01; *** P<0.001). Closest homologues in *Arabiopsis thaliana*: *Pl* PPO-7 (576 identity): no homology; closest homologue *Sesamum indicum* polyphenol oxidase 1, chloroplastic-like (66% identity); *Pl* LOX2-2 (907 nucleotides) lipoxygenase *At*LOX2 (55% identity); *Pl* PR1 (161 nucleotides): basic pathogenesis-related protein 1 (59% similarity); *Pl* PR2-1 (341 nucleotides): beta-1,3 glucanase 1, PR-2 (53% identity).



Supplementary Figure S5.2: The effects of soil conditioning by twelve common grassland species on biomass of *Mamestra brassicae* (A), and herbivore consumption (B), feeding on *Plantago lanceolata*. Error bars represent standard errors. For each treatment combination, n=5. Soils were conditioned by either forb or grass species. Abbreviations: PL = *Plantago lanceolata*, CC = *Crepis capillaris*, TO = *Taraxacum officinale*, MA = *Myosotis arvensis*, GEM = *Geranium molle*, GS = *Gnaphalium sylvaticum*, AO = *Anthoxanthum odoratum*, AP = *Alopecurus pratensis*, HL = *Holcus lanatus*, AC = *Agrostis capillaris*, BM = *Briza media*, FO = *Festuca ovina*.



Supplementary Figure S5.3: Correlation between mean caterpillar biomass and consumption area in the shoot of *Plantago lanceolata*. Each data point represents the average caterpillar biomass and consumption area for one conditioned soil. For each average, n=5.

Species-specific plant–soil feedbacks alter herbivore-induced gene expression and defense chemistry in Plantago lanceolata

Chapter 6

Foliar-feeding insects acquire microbiomes from the soil rather than the host plant

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Abstract

Microbiomes of soils and plants are linked, but how this affects microbiomes of aboveground herbivorous insects is unknown. We first generated plant-conditioned soils in field plots, then reared leaf-feeding caterpillars on dandelion grown in these soils, and then assessed whether the microbiomes of the caterpillars were attributed to the conditioned soil microbiomes or the dandelion microbiome. Microbiomes of caterpillars kept on intact plants differed from those of caterpillars fed detached leaves collected from plants growing in the same soil. Microbiomes of caterpillars reared on detached leaves were relatively simple and resembled leaf microbiomes, while those of caterpillars from intact plants were more diverse and resembled soil microbiomes. Plant-mediated changes in soil microbiomes were not reflected in the phytobiome but were detected in caterpillar microbiomes, however, only when kept on intact plants. Our results imply that insect microbiomes depend on soil microbiomes, and that effects of plants on soil microbiomes can be transmitted to aboveground insects feeding later on other plants.

Introduction

Soil microbiomes harbor an extremely rich diversity of bacteria and fungi (Lozupone & Knight, 2007; Delgado-Baquerizo et al., 2018). Plants also have microbiomes, and as they are rooted in the soil, a subset of the soil microbiome colonizes the roots (Lundberg et al., 2012; Bulgarelli et al., 2012). Consequently, aboveground plant parts, such as stems and leaves, are inhabited by specific commensal, symbiotic or pathogenic bacteria and fungi that, at least partly, originate from the roots and soil (Bai et al., 2015; Chi et al., 2005). Insects are also associated with a variety of microbes (Rosenberg & Zilber-Rosenbberg, 2016; Gilbert, Sapp & Tauber, 2012; Chen et al., 2016; Hammer et al., 2017). These microbes can act as pathogens causing diseases (Fisher et al., 2012) or can be beneficial for defense, detoxification, or digestion of food (Frago, Dicke & Godfray, 2012; Douglas, 2015; Hammer & Bowers, 2015; Shao et al., 2015). Herbivorous insects ingest microorganisms that are present in the plant, and hence microorganisms that originate from the soil, via the plant (Chi et al., 2005), can be incorporated in the microbiome of the insect Sugio et al., 2015). However, recent studies suggest that many of these microbes may not persist in the caterpillar gut (Hammer et al., 2017). Studies using animals other than insects have shown that an important part of the microbiome originates from non-dietary sources (Turnbaugh et al., 2007; Ross et al., 2018). Moreover, several studies have shown that herbivorous insects can take up specific symbiont bacterial species from the environment, and also directly from the soil (Kikuchi, Hosokawa & Fukatsu, 2007; Kikuchi et al., 2012). Whether herbivorous insect microbiomes as a whole are also influenced by the soil environment is unknown. An intriguing possibility is that changes in soil microbiomes can lead to changes in insect microbiomes and alter the performance of insects, mediated via the microbiome of the plant, or through direct soil-insect interactions.

Plants have aboveground and belowground parts and act as the primary providers of resources for most other aboveground and belowground dwelling organisms (Wardle et al., 2004). Moreover, an overwhelming amount of research over the past two decades has shown that plants are pivotal in mediating interactions between these aboveground and belowground organisms. For instance, root-associated organisms can influence foliar feeding insects on the same plant (Pineda et al., 2010; Koricheva et al., 2009). Plants also change the microbiome of the soil they grow in, and this depends on plant traits such as plant growth form (grasses and forbs) and growth rate (Cortois et al., 2016; Heinen et al., 2018b). Other plants that grow later

in these conditioned soils, and the insects feeding on those plants, respond to the changes in soil microbiomes (Heinen et al., 2018b; Kostenko et al., 2012). So far, most research has focused on the role of systemic changes in the chemical composition of aboveground and belowground plant parts (Bezemer & Van Dam, 2005). The role of changes in plant and insect microbiomes in these aboveground-belowground interactions is poorly understood, and how this is influenced by plant-mediated changes in soil microbiomes is unknown.

We hypothesize that plant-mediated changes in soil microbiomes will affect microbiomes of caterpillars feeding on plants that grow later in these soils, through modifications of the microbiomes of their host plants. We expect that plant growth form and growth rate are important drivers of soil microbiomes and that these microbiomes will affect the root and subsequently the shoot microbiome of our test plant species (*Taraxacum officinale*; Asteraceae), eventually altering the caterpillar (*Mamestra brassicae*; Lepidoptera; Noctuidae) microbiome. We use two parallel assays (Supplementary Figure S6.1) to disentangle the effects of the soil microbiome on the caterpillar microbiome mediated via the plant from the possible direct effects via the soil. Using these two parallel assays, we show that the microbiome of an aboveground insect herbivore is shaped not by the microbiome of its host plant, but directly by the microbiome of the soil its host plant grows in.

Results

Composition of soil, plant, and insect microbiomes

Briefly, microbiomes in the soil, plant and insect compartments were characterized by Illumina MiSeq sequencing, using 16S rRNA and ITS2 regions (for bacteria and fungi respectively). Rhizosphere soil contained the highest diversity of both bacteria and fungi, and leaves were the least diverse compartments (Figure 6.1a, b; Supplementary Figure S6.2). We use two parallel assays (Supplementary Figure S6.1) to disentangle if the microbial diversity in caterpillars is affected by plants or by soils. Caterpillars that were fed detached leaves had a significantly lower diversity of both bacteria and fungi in terms of absolute diversity and a lower number of fungal phyla and bacterial classes than caterpillars fed on intact plants (Figure 6.1a, b; GLM: bacteria: $F=7.56$, $P<0.001$; fungi: $F=8.11$, $P<0.001$). Both for bacteria and fungi, the community structure found in caterpillars fed on intact plants and in caterpillars fed on detached leaves differed significantly (PERMANOVA: bacteria: $F=30.05$, $R2=0.19$, $P<0.001$;

fungi: $F=43.11$, $R2=0.25$, $P<0.001$) and there was a little overlap between the two types of microbiomes (Figure 6.1c, d). Remarkably, microbiomes of caterpillars kept on intact plants resembled those found in soils much more closely than microbiomes of leaves or caterpillars fed on detached leaves (Figure 6.1c, d). There were no significant differences in microbiomes of leaves collected from plants that had caterpillars on them, and leaves from plants that were kept without caterpillars and that were used to collect leaves from for the detached plant assay (Figure 6.1c, d). Not only did the total microbial community composition differ between the caterpillars fed on intact plants and those fed on detached leaves, the composition in terms of phylum and class levels also differed. The bacterial phyla Actinobacteria and Chloroflexi, and the fungal classes Eurotiomycetes, Sordariomycetes, and Dothideomycetes, were more abundant in caterpillars fed on intact plants, while Betaproteobacteria and a group of unclassified fungal OTUs were more abundant in the caterpillars that fed on detached leaves (GLM: FDR adjusted $P<0.05$ for all cases; Supplementary Figure S6.3). The leaf microbiome consisted almost entirely of a group of unclassified fungal OTUs and members of the bacterial phylum Gammaproteobacteria (Supplementary Figures S6.4 and S6.5), both groups were also found more commonly in microbiomes of caterpillars fed on detached leaves, thus explaining the observed clustering (Figure 6.1c, d). Root microbiomes comprised a subset of the soil community, and especially Gammaproteobacteria, Firmicutes, Bacteroidetes, Sordariomycetes, Agaricomycetes and Glomeromycotina were enriched inside the roots (Figure 6.1c, d; Supplementary Figures S6.4, S6.5).

Shared microbes between soils, leaves, and caterpillars

Caterpillars fed on intact plants and detached leaves shared a common core microbiome which was also present in the leaves (20.3% of their microbiome) and in the roots (19.1%) (Figure 6.2a–c), but also harbored unique microbes; 16.7% of the caterpillar microbiome was found only in caterpillars. This core microbiome of caterpillars consisted predominantly of Proteobacteria, Acidobacteria, Firmicutes, and unclassified fungi (Supplementary Figures S6.6, S6.7). Remarkably, for caterpillars fed on intact plants, a large proportion of the OTUs found in caterpillars, was also detected in the soil (75%; represented as numbers 1 and 4 in Figure 6.2a). Microbiomes of caterpillars fed detached leaves had virtually no additional OTUs that were not also found in caterpillars kept on intact plants (Figure 6.2c), but the microbiomes of the latter contained three times more OTUs.

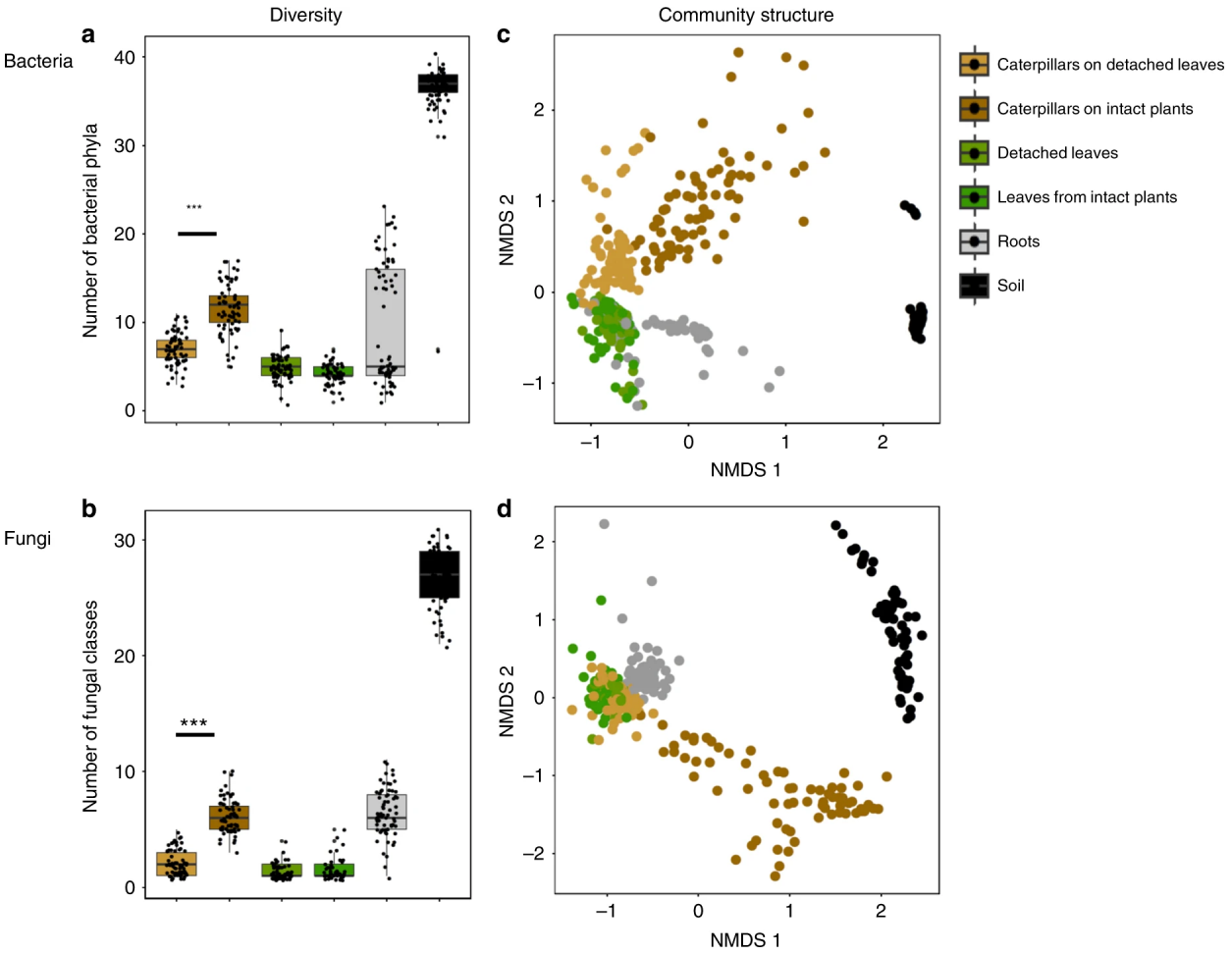


Figure 6.1: Diversity and community structure of bacteria and fungi in caterpillars, leaves, roots and soil. a number of bacterial phyla and b number of fungal classes of caterpillar, leaf, root and soil samples. Caterpillars were kept on intact plants or on detached leaves. The Tukey box-and-whisker-plots depict median number of phyla and classes in each compartment and variation is shown in the scatter. The raw (Chao1) diversity data is presented in Supplementary Figure S6.2, and phyla and their relative abundance in Supplementary Figure S6.3 (bacteria) and Supplementary Figure 4 (fungi). Asterisks (***) indicate significant differences of GLM at the level of $p<0.001$. c, d Non-metric multidimensional scaling (NMDS) of bacterial (c) and fungal (d) communities. The clustering is based on Bray-Curtis similarity and the resulting 2D stress for the best solution is 0.16 (bacteria) and 0.19 (fungi). Source data for a and b are provided in a Source Data file.

The main groups of shared OTUs between soils and caterpillars kept on intact plants were Actinobacteria (12.6% of OTUs), Eurotiomycetes (21.8%) and unclassified fungal OTUs (22.3%) (Supplementary Figure S6.6). Furthermore, the fungal class Eurotiomycetes and bacterial phylum Actinobacteria were represented in a disproportionally high ratio in caterpillars that

were kept on intact plants, compared to their abundance in soil (Supplementary Figure S6.4, 6.5).

Soil legacy effects on soil, plant, and insect microbiomes

We investigated the legacy effects created by field-grown plant communities, on the composition of microbial communities in soils, dandelions grown in those soils, and caterpillars reared on these plants, in two parallel assays (Supplementary Figure S6.1). The composition of the plant community (fast- and slow-growing grasses or forbs) that conditioned the soils that were used, influenced the fungal and bacterial community structure in these soils (Figure 6.3a, e). Surprisingly, this did not alter the root- or leaf -associated microbiomes in the dandelion plants that were growing in these soils (Figure 6.3c, d, g, h). However, we did detect these soil-derived plant community effects in caterpillar microbiomes, but only when the caterpillars were fed on intact plants (Figure 6.3b, f), suggesting that, even though they are plant feeders, the caterpillars had been in direct contact with the soil. In the caterpillars fed on intact plants the fungal class Eurotiomycetes and the bacterial phyla Bacteroidetes, Alphaproteobacteria and Betaproteobacteria were significantly affected by characteristics of the plant community that had conditioned the soil (Supplementary Figure S6.8).

Plant and insect biomass and abiotic soil characteristics

Shoot and root biomass of the test plants were on average higher in soils of fast-growing grass communities, but lower in soils of slow-growing grass communities than in other soils, both in test plants of the intact plant assay (Supplementary Figure S6.9A, C) and of the detached leaf assay (Supplementary Figure S6.9B, D). Caterpillar biomass was highest in soils of fast-growing forb communities, and lowest in soils of slow-growing forb communities but only when caterpillars were fed on intact plants (Supplementary Figure S6.10). Soil chemical parameters did not differ between soils, except that nitrogen availability was higher in soils from grass communities than in other soils (Supplementary Figure S6.11, Supplementary Table 6.1). There was no relationship between caterpillar biomass and plant biomass, and plant, and caterpillar performance did not correlate with soil chemical parameters (Supplementary Figure S6.12). We further related the abundances of fungal classes and bacterial orders in the caterpillars to the performance of the caterpillars. There was a negative relationship between the biomass of caterpillars that were kept on intact plants and the relative abundance of the fungal classes

Chaetotyriales, and between the number of surviving caterpillars and the relative abundance of Sordariales, Pseudomonadales and Burkholderiales. Caterpillar biomass and survival were positively correlated with two fungal classes and three bacterial orders (Figure 6.4). For the caterpillars that were fed detached leaves, there were no significant correlations between caterpillar biomass and the relative abundance of any fungal orders or bacterial classes (Figure 6.4).

Discussion

In this study, we tested the hypothesis that plants would acquire a subset of their phytobiome from the soil and that this would subsequently shape the microbiome of a plant-associated caterpillar. Remarkably, our results show that aboveground caterpillars acquire a large part of their microbiome, not from the plant they are feeding on, but directly from the soil. Over the past two decades a large number of studies have reported that soil microbiota can influence the performance of aboveground plant-feeding insects (Hooper & Gordon, 2001; Frago, Dicke & Godfray, 2012; Hammer & Bowers, 2015), but this has been solely attributed to systemic chemical changes in the host plant (Etalo, Jeon & Raaijmakers, 2018; Pineda et al., 2013). We now argue that these belowground-aboveground effects may be partly due to direct interactions between insects and soil microbiomes. Previous studies have already shown that insects can selectively acquire symbiotic bacteria from the genus Burkholderia from the soil (Kikuchi, Hosakawa & Fukatsu, 2007; 2011; Kikuchi et al., 2012). Our results now show that entire microbiomes of caterpillars on intact plants are affected by soils, and that they are enriched in particular bacterial and fungal genera, disproportionate to their relative presence in soils. When the caterpillars were fed detached leaves, this was not observed. Both Eurotiomycetes and Actinobacteria, the genera found disproportionally more in the caterpillars on intact plants than in soils and in caterpillars fed detached leaves, are known to act as insect symbionts and produce antibiotic compounds (Shao et al., 2015; Geiser et al., 2006; Salem et al., 2013). Furthermore, caterpillars that were in contact with soils had acquired species of yeasts commonly found in soils but that have recently been identified as symbionts of insects (Matsuura et al., 2018) and found in large numbers in human guts (Nash et al., 2017). This suggests that leaf eating insects may actively acquire more species of beneficial microbes from the soil than what is known from literature so far (Kikuchi et al., 2012).

