From the root of variation: A metabolomics perspective to plant soil-feedback

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For my grandparents

Contents

Chapter 1

General introduction

Soils are a critical component for most life on earth and are not only a substrate for plants to grow in, but also harbour a plethora of macro and microorganism, that influence plants (Van der Heijden et al., 2008). These interactions greatly affect plants in many ways and are decisive factors for a plant's survival. For the last decades, differences in soil abiotic factors, such as humidity and nutrients, and their effect on plant performance have been thoroughly investigated (Chadha et al., 2019; Patterson, 1995; Schulze, 1986). More recently, this focus has shifted to examining the effects of soil microbes on plants. Organisms such as plant growth promoting bacteria or mycorrhizal fungi can enhance plant growth or induce resistance against plant antagonists such as insect herbivores or pathogens (Pineda et al., 2010; Van der Putten et al., 2016). On the other hand, soil also contains detrimental organisms such as pathogenic fungi and bacteria which negatively influence plant growth. The interplay between these "positive" and "negative" soil-born organisms is crucial for the performance of a plant (Van der Putten et al., 2016)

Plant soil feedbacks

Interactions of plants and soil microbes are complex and the growth of a plant in a soil leads to species-specific, abiotic and biotic changes in the soil. These species-specific changes remain present in the soil and can thereby influence the performance of plants growing later in the same soil (Bezemer et al., 2010, 2006; Van de Voorde et al., 2011). This process is called plant-soil feedback (PSF) (Bever, 1994; Van der Putten et al., 1993). If the biomass of a plant increases, relative to a control situation, when grown in a soil conditioned by a plant species, we refer to this as positive plant soil feedback. In contrast, if the biomass is lower it is referred to as negative feedback (Bever, 1994; Van der Putten et al., 2013). Most plant species exhibit negative conspecific PSFs, i.e. grow less well in "own" soil conditioned by an individual of the same species than in "foreign" soil (Bever, 2003; Kulmatiski et al., 2008; Petermann et al., 2008). The scientific interest in PSF has increased rapidly in the last decade (Forero et al., 2019; Smith-Ramesh and Reynolds, 2017). Nevertheless, so far the main focus has been on the effect of conspecific PSF although heterospecific PSFs may as well be of great importance in mixed plant communities (Van der Putten et al., 2013; van de Voorde et al., 2012a). While the traditional definition of PSF is strongly related to biomass, other traits such as herbivore resistance or the concentration of secondary compounds in the roots or shoots can also be largely influenced by PSF (Huberty et al., 2020b; Ristok et al., 2019; Zhu et al., 2018).

The mechanisms that can cause PSFs are numerous. For example, plant pathogens can accumulate in soils conditioned by certain plants species and this can lead to a reduction in biomass in the feedback phase (Mills and Bever, 1998; Nijjer et al., 2007; Van der Putten et al., 2013). Alternatively, plants may accumulate symbionts, which can cause an increase in biomass of the later growing plant e.g. through releasing enzymes cause an increase in nutrient availability in the soil (Reynolds et al., 2003; Van der Putten et al., 2013). Chemicals released by plant roots can cause shifts in the microbial community in the soil, which can influence later growing plants, while these chemicals in the soil can also directly influence the growth of plants in the feedback phase (Bais et al., 2003; Hu et al., 2018; Inderjit et al., 2011; Vivanco et al., 2004). PSF can also arise from extracellular self-DNA which can reduce plant growth (Mazzoleni et al., 2015). Furthermore, while plants grow in soils they deplete nutrients in the soil and this can negatively affect later growing plants (Lepinay et al., 2018). Of all these mechanisms shifts in microbial communities are among the primary causes for PSF effects.

While many studies focus on PSF effects on the biomass of plants, to better understand the impacts of PSFs on deleterious and beneficial organism the plant is associated to (e.g. pathogens above and belowground, herbivores, beneficial microbes, pollinators), it is of great importance to also understand how PSFs influence the chemical composition of a plant. The overarching aim of this thesis is to examine how plant-mediated changes in the soil, influence the metabolome of other, later growing plants.

Microbial influence on the metabolome of plants

Several studies have examined the effects of soil microorganisms on plant metabolomes. In a study published in 2014, Schweiger et al. showed that the leaf metabolome of several plants (*Plantago lanceolata*, *P. major, Veronica chamaedrys*, *Medicago truncatula* and *Poa annua)* differed when these plants were inoculated with a generalist arbuscular mycorrhizal fungus (AMF). However, the responsiveness of the metabolome to mycorrihizal inoculation largely differed between plant species. *M. truncatula* was the most responsive to AMF inoculation with 14.7% of chemical features (putative compounds) changed. On the other hand, the grass *P. annua* showed a minimal response with only 1.7% of the features being modulated by AMF. In all other species around 5% of features were modulated due to AMF. Almost all changes associated with AMF inoculation were quantitative (i.e. variation in concentration) and only fewer than 1% of the features were only present in either the inoculated or the non-inoculated plants (qualitative). The direction of the quantitative changes as well as the individual features that changed varied between species.

Hill and colleagues (2018), investigated the metabolomic responses of root and shoot tissues of the plant *Jacobaea vulgaris* after inoculation with the AMF *Rhizophagus irregularis*. They showed that 33 compounds detected in the roots, most of them blumenols, significantly increased in plants that were exposed to AMF. The concentration of four pyrrolizidine alkaloids (PAs) increased in the roots after exposure to AMF but not in the shoots. This study showed that changes in the soil, e.g. presence or absence of AMF, can have distinguished effects on the metabolome of plants above and belowground.

Other studies investigated the effects of rhizobacteria and endophytes on plant chemistry, however those studies did not use soil that was inoculated but directly inoculated the plants under investigation. Pandey and colleagues (2016) showed that the inoculation of *Catharanthus roseus* with fungal endophytes upregulated terpenoid indole alkaloid (TIA) pathway genes potentially leading to changes in indole alkaloid concentrations above ground. Another study showed that flavonoids and anthocyanins in the leaves were found in higher concentrations in *Epichloe festucae*, when the plant was infected with endophytes (Dupont et al. 2016). Inoculation of sterile in vitro-grown poplar plants with *Paenibacillus sp.* changed the metabolomic profile of the shoots (Scherling et al. 2009). Specifically, eleven metabolites were different between non-inoculated and inoculated plants. Concentrations of asparagine, urea and threitol increase upon inoculation and organic acids as well as amino acids and fructose-6-phosphate were found in lower concentrations.

Effects of soil inoculation on the metabolome of plants

Badri and colleagues (2013) inoculated *Arabidopsis thaliana* with different soil slurries and analysed how these influenced the growth of the plant, the leaf metabolome, and the feeding behaviour of the caterpillar *Trichoplusia ni*. As inocula the autors used soil slurries collected from fields with different agricultural histories that were either filtered or non-filtered in order to differentiate between the influence of microbial and chemical factors of the soil. Arabidopsis biomass above and belowground as well as the leaf metabolome changed depending on the legacy of the soil. The authors pyrosequenced the soils and analysed the nutrient content of the inoculates. Inoculation altered the concentrations of different chemical groups (e.g. sugars, amino acids, phenolics, sugar alcohols) and some of the inocula caused a decrease in the larval weight of *T.ni* when they fed from these plants. Since the nutrients in the inoculates did only vary mildly the observed changes were mostly likely due to differences in the composition of microbial communities within the inoculates. The decreased larval weight was negatively correlated with an increase in the abundance of bacterial taxa (e.g. Balneimonas, Skermanella, Nocardioides) in a principal component analysis. This study showed for the first time that the microbial composition of the soil is important for plant growth, herbivore performance and metabolome composition.

Kos and colleagues (2015c) studied how soil conditioned by different plant species influenced the growth, the chemical composition in the phloem and aphid performance on the plant *J. vulgaris.* They showed that growth by different plant species caused changes in soil fungal communities and that the biomass, the concentration of amino acids and PAs in the phloem, as well as the performance of a generalist aphid and a specialist aphid all varied depending on the soil in which *J. vulgaris* was growing. In this study the chemical analysis focused on specific compounds. However, as only targeted compounds were analysed, these studies may have overlook changes that occur in the non-targeted part of the metabolome of plants. Changes in these plant compounds could also influence interactions of these plants with other organisms. Furthermore, targeted approaches require previous knowledge of the metabolomic composition of the plant species under investigation which is not always available. Ecological studies often encompass different plant species or genotypes. Variation in targeted compounds such as secondary compounds might even vary considerably between different genotypes of one species although variation in primary compounds which are likely also involved in the interactions between plants and other organisms, is less pronounced among genotypes and more universal among plant species (Castro-Moretti et al., 2020). While this seems generally true for plants grown under controlled conditions, more variation is observed for plants collected in the field (Nagler et al., 2018). Interestingly changes in the metabolome of the plants growing in soil with particular microbes are not limited to secondary plant metabolites. Changes in the microbial communities in the soil can result in different concentrations of primary compounds in plants (Sampaio, Edrada-Ebel, & Costa, 2016). Technical advances make it now possible to examine entire metabolomic profiles and therefore can provide a more complete picture of the response of a plant to changes in its environment.

The plant metabolism

The metabolism of a plant consists of a complex of dynamic processes, such as photosynthesis, respiration and the synthesis and degradation of organic compounds and all of these processes are linked to each other (Ratcliffe and Shachar-Hill, 2001; Vickery and Vickery, 1981). The foundation of all these processes is photosynthesis which produces substrates for respiration. By that it provides the building blocks of organic compounds such as amino acids, nucleic acids, proteins, carbohydrates, organic acids, lipids, and secondary compounds. Traditionally the plant metabolome is divided into the primary metabolism, which is required for a plant's survival and the secondary metabolism which main purpose is the defence of the plant (Berenbaum, 1995). Secondary plant compounds are chemically highly diverse and differ largely between different plant species (Moghe and Last, 2015; Weng et al., 2012) Primary metabolites, in contrast, are traditionally thought to be conserved across different plant species (Pichersky and Lewinsohn, 2011). However, primary metabolites can also play a role in defence, for example against pathogens and herbivores (Berenbaum, 1995). Plant hormones often act as signalling molecules and prime primary pathways in the plant. Furthermore, they can regulate plant development and its metabolic status (Beckers et al., 2016). The composition of the chemical defence compounds in a plant depends on the environment of the plant. For example, microorganisms in the soil can trigger the production of defence compounds in above and belowground plant tissues (Schweiger et al., 2014; Zhu et al., 2018). Defences are typically divided into constitutive and induced defences (Hulten et al., 2006). Constitutive defences describe defences with compounds which are present in the plant all time. Induced defences describe compounds of which production increases upon a stress (e.g. herbivores or pathogens) leading to higher concentrations of these compounds in the plant.

Variation in space

Plants protect themselves from pathogens and herbivores above and belowground by producing chemical compounds and adapting their chemical composition to the local environment. However, the threats and the beneficial organisms, that plants can deter or attract with certain compounds vary considerably over space. Since plants cannot escape those unfavourable conditions, they must cope with the situation the face, and they do so by adapting their chemical composition to the local circumstances. For example, Kleine an Müller (2011) showed that the chemical composition of *Tanacetum vulgare* varied strongly within a small spatial scale of 1.5 meters, probably due to selection pressures of different specialist and generalist herbivores in the area, and these chemical changes can lead to species-specific distribution of herbivores over space. Not only herbivores differ on small spatial scales but also the soil microbial community varies largely between sites and within single locations. The microbiome of the soil can differ at scales from millimetres to centimetres (Ettema and Wardle, 2002; Fierer, 2017; O'Brien et al., 2016). Soil microbial communities are known to affect the growth of plants and the composition of plant communities (Heinen et al., 2020; Wang et al., 2019b). However, if and how strongly these spatial differences in the microbiome are related to differences in chemical composition of plants remains unknown so far.

Variation of PSF in time

All the mentioned causes for PSF, such as changes in the microbial communities in the soil, changes in soil chemistry, or changes in the concentration of plant exudates and self DNA, vary over time (Hannula et al., 2019a; Hu et al., 2018; Mazzoleni et al., 2015; Waring et al., 2015). We may expect that the longer a specific plant grows in the soil, the stronger the influence of this plant on the soil in which it is growing. These soil legacies can then influence properties of plants which grow in the same soil afterwards. Through these changes in the soil over time, the duration that a plant conditions the soil could potentially lead to alterations of the metabolome of plants grown in theses soils and the performance of insects on these plants. This is relevant for predicting the effect of PSFs on ecosystems and to understand how plants change the growth of other plants via the soil. A current debate among ecologists focuses on the reproducibility of PSF (De Long et al., 2019). Experiments that study the strengths of PSFs at different time points are urgently needed. Recently, we sampled the soils of six forb and grass species over the course of one year and followed bacterial and fungal communities over time (Hannula et al., 2019a). In that study we show that fungal communities in the soil are more stable over time than bacterial communities, which showed considerable changes between time points. Fungal communities also varied, but these changes were more related to the functional groups (grass/forb) of the monocultures that were growing in the soil than to temporal changes (Hannula et al., 2019a). In this thesis, I used these soils conditioned by monocultures to investigate how the soil legacies influence the metabolome and the biomass of *J. vulgaris* plants growing in these soils over the course of a year to examine how stable PSF patterns are over time.

Methods to analyse metabolomes

The effect of bioprocessing is reflected in the metabolic pool, the so-called metabolome. Traditionally, responses of plants to environmental challenges, such as changes in abiotic or biotic factors in the environment, are measured by recording the changes of a few targeted metabolites focusing on some plausible biosynthetic pathways. However, these targeted approaches can miss alterations in the plant simply because the change caused by the environmental challenge might not or not only occur in the targeted compounds. In contrast to these targeted analyses that require at least some prior knowledge about the species under investigation, untargeted metabolomics enable to explore changes in the whole metabolome without prior chemical knowledge. Up to today most plant metabolomics research focuses on model plant species (e.g. *Arabidopsis thaliana*, tomato and maize) (Kuhlisch and Pohnert, 2015) and a few secondary groups of compounds (Pyrrolizidine alkaloids, Glucosinolates). However, for many plant species no prior knowledge about their chemical composition is available. Therefore, untargeted metabolomics represent an ideal tool to investigate the chemical composition of non-model species and by that opens up a new tool to ecologists to analyse how interactions in nature influence the metabolome of plants.

Metabolomics is a field that emerged due to the technological advances in chromatography during the last decades with the ultimate goal to extract and detect all compounds in an organism. It is currently used in a broad range of life sciences ranging from medicine to biology. It is the omics technique which investigates the end product of the whole cellular machineries and is therefore the omics technique which gives the most realistic picture of the situation that an organism interacting with a plant will encounter.

Ecometabolomics is a young research branch of metabolomics and describes the use of metabolomics techniques to assess the effect of an environmental gradient or stress on the metabolic state of an organism. The application of metabolomics techniques in ecology focus on understanding the biochemical mechanisms that drive species interactions with other organisms and with the environment (Bundy et al., 2009; Peñuelas and Sardans, 2009; Sardans et al., 2011; Van Dam and Meijden, 2011). Today it is also commonly used to explore the metabolic response of one or more organisms which interact and their variation in time and space (Peters et al., 2018). Metabolites within plants are needed to survive, reproduce, and interact with other individuals and organisms in the environment and are adjusted to the environmental conditions continuously. They represent the language that plants speak and adjustments to the current specific situation can be measured and identified with metabolomics techniques. Metabolomic analyses can be based on metabolic fingerprinting represents an untargeted approach supplying an overview of the differences in the metabolome but not necessarily identifying compounds within the organism, or on a semi-targeted approach, metabolite profiling, which focuses on specific groups of metabolites within samples (Hall, 2011).

Despite the continuous advancements in techniques and analysis used in metabolomics this young research field is still facing multiple challenges. The ultimate goal of metabolomics is to extract and detect all compounds in a certain organism at a given time (Stobiecki and Kachlicki, 2005). However up to today the field is a long way from reaching this ultimate goal. The first analytical step in metabolomics studies, untargeted and targeted, is the extraction of the compounds within the organism with an extraction solvent. However, no solvent can truly extract all compounds. Water is a universal solvent which, in plant metabolomics, is often used for the extraction of primary compounds such as sugars amino acids, organic acids and phosphorylated compounds (Cocuron et al., 2014). Other solvents such a methanol are more effective in the extraction of secondary compounds from plant leaves (Cocuron et al., 2019). A solution that is often applied is to extract with mixtures of methanol and water (Kim et al., 2010). The next step is to choose the suitable method to analyse the extract sample. The most common methods used for metabolomics analysis are liquid chromatography coupled with mass spectrometry (LC-MS), gas chromatography coupled with MS (GC-MS) and nuclear magnetic resonance spectroscopy (NMR) (Peters et al., 2018). All of the methods bear advantages and disadvantages. LC-MS allows for detection of polar to medium polar compounds, but often lacks a suitable database and absolute quantification of compounds is not possible. In GC-MS the samples need to be derivatized and heated in order to be detectable and injectable. This process can alter compounds in the samples, and detected compounds might differ from the ones originally contained in the sample. The size of compounds analysed with GC-MS is also limited, since they need to be dissolvable in the gas phase, which excludes large compounds, because of their high molecular weight. NMR covers a broad range of compounds which can be detected, but the identification of individual signals is challenging and requires experience due to overlapping signals in mixtures. As opposed to other methods absolute quantification is possible with NMR.

Especially ecometabolomics requires the analyses of complex chemical samples, and many different factors might influence the composition of the plant under investigation. In the light of this complexity multiplatform approaches can be useful to cover a large spectrum of compounds with different chemical properties.

Thesis outline

In this thesis, I examine if plant-induced changes in the soil influence the metabolome of plants which grow later in these soils, and how these effects differ in time and space. Furthermore, I examine how multiplatform metabolomics can be used for ecometabolomics studies.

A graphical abstract of the research questions addressed in the experimental chapters 2 to 5 can be found in Fig. 1.1.

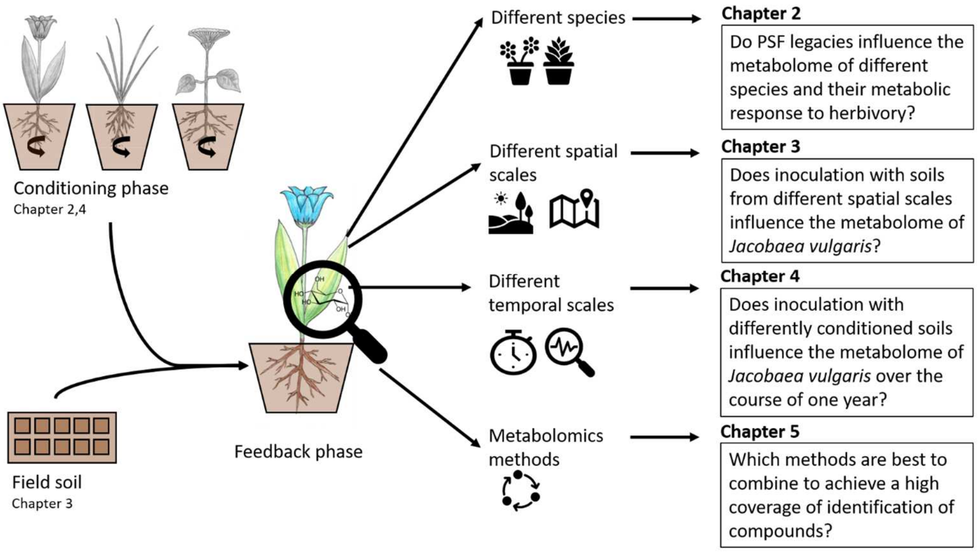


Figure 1.1 Schematic overview of the experimental chapters of this thesis. Plant soil feedback (PSF) experiments were carried out by first conditioning the soil in the conditioning phase. This led to abiotic and biotic changes in the soil with which sterilised soils were inoculated in the feedback phase. We evaluated how these changes influenced the metabolome of 12 different species and their response to herbivory in chapter 2. In chapter 3 the variation of inoculation with field soil collected across different spatial scales on a model species (*Jacobaea vulgaris*) was accessed. In chapter 4 temporal variation of PSF and their effects on metabolomes of *Jacobaea vulgaris* was accessed. In chapter 5 the possible methods for metabolomics are compared and combined on the example of *Taraxacum officinale*

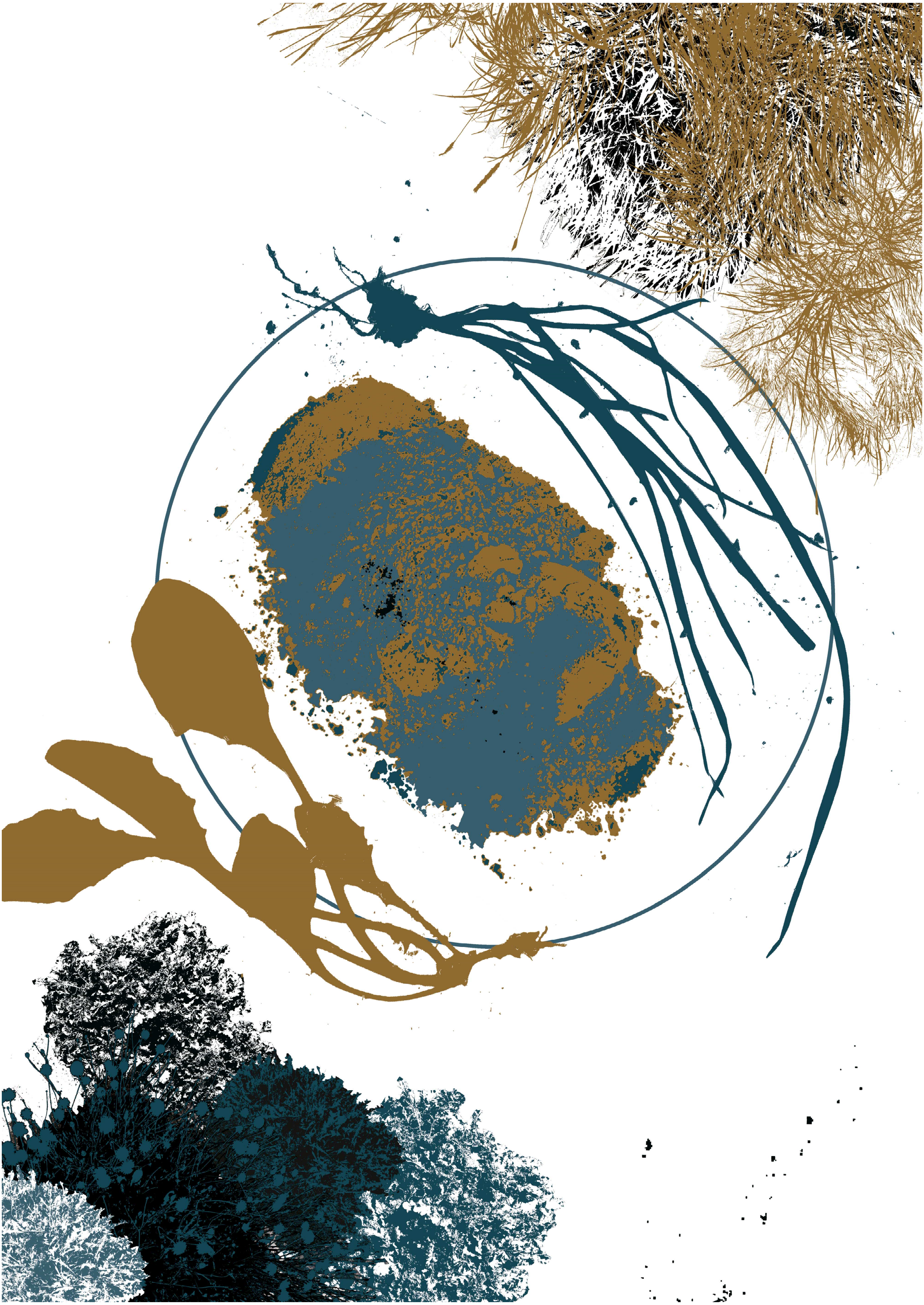
In **Chapter 2**, I examine the effect of PSFs on the metabolomics of a number of forb and grass species. In a fully crossed design, we grew 12 species in soils inoculated with conditioned soils of all 12 plants species. We then exposed the plants to herbivory by a caterpillar (*Mamestra brassciae*) or not. The plants were then harvested, and the shoots were analysed with untargeted metabolomics with NMR. The metabolic response to the soils was species specific. Surprisingly, the soil conditioning predominately influenced the concentration of primary compounds such as sugars and amino acids. Remarkably, the soil treatment explained more of the variation of the metabolome of the plants than herbivory, for 7 of the 12 plant species, even though herbivory is generally known to have a large impact on the metabolome of plants. This chapter proves that PSFs influence the metabolome of plants. However, the sensitivity of the metabolome and the compounds that change are species specific. Moreover, even though I expected strong interactions between the effects of the soil and herbivory treatments, in this study, only for one species did the metabolic response to herbivory depend on the soil treatment.

For the study presented in **Chapter 3**, I collected soil from four different natural grasslands in the Netherlands. In each grassland, multiple soil samples were collected according to a spatial gradient. I then grew identical clones of *J. vulgaris* in sterilized soil inoculated with the different soil samples. Based on current knowledge I expected the soil samples to inhabit different microbial communities and I hypothesized that metabolomic profiles of these *J. vulgaris* plants will depend on the soil origin of the inocula and differ between sites. Within each site I expected that the metabolomic profile of soil samples collected close together (30 cm) would be more similar than samples collected further apart (100 m). Inoculation with soils from one of the four grasslands altered the metabolome of *J. vulgaris*, compared to plants grown in sterile soil but I did not detect a distance relationship. However, similarity analysis revealed that the metabolomes of plants grown with soil inocula that originated from the same site were more similar than the metabolomes of plants grown with inocula collected from different sites.

In **Chapter 4**, I investigate the temporal dynamics of PSF effects on plant metabolomics by repeatedly examining plant growth, and metabolomic composition of genetically identical *J. vulgaris* plants and by determining herbivore performance on these plants for an entire year. We set up an experiment in which we conditioned soils with monocultures of six different plant species (including *J. vulgaris*) in large containers outdoors in a common garden. Every two months I collected soil from these containers and grew genetically identical *J. vulgaris* plants in sterile soil inoculated with soil from the containers in a climate-controlled growth chamber. After 6 weeks of growth I determined the shoot and root biomass and the performance of *M. brassicae* on leaves of the plants and analysed the shoot metabolome with untargeted metabolomics. The relative response, in terms of biomass and herbivore performance, to the soil conditioning treatments was similar over the course of the year. However, the effects of soil conditioning on the metabolome varied greatly over time. Furthermore, we show that the changes in the metabolome are more strongly correlated to changes in the bacterial communities than the fungal communities of the soil. The changes in the microbiome of the soil and the metabolome were correlated strongest after 8 months of conditioning.

In **Chapter 5**, I used three commonly used methods in metabolomics. ^{1}H NMR, LC-MS and HPTLC to do metabolic fingerprinting of *Taraxacum officinale* plants that either experienced herbivory of not. All three methods revealed that the metabolome clearly differed between plants which experienced herbivory or not. However, the identification of compounds was hindered by the chemical complexity of the extracts. Therefore, in a next step we used HPTLC as a preparative tool, enabling us to separate the extracts into fractions with distinct chemical properties. In the next step these fractions were analysed with LC-MS and GC-MS. By that we were able to detect campesterol which changed depending on the treatment and could not be detected with any of the conventional methods. We show that each chemical method bears advantages and disadvantages and that HPTLC can be a useful tool to concentrate compounds from mixtures and therefore enabling the identification of compounds, which would have been overlooked otherwise.

In **Chapter 6**, I discuss the results of the experimental chapters in a broader context and highlight a number of potential areas for future research.



Chapter 2

Aboveground plant metabolomic responses to plant-soil feedbacks and herbivory

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Abstract:

1. Understanding the causes of variation in foliar plant metabolomes is essential for our understanding of ecological interactions between plants and other organisms. It is well-accepted that foliar herbivory alters metabolites in leaves. However, soil (micro)organisms can also induce such changes.
2. We generated plant-specific soil legacies by growing twelve plant species individually in a common starting soil. Then we planted all plant species in all soils and exposed a subset to foliar herbivory. We then used ^{1}H nuclear magnetic resonance (NMR) to analyse the shoot metabolomes of all responding plants.
3. Aboveground herbivory and soil legacies altered shoot metabolomes. In most plant species, soil legacy more strongly affected shoot metabolomes than foliar herbivory.
4. *Synthesis*. Our results show that plant-induced changes in soil alter metabolomes of plants that grow later in those soils. Such belowground legacy effects can have far-stretching consequences for aboveground multitrophic interactions as these often depend on the plant chemical composition. Recently, plant-soil feedbacks have received considerable attention in ecological studies, and our study now highlights that these feedbacks can be an important determinant of the often-unexplained intraspecific variation in chemical composition amongst plants.

Key words: Above-belowground interactions, Ecometabolomics, Ecological omics, Insect herbivory, Nuclear Magnetic Resonance spectroscopy, Plant-soil interactions, Soil Legacy effect.

Introduction

In natural systems all organisms actively interact with other organisms and their surroundings. This is especially true for plants which are sessile and hence forced to cope and interact continuously with a range of biotic and abiotic challenges above- and belowground. Chemical compounds play a key role in many of these biotic interactions, and one of the challenges in chemical ecology is to disentangle the drivers of variation in chemistry within and between plants and the role these compounds play in ecological interactions (Dyer et al., 2018). A large body of literature has shown that foliar herbivory by insects typically leads to changes in the composition or concentration of specialized compounds of plants, and that these changes generally increase the resistance of the plant to herbivory (Karban & Baldwin, 1997). In the soil, plant roots interact with soil-dwelling organisms such as microbes (e. g. pathogens, mycorrhizal fungi), nematodes and root feeding insects, and an increasing number of studies is showing that specific soil organisms such as insects or microbes can induce changes in the composition of plant derived chemicals in the foliage (Bezemer & Van Dam, 2005; Etalo, Jeon, & Raaijmakers, 2018; Pieterse et al., 1998). Most of these studies focus on changes in one or a few specific groups of chemical compounds, but the plant metabolome consists of thousands of compounds. Several authors have argued that changes in the chemical composition within a plant may be more important for the interactions between plants and other organisms than changes in specific compounds (Berenbaum & Zangerl, 1993; Liu, Vrieling, & Klinkhamer, 2017; Nelson & Kursar, 1999). However, how plant metabolomes change in response to herbivory or interactions with belowground organisms, and how common these responses are among plant species, is poorly understood.

The impact of a particular soil-dwelling organism on plant chemistry can be influenced strongly by antagonistic and symbiotic interactions that occur with other soil organisms. Hence effects of an isolated group of soil organisms, although very important for mechanistic understanding of specific interactions, may not be a good representation of the effects of those organisms in entire soil communities and might be a too simplistic approach. Interestingly, several studies have shown that changes in the composition of entire soil communities can also lead to altered plant growth, plant chemistry and interactions with aboveground insects (Badri, Zolla, Bakker, Manter, & Vivanco, 2013; Bezemer et al., 2013; Heinen, Sluijs, Biere, Harvey, & Bezemer, 2018; Hu et al., 2018; Kos, Tuijl, De Roo, Mulder, & Bezemer, 2015c; Ristok et al., 2019; Zhu et al., 2018). However, the wealth and complexity of interactions that occur within these soil communities make it empirically challenging to manipulate their composition. Plants are sensitive to changes in the soil, but also greatly impact the community in the soil in which they grow, and plant species differ greatly in how they alter soil communities. Hence, by growing a plant in soil, the composition of the soil community will change, and this will then influence the performance of another plant that grows later in this soil, a phenomenon that is called plant-soil feedback (PSF) (Van der Putten et al., 2013). These PSFs can provide a useful approach to study the impact of entire soil communities on plant chemistry. However, even though plant-soil feedbacks are receiving considerable attention, so far most of that work has focused on changes in the biomass of the responding plants and not much attention has been paid to the effects on plant metabolomic profiles (Kulmatiski, Beard, & Heavilin, 2012; Van der Putten et al., 2013).

Plant species differ greatly both in how they influence the soil and in how they respond to changes in the soil, and one of the challenges in PSF research is to unveil commonalities in how plants change the soil they grow in, or respond to these changes (Cortois, Schröder-Georgi, Weigelt, Van Der Putten, & De Deyn, 2016; Heinen et al., 2018). Grasses and forbs are two groups of plants that differ morphologically and functionally, and these two groups also differ in how they interact with soil biota. Grasses generally produce more fine roots and denser root systems than forbs. Hence, per unit soil, grasses may have a larger influence on soil organisms than forbs and may respond stronger to changes in the soil (Brundrett, 2002). Interestingly, forbs often grow better in grass soil than in forb soil (Heinen et al., 2018; Ma, Pineda, Van der Wurff, Raaijmakers, & Bezemer, 2017; Wubs & Bezemer, 2016). Some studies indicate that grass soils may provide the plants with greater resistance against aboveground herbivores (Heinen et al., 2018; Kos, Tuijl, De Roo, Mulder, & Bezemer, 2015b; Latz et al., 2012). Several studies have shown that microbiomes in soils conditioned by grasses differ considerably from those conditioned by forbs (Bezemer, Jing, Bakx-Schotman, Bijleveld, & Bijleveld, 2018; Heinen et al., 2018; Kos, Tuijl, et al., 2015c; Latz, Eisenhauer, Rall, Scheu, & Jousset, 2016; Latz, Eisenhauer, Scheu, & Jousset, 2015). For example, many grass species accumulate plant growth promoting rhizobacteria belowground (Latz et al., 2012). These bacteria can prime plants that grow later in that soils, resulting in changes in the composition or concentration of defence compounds in aboveground tissues (Pangesti et al., 2015; Van Oosten et al., 2008). So far, studies that quantify the changes in metabolomes of grass and forb species in response to changes in the soil are scarce. A study with arbuscular mycorrhizal fungi (AMF) showed that metabolomes of forbs change more in response to AMF addition than that of a grass species (Schweiger, Baier, Persicke, & Müller, 2014). However, whether metabolomes of grasses and forbs change consistently, whether there are common responses to soils in which first grasses or forbs have been grown, and how these changes are influenced by aboveground herbivory is not known. PSF studies often distinguish between conspecific and heterospecific soils. Most studies show that plants grow worse in their own soil than in soil from other plant species (Kulmatiski et al. 2008). How this will influence the metabolomes of the plants is less well known.

Ecometabolomics is an emerging field in metabolomics that uses untargeted biochemical approaches to measure thousands of metabolites to understand interactions among organisms (Peters et al., 2018). The metabolome of a plant is very diverse and so are the chemical properties of the metabolites. This makes it challenging to study and compare plant metabolomes. Most studies on plant metabolomics use different extraction and analysis methods to determine different types of compounds within a plant, such as liquid chromatography–mass spectrometry (LC-MS) and gas chromatography–mass spectrometry (GC-MS). With nuclear magnetic resonance techniques (NMR) a broad range of metabolites can be measured within plants with a single extraction method. The extraction is simple, and the results are highly reproducible (Kim, Choi, & Verpoorte, 2010; Verpoorte, Choi, & Kim, 2007). Hence, NMR is a very useful technique to study interactions among plants and other organisms.

Here we studied how soil legacies generated by six forb and six grass species altered the metabolomes of these forbs and grasses growing later in each soil. We used twelve species and each of these species was grown in all twelve conditioned soils. In addition, the response plants were either exposed to aboveground herbivory treatment by the polyphagous chewing herbivore *Mamestra brassicae* (Lepidoptera: Noctuidae) or kept as no herbivory controls. We investigated the metabolomic changes in the shoots of the response plants that were growing in the different soils enabling us to detect changes in the metabolome due to soils and examine whether these patterns stay the same under herbivory or not. We hypothesized that 1) soil-borne legacies created by different plant species will influence the metabolome of other plant species that grow later in the same soil, and that the response plants will respond differently to soils and herbivory. Furthermore, we hypothesize that 2) grasses and forbs will have different metabolomic profiles, that grasses and forbs will differ in their response to soil conditioning and herbivory, and that this response will depend on the functional group of the species that conditioned the soil; and 3) soil conditioning will alter the metabolomic response of a plant to aboveground herbivory.

Materials and Methods

Experimental design

We set up an experiment to investigate the metabolomic response of 12 different plant species to different soil legacies. We used a fully crossed design. For that we first conditioned soils by the 12 different plant species and used those soils with legacies to grow all species again on them. Furthermore, we exposed half of the grown plants to an aboveground insect herbivore. Per response species 4 replicates per soil and herbivory level were analysed. This resulted in 4 x 12 x 2 samples per response plant. As for some plants there was not enough material available, exact sample sizes varied slightly and are presented in the supporting information (Table S7). Due to other research plans six extra replicates were analysed for *Plantago lanceolata*. The number of replicates for each treatment combination ranged between 3 and 5. This sample size was chosen due to the availability of plants and greenhouse space. No data was excluded. The NMR samples were blinded for analysis.

Plants

Twelve different plant species from two functional groups (six grasses and six forbs) were used: Grasses: *Agrostis capillaris* (AC) (Poaceae), *Anthoxanthum odoratum* (AO) (Poaceae), *Alopecurus pratensis* (AP) (Poaceae), *Briza media* (BM) (Poaceae), *Festuca ovina* (FO) (Poaceae), *Holcus lanatus* (HL) (Poaceae), Forbs: *Crepis capillaris* (CC) (Asteraceae), *Geranium molle* (GM) (Geraniaceae), *Gnaphalium sylvaticum* (GS) (Asteraceae), *Myosotis arvensis* (MA) (Boraginaceae), *Plantago lanceolata* (PL) (Plantaginaceae), *Taraxacum officinale* (TO) (Asteraceae). All species are common grassland species that co-occur in the Mossel area from which the soil was collected. Seeds of all species were obtained from a provider of seeds of wild plant species (Cruydt-Hoeck; Nijberkoop, The Netherlands). Seeds were surface sterilized with 2% hypochlorite and rinsed with water afterwards and germinated in containers filled with sterile glass beads and demineralized water in a climate cabinet (16h: 8 h light: dark photoperiod, temperatures: day 21°C, night 15 °C). To adjust for differences in germination rates among species, germinated seedlings were kept at 4 °C and a photoperiod of 16 h upon germination.

Insects

Mamestra brassicae (Lepidoptera: Noctuidae), a generalist herbivore, which is native to the palearctic and known to feed on a wide range of plant families and species was used (Rojas, Wyatt, & Birch, 2000). The eggs of the *M. brassicae* (Lepidoptera: Noctuidae) were obtained from the Department of Entomology at Wageningen University. They were reared on *Brassica oleracea* var. gemmifer cv. Cyrus. Originally, they were collected from cabbage fields near the university.

Experimental conditioning phase

Soil (0-10 cm) was collected from a natural grassland "De Mossel" (Natuurmonumenten, Ede, The Netherlands). Soil was sieved (sieve mesh Ø 1.0 cm) to remove stones, dead plant material, roots and most macro-invertebrates. More soil was collected from the same site from the 10 cm to 20 cm layer. This soil was also sieved and then sterilized by γ-irradiation (> 25 KGray, Synergy Health, Ede, The Netherlands). The soil was collected deeper since this layer contained fewer roots and we did not need to sample the layer containing most of the microbiome influenced by plants since this soil was sterilized. For each of the 12 plant species, 60 square one-Liter pots (11x11 cm) were filled with 1050 gram live field soil (720 pots total). One seedling was planted into each pot in a greenhouse (L:D 16:8, day temperature 21 ± 1 °C, night temperature 16 ± 1 °C). To avoid dehydration and assure establishment of the seedlings, the seedlings were covered with shade-cloth during the first four days. To minimize emerging weeds and prevent fungus gnats from laying eggs a 1 cm-layer of silver sand was added. Seedlings emerging from the seedbank present in the live soil were removed on a daily basis and the plants were watered three times per week. After 10 weeks the aboveground biomass of all the pots was clipped and the roots were removed from the soil. The conditioned soil from each pot was kept at 4 °C. *A priori*, the sixty individual pots per species were divided over five separate replicates so that each replicate contained all soil from twelve independently conditioned pots resulting in 60 soils.

Experimental feedback phase

Each of the 60 soils was mixed with sterilized soil (1:2 conditioned: sterile v/v) to minimize potential differences in nutrient content among the conditioned soils and used to fill 24 pots (9x9cm; 650 g). All 12 plant species were grown on each conditioned soil in double and randomly allocated to one of two herbivory levels (present, absent), resulting in 12 response species x 12 conditioning species x 5 replicates x 2 treatments = 1440 pots. The plants were grown in a greenhouse under the same conditions as the plants in the conditioning phase. After 4 weeks, all 1440 pots were caged (9 cm diameter, 30 cm height) with a plastic tube made of transparent plastic with insect mesh fitted on top. In one of the two pots a freshly hatched *Mamestra brassicae* caterpillar was introduced. After 7 days of feeding the caterpillars were removed and feeding marks were observed in all treated plants except for 7 individuals. Those individuals were excluded from later metabolomics analysis. Upon harvest all plants were at the vegetative state, except for *Plantago lanceolata.* For this species only the leaves were sampled. The area that the caterpillars fed (damage) was recorded. All plants were then clipped, and fresh shoot biomass was recorded. The shoots were then immediately wrapped in aluminum foil and flash-frozen in liquid nitrogen and stored at -80 °C until lyophilization. *Gnaphalium sylvaticum* grew much slower than the other species and therefore the herbivory treatment was applied exactly one week later than the other species and the shoots were harvested one week later.

Metabolomics ^1H NMR analysis:

^{1}H NMR was chosen for untargeted metabolomics due to its high reproducibility and ability to detect a large dynamic range of molecules. The samples were lyophilized for 5 days. Lyophilized material was ground with a Retsch Mixer Mill MM 400 for 4-5 minutes, depending on the structure of the plant material, at a frequency of 30 Hz. Dried powdered samples were weighed (20.21 ± 0.05 mg) and put in 1.5 mL-microtubes. For the extraction 300 µL of CH_3OH-d_4 (Sigma, St Luis, MI, USA) was added to the samples followed by 300 µL of D_2O buffer with 0.01% TSP. The vials were sonicated for 10 minutes and then centrifuged for 10 minutes at 13000 ppm in a Heraeus Pico 17 Microcentrifuge. 250 µL of the clear supernatant was then filled in a disposable 3mm-NMR tube (Bruker, Karlsruhe, Germany) using a glass pipette.

^{1}H NMR spectra were recorded on a Bruker AV-600 MHz NMR spectrometer (Bruker, Karlsruhe, Germany) operating at a ^{1}H NMR frequency of 600.13 MHz. As internal lock we used CH_3OH-d_4. Each ^{1}H NMR spectrum consisted of 128 scans taking 10 min and 26 sec acquisition time with the following parameters: 0.16 Hz/point, pulse width (PW) = 30 ° (11.3 µs), Relaxation delay (RD) = 1.5 sec.

A presaturation sequence was used to supress the residual H_2O signal by low power selective irradiation at the H_2O frequency during the recycle delay. FIDs were Fourier transformed by a line broadening of 0.3 Hz. The spectra were then manually phased, baseline corrected and calibrated to TSP at 0.00 ppm, using TOPSPIN (v 3.0. Bruker).

Bucketing was done with AMIX software (v. 3.9.12 ,Bruker BioSpin GmbH, Reinstetten, Germany) with scaling to total intensity. Bucketing is a common data pre-processing technique used to minimize the effect of small shifts in signals (Kim, Choi, & Verpoorte, 2010). Spectral intensities were reduced to integrated regions of equal width (0.04 ppm). During analysis regions between 4.70 - 4.9 ppm and between 3.28 - 3.34 ppm were excluded because of the residual signals of the solvents. The ^{1}H NMR data matrix consisted of 241 buckets per analysed sample. A bucket contains the intensity of the ^{1}H NMR signal within a given range of the chemical shift. In ^{1}H NMR the intensity of the signal within each bucket directly represents the molar levels of a compound in a plant. The H atoms within one molecule can lead to signals in different buckets of the ^{1}H NMR profile. Depending on the chemical environment (neighbouring atoms) ^{1}H atoms show a different chemical shift in the ^{1}H NMR (low electron density-higher chemical shift, high electron density-low chemical shift). Neighbouring atoms do not only change chemical shift, but they can also change the pattern in which a signal is split (splitting pattern). Position and number of chemical shifts can be used as diagnostics for the structure of a molecule. The identification of the signals was done by investigating the splitting pattern and the chemical shift of signals and comparing it with an internal database and as described in Kim, Choi, & Verpoorte, 2010.

Data analysis

Data were analyzed using multivariate and univariate statistics. The elucidation of all signals in the NMR to compound identities' is time consuming. Here, we focused on the signals that the statistics showed to be different between treatments. All statistical analyses were performed in R Studio (R Studio Team, 2016) using the packages 'vegan' (Oksanen et al., 2018) and the function 'pairwise.Adonis'(Martinez, 2017).

To visualize the separation between metabolomes of the different response species and the different treatments an ordination using non-metric multi-dimensional scaling (NMDS) based on a Bray-Curtis dissimilarity matrix was used. Data was standardized before analysis using Wisconsin standardization and square transformation. NMDS uses a dissimilarity matrix to produce an ordination that optimally represents the pairwise dissimilarity between objects in a low-dimensional space. Bray-Curtis dissimilarity matrices are commonly used in community composition analysis and are based on intensities rather than presence/absence data.

In order to investigate if soil conditioning led to changes in the metabolome of the response plants, we conducted permutational multivariate analysis of variance (PERMANOVA). In all cases the permutations were set to 999 and analyses were based on Bray Curtis dissimilarities. The betadisper function in the vegan package was used to test for dispersion differences between groups. Post-hoc tests for all PERMANOVAs were performed with the pairwise Adonis function in R. Benjamini & Hochberg corrections for false discovery rates were used to correct the P values for multiple post-hoc testing and minimize type-1 errors (Benjamini & Hochberg, 1995).

We first ran a PERMANOVA with as fixed factors "response plant identity", "conditioning plant identity" and "herbivory". For this model all data were used. We then tested in separate PERMANOVAs the effects of "response functional group" and/or "conditioning functional group" as well as “self and non-self soil”. For these models means of every bucket per species grown in all soils were used so that plant species were used as replicates for functional groups (in these analyses the replicates within species were considered pseudo replicates). Subsequently, we ran a PERMANOVA for each response plant species separately, including as fixed factors "conditioning plant identity" and "herbivory". For each response plant species, we also tested the effects of "conditioning functional group" and "herbivory" as well as "self and non-self soil" as described above.

Results

Overall comparisons of metabolomes

Aboveground metabolomes distinctly differed among the twelve plant species and between grasses and forbs (Fig.2.1, Supporting information Table S2.1) and the profiles were strongly influenced by herbivory, soil legacies and the interactions between these two factors (Supporting information Table S2.1). However, the metabolic responses to soil legacies were species-specific and did not differ consistently between soil legacies from grasses and forbs (Supporting information Table S2.1; no significant soil conditioning effects in Model 2 and 4). Overall, the metabolic responses did not vary between plants grown in soil conditioned by the same plant species (self-soil) or in soils conditioned by other plant species (non-self) (Supporting information S2.1, Model 5 and 6). Herbivory strongly influenced the metabolome of the response plants for all species, but the area of leaf material consumed varied significantly between the plant species (Supporting information Fig. S2.5).

