Soil legacy effects on aboveground plant-insect interactions

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Chapter 1

General Introduction

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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.

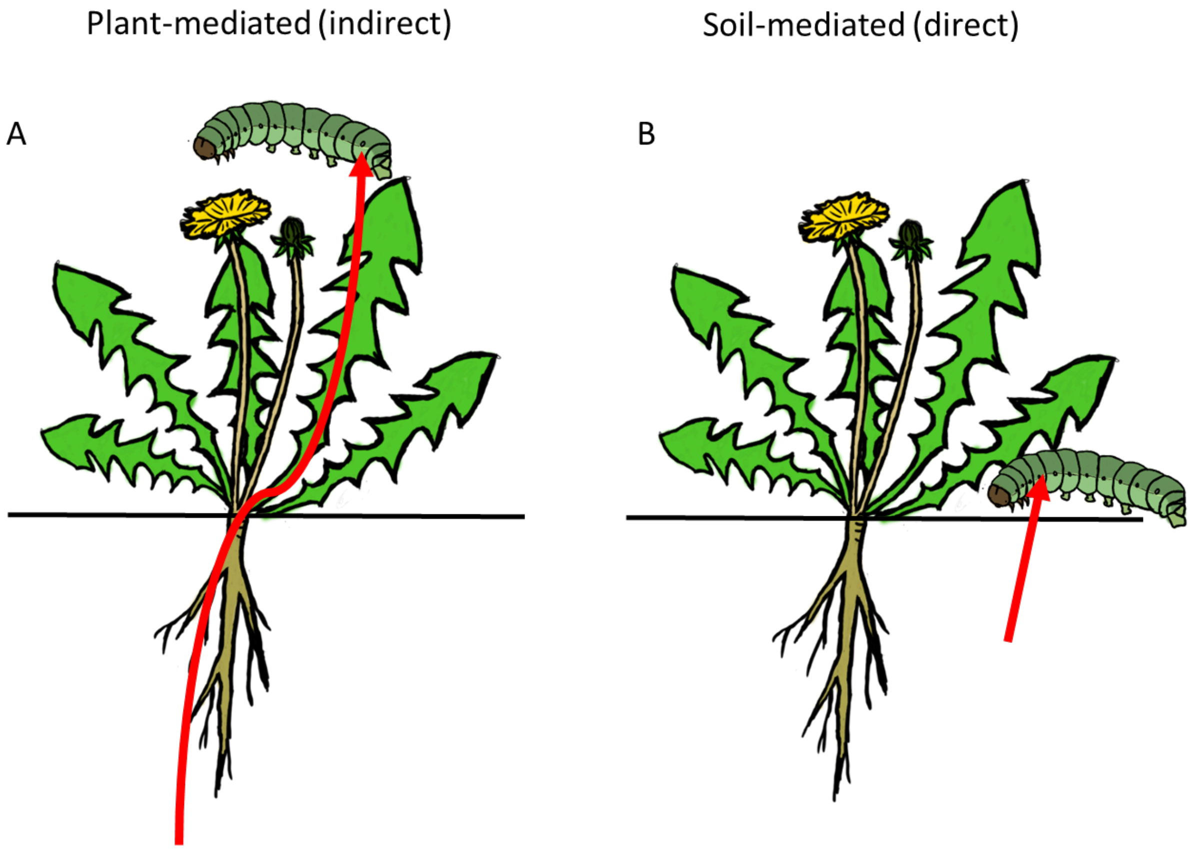

Figure 1.1: Schematic representation of two hypothetical pathways via which soil microbiomes may affect the caterpillar microbiome. A) In the plant-mediated pathway, soil microbes are transferred from the soil to the root to the shoot parts, where they are ingested by the caterpillar and end up inside 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.