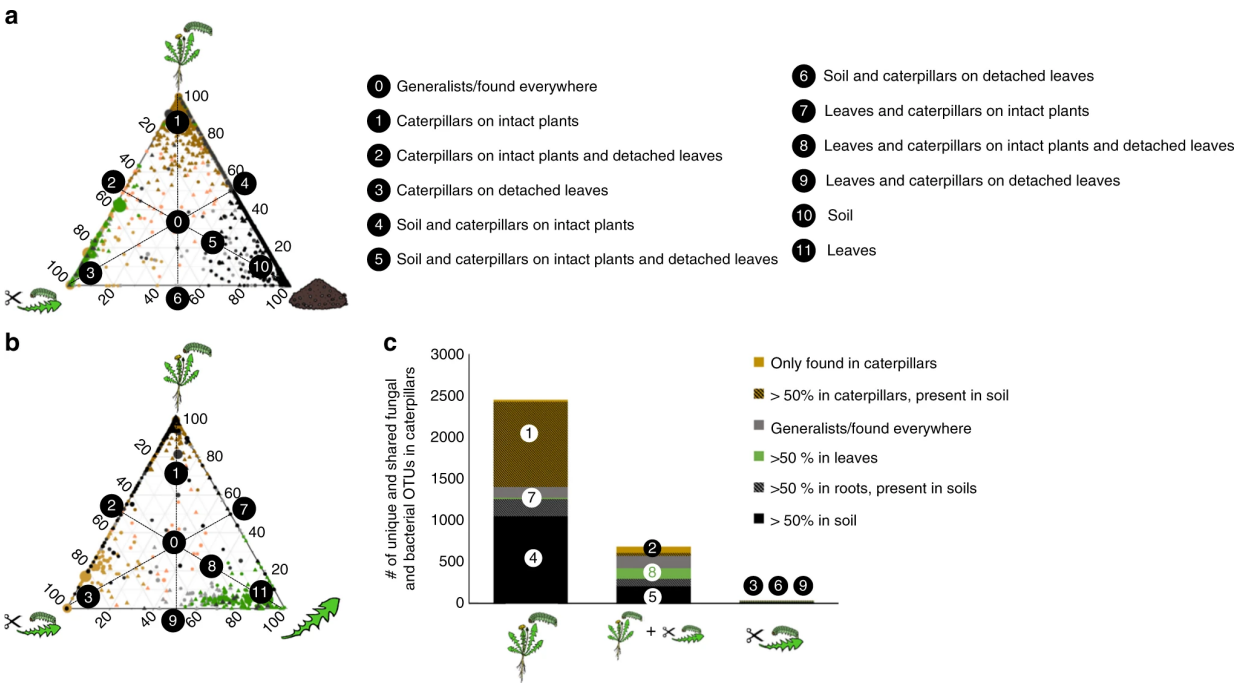


Figure 6.2: Bacterial and fungal OTUs shared among caterpillars, plants and soil. a, b Ternary plots of OTUs found in caterpillars. Each symbol represents a single OTU; circles represent bacterial OTUs and triangles fungal OTUs. Only OTUs found in at least 10% of the samples are included in the Figure. The size of each symbol represents its relative abundance (weighted average) and its color the compartment where it is primary found. Green depicts OTUs found >50% in leaves, brown depicts OTUs found >50% in caterpillars (dark brown OTUs in caterpillars on intact plants and light brown on detached leaves), black depicts OTUs found >50% in soil, grey OTUs found >50% in roots. Grey symbols represent general OTUs found in all compartments. The position of each symbol represents the contribution of the indicated compartments to the total relative abundance. The 50% lines are drawn in the Figure and most important compartments are marked with numbers (0–9). a Depicts OTUs shared between soil (right side), caterpillars on intact plants (top) and caterpillars on detached leaves (left) and b depicts OTUs shared between plants (right), caterpillars on intact plants (top) and caterpillars on detached leaves (left). c The total number of unique and shared OTUs of caterpillars on intact plants and caterpillars on detached leaves. Both fungi and bacteria are included in the Figure and their identity on the phylum/class level is shown in Supplementary Figure S6.6. The color of the compartment where the OTUs are predominantly found and the corresponding region in panel a and b is also shown

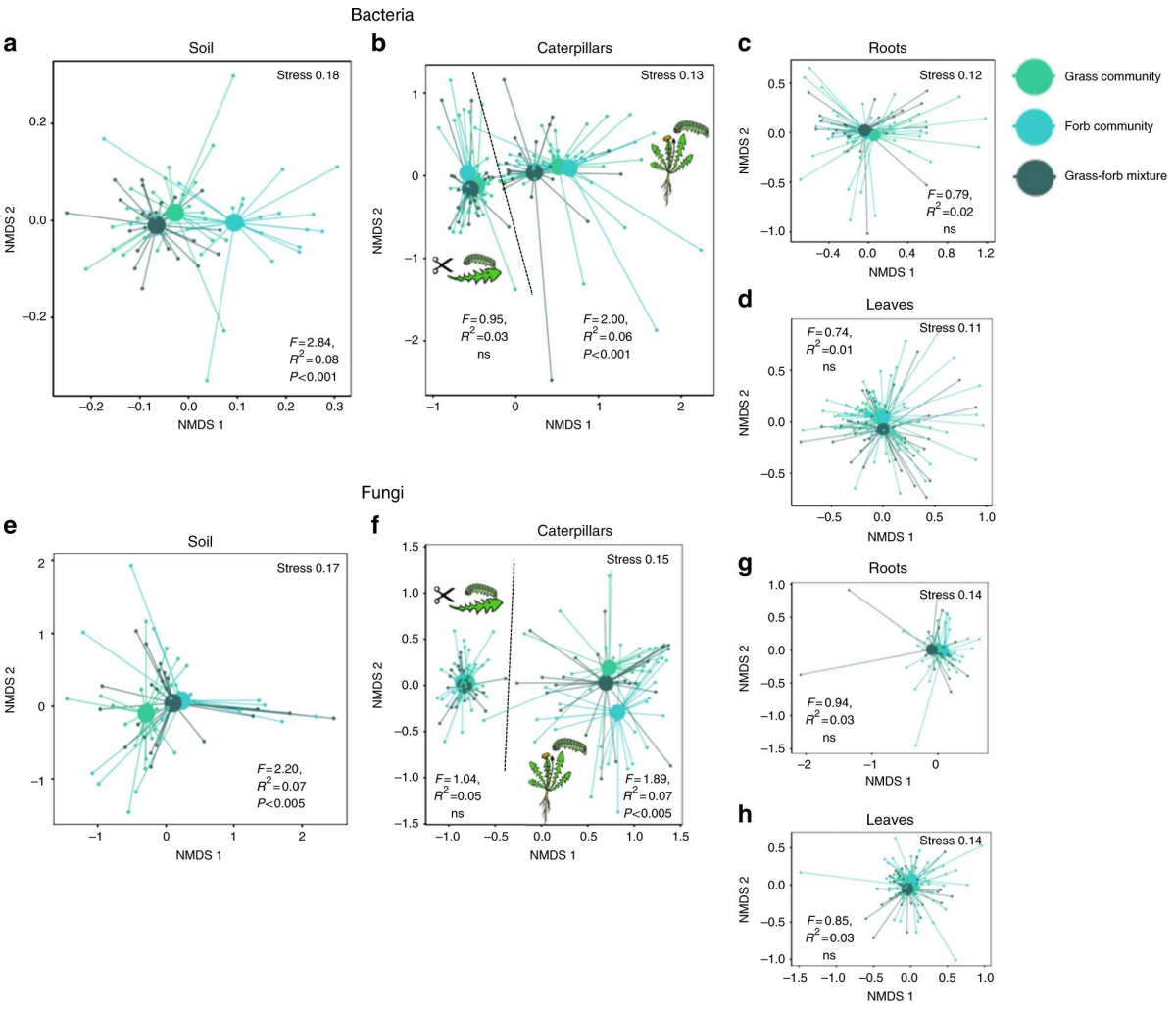


Figure 6.3: Legacy effects of plant communities on microbiomes. Plant community identity effects on bacterial a–d and fungal (e–h) communities in caterpillars, leaves, roots, and soil. NMDS plots are presented based on Bray–Curtis similarity. The 2D stress value for each panel ranges between 0.11–0.18. Soils originating from grass communities are presented with light green symbols, soils from forb communities with turquoise symbols and soils from mixed grass and forb communities with dark green symbols. In each panel, smaller symbols depict individual samples, centroids are depicted with larger markers. Significance of the plant community treatment effect based on a PERMANOVA is also presented in each panel. a, e represent the composition of microbiomes in soils, b, f microbiomes in caterpillars both on intact plants and on detached leaves. c, g microbiomes in roots and d, h microbiomes in leaves. The effect of plant community growth rate (fast- and slow-growing communities) is shown in Supplementary Figure S6.14

However, we observed both positive and negative relationships between the relative abundance of soil microorganisms and the performance of the caterpillars, indicating that the acquisition of microbes from the soil by insects may not always be beneficial. Recent work indicates that caterpillar microbiomes may be transient (Hammer et al., 2010). Our findings that soils shape insect microbiomes now offer a viable explanation why these microbiomes are variable even within a single insect species. Caterpillar microbiomes reflect their (soil) environment and as soil microbiomes vary temporally and spatially (Hannula De Boer & Van Veen, 2012), this may also affect the microbiomes of the caterpillar. An important question that remains to be answered is how persistent these soil effects on insect microbiomes are and to what extent they change when insects encounter new soil microbiomes as they move or grow.

Remarkably, our results also show a link between the composition of the plants that previously grew in the soil and insect microbiomes. The consequences of (microbial) soil legacy effects for plant growth and plant-insect interactions have received considerable attention recently (Heinen et al., 2018b; Pineda, Kaplan & Bezemer, 2017)7. Our study now shows, for the first time, that such soil legacy effects can influence the performance of aboveground insects as well as their microbiomes. However, interestingly, these legacy effects on caterpillar performance and insect microbiomes were only observed in caterpillars that were fed on intact plants, and not when they were fed on detached leaves. This is important, as it suggests that soil legacies may not only influence insects mediated via plant quality, but that there may be a direct link between soils and insects, via the microbiome.

It is important to note that the test plant and insect microbiomes were investigated under artificial conditions in the greenhouse. Under natural conditions, insects may acquire a higher proportion of their microbiomes from dietary sources than we observed in this study. For instance, leaf microbiomes of host plants may be enriched by environmental microbiomes, e.g. via rain splash or wind38. As such, in natural settings, the dynamics of microbiome acquisition may vary from those observed in this study. Polyphagous caterpillars, such as the one used in this study, can often be found on soil e.g. because they move up and down the plant and regularly change host plants (Heinen et al., 2018b). Hence, they may also have more frequent contact with the soil under natural conditions than in the artificial greenhouse setting with individually potted plants that we used in this experiment.

A potential caveat in our study is that instead of a bottom-up pathway, the caterpillar microbiomes may have caused changes in the composition of the soil or leaf microbiomes e.g. excreted via their frass. However, we consider this unlikely for two reasons. First, there were no differences in microbial composition between the leaves that were in contact with caterpillars (and their frass) and leaves from the plants which had no insects. Second, insects weighed only 15 mg at the end of the experiment and the amount of frass produced by these small insects was marginal relative to the amount of soil used in each pot. However, studies with soil and insect microbes, labeled with isotopic tracers should further examine the direct and indirect interactions between soil, plant and insect microbiomes. Future studies should also address the functional consequences of soil legacy effects on microbiomes of aboveground insects and how widespread this phenomenon is among insect taxa.

A second caveat is that differences in size of the caterpillars in the two parallel assays may have contributed to the observed differences in caterpillar microbiomes. In the detached leaf assay, caterpillars were reared to L3 stage, until there were no more suitable leaves available on the source plants. At this point, the caterpillars in the parallel intact plant assay were considerably smaller (L2). As it is known that insect microbiomes differ between larval stages (Chen et al., 2016; Kikuchi, Hosakawa & Fukatsu, 2011; Hammer, McMillan & Fierer, 2014), the intact plant assay was continued until the caterpillars had molted to L3. Although the caterpillars were bigger on whole plants than on detached leaves (Supplementary Figure S6.13) when they were collected, their average biomass differed only by 4.4 mg. *M. brassicae* is known to grow well over 200 mg on various plant species that grow in similar soil types (Heinen et al., 2018b). Therefore, it is unlikely that these differences are the main driver of the observed differences in microbiomes. The small size of the caterpillars did not allow for proper removal of the gut, which is the reason why we extracted caterpillar-associated microbiomes from whole caterpillars (Douglas, 2015). However, we used generally accepted methods in microbial ecology to sterilize surfaces (Lundberg et al., 2012) to thoroughly clean the insect cuticle. We detected various cuticle-associated insect pathogens in the soils, which also correlated negatively with insect performance, but we did not observe these pathogens in the insect samples, suggesting that our sterilization procedure was effective in eradicating cuticle-bound microbes and thus that it likely reflects the internal insect microbiome.

We conclude that soil and insect microbiomes are linked, but that this is not mediated by the host plant, and that the role of soil microbiomes in modulating aboveground food-webs should be re-evaluated. Until now this has been overlooked, and the current results stress that studies on the composition and functioning of the microbiomes of plant-feeding insects should be carried out under conditions in which insects have access to the soil and soil microbiome that the host plant is growing in. Finally, an increasing number of studies is now showing that insect microbiomes may be important for insect fitness. We stress that these insect microbiomes can be the consequence of legacy effects of previous generations of plants on soil microbiomes.

Methods

Field design and soil sampling

To create specific soil legacies, field plots were set-up in an existing grassland in the nature area De Mossel (N 52° 3′, E 5° 44′, Natuurmonumenten, Ede, The Netherlands). Each field plot measured 80×250 cm, and between plots there were 1-m-wide paths that were mown regularly. In May 2015, the vegetation (sods) of each plot was removed at 4 cm depth to remove the majority of the roots. The plots were subsequently sown with fast- and slow-growing grass and forb species that are common in this grassland ecosystem. Each plot was sown with three grass species, three forb species, or with a mixture of three grass and three forb species. The total seed density in each plot was 12450 seeds, equally divided over the species in the community. There were three different fast- and three different slow-growing grass, forb and mixed communities (totalling 18 communities, see table S6.2 and S6.3) and there were four replicate plots for each community (72 plots in total). To maintain the composition of the sown communities, plots were hand-weeded regularly in 2015 and 2016.

In February 2017, live field soil was collected from each plot from the top 10 cm of the soil, as most of the roots are concentrated in this top layer40. Soils were sieved to remove roots, stones and most macro-invertebrates (sieve mesh Ø1.0 cm). Live soils were then mixed with sterilized bulk field soil (1:2 live:sterile v/v). Sterilized soil was obtained by γ-irradiation (>25 Kgray, Synergy Health, Ede, The Netherlands), of homogenized soil that was collected from the same field site. 11×11 cm square pots were filled with 1000 g of mixed soil. Two pots were filled with the same soil for each of the replicates in this experiment.

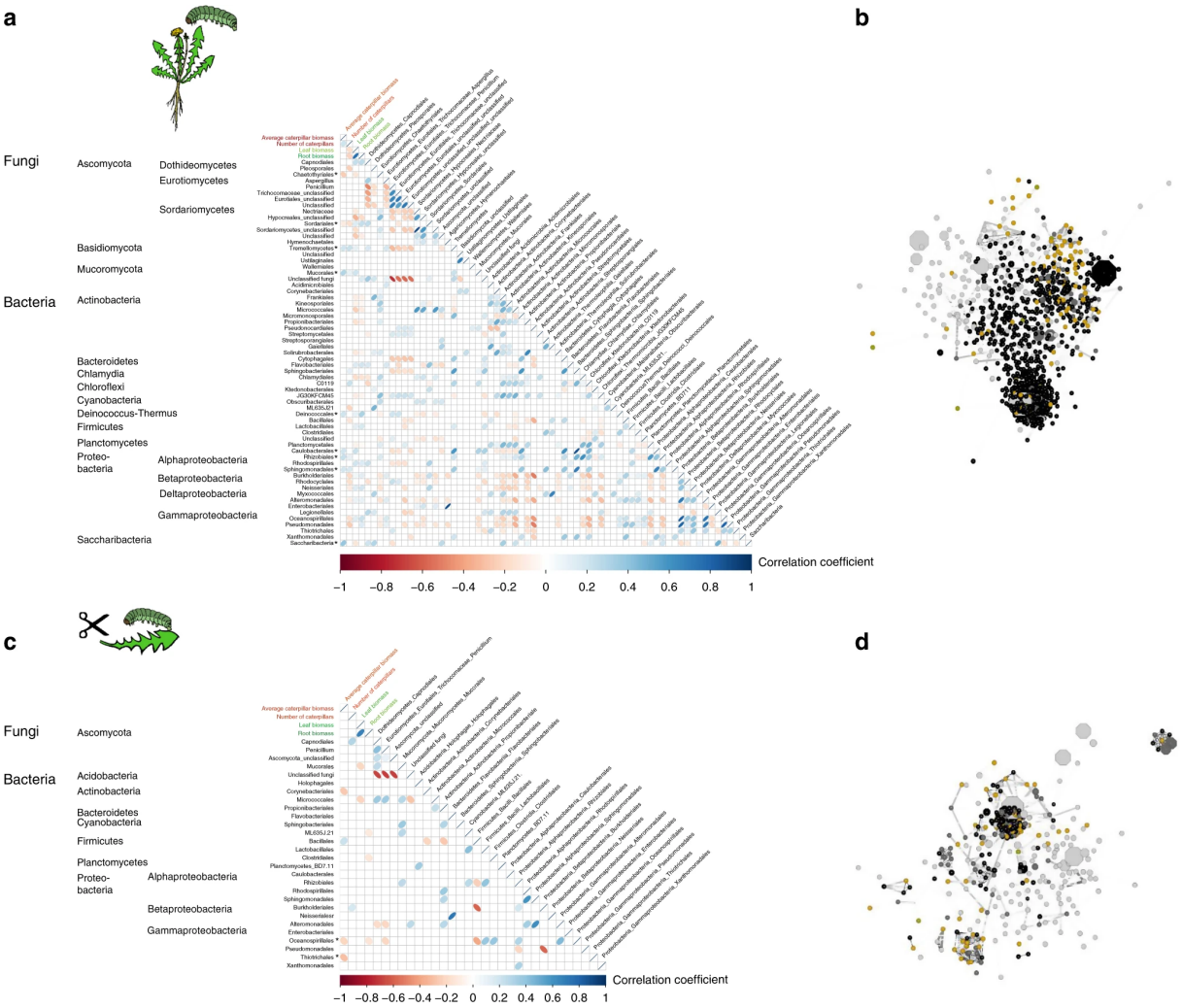


Figure 6.4: Correlations between caterpillar parameters, plant parameters, and relative abundance of fungal and bacterial taxa in the caterpillars. a fungal orders and bacterial classes detected in caterpillars fed on intact plants, and c on detached leaves. Correlations are based on linear Pearson correlation coefficients against each other and average caterpillar biomass (red), caterpillar survival (red), and leaf- and root biomass (green). The scale color of the filled squares indicates the strength of the correlation (r) and whether it is negative (red) or positive (blue). All correlations are corrected with FDR and only significant correlations with $p<0.05$ are shown. If the correlation is not significant, the box is left white. Asterisks next to names of taxa mark significant correlation between this taxon and caterpillar performance. b and d represent a network of all significant co-occurrences (Spearman rank correlation coefficient with Bonferroni correction, $p<0.01$) of OTUs in caterpillars on intact plants (b) or on detached leaves (d). The size of the nodes represents the relative abundance of the OTUs (weighted average) and the color represents the compartment where it is primary found. Green depicts OTUs found mostly in leaves, brown OTUs in caterpillars (dark brown OTUs of caterpillars on intact plants and light brown OTUs of caterpillars on detached leaves), black depicts OTUs found primarily in the soil and grey OTUs that are general in all compartments

A priori, one of the two pots was assigned to the detached-leaf assay while the other was assigned to the intact-plant assay. There were 18 plant community-conditioned soils, four independent field plot replicates, and two types of bioassay resulting in a total of 144 pots (Supplementary Figure S6.1A, B). After filling, pots were acclimatized in a climate-controlled greenhouse (light regime 16:8, L:D, day temperature 21°C, night temperature 16°C, relative humidity 50%) for 1 week, allowing the soil microbial communities to recover.

Test plants

Common dandelion (*Taraxacum officinale*, Asteraceae) was used as a model species. Dandelion is a perennial lactiferous plant with a broad geographical distribution that occurs in most of the temperate and subtropical regions of the world41. Several recent studies have used dandelion to address various ecological questions42,43. In this study, seeds of *T. officinale* were genetically identical, as they were obtained from a single clonal (apomictic) maternal line. Before germination, seeds were surface-sterilized using 2.0% bleach solution and then thoroughly rinsed with demineralized water. Seeds were geminated on sterile glass beads in a climate cabinet (light regime 16:8, L:D, day temperature 21°C, night temperature 16°C).

We transplanted one *T. officinale* seedling per pot when the seedlings were one-week-old. Dandelion leaves grow upwards in pots and thus, the rosettes are not in direct contact with the soil (Supplementary Figure S6.1C). Pots were randomly distributed in the greenhouse and plants were grown for five weeks under controlled conditions (light regime 16:8, L:D, day temperature 21±1°C, night temperature 16±1°C, relative humidity 50%). The plants were watered with demineralized water three times per week to keep a constant soil moisture level. Each plant received 60ml of 50% diluted Hoagland (1:1 Hoagland:demineralized water, v/v) nutrient solution in week 3 and 4, to mitigate the effects of nutrient limitation. The plants were used for assays when they were five weeks old.

Insect-plant assays

Eggs of the polyphagous cabbage moth, *Mamestra brassicae* (Lepidoptera: Noctuidae) were obtained from the Department of Entomology at Wageningen University, The Netherlands. The larvae were originally collected from organic cabbage fields near the university. The cabbage moth had been mass-reared for several generations on Brussels Sprouts, *Brassica oleracea* var. *gemmifera* cv. Cyrus. The eggs laid by a cohort of females were surface-sterilized using 2.0%

bleach solution and rinsed with demineralized water and then dried with sterile filter paper. The eggs were subsequently transferred to sterile petri-dishes and kept in a climate cabinet (light regime 16:8, L:D, temperature 21°C). Upon hatching, *M. brassicae* larvae were fed on artificial diet (Supplementary Table 4) until they reached the second larval instar stage.

We tested the effects of each of the soils on *M. brassicae* caterpillars in two parallel assays in order to disentangle the plant-mediated and the direct soil effects on caterpillar microbiomes. The outline of these two assays is shown in Supplementary Figure S6.1D. The assays were performed parallel to each other and we used second instar *M. brassicae* larvae, randomly selected from several hundred mass-reared larvae which were grown under sterile conditions. In one assay, caterpillars were fed with leaves clipped from plants that were growing in the different soils, and in the other assay they were fed on intact caged plants growing in soil from the same origin. For the first assay we cut the largest fully expanded leaf of each plant using sterile curved razor blades and placed it on a sterile petri-dish with the petiole covered with a piece of wet cotton that was soaked in demineralized water to prevent dehydration during the assay. Five *M. brassicae* caterpillars were placed in each petri-dish that contained one detached-leaf. After±24h, the leaf was removed and replaced by a newly collected leaf originating from the same plant. We conducted the detached-leaf assay for 5 days due to the limited availability of suitable leaves after which the caterpillars were collected and their biomass was measured. Caterpillars from this experiment were collected to be used for molecular analysis. In the second assay, *T. officinale* plants were transferred individually to fine-meshed (300µm) polyester sleeves and five *M. brassicae* larvae were placed on each individual plant. As growth of the caterpillars was much faster on the detached leaves (which we may speculate to be due to the absence of herbivore-induced defences in these plants44) and caterpillar microbiomes are known to differ between larval stages45, we kept the insects on the plant until they were of the same larval stage (L3) and visually similar in size (Supplementary Figure S6.13). Thus, in the intact-plant assay the caterpillars were allowed to feed and move freely on the plant for 14 days. Caterpillar mortality was recorded and fresh biomass of each individual caterpillar was measured and averaged per cage. Shoot and root biomass was collected after the insects were removed from the plants and dry weight was measured after oven drying (60°C for 4 days).