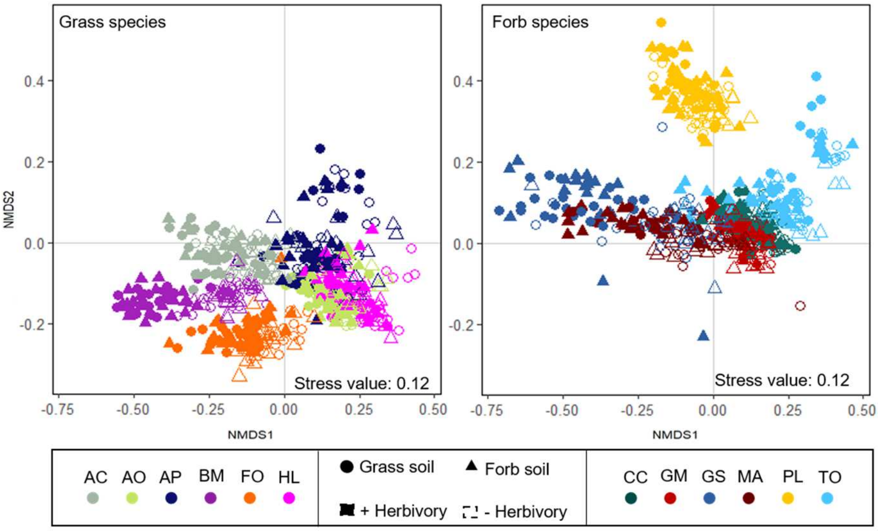

Figure 2.1 Metabolomic profiles of grasses and forbs grown in differently conditioned soils. Ordination using non-metric multidimensional scaling (NMDS) of metabolomes of the 12 different plant species (depicted in different colours) based on Bray-Curtis dissimilarities. The NMDS was conducted for all species growing in all soils but for clarity only the datapoints for grasses (left) and forbs (right) are shown, respectively. The plants were grown in soils conditioned by forbs (triangles) or grasses (circles) and subjected to herbivory (closed symbols, + herbivory, open symbols, - herbivory) by Mamestra brassicae caterpillars. The stress value is a measure of goodness of fit. Iterations were set at 31. The species used were *Agrostis capillaris* (AC), *Alopecurus pratensis* (AP), *Anthoxanthum oderatum* (AO), *Briza media* (BM), *Festuca ovina* (FO), *Holcus lanatus* (HL), *Crepis capillaris* (CC), *Geranium molle* (GM), *Gnaphalium sylvaticum* (GS), *Myosotis arvensis* (MA), *Plantago lanceolata* (PL), *Taraxacum officinale* (TO).

Species-specific metabolomes

We subsequently analyzed the effects of conditioning and herbivory for all plant species separately. Plant metabolic responses to soil legacies and herbivory largely differed (Fig. 2.2). For most response species, soil legacies and herbivory significantly influenced the metabolome. However, the effect of soil legacies on the aboveground metabolome was stronger than the effect of herbivory for seven of the twelve species (Fig. 2.3, Supporting information Table S 2.2). Moreover, the plant species that conditioned the soil explained a higher proportion of the variance in metabolic profiles than their functional group, i.e., whether they were a grass or a forb (Supporting information Table S2.1 and Table S2.2, Fig. S2.1). The conditioning plant species differed in the extent to which they influenced the metabolomes of the response species through the soil. Soil legacies of the grasses *Anthoxanthum odoratum* and *Briza media* and of the forb *Geranium molle* most strongly influenced the metabolomic profiles across the tested plant species, while soil of the grass *Agrostis capillaris* had the weakest effect (Supporting information Fig. S2.2). Only for the test plant *Holcus lanatus*, we observed a significant interaction between soil and herbivory, indicating that only for this species, the metabolic response to herbivory depended on the soil the plant was growing in (Supporting information Table S2.2). The variation in metabolomes was also partly explained by the biomass of the shoots of the response plant for all species, except for *Holcus lanatus* and *Gnaphalium sylvaticum* (Supporting information Table S2.3). The metabolome of *Agrostis capillaris, Alopecurus pratensis* and *Taraxacum officinale* varied significantly depending on whether the plants were grown in self-soil or non-self soil (Table S2.2).

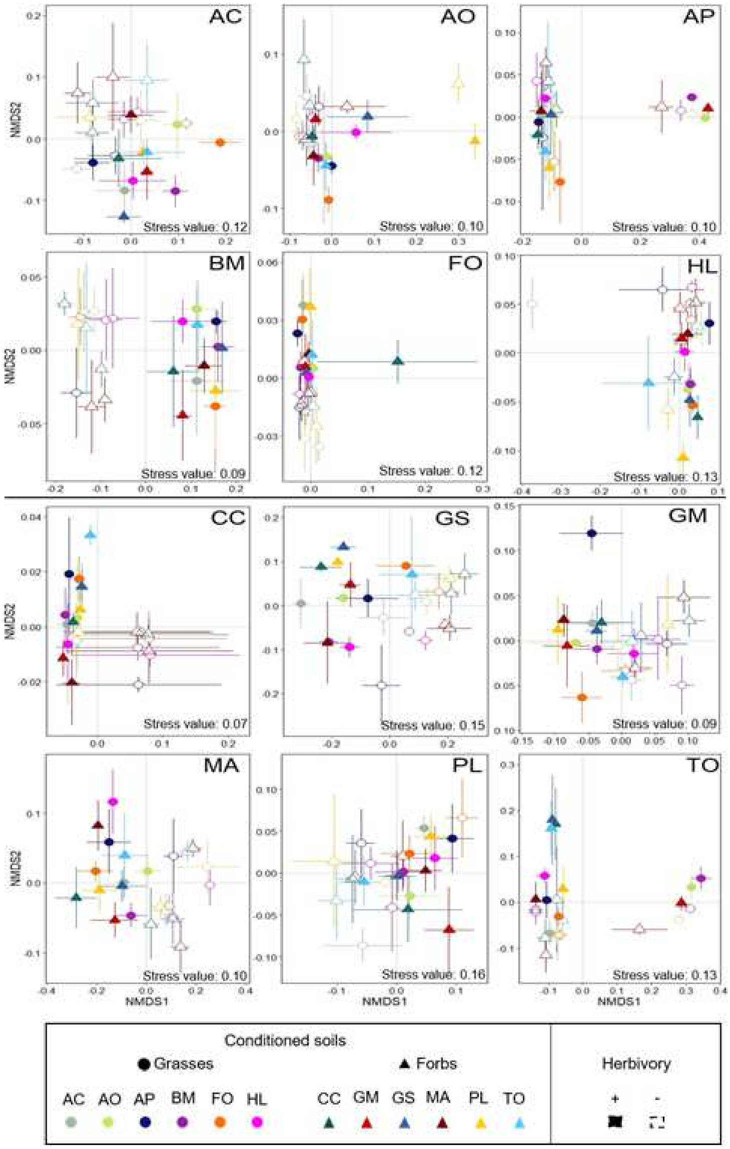

Figure 2.2 Responses of the metabolomes of the 12 test species to conditioned soil and herbivory. Ordination using non-metric multidimensional scaling (NMDS) of metabolomes of each response species (species abbreviations are in the right corner of each panel) based on Bray-Curtis dissimilarities. Depicted are the centroids ± SE. The top six panels are grass species and the lower panels are forbs. The plant species that conditioned the soil is depicted by colours and their functional group by symbols (triangles: forbs, circles: grasses). Open symbols depict plants that did not experience herbivory by Mamestra brassicae (H-) and filled circles ones that were subjected to herbivory (H+). The stress value is a measure of goodness of fit. The species used were *Agrostis capillaris* (AC), *Alopecurus pratensis* (AP), *Anthoxanthum oderatum* (AO), *Briza media* (BM), *Festuca ovina* (FO), *Holcus lanatus* (HL), *Crepis capillaris* (CC), *Geranium molle* (GM), *Gnaphalium sylvaticum* (GS), *Myosotis arvensis* (MA), *Plantago lanceolata* (PL), *Taraxacum officinale* (TO).

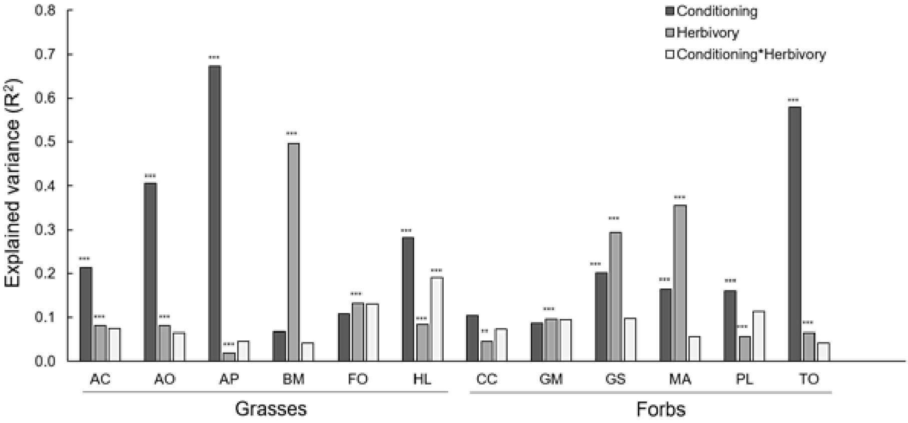

Figure 2.3 Variation in shoot metabolic profiles explained by soil conditioning and foliar herbivory**.** Variance explained by soil (conditioning; dark grey), herbivory by *Mamestra brassicae* (present or absent; light grey) and their interaction (white) for each of the 12 plant species grown on 12 different soils. Variance explained were obtained as R^2 from PERMANOVA models *, **, *** indicate significant effects in the PERMANOVA tests at $P < 0.05$; $P < 0.01$, $P < 0.001$, respectively. The species used were *Agrostis capillaris* (AC), *Alopecurus pratensis* (AP), *Anthoxanthum oderatum* (AO), *Briza media* (BM), *Festuca ovina* (FO), *Holcus lanatus* (HL), *Crepis capillaris* (CC), *Geranium molle* (GM), *Gnaphalium sylvaticum* (GS), *Myosotis arvensis* (MA), *Plantago lanceolata* (PL), *Taraxacum officinale* (TO).

NMR Signals

Detailed inspection of the NMR signals shows that soil conditioning and herbivory influenced a large number of the signals (buckets, see Supporting information methods) but that these responses were species-specific (Supporting information Table S2.5). NMR spectra are divided into "buckets" of a specific size (0.04 ppm). The intensity within one bucket is the mean of all signals that fall into that bucket.

Of the total of 241 buckets, 83 buckets were associated to a specific metabolite or metabolite group. Overall, soil legacies mainly affected sugars (sucrose, glucose), sugar alcohols (inositol, mannitol), amino acids (glutamine, glutamate), other acids (fumaric acid, 3-caffeoyl-quinic acid, malate) flavonoids and lipids (Supporting information Table S2.5). Herbivory mainly influenced the concentration of sugars and specialized compounds (flavonoids, chlorogenic acid, phenylpropanoids; Supporting information Fig. S2.3 and Table S2.5). The variability of the metabolome of the response plants due to the treatments differed between response plant species (Supporting information Fig. S2.3). Chemical diversity calculated as Shannon index varied across the response species, herbivory treatments and also differed intraspecifically between response species grown on soils conditioned by the different plant species (Supporting information Fig. S2.4, Table S2.6). Diversity of the metabolome was higher in all plants which did receive a herbivore treatment. We further investigated changes in particular specialized metabolites due to growth in the different soils and in the presence and absence of herbivory. Specialized metabolites changed most due to herbivory. In *Plantago lanceolata* aucubin was affected by both the soil treatment and by herbivory. Its concentration was significantly higher in plants that experienced herbivory. In *Holcus lanatus* concentrations of catechin were significantly higher upon herbivory treatment and the concentration of flavonoids was lower upon herbivory. In *Festuca ovina* chlorogenic acid and phenylpropanoids concentrations were significantly higher upon herbivory. More details can be found in Table S2.5.

Discussion

Our study shows that the composition of the metabolome of eight out of the twelve response species depended on the soil the plant was growing in. Hence, we provide strong evidence that plant-soil feedbacks alter shoot metabolomes. The effects were better explained by the species identity of the plant that conditioned the soil, than by the functional group of the plant that conditioned the soil. This indicates that the effect that a plant has on the metabolome of another plant via its effect on the soil is species specific. Although it is well known that foliar herbivory causes considerable changes in plant metabolomes (Marti et al., 2013; Widarto et al., 2006), remarkably, we observed here that for most response plant species, soil conditioning explained more of the variation in the plant metabolome than did aboveground herbivory. We note that the herbivory and soil treatments differed in duration and that we cannot exclude that this has influenced the outcome of our experiment. However, as the intensity of the treatments also differs similar durations would not resolve this issue and the comparison between the two treatments in our study should be done cautiously. These findings show that the foliar chemistry of plants greatly depends on the soil that the plant grows in. Our study adds a new perspective to the rapidly growing field of plant-soil feedbacks. We now show that these feedbacks do not only alter plant biomass, but also can alter entire plant metabolomes, with potential consequences for aboveground plant-insect interactions. Previously, effects of plant-soil feedbacks have been shown for specific plant compounds in studies that tested the effects of plant-soil feedbacks on specific plant species (e.g, Bezemer, Harvey, Kowalchuk, Korpershoek, & Van der Putten, 2006; Kos, Bukovinszky, Mulder, & Bezemer, 2015a; Kostenko, Van de Voorde, Mulder, Van der Putten, & Bezemer, 2012; Ma et al., 2017; Zhu et al., 2018). To our knowledge, our study is the first to test entire metabolic profiles for a larger number of plant species growing on a range of soil legacies. This enables us to make broader conclusions about plant-soil feedback effects on plant chemistry. Interestingly, our metabolomics approach now shows that the influence of soil conditioning on plant chemistry is not limited to specialised compounds, but instead, that these soil effects predominantly lead to changes in primary compounds such as sugars. This result highlights the importance of metabolomics approaches to study aboveground-belowground interactions and plant-soil feedbacks. With the method that we used it is possible to identify specialized compounds, however, since primary compounds are more common among all plant species the probability to assign these to a particular compound is higher.

We hypothesized that the metabolomic response of a plant to foliar herbivory would depend on the soil in which this plant species is grown. For example, plant growth promoting rhizobacteria and plant growth-promoting fungi that are stimulated during soil conditioning can induce or prime the defence system of a plant that grows later in the soil, a process called induced systemic resistance (ISR) (Van Loon, Bakker, & Pieterse, 1998). Several studies have shown that ISR can alter the response of a plant to foliar herbivory (Martinez-Medina et al., 2016; Pieterse et al., 2014; Pineda, Zheng, Van Loon, Pieterse, & Dicke, 2010). Therefore, in soils in which many plant growth promoting fungi and bacteria accumulate, plants can potentially respond differently to herbivory. Alternatively, soil conditioning may increase soil pathogen densities, and pathogens can also induce plant defence responses, especially related to salicylic acid (SA), which via crosstalk with the jasmonic acid (JA) pathway may influence the response of the plant to herbivory (Pineda et al., 2010). Plants grown in soils with a high load of soil pathogens, and therefore an activated SA pathway, could show an attenuated response of their JA pathway upon herbivory. However, in our study only for *Holcus lanatus* did herbivore-induced changes in the metabolic profile depend on the soil the plant was growing in. If generally true, this suggests that aboveground plant metabolomic responses to antagonists are not so sensitive to changes in the plant due to belowground plant-soil interactions

The concept of plant-soil feedback assumes that a plant changes the abiotic and/or biotic properties of the soil it grows in and that these changes then influence the growth of another plant that grows later in the soil. While we detected clear differences among the soils in how they influenced the metabolomes, we do not know the causal agents of these changes in the soil. Previous work in our laboratory using the same plant species growing in similar soil (but collected from the same field site several months earlier) showed that the composition of the bacterial and fungal communities depended on the species that conditioned the soil, and that there were clear differences between grass and forb conditioned soils (Heinen et al., 2018). Whether, the impact of the soils on plant metabolomes was due to e.g. pathogens, mycorrhiza or plant-growth promoting rhizobacteria remains to be tested. Other studies have shown for example, that mycorrhiza (Schweiger et al., 2014) and beneficial soil bacteria can alter plant metabolomes (Zhou, Huang, Guo, dos-Santos, & Vivanco, 2018). We mixed the conditioned soil with sterilized soil to diminish the potential differences in conditioned soils in e.g. soil nutrient availability. However, we cannot be sure that the effects of soil conditioning are only due to the differences in the microbial communities in the conditioned soils. For example, allelopathic compounds present in the conditioned soils or extracellular DNA could also influence on the performance of the plant that grows later in the soil (Mazzoleni et al. 2014, van der Voorde et al. 2012).

In our study, conditioned soil mainly influenced the concentration of primary plant compounds, such as organic acids and sugars. Similarly, Badri, Zolla, Bakker, Manter and Vivanco (2013) showed that amino acids, phenolics, sugars and sugar alcohols changed in the metabolome of *Arabidopsis thaliana,* if the soil the plant was growing in was inoculated with soil slurries from various sources. A common function of sugars is that they are used as building blocks for cell walls and therefore they play a vital role in cell wall biosynthesis (Loewus & Murthy, 2000). The composition and quantity of primary compounds in a plant may be directly related to changes in assimilation patterns in the plant but can also influence interactions between the plant and its environment. For example, variation in the primary compounds can be important in defence against oligophagous herbivores (Berenbaum, 1995). Furthermore, belowground infestations with pathogens, which may have accumulated in conditioned soils, can result in increased concentrations of defence compounds in the roots and this can result from reallocation of defence compounds from above- to belowground tissues, altering concentrations of primary and specialized compounds in the foliage (Bezemer

& Van Dam, 2005; Biere & Goverse, 2016). In our study the reduced concentration of sugars in plants grown on particular soils suggests that there were pathogens present in those soils, but this needs to be tested in further studies. From a plant-herbivore or plant-pathogen perspective, it is interesting that one of the sugar alcohol for which the signal varied among soils is inositol which can act as a feeding stimulant for insects (Thorsteinson & Nayar, 1963). Moreover, inositol as well as glucose are known to activate genes in the pathogen *Pseudomonas syringae* that are related to toxin production (Li, Starratt, & Cuppels, 1998).

Concentrations of species-specific specialized metabolites changed mainly in response to herbivory. The concentrations of particular specialized metabolites detected in a wider range of plant species such as chlorogenic acid and phenylpropanoids also changed in plants exposed to herbivory. This is probably due to the activation of the JA pathway triggered by herbivory.

In conclusion our results show that plant-induced changes in the soil change the metabolome of plants that grow later in the same soil. These soil legacy effects can be as large or even larger than the well-established effects of foliar herbivory on plant metabolomes. This is highly relevant for understanding how plant-soil feedbacks influence aboveground herbivores. Further, we show that the changes in plant metabolomes depend on both the species that conditioned the soil and the species that grows later in that soil. However, these effects are not explained well by whether the conditioning or response plants are grasses or forbs. Further studies should investigate if the soil-mediated effects on metabolomic changes remain strong when the soil is conditioned by multiple species, and when response plants are exposed to other stresses than foliar herbivory. This work lays the foundation for further work investigating the plant physiological responses to environmental challenges related to soil.

Acknowledgements

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Data accessibility statement

Data of this study are available from figshare 10.6084/m9.figshare.8834537

Author contributions

TMB and RH designed and planned the research. TMB and RH performed the experiment. MH prepared the samples for metabolomics analysis and MH and YHC carried out metabolomics analysis. MH, TMB and YHC analysed the data and MH and TMB led the writing of the manuscript. All authors contributed critically to the manuscript and read and approved the final manuscript.

Supporting Information

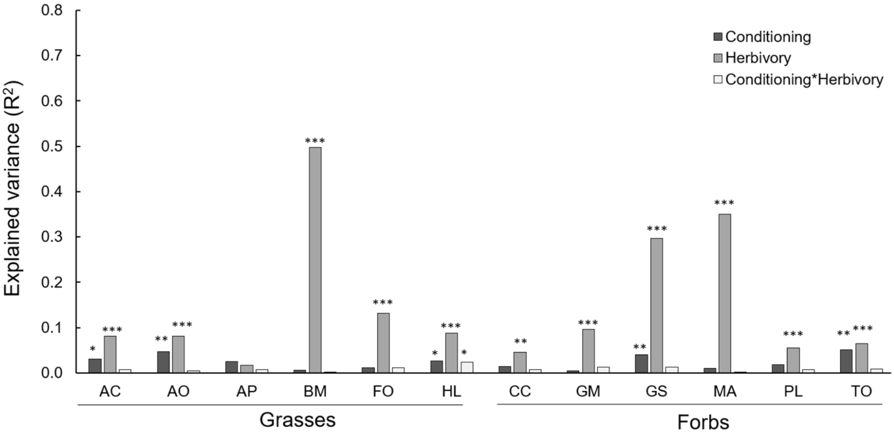

Figure S2.1 R^2 from PERMANOVA models for each plant species grown on soils conditioned by the functional groups (grass and forb) (conditioning) depicted in dark grey bars, and for herbivory of *Mamestra brassicae* (present or absent) in light grey bars and their interaction in white bars. Response species abbreviations are *Agrostis capillaris* (AC), *Alopecurus pratensis* (AP), *Anthoxanthum oderatum* (AO), *Briza media* (BM), *Festuca ovina* (FO), *Holcus lanatus* (HL*), Crepis capillaris* (CC), *Geranium molle* (GM), *Gnaphalium sylvaticum* (GS), *Myosotis arvensis* (MA), *Plantago lanceolata* (PL), *Taraxacum officinale* (TO). * indicates significance of the PERMANOVA tests at * $P < 0.05$; **$P < 0.01$,*** $P < 0.001$.

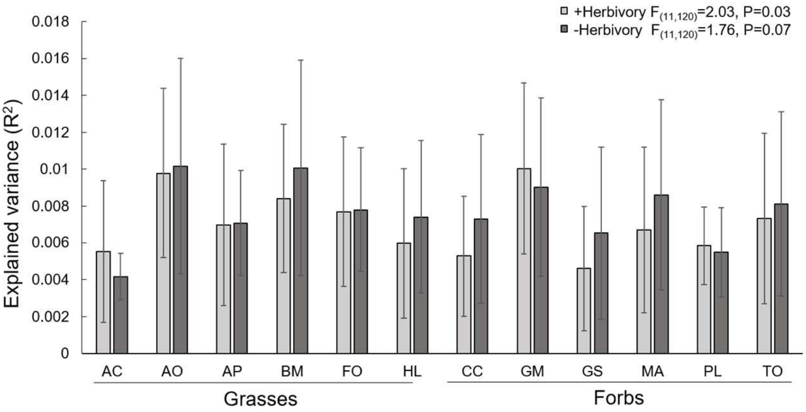

Figure S2.2 Mean R^2 ±SE of all pairwise comparisons for each response species of all pairwise comparisons involving a specific conditioning species with herbivory (light grey) and without herbivory (dark grey). Differences between the means were tested with an Analysis of variance (ANOVA) with species as fixed factor for both herbivore and non-herbivore treated groups separately. Response species abbreviations are *Agrostis capillaris* (AC), *Alopecurus pratensis* (AP*), Anthoxanthum oderatum* (AO), *Briza media* (BM), *Festuca ovina* (FO), *Holcus lanatus* (HL), *Crepis capillaris* (CC), *Geranium molle* (GM), *Gnaphalium sylvaticum* (GS), *Myosotis arvensis* (MA), *Plantago lanceolata* (PL), *Taraxacum officinale* (TO).

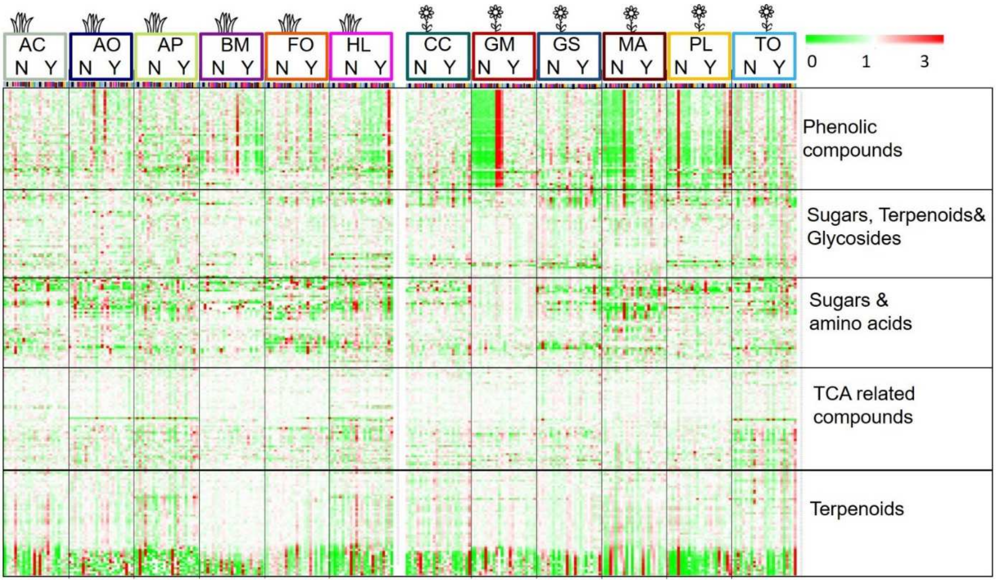

Figure S2.3 Heatmap showing changes in intensity of each bucket (rows) in relation to the bucket mean for a plant species when grown on different soils with or without herbivory (for further details see Supplementary Materials and Methods). Maximum values displayed were set to 3 but values ranged from 0-12. The white colour indicates no change relative to the mean for this bucket. Green indicates a decrease and red an increase relative to the mean for this bucket. Plants were exposed (Y) or not (N) to *Mamestra brassicae*. For each response species the columns depict the plant species that conditioned the soil for plants in presence and absence of herbivory. The species that conditioned the soil are depicted using the colour scheme of Figure 1. The tentative groups of plant compounds that are associated with the buckets in that region are also presented. Response species abbreviations are *Agrostis capillaris* (AC), *Alopecurus pratensis* (AP*), Anthoxanthum oderatum* (AO), *Briza media* (BM), *Festuca ovina* (FO), *Holcus lanatus* (HL), *Crepis capillaris* (CC), *Geranium molle* (GM), *Gnaphalium sylvaticum* (GS), *Myosotis arvensis* (MA), *Plantago lanceolata* (PL), *Taraxacum officinale* (TO).

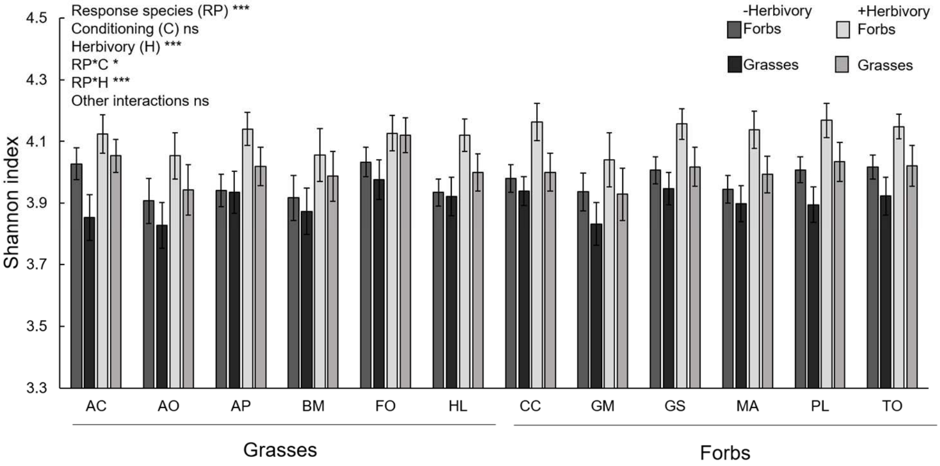

Figure S2.4 Mean Shannon index ±SE of all the metabolomes for each response species for soil conditioned by forbs and grasses with herbivory by *Mamestra brassicae* (light grey) and without herbivory (dark grey). Differences between the means were tested with an Analysis of variance (ANOVA) with response species, conditioning (forb, grass) and herbivory as fixed factors .Response species abbreviations are *Agrostis capillaris* (AC), *Alopecurus pratensis* (AP*), Anthoxanthum oderatum* (AO), *Briza media* (BM), *Festuca ovina* (FO), *Holcus lanatus* (HL), *Crepis capillaris* (CC), *Geranium molle* (GM), *Gnaphalium sylvaticum* (GS), *Myosotis arvensis* (MA), *Plantago lanceolata* (PL), *Taraxacum officinale* (TO). * indicates significance of the ANOVA tests at * P < 0.05; **P < 0.01,*** P < 0.001.

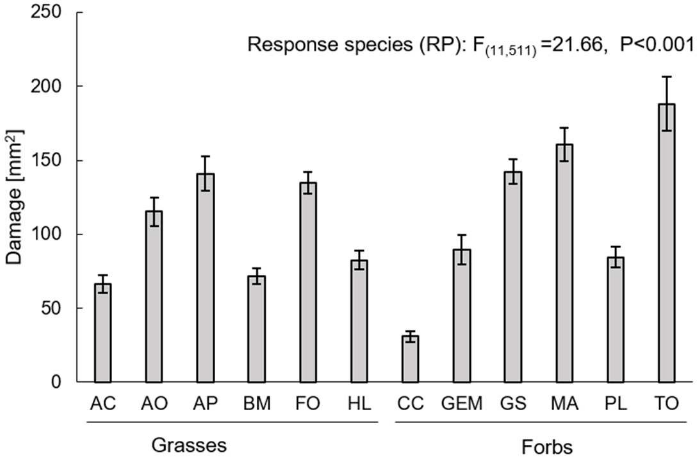

Figure S2.5 Mean ±SE area eaten by *Mamestra brassicae* feeding on different plant species. Differences between the means were tested with Analysis of variance (ANOVA) with response species as fixed factor. Response species abbreviations are *Agrostis capillaris* (AC), *Alopecurus pratensis* (AP*), Anthoxanthum oderatum* (AO), *Briza media* (BM), *Festuca ovina* (FO), *Holcus lanatus* (HL), *Crepis capillaris* (CC), *Geranium molle* (GM), *Gnaphalium sylvaticum* (GS), *Myosotis arvensis* (MA), *Plantago lanceolata* (PL), *Taraxacum officinale* (TO).

Table S2.1 Results of permutational multivariate analyses of variance (PERMANOVA) testing differences in the metabolomes of the twelve plant species grown in the twelve differently conditioned soils. Four models were used to test the effects of species or functional group identity of the response plant (RP), species or functional group identity of the plant that conditioned the soil (Conditioning (C)) and herbivory (H). Model 1 tested the effects of species identity of the response plants and species identity of the conditioning plant; Model 2 tested the effects of the functional group identity (grass/forb) of the response plants and the species identity of the conditioning plant; Model 3 tested the effects of species identity of the response plants and the functional group identity of the conditioning plant; Model 4 tested the effects of the functional group identity of both the response plants and the conditioning plant. Model 5 tested the effects of species identify of the response plant and conditioning by their own plant species (self) and all other soils (non-self) and herbivory. Model 6 tested the effect of the functional group of the response plant species and the conditioning (self/non-self) and herbivory. For Model 1 all replicates were used, for the other models means per species were used so that species were used as replicates for functional groups. Presented are F-values, degrees of freedom (df) and significance * $P < 0.05$; **$P < 0.01$, *** $P < 0.001$. Permutations were set to 999.

	Model 1	Model 2	Model 3	Model 4	Model 5	Model 6
Response plant (RP)	$F_{(11,856)}$ =506.18***	$F_{(1,240)}$ = 32.81***	$F_{(11,240)}$ = 169.01***	$F_{(1,280)}$ = 37.46***	$F_{(11,240)}$ =163.20***	$F_{(1,280)}$ =37.46***
Conditioning (C)	$F_{(11,856)}$ = 7.81***	$F_{(11,240)}$ = 0.34	$F_{(1,240)}$ = 1.91	$F_{(1,280)}$ = 0.28	$F_{(1,240)}$ = 2.05	$F_{(1,280)}$ = 0.31
Herbivory (H)	$F_{(1,856)}$ =161.05***	$F_{(1,240)}$ = 7.35***	$F_{(1,240)}$ = 57.17***	$F_{(1,280)}$ = 8.39***	$F_{(1,240)}$ =55.21***	$F_{(1,280)}$ =8.39***
RP*C	$F_{(121,856)}$= 4.53***	$F_{(11,240)}$ = 0.07	$F_{(11,240)}$= 1.52***	$F_{(11,280)}$ = 0.09	$F_{(11,240)}$= 0.90	$F_{(1,280)}$= 0.19
RP*H	$F_{(11,856)}$ = 12.88***	$F_{(1,240)}$ = 0.64	$F_{(11,240)}$= 4.29	$F_{(1,280)}$ = 0.73*	$F_{(11,240)}$ = 4.14***	$F_{(1,280)}$ = 0.73
C*H	$F_{(11,856)}$ = 1.12***	$F_{(11,240)}$ = 0.05	$F_{(1,240)}$ = 1.20**	$F_{(1,280)}$ = 0.18	$F_{(1,240)}$ = 0.63	$F_{(1,280)}$ = 0.10
RP*C*H	$F_{(121,856)}$= 1.20***	$F_{(11,240)}$ = 0.07	$F_{(11,240)}$= 1.52	$F_{(1,280)}$ = 0.07	$F_{(1,240)}$= 0.33	$F_{(1,280)}$= 0.02

Table S2.2 Results permutational multivariate analyses of variance (PERMANOVA) testing for differences in the metabolome of all plant species (response plants) separately. With the fixed factors species which conditioned the soil (Conditioning (C)), Herbivory (H)(by *Mamestra brassicae* absent or present) and their interaction on the left side of the table or the functional group which conditioned the soil, herbivory and their interaction in the middle. The right side of the table shows the effect of self/non-self conditioning of the soil, herbivory and their interaction. In the lowest part of the table results within functional groups are shown. Depicted are degrees of freedom (df), F and significances of the tested factors * $P < 0.05$; **$P < 0.01$,*** $P < 0.001$. Permutations were set to 999. Response species abbreviations are *Agrostis capillaris* (AC), *Alopecurus pratensis* (AP*), Anthoxanthum oderatum* (AO), *Briza media* (BM), *Festuca ovina* (FO), *Holcus lanatus* (HL), *Crepis capillaris* (CC), *Geranium molle* (GM), *Gnaphalium sylvaticum* (GS), *Myosotis arvensis* (MA), *Plantago lanceolata* (PL), *Taraxacum officinale* (TO)

	Conditioning Species			Conditioning functional group			Conditioning (self/non-self)$^{b)}$		
Response plant	Conditioning(C)	Herbivory(H)	C*H	Conditioning(C)	Herbivory(H)	C*H	Conditioning(C)	Herbivory(H)	C*H
AC	$F_{(11, 70)}$= 2.16***	$F_{(1, 70)}$= 19.02***	$F_{(11, 70)}$=0.75	$F_{(1, 90)}$=3.17*	$F_{(1, 90)}$= 8.23**	$F_{(1, 90)}$=0.75	$F_{(1, 90)}$=2.36*	$F_{(1, 90)}$= 8.29***	$F_{(1, 90)}$=0.61
AO	$F_{(11, 72)}$= 5.91***	$F_{(1, 72)}$= 12.93***	$F_{(11, 72)}$=0.95	$F_{(1, 92)}$=4.96**$^{a)}$	$F_{(1, 92)}$= 8.53**	$F_{(1, 92)}$=0.43	$F_{(1, 92)}$=1.18	$F_{(1, 92)}$= 8.21***	$F_{(1, 92)}$=0.55
AP	$F_{(11, 69)}$= 1.58***$^{a)}$	$F_{(1, 69)}$= 0.04**	$F_{(11, 69)}$=1.10	$F_{(1, 89)}$=2.4	$F_{(1, 89)}$= 1.58	$F_{(1, 89)}$=0.73	$F_{(1, 89)}$=2.87*	$F_{(1, 89)}$= 1.57	$F_{(1, 89)}$=0.29
BM	$F_{(11, 72)}$= 1.12	$F_{(1, 72)}$= 90.76***	$F_{(11, 72)}$=0.69	$F_{(1, 92)}$=1.01	$F_{(1, 92)}$=92.01**	$F_{(1, 92)}$=0.24	$F_{(1, 92)}$=1.71	$F_{(1, 92)}$=92.95***	$F_{(1, 92)}$=0.49
FO	$F_{(11, 72)}$= 1.13	$F_{(1, 72)}$= 15.09***	$F_{(11, 72)}$=1.36	$F_{(1, 92)}$=1.2	$F_{(1, 92)}$=14.34**	$F_{(1, 92)}$=1.19	$F_{(1, 92)}$=0.74	$F_{(1, 92)}$=14.21***	$F_{(1, 92)}$=0.76
HL	$F_{(11, 71)}$= 4.08***	$F_{(1, 71)}$=113.28***	$F_{(11, 71)}$=2.75***	$F_{(1, 91)}$=2.79*	$F_{(1, 91)}$= 9.31**	$F_{(1, 91)}$=2.56*	$F_{(1, 91)}$=1.65	$F_{(1, 91)}$= 8.92***	$F_{(1, 91)}$=1.14
CC	$F_{(11, 72)}$= 0.88	$F_{(1, 72)}$= 4.28**$^{a)}$	$F_{(11, 72)}$=0.61	$F_{(1, 92)}$=1.36	$F_{(1, 92)}$= 4.51*$^{a)}$	$F_{(1, 92)}$=0.67	$F_{(1, 92)}$=0.43	$F_{(1, 92)}$= 6.11**	$F_{(1, 92)}$=1.07
GM	$F_{(11, 71)}$= 0.78	$F_{(1, 71)}$= 9.39***	$F_{(11, 71)}$=0.85	$F_{(1, 91)}$=0.51	$F_{(1, 91)}$= 9.84**	$F_{(1, 91)}$=1.28	$F_{(1, 91)}$=1.82	$F_{(1, 91)}$= 9.84**	$F_{(1, 91)}$=1.28
GS	$F_{(11, 66)}$= 2.95***	$F_{(1, 66)}$= 47.61***	$F_{(11, 66)}$=1.44	$F_{(1, 89)}$=5.21**$^{a)}$	$F_{(1, 89)}$=39.17**	$F_{(1, 89)}$=1.70	$F_{(1, 89)}$=2.18	$F_{(1, 89)}$=37.80***	$F_{(1, 89)}$=0.72
MA	$F_{(11, 71)}$= 2.51***	$F_{(1, 71)}$= 59.41***$^{a)}$	$F_{(11, 71)}$=0.85	$F_{(1, 91)}$=1.45	$F_{(1, 91)}$=50.01**$^{a)}$	$F_{(1, 91)}$=0.89	$F_{(1, 91)}$=1.98	$F_{(1, 91)}$=50.82***	$F_{(1, 91)}$=1.68
PL	$F_{(11, 78)}$= 1.69**	$F_{(1, 78)}$= 6.57***	$F_{(11, 78)}$=1.2	$F_{(1, 98)}$=1.97	$F_{(1, 98)}$= 5.93**	$F_{(1, 92)}$=0.74	$F_{(1, 98)}$=1.34	$F_{(1, 98)}$= 5.84***	$F_{(1, 92)}$=1.32
TO	$F_{(11, 72)}$=12.09***$^{a)}$	$F_{(1, 72)}$= 14.86***	$F_{(11, 72)}$=0.87	$F_{(1, 92)}$=5.34**	$F_{(1, 92)}$= 6.80**	$F_{(1, 92)}$=0.87	$F_{(1, 92)}$=2.87*$^{a)}$	$F_{(1, 92)}$= 6.60***	$F_{(1, 92)}$=0.54
Grass	$F_{(11,546)}$= 0.90	$F_{(1,546)}$= 12.68***	$F_{(11,546)}$=0.25	$F_{(1,566)}$=0.42	$F_{(1,566)}$=12.85**	$F_{(1,566)}$=0.46	$F_{(1,566)}$=1.07	$F_{(1,566)}$=12.88***	$F_{(1,566)}$=0.20
Forb	$F_{(11,550)}$= 0.76	$F_{(1,550)}$= 16.44***	$F_{(11,550)}$=0.20	$F_{(1,570)}$=0.97	$F_{(1,570)}$=16.75**	$F_{(1,570)}$=0.34	$F_{(1,570)}$=0.67	$F_{(1,570)}$=16.75***	$F_{(1,570)}$=0.20

$^{a)}$ Dispersion between groups was not equal (significant in BETADISP) and therefore these differences can be caused by different dispersion of the groups or by biological differences. See Table S4.for details .$^{b)}$ The experimental design is unbalanced for this factor.

Table S2.3 Redundancy analysis (RDA) results for each response plant with fresh shoot biomass as a fixed factor and with fresh shoot biomass and conditioning species as fixed factor. Permutations were set to 999. For abbreviations of species see material and methods. Bold letters indicate significances below <0.05 of for permutation test on all axis. Response species abbreviations are *Agrostis capillaris* (AC), *Alopecurus pratensis* (AP*), Anthoxanthum oderatum* (AO), *Briza media* (BM), *Festuca ovina* (FO), *Holcus lanatus* (HL), *Crepis capillaris* (CC), *Geranium molle* (GM), *Gnaphalium sylvaticum* (GS), *Myosotis arvensis* (MA), *Plantago lanceolata* (PL), *Taraxacum officinale* (TO)

		Fixed factor: Biomass		Fixed Factor: Biomass and Conditioning species	
Response species	Herbivory	Explained variation (%)	Permutation test	Explained variation (%)	Permutation test
AC	N	5.08	**F= 2.4, P=0.033**	36.83	**F=1.7, P=0.005**
	Y	14.88	**F= 7.9, P<0.001**	40.14	**F=1.9, P=0.003**
AP	N	10.12	**F= 5.0, P<0.001**	43.08	**F=2.1, P<0.001**
	Y	4.23	**F= 2.0, P=0.047**	39.82	**F=1.9, P<0.001**
AO	N	9.25	**F= 4.7, P<0.001**	45.95	**F=2.5, P<0.001**
	Y	2.81	F= 1.3, P=0.173	38.27	**F=1.8, P<0.001**
BM	N	1.89	F= 0.9, P=0.526	24.79	F=1.0, P=0.601
	Y	6.89	**F= 3.4, P=0.004**	25.84	F=1.0, P=0.443
FO	N	7.09	**F= 3.5, P=0.001**	34.10	**F=1.5, P=0.004**
	Y	18.49	**F=10.4, P<0.001**	43.02	**F=2.2, P<0.001**
HL	N	2.46	F= 1.2, P=0.280	43.38	**F=2.2, P<0.001**
	Y	2.44	F= 1.1, P=0.300	45.95	**F=2.4, P<0.001**
CC	N	11.18	**F= 5.8, P<0.001**	39.25	**F=1.9, P<0.001**
	Y	16.65	**F= 9.2, P<0.001**	42.22	**F=2.1, P<0.001**
GM	N	7.65	**F= 3.8, P=0.003**	46.18	**F=2.5, P<0.001**
	Y	5.00	**F= 2.4, P=0.025**	36.04	**F=1.6, P=0.002**
GS	N	2.46	F= 1.1, P=0.307	36.25	**F=1.6, P=0.021**
	Y	3.87	F= 1.7, P=0.113	48.59	**F=2.4, P<0.001**
MA	N	6.76	**F= 3.3, P=0.016**	46.92	**F=2.6, P<0.001**
	Y	9.67	**F= 4.8, P<0.001**	46.07	**F=2.4, P<0.001**
PL	N	4.73	**F= 2.4, P=0.015**	36.14	**F=1.7, P<0.001**
	Y	1.86	F= 0.9, P=0.463	27.24	F=1.2, P=0.077
TO	N	14.57	**F= 7.8, P<0.001**	45.73	**F=2.5, P<0.001**
	Y	23.28	**F=14.0, P<0.001**	54.42	**F=3.5, P<0.001**

Table S2.4 Outcomes of permutation test (999 permutations) of the spread of the Bray Curtis dissimilarities tested with betadisper for conditioning functional groups, conditioning species self/non-self soil and herbivory. Shown are degrees of freedom (Df), F-values and P-values for all tested species. * p<0.05, **p<0.01 Response species abbreviations are *Agrostis capillaris* (AC), *Alopecurus pratensis* (AP*), Anthoxanthum oderatum* (AO), *Briza media* (BM), *Festuca ovina* (FO), *Holcus lanatus* (HL), *Crepis capillaris* (CC), *Geranium molle* (GM), *Gnaphalium sylvaticum* (GS), *Myosotis arvensis* (MA), *Plantago lanceolata* (PL), *Taraxacum officinale* (TO)

	Conditioning Functional group				Conditioning Species				Conditioning Self/non-self			Herbivory			
Response plant	Df	F	P		Df	F	P		Df	F	P	Df	F	P	
AC	1,92	0.79	0.345		11,82	0.62	0.799		1,92	2.87	0.09	1,92	0.04	0.819	
AO	1,94	8.75	0.004	**	11,84	0.38	0.977		1,94	1.63	0.18	1,94	0.45	0.502	
AP	1,91	2.78	0.105		11,81	2.07	0.038	*	1,91	3.87	0.05	1,91	0.16	0.701	
BM	1,94	0.21	0.622		11,84	0.61	0.833		1,94	0.01	0.92	1,94	3.57	0.077	
FO	1,94	0.28	0.640		11,84	0.81	0.660		1,94	0.46	0.47	1,94	0.20	0.669	
HL	1,93	0.29	0.621		11,83	1.59	0.106		1,93	1.30	0.26	1,93	0.23	0.618	
CC	1,94	0.15	0.698		11,84	0.37	0.973		1,94	0.61	0.48	1,94	5.56	0.022	*
GM	1,93	0.40	0.946		11,83	0.40	0.975		1,93	0.34	0.591	1,93	3.44	0.055	
GS	1,88	4.61	0.027	*	11,78	1.43	0.185		1,88	0.68	0.43	1,88	1.00	0.333	
MA	1,93	0.86	0.371		11,83	0.68	0.780		1,93	0.22	0.65	1,93	4.32	0.031	*
PL	1,100	0.61	0.432		11,90	0.97	0.492		1,100	0.46	0.49	1,100	0.11	0.743	
TO	1,94	0.46	0.501		11,84	2.57	0.012	*	1,94	3.43	0.06	1,94	1.44	0.243	

Table S2.5 Results from two-way Analysis of variance (ANOVA) testing the intensities of all buckets (N=246) and their Chemical shift (ppm) individually for the effects of the fixed factors: soil conditioning (C), Herbivory (H) (by *Mamestra brassicae* absent or present) and their interaction for species all response species analysed separately**.** Depicted are P-values after False discovery rate correction with * $P < 0.05$; **$P < 0.01$,*** $P < 0.001$. Signals of buckets that were significantly influenced by the treatment were identified by their splitting pattern and their chemical shift and compared to an internal database (see [1]for details). Some compounds were only identified in certain species which are indicated in parenthesis. Response species abbreviations are *Agrostis capillaris* (AC), *Alopecurus pratensis* (AP*),* *Anthoxanthum oderatum* (AO), *Briza media* (BM), *Festuca ovina* (FO), *Holcus lanatus* (HL), *Crepis capillaris* (CC), *Geranium molle* (GM), *Gnaphalium sylvaticum* (GS), *Myosotis arvensis* (MA), *Plantago lanceolata* (PL), *Taraxacum officinale* (TO).

See supplementary data file: Table.S5.xlsx in the online version of the published paper

Table S2.6 Results of analysis of variance (ANOVA) testing differences in Shannon diversity indexes for the twelve plant species grown in the twelve differently conditioned soils. Four models were used to test the effects of species or functional group identity of the response plant (RP), species or functional group identity of the plant that conditioned the soil (Conditioning (C)) and herbivory (H). Model 1 tested the effects of species identity of the response plants and species identity of the conditioning plant; Model 2 tested the effects of the functional group identity (grass/forb) of the response plants and the species identity of the conditioning plant; Model 3 tested the effects of species identity of the response plants and the functional group identity of the conditioning plant; Model 4 tested the effects of the functional group identity of both the response plants and the conditioning plant. For Model 1 all replicates were used, for the other models means per species were used so that species were used as replicates for functional groups. Presented are F-values, degrees of freedom (df) and significance * $P < 0.05$; **$P < 0.01$, *** $P < 0.001$.

	Model 1	Model 2	Model 3	Model 4
Response plant (RP)	$F_{(11,856)}=170.94$***	$F_{(1,240)}=8.43$**	$F_{(11,240)}=81.50$***	$F_{(1,280)}=9.52$**
Conditioning (C)	$F_{(11,856)}=4.94$***	$F_{(11,240)}=0.50$	$F_{(1,240)}=0.75$	$F_{(1,280)}=0.18$
Herbivory (H)	$F_{(1,856)}=145.33$***	$F_{(1,240)}=14.64$***	$F_{(1,240)}=68.64$***	$F_{(1,280)}=16.53$***
RP*C	$F_{(121,856)}=3.14$***	$F_{(11,240)}=0.08$	$F_{(11,240)}=2.00$*	$F_{(11,280)}=0.53$
RP*H	$F_{(11,856)}=7.77$***	$F_{(1,240)}=0.26$	$F_{(11,240)}=3.72$***	$F_{(1,280)}=0.30$
C*H	$F_{(11,856)}=0.72$	$F_{(11,240)}=0.07$	$F_{(1,240)}=0.01$	$F_{(1,280)}=0.01$
RP*C*H	$F_{(121,856)}=1.07$	$F_{(11,240)}=0.17$	$F_{(11,240)}=0.76$	$F_{(1,280)}=0.46$

Table S2.7 Number of replicates analysed in the experiment. Response species abbreviations are *Agrostis capillaris* (AC), *Alopecurus pratensis* (AP*), Anthoxanthum oderatum* (AO), *Briza media* (BM), *Festuca ovina* (FO), *Holcus lanatus* (HL), *Crepis capillaris* (CC), *Geranium molle* (GM), *Gnaphalium sylvaticum* (GS), *Myosotis arvensis* (MA), *Plantago lanceolata* (PL), *Taraxacum officinale* (TO).

	without herbivory													with herbivory												
	Soils conditioned by													Soils conditioned by												
Response plant	AC	AO	AP	BM	CC	FO	GM	GS	HL	MA	PL	TO	Total	AC	AO	AP	BM	CC	FO	GM	GS	HL	MA	PL	TO	Total
AC	3	4	4	4	4	4	4	4	4	4	4	4	47	4	4	4	4	4	4	4	3	4	4	4	4	47
AO	4	4	4	4	4	4	4	4	4	4	4	4	48	4	4	4	4	4	4	4	4	4	4	4	4	48
AP	4	4	4	4	4	3	4	3	4	4	4	4	46	4	4	4	3	4	4	4	4	4	4	4	4	47
BM	4	4	4	4	4	4	4	4	4	4	4	4	48	4	4	4	4	4	4	4	4	4	4	4	4	48
CC	4	4	4	4	4	4	4	4	4	4	4	4	48	4	4	4	4	4	4	4	4	4	4	4	4	48
FO	4	4	4	4	4	4	4	4	4	4	4	4	48	4	4	4	4	4	4	4	4	4	4	4	4	48
GM	4	4	4	4	4	4	4	4	4	4	4	4	48	4	4	4	3	4	4	4	4	4	4	4	4	47
GS	4	4	4	3	3	4	4	4	4	4	4	4	46	4	4	4	4	3	4	3	3	4	4	3	4	44
HL	4	4	4	3	4	4	5	4	4	4	4	4	48	4	4	4	4	4	4	3	4	4	4	4	4	47
MA	4	4	4	4	4	4	4	4	4	4	4	4	48	4	4	4	4	3	4	4	4	4	4	4	4	47
PL	4	4	4	5	4	4	4	4	5	4	4	4	50	5	4	4	4	4	4	4	4	5	5	4	5	52
TO	4	4	4	4	4	4	4	4	4	4	4	4	48	4	4	4	4	4	4	4	4	4	4	4	4	48

Methods S1 Additional data analysis

To visualize the metabolic variation of all species, a heatmap was created with WebMeV (Multiple Experiment Viewer v. 4.9.0. Saeed et al., 2003). For each response species, the mean of each bucket was calculated and then the relative intensity for each sample to this mean was calculated. Means of these relative intensities for each conditioned soil/herbivory combination were then expressed. Values close to 1 represent bucket intensities which are similar to the overall mean of this bucket for this response species. Values close to 0 represent buckets that have an intensity that is lower than the mean of this bucket for this response species, while values higher than 1 represent buckets with a higher intensity then the mean. To determine which signals from the NMR differed most between the soils and between the herbivory and non-herbivory treatment we conducted a two-way Analysis of variance (ANOVA) for every bucket with conditioned soil and herbivory as fixed factor for each species. The p-values were corrected for the multiple testing correction for false discovery rates (Benjamini & Hochberg 1995).To compare how strongly the differently conditioned soils influenced the metabolomes of the different species, for each response species we calculated all pairwise comparisons between metabolomes of plants growing in all soils based on the dissimilarity matrix and then calculated the mean R^2 of all pairwise comparisons involving a specific conditioned soil. This was done separately for the herbivory and no-herbivory datasets. Redundancy analysis (RDA) was used to test if variation in the metabolomes of the response plants could be significantly explained by the above ground fresh biomass of the response plants using plant shoot biomass as an explanatory variable. To compare the chemical diversity of the different plant species and between the different soils we calculated the Shannon index and used it as input for the analysis of variance (ANOVA) with in model 1 fixed factors "response plant identity", "conditioning plant identity" and "herbivory". For this model all data were used. We subsequently tested in separated ANOVAs the effect of “response functional group” and/or “conditioning functional group”. For these models the mean Shannon index per group was used. In this way plant species were used as replicates.

Chapter 3

Soil inoculation alters leaf metabolic profiles in genetically identical plants

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Abstract:

Abiotic and biotic properties of soil can influence growth and chemical composition of plants. Although it is well-known that soil microbial composition can vary greatly spatially, how this variation affects plant chemical composition is poorly understood.

We grew genetically identical *Jacobaea vulgaris* in sterilized soil inoculated with live soil collected from four natural grasslands and in 100% sterilized soil. Within each grassland we sampled eight plots, totalling 32 different inocula. Two samples per plot were collected, leading to three levels of spatial variation: within plot, between and within grasslands. The leaf metabolome was analysed with ^{1}H Nuclear magnetic resonance spectroscopy (NMR) to investigate if inoculation altered the metabolome of plants and how this varied between and within grasslands.

Inoculation led to changes in metabolomics profiles of *J. vulgaris* in two out of four sites. Plants grown in sterilized and inoculated soils differed in concentrations of malic acid, tyrosine, trehalose and two pyrrolizidine alkaloids (PA). Metabolomes of plants grown in inoculated soils from different sites varied in glucose, malic acid, trehalose, tyrosine and in one PA. The metabolome of plants grown in soils with inocula from the same site was more similar than with inocula from distant sites.

We show that soil influences leaf metabolomes. Performance of aboveground insects often depends on chemical composition of plants. Hence our results imply that soil microbial communities, via affecting aboveground plant metabolomes, can impact aboveground plant-insect food chains but that it is difficult to make general predictions due to spatial variation in soil microbiomes.

Key Words: Ecometabolomics, aboveground-belowground interactions, plant-soil interaction, Nuclear magnetic resonance spectroscopy, *Jacobaea vulgaris*.

Introduction

Plants are sessile and this limits their capacity to escape unfavourable conditions in their surroundings. As plants cannot escape from exposure to organisms such as pathogens, herbivores and symbionts aboveground as well as belowground, they produce a vast array of chemical compounds to protect themselves. However, such compounds can also be used by these organisms as e.g. feeding stimulants or for host recognition (Macel 2011). Therefore, chemical variation among plants is a key factor in understanding interactions between plants and their environment (Dyer et al. 2018). The potential threats to plants vary spatially, both between and within different sites. This raises the question if plants can adjust their chemical composition according to the pests and pathogens they are confronted with at a local scale. For insect herbivores there is ample evidence that this is indeed the case (Kleine and Müller 2011). The soil microbial community also varies greatly between sites and even spatially within a single location. It is well-known that variation in soil such as changes in the microbial community affects the growth of plants and the composition of plant communities (Van der Putten et al. 2016; Wang et al. 2019a,b), how such changes in the soil, that we expect to be the result of soil inoculation, affect the chemical composition of plants is less well understood (Bezemer et al. 2005).

Several studies have shown that the foliar chemistry of plants may vary depending on the soil in which they grow (e.g. Kos et al. 2015a; Ristok et al. 2019; Zhu et al. 2018). This can be attributed to differences in abiotic properties of soils, such as nutrient or water availability, but also to differences in soil biota, for example, the presence of arbuscular mycorrhizal fungi (AMF) or beneficial rhizosphere bacteria in the soil (Schweiger et al. 2014; Zhou et al. 2018). Hill et al. (2018) recently showed that 33 compounds in the root metabolome of *Jacobaea vulgaris* changed in plants after exposure to the arbuscular mycorrhizal fungus (AMF) *Rhizophagus irregularis*, even though no compounds changed in the leaf metabolome. These studies examined the effect of one isolated group of soil organisms on the chemical composition of plants. Other studies focused on the total microbial community: PAs and amino acid composition and concentration in *J. vulgaris*, for example, depend on the microbial community of the soil in which the plant grows (Kos et al. 2015c; Kostenko et al. 2012; Wang et al. 2019b). Recently, Ristok et al. (2019) reported that changes in plant species richness lead to soil biotic legacies that subsequently elicit changes in the metabolomes of later growing plants. In most of these studies, test plants were grown in sterilized bulk soil that was inoculated with a small portion of live soil collected from potted plants or monocultures, or with watery extracts of live soil from potted plants. In this way all plants are grown in soil with comparable abiotic conditions but with different soil microbiomes (Wang et al. 2019b).

Soil biota can influence the plant metabolome either directly by triggering a response in the plant, such as induced systemic resistance (Van de Mortel et al. 2012), or indirectly by influencing the growth of the plant, since the composition of many plant compounds is related to the growth of the plant and associated characteristics such as the shoot/ root ratio. For example, *J. vulgaris* plants with lower biomass often have higher concentrations of pyrrolizidine alkaloids (PAs) than plants with higher biomass, because the concentration of these toxic PAs is diluted in larger plants (Hol 2011).

Microbiomes in the soil are altered by both biotic and abiotic factors of the soil and are highly dynamic (O'Brien et al. 2016). Hence, even closely located sampling points can harbour soil microbiomes that differ greatly as the abiotic and biotic properties of the soil might differ even at a scale of millimeters or centimeters (Ettema and Wardle 2002; Fierer 2017). To what extent those potential spatial differences in microbiome composition in the soil influence the chemistry of plants growing in those soils is poorly understood. In this study we used *J. vulgaris*, a monocarpic perennial herb, native to Europe and Asia and invasive in North America, Australia and New Zealand (Bain 1991). *J. vulgaris* can grow in a broad range of soils and in a range of diverse habitats, such as sand dunes, woodlands and grasslands (Bezemer et al. 2006). Pyrrolizidine alkaloids are one of the major groups of secondary metabolites in *Jacobaea* species and are known to influence interactions of the plants with insects (Macel 2011). Several studies in which *J. vulgaris* was grown in sterilized soil inoculated with soil collected from different locations within a single grassland show that plant biomass varies depending on the soil sample that was used as inoculum (Kos et al. 2013; Bezemer et al. 2005). As the concentration of PAs in this species is linked to biomass (Hol 2011) this suggests that this group of compounds may also vary among those spatially collected soil inocula.

Up to now, most studies that examined the effects of soil inoculation on plant chemical compounds used targeted approaches (e.g. Zhu et al. 2018; Kos et al. 2015c). The metabolome of a plant, however, is highly diverse, and changes in one or a few specific compounds or groups of compounds are unlikely to represent a realistic picture of the metabolic changes that occur within the plant. Therefore, untargeted metabolomic approaches are preferred to investigate the chemical response of plants to soil inoculation. In this study, we inoculated sterilised soil with soil collected from four natural grasslands in The Netherlands. Within each grassland we collected soils from different locations at fixed distances and collected two samples within each plot so that there were three different spatial scales in our experimental design (plot, within sites and between sites). These soils presumably differed in microbiome composition, but we did not measure that in this present study. We grew *J. vulgaris* in sterilized bulk soil inoculated with the different soils and used genetically identical *J. vulgaris* plants. We measured the leaf metabolome using ^{1}H Nuclear magnetic resonance spectroscopy (^{1}H-NMR), which enabled us to detect a large range of chemical compounds, including both primary and secondary metabolites as well as polar and non-polar metabolites.

We hypothesize that (i) the metabolic composition of *J. vulgaris* will vary between inoculated and uninoculated soils; that (ii) metabolomes will vary among the sites the soil was collected from; and (iii) that metabolomes of plants growing in inoculated soils collected from the same grassland will be more similar than when the inocula originate from different grasslands.

Methods and materials

Inoculation soil was collected in early March 2017 from four different natural grasslands in the Netherlands at sandy soils. Two sites A (N52 °09.259’ E4 °22.847’) and B (N52 °09.770’ E4 °23.520’) were natural grasslands near the Dutch coast, and two sites C (N52 °01.613’ E5 °48.379’) and D (N52 °00.694’ E5 °46.877’) were natural grasslands at the Veluwe area in the mainland of the Netherlands. All sites were nature areas on sandy soils that were formerly used for agricultural purposes. At each site, soil samples were collected along two transects that were laid out in a 100 by 200 m area in which no visible gradient in vegetation was observed. Each transect consisted of four plots (30 x 30 cm) at 0 m, 20 m 60 m and 100 m distance. The distance between the two transects was 200 m. Two soil cores were taken in each plot at 15 cm depth with a soil auger (⌀ 7 cm). Each sample was kept separate so that there were 16 samples per grassland. The samples were sieved individually through a sterilized sieve (1 cm) and stored at 4 °C.

Bulk Soil

For sterilized bulk soil we collected 300 kg soil from a natural grassland at the Veluwe "De Mossel" (Ede, The Netherlands). This soil was sieved through a 1 cm sieve, homogenized and sterilised by γ-irradiation (> 25 KGray, Synergy Health, Ede, The Netherlands). The soil is a sandy loam soil (85% sand, 10% silt, 3% clay, 3% organic matter, pH 4.5, N total 1332 mg/kg; P plant available 4 mg/kg, K plant available 41 mg/kg, Mg plant available 55 mg/kg; S Total 208 mg/kg).

To preclude variation in the metabolome due to genetic differences we used tissue cultured plants in this study. In a climate room 200 *J. vulgaris* cuttings from a single genotype were asexually propagated in tissue culture using MS medium (Murashige and Skoog medium) with 100 mg/L benzylaminopurine (BAP) (16:8 h light:dark photoperiod, 20° C). To produce roots the cuttings were grown in MS medium without BAP for 10 days. The genotype that was propagated was formerly collected from Meijendel (Wassenaar), The Netherlands.

Experimental Phase

For each pot 45 g of (live) soil was mixed with 405 g sterilized bulk soil (1:9 ratio). Each mixture was prepared individually and homogenized in a new plastic bag. There were 64 (4 sites × 8 plots × 2 samples) mixtures of different soils. In addition, five pots were filled with 450 g sterilized soil and used as control giving a total of 69 pots. The pots were randomly placed in the climate room (16h: 8 h light: dark photoperiod, 20 °C) and covered with plastic foil for 5 days to maintain humidity and allow the microbial community to establish before proceeding with planting.

The size of the 200 *J. vulgaris* plantlets was visually inspected and 69 similar sized (longest leaf ± 4 cm) plantlets were selected and one plantlet was transplanted into each pot. Seedlings that emerged from the soil were removed every 2 days. The pots were placed in blocks in the climate room and the position of pots within each block was randomised once a week. To control the moisture of the soils and to account for potential differences in water usage depending on the inocula, the pots were individually reset to the same humidity (pot weight) twice per week. During other days, all plants received the same amount of water. Six weeks after planting shoots were clipped to determine biomass and used for metabolomics analyses. The leaves were immediately wrapped in aluminium foil and flash frozen in liquid nitrogen and stored at -80 °C until lyophilisation. Shoots were lyophilised for 72 hours. To investigate if the nutrient content of the soils differed between the inocula, the soil from each pot was dried at 40 °C for a subset of 37 samples in the oven for soil chemical analysis (see Soil Chemical Analysis for details). The roots were carefully washed, dried and weight. After the lyophilisation shoot dryweight of each plant was determined. All lyophilised plant material was stored at room temperature in plastic bags with silica gel.

Soil Chemical Analysis

Soil chemical analysis were conducted on a subset of the samples collected after the plants had grown in the soil. For the analysis we randomly selected soils from 4 of the 8 plots per grassland. Both replicates of each plot (4 grassland sites x 4 random plots x 2 replicates) were analysed as well as soil from five control pots. Oven-dried soil samples (40 °C) were sieved through a 2 mm sieve and 3 g of dry soil was added to 30 mL of 0.01 M $CaCl_2$ and shaken for 2 h at 250 rpm. Soil samples were centrifuged for 5 min at 3000 rpm and 15 mL of the supernatant was filtered through a syringe filter (cellulose acetate membrane). 12.86 mL of this filtrate was vortexed, and Fe, K, Mg, P, S and Zn were measured the following day (ICP-OES, Thermo Scientific iCAP 6500 Duo). The remaining filtrate was used to measure $NO_2 + NO_3$ and NH_4 on a QuAAtro Autoanalyzer (Seal analytical).

Metabolomics ^1H NMR Analysis

The extraction of the leaf samples was done following an adapted version of the protocol described by Kim et al. (2010). The lyophilised plant material was ground in a micro tube (1.5 ml) with one metal ball bearing and placed in a TissueLyser (Retsch Mixer Mill MM 400) for 3 min at 30 s^{-1}. Then, 20 mg±1 mg was transferred to a 1.5 ml microtube and 300 µl CH_3OH-d_4 (Sigma, St Luis, MI, USA) followed by 300 µl KH_2PO_4- D_2O buffer with 0.01 % TSP was added to the weighed plant material. The samples were then sonicated for 10 minutes and were centrifuged at 13.000 ppm for 10 minutes. 250 µl of the supernatant was collected and transferred to an NMR tube (103.5 × 3 mm, inside-ø 2.24 ± 0.05 mm).

^1H NMR spectra were recorded on a Bruker AV-600 MHz NMR spectrometer (Bruker, Karlsruhe, Germany), operating at a frequency of 600.13 MHz. As an internal lock we used CH_3OH-d_4. ^1H NMR spectra were recorded with pulse width (PW) = 30 ° (11.3 µs), Relaxation delay (RD) = 1.5 sec and 128 scans with a total of 10 min and 26 sec acquisition time with

0.16 Hz/point. A presaturation sequence was used to reduce the signal of H_2O frequency during the recycle delay. FIDs were Fourier transformed by a line broadening of 0.3 Hz. Spectra were then manually baseline corrected, calibrated to TSP at 0.00 ppm, and phased in TOPSIN (v.3.0. Bruker). Then the data was bucketed with scaling to total intensity and a bucket width of 0.04 ppm in AMIX software (v. 3.9.12 Bruker BioSpin GmbH, Reinstetten, Germany). Bucketing or binning is commonly used in metabolomics to reduce the effect of small shifts of signals between samples (Kim et al. 2010). Residual signals from solvents in regions between 4.70 – 4.90 ppm and 3.28 – 3.34 ppm were excluded. The pre-processing therefore leads to a data matrix with 246 buckets per sample. Each bucket contains the signals from the NMR within the range of 0.04 ppm and directly represents the molar level of a compound leading to a signal in this region of the NMR. In ^{1}H-NMR all H atoms within one molecule lead to signals. Therefore, molecules consisting of more than one H atom lead to signals in several buckets. The chemical shift of the signal depends on the chemical environment of the H atom and is defined by the neighbouring atoms of the H atom. Furthermore, the neighbouring atoms influence the splitting pattern of a signal in the NMR. Here we used the chemical shift and the splitting pattern to identify the compounds in the NMR and compared them to an internal database (for details see Kim et al. 2010). The compounds were putatively identified. PAs could not be specified in depth with NMR and are therefore only referred to as PAs.

Data Analysis

If not mentioned otherwise all analyses were performed in R Studio (RStudio Team, 2016) using the package 'vegan' (Oksanen et al. 2018) and the function and the function 'pairwise.Adonis'(Martinez 2017). Volcano plots were made using Metaboanalyst (Chong et al. 2018). Co-correspondence analysis (CoCA) was done in in CANOCO 5 (Šmilauer and Lepš 2014).

We visualised the foliar metabolome changes (intensity in buckets) due to inoculation by non-metric multi-dimensional scaling (NMDS) based on Bray-Curtis dissimilarities. NMDS is a method that uses a dissimilarity matrix to produce an ordination which represents the dissimilarities between objects in a low-dimensional space. We used Bray-Curtis dissimilarities as this method uses intensities of the measured signals rather than presence/absence data.

To examine if the different inocula varied in how they changed the metabolome of *J. vulgaris* we conducted a permutational analysis of variance (PERMANOVA) based on Bray Curtis dissimilarities. Permutations were set to 999. For this analysis the data of the plants grown in 100% sterilized soil was removed. We conducted a PERMANOVA with the fixed factor "site" and the covariate "shoot dry biomass". With a second PERMANOVA we analysed if changes in the metabolome are linked to root biomass by including the factors "site" and the covariate "root dry biomass". To investigate the biomass effect on the metabolome, we conducted a Pearson correlation analysis of the intensity of signals in each bucket with the shoot biomass of the plants. P-values were then corrected for multiple testing by false discovery rate (FDR) (Benjamini and Hochberg 1995).

We used variance partitioning (Multivariate redundancy analysis RDA) using the function "varpart" to disentangle the effects of site and shoot biomass on the metabolome of *J. vulgaris*. For this the data of the plants grown on 100% sterile soil was removed from the dataset. We tested the significance of the marginal and conditional effects of both predictors with a Monte Carlo permutation test (999 permutations).

To investigate if inoculation influenced the chemical diversity of the plants growing in the soils, we calculated the Shannon evenness of the plant metabolomes. A one-way analysis of variance (ANOVA) with a Dunnett post hoc test was conducted to compare the Shannon evenness of plants grown in pots inoculated with soil from different sites (four levels) and plants grown in sterilized soil (1 level).

To analyse site-specific effects on the Shannon evenness an ANOVA with fixed factor "site" and biomass of the shoot as a covariate was conducted. For this analysis the data of the plants grown in 100% sterilised soil was excluded.

To visualise the metabolomic differences of plants grown in sterilised soil and inoculated soil we used volcano plots. For the volcano plots the $\log_2$ fold-change between plants grown in sterilized soil and inoculated soil was calculated per site. For each bucket an ANOVA was used to compare plants grown in sterilized and inoculated soil and the P-values were $\log_{10}$-transformed. Then the $\log_2$ fold-change was plotted against the log P-values. This enabled us to visualise which signals in the NMR differed most significantly (fold-change) between plants grown in inoculated and sterilized soil.

To examine the effect of different spatial scales we used the Bray-Curtis dissimilarity for all pairs of two plants growing in soil from the same plot at the same site (plot scale, eight per site); one random pair of the same plot with a plant growing in soil from different plots at the same site (site scale, 16 per site) and random pairs of each plant with plants growing in plots from different sites (large scale, 16 per site). The Bray Curtis dissimilarities were then analysed with ANOVA with as fixed factors scale (plot scale, site scale, large scale) and site (A, B, C, D).

Shoot biomass and root biomass of plants grown in pots inoculated with soil from the four different sites and plants grown in sterilized soil were analysed using one-way ANOVA followed by a Dunnett post hoc test. To compare the site-specific effects of the different soil inocula on the biomass of *J .vulgaris* plants, plants grown in 100% sterilized soil were removed from the dataset and with a new ANOVA the effect of the soil from different sites as well as the plots within each site was compared (plot nested within site). With a Tuckey post-hoc test treatments were compared to each other. All data was checked for homogeneity of variance and normal distribution of the residuals. Shoot biomass was square root-transformed to obtain normality of the residuals.

Soil parameters were analysed using ANOVA with site as fixed factor. For the soil parameters the plot effects could not be accessed because we only measured soil parameters for a subset of samples from each site.

The relationship between soil characteristics and leaf metabolome composition, was analysed using a co-correspondence analysis (CoCA) in CANOCO 5 (Šmilauer and Lepš 2014) whereby the soil abiotic parameters where centred and standardized. A Monte Carlo permutation test with unrestricted permutations for all axes was done as described in Šmilauer and Lepš (2014).

A metabolic pathway of *S. vulgaris* was constructed with the help of KEGG reference pathways (Kanehisa and Goto 2000) and mean values for the intensity of the signals of buckets associated to the compounds displayed in the pathway were calculated and displayed in the metabolic pathway map.

Results

The composition of the leaf metabolomes of *J. vulgaris* varied significantly among the four sites from which the inocula originated (Fig. 3.1, Table 3.1) and was significantly related to shoot biomass (Table 3.1, Supporting information Fig. 3.1). In a PERMANOVA, site explained 13% of the variation in the leaf metabolome and shoot biomass 18% (Table 3.1). In a pairwise comparison the metabolome of plants grown in inoculum A was different from metabolomes of plants grown in the other soils (Supporting information Table 3.1, Table 3.2). Variance partitioning showed comparable results with 5% of the metabolome variation solely being explained by the different sites and 22% by shoot biomass (Table 3.2). All marginal and conditional effects of the predictors were significant. A PERMANOVA which included root biomass instead of shoot biomass showed that root biomass did not significantly explain variation in the metabolome (Supporting information Table 3.3)

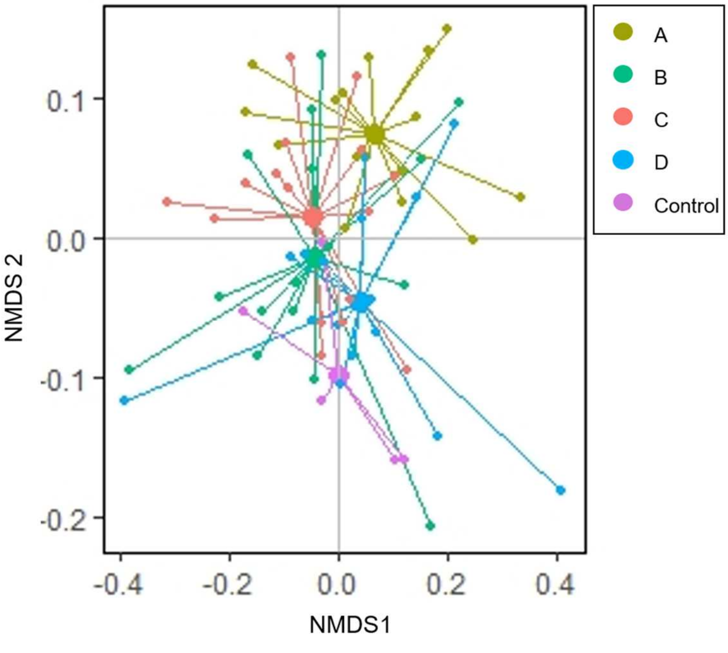

Figure 3.1 Non-metric multidimensional scaling (NMDS) plot of the metabolome of *Jacobaea vulgaris* grown in inoculated soil from different sites (A, B, C, D) and in 100% sterilized soil using Bray-Curtis dissimilarities. Shown are centroids (large circles) and individual samples (small circles) for each site and for the control. The stress is a measurement for the fit of the model and was 0.10

^{1}H signals in the metabolomes that differed between sites from which the soil inocula was collected from, were glucose, malic acid, trehalose, tyrosine, unknown/unidentified PAs (PA A) and two other unknown compounds (Supporting information Fig. 3.2a). The intensity of multiple signals in the NMR depended on shoot biomass (Supporting information Fig. 3.2c, Fig. 3.3). Changes in the intensities of amino acids, sugars (mannitol, glucose, raffinose and other signals related to sugar compounds which could not be determined more precisely) were related to biomass. 80 out of the 96 significant correlations between signal intensity and biomass were negative, strongly showing the dilution effect. This effect was especially strong for amino acids, phenolic compounds and terpenoids (all negative) while the opposite was found for the sugars (with significant positive correlations) (Supporting information Fig. 3.3).

We subsequently compared metabolomes of plants grown in inoculated soil with plants in sterilized soil for each site separately. The concentration of PA A was lower in plants grown in inoculated soils than in 100% sterilized soil for sites A, C and D (Supporting information Fig. 3.4). The concentrations of trehalose, tyrosine and inositol were significantly higher, and the concentration of PA A was lower in plants grown in inoculated soil than in 100% sterilized soil for site A. Malic acid was lower in plants grown in inoculated soil from site A than in plants grown in 100% sterilized soil. Overall, inoculation with soil from site A led to most changes in the metabolome (Fig. 3.2, Supporting information Fig. 3.4, Table 3.2).

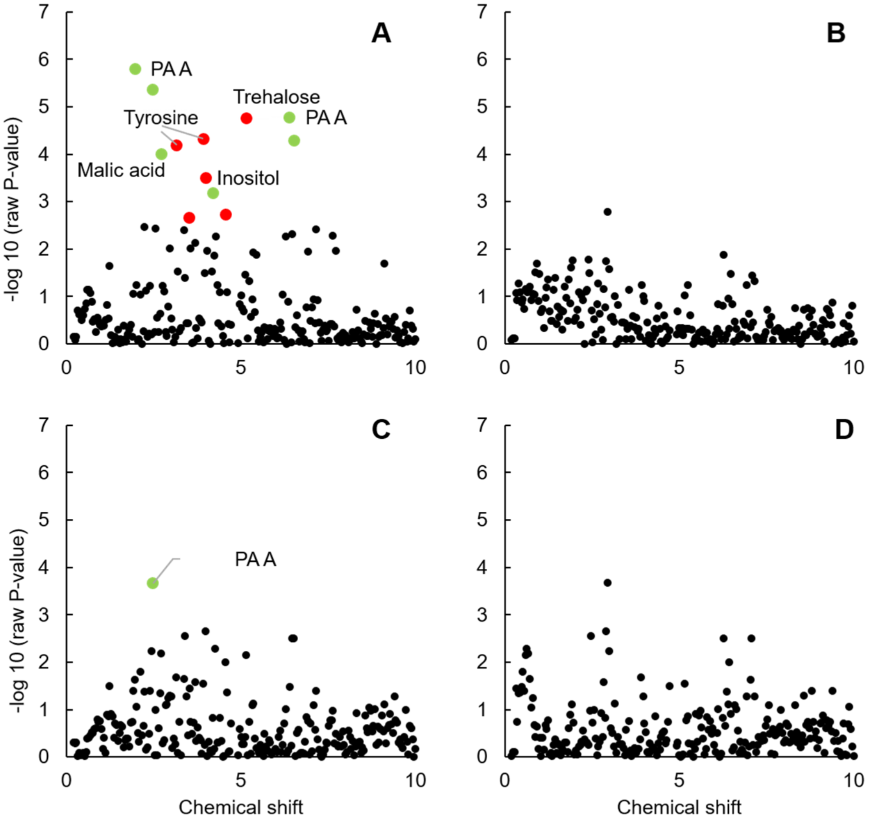

Figure 3.2 Negative logarithm of P-values of a t-test testing for differences within each bucket (each chemical shift) in the intensity of the signals representing the metabolome of *Jacobaea vulgaris* grown in 100% sterilized soil and in soil inoculated with soil collected from site A, B, C and D. Red coloured dots represent buckets in which the signal showed a higher intensity and green dots buckets with a lower intensity in plants grown in inoculated soil than in 100% sterilized soil. For signals that showed a P-value <0.05 after false discovery correction (FDR) identifications are indicated. Signals were tentatively associated to compounds: pyrrolizidine alkaloid A (PA A) 1.96, and 6.4 ppm, trehalose 5.16 ppm, tyrosine 3.16 and 3.92 ppm, malic acid 2.72 ppm, inositol 4.00 ppm. Coloured dots without a description are from signals which could not be assigned to a specific compound.

Inoculation led to changes in the metabolome of *J. vulgaris* in various parts of the metabolic pathway (Fig. 3.3). Concentrations of certain amino acids (ARG, GLU, THR, ALA, LEU) were reduced in plants grown in sterilized soil while the concentrations of the amino acids TYR and HIS were higher in plants grown in sterilized soil. Compounds related to the sugar metabolism and tricarboxylic acid cycle (TCA) were also influenced by inoculation.

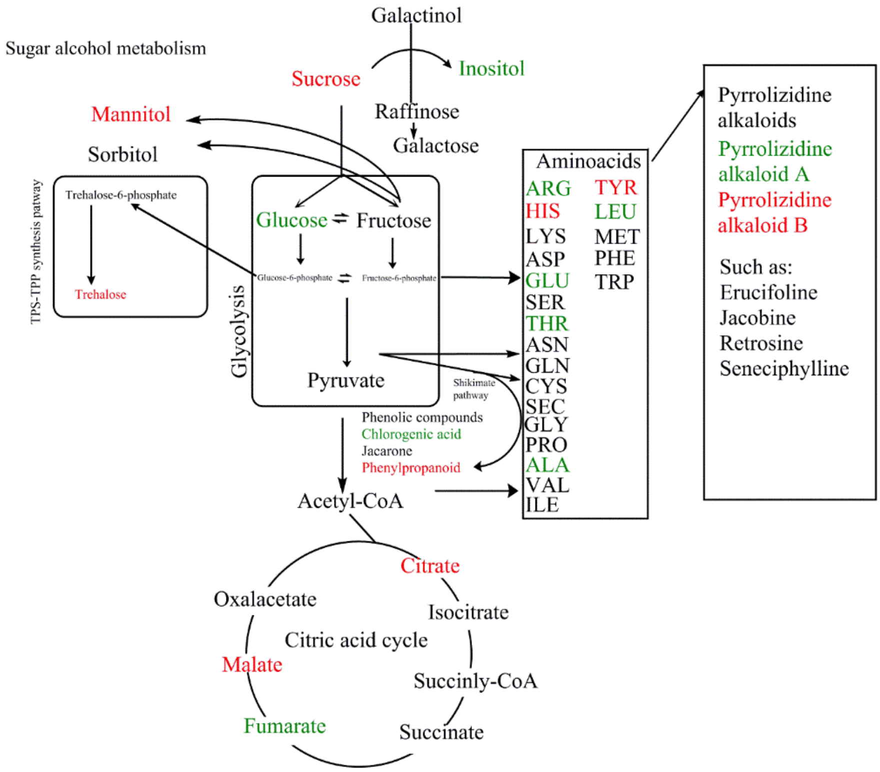

Figure 3.3 Metabolic pathway of *Jacobaea vulgaris* displaying changes in the pathway due to growth in inoculated or in 100% sterilized soil. Green font indicates higher and red font lower concentrations of compounds in plants grown in 100% sterilized soil than in inoculated soil. This pathway only depicts the main pathways; not all reactions and intermediates are depicted. The compounds displayed were associated to signals as follows: glucose 5.2 ppm, sucrose 6.60 ppm, citrate 2.52 ppm, malate 4.32 ppm, mannitol 3.84 ppm, inositol 4.04 ppm, malic acid 2.64 ppm, pyrrolizidine alkaloid A 6.42 ppm, pyrrolizidine alkaloid B 2.56 ppm, ARG 1.7 ppm, HIS 8.1 ppm, GLU 2.4 ppm, THR 1.3 ppm, ALA 1.5 ppm, TYR 3.16 ppm, LEU 0.9 ppm, fumarate 6.73 ppm, phenylpropanoid 6.45 ppm. Compounds presented in black could not be identified

The dissimilarity in metabolome composition did not vary significantly between sites (ANOVA: $F_{(3,148)}$=0.44 P=0.724). However, the Bray-Curtis dissimilarity differed between the different spatial scales (plot scale, site scale, large scale) (ANOVA: $F_{(2,148)}$=4.59, P=0.012) (Fig.3.4). The Bray-Curtis similarity was on average highest when two samples were compared of plants grown with inocula collected from the same plot. The chemical diversity of the metabolome, measured as Shannon evenness did not differ significantly between sites (ANOVA: $F_{(3,59)}$=2.31, P=0.086) (Supporting information Fig. 3.5) but did depend on the shoot biomass of the plants (ANOVA: $F_{(1,59)}$=17.91, P<0.001).

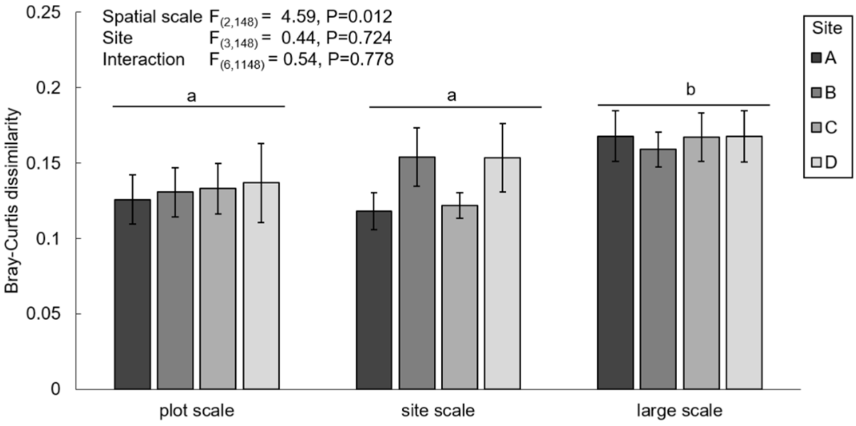

Fig. 3.4 Mean Bray-Curtis dissimilarity (± SE) of the leaf metabolome of *Jacobaea vulgaris* plants grown in inoculated soil originating from the same plot (plot scale), from the same site but from different plots (site scale), or from different sites (large scale). The values for each site are presented in different shades of grey. Results from an analysis of variance (ANOVA) with fixed factors "spatial scale" (plot, site, large) and "site" are indicated in the left upper corner.

There was no effect of site on shoot biomass (ANOVA: $F_{(3,32)}$=2.13, P=0.115) but root biomass differed significantly between sites (ANOVA: $F_{(3,32)}$=4.09, P=0.025) (Fig. 3.5). After plant growth, soil characteristics in all pots were similar and did not differ between treatments (Supporting information Table 4), and there was no relationship between soil characteristics and metabolome composition (CoCa Test on all axes: trace=0.0001, P=0.757).

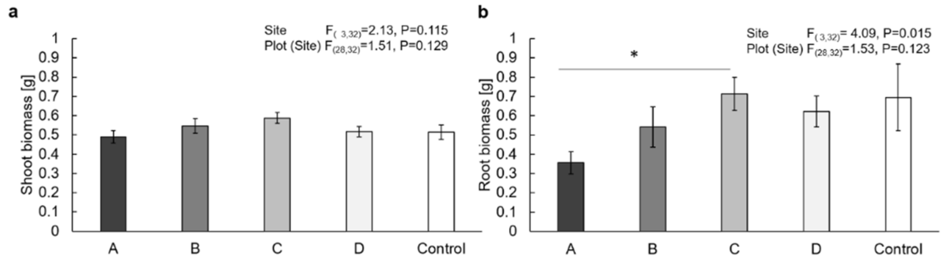

Fig. 3. 5 Mean dry biomass ±SE of a) shoots and b) roots of *Jacobaea vulgaris* grown in inoculated soils from 4 sites (A, B, C, D) and in 100% sterilized soil (control). Results of an analysis of variance (ANOVA) with factors "site" and "plot nested within site" are also depicted. An ANOVA followed by a Dunnett post-hoc test indicated that neither root nor shoot biomass of control plants significantly differed from the plants grown in inoculated soil

Discussion

Our study shows that soil inoculation changed the metabolome of *J. vulgaris*. Further the metabolome alternations varied among the sites from which the inocula were collected. Moreover, metabolomes were more similar when plants were compared that had been grown with soil collected from different plots but from the same site than from plots that originated from different sites. We investigated the metabolomic changes with an untargeted metabolomics approach and then focused on the compounds that were related to the differences between plants grown in sterile and inoculated soils. Interestingly there were no distinct differences in metabolomes for all sites, but in plants in which metabolites were changed through inoculation, this consistently led to changes in the same compounds.

In accordance with our first hypothesis inoculation of sterilized soils with live soil lead to changes in the shoot metabolome of *J. vulgaris,* in particular in primary metabolites such as sugars. Other work has shown that phenolics and PA concentrations in *J. vulgaris* vary depending on the soil in which the plant was grown (Joosten et al. 2009; Wang et al. 2019b). With this study we now show that also other metabolites change upon inoculation and by not only targeting specific compounds or groups of compounds we can now provide the first fully untargeted metabolomics analysis of the response of *J. vulgaris* to soil inoculation. Our study provides evidence that metabolomes of plants can be modulated by inoculation of soils. The concentration of one PA decreased in almost all plants grown in soils inoculated with live soil compared to those grown in pure sterilized soil. An earlier study showed that damage at the roots or shoots of *J. vulgaris* leads to a decrease in concentrations and to changes in the composition of PAs (Kostenko et al. 2013b). This suggests that certain PAs are involved in root defences upon attack and is probably related to the fact that certain PAs are synthesized in the roots (Hartmann 1999) and therefore more likely influenced by interactions in the soil than compounds which are synthetized in the shoots. Therefore, in our study the decrease of PA A in the shoots of the plants grown in inoculated soil could point at increased defence of the roots. Malic acid was also lower in plants grown in inoculated soils (A&C) than in plants grown in sterilized soil. Malic acid has many ecological functions. In root exudates it can attract beneficial rhizobacteria (Rudrappa et al. 2008) but also pests such as wireworms (*Agriotes* spp.) (Thorpe et al. 1947). Aboveground exogenous application of malic acid on shoots leads to increased chlorophyll contents (Darandeh and Hadavi 2012), however how changes in malic acid influence above ground interactions is not clear. All the other compounds which changed in concentrations are known to influence interactions with herbivores aboveground.

Inoculation with soil from certain sites, but not all sites, lead to metabolomic changes in the leaves of *J. vulgaris* when compared to the patterns observed in 100% sterilized soil. This may be relevant for our understanding of plant-insect interactions in the field for this species. In nature the microbial composition of soils changes at small spatial scales (Ettema and Wardle 2002; Fierer 2017) and our study now shows that such differences can potentially lead to changes in the metabolic characteristics of plants causing spatial heterogeneity in chemical composition among plants in the field. These metabolomic changes can influence the behaviour of enemies and beneficial organisms above and belowground in the field by attracting or deterring them (Van Dam et al. 1995; Vrieling et al. 1990, Kostenko and Bezemer 2013a). Hence, spatial variation in the composition of the microbiome in the soil may be one of the reasons for the often-unexplained chemical variation among plants in the field (Kostenko and Bezemer 2013a). However, this remains to be tested in more natural setups.

The concentrations of several compounds such as trehalose, tyrosine and an unknown PA (PA B) increased if *J. vulgaris* grew with inoculum from site A. Trehalose is involved in stress responses in plants and its effects can be either protective or adverse both in response to abiotic and biotic stress (Fernandez et al. 2010). Interestingly, trehalose can be produced by microorganisms such as endophytes and can change the plant's ability to cope with stress

(Vílchez et al. 2016). Therefore, the higher concentrations of trehalose in plants grown with inocula from site A might hint at a specific community of endophytic bacteria that is transferred from the soil to the plant. The synthesis of phenolics, lignins and flavonoids in cell walls all require tyrosine as a precursor (Walling 2000) and therefore changes in tyrosine concentrations can have far reaching consequences for cell wall properties. Furthermore, insects with sclerotized cuticles, such as Coleoptera, require tyrosine for the synthesis of their cuticle (Andersen et al. 2010). Tyrosine is a limiting resource for insects with a sclerotized cuticle and changes in tyrosine content in the food source brought by changes in the soil therefore have the potential to influence the fitness related characteristics of herbivorous insects. We show that different parts of the metabolome of *J. vulgaris*, such as primary and secondary compounds can be influenced by the soil in which a plant is grown. These results emphasise the importance of untargeted metabolomic fingerprinting approaches to investigate the chemical response of plants to interactions with the soil rather than focusing on a few target compounds in the plant. Most studies, so far, have focused on secondary compounds however recently the importance of investigating the response of all plant compounds to abiotic and biotic factors is increasingly acknowledged (Peters et al. 2018). Not only can changes in primary compounds scale up to changes in secondary compounds over time, but these compounds themselves can also influence interactions of plants with organisms in their surroundings both above and belowground (Berenbaum 1995; Hervé and Erb 2019; Zhou et al. 2015).

All soils in which *J. vulgaris* was grown consisted of 90% sterilized bulk soil. This greatly reduced the potential effects of nutritional differences among soil inocula on the metabolome. This is confirmed as there were no differences in soil chemical characteristics among the soils in which *J. vulgaris* had grown. It is important to note that sterilized soil does not stay sterile and that microbiomes certainly were also present in pots with 100% sterilized soil. However other studies have shown that the composition of these soil microbiomes varies greatly from those in inoculated soils (e.g. Ma et al. 2018). Therefore, we propose that the differences that we observed in metabolome composition were caused by differences in soil microbial communities in the different inocula. Different microbial communities may vary in their direct effects on the plant (e.g. mutualists or pathogens) or indirectly affect the plant via influencing abiotic characteristics of the soil such as the nutrient availability or pH in the soil. Differences that we observed between metabolomes of plants grown in sterilized and live soil can be due to absence of specific (groups of) microbes in the sterilized soil. For example, the AMF *Rhizophagus irregularis,* can cause changes in the metabolome of *J. vulgaris* (Hill et al. 2018). In that study no changes were detected in the shoot metabolome but other studies with different plant species have shown that beneficial bacteria and AMF can influence foliar metabolomes (Zhou et al. 2018; Schweiger et al. 2014). Our results show that soils can influence the metabolome of plants and that these changes are probably caused by different microorganisms that are present in the different inocula. However, we can only speculate about the potential causes of these changes since we did not measure the microbial composition present in the soil.