Soil, plant, and caterpillar sampling for microbiome analysis

We collected samples of surface-sterilized caterpillars, and leaves for analysis of the microbiomes3 from both assays. Leaves were collected from three leaf discs from each of three individual fully expanded leaves using a sterile 25 mm sample puncher. In the intact plant-assay leaves with clear signs of caterpillar feeding damage were selected for the analysis. Leaves for the detached leaves were selected from the corresponding plants at the same time point. The leaf discs were flash-frozen in liquid nitrogen and then stored at −80 °C until processing.

From the intact plant assay we further collected and surface-sterilized roots and rhizosphere soil. All caterpillar and root samples were surface-sterilized by dipping them in 2.0% bleach for 30 sec and then rinsed with autoclaved demineralized water. The caterpillars and roots were subsequently transferred to a new 15 mL falcon tube filled with 10 mL autoclaved Dulbecco's phosphate buffered saline (DPBS, Sigma-Aldrich, Darmstadt, Germany) and then sonicated in a BRANSONIC ultrasonic cleaner (Bransonic ultrasonics, Danbury, USA) for 10 min (ten cycles of 30s ultrasonic burst, followed by 30s rest) in order to disrupt microbes that were attached to the exterior surfaces3. After sonication, the caterpillars and roots were rinsed with autoclaved demineralized water three times and then stored at −80 °C until processing. Leaf, root and caterpillar samples were lyophilized prior to DNA extractions. Rhizosphere soils were collected from the intact-plant assay by first removing the bulk soil by shaking the root system and then gently removing the remaining soil above a sterile tray. This soil was stored in -80°C until processing.

Soil chemical analysis

For soil chemistry measurements, the soil samples were air dried at 40 °C and sieved through a 2 mm sieve. For extraction, 3 g dry soil was combined with 30 ml of 0.01 M $CaCl_2$ and shaken for 2 h at 250 rpm. After centrifugation at 3000 rpm for five minutes, 15 mL of the supernatant was filtered through a syringe filter with cellulose acetate membrane. Then 12.87 mL of filtrate and 130 µL HNO_3 were vortexed and extractable elements (Fe, K, Mg, P, S, and Zn) were measured the next day (ICP-OES, Thermo Scientific iCAP 6500 Duo). The remaining part of the filtrate was used to measure pH, and measure $NO_2 + NO_3$ and NH_4 on a QuAAtro Autoanalyzer (Seal analytical).

Molecular analysis of soils, plants, and caterpillars

For root, leaf and caterpillar samples, bead beating and DNA extraction were performed with the MP Biomedical FastDNA™ Spin Kit. For the soil samples, DNA was extracted using Qiagen DNeasy PowerSoil Kit. Approximately 10 ng of template DNA was used for PCR using primers ITS4ngs and ITS3mix targeting the ITS2 region of fungi46. For bacteria we used primers 515FB and 806RB47 targeting the V4 region of the 16S rRNA gene. Presence of PCR product was checked using agarose gel electrophoresis. The PCR products were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter). Adapters and barcodes were added to samples using Nextera XT DNA library preparation kit sets A-C (Illumina, San Diego, CA, USA). The final PCR product was purified again with AMPure beads, verified using agarose gel electrophoresis and quantified with a Nanodrop spectrophotometer before equimolar pooling. Separate libraries were constructed for bacteria and fungi, and from rhizosphere soil samples (72 samples per library) and a combination of samples derived from leaves, caterpillars of the plants allocated to the detached leaf and intact plant bioassays, and roots (360 samples). This made the total data collected to be 4 runs on a MiSeq. Libraries were sequenced at McGill University and Genome Quebec Innovation Center. For all compartments, extraction negatives were used and further sequenced. A mock community, containing 10 fungal species, was included to compare between sequencing runs and to investigate the accuracy of the bioinformatics analysis.

Bioinformatic and statistical analysis

The bacteria data were analysed using an in-house pipeline (De Hollander, 2017) using the SILVA database with SINA classifier. The PIPITS pipeline (Gweon et al., 2015) was used to classify fungi. Taxonomy was assigned using the rdp classifier against the UNITE fungal ITS database (Abarenkov et al., 2010). Finally, the OTU table was parsed against the FunGuild (v1.1) database to assign putative life strategies to taxonomically defined OTUs (Nguyen et al., 2016). All singletons and all reads from other than bacterial or fungal origin (i.e. plant material, mitochondria, chloroplasts and protists) were removed from the dataset. The resulting data included approximately 10 million good quality (QC over 28, overlap over 25 bp, length over 100 bp, no chimeras) paired sequences for bacteria and 7.9 million sequences for fungi.

Samples that had over three times lower or higher number of reads than average in the same compartment were removed from the dataset. This resulted in removal of 1–10 samples out of 72 depending on organisms and compartment (Table S6.5). Furthermore, sequence count in a sample was used as a co-variate in the model when Chao1 and relative abundances of fungal classes and bacterial phyla were analysed to prevent the sequencing depth having effect on the results. Data was normalized using the cumulative sum scaling (CSS) after exploring several other normalization options (Weiss et al., 2017). We used the Adonis function with Bray-Curtis dissimilarity (permutational MANOVA using distance matrices; R package Vegan (Oksanen et al., 2006) to test whether microbial composition differed between sample types and plant community legacies, including species identity as an explanatory variable and the matrix of community dissimilarities among samples as the response. Separations among treatments were visualized using non-metric multidimensional scaling (NMDS) of a Bray-Curtis dissimilarity matrix using square transformation and Wisconsin standardization. For the OTU level analysis, the presence of each OTU in each compartment was individually calculated. As a rule, for an OTU to be present in a compartment, it needed to be present in more than 10% of the samples of the compartment. The ternary plots were created using package ggtern (Hamilton & Ferry, 2017). Generalized linear models (GLM) were used to compare the diversity and Chao1 index and the relative and absolute abundances (counts) of bacterial phyla and fungal classes between compartments and legacies. The Chao1 data was ln transformed prior to analysis to fulfil the requirements of normality. Sequence count was used as a co-variate in the analysis. To account for the overdispersion in the model when comparing different compartments, we used Poisson distribution in our generalized linear model (GLM) for the count data. Further, we fitted zero-inflated Poisson regression models (package PSCL in R) but with our data they were not superior to GLM with Poisson (Vuong test; $P>0.05$). The results of GLM were evaluated with a Chi-square test and a Tukey post-hoc test. To analyze the effects of different soil legacies on bacterial and fungal taxa and on caterpillar biomass, linear mixed effects models (LME) were used from the package nlme as the data within each compartment were generally normally distributed. All p-values derived from multiple calculations were corrected with Benjamini & Hochenberg which relies on calculating the expected proportion of false discoveries among rejected hypotheses to control for false discovery rate (FDR) (Benjamini & Hochberg, 1995). All numerical data were checked for (multivariate) normality and log-transformed if necessary. To create networks the co-occurrence of each OTU present in more than 10% of the samples of

the caterpillars was calculated using Spearman rank correlation coefficients following a Bonferroni correction ($P<0.05$) as a cut off for a significant correlation between two OTUs (Morrien et al., 2017). The networks were visualised in Cytoscape (Shannon et al., 2003). All statistical analyses were performed in R version 3.4.4 (R Development Core Team, 2008).

Data availability

Paired-end DNA sequencing reads for this project have been deposited in the European Nucleotide Archive under accession number PRJEB27512 [https://www.ebi.ac.uk/ena/data/view/PRJEB27512]. Plant and caterpillar growth data and soil chemistry data are deposited in Dryad [https://doi.org/10.5061/dryad.99504fd].

Supplementary information Chapter 6

Supplementary table S6.1: Effect of plant community type (communities with grasses only, with forbs only, and with mixtures of grasses and forbs), growth rate (fast- and slow-growing plant communities), and their interaction (C x GR) on soil chemistry during the conditioning phase in the field. Mean values are presented in Supplementary Figure S6.11. Effects that were significant after correction for FDR are marked in bold.

	Community (grass/forb/mixture)	Growth rate (fast/slow)	C x GR
	F (p)	F (p)	F (p)
pH	1.9 (0.153)	0.0 (0.848)	1.3 (0.285)
NO_2+NO_3	7.2 (0.002)	4.8 (0.312)	0.7 (0.481)
NH_4	0.1 (0.950)	1.8 (0.189)	0.5 (0.597)
Fe	2.0 (0.141)	1.1 (0.297)	2.1 (0.132)
Zn	0.6 (0.562)	1.1 (0.289)	1.3 (0.274)
P	0.2 (0.804)	0.0 (0.914)	0.5 (0.626)
S	1.7 (0.197)	0.0 (0.897)	0.2 (0.836)
K	0.1 (0.097)	5.1 (0.059)	0.0 (0.024)
Mg	0.1 (0.930)	2.0 (0.167)	0.2 (0.802)

Supplementary table S6.2: List of plant species sown in the field plots.

Fast-growing grasses	Slow-growing grasses	Fast-growing forbs	Slow-growing forbs
Dactylis glomerata (Dg)	*Arrhenaterum elatius* (Ae)	*Plantago lanceolata* (Pl)	*Tripleurospermum maritimum* (Tm)
Holcus lanatus (Hl)	*Briza media* (Bm)	*Rumex acetosella* (Ra)	*Clinopodium vulgare* (Cv)
Alopecurus pratensis (Ap)	*Trisetum flavescens* (Tf)	*Achillea millefolium* (Am)	*Geranium molle* (Gem)
Agrostis capillaris (Ac)	*Anthoxanthum odoratum* (Ao)	*Taraxacum officinale* (To)	*Myosotis arvensis* (Ma)
Lolium perenne (Lp)	*Deschamptia flexuosa* (Df)	*Epilobium hirsutum* (Eh)	*Galium mollugo* (Gam)
Phleum pretense (Pp)	*Festuca ovina* (Fo)	*Crepis capillaris* (Cc)	*Gnaphalium sylvaticum* (Gs)

Supplementary table S6.3: Composition of the sown grass, forb and mixed communities consisting of fast and slow growing plants. Species abbreviations are explained in Table S1.

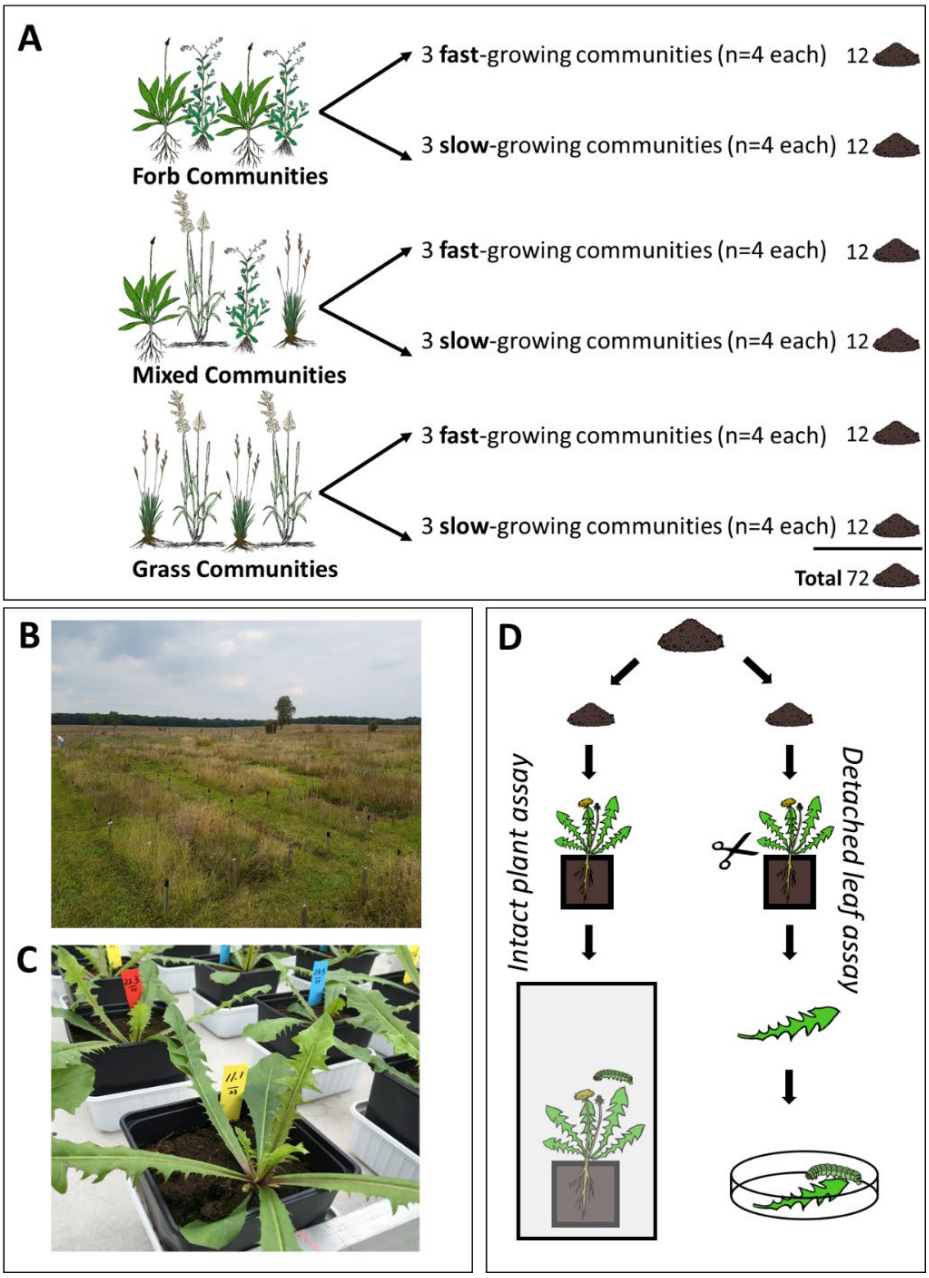
Type	Community	Grasses			Forbs		
Fast-growing grasses	1	Dg	Hl	Ap			
	2	Ac	Lp	Hl			
	3	Pp	Dg	Lp			
Fast-growing forbs	4				Pl	Cc	Ta
	5				Ra	Cc	Am
	6				Am	Eh	To
Fast-growing mixtures	7	Dg	Hl	Ap	Pl	Cc	Ta
	8	Ac	Lp	Hl	Ra	Cc	Am
	9	Pp	Dg	Lp	Am	Eh	To
Slow-growing grasses	10	Ae	Bm	Fo			
	11	Bm	Tf	Ao			
	12	Ao	Df	Tf			
Slow-growing forbs	13				Tm	Cv	Gem
	14				Cv	Gs	Ma
	15				Tm	Ma	Gam
Slow-growing mixtures	16	Ae	Bm	Fo	Tm	Cv	Gem
	17	Bm	Tf	Ao	Cv	Gs	Ma
	18	Ao	Df	Tf	Tm	Ma	Gam

Supplementary table S6.4: Recipe for the artificial diet that was used to feed *Mamestra brassicae* in the first larval stage.

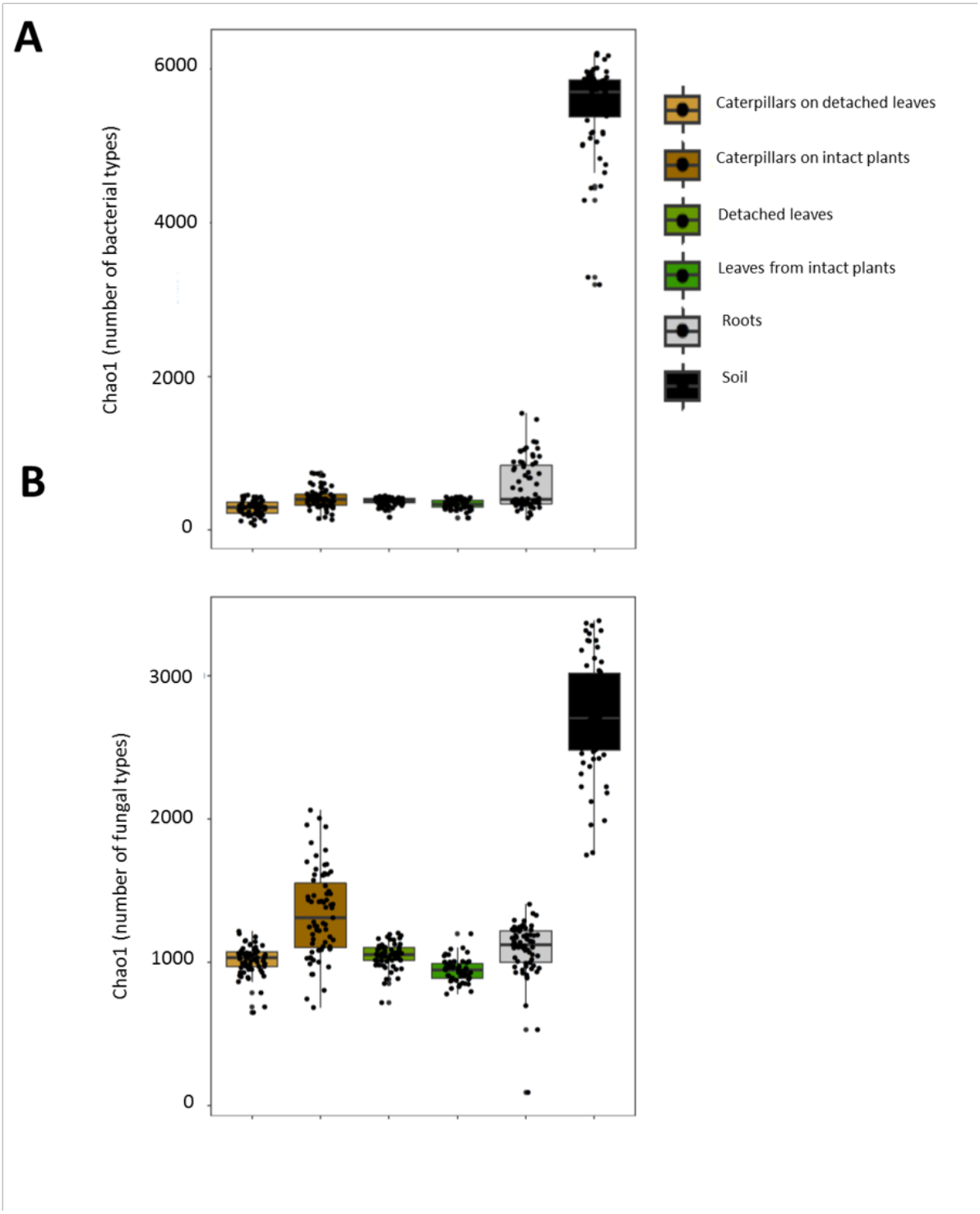
Ingredients
5L water
140g agar
800g corn flour
250g beer yeast
150g wheat germs
10g sorbic acid
40g ascorbic acid
8g nipagin (methyl-4-hydroxybenzoate)
0.5g streptomycin
Preparation
Bring 4L water to a boil, while dissolving the agar in 1L cold water. When boiling, turn down the heat and add corn flour, yeast and wheat germs and stir until homogenized. Add sorbic acid and nipagin until homogenized. Add ascorbic acid and streptomycin and stir until homogenized. Freeze in small portions and thaw before use for rearing.

Supplementary table S6.5 Number of samples left in each compartment after filtering the samples with too few or too many reads.

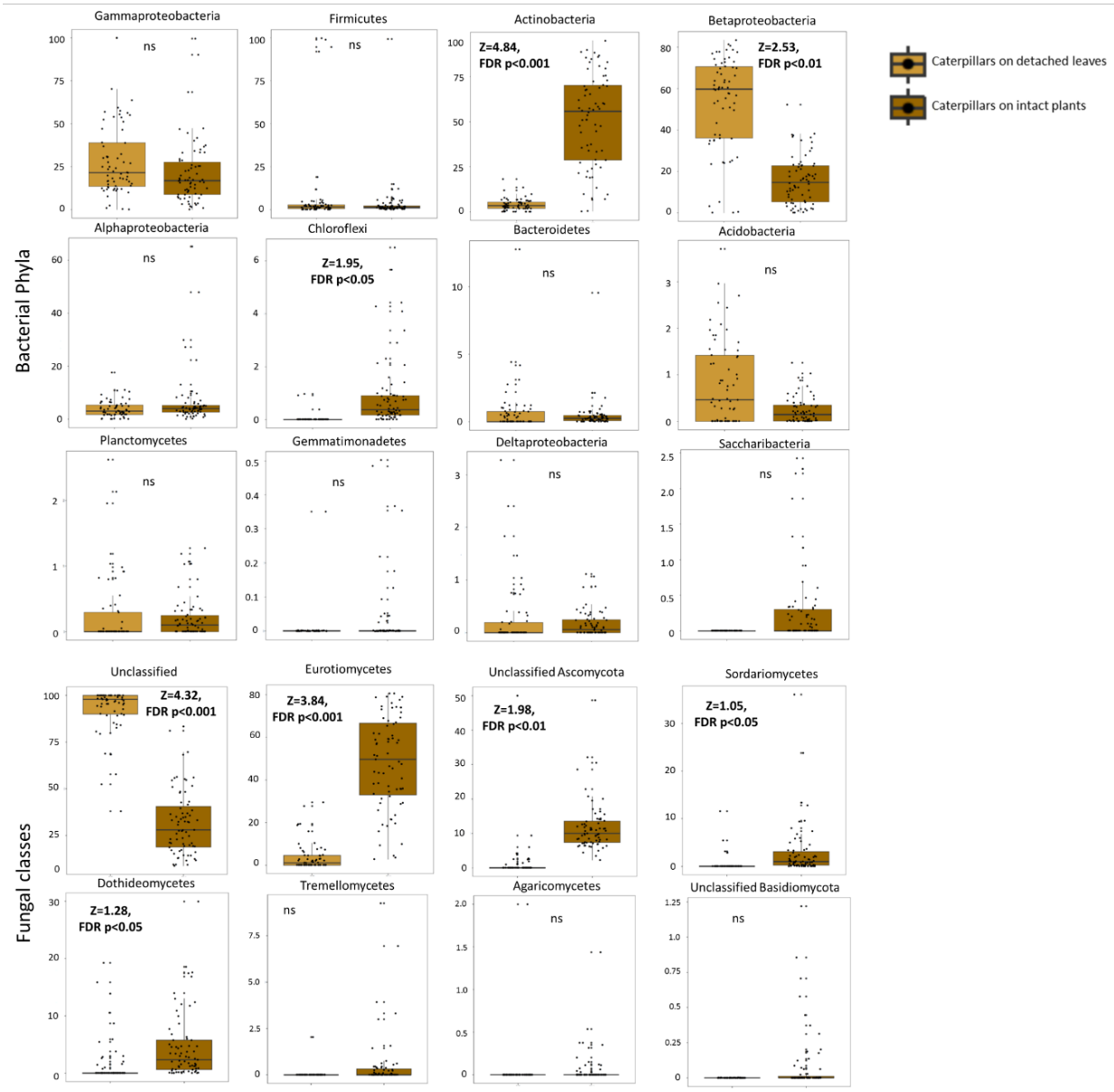
	Compartment	Fungi (n=72)	Bacteria (n=72)
Intact plant assay	Caterpillars	71	68
	Leaves	62	65
	Roots	67	70
	Soil	65	68
Detached leaf assay	Caterpillars	68	69
	Leaves	64	70



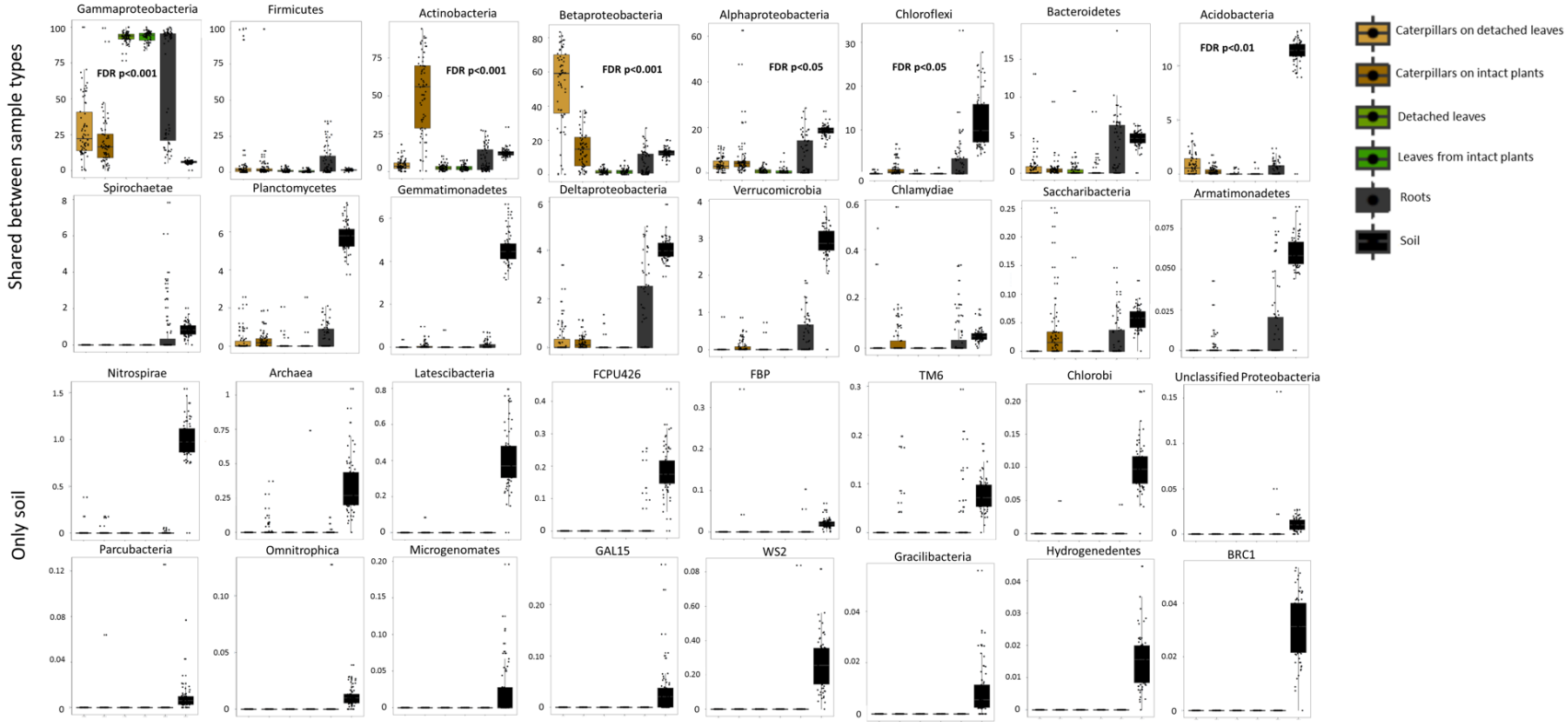
Supplementary Figure S6.1: A Experimental design of the field experiment from which the soils were collected. Plots sown with plant communities that consisted of only forbs, forbs and grasses, or only grasses. For each of these categories, there were three randomized slow-growing plant communities, or three randomized fast-growing plant communities (see Tables S6.2 and S6.3 for species composition). Each of the individual communities was replicated four times over four blocks in the field. **B.** Picture of the field experiment at 'De Mossel', Ede, The Netherlands in September 2017. **C.** *Taraxacum officinale* has a rosette growth-form but leaves generally grow upright. Except for the first few true leaves, most leaves are never in touch with the soil. **D.** Schematic overview of experimental procedure. Each donor soil was divided over two pots and one individual *T. officinale* was planted in each pot. At the onset of the caterpillar assays one plant was caged with caterpillars (intact plant assay). From the other plant, leaves were clipped and fed to caterpillars in large petri dishes (detached leaf assay).



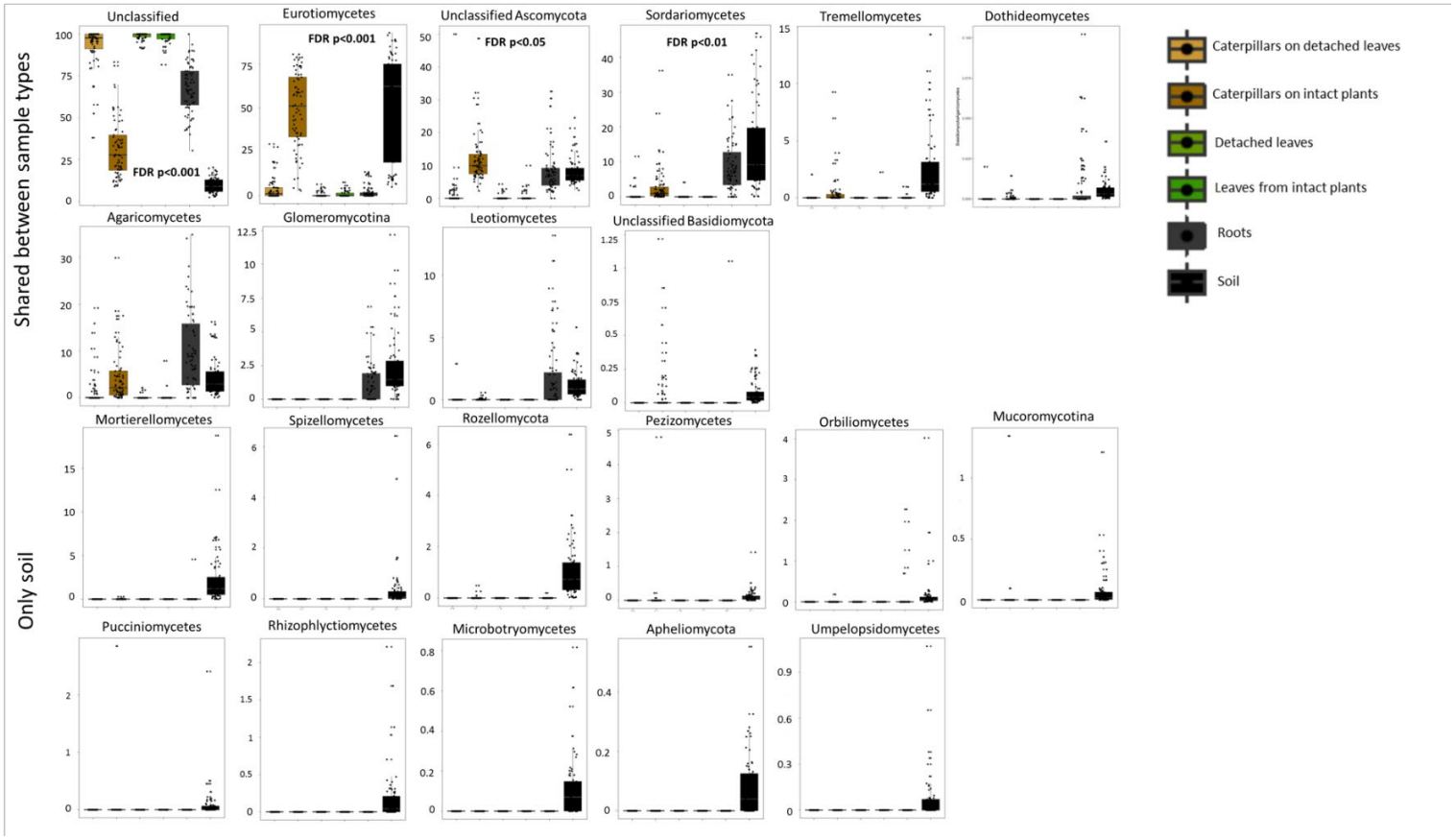
Supplementary Figure S6.2: OTU Richness of **A.** bacteria and **B.** fungi. The Chao1 index is shown for caterpillars on intact plants (dark brown), caterpillars on detached leaves (light brown), leaves from plants from the "intact-plant assay" (dark green) and leaves from plants from the "detached-leaf assay" (light green), roots (grey) and soil (black). The Tukey box-and-whisker plots depict median number of phyla and classes in each compartment and variation is shown in the scatter.



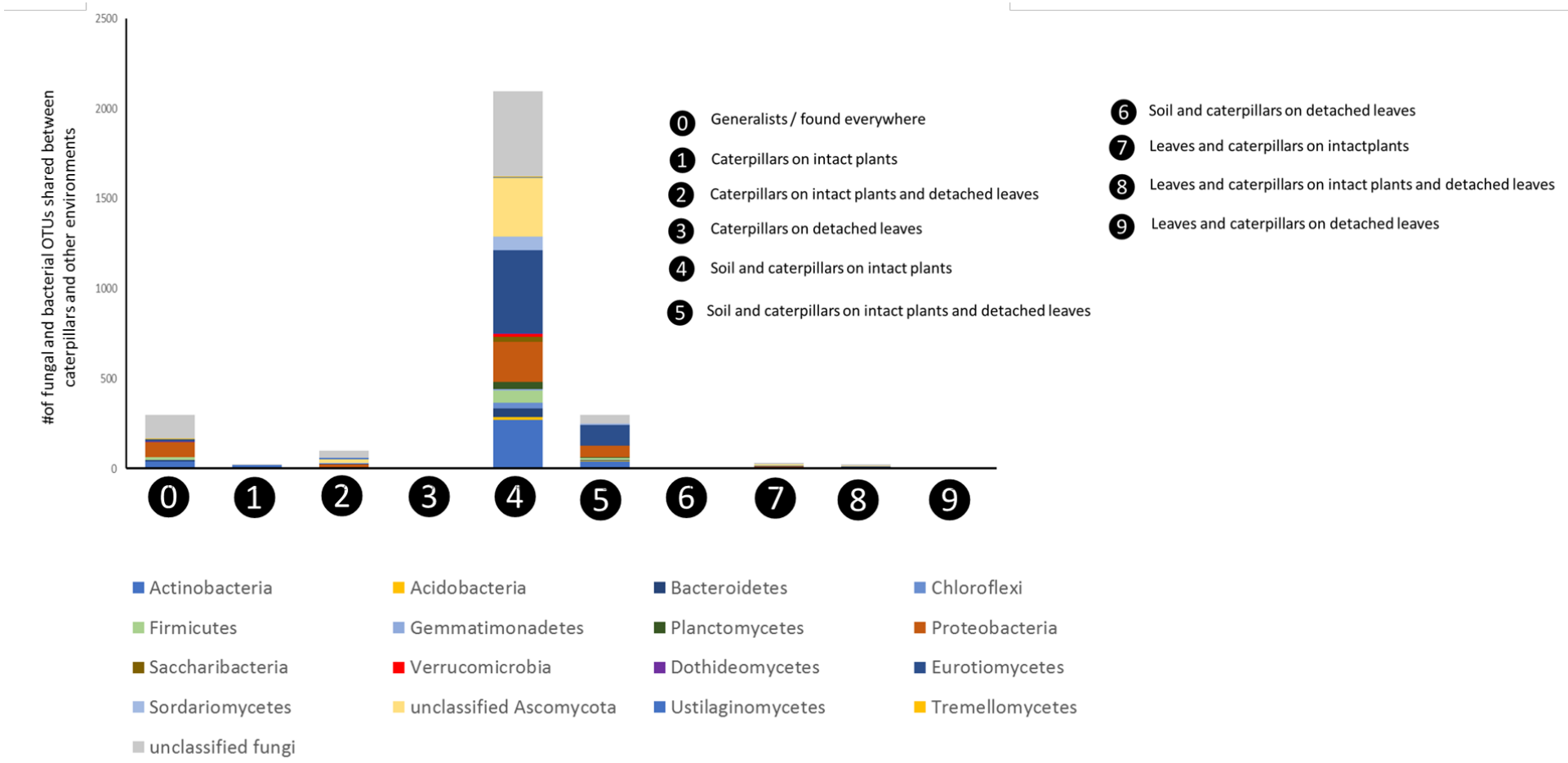
Supplementary Figure S6.3: Relative abundance of bacterial phyla and fungal classes inside caterpillars kept on intact plants (dark brown) and caterpillars fed detached leaves (light brown). The box plots depict median relative abundance of phyla and classes in caterpillars on detached leaves and on intact plants and variation is shown in the scatter. The Tukey box-and-whisker plots of relative abundances of bacterial phyla and fungal classes are organized by abundance, in decreasing order. The z-values derived from a GLM model and the FDR corrected p-values for bacterial phyla and fungal classes that significantly differ between the caterpillars on intact plants and on detached leaves are presented in the panels.



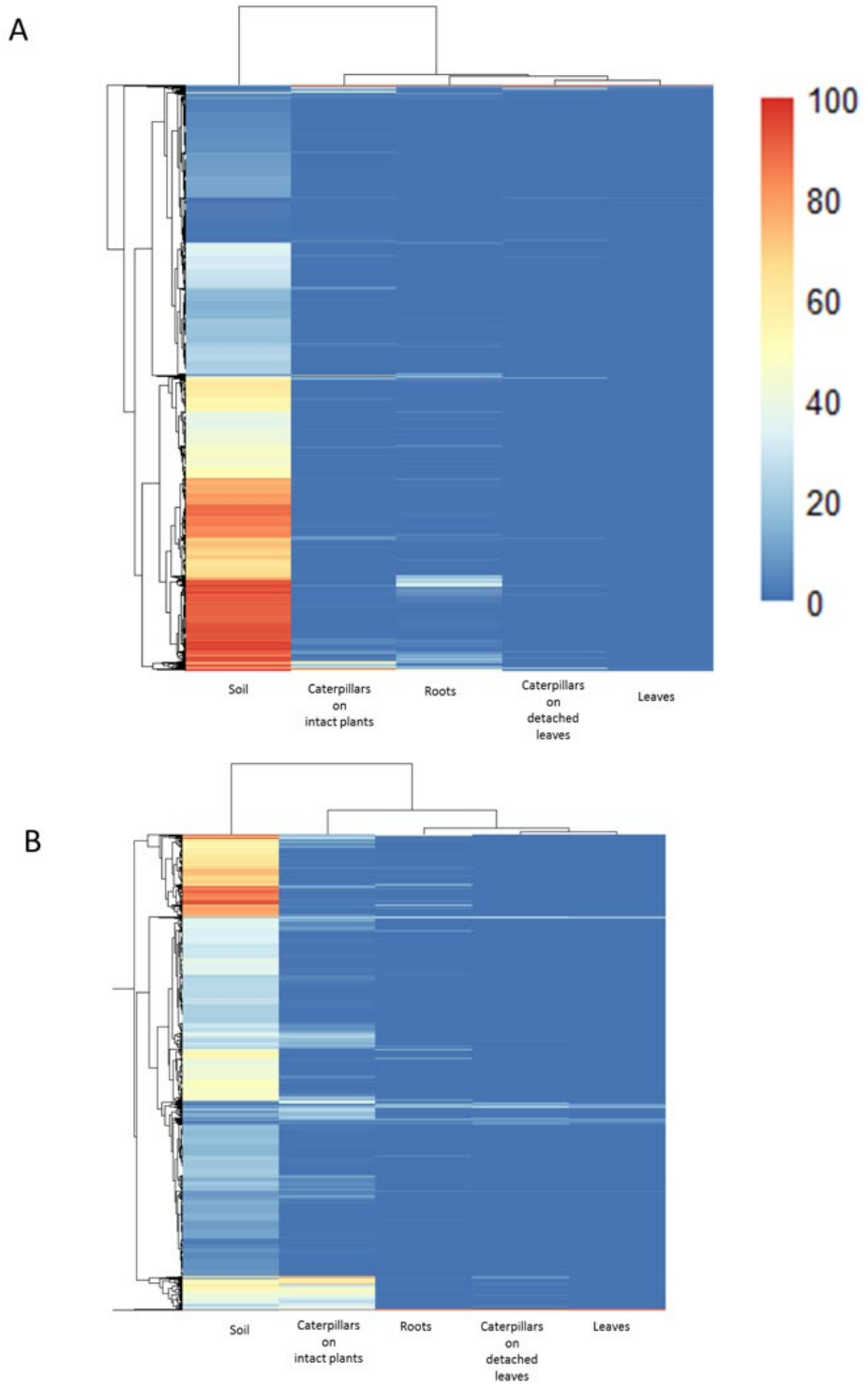
Supplementary Figure S6.4: Relative abundance of bacterial phyla in caterpillars, leaves, roots and soil. The upper 16 panels represent phyla shared between multiple sample types and the lower 16 panels are rare in other environments than soil (Figure 6.1A). The light brown color represents microbes in caterpillars fed on detached leaves, dark brown represents microbes in caterpillars kept on intact plants, light green represents microbes in leaves from plants of the detached-leaf assay plants; dark green represents leaves from plants from the intact-plant assay, grey represents microbes inside the roots, and black represents microbes in the soil samples. The Tukey box-and-whisker plots depict median relative abundance of each phyla and variation is shown in the scatter. The phyla are ordered based on their relative abundance from highest to lowest. Significant FDR corrected p-values derived from a chisquare test of the GLM model are presented in the panels for samples present in all compartments.