Changes in plant metabolomes can arise from differences in the biomass of the plant (Lisec et al. 2008). In our study, we did not find a significant difference in shoot biomass among plants grown in inoculated soils from different sites. However, there was a strong relationship between plant shoot biomass and the leaf metabolome. All signals not associated to sugars were negatively or not correlated with shoot biomass. This can due to several reasons. First there can be a dilution effect of all other compounds in the metabolome due to increased biomass. A higher photosynthetic activity can lead to a higher sugar content and the production of other compounds may lack behind. Second, plants that produced most biomass aboveground grew in 100% sterilized soil. Twenty to forty percent of a plant's carbon fixed through photosynthesis is exuded into the soil by the roots (Badri and Vivanco 2009). We speculate that plants grown in 100% sterilized soil and with inocula from sites B, C and D may have spent less carbon for exudation to maintain their soil microbiome and might therefore have higher concentrations of sugars. This remains to be tested in future research. Interestingly, root biomass varied significantly depending on the origin of the inoculum. This indicates that inoculation, and presumably, differences in soil microbial communities impact root growth of *J. vulgaris* much more than shoot growth (Bezemer et al. 2013). But root biomass did not significantly explain variation of the metabolome. This shows that plant soil interactions which influence the root biomass without changing shoot biomass can still influence the metabolome of the shoots.

We found evidence for our third hypothesis that the dissimilarity in metabolome composition will depend on the spatial distance. The metabolomes of plants grown in soils inoculated with inocula collected from the same plot were more similar than those of plants that were grown with inocula from more distant sites. Therefore, our study provides some evidence for a spatial soil effect. We speculate that this is linked to higher similarity in the microbial community in the soil on plot level than at larger spatial scales, as shown previously in other studies (Brockett et al. 2012; Constancias et al. 2015; Oda et al. 2003; Wang et al. 2017; Xue et al. 2018). Previous research has highlighted that spatial heterogeneity in abiotic factors and spatial distance can explain the composition of microbial communities on local, regional and intercontinental scales (Hanson et al. 2012; Vos et al. 2013). With our study we show that the spatial distribution of soil can also cause spatial differences in the metabolomes of plants growing in these soils. Further studies should examine how spatial variation and the interplay of abiotic and biotic factors in the soil influence the chemistry of the plants grown in these soils in nature.

In conclusion we show that soil inoculation leads to changes in the composition of the leaf metabolome of *J. vulgaris*. In natural systems such differences could lead to variation in susceptibility to pathogens, and herbivores above and belowground and ultimately influence the abundance of these higher trophic levels.

Acknowledgments

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Author contribution

The idea for the experimental design arose through discussions of TMB, PGLK, KV and MH. TMB JvK and MH designed and planned the research. JvK and MH performed the experiment. BM, YHC and MH carried out the NMR and MH, YHC and TMB analyzed the NMR data. MH wrote the first version of the manuscript and MH and TMB led the writing of the manuscript. All authors contributed critically to the manuscript and read and approved the final manuscript.

Data availability

Data available from om Dryad Digital Repository (https://doi.org/10.5061/dryad.p2ngf1vm5).

Supporting information

Table S 3.1 Results of permutational multivariate analysis of variance (PERMANOVA) testing the effect of inoculation with soil from four different sites (A, B, C, D) and plants grown in 100% sterilized soil, and shoot biomass on the metabolome of *Jacobaea vulgaris.* Presented are F-values with degrees of freedom (df), explained variance (R^2) and P-values. Permutations were set to 999. Significant factors are indicated in bold

Factor	F-value	R^2	P-value
Site	$F_{(4,59)}=3.57$	0.14	0.002
Shoot biomass	$F_{(1,59)}=18.46$	0.19	0.001
Site * Shoot biomass	$F_{(4,59)}=1.96$	0.08	0.027

Table S 3.2 Results of a pairwise comparison of metabolomes with the function pairwiseadonis. Depicted are differences in the metabolome of *Jacobaea vulgaris* grown in inoculated soil from different sites (A, B, C, D) and in 100% sterilized soil (Control). Depicted are degrees of freedom (df), F values, explained variance (R^2) and P-values (non-adjusted and adjusted). P-values were adjusted with FDR correction for multiple comparisons. Significant factors are indicated in bold

Pair			df	F	R^2	P	Adjusted P
A	**vs**	**D**	**31**	**3.76**	**0.11**	**0.016**	**0.040**
A	**vs**	**C**	**31**	**4.26**	**0.12**	**0.004**	**0.037**
A	**vs**	**B**	**31**	**4.74**	**0.14**	**0.011**	**0.037**
A	**vs**	**Control**	**20**	**4.06**	**0.18**	**0.011**	**0.037**
D	vs	C	31	3.11	0.09	0.039	0.078
D	vs	B	31	1.58	0.05	0.182	0.260
D	vs	Control	20	0.68	0.03	0.569	0.569
C	vs	B	31	0.75	0.02	0.532	0.569
C	vs	Control	20	2.62	0.12	0.054	0.090
B	vs	Control	20	0.96	0.05	0.392	0.490

Table S 3.3 Results of permutational multivariate analysis of variance (PERMANOVA) testing the effect of inoculation with soil from four different sites (A, B, C, D) and root biomass on the metabolome of *Jacobaea vulgaris*. Presented are F-values with degrees of freedom (df), explained variance (R2) and P-values. Permutations were set to 999. Significant factors are indicated in bold

Factor	F-value	R^2	P-value
Site	**$F_{(3,56)}$=3.13**	**0.13**	**0.005**
Root biomass	$F_{(1,56)}$=2.69	0.04	0.054
Site * Root biomass	$F_{(3,59)}$=1.40	0.06	0.183

Table S 3.4 Mean concentrations of soil characteristics (±SE) for each plot (n = 2) measured after growth of *Jacobaea vulgaris*. Four out of eight plots were randomly selected for each site for the analysis. The differences between the soil characteristics were compared with analysis of variance (ANOVA) with fixed factor "soil" (A, B, C, D, Control (100% sterilized soil)). All chemicals were measured in mg/kg

Site	Plot	Fe	K	Mg	NO_2+NO_3	NH_4-N	P	S	Zn
A	1	0.14±0.002	9.35±3.15	29.05±0.65	5.04±0.19	1.01±0.01	3.11±0.47	3.63±0.30	0.63±0.23
	6	0.17±0.013	6.30±1.00	32.10±0.10	5.69±0.86	1.64±1.24	4.47±0.30	3.22±0.15	1.51±0.26
	7	0.15±0.016	7.65±1.55	30.50±0.50	4.92±1.49	1.23±0.73	3.45±0.32	3.32±0.53	0.86±0.19
	8	0.14±0.007	7.60±1.90	30.80±0.40	5.41±1.04	0.94±0.48	4.00±0.13	3.88±0.30	0.85±0.16
B	3	0.15±0.012	7.15±0.35	28.00±1.50	4.96±1.20	1.28±0.11	3.80±0.03	2.92±0.21	1.15±0.05
	5	0.17±0.015	10.55±1.25	32.55±0.35	3.40±1.52	1.89±0.44	3.12±0.21	3.12±0.38	0.82±0.13
	6	0.17±0.034	12.60±0.10	30.55±0.75	2.16±2.02	1.61±0.80	2.12±0.07	2.71±0.12	0.57±0.28
	8	0.17±0.034	8.25±2.35	33.00±3.20	5.48±1.23	1.04±0.73	2.92±0.68	3.58±0.99	1.04±0.60
C	4	0.17±0.003	8.75±2.35	31.60±0.20	3.63±2.54	1.17±0.53	3.70±0.31	3.21±0.44	1.39±0.14
	5	0.16±0.005	7.40±0.30	30.75±1.55	10.39±5.24	1.30±0.38	3.62±0.21	2.96±0.01	1.31±0.27
	7	0.14±0.005	6.05±0.45	27.55±3.25	5.20±0.48	1.18±0.78	3.42±0.91	2.81±0.35	1.04±0.31
	8	0.14±0.016	10.75±2.55	26.55±1.05	2.68±1.12	3.11±0.65	2.25±0.10	3.25±0.01	0.64±0.15
D	3	0.17±0.012	9.85±1.55	30.40±5.10	6.60±0.87	2.06±0.79	3.86±0.86	5.19±2.89	1.70±0.38
	4	0.17±0.017	6.20±0.60	32.05±0.85	4.67±1.09	0.78±0.24	3.59±0.55	3.40±0.53	1.27±0.23
	5	0.21±0.017	8.40±0.10	28.70±1.40	2.92±0.97	2.15±0.29	2.98±0.11	3.16±0.07	1.20±0.28
	8	0.16±0.002	8.35±0.75	27.90±0.80	8.69±6.34	2.24±0.99	3.29±0.09	3.32±0.16	1.04±0.22
Control		0.16±0.019	6.70±0.32	31.58±1.09	5.74±0.52	1.51±0.48	3.88±0.34	3.48±0.37	1.06±0.34
ANOVA		$F_{(4,31)}$=1.54 P=0.214	$F_{(4,31)}$=1.29 P=0.295	$F_{(4,31)}$=0.86 P=0.499	$F_{(4,31)}$=0.39 P=0.817	$F_{(4,31)}$=0.51 P=0.728	$F_{(4,31)}$=1.69 P=0.177	$F_{(4,31)}$=0.75 P=0.566	$F_{(4,31)}$=0.98 P=0.430

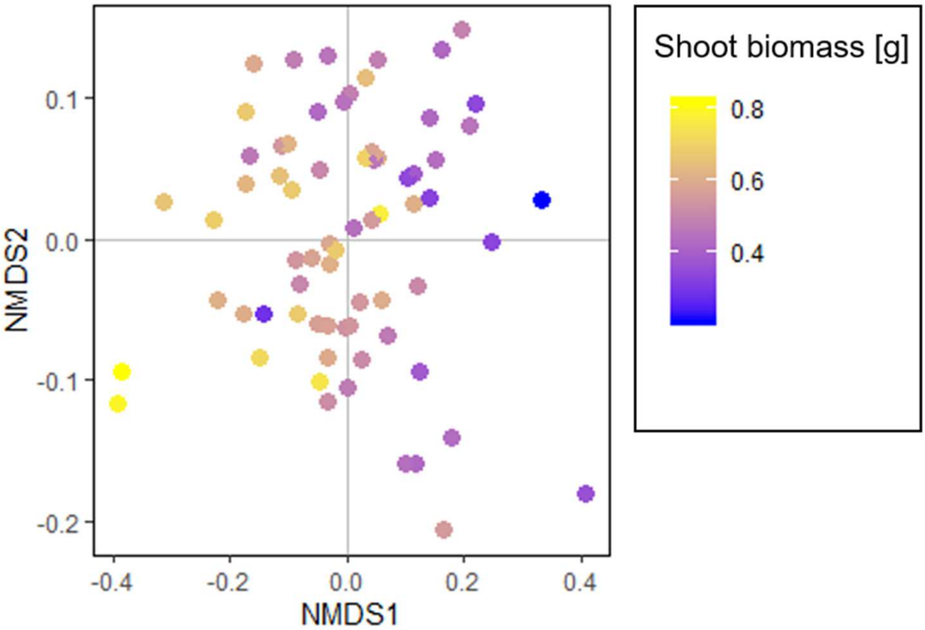

Figure S 3.1 Non-metric multidimensional scaling (NMDS) of the metabolome of *Jacobaea vulgaris* grown with inocula collected from different sites (A, B, C, D) and in 100% sterilized soil using Bray-Curtis dissimilarities. The individual samples are coloured by shoot biomass. The stress is a measurement for the fit of the model and was 0.10

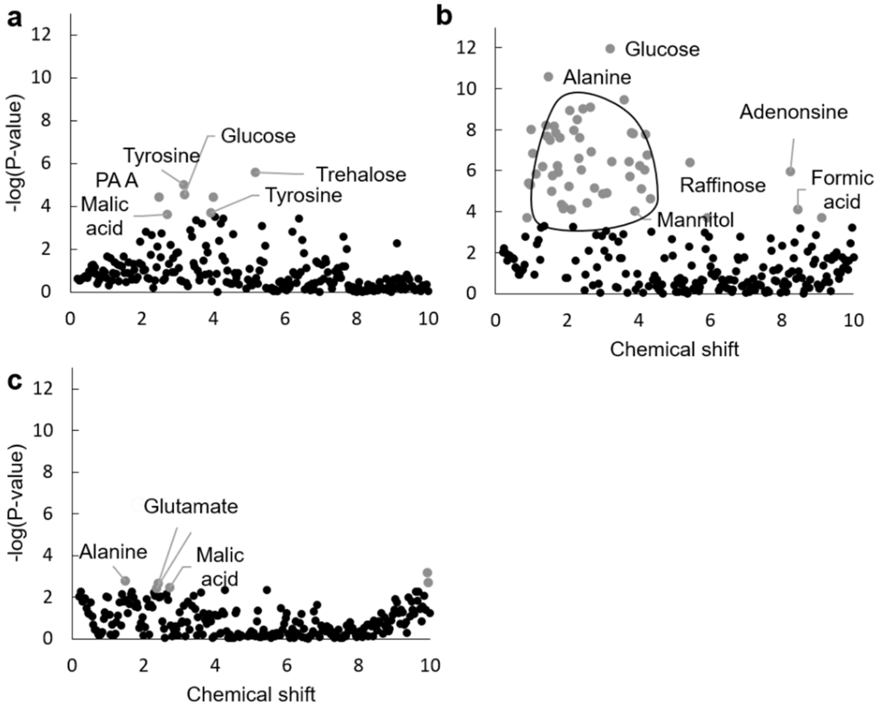

Figure S 3.2 Negative logarithm of p-values from an analysis of variance (ANOVA) testing for differences in intensity of signals in each bucket (chemical shift) of the metabolome of *Jacobaea vulgaris* for (a) the site from which the soil inocula was collected from, (b) shoot biomass, and (c) and the interaction between site and shoot biomass. Grey dots represent signals for which P-values were smaller than 0.05 after false discovery correction (FDR). Putative identifications of significant signals are indicated in the panels. No description indicates that no specific compound could be assigned to this signal. The chemical shift of the signals that were associated to the different compounds were: trehalose 5.16 ppm, tyrosine 3.16 and 3.9 ppm , glucose 3.2 ppm, pyrrolizidine alkaloid A (PA A) 2.48 ppm, malic acid 2.72 ppm, alanine 1.48 ppm, raffinose 5.44 ppm, adenosine 8.24 ppm, formic acid 8.44 ppm, mannitol 3.88 ppm, glutamate 2.36 ppm glutamine 2.4 ppm. The circle in (b) describes signals with a chemical shift in the region which is associated with signals from sugars

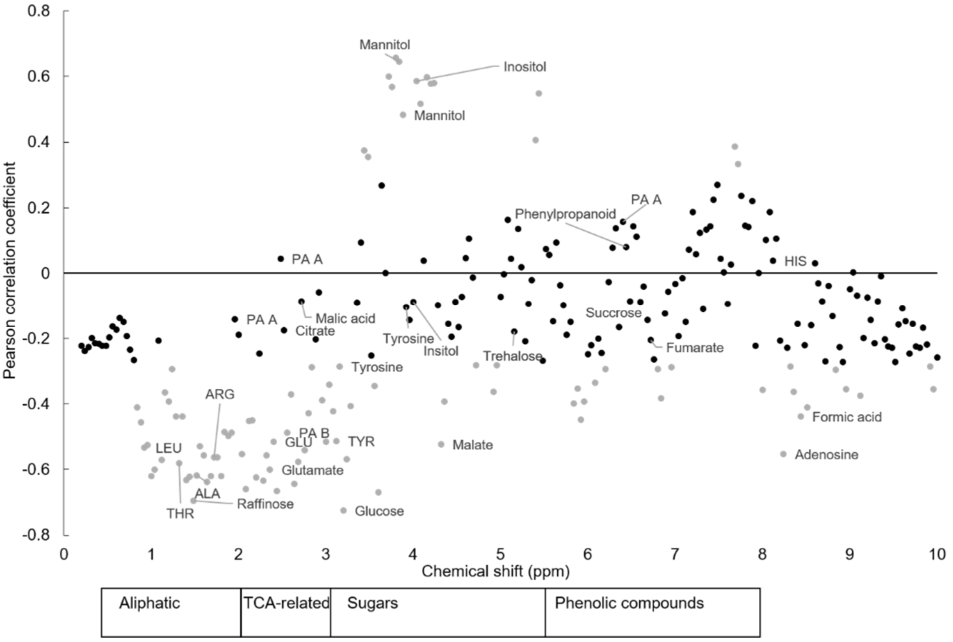

Figure S 3.3 Pearson correlation coefficient between shoot biomass of *Jacobaea vulgaris* and the intensity of the signals of the different chemical shifts (buckets). Grey dots indicate significant correlations after false discovery correction (FDR). Chemical shifts of buckets are indicated by numbers. A description of compounds which are often associated to a specific chemical shift is also presented. Two different pyrrolizidine alkaloids are abbreviated with PA A respectively PA B. Abbreviations for amino acids are: LEU= Leucine, ALA=Alanine, THR=Threonine, GLU=Glutamic acid, TYR=Tyrosine, HIS=Histidine.

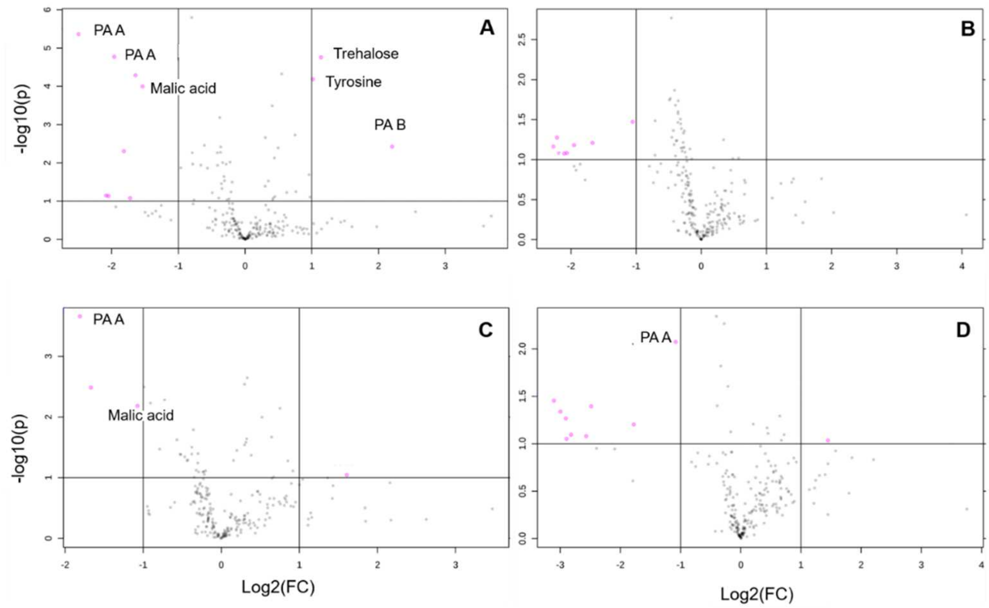

Figure S 3.4 Volcano plots depicting metabolomic differences of *Jacobaea vulgaris* grown in inoculated soil from four sites (A, B, C and D) compared to 100% sterilized soil. Colour dots in the volcano plot outside the cut-off lines depict signals with a fold-change (FC) >2 and P < 0.1 based on a t-test (unadjusted). The signals within these buckets were putatively assigned to compounds with signals identified as pyrrolizidine alkaloids A (PA A) at 2.48 and 6.52 ppm, malic acid at 2.72 ppm, trehalose at 5.16 ppm, tyrosine at 3.16 ppm and pyrrolizidine alkaloid B (PA B) at 2.56 ppm. Signals which could not be assigned to a specific metabolite were found at 6.48, 6.52 ppm and 0.4-0.6 ppm. Negative fold-changes represent signals that show a higher intensity in plants grown in 100% sterilized soil (left side) than in inoculated soils. Positive fold-changes represent signals that show a higher intensity in plants grown in the inoculated soil compared to the 100% sterilized soil

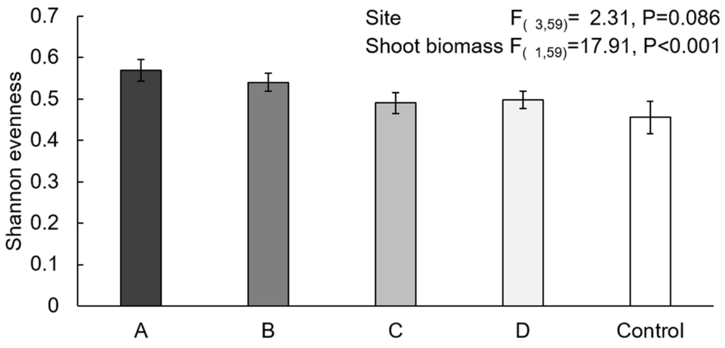

Figure S 3.5 Mean Shannon evenness (±SE) of Jacobaea vulgaris leaf metabolomes of plants grown with inocula from 4 different sites (A, B, C, D) and in 100% sterilized soil with shoot biomass as covariate. The results of an analysis of variance (ANOVA) testing for the effects of site, plots nested within site, and shoot biomass is also presented. For this analysis the 100% sterilised soil treatment was removed


1 year

Chapter 4

Temporal dynamics of plant-soil feedbacks on soil microbiomes, leaf metabolomics and plant-insect interactions

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Manuscript

Abstract:

1. Most plant-soil feedback (PSF) studies condition the soil for a short period of time only before initiating the feedback phase. However, the strength and even the direction of the PSFs may change depending on the time of conditioning.
2. We collected soil from monocultures of six plant species, growing in 200 L containers outdoors and examined temporal changes in the PSF for one of the species, *Jacobaea vulgaris.* Soil was collected every two months and genetically identical *J. vulgaris* clones were grown in sterilized bulk soil inoculated with 10% live soil in a climate-controlled growth chamber. Each time we measured nutrient availability in the soil, plant biomass and the performance of a generalist insect herbivore (*Mamestra brassicae*). At two, eight and twelve months we analysed the foliar metabolic composition of the test plants with ^{1}H-Nuclear magnetic resonance spectroscopy (NMR) and the bacterial and fungal community composition and abundance in the monoculture soils.
3. Plant growth and the strength of PSF varied over the course of the experiment but at each time point, *J. vulgaris* produced less biomass in its own soil than in the soils conditioned by other species, and most biomass when grown in 100 % sterilized soil. Metabolomic profiles in the test plants varied mainly with collection time. Performance of *M. brassicae* differed among soils but only after soils had been conditioned for twelve months. PSFs could not be explained by nutrient availability in the monoculture soils. Bacterial and fungal communities changed over time, and *J. vulgaris* biomass was related to composition of bacterial and fungal communities but only at twelve months. Furthermore, after twelve months, metabolomic composition could be explained by the fungal community composition in the soil.
4. *Synthesis.* Our results show that for *J. vulgaris*, the direction of the PSF does not change with time of conditioning but that the magnitude of the PSF varies over time. Leaf metabolomes are sensitive to plant-induced changes in the soil but the temporal dependency on these changes in the soil remain difficult to predict.

Key Words: Ecometabolomics, Insect herbivory, *Mamestra brassicae*, Plant-herbivore interactions, Plant-soil interactions, Soil legacy effects, Temporal variation.

Introduction

Soil is a medium in which plants grow, and greatly impacts the plant that grows in it, as well as other plant-associated organisms. For example, plant biomass, foliar metabolomes and the performance of herbivorous insects all depend on the microbial community that is present in the soil (Heinen, Sluijs, Biere, Harvey, & Bezemer, 2018; Huberty et al., 2020b; Zhu et al., 2018). However, plants also impact biotic and abiotic parameters in the soil, such as the microbial community. Via their effects on the soil, plants can influence the performance of other plants that grow later in this soil (Plant-soil feedback (PSF); Bever, 1994; Van der Putten et al., 2013). While many studies have provided evidence for PSFs, the temporal dynamics of PSF processes are still poorly understood (Bezemer, Jing, Bakx-Schotman, Bijleveld, & Bijleveld, 2018; Hawkes, Kivlin, Du, & Eviner, 2013; Lepinay et al., 2018).

Most knowledge of PSFs is based on greenhouse studies in which soil is conditioned by a plant for several weeks only. The impact of a plant on the soil microbial community can change considerably over time (Hannula et al. 2019a), but how the duration of conditioning by the first plant affects the performance of the succeeding plant and the herbivores on the plant is not well-known. If pathogens accumulate over time in the soil, the negative soil-mediated effect on another plant may increase over time (Luo et al., 2019), but this could also result in temporal variation in the defence induction patterns of plants (Heil & Bostock, 2002). This could lead to more pronounced metabolomic changes in plants in response to soil conditioning over time, and to stronger responses of insect herbivores to soil conditioning. In this study, we collected soil from monocultures every two months over the course of a year. We measured microbial composition and nutrient availability in these soils, and examined the temporal changes in, and relationships between PSFs, leaf metabolomics and herbivory, using identical clones of a common forb, *Jacobaea vulgaris*.

Soil properties that are important for plant growth such as nutrient availability and the composition of soil microbial communities all change over time. This could be due to changes in the climate, but also due to plant-mediated effects. Rhizodeposition patterns, for example, change with the age of the plant (Dechassa & Schenk, 2004) and the chemical composition of roots can vary over time due to changes in plant phenology or environmental temperatures (Huang, Bont, Hervé, Robert, & Erb, 2020). In PSF experiments that start with bare soil, over time, root biomass will increase, and therefore the root surface that is in contact with the soil increases. This suggests that the influence of the plant on soil nutrients and microbial communities in the soil will increase over time (Latz, Eisenhauer, Scheu, & Jousset, 2015; Micallef, Channer, Shiaris, & Colón-Carmona, 2009). We therefore expect that the strength of PSFs will become stronger with increasing time of conditioning. Such temporal effects on biomass have been reported for the duration of short (two to eight weeks) conditioning periods (Lepinay et al., 2018). However, how these effects change over the course of a year, and how this influences on plant metabolomes and plant-herbivore interactions is unknown.

Previous work has shown that the growth and chemical composition of the plant *J. vulgaris* depends on microbial characteristics of the soil in which the plant grows (Bezemer et al. 2006; Joosten et al. 2009, Kostenko et al. 2012; Bezemer et al. 2013, Huberty et al. 2020a). This species exhibits a strong negative conspecific feedback, and generally grows better in soil conditioned by grasses than in soil conditioned by forbs (Van de Voorde et al. 2012; Wubs and Bezemer 2018). Aphids and caterpillars feeding on the foliage of the plant also respond to soil legacies and this has been shown to be related to changes in plant chemical composition (Kostenko et al. 2012; Kos et al. 2015). As the metabolic changes in *J. vulgaris* are related to changes in the soil (Huberty et al. 2020a) and plant- mediated effects on the soil during the conditioning phase change over time (Hannula et al. 2019a), we expect that plant-herbivore interactions on the succeeding plant will also be time-dependent.

In this study we test in a controlled set-up how inoculation with soil from six monocultures (one conspecific and five heterospecific soils) over time influences growth and chemical composition of genetically identical clones of *J. vulgaris,* and the performance of a generalist herbivore, *M. brassicae*. The soils were collected every two months after establishment of the monocultures, and at each time point abiotic properties of the soil were determined. Furthermore, *J. vulgaris* was grown each time in identical sterilized bulk soil inoculated with 10% monoculture soil and in 100% sterilised soil in a growth cabinet. Metabolic profiles of plants were analysed at two, eight and twelve months and related to existing analyses of the bacterial communities of the same soils (Hannula et al. 2019a) using correlation approaches. Furthermore, the abundance of bacteria and fungi in the soil was determined with qPCR.

We address the following questions (i) Does the influence of PSF on plant biomass, herbivore performance and metabolomic profiles get stronger with increasing age of the conditioning plant community? ii) Does the sign and magnitude of PSF change over time? iii) Do temporal changes in potential causal agents of PSF (microbes, nutrients) explain the observed soil-plant-insect patterns?

Material & Methods

Jacobaea vulgaris is a biennial herb which is native to Europe and Asia and invasive in North America, Australia and New Zealand (Bain, 1991). *J. vulgaris* can grow in diverse habitats such as sand dunes, woodlands and grasslands and therefore has the capacity to grow in a broad range of soils (Bezemer, Harvey, Kowalchuk, Korpershoek, & van der Putten, 2006). *J. vulgaris* growth is negatively affected by growing in its own soil than in soils conditioned by other plant species, likely caused by an accumulation of soil pathogens in its own soil (Bezemer, Harvey, Kowalchuk, Korpershoek, & van der Putten, 2006; Van de Voorde, Van der Putten, & Bezemer, 2011; Wubs & Bezemer, 2016) . Furthermore, the microbial composition of the soil was shown to influence the concentration and composition of secondary metabolites such as pyrrolizidine alkaloids and primary metabolites such as amino acids (Kos, Tuijl, De Roo, Mulder, & Bezemer, 2015; Kostenko, van de Voorde, Mulder, van der Putten, & Bezemer, 2012; Wang et al., 2019) and the resistance of *J. vulgaris* to herbivores (Kostenko et al., 2012).

Monocultures

Thirty containers (48 cm x 80 cm x 50 cm), were filled with 200 L soil on the 3rd and 4th of April 2017. The soil was sieved through a 32 mm sieve in order to remove large stones and roots. The soil originated from a grassland near Lange Dreef, Driebergen, The Netherlands (52° 02´ N, 5° 16´ E) and is described as holtpodzol, sandy loam (84% sand, 11% silt, 2% clay, ~3% organic matter, 5.9 pH, 1.15 g N kg^{-1}, 0.06 g P_2O_5 kg^{-1}, 0.94 g K kg^{-1}). To inoculate the soil with a diverse microbial community, the soil in each container was topped with 5 cm of soil collected from a natural grassland "De Mossel" (Natuurmonumenten, Ede, The Netherlands, 52° 04´ N, 5° 45´ E) which was sieved through a 10 mm sieve. These soils are characterized as holtpodzol, sandy loam (94% sand, 4% silt, 2% clay, ~5% organic matter, 5.2 pH, 1.06 g N kg^{-1}, 0.08 g P_2O_5 kg^{-1} , 0.74 g K kg^{-1}).

Seeds of the species used in this experiment were sown in steamed potting soil and grown for 3 weeks in a greenhouse (70% relative humidity, light/dark 16/8h, 21/16 °C) and with supplemented light from 400 W metal halide lamps (225 µmol m-2 s^{-1} photosynthetically active radiation, 1 lamp per 1.5 m^2). All seeds originated from Cruydt-Hoeck (Nijberkoop, The Netherlands), except for *Jacobaea vulgaris*, which were collected in the mossel area in 2014. The plant species we used were three grasses, *Holcus lanatus, Festuca ovina, Alopecurus pratensis,* and three forbs, *Hypochaeris radicata, Jacobaea vulgaris, Taraxacum officinale.*

The seedlings were planted at a density of 100 seedlings per container on the 1st of May 2017. Plants that died were replaced within the first two weeks. Seedlings from other species were weeded out regularly. For each plant species 5 containers were established. The containers were placed in a randomized block design in a common garden at the Netherlands Institute Of Ecology (NIOO-KNAW) in Wageningen, the Netherlands (51° 59´ N, 5° 40´ E). The containers were watered regularly during the summer months.

Soil sampling

Every two months, four soil cores (0.7 cm diameter, 10 cm depth) were taken from each container. The soil was mixed and large pieces of roots were removed. Details about the sampling dates are provided in the supporting information (Supporting information Table S4.1). Photos of the monocultures at each sampling point are presented in Supporting information Fig. S4.1 of the supporting information (Supporting information Fig. S4.1). After the final sampling point (May 2018) total aboveground biomass was removed from each container, dried at 40°C and total shoot biomass per container was determined (Supporting information Fig. S4.2). Due to a technical failure of the growth cabinet used for the plant growth experiment (see below) in January 2018 the six-month sampling event was excluded from further analysis.

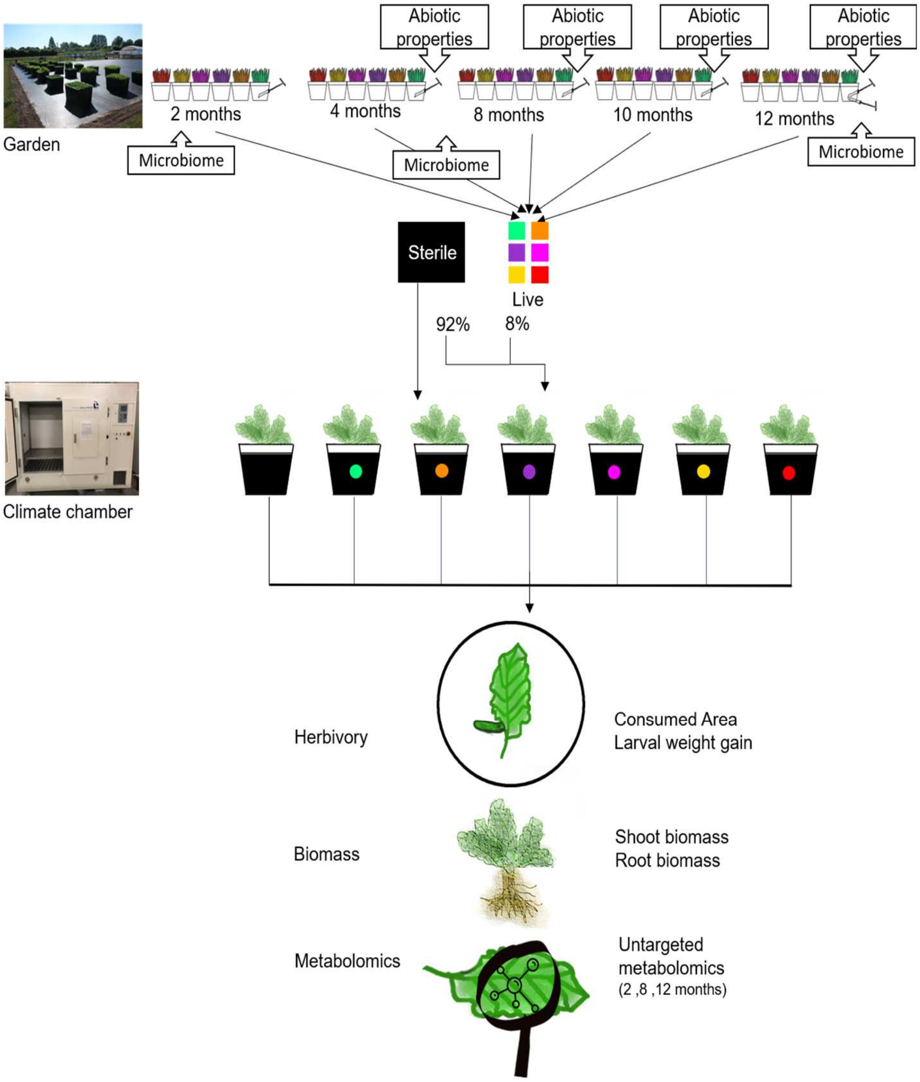


Figure 4.1 Conceptual framework of the experimental design: Soils were conditioned in large containers by monocultures by six plant species in a common garden over the course of one year. Soil samples were collected five times and soil abiotic characteristics were measured. The soil was mixed with sterilized bulk soil and *J. vulgaris* was grown in a climate chamber in different rounds. After 6 weeks of growth one leaf was clipped and used for a detached leaf assay with *Mamestra brassicae*, biomass of shoot and roots was determined and leaf metabolomics analysis was done for the 2, 8 and 12 months rounds. Samples to determine fungi and bacteria communities in the soil were taken at 2, 6, 12 months. Samples to quantify bacteria and fungi were taken at 1, 2, 3, 4, 5, 6, 9, 10, 12.

Soil abiotic characteristics

Every two months, the soil abiotic characteristics were determined for each soil, starting at four months. For each soil, a subsample was dried (40°C) and sieved through a 2 mm mesh. 30 ml of 0.01 M $CaCl_2$ was added to 3 g of soil and the mixture was shaken for 2 hours at 250 rpm and centrifuged at 3000 rpm for 5 minutes. 15 ml of the supernatant was filtered through a Whatman Puradisc Aqua 30 syringe filter with cellulose acetate membrane. To measure soil extractable nutrients (i.e., Fe, K, Mg, P, S, Zn), 12.87 ml of this filtrate were transferred to a 15 ml tube and 130 µL HNO_3 were added, vortexed and analysed by inductively coupled plasma - optical emission spectrometer (ICP-OES, Thermo Scientific iCAP 6500 Duo Instrument with axial and radial view and CID detector microwave digestion system). The remaining of the filtrate was transferred to a 15 ml tube and pH was determined. NO2+NO3 and NH4, were measured on a QuAAtro Autoanalyzer (Seal analytical, Mequon, Wisconsin, USA). For each sampling round, five samples from the sterilized bulk soil from the bag that was used for the plant growth experiment (see below) was also analysed except for the first round of analyses (4 months).

Soil bacterial and fungal communities

During the course of the experiment soil samples were collected every month from each container for molecular identification of the bacterial and fungal communities. These samples have been sequenced and the results have been published elsewhere (Hannula et al. 2019a). Here we use the data from soil samples collected at 2, 6, 12 months this coincided with the two of the three sampling rounds for which leaf metabolomics were analysed (See below). For one of the rounds of the feedback experiment (8 months) the soil microbial sampling of 6 months was used as there was no sampling at 8 months (Supportive information Table S4.1). A detailed description of the collection of the soils and the data processing is found in the Supplementary methods and in Hannula et al 2019a. The quantity of bacteria and fungi was determined by qPCR against a known standard as described in Hannula et al. 2019a. Here we use data from all time points.

Plant soil feedback test

For the PSF tests we used clonal *J. vulgaris* grown in tissue culture for 17 years and formerly collected from Meijendel, the Netherlands. Per time point we asexually propagated a total of 60 *J. vulgaris* plants on MSO medium with 100 mg/L benzylaminopurine (BAP) in a climate room (16h: 8 h light: dark photoperiod, 20°C). After 4 weeks of growth they were individually put on MSO medium without BAP for 10 days to form roots. Time intervals of growth on the two different media were the same for all time points. For each time point 35 equally sized (approximately 4 cm) *J. vulgaris* plantlets were selected for the PSF experiment and one plantlet was planted per pot.

The plants were grown in pots filled with 50 g (dry weight) of soil collected from the containers mixed with 410 g sterilized bulk soil. To calculate how much wet soil from each sample was needed, a subsample was weighed, dried (40 °C) and soil moisture was determined. After collection and prior to filling the pots, the soil samples were kept for 3 days at 4°C. The bulk soil consisted of homogenized soil that was used to fill the containers (Lange Dreef, Driebergen) that was gamma-irradiated (>25 Kgray). For each pot, the soil was mixed separately. At each round, five pots were filled with 100% bulk soil and served as sterile control. To allow the microbial community to establish before proceeding with the planting, the pots were put in a climate chamber without light for 7 days (16°C, humidity 70 %).

The PSF experiment was carried out in the growth cabinet each round to assure uniform conditions (16h: 8h light: dark photoperiod, 21, 16°C, humidity 70 % and PAR 290 µmol/m^2/s). During the six months round, the lights in the cabinet were accidentally switched off for an unknown period of time. Therefore, the resulting data from this sampling round were not included in further analyses. Plants were watered twice a week. To ensure similar soil moisture in all pots each time we weighed each pot and added water until it reached a weight of 505 gram. Water was autoclaved to avoid introduction of microbes through the water. After 6 weeks of growth the plants were harvested. For this, plants were carefully removed from the pots and roots were washed. After that the two largest leaves of each plant were cut and fresh weight was recorded. For the time points at which metabolomic profiles were measured, the largest leaf was immediately wrapped in aluminium foil and then flash-frozen in liquid nitrogen and stored at -80°C. The second largest leaf was always used for a non-choice bioassay using the caterpillar *M. brassicae* (see below). The roots were cut off the shoots and wrapped in aluminium foil and flash frozen in liquid nitrogen. The remainder of the shoots were put in a bag and frozen at -80°C. All frozen samples were lyophilised for 7 days and then stored in an exicator with silica gel upon use.

Herbivory assay

The stem of the second largest leaf of each plant was wrapped in wet cotton wool and parafilm to keep the leaf hydrated. *Mamestra brassicae* (Lepidoptera: Noctuidae) eggs were obtained from the University of Wageningen, the Netherlands and upon hatching the caterpillars were reared on artificial diet (as described in Hannula et al., 2019b). For each round, we recorded the biomass of each of the 35 L2 caterpillars prior to placing them individually on one of the leaves in a petri dish (100 x 15 mm). The petri dishes were randomly ordered and placed in a climate chamber with no light, 20°C, humidity 70 %. After 4 days the caterpillars were weighed again and leaf consumption of each leaf was assessed by drawing the consumed area of every leaf on an acetate sheet and scanning the sheet with an Epson STD4800. Food consumption (in mm^2) was then accessed with the program WinFOLIA (Version: 2016b Pro, Regent Instruments Canada Inc.). Mean weight gain per day of *M. brassicae* was also calculated. A few leaves desiccated (1 for 4 months, 1 for 8 months, 3 for 12 months). These samples were excluded from analysis.

Metabolomics ^1H NMR Analysis

Metabolomic profiling was performed using leaf samples from the two, eight- and twelve-month conditioning rounds. For the metabolomics analysis the samples were extracted according to an adapted version of the protocol described by Kim et al. (2010). In short, the lyophilised leaf was ground with a metal ball bearing in a TissueLyser (Retsch Mixer Mill MM 400) for 3 min at 30 s^{-1}. Of this powder 20 mg was weighed and extracted with 600 µl of Methanol-d_4, sonicated for 10 min and centrifuged for 10 min at 13.000 ppm. 250 µl of supernatant was transferred to an NMR tube (103.5 × 3 mm, inside-ø 2.24 ± 0.05mm).

We used a Bruker AV-600 MHz NMR spectrometer (Bruker, Karlsruhe, Germany), operating at a frequency of 600.13 MHz to record the ^1H NMR spectra. CH_3OH-d_4 was used as internal lock and ^1H NMR spectra were recorded with pulse width (PW) = 30 ° (11.3 µs), Relaxation delay (RD) = 1.5 sec and 128 scans with 10 min and 26 sec acquisition time with 0.16 Hz/point. To reduce the signal of H_2O frequency during the recycle delay we used a presaturation sequence. FIDs were Fourier transformed by a line broadening of 0.3 Hz.

We manually baseline corrected the spectra and calibrated them to the solvent at 0.60 ppm before phasing them in TOPSIN (v.3.0. Bruker). The data was bucketed with scaling to total intensity and a bucket width of 0.04 ppm in AMIX software (v. 3.9.12 Bruker BioSpin GmbH, Reinstetten, Germany). This is a pre-processing step which is often used in metabolomics to reduce the effect of small shifts of signals between samples (Kim, Choi, & Verpoorte, 2010). The residual signals from the solvents in regions between 4.70 – 4.90 ppm and 3.32 – 3.28 ppm were excluded. Pre-processing led to a data matrix with 246 buckets per sample. Each bucket contains the intensity of the signal from the NMR within the range of the buckets and corresponds directly to the molar level of a compound leading to a signal in this region of the NMR. Molecules which have more than one H atom will therefore lead to signals in several buckets across the NMR spectra. The chemical environment of the H atom is defined by the neighbouring atoms and determines the chemical shift and the splitting pattern of a signal.

Statistical analysis

All analyses were performed in R Studio (RStudio Team, 2016) using the packages “vegan” (Oksanen et al., 2018) and “mixomics” (Cao et al., 2020). Networks were constructed in R Studio and processed in Cytoscape version 3.7.2 (Shannon et al., 2003). Heatmaps were created with metaboanalyst (Chong et al., 2018).

Dry weight of shoots and roots, weight gain of *M. brassicae* and the total area eaten were analysed each with a two-way ANOVA with the factors “Time” and “Monoculture”. Since each round a new experiment was started and the individuals measured were not the same, time was not included as a repeated measure factor in these analyses. Data were then analysed per round. Plants grown only in sterile soil were excluded from the dataset for this analysis. For each round we reran the ANOVA including the 100% sterilized soil, and tested with a Dunnett-t test if the monoculture soils significantly from the sterile soil. All assumptions of ANOVA were fulfilled.

Soil abiotic characteristics were analysed each with an ANOVA with the factors “Time” and “Monoculture”. For that the soil the samples were always taken from the same replication unit, the containers, and therefore time was included as a repeated factor in this analysis. Data were then analysed per round. Plants grown only in sterile soil were excluded from the dataset for this analysis. For each round we reran an ANOVA including the 100% sterilized soil, and tested with a Dunnett-t test if the monoculture soils significantly from the sterile soil. All assumptions of ANOVA were fulfilled.

Metabolomic analysis

We visualised metabolomic changes in the leaves with Principal component analyses (PCA), one PCA was constructed for all data, followed by individual PCA for each of the three rounds separately. Statistical significance was inferred from a permutational analysis of variance (PERMANOVA) based on Bray Curtis dissimilarities with the factors “Monoculture”, “Time” and “Shoot biomass”. Then separate PERMANOVAs for each round, with the factors “Monoculture”, and “Shoot biomass” were conducted. For these analyses the metabolome data were normalised by the control by dividing for each sample the intensity of each bucket by the mean intensity of that bucket for plants grown in sterilized soil. Permutations were set to 999. A heatmap showing the 40 signals within the buckets, which differed the most between the plants grown in the different soils (tested with ANOVA) was constructed for each of the three rounds. For the eight months round only two of the five *J. vulgaris* plants grown in conspecific soil survived. Therefore, *J. vulgaris* soil was excluded for this round in the heatmap.

Bacterial and fungal community analysis

Sequences of bacteria and fungi were analysed with the PIPITS and the Hydra pipeline (Gweon et al., 2015; Mattias de Hollander, 2017). The data was filtered before being used for statistical analysis. An abundance threshold of 0.01 and a persistence of 10 was used. Algorithms were used to identify OTUs. For bacteria and fungi, we used SILVA (Quast et al., 2013) UNITE (Nilsson et al., 2019) respectively. Changes in the composition of the fungal and bacterial community were depicted with a PCA. Statistical significance was inferred from a permutational analysis of variance (PERMANOVA) based on Bray Curtis dissimilarities with the factors “Monoculture” and “Time”. Data were then analysed per round with the factor

"Monoculture". Bacterial and fungal copies (qPCR) were each analysed with an ANOVA with the factors "Time" and "Monoculture". Then they were each analysed per sampling round with an ANOVA with the factor "Monoculture". Fungal copies were log transformed to meet the assumption of normality.

Relationship between soil characteristics and plant and herbivore responses

The relationship between the composition of soil abiotic characteristics and features measured in the plant (shoot & root biomass, *M. brassicae* weight gain per day and damage) was analysed with redundancy analysis (RDA). The relationship between the metabolome and the abundance of bacteria/fungi was measured each with and RDA. Soil characteristics were standardised prior to the analysis. Univariate variables measured in the plant (shoot & root biomass, *M. brassicae* weight gain per day and damage) and abundance of bacteria and fungi in the soil was analysed with a Pearson correlation.

The relationship between fungal community, bacterial community, soil abiotic characteristics, and leaf metabolome composition was analysed with co-inertia analysis. Significances were tested with a permutation test with 999 permutations. For these analyses the filtered OTUs were used.