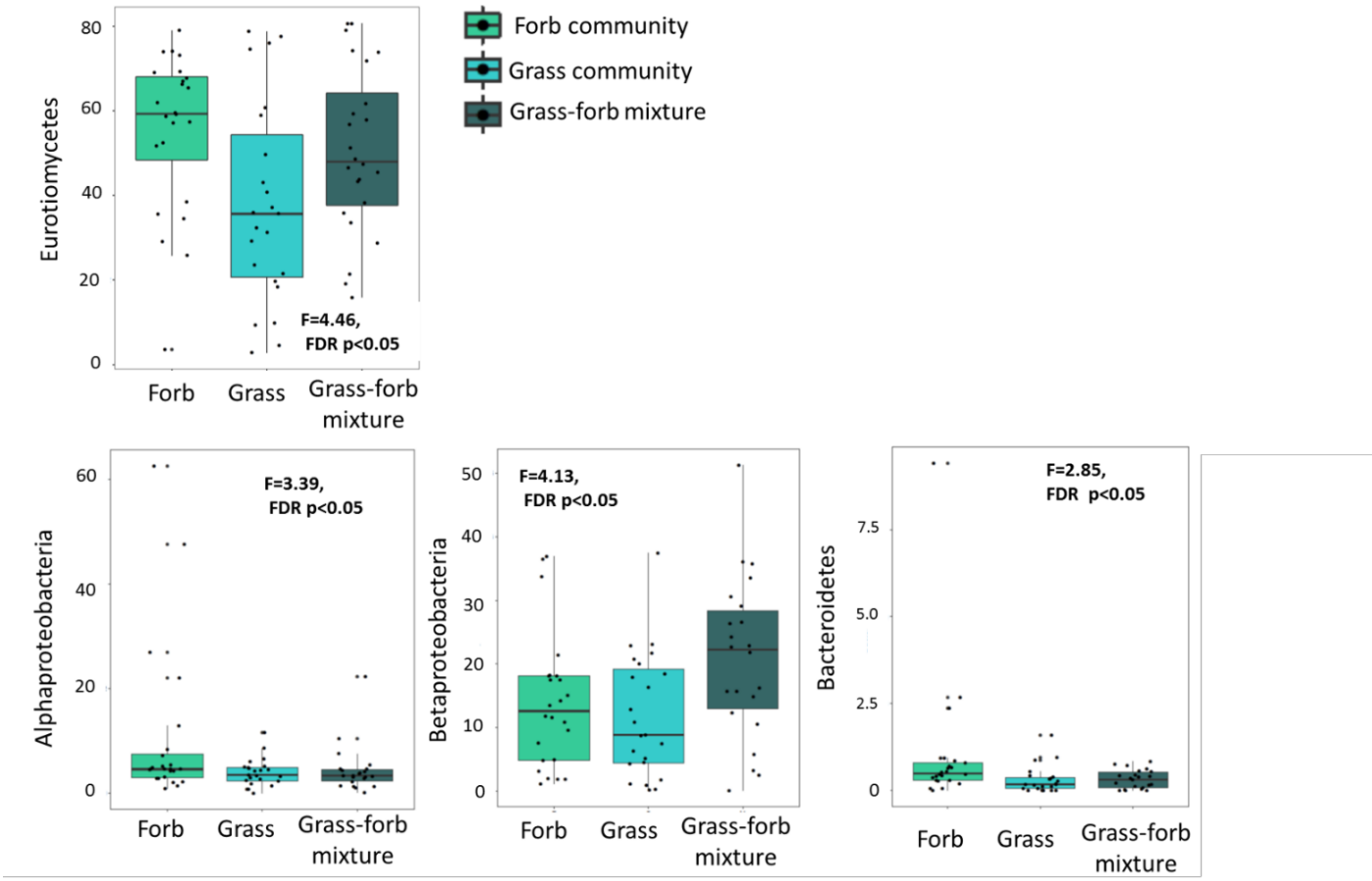
Supplementary Figure S6.5: Relative abundance of fungal classes in caterpillars, leaves, roots and soil. The first 10 panels are shared between multiple sample types and the last 11 are rare in other environments than soil (Figure 6.1B). Bars with a light brown color represent microbes in caterpillars fed on detached leaves, dark brown represents microbes in caterpillars kept on intact plants, light green represents microbes in leaves from plants of the detached-leaf assay plants; dark green represents leaves from plants from the intact-plant assay, grey represents microbes inside the roots, and black represents microbes in the soil samples. The Tukey box-and-whisker plots depict median relative abundance of each class and variation is shown in the scatter. The classes are ordered based on their relative abundance from highest to lowest. Significant FDR corrected p-values derived from a chisquare test of the GLM model are presented in the panels for samples present in all compartments.



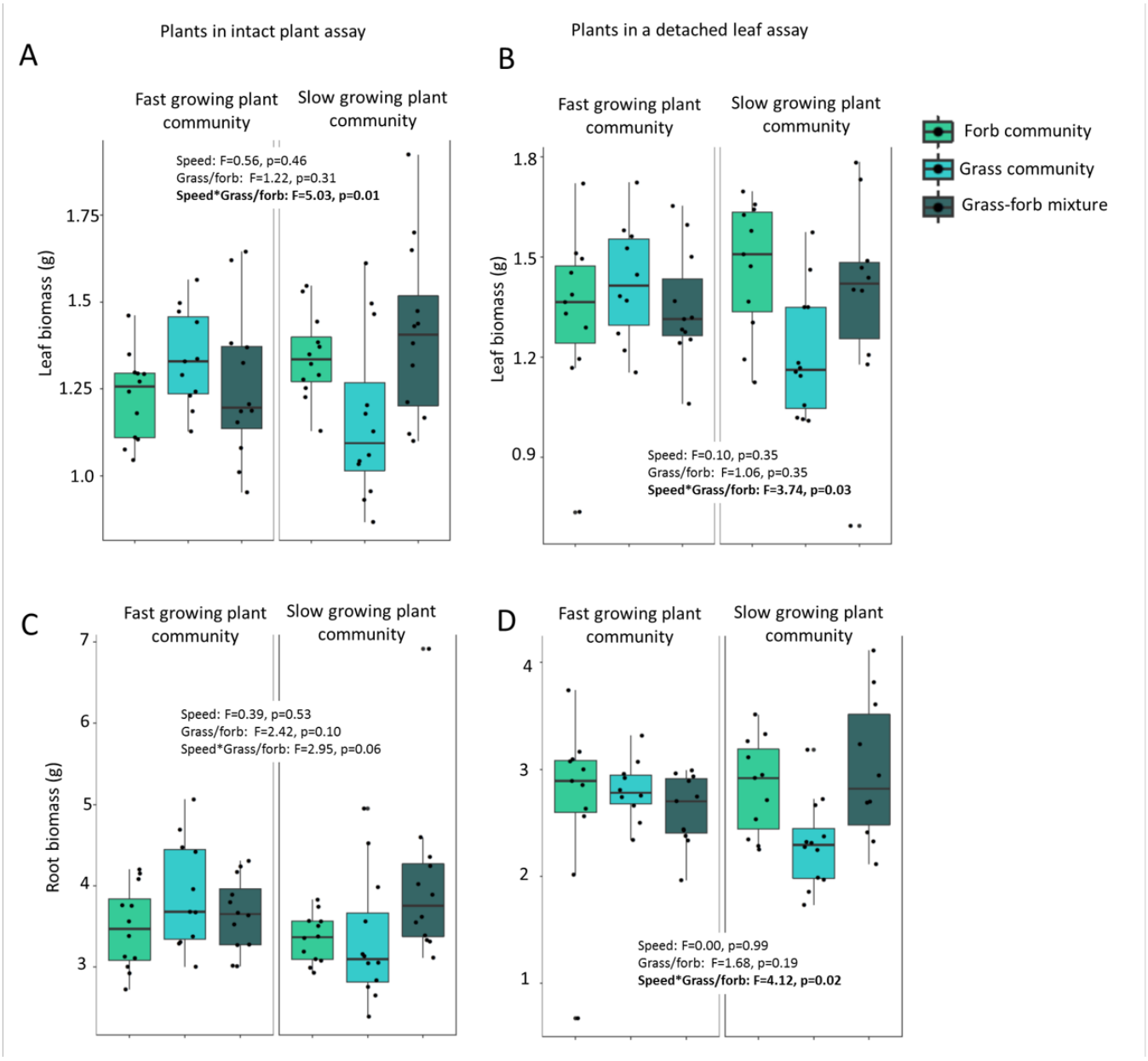
Supplementary Figure S6.6: The identity and the number of the OTUs shared between the environments (0-9) depicted in Figure 6.2C. Only phyla and classes with more than 5 OTUs present are presented in the Figure.



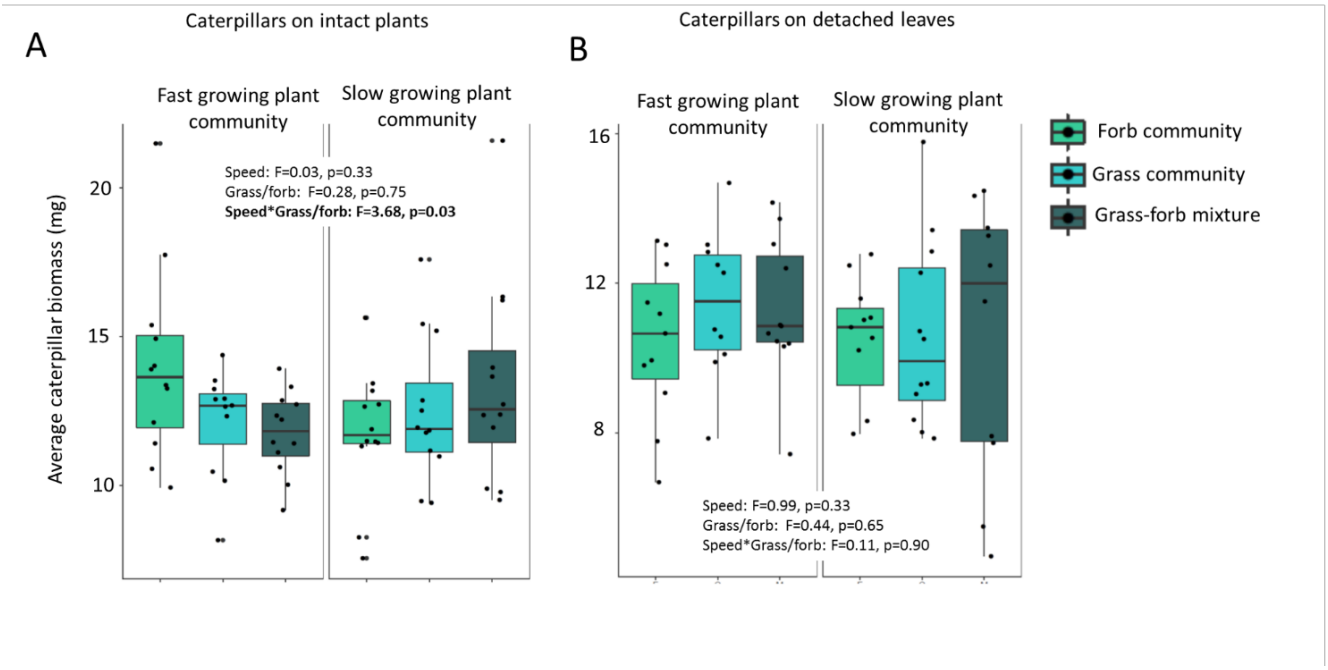
Supplementary Figure S6.7: Heat maps showing all **A.** bacterial and **B.** fungal OTUs with average abundance of more than <0.1% presence in samples (as % of samples present) in different compartments (soil, caterpillars on intact plants, caterpillars on detached leaves, roots and leaves), and how compartments cluster with each other. The red color indicates that a class is found in 100% of the samples while blue colors indicate that it is found in 0-30% of samples.



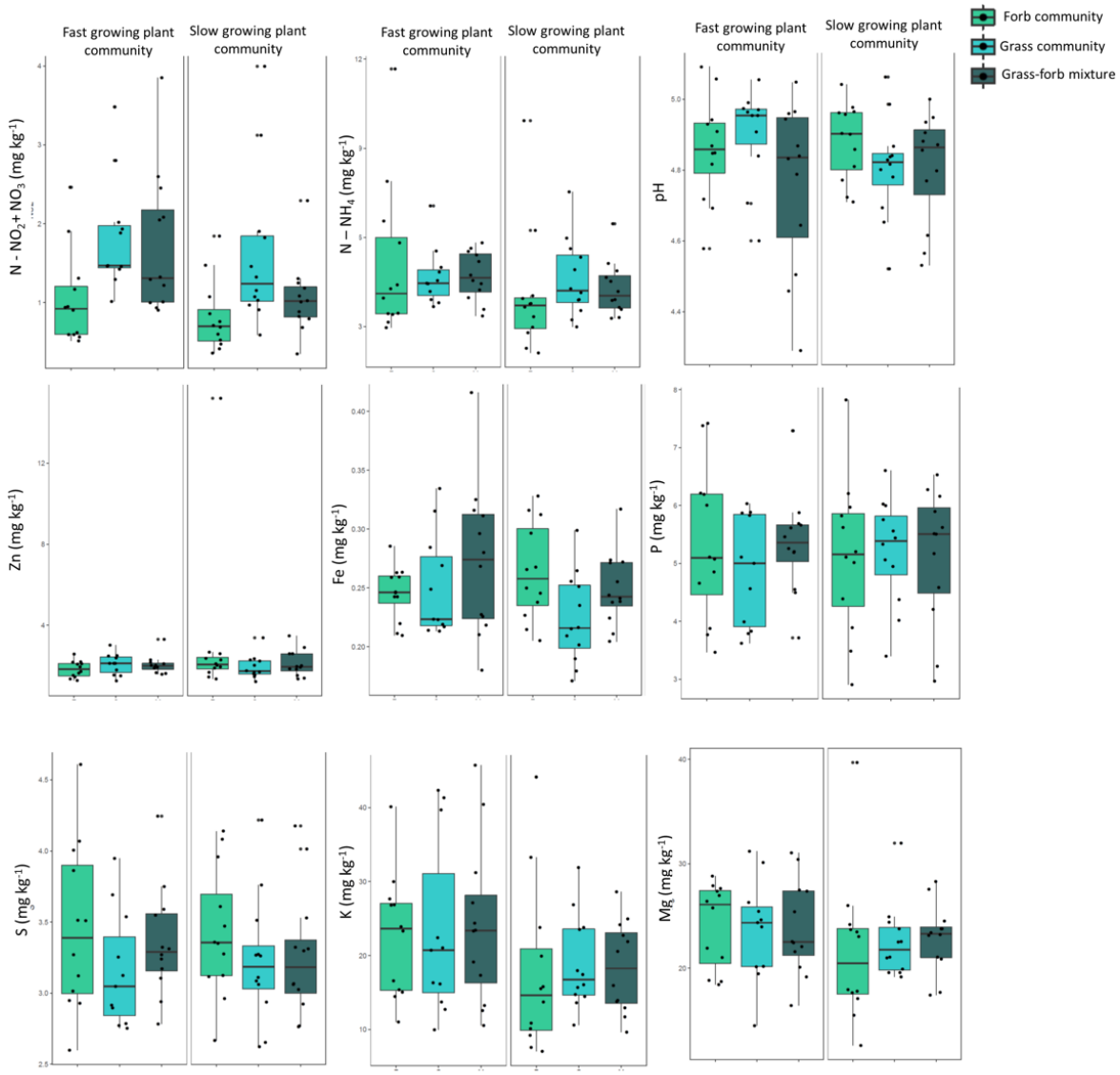
Supplementary Figure S6.8: Fungal classes and bacterial phyla in caterpillars kept on intact plants that are significantly affected by the type of the plant community that previously grew in the soil, as presented in Figure 6.3B&F. Relative abundances are depicted and they are presented in order of abundance. The Tukey box-and-whisker plots depict median relative abundance of phyla and classes and variation is shown in the scatter. Statistical results of ANOVAs on the relative abundances are also presented. Light green represents soil from grass communities, turquoise represents soil from forb communities and dark green represents soil from mixed communities.



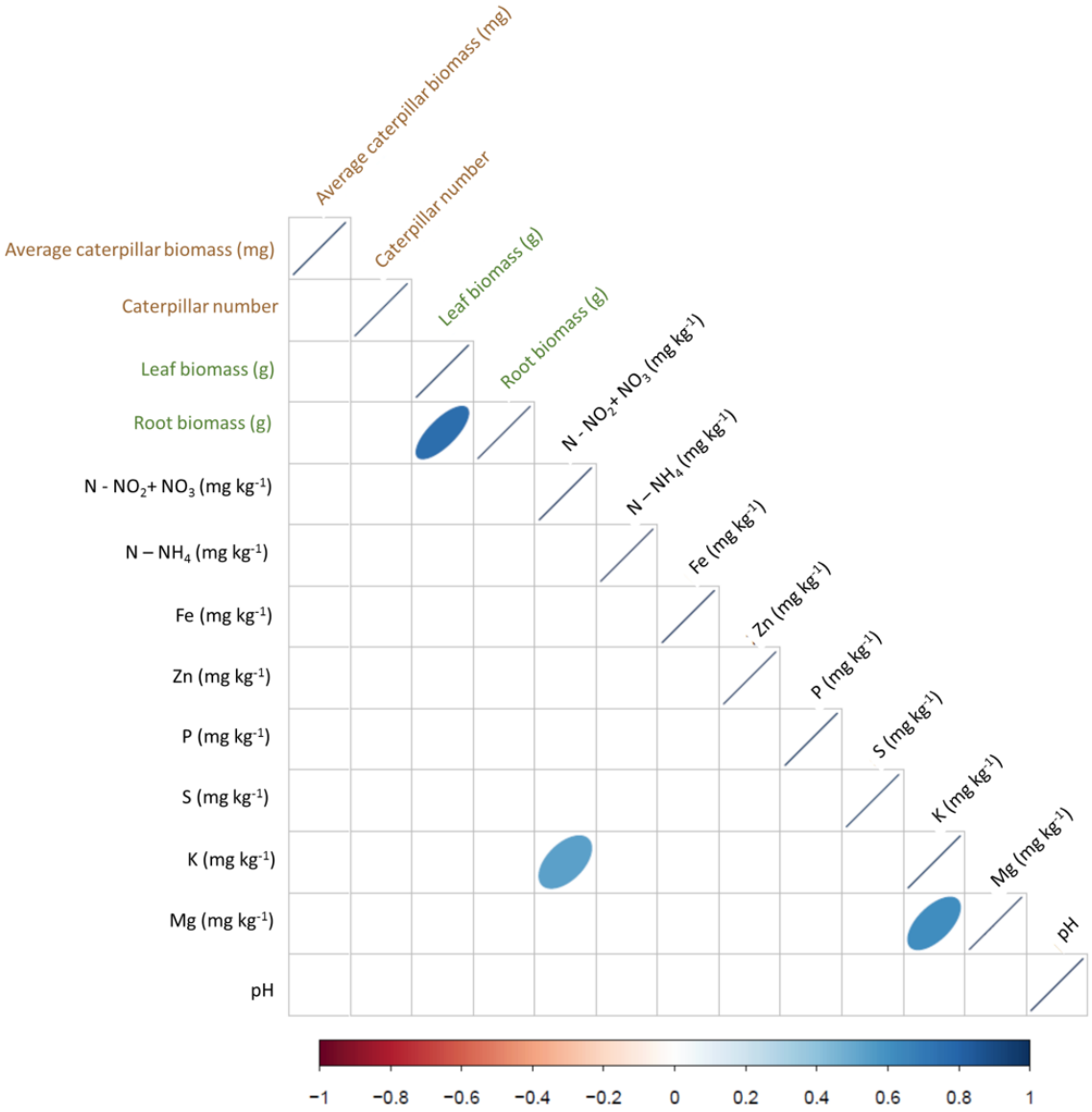
Supplementary Figure S6.9: Average leaf (**A & B**) and root (**C & D**) biomass of dandelion plants from the assay with intact plants with caterpillars (**A & C**) and from the assay with detached leaves (**B & D**) grown in soils with a legacy of fast or slow growing plants, and a legacy of forb, grass or mixed plant communities. The Tukey box plots depict median biomass of dandelion in different legacies and variation is shown in the scatter. Light green represents soil from grass communities, turquoise represents soil from forb communities and dark green represents soil from mixed communities. F-values and P-values from a GLM are also presented and significant p-values are marked in bold.



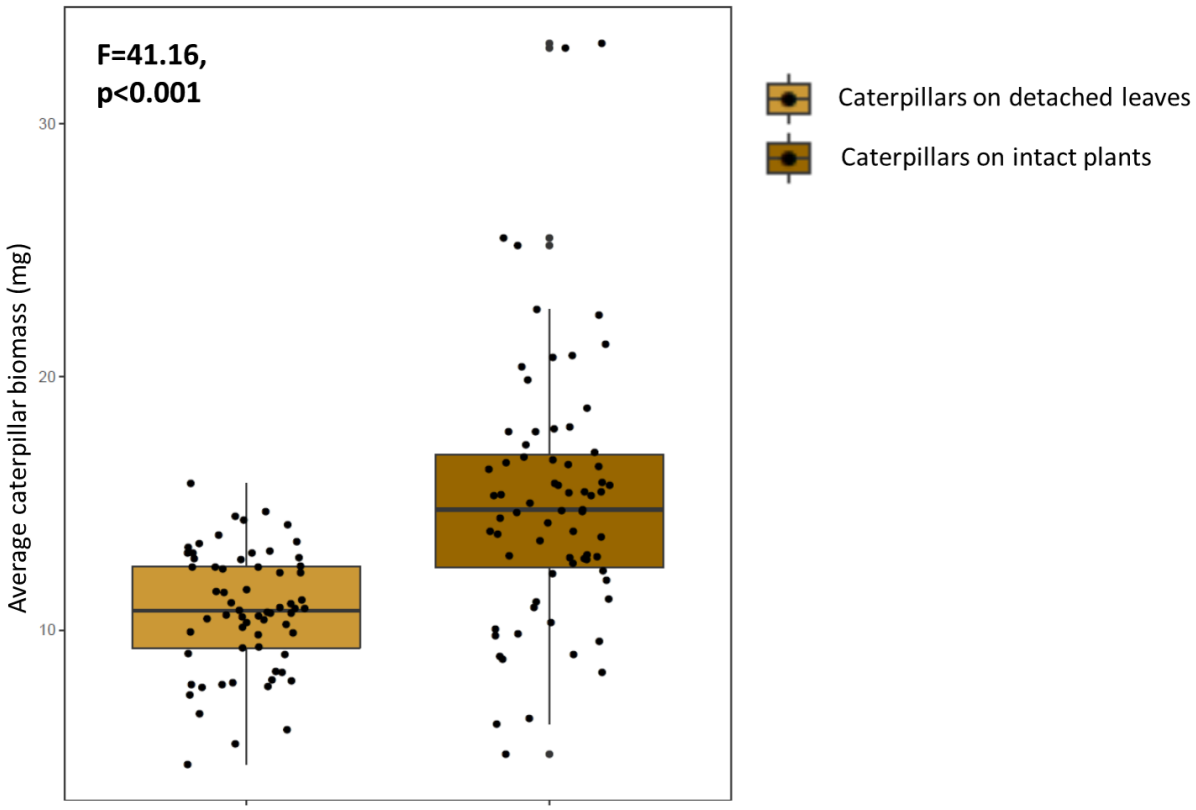
Supplementary Figure S6.10: Average caterpillar biomass on intact plants (**A**) and on detached leaves (**B**) from plants grown in soils with a legacy of fast or slow growing plants, and a legacy of forb, grass or mixed plant communities. The Tukey box plots depict median biomass of caterpillars in different plant legacies and variation is shown in the scatter. Light green represents soil from grass communities, turquoise represents soil from forb communities and dark green represents soil from mixed communities. F-values and p-values from a GLM are also presented and significant p-values are marked in bold.



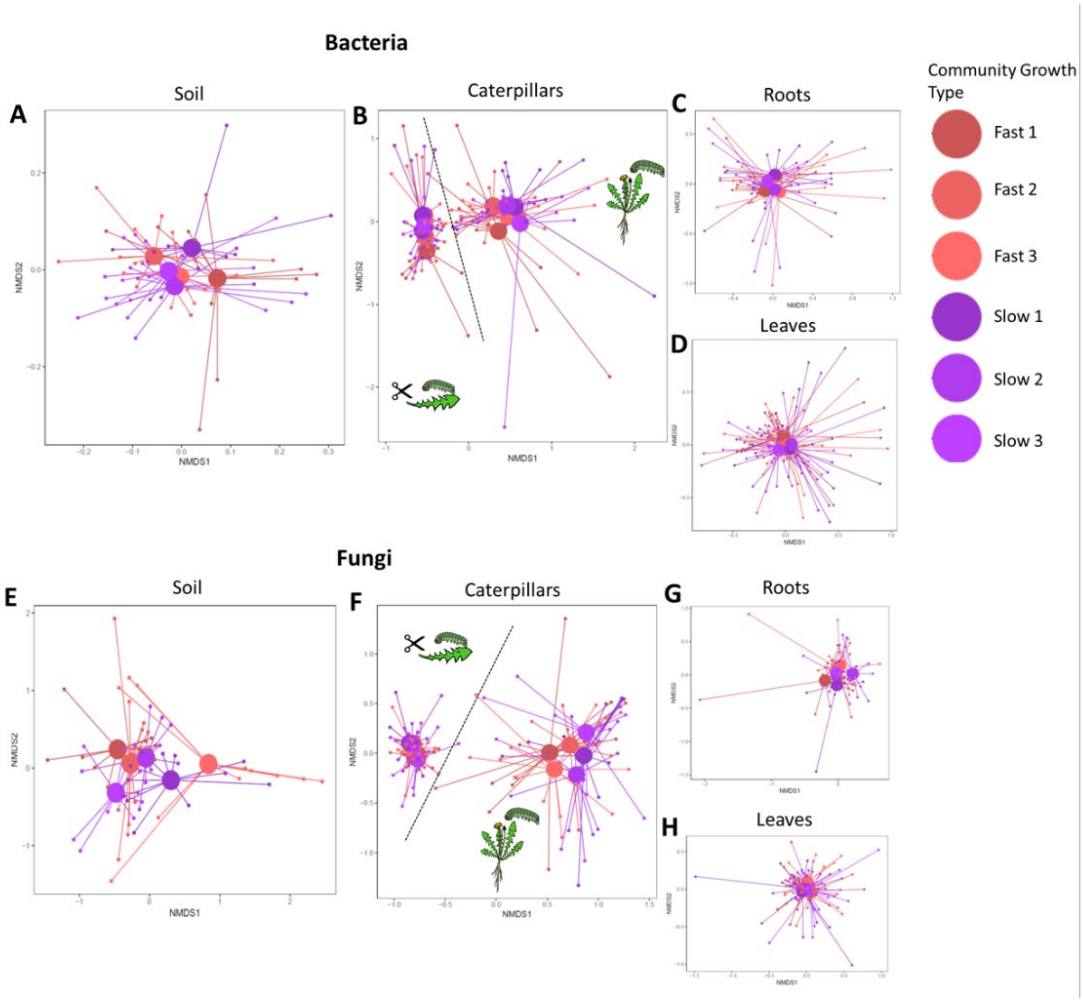
Supplementary Figure S6.11: Chemical composition of soils with a legacy of fast or slow growing plants, and a legacy of forb, grass or mixed plant communities. The Tukey box-and-whisker plots depict median measurement of chemistry in soils with different plant legacies and variation is shown in the scatter. Light green represents soil from grass communities, turquoise represents soil from forb communities and dark green represents soil from mixed communities. The results from a GLM are presented in supplementary table S6.1.



Supplementary Figure S6.12: Correlation matrix for soil chemistry variables and caterpillar and plant performance. Correlations are based on Pearson correlation coefficients. Average caterpillar biomass (brown), caterpillar survival (brown) per plant, and leaf- and root biomass (green) per soil sample was used. The scale color of the filled squares indicates the strength of the correlation (r) and whether it is negative (red) or positive (blue). All correlations are corrected for FDR and only significant correlations with $p<0.05$ are shown. If the correlation is not significant, the box is left white.



Supplementary Figure S6.13: Tukey box-and-whisker plot showing median caterpillar biomass after feeding on whole plants for 14 days (dark brown) or detached leaves for 5 days (light brown). The F-value and p-value of a GLM are also presented.



Supplementary Figure S6.14: Effects of plant community growth rate (fast or slow) on the community composition of bacteria (A-D) and fungi (E-H) in caterpillars, leaves, roots and soil. NMDS plots are based on Bray-Curtis similarity. The 2D stress value for each panel ranges between 0.11-0.18. **A-F** microbiomes originating from soils conditioned by fast growing species are represented by markers in shades of red and microbiomes originating from soils conditioned by slow growing species are represented by markers in shades of blue. The centroids are marked with larger markers; smaller markers depict individual samples. **A&E** show the effect on soil microbiomes, **B&F** on microbiomes in caterpillars both on intact plants and on detached leaves, **C&G** on root microbiomes, and **D&H** on leaf microbiomes.

Chapter 7

General Discussion

Robin Heinen

Plants, when growing in the soil, can influence the community composition of organisms in the soil. Via this, plants can leave a biotic legacy in the soil that may persist over time, and affect the performance of plants grown later in that soil (Reynolds et al., 2003; Ehrenfeld et al., 2004; Kulmatiski et al., 2008; Van der Putten et al., 2013). More recent work has shown that plant-induced soil legacy effects (i.e. the effects of specific alterations in entire soil communities) can also influence plant-insect interactions (Kostenko et al., 2012; Kos et al., 2015; Wurst & Ohgushi, 2015). This is not surprising given the large impact that individual taxa of soil organisms have on plant-feeding insects (e.g., reviewed in Pineda et al., 2010; Gehring & Bennett, 2008; Hartley & Gange, 2008; Koricheva et al., 2008; Johnson et al., 2012; Soler et al., 2012; Wondafrash et al., 2013 and Heinen et al., 2018a, Chapter 2 of this thesis). In this thesis we set out to explore important questions in this novel field of soil legacy effects on aboveground plant-insect interactions.

In this PhD project, I first explored if plant species-specific soil legacy effects influence plant growth and insect herbivory across a range of twelve host plant species, individually potted in soils conditioned by all twelve species individually. I also investigated whether plant traits, in particular plant functional type and growth rate, played a role in mediating these soil legacy effects. Second, I investigated if, when plant communities are grown on soils with contrasting legacies, trait-mediated soil legacy affected plants and associated insects. Third, in an experiment that I did in collaboration with my colleague, Feng Zhu, I investigated potential mechanisms of how soil legacy effects can alter herbivory, specifically via plant secondary metabolism and phytohormonal pathways, in a focal plant species, *Plantago lanceolata*. Fourth, in an equal collaborative effort with Emilia Hannula, Feng Zhu and Martijn Bezemer, I tested how different microbial soil legacies affect the microbiomes of a focal plant, *Taraxacum officinale*, and a generalist insect herbivore feeding on its aboveground parts. Across all experimental chapters, we have observed that what grew in the soil in the past can have profound effects on the current composition of soil life, which in turn has an impact on establishing plants, and on aboveground insect herbivores.

Below, I will discuss and compare my findings from different chapters and place them in a broader context. As the hypotheses and questions tested across my chapters are variable in nature, I have tried to guide the discussion of these subjects under a series of specific headers that should be rather self-explanatory.

Soil legacy effects on aboveground plant-insect interactions.

A few studies that were published prior to this thesis reported plant-mediated soil legacy effects on plant-insect interactions for a well-known model plant system, ragwort, *Jacobaea* vulgaris (Bezemer et al., 2006a; Reidinger et al., 2012; Kostenko et al., 2012; Kos et al., 2015; Wang et al., 2019). Some of these studies have shown long-term legacy effects of sowing different plant diversity treatments in the field. These treatments affected plant-insect interactions that could, at least partly, be explained by composition of soil organisms (Bezemer et al., 2006a; Reidinger et al., 2012). Others have shown legacy effects of herbivory treatment on plants, via changes in soil fungal community composition, on future interactions between ragwort and a chewing herbivore (Kostenko et al., 2012). Lastly, one study showed that different plant species leave different fungal legacies in the soil, which affected the colony growth of two aphid species on ragwort (Kos et al., 2015). One of the main goals of this project was to investigate whether plant-mediated soil legacy effects on aboveground plant insect interactions can be considered a general phenomenon that occurs in a broad range of plant species, or that, instead, they are a rather rare event, that is strictly observed in a few select species (such as *Jacobaea vulgaris*). My findings suggest that plant growth and insect herbivory can be affected by plant-mediated (microbial) soil legacy effects across a broad range of plant species. I individually potted twelve plant species on soils with legacies that were created by all twelve of these species. For all of the twelve responding plant species I observed that there was at least one (but often more) soil with a plant-mediated legacy that resulted in a significant effect on insect herbivory (in terms of leaf consumption or growth of *Mamestra brassicae*), being either higher or lower than the average for that plant species (Chapter 3, Supplementary Figure S3.6). For plant biomass, in ten out of twelve species there were one or more soils in which plants performed significantly better or worse than average for that plant species (Chapter 3, Supplementary Figure S3.6). I believe that this is a first indicator that in most plant species, specific soil legacy effects may play a role in shaping aboveground plant-herbivore interactions, be it through their effects on plant growth or via physiochemical plant responses. We further analyzed the average effects that plant species have, via their soils, on all twelve plant species, and aboveground insects feeding on them, which indicated that plant species had very different legacy effects on herbivore performance and plant growth (Chapter 3, Figure 3.5). In Chapter 4, when the same set of plants were grown in the soils with different legacies

as a community, I observed that herbivore feeding behaviour was affected by the legacy of plants grown previously on that soil in three out of six experimental communities (Chapter 4, Figure 4.2). Lastly, in Chapter 6, I used soil legacies originating from different plant communities that were grown in the field. Soils from these different plant communities significantly affected growth of *Taraxacum officinale* (Chapter 6, Supplementary Figure S6.9), and the insect herbivore *M. brassicae* (Chapter 6, Supplementary Figure S6.10). My conclusion is that for most plant species, there are specific soils with microbial legacies that can affect their growth and their interactions with aboveground insect herbivores and thus the concept could be considered a general phenomenon and present in many plant species.

Evidence is also accumulating from other studies that plant-mediated soil legacy effects may influence plant-insect interactions in various other wild plant species, as has been observed in brassicaceous plants (Badri et al., 2013; Hubbard et al., 2018), or in agricultural crops (Carillo et al., 2019; Hu et al., 2018). For instance, a recent study demonstrated how maize plants can steer their local soil microbiome, mediated by specific plant secondary metabolites called benzoxazinoids (Hu et al., 2018). These soil microbiomes, in turn, negatively affected maize growth and growth of the fall armyworm, *Spodoptera frugiperda,* that fed on the plants. When benzoxazinoid knockout maize plants were used to condition soils, the observed effects on subsequent plant and insect growth in these soils were less negative. If the benzoxazinoid knockout maize plants were combined with a benzoxazinoid application, the results mirrored the soil legacy effects of wildtype maize. The fact that all of these effects could only be observed in live soils, but not in sterilized soils, strongly indicates that the soil microbiome was, at least partly, responsible for these effects. Negative soil legacy effects (or specific organisms present in soil biomes) may lead to suppressed plant growth and increased defense induction (Van der Putten et al., 2001; Hu et al., 2018). This idea is also in line with my own findings in Chapter 3. I observed a positive correlation between the legacy effects that soils have on subsequent plant growth and the legacy effects that those soils have on insects feeding on these plants. Soils that yield more vigorous plants, generally also yield an increase in herbivore performance (in line with the vigour hypothesis, Price, 1991).

There is one recurring problem that many ecologists - including myself - encounter when they study soil legacy effects. Usually, only some of the used soils, often with specific microbiomes, will exert a significant effect on plant growth or plant-insect interactions. Although, as I have

already discussed above, most plant species and insects that interact with them can respond to such specific soil legacy effects, one important fact is that in any of these plant species, many of the soils have hardly any effect. Consequently, by using study designs that incorporate more different soils, the odds of incorporating a soil with a strong soil legacy effect will be increased. I used twelve soil legacies in most of my studies and by this, by chance I always included some soils that had strong legacy effects on plant-insect interactions in most of the response plant species. Similarly, Badri and colleagues (2013) found that several of their 11 used soil inocula that were applied to *Arabidopsis thaliana* had very little effect on growth of an insect herbivore, *Trichoplusia ni* feeding on these plants, compared to controls that did not receive any microbial inoculum. Several other inocula, on the other hand, strongly inhibited growth of the herbivore. Two important conclusions can be made. First, most plant species will experience soil legacy effects, but the degree of sensitivity may be species-specific. Second, most plant species will have average growth or herbivory levels on some soils. In other words, some soils have no clear soil legacy effects on the test plant species (but may have effects on others). In many studies soil legacy effects are not observed, and this is in part in those studies only very few soils were tested. This is important for the conclusions that can be drawn. For instance, Vaello et al. (2018) found no evidence for plant-mediated soil legacy effects on thrips or aphids feeding on bell pepper plants. However, this study included only two specific soils, conditioned by *Achillea millefolium* and *Lolium perenne* (Vaello et al., 2018). I am personally cautious to draw strong conclusions on the impact of soil legacy effects, based on the use of only two donor soils. The fact that soil legacy effects were not observed in studies using two soils, does not mean that they will not ever occur or could potentially be important or interesting from an applied perspective, in that plant model system. Similarly, a recent study found no effects of soil legacies on performance of the tobacco hornworm, *Manduca sexta*, grown on several tomato cultivars (Carillo et al., 2019). However, for each cultivar the authors used tomato ('own') and non-tomato ('other') soils. This approach is very common in plant-soil feedback literature (Brinkman et al., 2010) and builds on the concept of accumulation of species-specific pathogens in 'own' soils, relative to soils that are conditioned by 'other' species, and effects are often expressed as ratios between plant performance in the two soil backgrounds (Van der Putten et al., 2013). The approach is solid, but the conclusions that can be drawn are also limited by the design, and the potential soil legacy effects present in the 'other' soils are often ignored. Moreover, what has grown on the 'other' soil, determines the outcome of the ratio between

'own' and 'other' soils. It has been shown for various plant species how wide-ranged soil legacy effects can be on plant growth (e.g., Van de Voorde et al., 2011; Ma et al., 2017) and based on my findings I believe the same to be true for soil legacy effects on plant-insect interactions. Thus, the important question is how can we predict which soils will have strong legacy effects and which ones do not?

Predicting soil legacy effects using plant traits

Part of the rationale for our species selection was to be able to identify broader ecological patterns in soil legacy effects. To achieve this, we selected plant species that were contrasting in growth rate and of different functional types. Understanding how plants with contrasting characteristics would differentially influence the soil, could help us, eventually, to predict what a plant with a certain set of traits would change in the soil, and how this would affect subsequent plant-insect interactions.

Growth rate

Ecological theory predicts that due to limited resources, there are trade-offs between growth and defense. As a result, fast-growing plants (which invest most of their resources in growth) will be less well-defended against invaders, than slow-growing plants (which invest only a small proportion of their available resources into growth). This concept, also known as resource availability hypothesis or the growth-defense trade-off, (Coley et al 1985; Herms & Mattson, 1992) has been hypothesized to play an important role in defenses belowground (Lemmermeyer et al., 2014). Following this concept, we would expect that fast growers would accumulate more pathogens in the soils around their roots than slow growers and that this would also affect the composition of soil microbial communities (Van der Putten et al., 2013). We expect that these microbial shifts in the soil will result in legacy effects on plant growth (plant-soil feedbacks; Kulmatiski et al., 2008; Van der Putten et al., 2013), but also on insect herbivores feeding on these plants (Kos et al., 2015).

In chapter 4, we observed that twelve plant species create soil legacies that are significantly different in their individual microbial composition (Heinen et al., 2018b). This was the case for both soil bacteria and soil fungi, although our results suggest that soil fungi are more strongly affected by plant growth than bacteria and may be more important in driving soil legacy effects on plants (see **Figure S4.4,** Mommer et al., 2017; Semchenko et al., 2018). However, in this

experiment, the categorization of plant species based on their growth rate did not affect the community composition of bacteria or fungi. Furthermore, when plants were grown together on the different soil legacies in communities, effects of growth rate of the conditioning plant, via the soil, on responding plant communities were not significant. However, based on the lack of growth rate effects on soil microbial community composition in this specific study, this is perhaps not so surprising. Based on the results of chapter 4 alone, my conclusion is that the hypothesis that plants with different growth rates create different soil legacy effects should be rejected.

We do find evidence in chapter 3 that growth rate of the plants that condition the soil can affect plants that grow later in the same soil when plants are grown individually. Moreover, insects follow very similar response patterns as plants. However, we also show here that the effects of growth rate depend on the functional type of the conditioning plant. Interestingly, we observe that soils that are conditioned by fast-growing forbs, have negative effects on plant growth, whereas soils that are conditioned by slow-growing forbs have positive effects. This is exactly what one would expect based on our hypothesis that fast growers would accumulate more pathogens (=negative effects) than slow growers (=positive effects) (Lemmermeyer et al., 2014; Bergmann et al., 2016). However, when we take a closer look at the soils that were conditioned by grasses, a different pattern emerges. In grasses, the effect of growth rate on soil legacy effects is in the opposite direction; i.e., fast-growing grasses have more positive soil legacy effects on plant growth than slow-growing grasses. These findings were corroborated in chapter 6. In this chapter we also observed that the responding plant species (*Taraxacum officinale)* reacted differently to microbial soil legacies of fast versus slow growing conditioning species, depending on whether they were forbs or grasses. For this study, we collected soils from a field experiment with plots with fast- and slow-growing grass and forb communities. Why soil legacy effects created by slow- and fast-growing species differ between forbs and grasses is difficult to explain. One explanation can be that grasses have very specific chemical exudation mechanisms that attract a specific group of rhizobacteria that produce pyrrolnitrin, which has antifungal biocontrol properties (Latz et al., 2012;2015). Via this pathway, some soil bacteria may suppress fungal pathogens (Hol, Bezemer & Biere, 2013; Schlatter et al., 2017; Tomashow, Kwak & Weller, 2019). We may speculate that larger grasses exudate more and thus attract more of these specific rhizobacteria, which may explain the effects of growth rate

of grasses in creating soil legacy effects. As we have not characterized the soil bacterial communities in Chapter 3, this requires further investigation. In Chapter 4 we did analyse the bacterial communities, but found no evidence that growth rate had any effects on bacterial communities (Figure S4.4).

The fungal communities of the conditioned soils that were used in chapter 3, have by now been sequenced. These results arrived from the sequence facility very recently, around the time of writing this section. We ran a very preliminary analysis to test whether the fungal communities in the soils from chapter 3 were affected by plant growth rate, functional type, or their interaction. In line with our observations in terms of plant biomass, we also observed main effects of growth rate and plant functional type, as well as a significant interaction between plant growth rate and functional type on the composition and diversity of soil fungal communities (Heinen et al,. *in preparation a*). Further analysis of these data is required in order to find out whether there are specific groups of soil fungi that may explain these plant responses.

Previous studies have found that root traits, such as specific root length (which describes the length of root system per gram root) and relative growth rate, correlated with soil legacy effects on plant growth, i.e., plant-soil feedbacks (Lemmermeyer et al., 2014; Bergmann et al., 2016). In these studies, indeed, plants with a higher growth rate or lower specific root length had more negative conspecific plant-soil feedback effects, i.e., they negatively affected growth of their own species. Another recent study showed opposite effects. Plants with higher specific root lengths had more negative conspecific plant-soil feedback effects (Cortois et al., 2016). All these studies included both grasses and forbs, and both groups were included jointly in the correlations that they present. This makes it hard to compare growth rate effects for the separate functional types. The contrasting effects of root traits on plant-soil feedbacks in previous studies, along with my contrasting findings of chapter 3 and 4, indicate that it is currently still difficult to reliably predict plant-soil feedbacks or other soil legacy effects using plant traits. This was also pointed out by Baxendale et al. (2014) who investigated how well a series of plant traits predicted plant-soil feedbacks. Interestingly, their study indicated that traits of plant communities much better predicted plant-soil feedbacks, than traits of individual plant species. Although this is in line with our findings that soils conditioned by plant communities in the field partially explained soil legacy effects (Chapter 6), it also once more

illustrates that plant-growth related traits do not have consistent effects on soils, between different studies, but also between effects of individual plants and of communities.