Circos plots were constructed to display correlations between bacteria, fungi and the NMR buckets which differed between the monocultures. For bacteria and fungi the OTUs were labelled to the finest taxonomic rank known. When possible, the buckets were assigned to chemical groups (sugars, phenolic compounds, TCA related compounds, aliphatic). Compounds that could be identified from the NMR spectra were assigned by chemical shift and splitting pattern. Buckets which could not be assigned to a chemical group were labelled with their chemical shift (ppm). To make the circos plots, the 40 variables in each measured community (bacteria, fungi, metabolome) that were most influential in a sparse Partial Least Squares (sPLS) were selected and correlated to each other. Only correlations with Pearson correlation coefficients higher than 0.8 were plotted. These correlations use the latent components as proxy (González, Cao, Davis, & Déjean, 2012). The components were set to two components each. This was done per round and for each monoculture soil. Correlation circle plots were constructed to display the correlation of the selected variables and the components of the sPLS.

To further explore the relationship between the metabolome of the plant and the bacterial and fungal communities in the soil, relevance network analyses were carried out. A sparse sPLS with regression (three sPLS components, 20 variables each) was run and then a network was constructed for all rounds and for each round separately.

In a last step, correlation networks were constructed. This approach is different from the approach described before since all variables are included in the analysis whereas in other analysis only variables selected through sPLS were included. For the correlation networks, all the variables of all measured communities (bacteria, fungi, metabolome) were correlated between and within each other.

Only correlations with Pearson correlation coefficients higher than 0.9 were extracted and plotted as a network in Cytoscape. All the above methods were also carried out testing the relationship between soil abiotic characteristics and the metabolome but there were no strong correlations (all pairwise comparisons were lower than 0.5) and therefore these data are not shown.

Results

Biomass

Shoot and root biomass varied greatly over time and between monoculture soils (Table 4.1, Fig. 4.2). Shoot and root biomass was lowest in soil collected after eight months, but this was also true for plants grown in sterile soil (Fig. 4.2). In the eight and ten month rounds, biomass did not differ between monocultures (Fig. 4.2, Table 4.1). Over time, *J. vulgaris* consistently showed poor growth on its own soil, compared to growth on other soils. Biomass did not vary much among the soils of the five other species. Overall, biomass was highest in 100% sterilized soil.

Table 4.1. Results of ANOVA testing the effects of monoculture soil (*Holcus lanatus, Festuca ovina, Alopecurus pratensis, Hypochaeris radicata, Jacobea vulgaris, Taraxacum officinale*) and the different rounds (2,3,8,10,12). Further the effect of monoculture soils for each round on shoot and root biomass of *Jacobaea vulgaris* and larval weight gain and food consumption of *Mamestra brassicae* was tested for each round separately. F-values, degrees of freedom (df) and P-values are presented. Significant P-values are presented in bold.

Round	Factor	Shoot	Root	Larval weight gain	Consumed area
	Round(R)	$F_{(4,120)}$=42.16, **P<0.001**	$F_{(4,120)}$=34.62, **P<0.001**	$F_{(4,98)}$=62.03, **P<0.001**	$F_{(4,98)}$=49.14, **P<0.001**
	Monoculture (M)	$F_{(5,120)}$= 5.39, **P<0.001**	$F_{(5,120)}$= 7.55, **P<0.001**	$F_{(5,98)}$=1.34, P=0.254	$F_{(5,98)}$=0.06, P=0.998
	R*M	$F_{(20,120)}$= 0.71, P=0.80	$F_{(20,120)}$= 0.64, P=0.87	$F_{(20,98)}$=1.16, P=0.30	$F_{(20,98)}$=1.38, P=0.15
2	M	$F_{(5,24)}$= 3.12, **P=0.026**	$F_{(5,24)}$= 2.96, **P=0.032**	$F_{(5,24)}$=1.38, P=0.27	$F_{(5,24)}$=0.89, P=0.50
4	M	$F_{(5,24)}$= 2.65, P=0.048	$F_{(5,24)}$= 5.15, **P=0.003**	$F_{(5,23)}$=0.65, P=0.66	$F_{(5,22)}$=2.25, P=0.09
8	M	$F_{(5,24)}$= 0.41, P=0.84	$F_{(5,24)}$= 0.50, P=0.77	$F_{(5,14)}$=0.62, P=0.69	$F_{(5,11)}$=0.43, P=0.81
10	M	$F_{(5,24)}$= 0.77, P=0.58	$F_{(5,24)}$= 0.45, P=0.81	$F_{(5,22)}$=2.41, P=0.07	$F_{(5,22)}$=0.12, P=0.99
12	M	$F_{(5,24)}$= 2.55, **P=0.050**	$F_{(5,24)}$= 3.44, P=**0.017**	$F_{(5,22)}$= 2.67, **P=0.049**	$F_{(5,19)}$=0.67, P=0.65

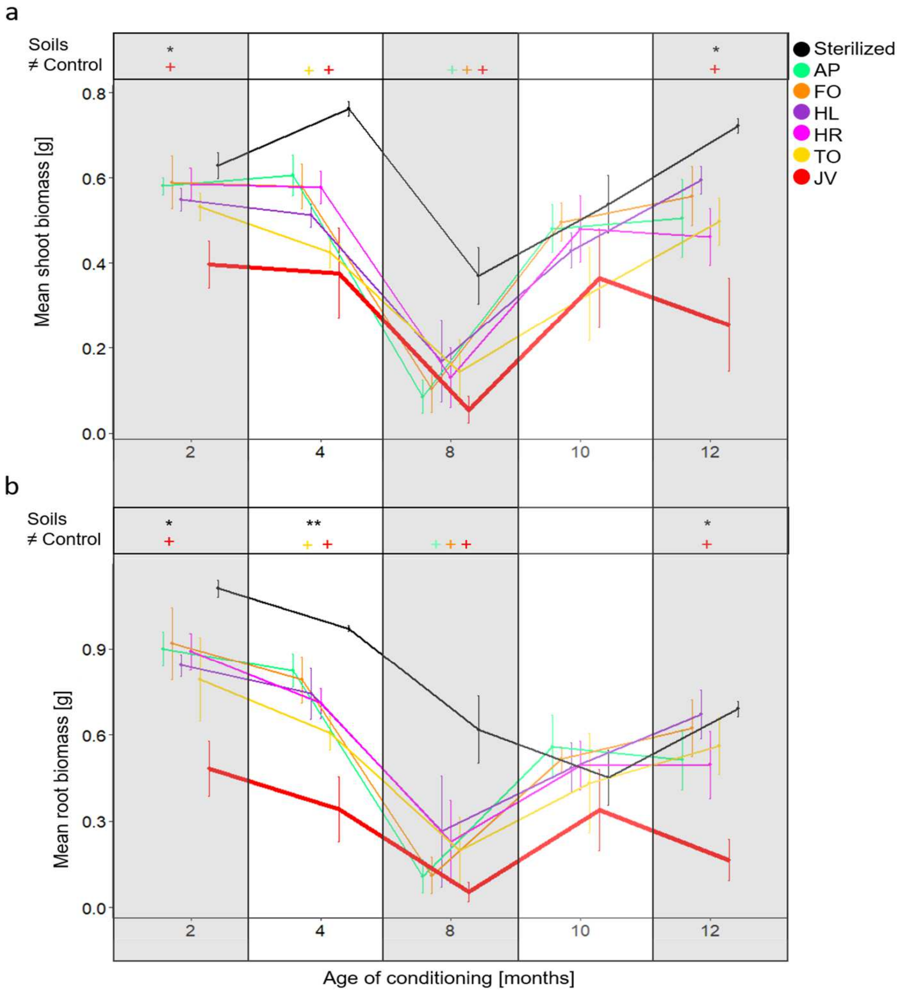


Figure 4.2 Mean (± SE) shoot (a) and root (b) biomass of *Jacobaea vulgaris* in monoculture soils collected over the course of one year (AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata* , TO= *Taraxacum officinale, JV= J. vulgaris* and in 100% sterilized soil (control). For each round, results of an ANOVA testing the effects of monocultures are also depicted in the figure (*p<0.05, **p<0.01). Plants grown in sterilized soil were excluded for this analysis. A Dunnet post hoc test, following a separate ANOVA was used for the comparison of each treatment with the100% sterilized soil was tested (P<0.05 is depicted as + with the colour indicating the species).

Herbivory

Weight gain and food consumption of *M. brassicae* varied greatly between rounds (Table 4.1) but there was no effect of monoculture soil (Fig. 4.3) except on weight gain in the 12 months round (Table 4.1).

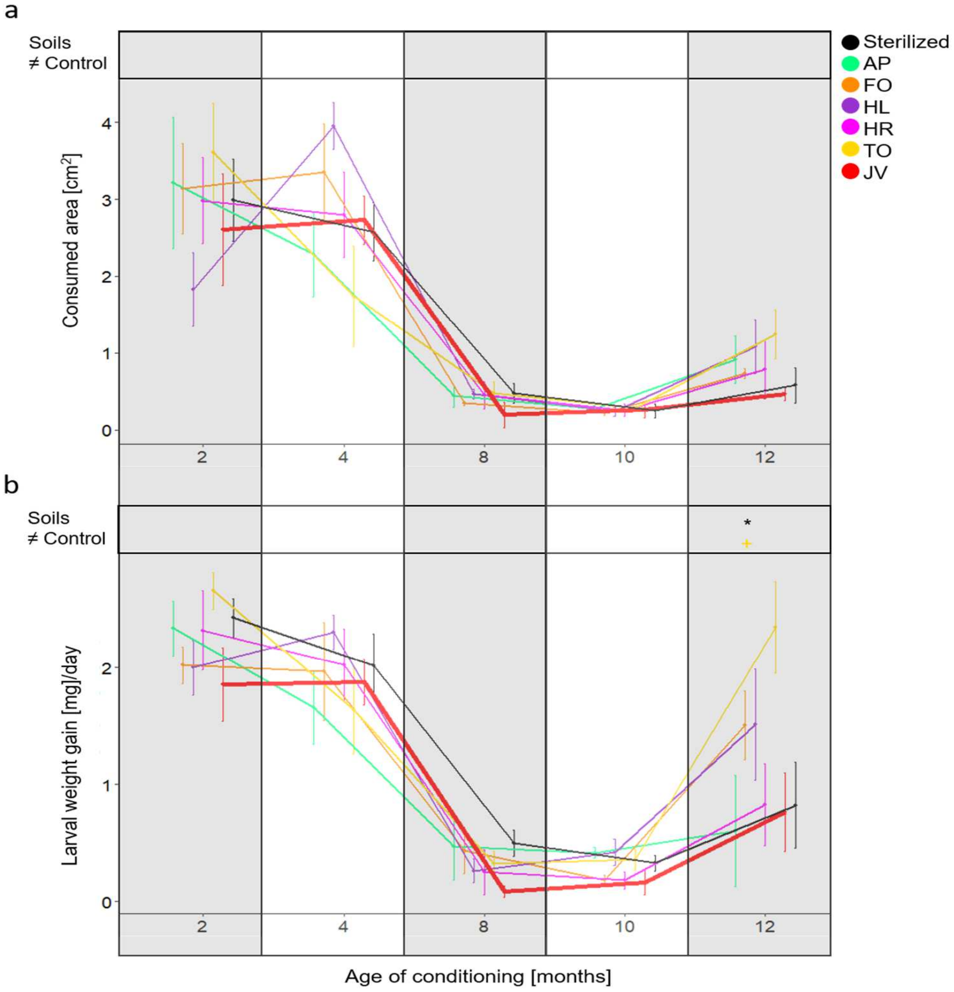


Figure 4.3 Mean (± SE) area consumed (a) and larval weight gain (b) of *M. brassicae* larvae on leaves of *Jacobaea vulgaris* grown in monocultures soils (AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata* , TO= *Taraxacum officinale, JV= J. vulgaris*) and in 100% sterilized soil (control) in each round. For each round, results of an ANOVA testing the effects of monocultures are also depicted in the figure (*p<0.05). Plants grown in sterilized soil were excluded for this analysis. A Dunnet post hoc test, following a separate ANOVA was used for the comparison of each treatment with the100% sterilized soil was tested (P<0.05 is depicted as + with the colour indicating the species).

Leaf metabolomes

The leaf metabolomes differed strongly among the three sampling rounds (Table 4.2 Fig. 4.4). The two month and eight month sampling rounds were separated most clearly along the first PCA axis, while the two and twelve month rounds were more similar in composition along the first axis but were separated along the second axis (Fig. 4.4). Separate PCAs for each round show that there is considerable variation in metabolome composition, but that the metabolomes of plants grown in sterilized soil varied less than those of plants grown in conditioned soils (Supporting information Fig. S4.3). The results of the overall PERMANOVA confirmed that metabolomes differed significantly between rounds - and were related to plant biomass, but did not differ between monoculture soils (Table 4.2). However, separate PERMANOVAs per round revealed that the soil effects were lower in the two month round than in the later rounds (measured as higher R^2; Table 4.2). The effect of total plant biomass on metabolomes also increased over time (Table 4.2). Phenolic compounds varied most strongly between the monoculture soil treatments, but formic acid was the only compound that responded consistently among rounds (Supporting information Fig. S4.4).

The variation in the composition of the metabolome was significantly related to the consumed area by caterpillars in the eight month round (Table S4.2).

Table 4.2. Results of permutational multivariate analysis of variance (PERMANOVA) testing the effect of monoculture soil (*Holcus lanatus, Festuca ovina, Alopecurus pratensis, Hypochaeris radicata, Jacobaea vulgaris, Taraxacum officinale*), round (2, 8, 12 months) and total plant biomass on the leaf metabolome of *J. vulgaris*. Further the effect of monoculture soils and plant biomass on the metabolome was tested for each round separately. The analyses are based on Bray-Curtis distances. Permutations were set to 999. Pseudo F-values, degrees of freedom (df), explained variance (R^2) and P-values are presented. Significant P-values are presented in bold.

Round	Factor	F	R^2	P
	Round (R)	$F_{(2,39)}=30.37$	0.35	**0.001**
	Monoculture (M)	$F_{(5,39)}=0.78$	0.02	0.58
	Total Biomass (B)	$F_{(1,39)}=35.83$	0.21	**0.001**
	M*R	$F_{(10,39)}=1.26$	0.07	0.28
	M*B	$F_{(5,39)}=1.28$	0.04	0.27
	R*B	$F_{(2,39)}=1.58$	0.02	0.21
2	M	$F_{(5,18)}=0.75$	0.12	0.63
	B	$F_{(1,18)}=1.70$	0.05	0.19
	M*B	$F_{(5,18)}=1.61$	0.26	0.15
8	M	$F_{(5,5)}=7.05$	0.29	**0.006**
	B	$F_{(1,5)}=57.15$	0.47	**0.001**
	M*B	$F_{(5,5)}=4.81$	0.20	**0.023**
12	M	$F_{(5,16)}=1.68$	0.22	0.16
	B	$F_{(1,16)}=11.42$	0.29	**0.001**
	M*B	$F_{(5,16)}=0.62$	0.08	0.77

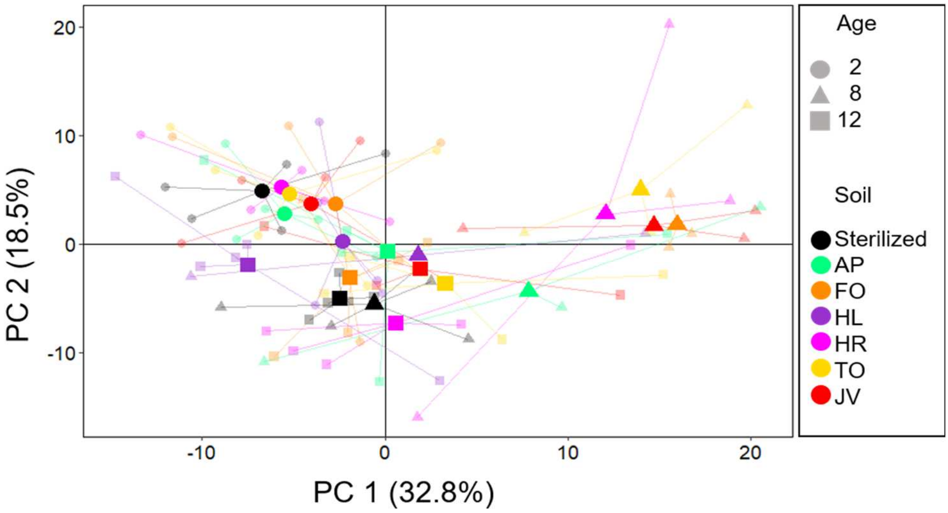


Figure 4.4 Principal component analysis (PCA) depicting the composition of the metabolome of *Jacobaea vulgaris* grown on in monoculture (AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata* , TO= *Taraxacum officinale,* JV= *J. vulgaris* and in 100% sterilized soil (control) in the different rounds (2 months circles, 8 months triangles, 12 months squares). Centroids connected to the sample scores of the replicates for the first two axes of an unconstrained principle component analysis (PCA) are presented. The percentage explained variance by each axis is also depicted. PCAs depicting metabolomic composition at each round are presented in Figure S4.3 of the supporting information.

Bacteria and fungi

Bacterial composition in the soil differed strongly between the three rounds but also between the different monocultures (Supporting information Fig. S4.5a Table S4.3). Fungal composition also differed strongly between monocultures and differed between the rounds (Supporting information Fig. S4.5b Table S4.3). The abundance of bacteria in the soil peaked at 6 months for five of the six species, but one month earlier in *J. vulgaris* monocultures. (Supporting information Fig. S4.6a, Table S4.4). Fungal copy numbers were higher in *T. officinale* and *J. vulgaris* monocultures after 6 and 12 months (Fig. S4.6b, Table S4.4).

Soil abiotic properties

Organic matter; iron (Fe) and sulphur (S) varied over time (Supporting information Fig. S4.7, Table S4.5). Potassium and to a lesser extent magnesium and phosphate availability was higher in monoculture soils of *J. vulgaris* and to a lesser for *T. officinale* soil than in soil conditioned by the other plant species (Supporting information Fig. S4.7; Table S4.5).

Relationships between soil and plant characteristics over time

Bacterial composition in the soil explained metabolome changes for the eight month round and this was true for fungi for the twelve month round (Table 4.3). The composition of both the bacterial and the fungal community significantly explained root biomass at the twelve month round and for the fungal community this was also true for shoot biomass. Soil abiotic characteristics did not explain metabolomic composition but were significantly related to larval weight gain after eight months and leaf consumption by *M. brassicae* after ten months (Table 4.3).

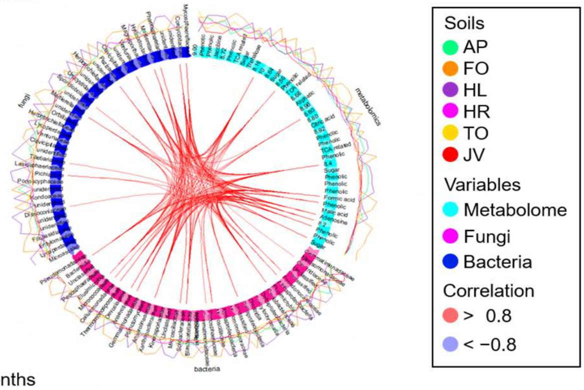
Table 4.3. Relationships between properties measured in the plant (shoot and shoot biomass, metabolome and insect performance) and soil characteristics (soil abiotic properties, bacterial composition, fungal composition, bacteria abundance and fungi abundance) overall and for each round. The relationships with metabolomes were analysed with coinertia analysis. For the coinertia analysis the RV coefficient and the significance tested with permutation tests (999 permutations) is presented. Relationships between univariate variables (abundance data) were analysed with Pearson correlations. The correlation coefficient is presented. All other relations were analysed with redundancy analysis (RDA). For the RDAs the explained variance and the significance tested with permutation tests (999 permutations) are displayed. *, ** indicate significant effects in the tests at $P < 0.05$; $P < 0.01$, respectively. Significant p-values are presented in bold.

Round	Variable	Abiotic characteristics	Bacteria community	Fungi community	Bacteria abundance	Fungi abundance
	Shoot biomass (SB)	**6.26*** **	**1.55*** **	**1.52** **	**0.18***	**-0.17***
	Root biomass (RB)	**4.11*** **	**2.62*** **	**1.81*** **	0.12	**-0.18***
	Metabolome (M)	0.14	**0.15***	0.11	0.02	0.03
	Larval weight gain (WG)	1.86	**1.89** **	**2.57*** **	0.15	-0.03
	Consumed area (CA)	0.90	**1.83***	**7.25 *** **	**0.27***	-0.14
2	SB	6.40[a]	3.40	4.16	0.27	-0.15
	RB	5.97[a]	3.10	4.42	0.33	-0.09
	M	0.14[a]	0.42	0.43	0.03	0.01
	WG	1.05[a]	3.53	3.68	-0.09	0.01
	CA	0.09[a]	3.35	3.57	0.23	0.06
4	SB	6.05			-0.24	**-0.44***
	RB	10.45			-0.48	-0.44
	M					
	WG	2.61			-0.17	0.01
	CA	3.79			-0.08	-0.22
8	SB	3.42	7.46[b]	7.06[b]	0.03	-0.03
	RB	2.73	7.37[b]	7.02[b]	-0.01	0.08
	M	0.26	**0.52***[b]	0.45[b]	0.03	0.06
	WG	**15.98***	6.81[b]	5.44[b]	-0.17	0.26
	CA	11.93	6.05[b]	6.76[b]	0.07	0.01
10	SB	12.48			-0,21	0.08
	RB	5.38			-0.22	0.10
	M					
	WG	2.62			0.08	0.15
	CA	**13.64***			0.08	0.05
12	SB	5.16	3.94	**5.27** **	0.01	-0.10
	RB	6.46	**4.08***	**4.64***	-0.01	-0.10
	M	0.18	0.35	**0.39***	0.03	0.07
	WG	6.06	3.66	4.07	-0.15	0.19
	CA	6.45	4.10	4.00	-0.23	0.12

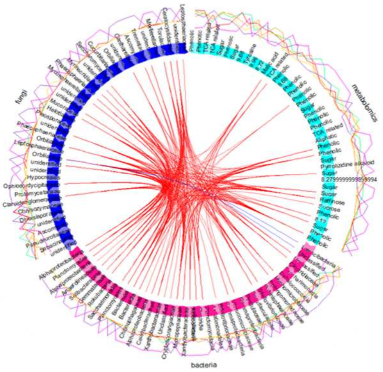
[a] Soil abiotics were not measured after 2 months and data collected for 4 months were used.
[b] Communities were measured in soil after 6 months of conditioning

The links between soil microbial communities and metabolomes varied between the three sampling rounds (Fig. 4.5). After two months we observed positive correlations between soil fungi, bacteria and metabolic compounds selected through sPLS. After eight months the number of positive correlations increased, while after twelve months there were fewer positive correlations and also negative correlations between fungi or bacteria and the metabolome appeared. The identity of metabolic compounds correlated with microbes varied between the rounds. However, in each round, changes in compounds related to the TCA-cycle such as malate and malic acid were linked to OTUs of bacteria and fungi. The patterns also varied between monocultures (Supporting information Fig. S4.8). Notably, there are more negative correlations between fungal OTUs and the metabolome for forbs than for grasses. While for most species the OTUs of fungi and the bacteria were linked to metabolome changes, for *H. lanatus* soil there were no links between changes in bacteria and the metabolome. For most monoculture soils there were more strong correlations between the metabolome and bacteria than between metabolome and fungi.

2 months



8 months



12 months

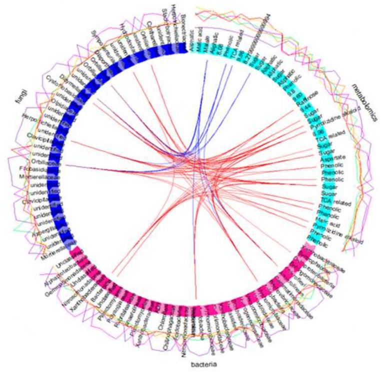


Figure 4.5 Circos plots visualising the correlation between bacterial and fungal communities in the soil and metabolomic changes in *Jacobaea vulgaris* plants grown in these soils for each round. Displayed are correlations that are higher than 0.8 (red) or lower than -0.8 (blue). Lines outside the circles show the concentration of each compound in the plants in six monocultures. For the bacteria and the fungi, the abundance of each OTU in each monoculture is displayed. Correlations within each measured community (metabolome, bacteria, fungi) are not depicted. Plant species abbreviations are AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata* , JV= *J. vulgaris*, TO= *Taraxacum officinale*.

Bacterial and fungal communities in the soil were strongly linked in all rounds, as depicted by the positive correlations in the correlation network (Fig. 4.6). In general, there were more strong positive correlations between the metabolome and bacterial OTUs than fungi. Strongest correlations were found after eight months. The correlations between fungi, bacteria and metabolome varied between monoculture soils (Supporting information Fig. S4.9). Notably, the number of negative correlations between bacteria and fungi in the soil and the metabolome was highest in soil of *J. vulgaris*. For soils conditioned by all species except *H. radicata* there were more positive correlations between the metabolome and bacteria than fungi.

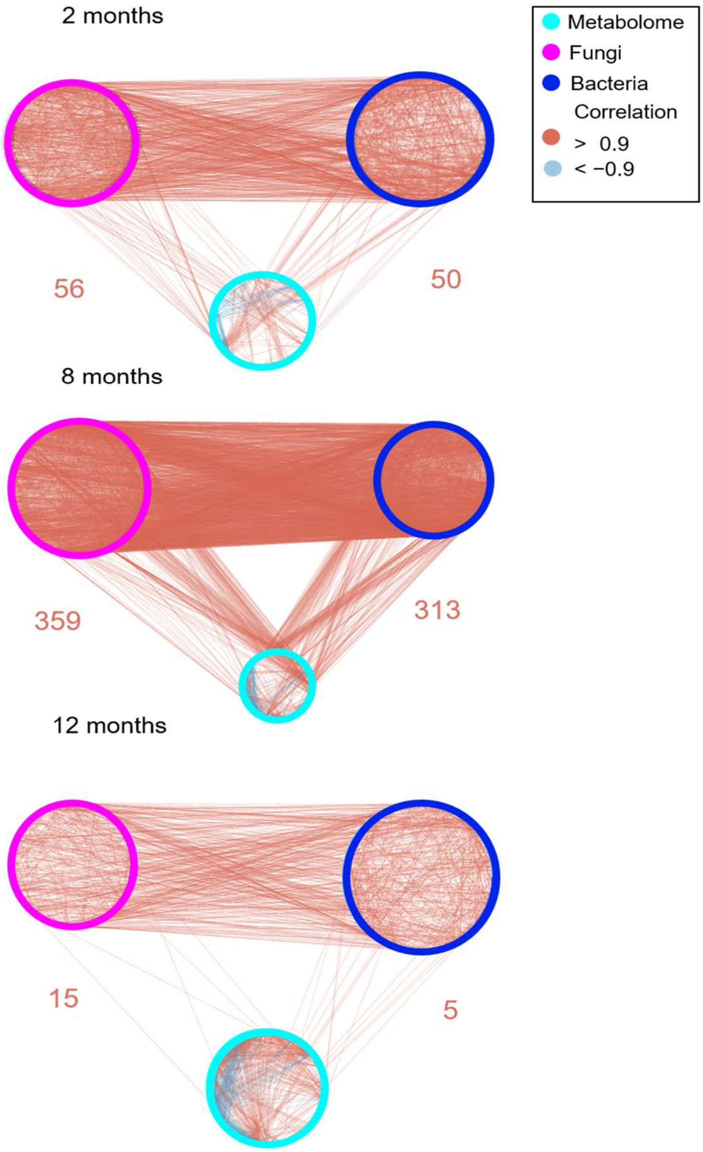


Figure 4.6 Correlation network between metabolomes fungi and bacteria for each round. Displayed are Pearson correlations between and within the metabolome of *Jacobaea vulgaris* and the bacterial and fungal communities in the soil. Depicted are correlations with a coefficient higher than 0.9 (red) or lower than -0.9 (blue).

We further investigated how changes in the metabolome are correlated to changes in the bacterial and fungal community in the soil using relevance networks (Supporting information Fig. S4.10, S4.11). These networks showed similar patterns as the circus plots and correlation networks. Fungal and bacterial links to metabolomic changes are strongest after eight months. The relevance networks were also constructed between the metabolomic changes and changes in soil abiotic characteristics in the soil. However, correlations between the metabolome and the abiotics were low and therefore none of the coefficients passed the threshold of 0.6.

Discussion

In this study we examined the growth pattern of *J. vulgaris* grown in sterilized soil and in soils conditioned by a conspecific and five heterospecific plant species for two, four, eight, ten and twelve months. Furthermore, we tested metabolomic changes in shoots of *J. vulgaris* caused by soils that were conditioned for different lengths of time and by different plant species. This enabled us to examine if soil legacies, in addition to influencing the growth of plants, also affects their chemical composition. At the same time, we tested if these potential metabolic changes lead to changes in the performance of the generalist herbivore, *M. brassicae*. Simultaneously we linked the potential causes of these metabolomics changes to bacterial and fungal communities, as well as the nutrients, in the soils which were used as inoculates. Our findings highlight two important aspects. First, although the magnitude of soil effects on biomass varies between different rounds of the same experiment, the patterns are generally very similar. Second, the influence of PSF on the metabolome is less stable between the different rounds of the experiment and is related to the time of conditioning. Metabolomic changes in the plants are linked stronger to microbial and fungal communities in the soils after eight months of conditioning than after two and twelve months of conditioning. Below these findings will be discussed in more detail.

Our results show that generally patterns of PSF on plant biomass stayed the same independently of age of the conditioning community. Biomass of *J. vulgaris* grown in soils collected at different times differed. In general, *J. vulgaris,* except after ten months of conditioning, showed the highest biomass above and belowground in 100% sterilized soil and the lowest in its conspecific soil. This increased biomass for plants grown in sterile soil was also found in multiple other studies (Joosten, Mulder, Klinkhamer, & Van Veen, 2009; Kos, Tuijl, De Roo, Mulder, & Bezemer, 2015a; Wang et al., 2019; Xue, Bezemer, & Berendse, 2018). Furthermore, similar to our findings other studies showed that *J. vulgaris* has a negative conspecific feedback (Bezemer et al., 2018; Van de Voorde, Van der Putten, & Bezemer, 2011). Our results now show that PSF is remarkably consistent, even if the conditioning plant community is aging. The strength of PSF moderately differs depending on the age of the community but the overall patterns stay the same over the course of a year. This is an important finding for PSF research since it highlights that the time of conditioning is not a major factor influencing the outcome of PSF experiments. We also showed that differences in the root biomass were more pronounced than differences in the shoot biomass. Biomass of the *J. vulgaris* roots decreased throughout the different rounds over the course of a year and did not reach the same biomass as in the first round of the experiment. Contrary to that, the shoot biomass of *J. vulgaris* in the feedback phase lowered until the age of 8 months and after that did reach the same magnitude again than in the beginning of the experiment. These findings strengthen the view that root biomass is more sensitive to PSF than shoot biomass. Other PSF studies also report this stronger effect of PSF on root than on shoot biomass (Kos et al., 2015a; Wang et al., 2019). This is probably caused by the proximity of the roots to the soil and therefore a more direct influence of the soil microbes on the roots than on the shoots. The species-specific differences of conditioned soils were less pronounced at the sampling at which the community was eight or ten months old, this might be because this sampling took place in early January and March. During winter, the functions of the microbial communities in the soil might get more similar since decomposition processes are getting dominant due to litter material which accumulated on the surface of the soil in the previous months. This could also be an explanation for the stronger links between the microbiome and the metabolome of *J. vulgaris* that we show in this study. In our study it is not possible to disentangle seasonal from time effects and future studies should address these points with long term experiments over multiple years.

We did not find any significant difference in the performance of *M. brassicae* feeding on the plants grown in differently conditioned soils except for the plants grown in soil inoculated with soil conditioned by *T. officinale* for twelve months, on which the daily weight gain was higher than on the plants in other experimental rounds. However, we did not find a relation between the metabolome composition and the herbivore performance after twelve months. Multiple studies have found effects of PSF on insect performance (Badri, Zolla, Bakker, Manter, &

Vivanco, 2013; Bezemer et al., 2013; Heinen, Biere, & Bezemer, 2019). In another study the abundance of the specialized aphid *Aphis jacobaeae* for example showed large differences on *J. vulgaris* grown on 10 differently conditioned soils (Kos et al., 2015b). However, a generalist aphid species (*Brachycaudus cardui*) did not show these differences in abundance due to PSF. This shows that the outcome of PSF studies investigating the herbivore reaction to PSF is highly variable depending on the used insect species, plant species and conditioning species. This might also be the reason that we did not find strong PSF effects on insect performance in our present study. Furthermore, a recent study showed that the biomass of *M. brassicae* fed on *T. officinale* grown in different soil legacies did differ if *M. brassicae* was feeding directly on the plant however in a detached leaf assay this was not the case (Hannula, et al., 2019a). This showed that the insects are directly affected by the soil microbiome. In our present study we only tested herbivore performance in a detached leaf assay and might therefore have missed legacy effects on insects which require contact with the soil.

The metabolome of *J. vulgaris* changed between the different rounds of the experiment and this occurred in conditioned soil but also in sterilized soil. While the metabolome of plants grown in sterilized soil was similar in the eight- and twelve-month sampling rounds, it was different at the two-month round. These differences can have various explanations. *J. vulgaris* might, although kept under the same conditions in a climate chamber without seasons, still have an imprinted seasonal rhythm which might lead to differences between seasons. To our knowledge there is no study testing these seasonal changes of metabolomes in plants growing in a controlled environment. However, some studies have demonstrated that in plants grown in natural conditions, abiotic properties are the best predictors of metabolomes (Peters, Gorzolka, Bruelheide, & Neumann, 2018; Sampaio, Edrada-Ebel, & Costa, 2016). Second, all sterilized soil used in the feedback rounds was irradiated at the beginning of the experiment and soil nutrient levels changed profoundly between the experimental rounds, but those changes do not match the patterns seen in the metabolome changes.

The causal agents for the observed PSF are mainly changes in community composition of bacteria and fungi of the soil over time. Soil abiotic conditions did not explain the patterns that were observed. We investigated the changes in the microbiome (bacteria and fungi) of the soils that were used as inoculates in the present study over the course of one year in Hannula et al. 2019a. In that study we could show that changes in bacterial communities were mainly explained by the time since the establishment of the plant community. Changes in fungal communities were mainly related to the plant species grown in the soil and their functional group (grass/forb). In our present study the biomass of *J. vulgaris* differed between the different rounds of experiments over time but in most rounds the species-specific effects on biomass stayed similar. Combined with the results of our previous study this points out that the metabolomic changes due to soils are mainly related to changes in bacterial communities in the soil which are mainly influenced by time. Our network analysis shows that the links between the metabolomic changes are stronger with bacterial OTUs than with fungal OTUs. PSF effects on biomass that vary but show similar patterns over time In our earlier study fungal communities in the soil mainly changed due to the plant species that was grown in the soil before. It could therefore be that the PSF effects on biomass are mainly triggered by fungi in the soil. Evidence for this in the present study however is weak since the fungal community of the soil only has a significant effect on the growth of the plants after twelve months of conditioning. However, at this time, the bacterial community also influenced plant biomass. We further found that the abundance of bacteria was highest six months after establishment of the communities. We found a similar pattern in the biomass responses of *J. vulgaris* with a drop in biomass after eight months of growth. Although the bacterial abundances were already lower again at eight months, the increase at six months could have led to changes in the soil that still influenced the growth of the plants at eight months. Higher bacterial abundances might go along with higher rates of exudation of the plants in order to maintain their microbial communities in the soils, this potential peak of exudation of the plants might have led to a chemical legacy in the soils, which we did not measure in this experiment, that led to a decrease of the biomass of *J. vulgaris* after eight months. Since the containers from which the soil samples in each round were collected were outside, we were not able to control for differences in herbivore loads of the different plant species in the different containers. A previous study has shown that above and belowground herbivory can influence soil legacies and the response of *J. vulgaris* to them (Wang et al., 2019). Therefore, it is possible that those potential differences in herbivore loads between species and the different rounds of experiments did enhance or decrease the effect of the inoculum on *J. vulgaris*.

In conclusion, we show that legacy effects of six different soils on above- and belowground biomass of a common focal plant differ in magnitude over time, but the overall response patterns stay remarkably similar over time. Interestingly, we find that plant metabolomic responses to soils are highly inconsistent and became more apparent in the later sampling rounds. This indicates that the effects of soil legacies on plant metabolomic profiles become more pronounced when the soil is conditioned for a longer period of time, which is in stark contrast with the relatively consistent responses in plant biomass. This strongly suggests that soil legacy effects may have farther-reaching impacts than plant growth alone. As plants are the primary resources for most organisms, soil legacy effects, through plant-metabolomic processes, may have lasting impacts higher in the ecological food web.

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Author contributions

M.H and T.M.B designed and planned the experiment. R.H, K.S, R.J, S.E..H and M.H carried out the experiments and kept the containers running for 1 year. M.H processed the metabolomics samples and analysed the results with T.M.B and Y.H.C. S.E.H. processed and analysed the sequencing data. M.H wrote the first version of the manuscript and all co-authors critically added to the manuscript.

Supporting Information

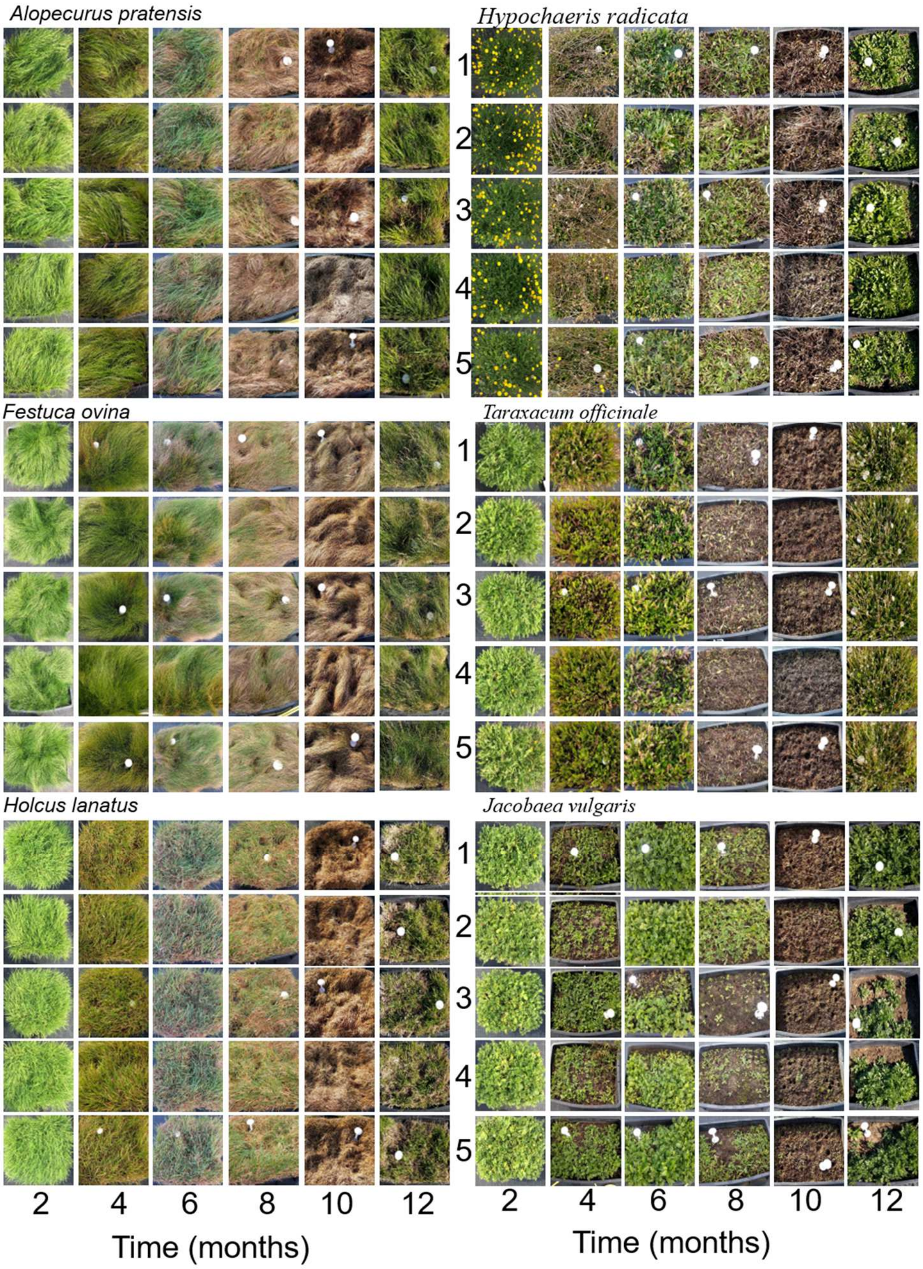


Figure S4.1 Photos depicting temporal aboveground changes in the monoculture vegetations. Numbers from 1-5 refer to the different replicates. The white marks in some of the photos are sensors which measured temperature and soil moisture.

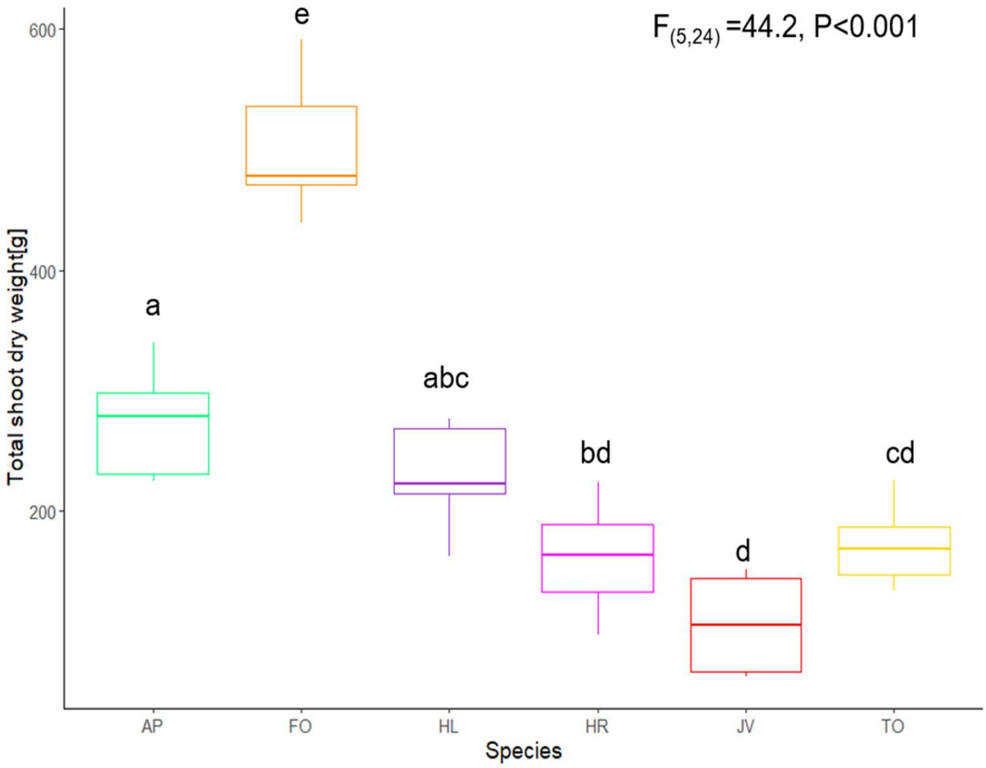


Figure S4.2 Mean shoot dry weight (±SE) of the different monocultures after 12 months (15 May2018). Tukey box-and-whisker plots display the median (horizontal line), the quartiles (box) of the data and the whiskers show all variation. Different letters above the bars represent significant differences ($p<0.05$) a Tukey HSD posthoc test. Plant species abbreviations are AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata*, TO= *Taraxacum officinale*, JV= *Jacobaea vulgaris*. Results of a One-Way ANOVA are also presented.

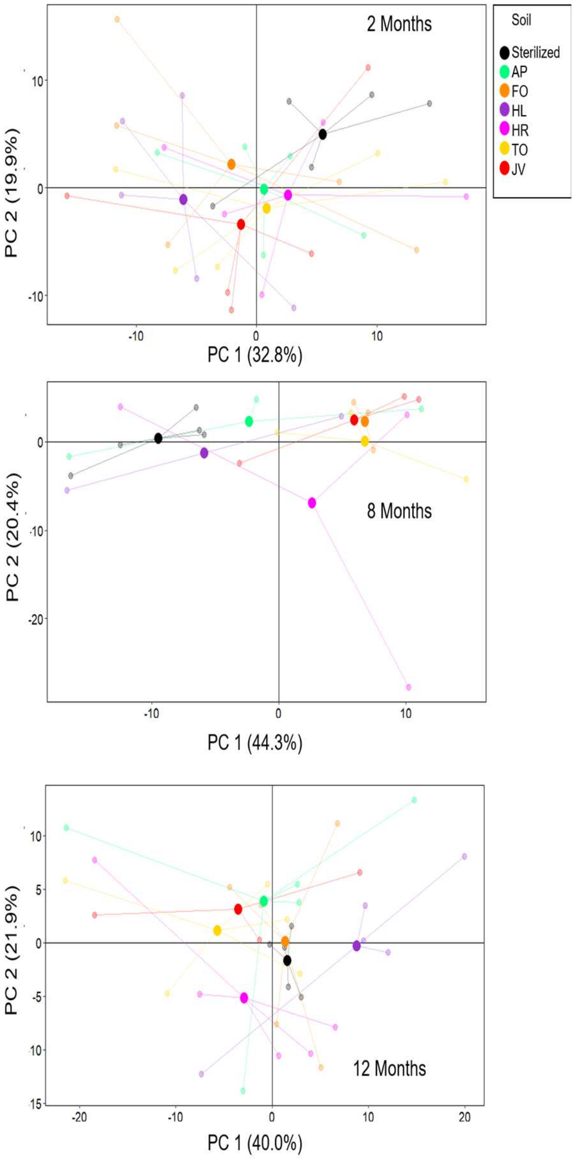


Figure S4.3 Composition of the metabolome of *Jacobaea vulgaris* grown in different soils (AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata*, TO= *Taraxacum officinale,* JV *J. vulgaris,* and 100% sterilized soil (control) at the three rounds. Centroids connected to the sample scores of the replicates for the first two axes of an unconstrained principle component analysis (PCA) are presented. The percentage explained variance by each axis is also depicted.