It may be that we are simply focusing on the 'wrong' plant functional traits. Of course, the theory of linking growth and defense is a solid and broadly accepted concept (Coley et al 1985; Herms & Mattson, 1992), but, as with so many ecological theories, the theory may not always apply to all organisms or all ecosystems (Lawton, 1993; Currie, 2019). I propose that other categories of plant traits, that better reflect how roots interact with their soil environment at the individual species level will have more predictive power. For instance, if one considers belowground defences, is it growth rate, per se, that influences soil organisms? I have used growth rate under the assumption that it correlates with defence. Perhaps measuring defense directly has stronger predictive power. Future studies may select plant species based on chemical composition of their rhizodeposits, the complexity of their exudate cocktails, or even net exudation rates. All of these factors are highly plant-species specific and appear to be driving factors in determining soil microbial composition (Bais et al., 2006; Lakshmanan, Selvaraj & Bais, 2014; Cordovez et al., 2019). Alternatively, selecting plant species based on their nutrient acquisition strategy and mutualistic status may also better predict what kind of legacy plants leave in the soil (Teste et al., 2017).

Functional type

I found evidence that plant functional type plays an important role in shaping microbial soil legacies. As presented in chapter 4, soil microbial communities of plant species of the same functional type clustered more closely together than they did to species that belong to a different functional type. Again, although significant for bacteria and fungi, the effect observed was much stronger for the latter group of soil organisms.

Based on my studies on plant growth in both chapters 3, 4 and 6, I conclude that the functional type of the conditioning plant plays an important role in creating the observed soil legacy effects on plant growth. In chapter 3, I observed that, on average, plants had more biomass in soils that were conditioned by grasses than in soils that were conditioned by forbs. However, it must be noted that in this study, there were also significant interactions with growth rate of the plant that conditioned the soil, which have been described in detail in the section on growth

rate above. I also provide evidence that individual species within communities can be affected by the functional type of the plants that conditioned the soil, although this did not affect overall plant community biomass production. In some of our test communities, the effects of conditioning plant functional type on the growth of plant species within the plant community resulted in shifts in the relative distribution of biomass across the plant species that grew within the plant community.

Plant functional type, via the soil, can also affect insects feeding on plants grown later in the same soil. As described above, when plants are grown individually, such as was done in chapter 3, insect biomass and consumption followed very similar patterns as observed for plant growth, revealing an interactive effect between functional type and growth rate of the plant that conditioned the soil. In chapter 4, where insects were kept on plant communities growing in different soils, overall, insects accumulated more biomass on plant communities that were grown in soils that were conditioned by forbs, than in soils that were conditioned by grasses (Heinen et al., 2018b). This finding is similar to earlier findings by Kos et al. (2015), who showed that colony growth of the aphid *Aphis jacobaeae* on ragwort plants was affected by the functional type of the plant that conditioned the soil. A potential - but as of now still speculative - mechanism could be that rhizobacteria - which can promote plant growth - are often enriched in grass soils (Latz et al., 2012;2015), but also have been shown to induce systemic resistance (Van Loon, Bakker & Pieterse, 1998; Pineda et al., 2010; Berendsen et al., 2012) and as such, may prime plants for defenses against future attack by herbivores. This could explain the negative effects that grass-conditioned soils had on insects in Chapter 4, but would not explain the observed insect responses (interaction growth rate and functional type) in Chapter 3 and 6.

Mechanisms linking soil legacies and plant-insect interactions

The field of above- and belowground interactions has received considerable attention in ecology in the past three decades and in these three decades it has become very clear that plants effectively connect the two spatially separated below- and aboveground worlds (Masters, Brown & Gange, 1993; Masters & Brown, 1997; Van der Putten et al., 2001; Johnson, Bezemer & Jones, 2008; Bardgett & Wardle, 2010). Organisms that interact with a host plant can trigger a plethora of physiochemical processes that are often induced systemically in the plant (Van Dam et al., 2003; Bezemer & Van Dam, 2005; Soler, Erb & Kaplan, 2012; Erb &

Reymond, 2019). Through such plant-mediated processes, aboveground organisms affect belowground organisms (Blossey & Hunt-Joshi, 2003), and belowground organisms affect aboveground organisms (Koricheva et al., 2009; Pineda et al., 2010; Soler et al., 2013). Above-belowground studies typically focus only on addition or removal of species or groups of soil organisms (Heinen et al., 2018a). How entire soil microbiomes may affect plant-insect interactions is less well-understood.

Plant-mediated effects

Most of the plant species that I selected for my studies are generally considered weeds. Weeds are not the most profitable plants for humans and this means that outside of fundamental ecology there is little incentive to gain a better understanding of their functioning. As such, not much is known about inducible defenses, or physiochemistry of many of these species.

For a few selected species from our list, there is information available on at least part of their secondary defense metabolism. Ribwort plantain, *Plantago lanceolata*, is one of these species and as this species was part of the larger experiments presented in chapter 3 and 4, we could use it to test how soil legacy effects alter levels of plant secondary defence metabolites. As part of its defense system, ribwort plantain produces two iridoid glycosides, aucubin and catalpol. Our work in chapter 5 shows that the levels of aucubin in shoot tissues were affected by species-specific (microbial) soil legacies, whereas those of catalpol were not. However, levels of catalpol were affected by the functional type of the plant species that grew previously on the soil, with levels of catalpol being higher in plantain growing in soils with a grass legacy. These findings are in line with other work that shows that soil microbiomes can affect secondary defense metabolism. For instance, Joosten et al. (2009) found that *Jacobaea vulgaris* plants expressed different levels of secondary metabolites called pyrrolizidine alkaloids when they were grown on soils with different soil microbial compositions. Very similar results were later obtained in the same model system (Kostenko et al., 2012; Bezemer et al., 2013; Kos et al., 2015; Wang et al., 2019). *Arabidopsis thaliana* also shows very specific plant-metabolic responses to different microbial soil inocula, specifically in levels of amino acids, phenolic compounds, sugars, and sugar alcohols (Badri et al., 2013). A recent study by Ristok et al. (2019) reported that soil legacy effects created by plant communities of different plant diversity (1, 4 or 8 species respectively) strongly affected shoot, and to a lesser extent, root metabolome composition in four common forb species. The shoot samples of the experiment that I

presented in chapter 3 have been used for metabolomic analysis by one of my colleagues on this project. Martine Huberty used nuclear magnetic resonance (NMR) to analyze the metabolomes of all 12 responding plant species, on all 12 conditioned soils when exposed to herbivory. In addition, she also used all plant-soil combinations, grown without exposure to herbivores. Her work shows that the entire metabolome of the plant is strongly influenced by the legacy in the soil in which it grows and that across plant species, soils have strong effects on levels of various sugars. More specifically, in seven out of twelve response species, the legacy of the soil in which the plant grows more strongly affects plant shoot metabolomes than insect herbivory (Huberty et al., submitted).

Based on earlier (RNAseq) work on *P. lanceolata*, by one of my co-supervisors (Arjen Biere, unpublished data) we had access to previously designed and tested primer pairs for two pathogenesis related genes (which are associated with the salicylic acid pathway), as well as for a polyphenol oxidase gene and a lipoxygenase gene (which are associated with the jasmonic acid pathway). The salicylic acid pathway is activated by biotrophic pathogens (that are often considered drivers of soil legacy effects), whereas the jasmonic acid pathway is activated by generalist chewing herbivores and necrotrophic pathogens. Therefore, this combination of target genes allowed us to investigate interactions between microbial soil legacies and herbivory. Interestingly, we found no soil legacy effects on the activation of pathogenesis related genes, which suggests that soils did not vary significantly in the level of activation of these defences by biotrophic pathogens. However, perhaps more interestingly, we observed an effect of soil legacies and herbivory on the transcription of the polyphenol oxidase gene Pl-PPO7. This gene was upregulated by chewing herbivory, as is expected for genes activated downstream of jasmonic acid signaling. However, we showed that the transcription levels also varied with soil. Further, levels of the lipoxygenase gene Pl-LOX were altered by an interaction between soil legacies and herbivory. This indicated that in some soils, plants express higher levels of this lipoxygenase transcript under herbivory than in control plants, but that in other soils, plants express higher levels of the transcript in the control plants than in plants that have been exposed to herbivory. So, not only do levels of secondary defense metabolites differ between soil legacies, soil legacies also interact with herbivory in their effects on the jasmonic acid pathway in the plant. This is important, because it indicates that the ability of a plant to defend itself against herbivores can be strongly altered by the legacy of the soil that it is growing

in. Other recent work has also indicated that soil microbiomes may mediate jasmonic acid responses (Young et al., 2018; Li et al., 2019). For instance, peanut plants grown in soils with a legacy of monocropping upregulated, among others, jasmonic acid marker genes, compared to plants grown in soils with a legacy of crop rotation (Li et al., 2019). This further strengthens our findings that soil legacy effects may interfere with jasmonic acid defences, and through this, may affect aboveground insect herbivores.

The accumulating evidence that soil legacies can change entire plant metabolomes and may also interfere with plant phytohormonal signaling pathways involved in defence, makes it likely and intuitive to conclude that the link between soil legacy effects on plants and insects is plant-mediated. However, as I will discuss further below, direct effects of microbial soil legacies may also play a role, and thus should not be overlooked.

Direct soil-insect effects

In chapter 6, we observed that caterpillar microbiomes strongly overlap with soil microbiomes (Hannula et al., 2019). We stumbled upon this more or less by surprise. In this experiment we originally wanted to test whether soil microbiomes would be transferred, from soil, to root, and further on to the shoot compartment in dandelion, *Taraxacum officinale*. Via this pathway, we expected that these microbiomes, as subsets of the soil, would end up in the caterpillar, as caterpillars generally are born without a microbiome. However, as they often have a microbiome in later stages, it has been suggested that the insects pick them up from their environment, e.g., through their diet (Douglas, 2015). To test this, we reared caterpillars on caged dandelion plants growing in soils with different soil legacies. However, in order to be absolutely sure that the microbes in the insects would be derived from the plant, we performed a parallel assay in sterile petri dishes. Here, we fed the caterpillars with leaves from a second set of plants with the same soil legacy treatments. To our surprise, leaf microbiomes were very low in diversity, and consequently, the caterpillar microbiome also was very low in diversity. However, the caterpillars from the caged plant assay had more diverse microbiomes, which resembled those of the soil. This effect was so strong that we could detect the plant community legacies that were present in the soil microbiome back in the caterpillar microbiomes. Caterpillars, we showed here, derive their microbiome, not only from their diet, but also from the soil. Caterpillar biomass was also affected by soil legacy treatments. However, we observed this only when the caterpillars had contact with the soil, and not in the petri dish assay. We also

observed various positive and negative correlations between various soil organisms and caterpillar biomass. I am aware that these should be treated with caution, as they do not imply causality. These correlations are interesting nonetheless and give rise to some speculation, and lead to the generation of new ideas and hypotheses to be tested in future studies.

Soil is full of organisms that can be detrimental to insects. For instance, soils are important reservoirs for various entomopathogenic organisms, including bacteria (e.g., Vodovar et al., 2006; Bode, 2009), fungi such as members from the genus *Beauveria* and *Metarhizium* (e.g., Meyling & Eilenberg, 2007; Vega et al., 2009), but also entomopathogenic nematodes (e.g., Kaya & Gaugler, 1993; Gaugler, 2002; 2017). There is a wealth of knowledge on the entomopathogenic effects of fungi and bacteria from feeding assays, when these organisms are present as plant endophytes (e.g., Lopez et al., 2014; Lopez & Sword, 2015) or applied to plant shoots (e.g., Shipp et al., 2003; Vandenberg et al., 1993; Zibaee et al., 2013). There is evidence that at entomopathogenic nematodes may affect aboveground insects, via intricate host search strategies, that include jumping onto potential host insects (Campbell & Kaya, 2002; Campbell et al., 2003). Very little is known about the ecological impacts that most other soil organisms may have on aboveground insects, via direct soil-insect contact. Soils are also full of organisms that have been shown to be beneficial to insects. For instance, various bacterial species from the genus *Enterococcus* commonly occur in insect gut microbiomes (Van Frankenhuyzen, Liu & Tonon, 2010; Chen et al., 2016; Jones et al., 2019), but also are often found in the soil. It has been shown that *Enterococcus* and other bacterial species can help the insect by detoxification of plant toxins, breakdown of plant material, or even have a protective function against potential invading pathogens (Van Frankenhuyzen, Liu & Tonon, 2010; Chen et al., 2016). An intriguing example of microbe-plant-insect interactions is also described in recent work (Kim et al., 2019). The authors show that species of the genus *Streptomyces* that are common in the soil, are transferred from rhizosphere to anthosphere in strawberry plants, where they are picked up with the pollen by bees. In the bee hive, the *Streptomyces* provide the bee colony with elevated resistance against insect pathogens (Kim et al., 2019). It is commonly assumed that insects can take up microbes from their environment, for instance via their diet, as recent studies even suggest that caterpillars may lack a permanent resident gut microbiome (Hammer et al., 2017). Only rarely has the direct link been made between insects and the soil as a source for their microbiome. However, in one study system involving stinkbugs,

it has been shown that the insects actively move to the soil and acquire symbiotic *Burkholderia* species (Kikuchi, Hosokawa & Fukatsu, 2007; 2011a,b). Acquisition of specific symbionts from the soil increases stinkbug fitness in terms of body size and biomass. This demonstrates that the use of soil as a reservoir of beneficial microbes could be one argument for insects to take up microbes from the soil. We cannot definitively conclude anything regarding the why and how, but that caterpillars take up soil microbes is evident from our study. Future work is needed in order to elucidate behavioural patterns, mechanisms, and the effects of these microbiomes on caterpillar health and fitness. Our work suggests that we may need to reconsider our understanding of soil-plant-insect interactions. The classic notion that soils affect plant quality and that these plant-mediated changes, in turn, affect insect performance may not be the full story. Instead, insect performance may be determined by indirect (plant-mediated) and direct (soil) effects that act on plant-insect interactions in parallel.

The broader role of soil legacy effects in ecology

In the work in this PhD project, I focused on the specific microbial legacy effects that are created by individual plant species. However, under natural conditions, plants rarely grow alone. As plants and their root systems are often tightly interwoven, it is more likely that plants alter soil legacies in conjunction with the other plant species that are growing in the same plant community. Humans have had a transformative effect on plant communities globally, for instance through deforestation and agriculture. As a result of human activity, other types of legacy effects may thus also manifest themselves in the soil, which has recently been shown to potentially affect future plant-insect interactions (De la Peña et al., 2016). For instance, recent work has revealed that soils with legacies of different land-use history may affect plant-insect interactions (De la Peña et al., 2016). Specifically, soils with a legacy of ancient forest had lower phosphate levels than post-agricultural soils, but also significantly differed in soil microbial community, and marginally differed in nematode community composition. A greenhouse study with soils from each land-use type revealed that the aphid *Rhopalosiphum padi* preferred their host *Deschampsia cespitosa* growing in soils from ancient forests, whereas *Aphis urticae* preferred their host *Urtica dioica* growing in post-agricultural soils, suggesting that soil legacy effects on aboveground plant-insect interactions are not necessarily following levels of soil nutrients, but are likely also mediated by soil organisms (De la Peña et al., 2016). As human activity, for instance through agricultural practice, deforestation/reforestation and nature

management has greatly influenced terrestrial ecosystems worldwide (Crutzen, 2006; Dirzo et al., 2014). The abiotic and biotic environments in our soils have changed concomitantly (e.g., Bell & Tylianakis, 2016; Vanwalleghem al., 2017; Poesen, 2018). Very little is known currently about how the soil legacies that humans have left, whether biotic or abiotic, affect aboveground insects.

Soils may also vary naturally in their biotic composition, as a result of geophysical location, physiochemical composition, or global patterns in vegetation (Orgiazzi, Bardgett & Barrios, 2016). Several recent studies indicate that soil microbiomes may determine the distribution of native and invasive plant species, especially under global warming scenarios (Wilschut et al., 2019; Ramirex et al., 2019). When, in response to warming, plants expand their ranges and invade new territories, they will consequently encounter novel organisms and establish novel interactions with organisms above- and belowground (Van der Putten, 2012). As a recent study shows, novel interactions belowground may also influence how plants deal with insect herbivores aboveground. Along the invasion transect of the invasive plant *Alternantha phyloxeroides*, soils contained fewer pathogens when they originated farther away from the original range (Lu et al., 2018). When *A. phyloxereides* and its non-invasive congener, *A. sessilis*, were grown in soils originating from this plant invasion transect, soils had no effect on insect herbivory in invasive plants, but native plants suffered more herbivory when they were grown in soils originating farther away from their native range (Lu et al., 2018). This suggests that, in some plant species, a release from co-evolved belowground enemies results in lower constitutive defenses, enabling aboveground herbivores to become successful, which may in turn limit plant distribution. In other plant species, enemy release may confer a competitive edge and allow a plant's successful invasion. The study by Lu (2018) is merely an example, and one of the first of its kind. Undoubtedly, range-shifts and the resulting shifting interactions between invasive plants and belowground organisms in the novel range, will also affect aboveground plant-insect interactions in other plant species.

Future directions

In this project, I have shown that plant species and the aboveground insect herbivores that feed on them can be affected by biotic legacy effects present in the soil that they grow in. Obviously, this story, involving soil organisms, host plants, and aboveground insect herbivores, is multi-faceted, and this scientific field is still rather 'young'. Therefore, there are many open questions

that still remain, as well as new questions that arise. In this section I will discuss some fundamental areas that I deem worthy of further exploration, and I will end with a brief note on application of soil legacy effects in agriculture.

My background and interests are in entomology and plant-insect ecology, and this field is generally characterized by its diversity in interactions. However, in this project, I have used only a single model insect species, *Mamestra brassicae*. Although this caterpillar is native in the area, it is not a model that represents the diversity that is found in the insect class. Plants often host a range of insect herbivores, simultaneously or sequentially. I think it is important to understand how soil legacy effects affect entire insect communities. For instance, a wealth of studies have shown that insects from different feeding guilds respond differently to changes in plant health status and quality (Bezemer & Jones, 1998; Awmack & Leather, 2002; Heinen et al., 2018b). Moreover, the degree of specialization that an insect exerts towards its host often plays a role in plant-insect responses (Ali & Agrawal, 2012). It is important to further study how belowground legacies may influence the composition of the insect community. Are polyphagous insect species more sensitive to soil legacy effects than oligophagous species? Do phloem feeders differ in their response from chewing herbivores, or for instance leaf miners? What are the consequences of soil legacy effects for higher trophic levels, such as predators and parasitoids? Or for pollinating insects? Future work should embrace the diversity of insects and study how insect communities respond to soils.

I also believe that it is important to place soil legacy effects in the context of current problems that we face in ecology. As already briefly discussed, the world is changing rapidly under the stress of a growing human population, urbanization and the ever-increasing need for production of food and feed. It is important to understand how anthropogenic global change affects the world's soils and how this impacts plants and the organisms that depend on them. Recent work indicates that global change has resulted in shifts in distribution for many plant species, resulting in novel interactions above- and belowground. However, relatively little is known about how interactions between plants and novel soils affect the novel interactions aboveground. Further, the effects of land use and management practices on soils can result in loss of soil biodiversity and how this may influence future plant interactions is not well-understood. We need to understand these systems in order to mitigate potential negative effects. Insects, as has been shown recently (e.g., Biesmeijer et al., 2006; Shortall et al., 2009;

Fox, 2013; Hallmann et al., 2017; Lister & Garcia, 2018), are in decline. Globally, but especially in areas of massive human activity, their numbers are in steep decline and an important question is what the role of soil is in shaping insect communities. Many insects live in the soil (Johnson et al., 2012; Soler et al., 2012) or spend a part of their life cycle in or in close contact with the soil (e.g., Reed, 1965). It is likely that soils affect insect biology to some extent. Perhaps we can lend insects a hand in their recovery by providing healthy soils that may facilitate insect performance or diversity?

We have only scratched the surface of direct soil legacy effects on insects. It is evident from my work that soils influence the composition of insect microbiomes and that the legacies in the soil can be traced back in microbiomes of aboveground insects. This is now a completely open field with exciting new questions. I have personally seen caterpillars crawl on soil surface in multiple independent studies, even when host plants were abundant. It is hard to explain why. Perhaps, being on the soil, below the plant canopy may provide a good refuge to escape predation or avoid heat of the day. Furthermore, many insect species, including many Noctuidae, pupate in the soil (Reed, 1965; Lee, Johnson & Wright, 1990). The question is whether caterpillars actively take microbes from the soil, or are they just invaded because they are in close contact with the soil sometimes? It would be interesting to investigate caterpillar behavior, for instance using camera tracking over time. When are insects feeding on soils? Does this behavior change with time and larval stage? Another important aspect is whether this behavior is adaptive and affects fitness parameters. Are there benefits of having a microbiome from the soil (i.e., is there a selection for mutualistic microbes)? What is the effect of soil entomopathogens? We have some indications that beneficial organisms and pathogens both may be affecting caterpillar performance, but future studies should experimentally manipulate presence and abundance of specific soil organisms to empirically assess their role in insect biology. In the longer term, it will be interesting to address the question what is the relative importance of the two pathways; *indirect* (plant-mediated) soil legacy effects and *direct* soil legacy effects, in determining insect herbivore performance

One final area that has received a lot of attention, is the application of knowledge on the soil microbiome, for instance in agriculture. A recent opinion piece has made such a plea for the application of plant-soil feedbacks to reduce insect pests in agriculture (Pineda, Kaplan & Bezemer, 2017). The idea of creating soils that have a positive effect on plant growth, and a

suppressive effect on pest insects, is appealing as a concept. Using plant-soil feedbacks is also practically challenging, as it would require the growth of an additional 'soil conditioning crop', which limits productivity of the cash crop in terms of growing seasons. However, I can see how, in the future, a designed microbial inoculum can achieve something with a similar effect, without affecting the production time. Concepts like these have already been worked out in the past, and relatively simple microbial mixtures, for instance containing plant-growth promoting rhizobacteria, rhizobia and mycorrhizal fungi, have been commercialized already. To date, the scientific evidence for the efficacy of these products is mixed (e.g. Mayer et al., 2010; Megali, Glauser & Rasmann, 2014; Megali, Schlau & Rasmann, 2015; Heinen et al., 2018a). There is a lot of progress to be made when it comes to design and application of soil microbiomes for use in crop protection. One aspect that needs to be dealt with is species-specificity. Different plant species host very different biotic interactions (Phillipot et al., 2013). Furthermore, each host plant species responds differently to soil microbiomes. One step forward would be to generate crop-specific beneficial microbiomes. Thus far, the available mixtures aim to benefit the full range of crops. A second aspect that requires attention is consistency across different locations and soil types. If a product is marketed, it should reliably do what it is supposed to do. Every time and regardless of where it is applied. Creating a soil microbiome that is stable and functions regardless of the receptor soil would be a major breakthrough. If these two hurdles can be overcome, and a consistent inoculum can be developed for certain crops, this would be a great alternative to insecticide use.