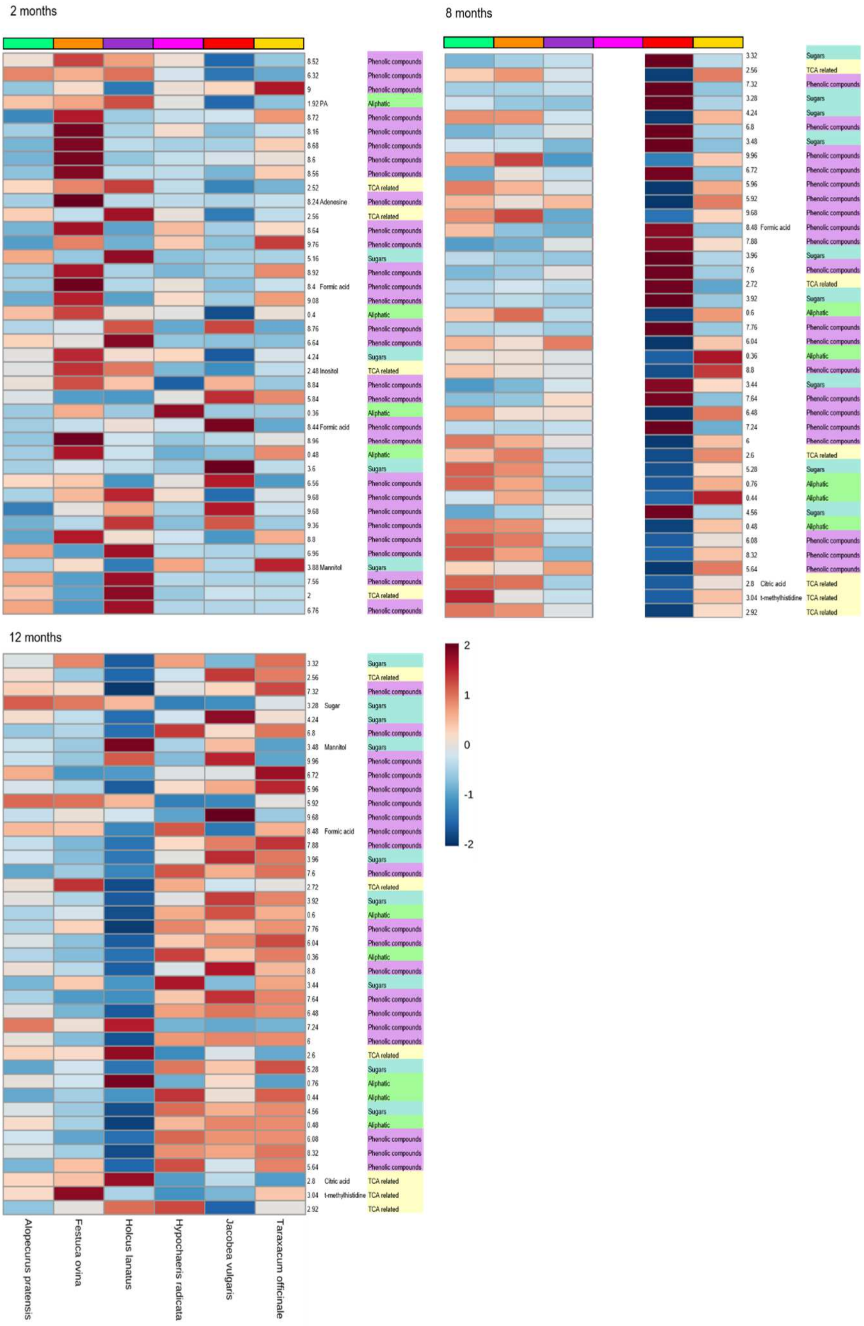


Figure S4.4 Heatmap showing the mean normalised intensity of the 40 buckets (rows) per timepoint (2, 8, 12 months) which varied most among the soils marked by colours above the heatmaps and indicated at the bottom of the heatmaps. Buckets were associated to compounds if possible and compound classes. The same compound classes are marked in the same colour.

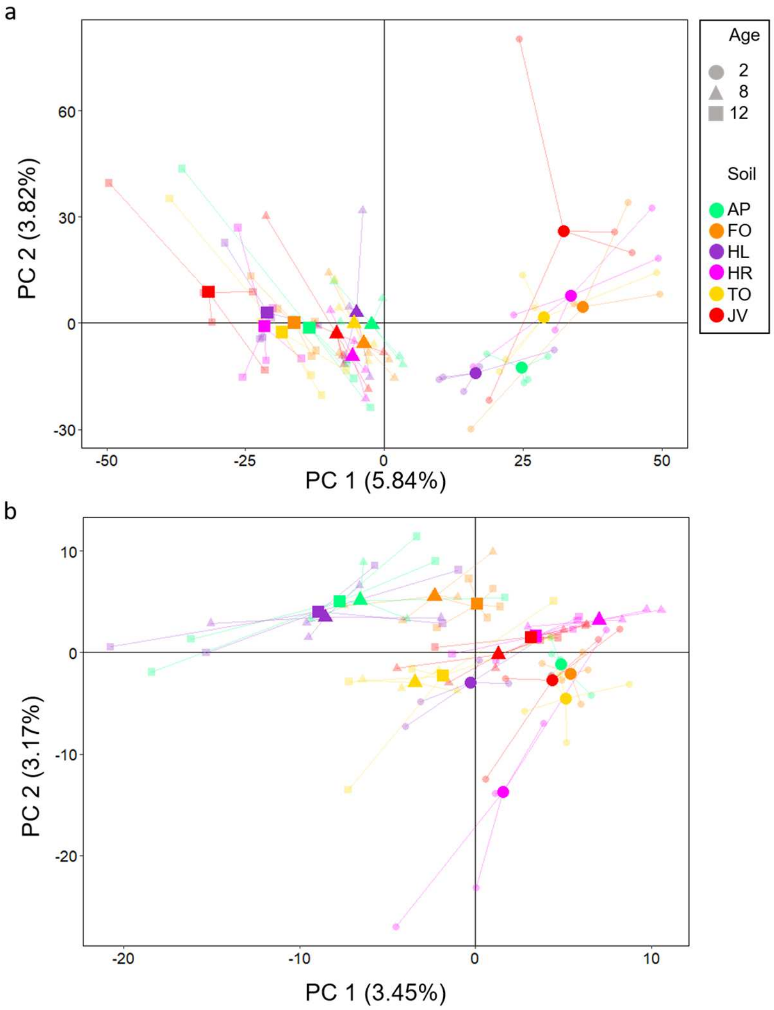


Figure S4.5 Composition of the bacterial a) and fungal b) communities in monoculture soils (AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata* , TO= *Taraxacum officinale,* JV= *Jacobaea vulgaris*) at the three rounds (different symbol shapes). Centroids connected to the sample scores of the replicates for the first two axes of an unconstrained principle component analysis (PCA) are presented. The percentage explained by each axis is also depicted.

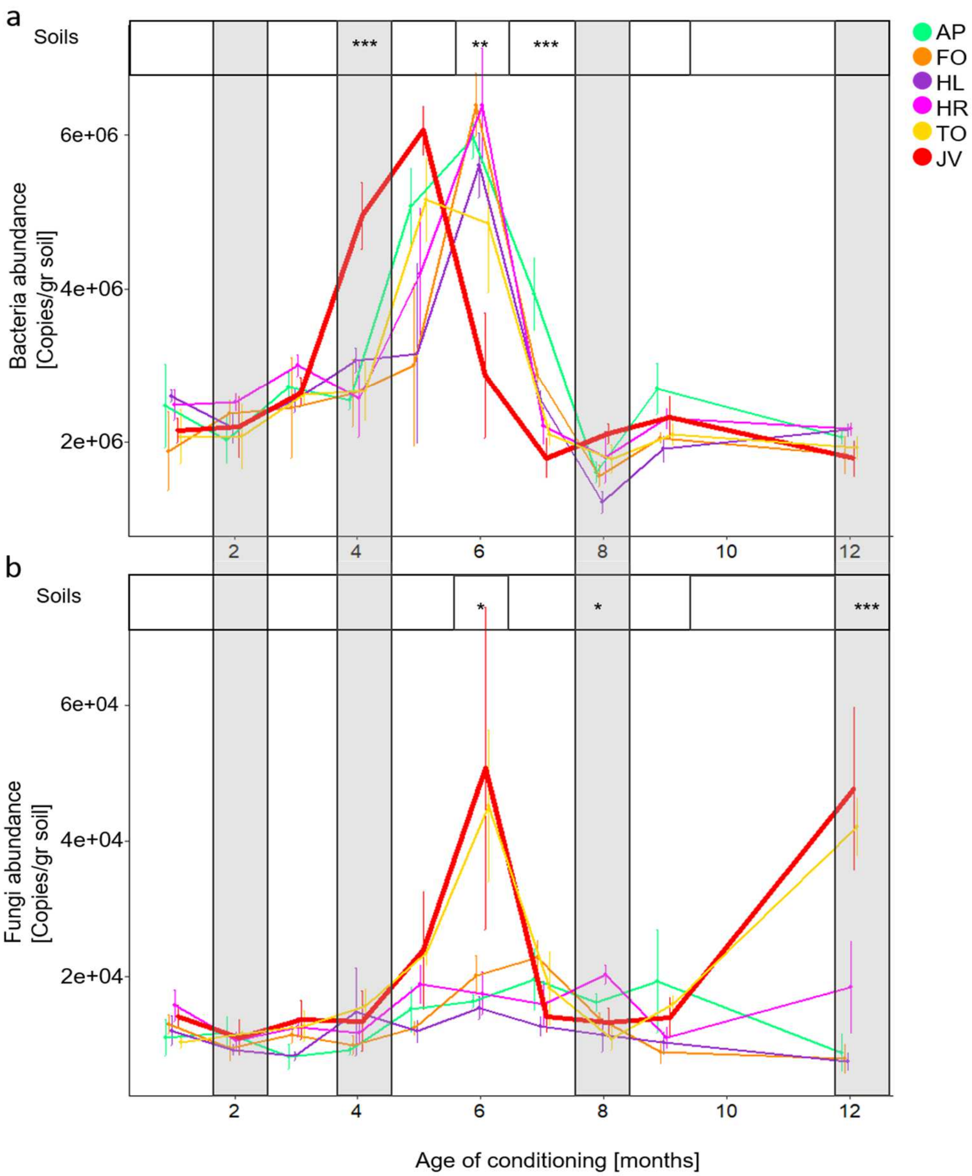


Figure S4.6 Mean temporal changes in bacterial (a) and fungal (b) abundance in the monoculture soils (copies per g of soil ± SE). AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata*, TO= *Taraxacum officinale* . *JV* = *Jacobaea vulgaris* at different timepoints (x-axis). Samples were collected monthly for nine months and after 12 months. For information, significance from ANOVA (Table S5) are depicted above the figures. *p<0.05, **p<0.01, ***p<0.001. Grey parts of the figures indicate sampling times that match with the PSF experiment.

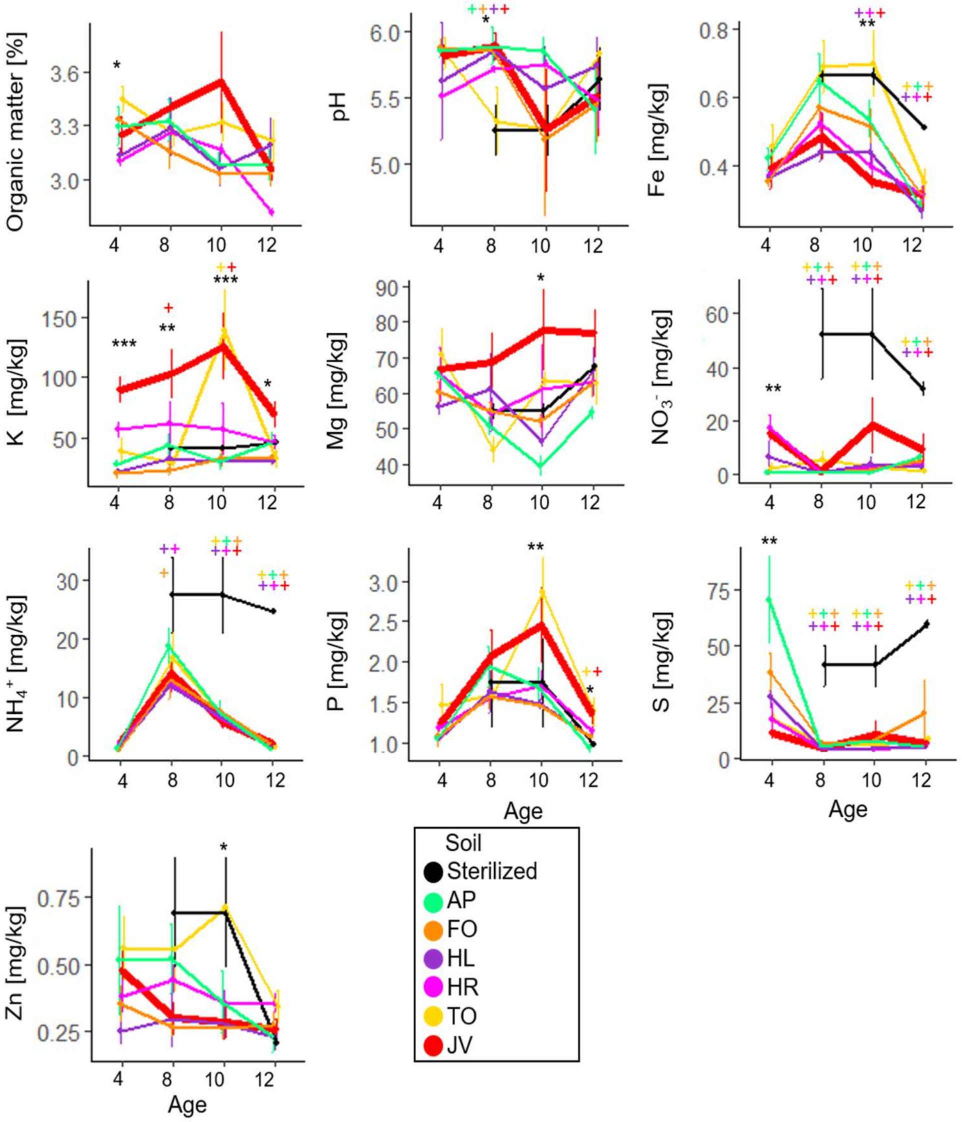


Figure S4.7 Soil abiotic properties measured over the course of the experiment. Points show the mean concentration [±SE] for the 5 containers per plant species (depicted by colours). Plant species abbreviations are AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata*, TO= *Taraxacum officinale*, JV= *Jacobaea vulgaris*. Control (100% sterilised) soil is depicted in black. Results of an ANOVA testing for differences between the conditioning species are indicated by asterisks * $p<0.05$, **$p<0.01$, ***$p<0.001$. Significant differences between the control and monoculture soil based on a Dunett post hoc are depicted as + with the colour indicating the species.

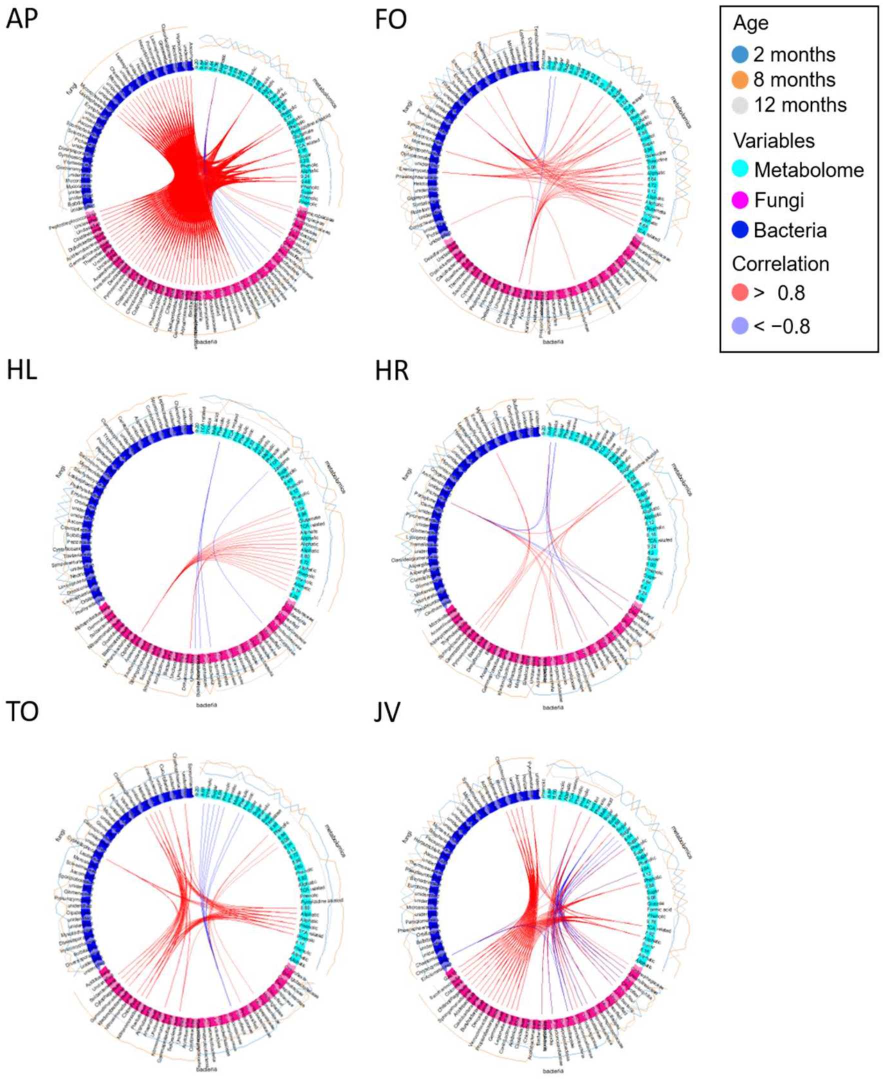


Figure S4.8 Circos plots visualising the correlation between bacterial and fungal communities in the soil and metabolomic changes in the plants grown in these soils for each monoculture. Displayed are correlations that are higher than 0.8 (red) or lower than -0.8 (blue). Lines outside the circles show the concentration of each compound in the plants in the three rounds. For the bacteria and the fungi, the abundance of each OTU in each round is displayed. Correlations within each measured community (metabolome, bacteria, fungi) are not depicted. Plant species abbreviations are AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata*, TO= *Taraxacum officinale*, JV= *Jacobaea vulgaris*.

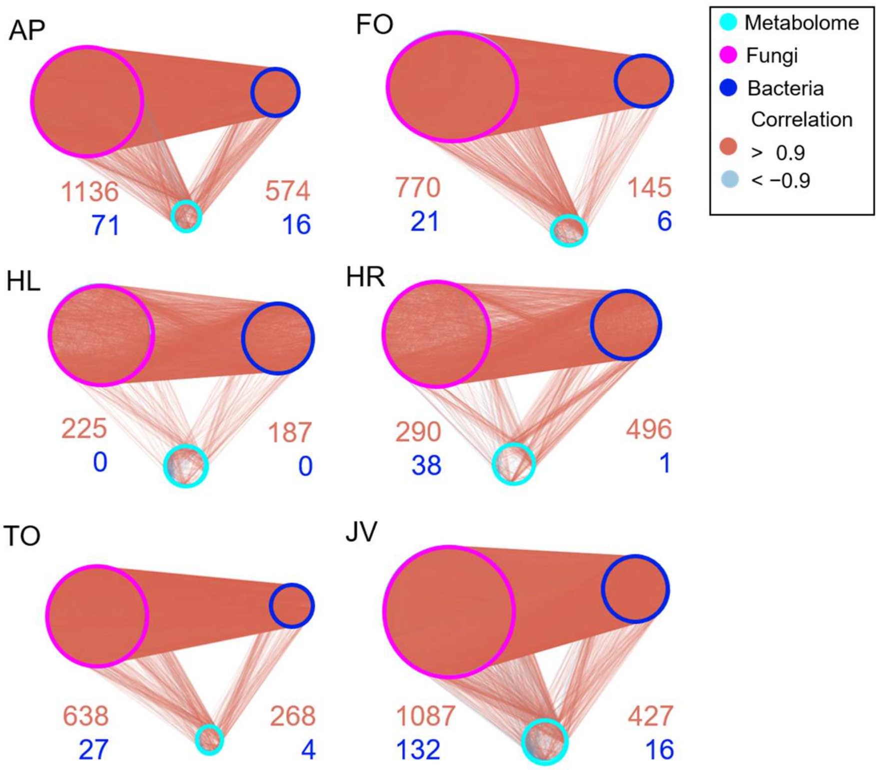


Figure S4.9 Correlation networks between bacteria and fungi in the soil and the metabolome of *Jacobaea vulgaris* for each monoculture separately. The networks are based on data from the three rounds (2,8,12 months) combined. Depicted are only correlations with correlation coefficients higher than 0.9 or lower than -0.9. Blue lines indicate negative correlations with correlation coefficients lower than -0.9 and red lines depict positive correlation with correlation coefficients higher than 0.9. Numbers depict the number of positive and negative correlations between bacteria or fungi and the metabolome. Plant species abbreviations are AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata*, TO= *Taraxacum officinale*, JV= *Jacobaea vulgaris*.

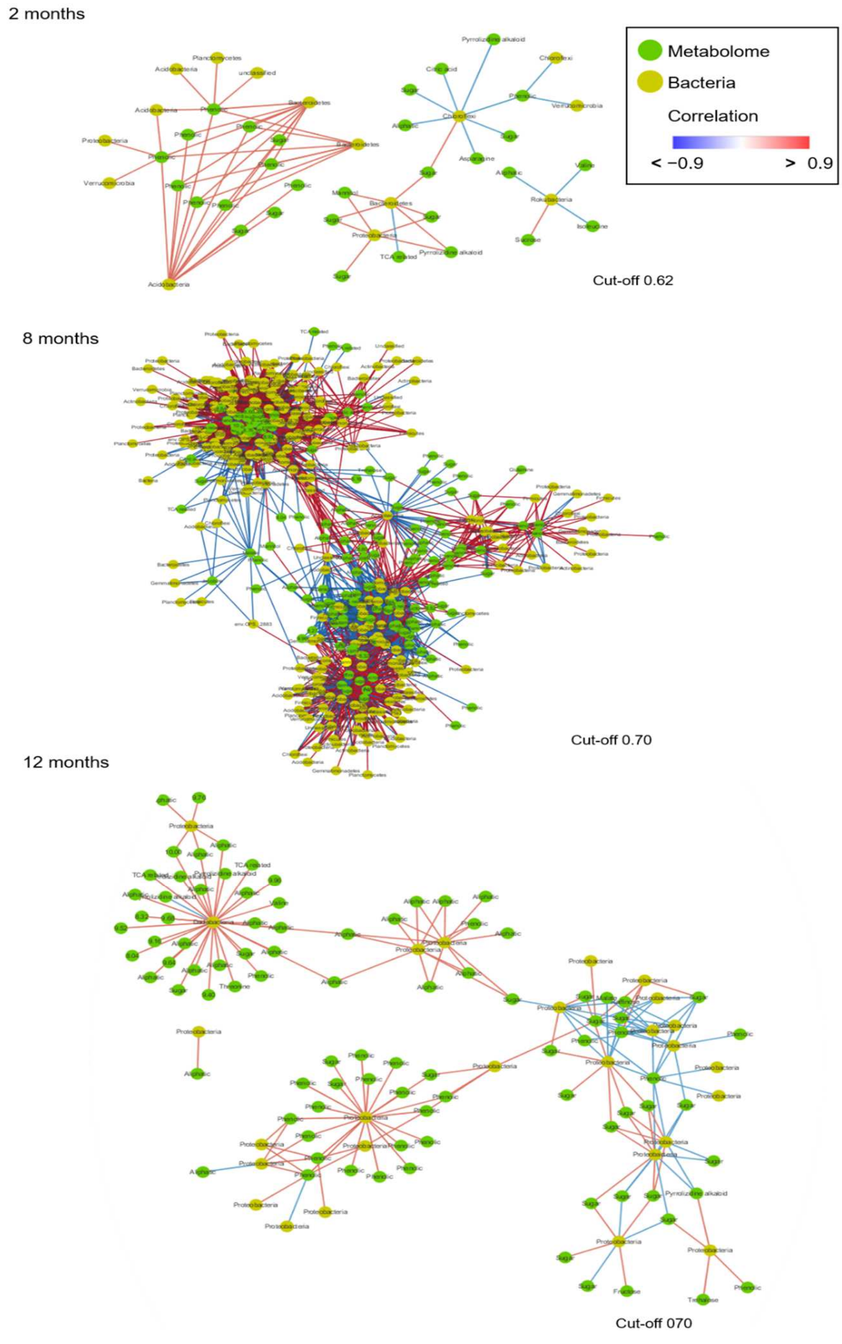


Figure S4.10 Relevance network visualising bacteria in the soil and compounds within the plant. The features used in this network were selected through Partial Least Squares (sPLS). Networks were constructed for each round separately. Each network displays correlations higher (red) or lower (blue) than the indicated cut offs (blue).

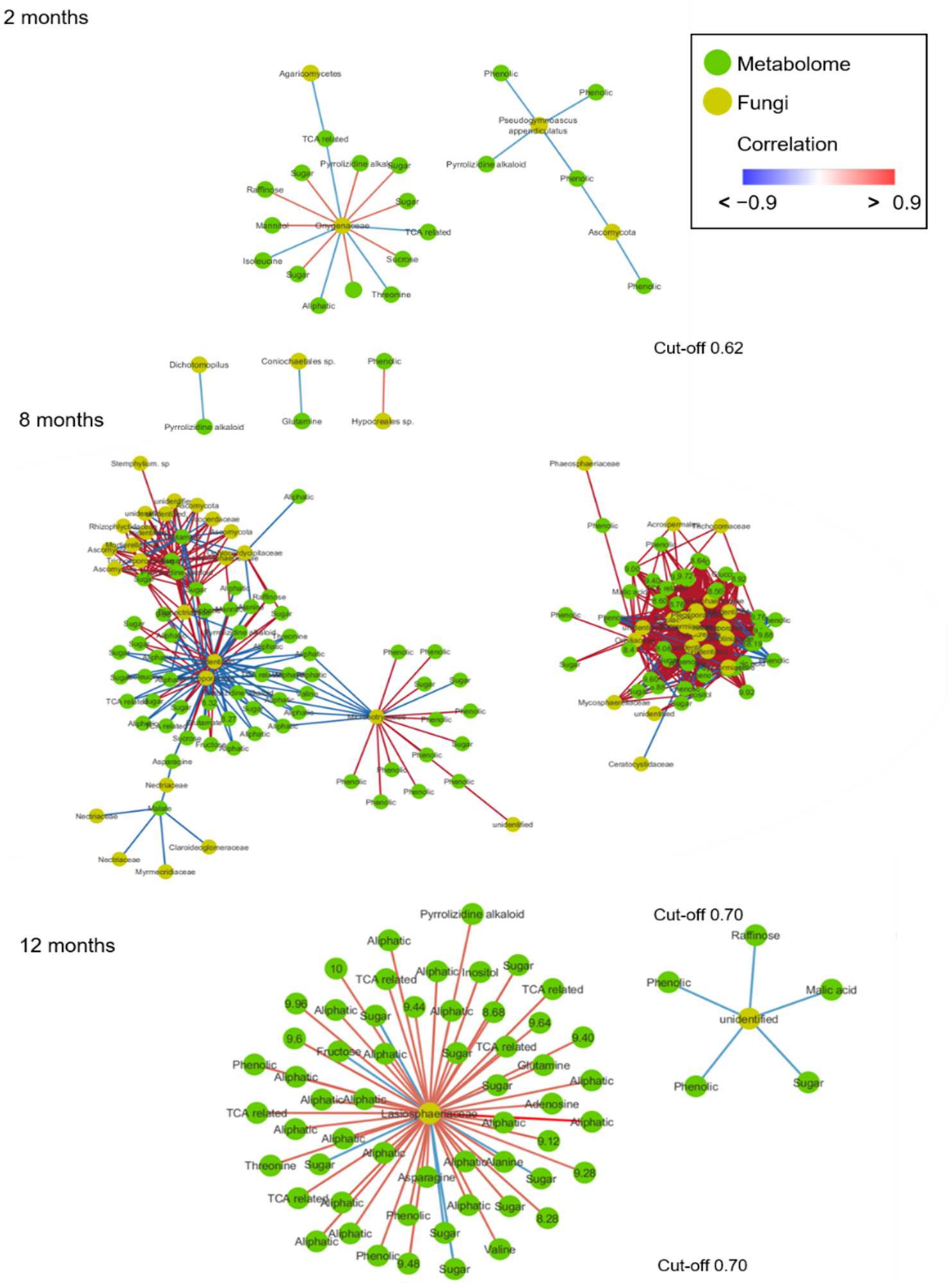


Figure S4.11 Relevance network visualising fungi in the soil and compounds within the plant. The features used in this network were selected through Partial Least Squares (sPLS) . Networks were constructed for each round separately. Each network displays correlations higher (red) or lower (blue) than the indicated cut offs (blue).

Table S4.1 Practical details about the experiment. Displayed are the dates of collection of the soil, planting of *Jacobaea vulgaris*, harvest for the plant soil feedback test and the collection dates of samples for microbial communities and abundance measurement.

	Plant soil feedback test			Microbial measurements	
Time	Collection of soil	Planting	Harvest	Collection of soil for microbial community	Collection of soil for abundance (qPCR)
1		No test		No sampling	01.06.2017
2	07.07.2017	17.07.2017	22.08.2017	03.07.2017	03.07.2017
3		No test		No sampling	03.08.2017
4	08.09.2017	20.09.2017	23.10.2017	No sampling	09.09.2017
5		No test		No sampling	09.10.2017
6		Discarded		07.11.2017	07.11.2017
7		No test		No sampling	07.12.2017
8	05.01.2018	12.01.2018	26.02.2018	No sampling	11.01.2018
9		No test		No sampling	06.02.2018
10	09.03.2018	16.03.2018	20.04.2018	No sampling	No sampling
12	06.05.2018	14.05.2018	18.06.2018	08.05.2018	08.05.2018

Table S4.2 Explained variance and the significance (permutation tests with 999 permutations) from a redundancy analysis (RDA) testing for the relationship between weight gain and consumed leaf area and the metabolome composition of *Jacobaea vulgaris* for all rounds together and for each of the three rounds separately. * indicates significance at P<0.05.

Round	Variable	Explained variance (%)
	Larval weight gain (WG)	2.04
	Consumed area (CA)	1.67
2	WG	2.97
	CA	4.27
8	WG	7.72
	CA	27.20*
12	WG	2.18
	CA	1.42

Table S4.3 Results of permutational multivariate analysis of variance (PERMANOVA) testing the effect of monoculture soil (*Holcus lanatus, Festuca ovina, Alopecurus pratensis, Hypochaeris radicata, Jacobaea vulgaris, Taraxacum officinale*) and round (2, 6, 12 months) on the bacterial and fungal composition of the soil. Further the effect of monoculture soils on the bacterial and fungal composition of the soil was tested for each round separately. The analyses are based on Bray-Curtis distances. Permutations were set to 999. Pseudo F-values, degrees of freedom (df), explained variance (R^2) and P-values are presented. Significant P-values are presented in bold.

		Bacteria			Fungi		
Round	Factor	F	R^2	P	F	R^2	P
	Round (R)	$F_{(2,68)}$=10.69	0.20	**0.001**	$F_{(2,69)}$=3.39	0.15	**0.001**
	Monoculture (M)	$F_{(5,68)}$= 1.81	0.08	**0.001**	$F_{(5,69)}$=5.28	0.09	**0.001**
	R*M	$F_{(10,68)}$= 1.13	0.10	0.10	$F_{(10,69)}$=1.83	0.16	0.10
2	M	$F_{(5,24)}$= 1.48	0.24	**0.001**	$F_{(5,24)}$=2.58	0.35	**0.001**
6	M	$F_{(5,23)}$= 1.37	0.23	**0.001**	$F_{(5,21)}$=3.02	0.42	**0.001**
12	M	$F_{(5,21)}$= 1.23	0.23	0.066	$F_{(5,24)}$=1.71	0.26	**0.001**

Table S4.4 Results of ANOVAs testing the effects of monoculture soil (*Holcus lanatus, Festuca ovina, Alopecurus pratensis, Hypochaeris radicata, Jacobaea vulgaris, Taraxacum officinale*) bacterial and fungal copies per gram of soil. Analysis were carried out for all rounds together and separately for each month. F-values, degrees of freedom (df) and P-values are presented. Significant P-values are presented in bold.

Time (months)	Variable	Bacteria (copies/gr soil)	Fungi (copies/gr soil)
	Round (R)	**$F_{(9,170)}$=12.93, P<0.001**	**$F_{(9,170)}$=4.43, P<0.001**
	Monoculture (M)	**$F_{(5,170)}$=3.08, P=0.002**	**$F_{(5,170)}$=4.34, P<0.001**
	R*M	**$F_{(45,170)}$=3.12, P<0.001**	$F_{(45,170)}$=1.24, P=0.17
1	M	$F_{(5,24)}$=0.64, P=0.68	$F_{(5,24)}$=1.04, P=0.42
2	M	$F_{(5,24)}$=0.41, P=0.84	$F_{(5,24)}$=0.40, P=0.84
3	M	$F_{(5,24)}$=0.38, P=0.86	$F_{(5,24)}$=1.89, P=0.13
4	M	**$F_{(5,24)}$=6.36, P<0.001**	$F_{(5,24)}$=0.54, P=0.74
5	M	$F_{(5,24)}$=2.27, P=0.08	$F_{(5,24)}$=2.57, P=0.05
6	M	**$F_{(5,24)}$=4.24, P=0.007**	**$F_{(5,23)}$=2.80, P=0.04**
7	M	**$F_{(5,24)}$=9.23, P<0.001**	$F_{(5,24)}$=1.14, P=0.37
8	M	$F_{(5,24)}$=2.36, P=0.071	**$F_{(5,24)}$=3.08, P=0.028**
9	M	$F_{(5,24)}$=1.85, P=0.14	$F_{(5,24)}$=1.54, P=0.21
12	M	$F_{(5,24)}$=1.04, P=0.42	**$F_{(5,24)}$=6.06, P<0.001**

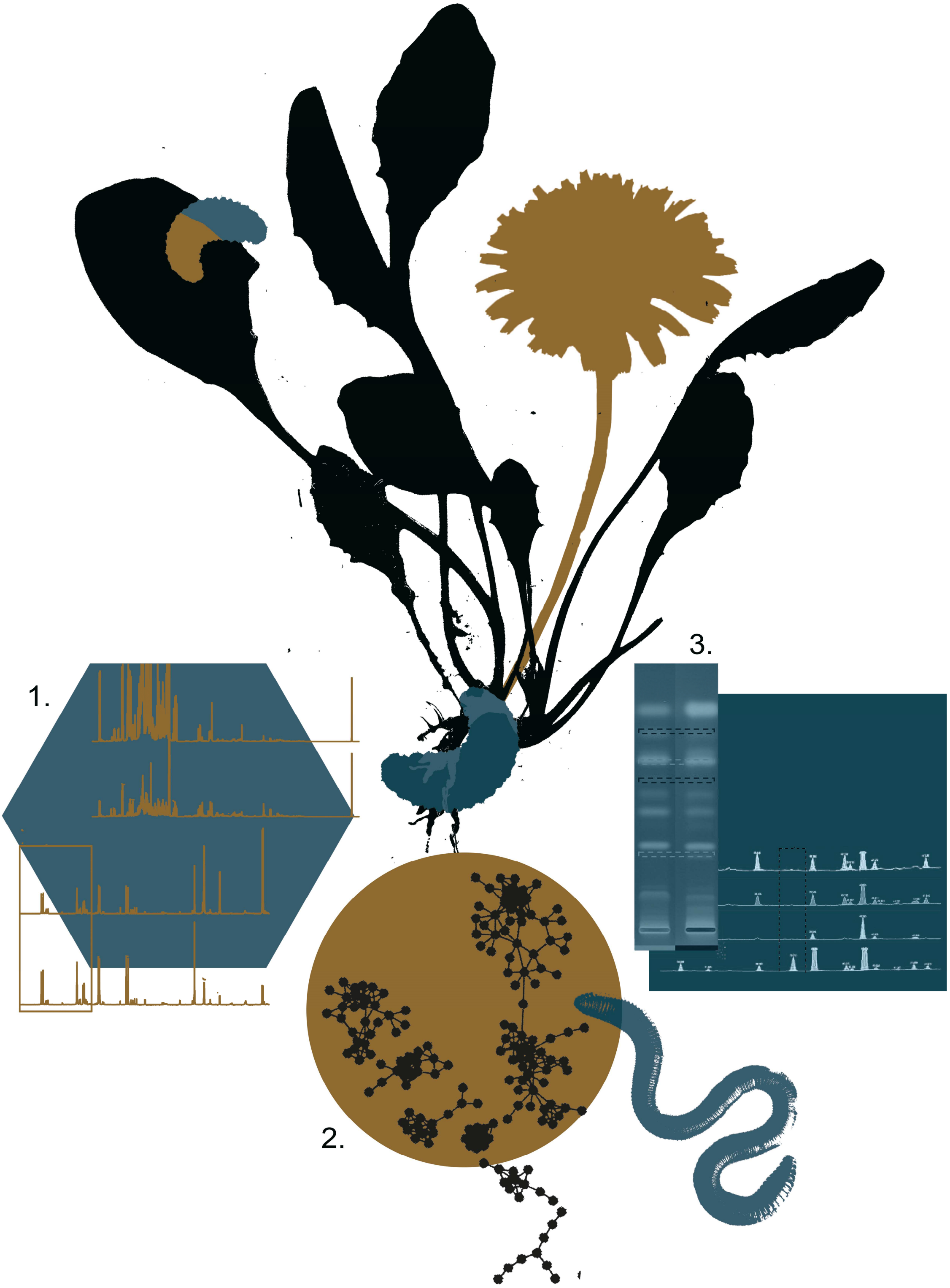
Table S5 Results of ANOVA testing the effects of monocultures on soil abiotic characteristic for each. All rounds were analysed combined and for each round separately. Depicted are F and p values. The degrees of freedom were (5,24) for all separate tests. For the overall test the degrees of freedom were round 4, monoculture 5 and for the interaction 20 with residuals of 90. Significant p-values are presented in bold.

Factors		Round (R)	Monoculture (M)	R*M	M	M	M	M
Round					4	8	10	12
OM (%)	F	**2.84**	**4.46**	**2.91**	**3.04**	0.66	2.48	2.21
	p	**0.03**	**0.001**	**<0.001**	**0.030**	0.66	0.062	0.088
pH	F	0.811	**3.00**	0.74	0.51	**2.91**	0.72	0.44
	p	0.522	**0.02**	0.77	0.77	**0.035**	0.62	0.82
Fe (mg/kg)	F	**12.51**	**2.46**	1.46	0.94	2.41	**4.93**	0.83
	p	**<0.001**	**0.04**	0.12	0.47	0.067	**0.003**	0.54
K (mg/kg)	F	0.23	**2.46**	**3.22**	**14.66**	**5.48**	**5.92**	**3.86**
	p	0.92	**0.04**	**<0.001**	**<0.001**	**0.002**	**0.001**	**0.011**
Mg (mg/kg)	F	0.15	0.89	1.06	1.12	2.06	**3.45**	2.13
	p	0.96	0.49	0.40	0.38	0.11	**0.018**	0.097
NO_3^-(mg/kg)	F	1.23	**2.85**	**2.64**	**4.56**	1.01	2.23	0.72
	p	0.30	**0.02**	**<0.001**	**0.005**	0.44	0.086	0.62
NH_4^+(mg/kg)	F	0.23	**9.11**	0.75	1.83	1.44	0.36	2.15
	p	0.92	**<0.001**	0.76	0.15	0.25	0.87	0.095
P(mg/kg)	F	0.98	**4.87**	**1.66**	1.68	1.12	**4.12**	**3.32**
	p	0.42	**<0.001**	**0.06**	0.18	0.38	**0.008**	**0.020**
S(mg/kg)	F	**2.79**	**6.74**	**3.60**	**5.66**	2.20	0.95	0.96
	p	**0.03**	**<0.001**	**<0.001**	**0.002**	0.088	0.47	0.46
Zn(mg/kg)	F	0.25	0.73	0.578	1.11	1.64	**3.85**	1.28
	p	0.91	0.601	0.92	0.38	0.19	**0.011**	0.30

Supplementary methods

Per container, for molecular analysis, six soil cores were collected (12 cm deep, 7 mm diameter) and homogenized. After that the soil samples were stored immediately at -20°C until they could be processed. 0.75 g or soil per container was weighed and extracted with the PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany). Then the DNA quantity was evaluated with a Nanodrop spectrophotometer (Thermo Scientific, Hudson, NH, USA). After that we used approximately 100 ng of DNA for the PCR. We used the primers ITS4ngs and ITS3mix targeting the ITS2 region of fungi (Tedersoo et al., 2015) and the primers 515FB and 806RB (Apprill, McNally, Parsons, & Weber, 2015; Caporaso et al., 2012; Parada, Needham, & Fuhrman, 2016) targeting the V4 region of the 16S rRNA gene in bacteria. The presence of PCR product was checked with an agarose gel electrophoresis. The PCR products were purified with Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA). With Nextera XT DNA library preparation kit set A & B (Illumina, San Diego, CA, USA), adapters and barcodes were added to the samples. Then the final PCR product was purified with AMPure beads and checked it with an agarose gel electrophoresis. It was then quantified with a Nanodrop spectrophotometer. Subsequently, the samples were equimolar pooled. All fungal samples (180) were pooled in one sequencing run. Bacterial samples were divided into two separate runs with 90 samples in each run. A mock community which contained ten fungal species as a control for the bioinformatics analysis, was used. Illumina MIseq PE 250 at McGill University and Genome Quebec Innovation Center was used to sequence libraries. Extraction negatives were used and further sequenced.

Quantitative PCR was used to determine the abundance of bacteria and fungi. qPCR was determined every month, up to 9 months and after 12 months (for sampling details see Supplementary Table S 4.1). To have similar amplification from all soils and to enhance the reaction T4 Gene 32 protein (Roche, Basel, Switzerland) was used. With a robot (Gorbett Research, Sydney, Australia) the reaction mixtures were dissolved in 20 µl volume that contained 0.3 µM of each primer, 0.25 µl T4 and 1.0-10.0 ng template DNA. The cycling conditions for fungi were: 40 sec at 95 °C, 1 min at 55 °C and 1 min at 72 °C and for bacteria 40 sec at 95 °C, 1 min at 53 °C and 1 min at 72 °C. Samples were analysed with a Rotor-Gene 3000 machine (Gorbett Research, Sydney, Australia). A serial dilution of plasmids extracted from the Rotor-Gene 3000 machine was used as reference to calculate the copy numbers. Part of these data was already published in a previous paper of our group (Hannula, et al., 2019a).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Chapter 5

Herbivory effects on the metabolome of *Taraxacum officinale* monitored in multi analytical platforms and their quality comparison

Martine Huberty, Lina M. Bayona, T. Martijn Bezemer, Young Hae Choi

Manuscript

Abstract:

Organisms interact with a multitude of environmental factors in their surroundings. These interactions are influenced by chemicals within the organisms, which are highly diverse. Conventional targeted chemical analysis can only describe a limited fraction of this diversity. Systems biology offers a suitable framework to explore the influence of these factors on the state of an organism. Metabolomics, in systems biology, aims to monitor the metabolome to reveal the state of organisms. The ultimate goal of metabolomics is the extraction, detection and identification of all metabolites within an organism. To overcome the limitation of a single analytical platform, it was suggested to combine various analytical platforms and integrate them. Prerequisite for this, is a thorough understanding of the individual methods, e.g. coverage and sensitivity of metabolites, which allow to choose the most suitable method. Here we analysed the response of *Taraxacum officinale,* as a model plant, towards herbivory with multiple analytical platforms and compared the outcome. With ^{1}H NMR we show that primary metabolites, e.g. sugars and amino acids, changed upon herbivory. Some abundant secondary metabolites such as flavonoids were detected, but identification of individual compounds requires a further step. We then analysed the samples by LC-MS to clarify changes in secondary compounds of plants in response to herbivory. With molecular networking based on LC-MS-MS spectra we found that mainly compounds within the groups of lipids and lipid-like molecules and phenolics were changed. In the last step, we used HPTLC as a supplementary tool to aid identification of metabolites. The metabolite that changed most was isolated and further identified as campesterol by GC-MS. In conclusion, we show that ^{1}H NMR is suitable to investigate the response of plants but mainly for metabolites present in high concentrations. LC-MS can be used to investigate changes in structurally-diverse low concentrated secondary metabolites. HPTLC offers a cost and time effective method to investigate targeted groups of compounds and can be used as preparative tool to concentrate certain compounds which would be present in too low concentrations otherwise. Up to today one optimal method for metabolomics does not exist and different methods give different insides which all together improve our understanding of how the metabolome changes.

Key Words-Ecometabolomics, integration, analytical platforms, HPTLC, ^{1}H NMR, LC-MS, GC-MS.

Introduction

All living organisms continuously interact with others in their surrounding or even within or on their own body. Most interactions are triggered or mediated by chemicals released or contained within an organism (Dyer et al., 2018). Especially for sessile organisms, such as plants, these are far more essential and inescapable. Basically, plants interact through any tissue with their surroundings, both above and belowground, therefore the chemicals involved in plant interactions can also maintain communication within the plant, transmitting signals from below to aboveground tissues and vice versa. These triggered interactions can lead to a whole cascade of physiological changes and influence bioprocessing revealed in genes, proteins and metabolites (Huberty et al., 2020; Zhu et al., 2018) .

As soon as systems biology emerged, attempts were initiated to analyse the profound impact of those interactions. Along with genomics, transcriptomics, and proteomics, metabolomics, as a part of systems biology, has its own contribution to this, aiming at a comprehensive characterisation of the metabolome of an organism at a certain stage in a specific condition (Stobiecki and Kachlicki, 2005). In particular, metabolomics can serve as a powerful tool to unveil environmental effects on organisms by investigating the final products of gene expressions. Despite recent efforts in the development of methodologies, the high diversity of compounds within a metabolome is still one of the biggest challenges in modern metabolomics. Even though the goal of any metabolomics investigation is comprehensive profiling, the outcomes usually contain a paucity of identified metabolites.

In a recent trend of metabolomics, multiple analytical platforms are implemented to address specific biological questions in an integrated manner, admitting that every metabolomics platform has its own advantages and disadvantages in terms of sensitivity and coverage. In general, the most common methods in metabolomics analysis are liquid chromatography coupled with mass spectrometry (LC-MS), gas chromatography coupled with MS (GC-MS) and nuclear magnetic resonance spectroscopy (NMR) (Peters et al., 2018). Each of them has its own realm for detection and identification of metabolites. Despite various positive properties of LC-MS, regarding its sensitivity and powerful analytical features to identify secondary metabolites, its low data-reproducibility, difficulty of ionization of certain metabolic groups, and low reliability of the databases are thought to be barriers to achieve a real metabolomics goal. Another MS-based technology, GC-MS, has a specialty for the analysis of primary metabolites *vis-á-vis* LC-MS ones. This leads to a preference in the field of metabolomics to use GC-MS as the first choice of method when details of amino acids, sugars and organic acids are of interest.

Inherently, GC-MS works require a derivatization to increase volatility of the samples to go through the columns at high temperature, in which some labile metabolites could be degraded. The greatest drawback of GC-MS is its limitation of molecular weight of metabolites to 600 – 700 MW.

Compared with the MS-based methods, NMR, particularly ^{1}H NMR, covers a broad range of metabolites for detection – especially, every proton in organic molecules no matter the position can be easily detected by ^{1}H NMR. This easiness facilitates any kind of overall metabolic description, especially of organisms for which there is no prior knowledge about the compounds in the organism. However, it is still confined to a rather rough metabolic profiling tool owing to its low sensitivity and large dependence on spectral databases for identification. Although the sensitivity has been improved recently, e.g. by cryo (cold)- and/or micro-probe, in the case of the requirement of spectral database there are not yet reliable databases, particularly for the large number of secondary metabolites. ^{1}H NMR therefore offers a great tool to analyse unknown samples and get a general overview of the metabolome. However, for complex mixtures it often suffers from a strong overlap of signals caused by the complexity of samples. This makes the identification of specific metabolites difficult. An overview of the strengths and weaknesses of all mentioned methods is found in Table 5.1

Table 5.1. Strength and weaknesses of each of the used metabolomic methods.

Method	Strength	Weakness	Metabolic Coverage
^{1}H NMR	• Broad range of compounds can be detected • Absolut quantification possible • High reproducibility	• Identification of compounds in mixtures requires experience • Low sensitivity -high detection limit • Low concentrated compounds (small peaks) often overlapped by other peaks.	• High concentrated metabolites (μmol level)
GC-MS	• Simple identification based on database • High sensitivity	• Samples need to be heated and derivatized • Required internal standards for quantification • Difficulty in the detection of glycosides	• Primary metabolites • Volatiles • Limitation to molecules with MW not over 600-700 MW
LC-MS	• High sensitivity • Simple detection of MW	• Required internal standard for quantification • Low reproducibility • Difficulty to build up database	• Most of secondary metabolites • Limitation of the detection of non-polar metabolites
HPTLC	• Low costs • Parallel analysis • Broad coverage of metabolite detection	• Low resolution • Semi-quantitation	• Most of metabolites can be detected by various chemical reactions

There have been many attempts to combine multi-analytical platforms for metabolomics research. In present-day metabolomics research, there are two kinds of approaches to this attempt: mathematical or statistical integration of different analytical results carried out in parallel analyses and targeting distinct groups of compounds with different methods. Recently, several publications have attempted to combine NMR and MS analysis to obtain a broader coverage of compounds which can be detected and identified. For this, samples were first analysed with ^{1}H NMR, TOCSY, HSQC and then with LC-MS and the data matrixes received by both methods were then combined with statistical tools such as correlation analysis (CoNaM) (Li et al., 2019). One example for these applications is a method called statistical heterospectroscopy (SHY) with which NMR and LC-MS can be combined and this was used in multiple studies to identify biomarkers in biological samples (Crockford Dj et al., 2006). The application could extend to other combinations like GC-MS and LC-MS data which can be fused and analysed as one data matrix (Tikunov et al., 2010). However, this statistical connection of two methods comes with certain limitations. Information might be lost during the process of combining data from different methods and the individual strengths of each method might be missed or diluted. Especially the combination of MS data and NMR data can be difficult since the sizes of the data matrixes differ strongly. The weight of the MS data on the outcome of the analysis might be higher due to its bigger size compared to NMR data.

Another way to exploit the potential of the different techniques available for metabolomics is to use each platform for the identification of specific compounds and use the individual strength of each method. Previously, (Kusano et al., 2011) assessed the diversity of the metabolome of genetically modified tomato plants and used for primary metabolites detection GC-MS, and for polar secondary metabolites LC-MS and for ionic compounds CE-MS. In another study they used as untargeted approach UPLC-FTMS and as targeted approach NMR for sugar amino acids and organic acids, LC/BFI-MS/MS for acylcamolines glycerophospoholipids sphingolipids and HPLC-UV for phenolics, LC MS/MS for alkaloids to investigate the metabolome of 20 taxonomically related benzylisoquinoline alkaloid-producing plants (Hagel et al., 2015). All these studies showed that the coverage of metabolites identified increases if certain targeted platforms are used.

Previous studies have therefore shown that the combination of platforms can yield a higher coverage however the limitations of these studies are that often only MS based platforms were used and therefore these studies often neglect the potential changes in primary metabolites. In this study we aimed to examine the feasibility of different metabolomics tools for the model plant *Taraxacum offincale.* Although some studies have investigated which method would be best used for which part of the metabolome, for our model species it was still unclear which compounds we could exactly identify by each method and how efficient each method is to discover differences between plants grown when exposed to stress. Herbivory is such a stress that is known to have a strong effect on the metabolome of plants (Papazian et al., 2019) and it therefore represents an ideal treatment to investigate the combinations of the platforms in our laboratory.

In a previous study where we grew plant species in sequential orders, we detected that the metabolome of 12 tested species greatly changed depending on which plant grew in the soil previously and whether the test plant was exposed to herbivory (Huberty et al. 2020). The treatments mainly influenced primary metabolites such as amino acids and sugars inferring that primary metabolites are essential for plant adaptations to these stresses or changes in the soil. Most secondary metabolites could not be attributed to the treatment of soils and herbivory even though major proximate roles of them are linked to a plant's survival and fitness upon environmental challenges. This might be because a single analytical tool (^{1}H NMR in the case) could pose a barrier to the detection of the low-concentrated secondary metabolites.

Taraxacum officinale was chosen as model plant for the current study since it is globally distributed and occurs in many different ecological systems (CABI, 2020) and it is often used in ecological studies. Furthermore, in our previous study, it was one of the plant species that showed the strongest reaction in its metabolome towards herbivory, and it has a high metabolic diversity and therefore a high potential to react to environmental stresses in different molecular ways.

In this study we investigated the metabolomic changes in *T. officinale* due to herbivory by *Mamestra brassicae*. *Mamestra brassicae* is a generalist herbivore, native to the palearctic and can feed on a wide range of plant families and species (Rojas et al., 2000). It is commonly used in ecological studies.

To get a broad overview of the metabolomic differences between the samples we used ^{1}H NMR. ^{1}H NMR offers a great tool to get an overall impression of the metabolomic changes in the plants and therefore represents a good first step for studies that investigate ecological interactions which influence on the metabolome was not investigated before. If this overall metabolomics approach shows that the metabolomes differ it is worth to further investigate the differences. The next step in this study was then to use methods for a more detailed metabolomics analysis. The samples were analysed with LC-MS/MS for secondary compounds and we used the ^{1}H NMR data to investigate changes in primary metabolites. LC-MS/MS was chosen as a tool to identify secondary compounds due to its high sensitivity. Finally, targeted analysis for more identifications of compounds were carried out. For this we combined LC-MS and GC-MS with HTPLC. HPTLC offers the possibility to split an extract into certain fractions, visually depict differences in the extracts and offers a quantitative and qualitative method for metabolomics. TLC offers many advantages over other metabolomic techniques. The initial costs for the equipment and the running costs are low and since it is possible to run several samples at the same time it offers a short handling time (Morlock et al., 2014). It can split and detect a broad range of chemicals which makes it an ideal tool for metabolomics. Moreover, it can be used as preparative tool to split up an extract and use different fractions for identification with other tools at hand or for biological tests. A range of different metabolomic groups can be visualised with different derivatisations methods (Fichou et al., 2016). Similar to other techniques, TLC has its disadvantages such as its relatively low resolution and reproducibility. However, in the last years those limitations were minimized by the development of more effective sorbents and an automatization of the crucial steps of the TLC (e.g. sample application, development and derivatisation as well as visualisation). This automated TLC is also known as high-performance thin-layer chromatography (HTPLC) (Salomé-Abarca et al., 2018). With this new approach we display an easy and reproducible way to separate, detect and visualise metabolomic variation of plant samples due to environmental challenges which they face.

One approach to tackle the diversity of metabolites in plants is to combine different chemical platforms to get information about the metabolites within a plant from different methods. Together, liquid chromatography and/or gas chromatography coupled with mass spectrometric detection (MS or MS/MS) and ^{1}H NMR represent a great toolbox to analyse plants metabolomes and offers detection of a broad range of metabolites with suitable resolution and sensitivity (Kim et al., 2010). However, all those methods have limitations and often do not lead to conclusive results since the data matrix is too complex to identify single compounds. In a traditional setup the extracts obtained from experimental plants would be split into different fractions with highly time-consuming preparative work, and it often leads to losses of extract during the process or the extracts would have to be purified of all compounds which might interfere with the analysis. With this study we highlight a comprehensive way to metabolic profiling of this specific interaction of a plant and its herbivore. We also will shed light on the different methods used by comparing their outcome for the same samples and with show the strengths and weaknesses of each method.

Material and Methods

Plant material

The plants were grown in soils in which previously other plants were grown. However, for this chapter we only investigated the effect of herbivory. The soils in which other plant species were previously grown were mixed for each of the plant species. One part of soil with two parts of sterile soil (1:2 conditioned: sterile v/v) were mixed to reduce potential differences in nutrient content of the soils. These mixed soils were then used to fill 2 pots (9 x 9cm; 650 g). *Taraxacum officinale* was grown in all soils in duplicate and one of the replicates was randomly assigned to one of two herbivory levels (absent or present). This led to 36 replicates x 2 treatments = 72 pots. After four weeks of growth all pots were caged with a transparent plastic tube (9 cm diameter, 30 cm height) closed with a mesh on top. We then put one freshly hatched *M. brassicae* in one of the two cages. After 7 days of feeding the larvae were removed and the shoots of all plants were immediately wrapped in aluminium foil and flash frozen in liquid nitrogen and stored at -80 °C.

Insects

M.brassicae (Lepidoptera: Noctuidae) is a generalist herbivore, native to the palearctic and that can feed on a wide range of plant families and species (Rojas, Wyatt, & Birch, 2000). Eggs were received from the Department of Entomology at Wageningen University (Wageningen, The Netherlands).

Metabolomics ^1H NMR analysis

The samples were first analysed with ^{1}H NMR. Samples were lyophilized for 5 days and grounded with a Retsch Mixer Mill MM 400 for 4-5 minutes, depending on the structure of the plant material, at a frequency of 30 Hz. These ground powered samples were then aliquoted and weighed (20.21 ± 0.05 mg). To the extracts we added 300 µL of CH_3OH-d_4 (Merck, Darmstadt, Germany) and 300 µL of D_2O buffer with 0.01% TSP to the samples. The vials were then sonicated for 10 minutes and centrifuged for 10 minutes at 13000 ppm in a Heraeus

Pico 17 Microcentrifuge. 250 µL of the clear supernatant was then transferred to a 3mm-NMR tube (Bruker, Karlsruhe, Germany) using a glass pipette.

The ^{1}H NMR spectra were recorded on a Bruker AV-600 MHz NMR spectrometer (Bruker, Karlsruhe, Germany) operating at an NMR frequency of 600.13 MHz. CH_3OH-d_4 was used as internal lock. Each ^{1}H NMR spectrum consisted of 128 scans taking 10 min and 26 sec acquisition time with the following parameters: 0.16 Hz/point, pulse width (PW) = 30° (11.3 µs), Relaxation delay (RD) = 1.5 sec.

To supress the residual H_2O signal, we used a presaturation sequence by low power selective irradiation at the H_2O frequency during the recycle delay. FIDs were Fourier transformed by a line broadening of 0.3 Hz. The spectra were phased manually, baseline corrected and calibrated to TSP at 0.00 ppm, using TOPSPIN (v 3.0. Bruker).

Bucketing was done with AMIX software (v. 3.9.12, Bruker BioSpin GmbH, Reinstetten, Germany) with scaling to total intensity and a bucket width of 0.04 ppm. Due to residual signals of solvents, the regions between 4.70 - 4.9 ppm and between 3.28 - 3.34 ppm were excluded. The ^{1}H NMR data matrix consisted of 246 buckets per analysed sample. The identification of the signals was done by examining the splitting pattern and the chemical shift of signals and comparing it with an internal database and as described in (Kim et al., 2010).

HPTLC analysis

For the HPTLC analysis 20 mg of the samples were weight and extracted with 600 µL of Methanol. Samples were then sonicated for 10 minutes and centrifuged at 13000 ppm for 10 minutes in a Heraeus Pico 17 Microcentrifuge. The supernatant was transferred into HPTLC glass vials. For the application, development, derivatization and the visualization we used an in house CAMAG HPTLC facility consisting of TLC sampler (version 4), Automatic Developing Chamber 2 (ADC 2), derivatiser (version 1.0 AT), TLC plate heater (version III), and TLC visualiser was used (CAMAG, Muttenz, Switzerland). We used Silica HPTLC plates (20 × 10 cm, F254, Merck, Darmstadt, Germany). We spotted 10 µL per sample on the plate in bands of 10 mm. Therefore, we applied 13 samples on one plate with a distance of 2 mm between bands. As a quality control we used a mix of all extracts and applied it in random positions on each plate. Therefore, each replicate was applied on a different plate. With a total number of samples of 72 this led to 6 plates. The samples were applied with the TLC sampler. The plates were then dried and developed in an automatic devolving chamber with the respective mobile phase. In order to split up polar and non-polar compounds in the extract we used two different mobile phases e.g. Ethyl acetate, acetic acid, formic acid, water (100:11:11:27), Toluene, Ethyl acetate (8:2). The chamber saturation time was automated (± 20 min) and the migration front was at 20 mm from the application point. After the plates had developed, they were sprayed with 2mL of p-anisaldehyde/sulfuric acid (Wagner et al. 1984) with an automated derivatizer. They were then placed on a TLC plate heater at 100°C for 3 minutes. After that images were recorded with a TLC visualiser at 366, 254 nm and white light. Before developing the plates, pictures of the empty plates were taken with the TLC visualiser in order to correct for potential impurities of the plates before the start of the experiment. These impurities were corrected for by visionCATS. The pictures were then exported into rTLC (version 1.0) and the data was extracted according to Fichou et al. (2016). The dimensions for the data extraction were the same as the settings in the CAMAG system. Pixel width was set to 128 units and band alignment and parametric time warping was used. The program extracts values of intensity of the colour of the picture for red blue and green and averages intensities of colour in the grey channel.

HPTLC as preparative method

After multivariate data analysis the HPTLC with the mobile phase toluene-ethyl acetate (8:2) was run again and all the steps were the same except that the plate was not derivatized. Instead the silica was scraped out at the Rfs that were of interest based on the results of the multivariate data analysis. Four bands (Fraction 1: Rf 0.27-0.31, Fraction 2: Rf 0.55-0.57, Fraction 3: Rf 0.62-0.63, Fraction 4: Rf 0.73-0.75). The scrape out silica was stored in a microtube. The HPTLC was repeated 4 more times to achieve a higher concentration of the isolated compounds. The silica was then immediately extracted with 500 µL acetonitrile and sonicated for 30 min. To exclude measuring compounds related to the silica, a control which only included the scraped-out silica after development of the plate without a sample was included and measured in later steps. The extract was then put through a filter (0.02 µm) and stored in a glass vial upon use for the LC-MS. For the identification with GC-MS we dried the samples that were used for GC-MS in the speedVac and rediluted them with 100 µL Pyridine and 100 µL BSTFA and TMCS. For the derivatization the tubes were put in a water bath of 80°C for 50 min.

UHPLC-QTOF-MS

The UHPLC-DAD-MS was performed using a Bruker OTOF-Q II spectrometer with electrospray ionization (ESI). For separation a Kintex C18 column (2.1 x 150 mm, 2.6µm) (Phenomenex, Utrecht, the Netherlands) with a gradient of water (A) and acetonitrile with 0.1% formic acid (B) with a flow rate of 0.300 mL/min. The gradient was 0-29 min 10% B 90%A; 30-31 min 90% B, 31-27 min 98% B, 38 -40 min 10% B. The temperature of the column was 40°C. The injection volume was set at 1 µL. The MS parameters were: Nebulizer gas 2.0 bar, drying gas 10.0 mL/min, gas temperature 250°C, capillary voltage 3500 V. The MS was operated in positive mode and data were collected between 100 and 1650 m/z. Each chromatogram was processed in Brucker Daltonics Profile Analysis version 2.1 and calibrated using the zone between 0.0 and 0.25 minutes. As a calibrant sodium formate solution was used. Data was saved as mzXML file. Data was then processed with XCMS online (Tautenhahn et al., 2012). For the molecular networking, the same samples were run again but in MS/MS detection mode.

Gas chromatography–mass spectrometry (GC-MS)

The derivatized samples were analysed with a 7890A gas chromatograph with 7693 automatic samples coupled to a 5975C mass single-quadrupole detector (Agilent, Folsom, CA, USA). A DB-5 GC column (30 m × 0.25 mm, 0.25 µm film thickness, J&W Science, Folsom, CA, USA) with an ionisation energy in EI of 70 eV. As carrier gas, He (99.9% purity) at a flow rate of 1mL/min was used. The oven temperature started at 60 °C for one minute and increased to 290 °C for 5 minutes with a rate of 7 °C/min. After that it was heated up to 310°C for 3 minutes with a rate of 5°C/min. The injector was put on splitless mode and 280°C. Peaks were identified by comparison of their iron spectra with the NIST library (version 2008) or by comparisons of their retention time and spectra with standard compounds.

Data analysis and statistics

To reduce the variation between plates the HPTLC data extracted was normalised to the signal of the pooled quality control sample for each plate. Orthogonal partial least square-discriminant analysis (OPLSDA) were done with SIMCA-P software (v15.0.2, Umeå, Sweden). As scaling method UV was used expect for the LC-MS data which was scaled with pareto. UV scaling was chosen for ^{1}H NMR since it yielded better fitting models.

To compare the effectivity of the different methods for detecting metabolome differences induced by herbivory we conducted a t-test for every feature/bucket and compared the signal for herbivory and non-herbivory samples. P-values were FDR corrected and the percentage of significant signals was calculated. These analyses were performed in R Studio (R Studio Team, 2016).

A molecular network was created using the online workflow (https://ccms-ucsd.github.io/GNPSDocumentation/) on the GNPS website (http://gnps.ucsd.edu) (Wang et al., 2016). For that all mzXML data were uploaded. The data was filtered by removing all MS/MS fragment ions within ± 17 Da of the precursor m/z. MS/MS spectra were window filtered by choosing only the top 6 fragment ions in the ± 50Da window throughout the spectrum. The precursor ion mass tolerance was set to 2.0 Da and a MS/MS fragment ion tolerance of 0.5 Da. A network was then created where edges were filtered to contain a cosine score higher than 0.7 and that included more than six matched peaks. Further, edges between two nodes were kept in the network when each of the nodes appeared in each other's respective top 10 of most similar nodes. Finally, the maximum size of a molecular family was set to 100, and the lowest scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the network were then searched against GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least 6 matched peaks.

To enhance chemical structural information within the molecular network, information from in silico structure annotations from GNPS Library Search, Network Annotation Propogation were incorporated into the network using the GNPS MolNetEnhancer workflow (https://ccms-ucsd.github.io/GNPSDocumentation/molnetenhancer/) on the GNPS website (http://gnps.ucsd.edu) (Ernst et al., 2019; Silva et al., 2018; Wang et al., 2016). Chemical class annotations were performed using the ClassyFire chemical ontology (Djoumbou Feunang et al., 2016). The definitions of the chemical groups can be obtained from: http://classyfire.wishartlab.com/tax_nodes. The outcome of all molecular networks was processed and visualised in Cytoscape version 3.7.2 (Shannon et al., 2003). For a detailed view of the network it is recommended to refer to the online version which is found under: https://gnps-cytoscape.ucsd.edu/process?task=f971cbc9d4ab42229397ccda76748ab2

Results and Discussion

In this study we investigated the chemical response of *T. officinale* to herbivory by *M. brassicae*. *Taraxacum officinale* grows in a large range of habitats and is common all over the world (CABI, 2020). We therefore choose this ubiquitous species as an example to demonstrate the power of the different metabolomics platforms, which we used. *Taraxacum officinale* is used in traditional medicine to treat diabetes mellitus, hepatic disease, rheumatic arthritis cancer and jaundice (Hfaiedh, Brahmi, & Zourgui, 2016; Liu, Xiong, Ping, Ju, & Zhang, 2010; Warashina, Umehara, & Miyase, 2012). Furthermore it can have anti-inflammatory and anti-oxidant properties (Chen et al., 2012; Hu and Kitts, 2003). Not many compounds have been identified so far in *T. officinale* but it is known to contain phytosterols sesquiterpene lactones, flavonoids and phenolic acids (Choi et al., 2018; Díaz et al., 2018). From previous studies it is known to contain saponins, flavonoids, alkaloids, steroids and phenols (Amin Mir et al., 2013; Schütz et al., 2006). *Taraxacum officinale* has also extensively been studied due to its pharmacologically relevant metabolites. For example, sesquiterpenes such as eudesmanolides, tetrahydroridentin B and taraxacolide-*O*-β-glucopyranoside (Hänsel et al., 1980) have been detected. Furthermore sterols such as , taraxasterol, β -taraxasterol, their acetates and their 16-hydroxy derivatives arnidol and faradiol, - and β -amyrin, β -sitosterol, β -sitosterol- β -d-glucopyranoside and stigmasterol, have been reported (Akashi et al., 1994; Burrows and Simpson, 1938; Hänsel et al., 1980). In roots tissue of *T. officinale* several phenolic compounds (e.g. chlorogenic acid, vanillic acid, caffeic acid) have been detected (Williams et al., 1996). In the shoots of *T. officinale* the most highly concentrate phenolic compounds are caffeic acid esters such as chlorogenic, dicaffeoyltartaric (chicoric acid) and monocaffeoyltartaric acids(Williams et al., 1996). Cumarins have also been identified in the aboveground parts of *T. officinale* (Williams et al., 1996).With this diversity of metabolites, it is an excellent species to demonstrate the potential of different metabolomics platforms.

To investigate the effect of herbivory on the metabolome of *T. officinale* we analysed the foliar extracts with different metabolomic platforms. ^{1}H NMR was used to get a broad overview of the metabolic state of *T. officinale* and to analyse differences in primary metabolites and abundant secondary metabolites. LC-MS was used to target secondary metabolites. HPTLC was used for metabolic fingerprinting and as a preparative tool for LC-MS and GC-MS to separate compounds depending on their properties (Fig. 5.1). With this integration of different metabolomics methods, we aimed to maximize the outcome of the metabolomic analysis.

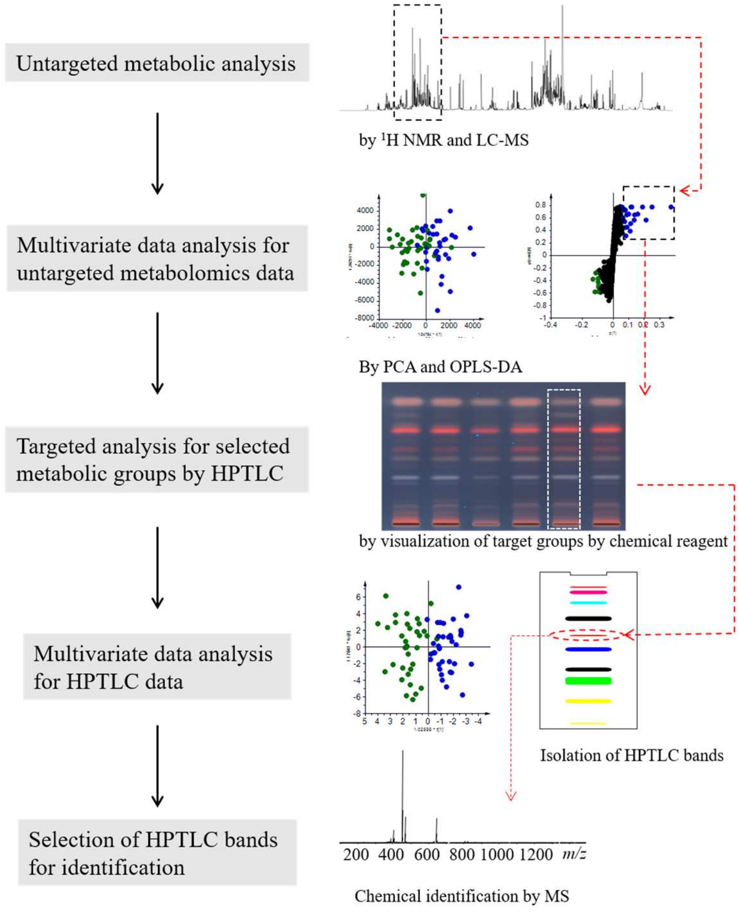

Figure 5.1 Integration plan of metabolomics tools. Figure is adapted from (Bayona et al., 2019)

^{1}H NMR: monitoring overall metabolomic changes

The typical ^{1}H NMR spectrum of *T. officinale* is depicted in Fig. 5.3c and an expansion in the region between δ 4.5-8.0 is found in Fig. 5.2. The most intense signals corresponded to sugars. Of these signals many were overlapping but some characteristic signals for sugars could be detected such as CH-4 for α-glucose δ 3.20 (d, *J* = 3.8 Hz) and CH-1 at δ 5.20 (d), CH-1 for inositol at δ 4.02 (t, *J* = 8.3 Hz), CH-3´ for sucrose at δ 4.16 (d, *J* = 3.9 Hz) and CH-1 at 5.40 (d). As well as amino acids such as threonine at δ 1.48 (d, *J* = 7.3 Hz, 1H) and alanine at δ 1.36 (d, *J* = 6.9 Hz). Furthermore, CH-8´ of chlorogenic acid (3-caffeoylquinic acid) at δ 6.48 (d, *J* = 16.0 Hz, 4H), citrate at δ 2.55 (d, *J* = 13.6 Hz) and malate at δ 2.77 (dd, *J* = 16.4, 4.5 Hz and δ 4.31 (dd, *J* = 6.9, 4.5 Hz,) could be identified. Characteristic signals for flavonoids are found in the region of δ 7-8.

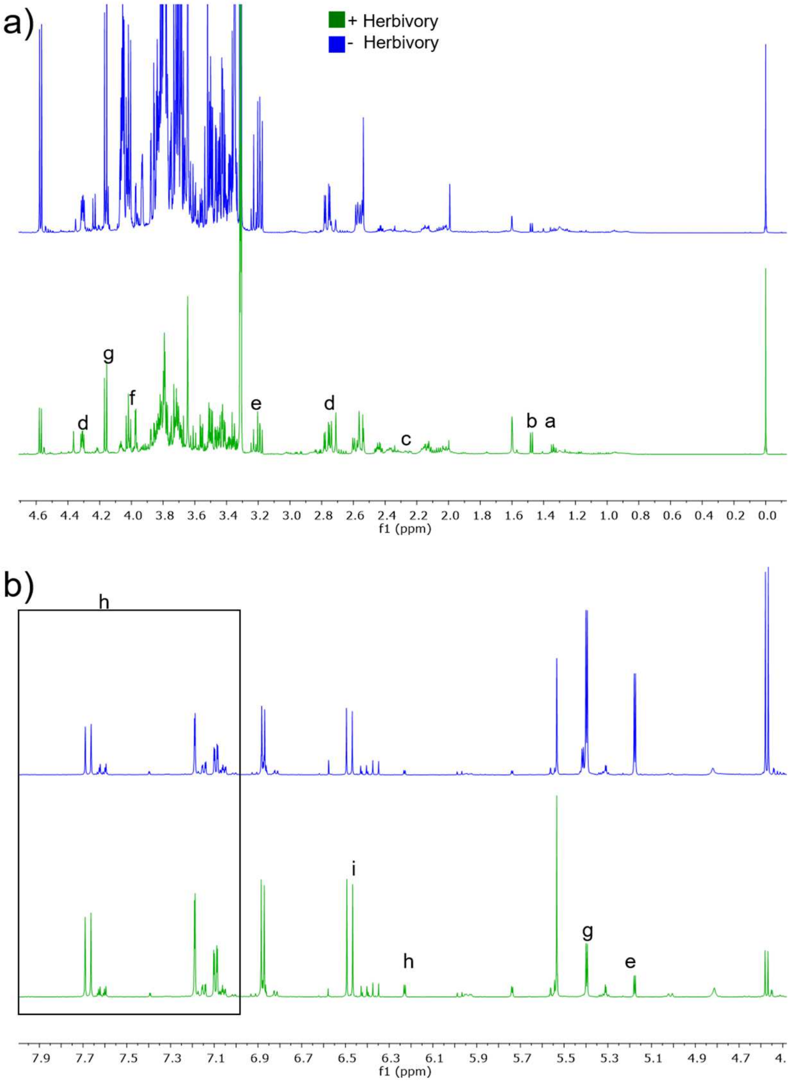

Figure 5.2 Expanded ^{1}H NMR spectra with signals related to a: alanine, b: threonine c: citrate, d: malate e: glucose, f: inositol, g: sucrose, h: flavonoids and i: chlorogenic acid (3-caffeoylquinic acid)

Principal component analysis (PCA) was used to investigate the influence of herbivory on the metabolome measured by ^{1}H NMR. PCA did not show any clear separation between the treatments and therefore we applied orthogonal partial least squares discriminant analysis (OPLS-DA) in the next step. The prediction power of the OPLS-DA was high with a cross-validation Q^2 (0.33) and a significant CV ANOVA ($p<0.001$) when data was UV-scaled. This scaling was used since it provided the best fit of the model. The score plot shows that the metabolome measured with ^{1}H NMR differs between the two herbivore treatments along the first axis (Fig. 5.3a). To identify the metabolites discriminant for the treatments the S-plot was constructed (Fig. 5.3b). The S- plot showed that alanine, threonine and flavonoids were especially high in plants exposed to herbivory, while glucose, inositol and signals related to the sugar region (δ 3.2- δ 4.0) in ^{1}H NMR were low. The intensity of the signals in 50.07% of the buckets changed significantly upon herbivory.

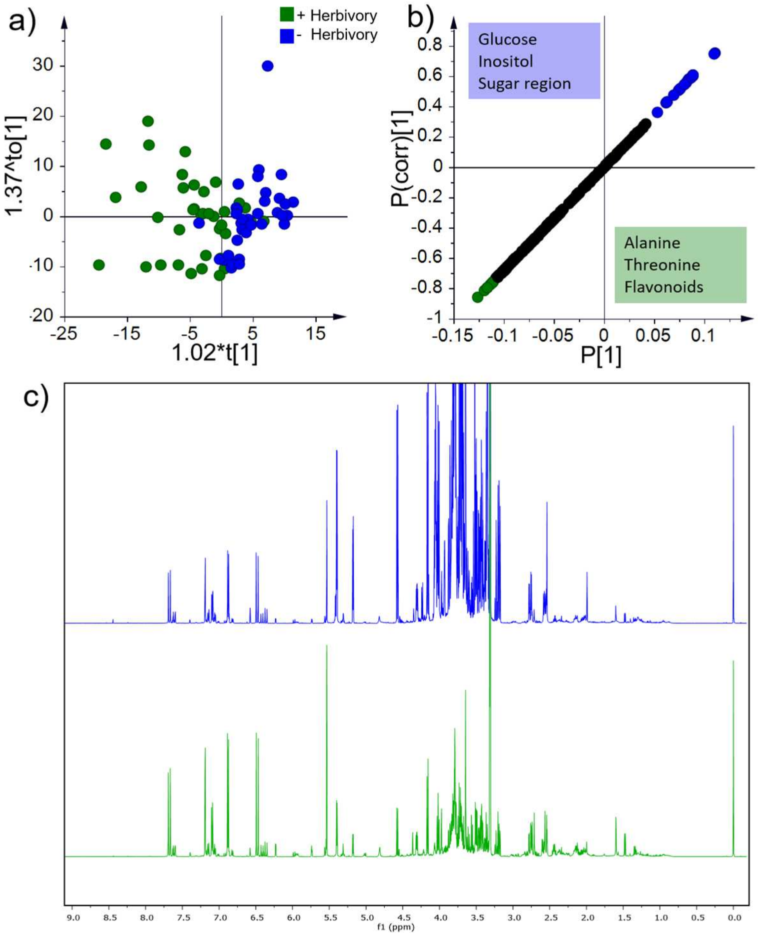

Figure 5.3 Orthogonal partial least squares discriminant analysis of the metabolomics data acquired with ^{1}H NMR. a) the sample plot and b) the S-plot. In a) colours depict the herbivory treatment (Yes/No) and in b) they depict the signals related with the corresponding treatment. Compounds associated with the treatments were tentatively identified. c) typical ^{1}H NMR of extracts from plants with or without herbivory. For expanded spectra in regions δ 0-4.6 and δ 4-8 refer to Figure 5.2

^{1}H NMR fingerprinting provided a broad overview and fingerprint of the metabolomic changes due to herbivory. However, the metabolites that differed between the treatments and that could be identified in the mixtures were all primary metabolites. Signals related to secondary metabolites were detected but could not be identified with ^{1}H NMR. In a biological framework differences in primary metabolites can be important for interactions of the plant with the environment. They were recognized in the last decades to influence herbivore performance especially of specialist herbivores that only feed on one plant (Berenbaum, 1995). For example, inositol concentrations were decreased upon herbivory, and inositol can act as a feeding stimulant for certain insects (Thorsteinson and Nayar, 1963). However, herbivory is known to also change secondary metabolites in plants. We assume that these changes in secondary metabolites might not be well visible with the current method because of potential overlap of signals.

^{1}H NMR is a suitable tool for an overall view of the metabolome changes induced by herbivory and metabolomic fingerprinting. It is a tool which is popular for profiling with the possibility to detect a large range of metabolites. However, ^{1}H NMR is mostly suitable to detect differences of abundant metabolites. The deconvolution of signals in mixtures and with that the identification of single metabolites is difficult. This was also the case in our study in which the identification was hindered by highly congested signals in ^{1}H NMR.

LC-MS: uncovering secondary metabolites

In the ^{1}H NMR analyses we found that flavonoids changed due to herbivory, however it was not possible to identify them in the ^{1}H NMR spectra. We therefore used LC-MS as a next step to analyse the metabolome focusing on secondary metabolites. We analysed all samples with LC-MS in order to achieve a higher coverage of different metabolites that can potentially be identified. Flavonoids were detected in the shoots of *T. officinale* in other studies (Amin Mir et al., 2013; Schütz et al., 2006).

A typical LC-MS chromatogram of a sample from a plant exposed to herbivory and a control plant is depicted in Fig. 5.4c. The PCA of the LC-MS data did not show any clear discrimination between the treatments. Therefore, an OPLS-DA was used. The model was validated with Q^2 =0.312 and a p-value in CV ANOVA lower than 0.01. The plants exposed to herbivory clearly sperate along the first axis in the score plot (Fig. 5.4 a). The S-plot showed an increase and decrease of metabolites caused by the treatment. However, none of the signals which differed between herbivory and non-herbivory plants (Fig. 5.4 b) were present in our LC-MS library and therefore we were not able to identify the metabolites. 16.56% of the features in LCMS changed upon herbivory and 50 signals (m/z) had a high VIP values (>2.5) in the OPLS-DA.

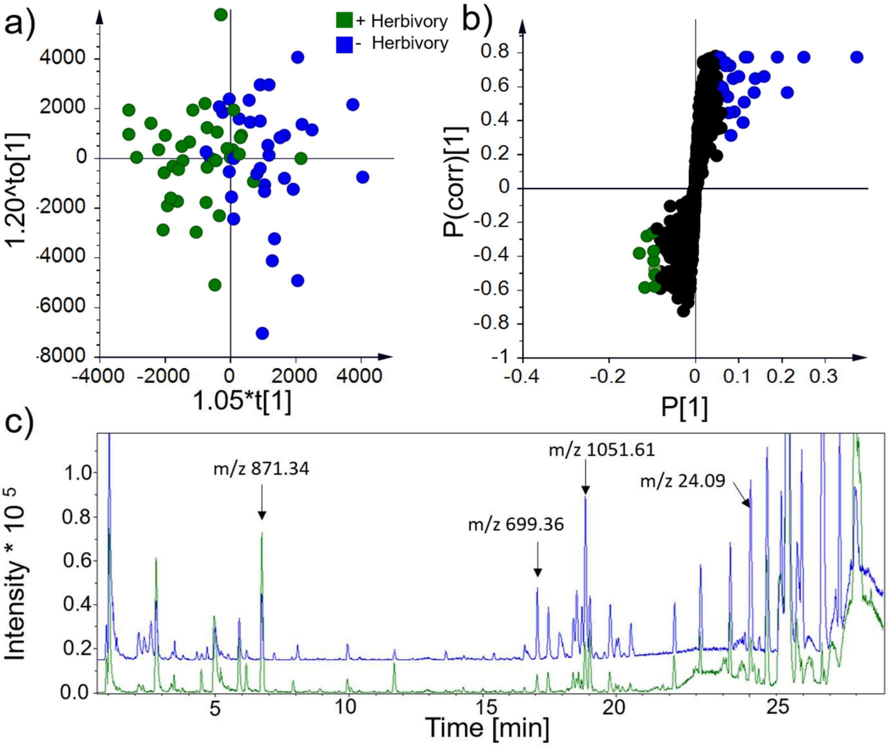

Figure 5.4 Orthogonal partial least squares discriminant analysis of the metabolomics data acquired with LC-MS. a) the sample plot and b) the S-plot. In a) colours depict the herbivore treatment (Yes/No) and in b) they depict the signals related with the corresponding treatment. C) A typical LC-MS chromatogram of plants treated with a herbivore or not. The signals having the strongest effect in b) are depicted in c).

Our results point at one of the major limitations of LC-MS, which is that only a limited number of libraries is available, making the identification of metabolites difficult, especially for plant species for which the metabolome has previously not been studied (Peters et al., 2018). One solution in the future for this problem can be molecular networking (Ramos et al., 2019; Silva et al., 2018; Wang et al., 2016).

A current trend in metabolomics is the use of molecular networks (MN). MN can be used to overcome the often-faced problem of LC-MS that the libraries only contain a small fraction of the detected metabolites. In molecular networks chemical space in MS/MS data is visually displayed. By this, spectra from related molecules (molecular network) can be put together in sets even if the spectras itself do not match with any known metabolite. In molecular networking each spectrum is represented in a node and the connections (edges) represent spectrum alignments. By comparison of the spectra with various databases they can be grouped into chemical groups, or depending on the spectra even identified within the network (Wang et al., 2016). The start of the Global Natural Products Social Molecular Networking (GNPS) in 2016 gave the scientific community the opportunity to share and curate data and use multiple Ms libraries. With that MN can help to increase the efficiency, in terms of identification, of LC-MS data.

LC-MS/MS was performed, and a feature-based network was constructed. The classification of the compounds was done according to ClassyFire. With this network we found that the compounds marked in the loading plot shown in Fig. 5.3b and related to the herbivory treatment mainly belonged to the group of lipids and benzenoids, including phenolics. Within the lipid class we found compounds that were glycerophospholipids, steroids and steroid derivates. In the group of the benzenoids, we detected phenolic glycosides and biphenyls and derivatives (Fig. 5.5). Furthermore, one signal could be associated to one triterpenoid and one trialkylamine. Compounds related to control plants were clustered as lineolic acid or one of its derivates and two glycerophospholipids.

Biologically these results show that there was an activation of plant defences upon herbivore. Triterpenoids are normally stored in a glycosylated form such as saponin. They are known to be defensive compounds against herbivores (Kuzina et al., 2009). Steroids and steroid derivatives were also influence by herbivory. Since they are produced from precursors of terpenoids the may be related to the changes in triterpenoids. Furthermore, a few of the compounds were identified as glycerophospholipids which are a component of cell walls and they could therefore indicate that cell wall processes change upon herbivory. This has previously been shown for mechanical wounding and herbivory in plants (Galati et al., 2019; Ryu and Wang, 1996).

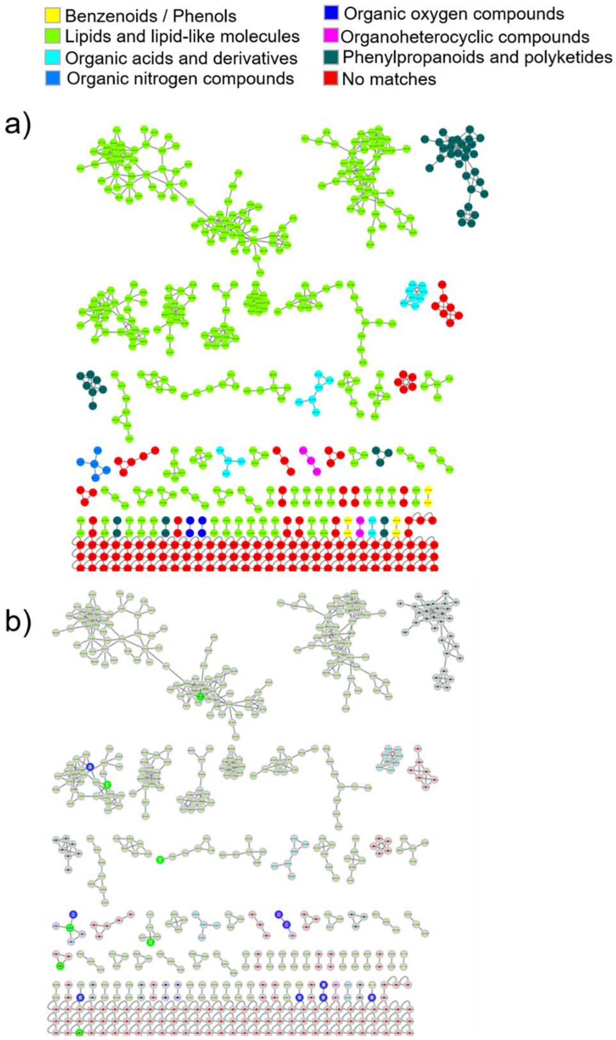

Figure 5.5 Feature based network. a) shows the network and colours depict the chemical class at which the compounds were assigned to. b) shows the same network but the compounds which were shown to differ between the treatments in Figure 5.4b. are depicted by the colour around the nodes. Green represents compounds associated with herbivory. Blue highlights compounds associated with the control plants.

The molecular networking made it possible to relate signals that changed due to the treatments with certain chemical groups. It is a highly promising tool and it is likely that this tool will be used more frequently in future studies. However, with this method it is not possible to identify single compounds.

HPTLC: metabolic fingerprinting and isolation of metabolites.

Traditionally facing many issues HPTLC has recently improved in its reproducibility and standardisation of the use (Audoin et al., 2014). These technical advances and the automatization have made it a suitable tool for metabolomics. It has been used for metabolomic fingerprinting of traditional medical plants (Ogegbo et al., 2012) and for chemical differentiation between different plant species (Maldini et al., 2019; Salomé-Abarca et al., 2018). It has also been used to differentiate between different collection periods of plants (Salomé-Abarca et al., 2018) and to examine the variation of the metabolome of rhubarb roots from different regions (Ge et al., 2018).. T our knowledge, HTPLC has not been used for the differentiation of plants experiencing environmental challenges. We therefore provide here the first study showing that HPTLC can be used as metabolomics fingerprinting platform in the context of ecometabolomics.

An advantage of HPTLC is that it can be used for metabolomic fingerprinting in a simple manner. In contrast to the other platforms it simultaneously offers the possibility to separate mixtures into fractions that can be analysed with other platforms. In order to do so we performed HPTLC with a polar and a non-polar mobile phase to target a large range of compounds with different polarities.

HPTLC offers the possibility to supplement the results from ^{1}H NMR and LC-MS by potentially increasing the concentrations of the compounds and by separating the compounds from the rest of the chemical matrix and thereby decreasing the overlap of signals in ^{1}H NMR. One of the advantages of HPTLC is that it produces highly reproducible data, in contrary to other metabolomics platforms (Jayachandran Nair et al., 2017). Furthermore, many samples can be analysed simultaneously on one plate and it can cover a wide range different metabolic groups that originate from numerous chemical reactions. In our example, the PCAs from the data of the two mobile phases did not show a separation between the treatments. Multivariate data analysis could only be validated for the HPTLC with the mobile phase (toluene: ethyl acetate) for non-polar compounds (with a Q^2 of 0.31 and a p-value under 0.001 in CV-ANOVA). The metabolome measured with HPTLC did differ between the treatments as shown in the sample plot of the OPLS-DA (Fig. 5.6a). In the next step we identified the compounds responsible for this difference (Fig. 5.6b) by using HPTLC as a preparative tool for other metabolomics techniques (LC-MS and GC-MS). On its own it is difficult to identify compounds with HPTLC but it offers an easy possibility to fractionate extracts and analyse those with other available methods. We analysed four fractions of the HPTLC with a second metabolomics platform in order to identify the compounds in each fraction. A typical representation of a HPTLC chromatogram of one extract from a plant with herbivory and one without herbivory is found in Fig. 5.6c). 3.90% of all signals changed significantly in HPTLC due to the treatment.

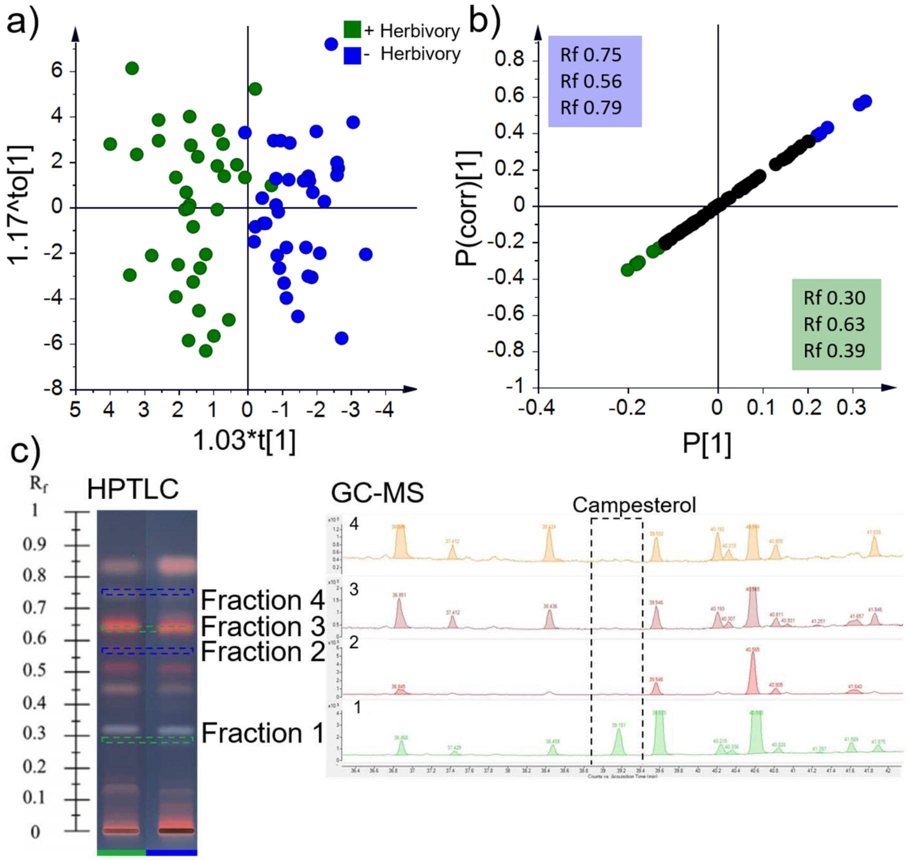

Figure 5.6 Orthogonal partial least squares discriminant analysis of the metabolomics data acquired with HPTLC with Toluene, Ethyl acetate (8:2) mobile phase. a) the sample plot and b) the S-plot. In a) colours depict the herbivore treatment (Yes/No) and in b) they depict the signals related with the corresponding treatment. Rf values for the compounds important for the separation in a) are given in b). c) A typical chromatogram of a high-performance thin layer chromatogram (HPTLC) of *Taraxacum officinalis* developed on a silica gel HPTLC plate using Ethyl acetate, acetic acid, formic acid, water (100:11:11:27) as mobile phase. For visualisation plates were sprayed with anisaldehyde-H_2SO_4 and the picture was taken under 366 nm light. The fraction was analysed by GC-MS separately and the chromatogram of each fraction is shown in c)

We analysed the scraped-out fraction with LC-MS and were able to detect peaks that were not detected in the crude extract, potentially due to a low concentration in the crude extract that we overcame by concentrating the compound through HPTLC. However, we were not able to identify the compounds which we detected in LC-MS. Therefore, we derivatized the same extracts for analysis in GC-MS.