Conclusion

Plants are important drivers of the biotic and abiotic conditions in the soil. Throughout their life cycle, they create legacies in the soil that may persist after they disappear. These soil legacy effects can have strong effects on future plants that grow in the same soil, but may also influence plant-insect interactions. My work in this thesis has shown that soil legacy effects on plant-insect interactions are common in twelve wild plant species. I have shown that growth-related traits can influence these effects to a certain extent, but that effects differ between plants that belong to different functional types. Further, my colleagues and I have revealed that soils may affect insect herbivores through indirect, plant-mediated pathways that involve secondary defense metabolism and phytohormonal defense pathways in plant tissues. However, we also highlight that soil legacies can directly affect insect

herbivores, via their gut microbiome. In conclusion, legacy effects in the soil can have strong effects on aboveground plant-insect interactions and our work underlines that the role of soil communities in shaping plant-insect interactions should not be overlooked in ecology.

Chapter 8

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Summary

Soils are highly diverse environments that contain many organisms that interact with the plants that grow in the soil. These organisms can have effects on plants that range from beneficial (e.g., mutualists) to detrimental (e.g., pathogens). Furthermore, a large group of organisms does not directly interact with plants, but are still essential parts of the soil, by breaking down organic matter and making nutrients available to the plant. In recent decades, it has also become very clear that soil organisms can affect organisms that interact with the plant aboveground. The field of above-belowground interactions has since become well-studied for many individual groups of soil taxa and aboveground insects. The implications of entire soil communities for aboveground plant-insect interactions has only recently received more attention.

Plants also have a strong effect on the organisms around their roots. Via the exudation of carbon and other compounds from their roots, they may repel some organisms and attract others. As a result, the soil microbiome often reveals plant species-specific patterns. These patterns in soil communities may persist in the soil for a long time, as soil legacies. It has been shown that these specific soil legacies can alter the growth of plants that grow later in the same soil (a process better known as plant-soil feedback). Pioneering work published before this PhD thesis, revealed that the effects of entire soil communities, in the form of plant-specific soil legacies, can also have strong effects on chewing and phloem-feeding insect herbivores.

In this thesis, we set out to explore how general these soil legacy effects occur in a broad range of plant species and a common polyphagous chewing herbivore, the cabbage moth (*Mamestra brassicae*). Furthermore, I assessed whether these species-specific legacy effects on plant-insect interactions could be predicted using plant growth rate (fast/slow) and plant functional type (grass/forb). Using twelve plants consisting of combinations of fast- and slow-growing grasses and forbs, I created soils with different legacies and grew all twelve plant species on all these soils, either individually (Chapter 3), or in communities (Chapter 4). In the response phase, cabbage moth caterpillars were introduced, after which I measured their growth and leaf consumption, as well as individual plant biomass responses. These two experiments revealed that soil legacy effects on plant-insect interactions are common in individual plants, as well as in plant communities, and can, in part, be explained by plant functional type and interactions between plant functional type and plant growth rate.

Most previous above-belowground research has focused on mechanisms that are mediated via the shared host plant. In Chapter 5, we investigated whether soil legacy effects could alter herbivore-induced plant defenses in a focal plant species, *Plantago lanceolata*. Here, it was shown that levels of secondary metabolites (iridoid glycosides) can differ considerably between soils. Furthermore, using gene expression assays of marker genes for the jasmonic acid and salicylic acid pathways - two important herbivore-induced phytohormonal defense pathways – we show that the ability of a plant to defend itself against aboveground herbivory, depends largely on the legacy present in the soils it grows in.

Lastly, in Chapter 6, the role of the biotic component of the soil legacy itself, in aboveground plant-insect interactions, was studied. Previous work indicates that subsets of the soil microbiome can end up in the shoot microbiome. Through consumption, these microbes could end up in the insect herbivore gut. Indeed, some of the caterpillar microbiome was ingested via its diet, although this turned out to be a rather minimal source of microbes. Interestingly, caterpillars appeared to take up the majority of their microbiome from soil. Through this direct but previously overlooked pathway, soil legacy effects may play an important role in influencing aboveground insects.

In conclusion, I have shown in this thesis that soil communities can play an important role in mediating aboveground plant-insect interactions. Soil matters! Now, there are plenty of ways that soils may build up microbial legacy effects. Plant-specific legacies are just the beginning. Future studies should unravel how other legacy effects (e.g., agricultural land use, urbanization, biodiversity, historical abiotic differences or biogeographical differences) may affect plant-insect interactions consequently.

Samenvatting

Bodems zijn rijke ecosystemen die talrijke organismen bevatten die interacties aangaan met planten die in deze bodems groeien. Deze organismen kunnen een breed scala aan effecten veroorzaken, van positieve effecten (bijvoorbeeld mutualisten) tot negatieve effecten (bijvoorbeeld ziekmakers). Bovendien is er een grote groep organismen in de bodem aanwezig dat niet direct met planten interacteert, maar welke wel essentieel zijn voor het functioneren van bodemprocessen, bijvoorbeeld in het beschikbaar maken van voediingsstoffen via de afbraak van organisch materiaal (decomposeerders). In recente decennia is het duidelijk geworden dat deze bodemorganismen ook een belangrijke rol kunnen spelen in het leven van organismen die bovengronds van de plant voeden. Het wetenschappelijke veld van boven-ondergrondse ecologische interacties is een veel bestudeerde tak van ecologie en er is veel kennis vergaard over de effecten die specifieke groepen bodemorganismen op bovengrondse plant-insect interacties hebben. De effecten van gehele bodemgemeenschappen op bovengrondse plant-insect interacties heeft pas recentelijk meer aandacht gekregen.

Planten hebben zelf ook een sterk effect op de organismen die voorkomen in de bodem rondom hun wortels. Via de uitstoot van koolstofverbindingen en andere chemische stoffen uit hun wortels, kunnen ze bepaalde organismen aantrekken en anderen juist afstoten. Als resultaat dragen planten vaak een soort-specifiek patroon in het bodem-'microbioom'. Deze patronen in de bodem kunnen vaak lang aanhouden in de bodem, als ware bodem erfenissen. Het is een bekend fenomeen dat deze erfenissen de groei van planten die later in deze bodems groeien, sterk kunnen beinvloeden (een proces beter bekend als plant-bodem terugkoppeling). Pionierende studies voorafgaand aan deze PhD thesis liet zien dat deze bodem-erfenissen, via de plant, ook sterke effecten konden hebben op zuigende en kauwende herbivore insecten.

In deze thesis, heb ik bestudeerd hoe algemeen de effecten van deze plant-specifieke bodemerfenissen op bovengrondse plant-insect interacties daadwerkelijk zijn. Dit heb ik gedaan met behulp van een set van twaalf plantensoorten en de rupsen van een generalistische herbivoor, de kooluil. Ik heb onderzocht of we bepaalde patronen konden vinden in deze bodem effecten en of dit te voorspellen zou zijn aan de hand van kenmerken van de planten. Voor mijn plantenselectie, gebruikte ik snel- en langzaamgroeiende grassen en breedbladigen. Ik heb vervolgens bodemerfenissen gecreerd van iedere soort en de effecten bestudeerd op individuele planten (Hoofdstuk 3) en op plantengemeenschappen (Hoofdtstuk 4). In de respons

fase van de experimenten introduceerde ik rupsen van de kooluil op de planten en heb groei en consumptie van bladmateriaal gemeten. Deze twee studies lieten duidelijk zien dat bodemerfenissen een algemene rol spelen in het vormen van plant-insect interacties. Bovendien liet ik duidelijk zien dat deze effecten gedeeltelijk verklaard konden worden door de functionele groep van planten, maar ook door een interactie tussen de functionele groep en groeisnelheid van planten.

De meeste studies op het gebied van boven-ondergrondse ecologie hebben gefocusd op mechanismes die worden gereguleerd door de gedeelde waardplant. In Hoofdstuk 5, onderzocht ik of bodemerfenissen een rol speelden in de afweer van planten tegen insecten in een model plant, de smalle weegbree. Ik liet zien dat bodemerfenissen een substantiele invloed konden hebben op de concentraties van afweerstoffen in de bovengrondse weefsels. Bovendien, middels het gebruik van genexpressie analyses op merkers voor genen coderend voor jasmonzuur en salicylzuur (twee belangrijke verdedigingshormonen in het plantenrijk), heb ik aangetoond dat de potentie van een plant om zich te kunnen verdedigen tegen bovengrondse herbivore insecten, sterk afhangt van de erfenis in de boidem waarin de plant groeit.

Als laatst, in Hoofdstuk 6, bestudeerde ik de rol van de biotische component van de bodemerfenis zelf op plant-insect interacties. Eerder werk laat zien dat delen van het bodem-microbioom terecht kunnen komen in bovengrondse plantenweefsels. Via consumptie van die weefsels, zouden deze terecht kunnen komen in de darm van het insect. Ik vond inderdaad dat er een deel van het insecten-microbioom werd opgenomen via het dieet, maar wat opmerkelijker was, was dat het merendeel van het insecten-microbioom werd opgenomen uit de bodem. Via deze directe, maar doorgaans genegeerde weg, kunnen bodemerfenissen ook effecten hebben op insecten, zonder tussenkomst van waardplanten.

Ik heb in deze thesis laten zien dat bodemgemeenschappen een belangrijke rol kunnen spelen in het reguleren van bovengrondse plant-insect interacties. De bodem is belangrijk! Er zijn tal van wegen waarop bodemerfenissen een rol kunnen spelen. Plant-specifieke erfenissen zijn slechts een begin. Vervolgstudies zullen moeten aantonen wat de rol van andere erfenissen (bijvoorbeeld van landbouw, verstedelijking, biodiversiteit, historische of geografische verschillen) op plant-insect interacties kunnen zijn.

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Wim, your calm presence and accessible style is something that has inspired many scientists (including myself) over the years. I am grateful that I could be a small part of your department for a few years.

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Robin Heinen, 2019

Acknowledgements

Curriculum vitae

Robin Heinen was born on the 12th of August 1986, in Veenendaal, The Netherlands. He received his BSc in Biology (with specialization Animal Biology) from Wageningen University in December 2011. During his BSc thesis at the Department of Entomology, Robin focused on the effects of cannibalistic feeding behavior on fitness in mosquito larvae. After a break of one year, he started his MSc program in Biology (with specialization Bio-Interactions) in 2013. In 2013-14, he conducted his major MSc research at Plant Research International and the Department of Virology, where he focused on the role of various aphid species in the dispersal of potato viruses. In 2014, in his minor MSc research, he morphometrically analyzed ovipositors of parasitic Hymenoptera and related ovipositor characteristics to the toughness of the substrate that the ovipositors were adapted to. In 2015, he conducted an internship at the Netherlands Institute of Ecology (NIOO-KNAW), combining various projects investigating interactions between herbivorous insects and their host plants, as well as between herbivorous insects and their (hyper)parasitoids. In the same year, he started his PhD project at Department of Terrestrial Ecology at NIOO-KNAW, under supervision of Prof. dr. Martijn Bezemer, Prof. dr. Jeff Harvey and Dr. Arjen Biere. The focus of his PhD was on (microbial) soil legacy effects on aboveground plant-insect interactions. The findings of his PhD research are presented and described in this dissertation.

Publications and manuscripts

Heinen, R., Thakur, M.P., Hiddes-De Fries, J., Vandenbrande, S., Jongen, R., Steinauer, K., & Bezemer, T.M. (manuscript in preparation). Foliar herbivory creates soil legacy effects that alter insect herbivores via plant community biomass allocation.

Heinen, R., Hannula, S.E., Van der Sluijs, M., Zhu, F., Schrieks, J., Biere, A., & Bezemer, T.M. (preparing resubmission). Dissecting the effects of plant traits of conditioning and responding species on plant-soil feedbacks.

Heinen, R.#, Hannula, S.E.#, De Long, J.R.#, Huberty, M., Jongen, R., Kielak, A.M., Steinauer, K., Zhu, F., & Bezemer, T.M. (preparing resubmission *Ecology Letters*). Soil legacies mediated by plant community traits steer grassland vegetation. ***#Equal contributions***

Huberty, M., Choi, Y.H., **Heinen, R.,** & Bezemer, T.M. (under review *Journal of Ecology*). Aboveground plant metabolomic responses to plant-soil feedbacks and herbivory.

Hannula, S.E., Kielak, A.M., Steinauer, K., Huberty, M., Jongen, R., De Long, J.R., **Heinen, R.,** & Bezemer, T.M., (preparing resubmission *mBio*). Time after time: Temporal variation in the effects of plant species and plant functional groups on soil bacterial and fungal communities.

Heinen, R., Steinauer, K., De Long, J.R., Jongen, R., Biere, A., Harvey, J.A., & Bezemer, T.M. (under review *Arthropod-Plant Interactions*). Exogenous application of plant hormones in the field alters aboveground plant-insect responses and belowground nutrient availability, but does not lead to differences in plant-soil feedbacks.

De Long, J.R., **Heinen, R.,** Jongen, R., Hannula, S.E., Huberty, M., Kielak, A.M., Steinauer, K., & Bezemer, T.M., (under second review *Plant and Soil*). How plant-soil feedback maternal effects influence the next generation of plants.

Heinen, R., Biere, A., & Bezemer, T.M., (accepted with minor revisions, *Oikos*). Plant functional group and growth rate interactively shape soil legacy effects on individual plant-insect interactions

De Long, J.R., **Heinen, R.**, Steinauer, K., Hannula, S.E., Huberty, M., Jongen, R., Vandenbrande, S., Wang, M., Zhu, F., & Bezemer, T.M. (2019). Taking plant-soil feedbacks to the field in a temperate grassland. *Basic and Applied Ecology,* **40**, 30-42.

Hannula, S.E.#, Zhu, F.#, **Heinen, R.#**, & Bezemer, T.M.# (2019). Foliar-feeding insects acquire microbiomes from the soil rather than the host plant. *Nature Communications.* doi:10.1038/s41467-019-09284-w ***#Equal contributions***

Heinen, R. & Harvey, J.A. (2019). Spatial and temporal diversity in hyperparasitoid communities of *Cotesia glomerata* on garlic mustard, *Alliaria petiolata. Ecological Entomology.* doi:10.1111/een.12710

Harvey, J. A., Visser, B., Lammers, M., Marien, J., Gershenzon, J., Ode, P., **Heinen, R.**, Gols, R., & Ellers, J. (**2018**). Ant-like traits in wingless parasitoids repel attack from wolf spiders. *Journal of Chemical Ecology,* **44**(10), 894-904. doi:10.1007/s10886-018-0989-2

Zhu, F.#, **Heinen, R.#**, Van der Sluijs, M., Raaijmakers, C., Biere, A., & Bezemer, T. M. (**2018**). Species-specific plant soil feedbacks affect gene expression and defense chemistry in *Plantago lanceolata. Oecologia,* **188**(3), 801-811. doi:10.1111/1365-2745.12907 ***#Equal contributions***

Heinen, R., Biere, A., Harvey, J. A., & Bezemer, T. M. (**2018**). Effects of soil organisms on aboveground plant-insect interactions in the field: patterns, mechanisms and the role of methodology. *Frontiers in Ecology and Evolution. doi:10.3389/fevo.2018.00106*

Heinen, R., van der Sluijs, M., Biere, A., Harvey, J. A., & Bezemer, T. M. (2018). Plant community composition but not plant traits determine the outcome of soil legacy effects on plants and insects. *Journal of Ecology*, *106*(3), 1217-1229. doi:10.1111/1365-2745.12907

Harvey, J. A., Essens, T. A., Las, R. A., van Veen, C., Visser, B., Ellers, J., **Heinen, R.,** & Gols, R. (2017). Honey and honey-based sugars partially affect reproductive trade-offs in parasitoids exhibiting different life-history and reproductive strategies. *Journal of Insect Physiology*, *98*, 134-140. doi:10.1016/j.jinsphys.2016.12.003

Gussekloo, S. W., Berthaume, M. A., Pulaski, D. R., Westbroek, I., Waarsing, J. H., **Heinen, R.**, Grosse, I., & Dumont, E. R. (2017). Functional and evolutionary consequences of cranial fenestration in birds. *Evolution*, *71*(5), 1327-1338. doi:10.1111/evo.13210

Harvey, J. A., Fei, M., Lammers, M., Kos, M., Zhu, F., **Heinen, R**., Poelman, E.H., & Gols, R. (2016). Development of a solitary koinobiont hyperparasitoid in different instars of its primary and secondary hosts. *Journal of Insect Physiology*, *90*, 36-42. doi:10.1016/j.jinsphys.2016.05.006

Heinen, R., Gols, R., & Harvey, J. A. (2016). Black and garlic mustard plants are highly suitable for the development of two native pierid butterflies. *Environmental Entomology*, *45*(3), 671-676. doi:10.1093/ee/nvw024

PE&RC Training and Education Statement

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

Review of literature (6 ECTS)

- Effects of soil organisms on aboveground plant-insect interactions in the field: patterns, mechanisms and the role of methodology

Writing of project proposal (3 ECTS)

- Living legacies: influence of plant-mediated changes in soil communities on aboveground plant-insect interactions

Post-graduate courses (4.9 ECTS)

- Soil ecology course; PE&RC (2016)
- Workshop insect-microbe-plant interactions; COST Action, Idiv, Leipzig (2016)
- Introduction to statistics in R; PE&RC (2017)
- Generalized linear models; PE&RC (2017)
- Individual-based modelling; British Ecological Society, University of Reading (2017)

Laboratory training and working visits (3.2 ECTS)

- Network visit external lab; Potsdam University, Potsdam (2019)
- Network visit external lab; Institute of Plant Sciences, Bern (2019)

Invited review of (unpublished) journal manuscript (12 ECTS)

- Ecological Entomology: intrinsic competition parasitoids (2017)
- Soil Biology and Biochemistry: microinvertebrates and plant-soil feedbacks (2017)
- Economic Entomology: attractive intercropping to promote biocontrol of aphids (2017)
- Plant and Soil: plant-soil feedbacks in Mediterranean oaks (2017)
- Annals of Botany: plant-soil feedbacks in relation to herbivory in the Triadaca tree system (2017)
- PeerJ: companion crops to deter herbivores (2018)
- Functional Ecology: grazing effects on grasshopper behaviour in the field (2018)
- Scientific Reports: exploration of variability in plant-growth-promoting Rhizobacteria (2018)
- Ecological Entomology: soil humidity, soil nutrient and plant species effects on herbivore feeding preferences in Asteraceae (2018)
- Frontiers in Ecology and Evolution: above-belowground ecology (2019)
- Oikos: allelochemistry and plant competition (2019)
- Journal of Ecology: effects of plant diversity and soil microbes in overyielding in plant communities (2019)
- New Phytologist: soil microbial succession and effects on aboveground plant-insect interactions (2019)

Competence strengthening / skills courses (1.9 ECTS)

- Mindfulness workshop for work-life balance; WUR (2013-2014)
- Time Management; WUR (2015)

- Consultancy skills; WUR (2015)
- Workshop the Netherlands code of conduct for academic practice and principles of good academic research.; NIOO Research Integrity Board (2016)
- Summer school seminar scientific intergrity; EpiDiverse (2018)
- Discussion morning session implication plan S open access for the future of science; TE department NIOO (2019)

Discussion groups / local seminars / other scientific meetings (4.8 ECTS)

- Entomologendag; oral presentation; Ede (2015)
- Active participation in PhD discussion group; Terrestrial Ecology, NIOO (2015-2017)
- Entomologendag; Ede (2016)
- NERN Meeting; oral presentation (2017, 2018)

International symposia, workshops and conferences (9.1 ECTS)

- BES; poster presentation; Ghent (2017)
- Cost action annual meeting; oral presentation; Malta (2018)
- Entomological Society Europe meeting; oral presentation; Napels (2018)
- Ecological Society of America meeting; oral presentation; New Orleans (2018)
- Cost action annual meeting; oral presentation; Thessaloniki (2019)

Supervision of MSc students (3 ECTS)

- Herbivory effects on PSF
- Effects soil transplantation on ground-dwelling herbivores
- Herbivore feeding preference as explanatory variables in plant-soil feedback studies