We analysed the fractions of the extract differing between plants exposed to herbivory or not with GC-MS in order to identify the compounds responsible for the separation in the OPLS-DA. In the GC-MS we were able to identify several compounds in Fraction 1, indicating that the sample was not pure. We identified phytosterols including β-sitosterol, stigmasterol and campesterol. With this information we investigated our ^{1}H NMR and LC-MS spectra again and found a signal in the ^{1}H NMR related to sterols (δ 0.8 (s). In the mixture it would have not been possible to identify the specific sterols. However, this was not visible in the LC-MS, probability because of the non-polar properties of the sterols. With that we show that HPTLC can be hyphenated with GC-MS in order to identify compounds that might otherwise would have been missed out on.

We subsequently analysed one crude extract with GC-MS to see if the compounds identified in the fraction are visible in the crude extract. Stigmasterol and β-sitosterol were also found and could be identified in the crude extract. Campesterol could however not be identified in the crude extract because it the quantities in the crude extracts were too low. With the HPTLC step we managed to double the concentration of campesterol and with that concentration we were able to identify this compound.

From a biological view these results are highly interesting since arthropods cannot produce cholesterol de novo (Behmer and David Nes, 2003) and some caterpillars and grasshoppers are known to generate tissue cholesterol by metabolising sterols from plants that they feed on (Behmer et al., 1999). An increase in sterols in the plant would lead to an increase of sterols in the insect since the insect's uptake of sterols is non-selective (Behmer et al., 1999). High concentrations of sterols can lead to insect mortality (Behmer et al., 2011). In our study we now show that herbivory can lead to increased concentrations of sterols in plants. Feeding on the plant could therefore, in the long term, negatively affect the herbivore or even lead to the death of the insect. This needs to be tested in future studies. To our knowledge this is the first report of changes in sterol concentrations in *T. officinale* due to herbivory. This shows that the combination of methods that we propose enabled us to detect and identify compounds that would not have been visible without the HPTLC unless tedious fractionation steps were included.

Conclusion

In this study we show that herbivory by *M. brassicae* influenced the metabolome of *T. officinale*. To achieve a broad cover of different metabolites we combined different metabolomics platforms. ^{1}H NMR showed that herbivory leads to different concentrations of primary metabolites such as sugars and we also detected flavonoids in the ^{1}H NMR. ^{1}H NMR was therefore proven to be a suitable tool for metabolomic fingerprinting and provided an overview of the abundant secondary compounds. However, identifying secondary compounds with ^{1}H NMR still faces many difficulties. Therefore, the secondary metabolites were studied in more depth with LC-MS-MS, and with molecular networking we were able to group the signals from LC-MS into chemical groups. LC-MS-MS together with MN has great potential to make it possible to identify more metabolites in the future, especially since the libraries are getting more exhaustive. However even in LC-MS some metabolites which are only found in low concentrations in the plant, but potentially with a biological effect, cannot be detected. Lipid like metabolites mainly differed between the treatments. We then used HPTLC to combine metabolomic fingerprinting with the fractionation of the complex mixtures. HPTLC enabled us to detect campesterol which was not detected with the other methods. With that HPTLC represents a promising supplementary tool for metabolomics enabling scientists to simultaneously do metabolomic fingerprinting of many samples at once and the fractionation of complex mixtures.

From a biological perspective our results show that sugar concentrations, in general, decrease upon herbivory as has been previously shown in (Van dam and Oomen, 2008; Steinbrenner et al., 2011). Interestingly, we could show in our study by combining different platforms that many lipid-like compounds, such as steroids change upon herbivory.

Metabolomics is a relatively young research field in which the challenges are growing with each new publication. More and more complex systems are analysed, and the rise of ecometabolomics researching ecological interactions with metabolomics increase the need for methods to explore those complex chemistries. A way to tackle this challenge is to use multiple platforms - multiplatform metabolomics. This approach gives us the possibility to exploit the advantages of each method and provides a large coverage of different metabolites. With our study we show how to select and separate compounds influenced by the treatment of interest in a quick and uncomplicated way by using HPTLC to separate compounds and combining this with LC-MS.

We show that all single methods showed different metabolites which differed depending on the herbivory treatment. This shows that it remains important to use more than on established method for metabolomics. However, if only one method can be used, NMR is probably the best choice to depict overall changes in the metabolome.

Identifications of compounds in mixtures can be tedious and difficult. Our method of combining HPTLC to separate compounds and LC-MS to analyse the compounds afterwards enables the users to identify compounds related to their treatment of interest quicker.

Acknowledgments

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Chapter 6

General discussion

The overarching aim of this thesis was to study if and how soil-mediated feedbacks can influence the metabolome of plants growing in these soils. In Chapter 2 we studied how plant soil feedbacks influence metabolomes of plants. We created soils of six grasses and six forbs and tested the metabolomic response of these same plant species when grown in sterilized soil that was inoculated with soils from each of the species. Furthermore, we investigated if the changes of the metabolome upon herbivory were dependent on the soil legacies that the host plants experienced. Most of the tested plant species were sensitive to the soil effects. In Chapter 3 we collected soils from different spatial scales (different sites and different locations within each site) and investigated how those soils influenced the metabolome of *Jacobaea vulgaris*. Here, we showed that the similarity in metabolomics profiles is higher if plants grow with soil inocula collected closely together. This work also shows that there is a connection between soil microbial communities and aboveground plant metabolomic responses. In Chapter 4, we investigated how soils of six different plant species influenced the metabolome of *J. vulgaris* and the effects of those plant-induced changes on the soil on herbivore performance of *J. vulgaris* over the course of one year. We also measured the microbiome of the soils at different time points and showed that metabolomic variation in the plant is related to microbiome changes in the soil. This demonstrates that the impact of soil on the metabolome changes over time but, again, show that metabolome changes in aboveground plant tissues are linked to microbial changes in the soil. Lastly, in Chapter 5 we applied diverse analytical platforms to the metabolomics of a model plant and investigated how they can be integrated in order to enlarge metabolic coverage and strengthen identification power. We suggest a concept of multiplatform metabolomics in integrated manner: each method is employed in each stage (overviewing by NMR, targeted analysis by LC-MS, fingerprinting and preparative works by HPTLC).

In the following, I will address the findings of all chapters and discuss them in a broader context to conclude how this thesis advances the knowledge about PSF effects on metabolomes and the implications that these will have on science in the future.

Soil effects on the metabolome of plants

The main goal of this thesis was to study if plant-mediated changes in the soil, here called soil legacies, influence the metabolome of plants aboveground, and how this relates to the performance of insects that feed on those plants. Earlier studies in our group observed performance changes of insects due to soil legacies (Heinen et al., 2019; Kos et al., 2015c; Kostenko and Bezemer, 2013a). Further, I studied if a broad range of plant species is sensitive to soil legacies or if these soils induced changes occur only in certain species. Recently, several studies were published that examined the effects of soils on the metabolome of plants. For example (Ristok et al., 2019) showed that the diversity of the plant community that was grown in a soil can influence the belowground and aboveground metabolome of four different plant species and that those changes resulted in differences in the performance of a herbivore. Further Badri et al. (2013) showed that inoculation with soils with a different legacy of plants growth (either agricultural or not) influenced the metabolome of *Arabidopsis thaliana* and that those changes resulted in herbivore performance differences. Zhu et al. (2018) used a subset of the plants used in Chapter 2 of this thesis, and showed that the soil legacies of 12 plant species lead to changes in defence-related genes in the plant *Plantago lanceolata* and two iridoid glycosides were changed due to the different legacies. Specifically, Zhu et al. (2018) showed that genes related to the jasmonic acid (JA) pathway in the plant were altered by soil legacies whereas the expression of genes related to salicylic acid (SA) signalling were not altered by soil legacies. In other studies, one component of the soil (bacteria, fungi, chemical composition-exudates) was specifically changed and such changes also resulted in altered metabolomes of response plants (Hu et al., 2018; Mahmood and Kataoka, 2020; Schweiger et al., 2014; Zhou et al., 2018). These recent studies show that soils are factors determining plant metabolomes and that there is rising interest in these soil-mediated effects.

Soils influence the metabolome of most plant species

My thesis now adds to this body of knowledge by testing the effects of the inoculation with whole soil communities on the metabolome of various response plants. We could clearly show that the metabolome of the majority of the tested plant species was influenced by the legacy present in the soil in which they were grown. In Chapter 2, the metabolomic response of 12 plant species to soil legacies created by each of those plant species was studied and we found that only four of the 12 tested plants did not show metabolomic changes due to the different soil legacies **(Fig. 2.3)**. However, what also appears from my work is that the outcome of such experiments can differ, as we demonstrate e.g. in Chapter 4, where we show that the leaf metabolome of *J. vulgaris* did only differ significantly depending on the plant species which grew in the soil previously, after 8 months of conditioning **(Table 4.2)**. Similarly, in Chapter 3 we show that the metabolome of *J. vulgaris* is sensitive to the soil in which the plant growth, but that this is only true for some sites and not always **(Table S3.2).** All together this indicates that it is still difficult to predict if the metabolome of a plant will responsive to changes in the soil and which factors in the soil explain this pattern.

Which plant metabolites change due to soil-mediated effects?

In our studies we show that the soil in which a plant grows can influence the sugar content, and the concentration of amino acids and secondary compounds of the plant. In Chapter 2 and 3 we found that tyrosine and sugars concentration changed due to soil legacies. These compounds are related to cell wall processes (Loewus and Murthy, 2000; Walling, 2000). Future PSF studies should therefore investigate the impact of PSFs on cell wall components in plants. Cell wall thickness and composition can influence herbivore performance, and cell wall adaptations are a stress response that is commonly observed across the monocotyledon/dicotyledon barrier (Houston et al., 2016). Furthermore, we found that sugar and amino acid concentrations change depending on the soil, in which the plant grows. Although plant-herbivore research has focused recently on the role of plant defence compounds, primary compounds such as sugars can greatly influence herbivore performance on plants (Berenbaum, 1995). In Chapter 3 and Chapter 4, we also showed that the concentration of secondary compounds (e.g. Pyrrolizidine alkaloids) can change depending on the soil legacy, and hence, also the defence status of a plant can depen d on the soil in which it grows. This gives rise to many possible exploitations which are described in more detail in the future directions section of this chapter.

Chemical variation

Although we recorded strong effects of soil legacies and herbivory on the metabolome of twelve different plant species in Chapter 2, the part of the variation explained by soil and herbivory largely differed between species **(Fig. 2.3)**. This is interesting as it raises the question why certain plant species, such as *Taraxacum officinale*, are more sensitive to soil legacies, than others like *Geranium molle*. So far, it is unknown why sensitivity of plants to soil varies and we can only speculate about the underlaying reasons. It is plausible that soils conditioned by other plant species will have stronger effects for the unresponsive species. Maybe some of the species do occur more often together than others and are therefore more prone to the soil legacies that they were exposed to in nature. Another possibility is that the metabolome of some plants is overall more sensitive in, or specifically to changes that occur in the soil. It is notable that for some plant species only 20% while for others up to 70 % of the variation of the metabolome is not explained by soils and herbivory. It is important that PSFs explain so much of the variation for some plant species, but there also is an urgent need to further explore the relevant factors that cause chemical variation in the plant species that were relatively insensitive to soil. How do these species respond to other environmental stresses? Could their metabolomic capacity for changes to different stresses be measured? Do they rely less on induced defences upon herbivory than the others? All these questions are surely interesting and should be addressed in future studies.

Effects of metabolome changes on insect performance

Soil legacies can impact the metabolome of a broad range of plant species and via soils, plants potentially influence other plants growing in the same soil at a later timepoint. This can be a reason for effects of PSF on insect performance on those plants as found in several recent studies (Hannula et al., 2019b; Heinen et al., 2019; Pineda et al., 2020). However, when we tested if changes in the metabolome explained the performance of *M. brassicae,* we could only find such a relationship in soils conditioned for 8 months **(Table 4.4)**. Numerous previous studies have found a relationship between specific metabolites and insect performance (Hu et al., 2018; Kos et al., 2015c; Ma et al., 2017; Zhu et al., 2018). One possible reason why we did not find such relationship could be linked to the holistic approach we choose. We related the whole metabolome structure to the performance of the herbivore, something which, to our knowledge, has not been done so far. Most studies either apply untargeted metabolomic analyses, identify the signals which differ most between herbivory and non-herbivory, or carry out targeted metabolomics and correlate changes in these selected compounds to herbivore performance (Ma et al., 2017; Marti et al., 2013). In untargeted metabolomics, it is possible that the effect of a group of compounds or one specific compound is missed since the extraction and detection are targeted to detect as many compounds as possible which might result in less fine scaled results. On the other hand, several publications have pointed out that is highly likely that for interactions between plants and insect herbivores, many compounds may play a role and these compounds can interact in how they influence the herbivore (Berenbaum and Zangerl, 1993; Houston et al., 2016; Liu et al., 2017; Nelson and Kursar, 1999). Therefore, the approach we used might reveal the real interaction between a plant and a herbivore more closely than correlations based on one or a few specific compounds. It is important to note that in Chapter 4, the influence of PSFs on herbivore performance was surprisingly weak and this could be another reason why the metabolomic changes were not linked to the performance of the herbivore in most of the experimental PSF rounds. Other studies found strong effects of PSF on insect performance, but these effects strongly depend on the plant species and the insect species used (Pineda et al., 2017). Furthermore, soil-mediated effects were found to considerably differ depending on the conditioning plant species. An earlier study conducted in our group showed that PSF effects on growth, defence and susceptibility to soil-borne diseases differed among 37 conditioning plant species (Ma et al., 2017).

Therefore, it is possible that the choice of the herbivore and plant species, in this study, resulted in the finding that the metabolome and herbivory performance were not linked.

Ideally, studies should explore the metabolomic differences for a multitude of plant species grown in differently conditioned soils and treated with different herbivore species. Of course, reality applies restrictions to the dimensions of an experiment, and a trade of between complexity of the model and feasibility of the execution needs to be found. One solution for this could be to build a network of researchers working on PSF. Samples from the different experiments could be collected and combined for data analysis. However, such large-scale collaborative actions require standard protocols and procedures in how to carry out PSF experiments, and these are hard to agree on. The idea of standardized protocols for PSF research has been advocated several times in the recent literature (Brinkman et al., 2010; Gundale et al., 2019). Scientists that focus on investigating the biomass responses of plants, for example, could freeze-dry the harvested material instead of drying it in the oven, so that the samples could also be used for metabolomics analyses. This would offer the possibility to explore the reproducibility of the effect of soils on the metabolome across different experiments and the samples sizes will be much larger and conditions much broader than what a single study can provide at the moment. Different handling and procession of samples as well as additional data acquisition is always associated to additional costs. It is therefore important to agree on such an endeavour in the scientific community. This would be an important step towards making the soil-metabolome connection applicable for agriculture and horticulture and therefore can also greatly increase its relevance for society.

Different plant species lead to different microbiomes

Not only do the plant species differ in their response to PSF, they also have distinct species-specific effects on the soil in which they grow. For example, studies have shown that the microbiome of the soil differs depending on the species and/or the functional group of the plant species grown in it (Bezemer et al., 2018; Hannula et al., 2019a; Heinen et al., 2018; Latz et al., 2016, 2015). The reasons for these differences between the functional groups can be manifold. Grasses and forbs do not only differ morphologically but also in their root architecture. Grasses produce finer but denser roots compared to forbs. Therefore, grasses might have a larger area of contact with soil microorganisms than forbs. This might lead to a more distinct influence on the microbiome of the soil (Brundrett, 2002). Several studies have also reported that plant-growth-promoting-rhizobacteria accumulate in soils of grass species (e.g. Latz et al., 2012). These bacteria have the potential to change the composition or concentration of defence compounds in aboveground tissues (Pangesti et al., 2015; Van Oosten et al., 2008). Not only does the effect that plants have on soils differ between functional groups, often soils in PSF are categorized in conspecific and heterospecific soils. Most plants grow less well in their own soil and hence better in soils in which a plant that belongs to another species has grown before (Kulmatiski et al., 2008), probably due to the accumulation of species-specific pathogens in the soil (Reynolds et al., 2003; Van der Putten et al., 2013). Furthermore, some soils might contain a higher density of mycorrhizal fungi than other soils due differences in the capacity to accumulate mycorrhizal fungi by different plant species. Based on my work and that of others we can conclude that PSFs are highly plant species-dependent and, hence, this should always be taken into account in PSF studies.

What are the driving forces of soil-mediated effects on plants?

The mechanisms underlaying PSFs can be numerous and reach from changes in the microbiome of the soil and abiotic conditions of the soil to extracellular-self DNA of plants (Dostálek et al., 2016; Mazzoleni et al., 2015; Wang et al., 2019a). In this thesis we investigated the microbiome and the abiotic conditions of the soil as causal agents of PSFs, although we only analysed the microbiome of the soils in Chapter 4. The changes in the metabolome that we recorded in response to the soil inocula used in our studies, are likely due to differences in the microbiomes of the soils, since we mixed a small portion of the inoculum with a large portion of sterilized soil, in order to reduce the differences among the conditioned soils in nutrient availability. This is common practice in PSF studies, and in Chapter 3 **(Table S 3.4)** we compared the nutrient content of soils after *J. vulgaris* had been grown in these soils and did not detect differences in nutrient contents. Furthermore, we also studied the nutrient content of the soils used as inocula in Chapter 4 and did not detect significant effects of soil nutrients on the metabolome (**Table 4.3**). Therefore, we conclude that the differences in the plant metabolomes are caused by differences in the microbiota in the soils. This was actively tested in Chapter 4 where we show that the metabolome composition is influenced by the bacterial and fungal community. We correlated the fungal and microbial communities sequenced in the soil samples, with the metabolome composition of the plants which grew in sterilized soil inoculated with theses soil samples. This experiment demonstrated that the many metabolome changes are linked to specific bacteria and fungi in the soil. However which bacteria/fungi led to which alterations differed between the experimental rounds and more research is needed to better understand the influence of particular bacteria and fungi in the soil on the plant metabolome. For this it might be a good approach to start with a simple experiment in which sterilized soil is inoculated with cultures of the bacteria or fungi that were related to changes in the metabolites and then examine the metabolome response under these more controlled conditions.

How do soil microbiome changes lead to metabolome changes?

Differences in the metabolome of a plant due to the distinct soil legacies can be caused by direct or indirect effects of the microbiome in the soil on the plant. Bacteria and fungi in the soil can directly influence the metabolome of a plant by activating immune responses such as JA and SA pathways in the plant and trigger the induction of inducible defences. However, there are also many possibilities for how the microbiome of the soil can indirectly influence the metabolome. Certain bacteria and fungi are known to enhance growth of plants and that can result in changes in the metabolome of the plants or cause changes in the concentrations of compounds in shoots and roots (Hol, 2011). Plants with more biomass, for example, often contain higher concentrations of sugars than smaller plants. Endophyte communities in the plant also depend on the microbiome of the soil (Lundberg et al., 2012). Endophytes interact with the plant in various ways. They can produce beneficial compounds, helping the plant to protect itself, or themselves can process compounds produced by the plant. By this, compounds used by the plant for defence can become more toxic. Furthermore, a plant’s hormone signalling pathway can be manipulated and by that plants increase the resistance to pathogens (Khare et al., 2018; Vandenkoornhuyse et al., 2015). Furthermore, through enzyme exudation, certain communities of bacteria and fungi increase the availability of nutrients in the soil, and thereby change the nutritional status of the plant. Other microbes are known to control the abundance of pathogenic bacteria and fungi in the soil by the production of antibiotics, hydrogen cyanide or enzymes (Ferreira et al., 2019). It was beyond the scope of this thesis to pinpoint the specific mechanisms causing the changes in plant metabolomes. Future research is needed to identify these mechanisms. Multi -omics has great potential to investigate the transcriptional and genetic changes in the plant, and will make it possible to more exactly pinpoint specific mechanisms.

Spatial variation in soil effects on plants

The microbiome of the soil can differ at scales from millimetres to centimetres. This heterogeneity in the microbiome of the soil is driven by abiotic as well as biotic factors (Fierer, 2017). In Chapter 3 we studied if such spatial differences in the microbiome lead to differences in the chemical composition of plants. We showed that the metabolome of plants grown in soils with inocula collected in close proximity is more similar than the metabolome of a plant grown with inocula collected at larger distances **(Fig. 3.4)**. These spatial differences are probably linked to specific bacterial and fungal communities, known to strongly differ in abundance, even at small spatial scales. However, we can only speculate about the driving forces of these effects since we did not measure the microbiome of the soils used for inoculation. Nevertheless, the interaction of soil and plant at each specific spatial scale can be one explanation for the often-unexplained large chemical differences among plants of the same species in the field. This variation leads to several questions such as: is this also the case in intensively used agricultural landscapes such as monocultures of crops, and if so, can plants actively chose in which patch in the soil to grow their roots? This could be for example triggered by volatiles released by the bacterial communities in the soil and picked up by the plant. A first attempt to investigate this has been made by (Hendriks et al., 2015), who showed that spatial heterogeneity of PSFs influences root responses. However, in their setup it remains unclear if plants can actively choose the spots where to put their roots in a heterogenic environment. Finally, the spatial factor of the soil is enormously complex which makes it extremely challenging to measure, study and manipulate it.

The effect of temporal variation of plant soil feedbacks on plants

One of the common criticisms to PSF research has been its reproducibility and consistency over time. Two different phases of PSF studies can be influenced by time: the conditioning and the feedback phase. In this thesis we investigated how an increase in conditioning time influences the microbiome in the soil, the biomass and the metabolome of plants grown in these soils and their resistance to herbivory.

In Chapter 4 we investigated the changes of PSF over the course of a year with soils that were conditioned for different times while keeping the duration of the feedback phase constant **(Fig. 4.1)**. *J. vulgaris* produced most biomass in sterilized soil and least in its own soil **(Fig. 4.2).** This has been found also in other studies (Kos et al., 2015c; Van de Voorde et al., 2012, 2011). However, in Chapter 3, the biomass of *J. vulgaris* grown in 100% sterilised soil and inoculated soil did not differ **(Fig. 3.5)**. Another study in our group, showed that the positive effect of sterilized soil on the biomass of the plant (in this case Chrysanthemum) did reverse after one growth cycle and the plants performed better in soils which were inoculated with plant-conditioned inocula than in sterile soil (Ma et al., 2018). Also, in another study using *J. vulgaris* plants did not produce most biomass in sterilised soil (Joosten et al., 2009). Sterilized soil is a medium that can be easily colonized by bacteria and fungi from the environment as there is no microbiome present in the soil that these colonizers have to compete with.

In Chapter 4 we further investigated how the plant metabolome changes when the conditioning period increases. It is difficult to predict the effect of an inoculum on the metabolome of a plant as the specific connections between the microbes in the soils and the metabolome changes differed between the rounds of the experiments **(Table 4.2)**. In Chapter 4, this can be caused by the different ages of the soils that were used. This stresses the importance to replicate PSF studies investigating the metabolome of the plants under exactly the same conditions to understand how universal the influence of soils or soil microbes on specific metabolites is. As causal agents of the changes over time we investigated the fungal and bacterial communities in the soil and their changes over time in Chapter 4. In Hannula et al. (2019a) these microbial changes were examined in much more detail. There we show that the bacteria greatly differ over time independent of the plant species that conditions the soil, but that and fungi mainly differ depending on the conditioning species. In Chapter 4 we show that the metabolome changes in the plant were mostly related to bacterial changes in the soil. It seems that the effects of the soils on the biomass of *J. vulgaris* are mainly driven by fungi in the soil and therefore stay more similar over time, whereas the effects of bacteria are mainly on the metabolome, and hence the metabolome of *J. vulgaris* changed greatly depending on when the soil was collected. Whether this is true remains to be confirmed, but the potential implications warrant that these contrasting effects of bacteria and fungi on plant growth and chemistry should be examined in more detail in future studies.

Metabolomics – a key tool for today's ecologists

In this thesis I showed that metabolomics is a tool which enables scientists to investigate a language that is important of many interactions between plants and their environment – the metabolome. Ecometabolomics is a promising and new strategy which uses a metabolomic approach to address and understand ecological problems (Peters et al., 2018). In Chapter 2 we showed that it is important to not only investigate concentrations of targeted groups of compounds but instead to investigate changes in the whole metabolome that occur due to interactions of the plant with the soil and herbivore. However, even today, it is not possible to reach the ultimate goal of metabolomics: to extract, detect and quantify all compounds in an organism. To accomplish this goal, different platforms for metabolomics should be used together and their outcome linked. A study pointing in this direction is presented in Chapter 5.

Metabolomics is a new tool to explore complex ecological interactions, introduced only just a decade ago. Although its application has often yielded new and promising results, most studies on plant ecology still focus on targeted compounds. There can be many reasons for this, but one is that access to metabolomics facilities is often unavailable to ecologists. Interdisciplinary approaches have led to great success in tackling problems in science, and in my opinion, more courage is required to combine ecology with cutting-edge techniques from other fields. Even though it is often challenging to find a consensus between the ecologist's and chemist's view on the design and interpretation of an experiment, this thesis proofs that the outcome can be very rewarding. By using metabolomics approaches we could show in this thesis that the changes in the metabolome of plants do not always occur in the metabolomic groups that we expect. I propose that there is a lot of potential in other ecological research to discover pathways and compounds in plants that are influenced by treatments, but have been neglected so far and this is essential to obtain a realistic and holistic view of the processes that occur within the plant.

Although the techniques and the sensitivity of the tools used in metabolomics have greatly improved over the past years, it is still not possible to detect and identify all metabolites within an organism with a single metabolomics platform and one extraction process. Ecologists often conduct experiments with many biological replicates but with relatively low amounts of sample available per replicate, Ample amount of sample, is however, one of the main requirements of metabolomics facilities, and therefore often there is only enough material for one extraction process. We faced this problem in chapters 2, 3 and 4, and therefore aimed to provide a framework to overcome this issue in the last experimental chapter. One way to approach this is to use one extraction method, analyse the sample with a non-destructive platform and recover the sample to analyse subsequently on another platform. However, this is often extremely time consuming and instead of a single dataset, multiple datasets from different platforms have to be analysed and combined. There are methods available to link datasets statistically, however, this remains a challenging task, especially since the different matrixes have different sizes. Consecutive analysis is also not applicable for all metabolomics platforms and may require extensive laboratory work before it can be applied. For example, samples extracted for GC-MS cannot be used at another platform because derivatisation is required. Therefore, there is a clear need for a more optimized strategy for multi-platform metabolomics.

In general, NMR represents a method with a relatively simple sample extraction method, that still allows the identification of a large range of compounds. However, signals often overlap and it is difficult to identify secondary compounds with this method. In Chapter 5 we explore an alternative solution and use an intermediate step of HPTLC which can be used to examine global differences in the metabolome of plants between treatments. In this setup, compounds can be split according to their chemical properties and different reagents can be used to colour them according to their chemical group. Subsequently, data has to be statistically analysed to find groups which differ among the treatments. With the chemical information (e.g. polarity, chemical group) of these groups, the fractions can then be analysed with the suitable platform **(Fig. 5.1)**. Another method, to simplify metabolomics work in the future, especially to simplify the identification of compounds are MS-MS networks. These networks deploy similarities in information such as mass-to-charge ratio (m/z) and group compounds with similar chemical properties together which helps to group signals which would not have been identified otherwise **(Fig. 5.4)**. This is important since, for example, for LC-MS data a detailed library including all potential compounds is often not available.

Implications for ecology

Metabolomic changes are often related to a complex network of influencing factors. Regardless, in our experiments we could show the influence of soil on the metabolomes. Hence, soil characteristics may be one of the potential drivers of insect distributions observed in the field.

Practical implications

Although soil is acknowledged more and more as being important for plant growth, it is still often ignored in studies investigating plant responses. Greenhouse studies most often use potting soil and probably all plant ecologists – including myself – have done experiments using potting soil. But not only are the nutrient levels in potting soil often not representative for the nutrient levels occurring in natural soils but most certainly the microbial communities in these potting soils are also not comparable to naturally occurring bacterial and fungal communities in the soil. With this thesis I now show that the microbiome of the soil can not only influence the growth of plants but in most cases also impacts the metabolome of plants. Therefore, it is of great importance that ecologists investigating for example insect performance on plants take the microbiome of the soil into account. Furthermore, studies investigating the metabolome of plants often ignore soils as a component of the experiment and with that ignore the fact that the choice of the soil can influence the outcome of the study. The outcomes of my thesis exemplify that plant ecologists and plant chemists should think about the soils they use for experiments and consciously incorporate soils in the experimental design.

Future directions

Active manipulation of chemical composition

The findings of this thesis are a first step in understanding how soils change the metabolomes of plants. Thinking about this concept it would be possible to change the chemical composition of plants by inoculation of soils with certain bacteria or fungi. Of course, to achieve this further research is needed that investigates combinations of bacteria of fungi and different plant species and that identifies their specific effects on the metabolome. Furthermore, the applicability of soil inoculation to soils that already contain a microbiome will need to be tested. If soil microbiomes can be steered, inoculation could for example be used to enhance the taste of fruits and crops in a natural way. Furthermore, an intriguing question is how volatiles of plants change in response to variation in certain components of the soil, and how this then influences predator host location, or pollination rates which currently decline due to a decreasing number of insects (Cardoso et al., 2020). In agriculture it is already common to inoculate fields with beneficial bacteria in order to increase the yield of plants or to make them more resistant to herbivores (Compant et al., 2005). However not much is known about the effect of this on the metabolomes of plants. Therefore, it can be assumed that optimal conditions have not yet been discovered and that there is still room for improvement as well as targeted application of soils to trigger specific plant responses. With this thesis I provide more evidence that the soil microbiome is an important factor for the chemical composition of plants and this paves the way for future studies in the field.

Interacting spatial and temporal variation in PSF

In this thesis we investigated how soil - metabolome relationships change at different spatial and temporal scales in two separate chapters. The interaction of spatial and temporal variation in PSF is still lacking and this should be explored in future studies. One of the reasons for this is that long term experiments across large spatial scales are difficult to setup and maintain. However, the effort would be worth to learn more about the variation of the effects in nature and see if they act in similar or different ways at different spatial scales. A recent study (Zhang et al., 2020) investigated spatial dynamics in microbes in the soil over time. They showed that spatial or temporal heterogeneity is far more important for their composition than seasonality and this has been confirmed by other studies (Fierer and Jackson, 2006; Lauber et al., 2013). It is important to study if this also holds true for the metabolome of the plants that grow in these soils.

PSF under climate change

Climate change is impacting ecosystems all over the world, but what will happen to plant-soil interactions? To examine how climate change will influence the interactions of plants and microbes and the effect that they have on plant chemistry is an important future direction. Especially when those patterns are linked to how insects will perform on plants in the future climates. It is argued that genetic variability makes plants more resilient to climate change, however, a variation in the rhizosphere microbiome may also be important for creating plants that are resistant or well-adapted to climate change. Certain bacteria can increase resistance of plants to droughts or increase availability of nutrients to the plant (Vurukonda et al., 2016). Additionally, microbes in the soil differ in their sensibility towards temperature increases, which often go along with climates with increases in droughts (Oliverio et al., 2017). This might lead to unforeseen changes in the microbiome of the soil tand in metabolome changes in the plants grown in these soils. Another possible effect of climate change might be the loss of biodiversity not only aboveground but also belowground (Bardgett and van der Putten, 2014). On this line of reasoning, a loss of biodiversity below ground could result in a loss of diversity in the metabolites of a plant, which can have far stretching consequences. Another recent study (Ristok et al., 2019) supports this proposition. To predict the effects of such losses on the metabolome of plants, and with that on their interactions with the environment, will be an important next step.

Combining omics techniques

With the recent advancements in omics techniques, mixed-omics is becoming more and more common and is slowly turning into a useful tool for ecologists. Linking, for example, genomics and transcriptomics to metabolomics would help in our study system to understand which chemical pathways in plants are activated through the different microbiota of the soil. In a way, this thesis is a first attempt in fusing two omic techniques for our study system by combining the microbiome in the soil with the metabolome of the plants that grow in these soils in Chapter 4. In my opinion omics techniques will become more readily available to a broader scientific community in the near future, which will aid these scientists to better understand specific plant-microbe interactions, such as which genes are switched on in the plant due to which microbes in the soil, and as a consequence which metabolites change in the plant. This can lead to a new approach in ecology and help to truly understand the mechanisms behind different ecological processes.

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Summary

Soil is not only a substrate for plants to grow in, it is a substantial part of the system in which plants live and defines the identity of plants. While growing in a soil, plants change the abiotic and biotic properties of the soil. These effects on the soil are species specific and can influence, mainly through changes in the bacterial and fungal soil communities, the properties of plants growing in this soil later on. Those so-called plant mediated changes in the soil, or plant-soil feedback, are known to change the biomass of plants as well as the concentration of specific chemical compounds within the plant. This is probably also directly related to the performance of insects interacting with these plants. Up to today, studies that investigate the changes in the chemistry of plants due to soil legacies mostly use targeted approaches, focusing on one or a few specific compounds in plants. However, the plant metabolome is composed of hundreds to thousands of metabolites. Untargeted metabolomics aims to measure all metabolites within an organism and by that provides an overall view of its chemical state. Modern metabolomics provides the possibility to investigate the full metabolome response to ecological changes plants face, such as plant mediated changes in the soil. In this thesis, we harnessed the power of these metabolomics techniques to answer the question, if and how plant metabolomes change, depending on the soil.

First, we investigated if the effects of soils on metabolomes are a general phenomenon and common across different plant species. Additionally, we investigated if the effect which soils have on the metabolome are as pronounced as herbivory, which is well known to have strong impact on the metabolome. We set up a fully crossed design, in which we tested the metabolic response of 12 plant species to soil mediated plant legacies of all 12 plant species as well as herbivory. To evaluate the changes in the metabolome due to soil legacies and herbivory, the shoots of all plants were analysed with ^{1}H nuclear magnetic resonance (NMR). For 7 out of 12 plant species, soil explained most of the variance in the metabolome. This effect was even stronger than herbivory. We were able to show that the influence of soil on the metabolome of plants is common across different plant species and therefore soil might be one of the relevant factors to understand the often-unexplained intraspecific variation in the chemical composition of plants.

Bacterial and fungal communities in the soil do not only depend on the plant species growing in the soil, but they can also differ on spatial scales of millimetres to centimetres. In nature, these soil communities are known to be one relevant factor to explain composition of plants species and the insects that interact with them. However, so far it was unclear how this spatial variation of microbiomes affects the chemical composition of plants. To address this question, we set up an experiment, in which sterilized soil was inoculated with soils collected from different fields according to a spatial gradient. Identical clones of *Jacobaea vulgaris* were grown in these differently inoculated soils, weighed for biomass and analysed with ^{1}H NMR to assess the foliar metabolome. Metabolomic profiles of plants were more similar if the soil samples were collected closer together. However, only in samples from one of the four grasslands did the metabolome significantly differ from that of plants grown in sterilized soil. This proofs that soils of different spatial scales can indeed have a distinct effect on the metabolome, but also that not all soil inocula have an effect on the metabolome.

The effect of soil legacies does not only differ within space but the properties of the soil also depend on the time of conditioning, referring to the time of growth of the first plant species. To explore the variation of soil legacies over time, we repeatedly examined plant-soil feedback by measuring biomass, herbivory and metabolomic composition for genetically identical *J. vulgaris* plants in monoculture soils. This data was acquired for a full year. While plant biomass in monoculture soils was similar over the course of the year, the effects of the soils on the metabolome of plants varied greatly. As causal agent of these effects we assessed the fungal and bacterial communities in the soil. Bacteria communities manly differed between the time and fungal community composition did differ strongest between the different monocultures. The metabolome changes in the plants could be related to these community shifts in the soil. Variation in the metabolome of plants was mostly related to changes of bacterial communities in the soil and the effect of soils on biomass was probably due to changes in the fungal communities of the soil. This study provides evidence that there is a distinct difference between the effect of fungal and bacterial communities on plants over time.

In previous chapters of this thesis we used one common metabolomics technique - ^{1}H NMR, a platform that excels at giving a broad overview of the metabolome and detecting abundant compounds within the metabolome. However, the ultimate goal of metabolomics is to extract and detect all metabolites within an organism. For now, this is ambitious goal cannot be reached by one technique. In this last chapter we give an overview of the currently available platforms for metabolomics and explain on the example of *Taraxacum officinale* how the strengths of the different platforms can be combined. We used a combination of ^{1}H NMR, high-performance thin-layer chromatography (HPTLC) and liquid chromatography with tandem mass spectrometry (LC-MS-MS) to examine herbivory effects. We introduce the concept of HPTLC and demonstrate how it can be used for metabolomic fingerprinting as well as for the separation of compounds, which can be analysed subsequently with other methods. With HPTLC we were able to concentrate and identify a chemical compound, campesterol, which was not detected with conventional methods, but showed a significant response to herbivory treatment. This highlights the importance of translating cutting-edge metabolomics tools to ecology-based research.

In conclusion, in this thesis I have shown that changes, belowground, in the soil can have implications on the language of plants – the metabolome aboveground. It is important to note that these effects differ in time and space. I provide evidence that soils can be one important factor to explain the variation in plant chemistry in nature. However, this thesis only represents a first step towards a better understanding of how microbial communities influence the chemistry of plants, and with that also the insects interacting with this plant in nature. We demonstrate that there is large variation in the effects of soils on plant metabolomes and with this thesis I set the ground for future studies to explore this variation and the underlaying mechanisms. This will help us to get a better understanding of plant-soil interactions in nature and how to utilize these effects to overcome ecological and environmental challenges of the future.

Samenvatting

De bodem is niet alleen een substraat waar planten in kunnen groeien, maar het is ook een substantieel deel van het systeem waarin planten leven en het definieert de identiteit van planten. Wanneer planten in een bodem groeien, veranderen ze de abiotische en biotische eigenschappen van de bodem. Deze effecten op de bodem zijn specifiek per plantensoort en kunnen, grotendeels door middel van veranderingen in de compositie van bacteriële en schimmelgemeenschappen, de eigenschappen van planten die later in dezelfde bodem groeien veranderen. Deze door de plant veroorzaakte veranderingen in de bodem, ook wel plant-bodem wisselwerking genoemd, kunnen de biomassa van en de concentratie van specifieke chemische stoffen in de plant veranderen. Dit is waarschijnlijk ook direct gerelateerd aan hoe goed insecten die een interactie hebben met deze planten het doen. Onderzoek naar veranderingen in de chemie van de plant door bodem erfenissen is vaak doelgericht, met een gefocus op één enkele, of een kleine hoeveelheid specifieke stoffen. Het metaboloom van planten bestaat echter uit honderden tot duizenden verschillende metabolieten. Untargeted metabolomics richt zich op het meten van alle metabolieten die zich in een organisme bevinden en op deze manier kan een algemeen overzicht gecreëerd worden. Moderne metabolomics maakt het mogelijk om de volledige respons van het metaboloom op ecologische veranderingen die planten ondergaan te bestuderen, zoals veranderingen in de bodem. In deze thesis heb ik de mogelijkheden van deze metabolomics technieken benut om de vraag te beantwoorden of, en hoe, het metaboloom van planten verandert als de bodem verandert.

Ten eerste heb ik onderzocht in een aantal verschillende planten soorten of de effecten van bodems op het metaboloom vergelijkbaar zijn. Daarnaast heb ik onderzocht of het effect van bodems op het metaboloom evenveel effect heeft als dat van herbivorie door rupsen, waarvan bekend is dat het een grote impact heeft op het metaboloom. Hiervoor heb ik een volledig gekruist design gebruikt, waarin ik de metabolische respons van 12 plantensoorten gemeten heb als ze groeiden op bodems van elk van deze soorten. Om de veranderingen in het metaboloom door zogenaamde bodem nalatenschappen en insectenvraat te evalueren, zijn de scheuten van alle planten geanalyseerd met ^{1}H kernspinresonantie (ook wel ^{1}H NMR genoemd). Bij 7 van de 12 plantensoorten werd een signficant deel van de variantie verklaard door de bodembehandeling. Dit effect was sterker dan dat van insectenvraat. De invloed van de bodem op het metaboloom van planten bleek dus vrij algemeen. De bodem waarin een plant groeit zou daarom één van de factoren kunnen zijn die de vaak onverklaarbare intraspecifieke variatie in de chemische compositie van planten kan verklaren.

Bacteriële en schimmelgemeenschappen in de bodem zijn niet alleen afhankelijk van de plantensoorten die in de grond groeien, maar de dichtheid en samenstelling van bacterien en schimmels in de bodem kan sterk variëren over afstanden van millimeters of centimeters. In de natuur is de microbiele bodemgemeenschap belangrijk voor de samenstelling van planten- en insectengemeenschappen. Tot nu toe was het echter niet bekend hoe deze ruimtelijke variatie in het bodemmicrobioom de chemische compositie van planten kan beïnvloeden. Om deze vraag te kunnen beantwoorden, heb ik een experiment opgezet waarbij steriele grond werd geïnoculeerd met grond afkomstig van vier verschillende graslanden. De bodemmonsters werden in elk veld verzameld volgens een ruimtelijk patroon waardoor de afstand tussen de monsters bekend was. Identieke klonen van de plant jakobskruiskruid (*Jacobea vulgaris*) werden vervolgens gegroeid in potten met verschillend geïnoculeerde bodems. Met behulp van ^{1}H NMR werd het metaboloom van het blad bestudeerd. De metabolische profielen van de planten vertoonden leken meer op elkaar als de bodem monsters dicht bij elkaar genomen waren. Echter, slechts voor bodem van één van de vier graslanden was het metaboloom significant anders dan dat van planten gegroeid in steriele grond. Dit bewijst dat ruimtelijke variatie in bodems een effect kan hebben op het metaboloom van planten, maar ook dat niet alle bodem inocula het metaboloom beïnvloeden.

De invloed veranderingen in de bodem door planten die eerder in de grond groeiden, hangt ook af van hoe lang de eerste plantensoort in de grond gegroeid heeft. Om de variatie in bodem erfenissen vanuit een temporeel perspectief te onderzoeken, heb ik gedurende een jaar, herhaaldelijk plant-bodem wisselwerkingen geanalyseerd door de biomassa, de mate van herbivorie en de metabolische compositie te meten bij genetisch identieke *J. vulgaris* planten in monocultuurgronden. De biomassa van de planten in de verschillende bodems veranderde niet heel sterk over tijd, maar de effecten van de bodems op het metaboloom van de planten bleek sterk te verschillen gedurende het jaar. Aangezien bacteriële en schimmelgemeenschappen de oorzaak kunnen zijn van deze effecten, werden deze gemeenschappen geanalyseerd. Hieruit bleek dat bacteriële gemeenschappen het meest verschilden over de tijdspan van het onderzoek, terwijl de schimmelgemeenschappen het meest varieerden tussen verschillende monocultuurbodems. De metabolische veranderingen van de planten kon gerelateerd worden aan de veranderingen in de bacteriële gemeenschappen in de bodem. De effecten op de biomassa waren meer gerelateerd aan de verschillen in schimmelgemeenschappen in de bodems. Dit onderzoek levert bewijs dat er een duidelijk verschil is tussen de effecten van schimmelgemeenschappen en bacteriële gemeenschappen op planten over een tijdspanne.

De vorige hoofdstukken van deze thesis waren gebasseerd op een vaak gebruikte metabolomics techniek - ^{1}H NMR, een platform dat een globaal overzicht geeft van het metaboloom en de detectie mogelijk maakt van veel verschillende stoffen binnen het metaboloom. Het ultieme doel van metabolomics is echter de extractie en detectie van alle metabolieten binnen een organisme. Het is op dit moment nog niet mogelijk om dit te bereiken met één enkele techniek. In dit laatste hoofdstuk geef ik een overzicht van de platformen die nu beschikbaar zijn voor metabolomics en beschrijf ik met het voorbeeld van de paardenbloem (*Taraxacum officinale)* hoe de mogelijkheden en tekortkomingen van elk platform kunnen worden gecombineerd. Ik gebruikte een combinatie van ^{1}H NMR, high-performance thin-layer chromatography (HPTLC) en vloeistofchromatografie, gekoppeld aan massaspectrometrie (LC-MS-MS) om de effecten van insectenvraat op paardenbloem te bestuderen. Het concept van HPTLC wordt geintroduceerd en ik demonstreer hoe het gebruikt kan worden voor metabolische fingerprinten en voor de scheiding van stoffen, die daarna geanalyseerd kunnen worden met andere methoden. Met HPTLC was het mogelijk om een chemische stof te concentreren en identificeren genaamd campesterol, die met conventionele methoden nog niet eerder was gedetecteerd, maar wel een significante reactie vertoonde op herbivorie. Dit benadrukt het belang van de nieuwe metabolomics technieken in ecologische onderzoeken.

Zesummefaassung

Buedem ass net nëmmen een Substrat vir Planzen vir dran ze wuessen, en ass och een essentiellen Bestanddeel vun dem System an dem Planzen liewen an kann een vun den Faktoren sënn déi d´ Identitéit vun Planzen formen. Wärenddeem eng Planz an engem Buedem wiisst ännert se déi liewend Bestanddeeler an d´Närstoffer am Buedem. Des Effekter ob den Buedem ënnerscheeden sech tëschend dënnen verschiddenen Planzenzorten an kennen haaptsächlech duerch Bakterien an Pilzen am Buedem erkläert ginn. Des duerch den Buedem transferéiert Ännerungen am Buedem, heeschen an der Wëssenschaft och Buedem Vermiechtnes oder Planzen Buedem Reckkopplung. Sie sënn dofir bekannt, d´ Biomass vun Planzen ze beaflossen sou wei och d´ Performance vun Insekten ob dësen Planzen an d´ chemesch Zesummesetzung vun Planzen, den Metabolome. Dei meescht Studien déi des chemesch Effekter ënnersichen maachen dëst mat gezielten Analysen bei deenen d´Konzentratioun vun engem bestëmmten chemeschen Stoff oder enger Grupp Stoffer bestëmmt gëtt. Mee den Metabolismus vun enger Pflanz besteet aus honnerten bis dausenden verschiddenen chemeschen Stoffer. Ziel vun ongezielt Metabolomics Techniken ass all chemescher Stoffer an engem Organismus ze extrahéieren an ze moossen wei des sech als Reaktioun ob Ëmweltaflëss, wei zum Beispill verschidden Biedem, auswierken. An dëser Arabescht benotzen ech modern Metabolomics Techniken vir d´Fro ze beäntwerten ob Biedem mat ënnerschiddlechem Microbiome een Effekt ob den Metabolome, an domat Chimie vun Planzen hunn an wann dat de Fall ass wie eng chemesch Stoffer sech duerch d´Biedem an der Planz änneren.

Als éischt hunn mir ënnersicht ob d´Planzen vermëttelten Verännerungen am Buedem een Afloss ob den Metabolome vun Planzen hunn an ob dat een generellen Phenomen ass an sou mat deselwechte Effekt ob vill verschidden Planzenaarten huet. Zousätzlech hunn mir ënnersicht wei staark den Effekt vun dësen Ännerungen am Buedem ob den Metabolome ass. Dofir hunn mir d´Stäerkt vun den Effekter mat den Effekter vun engem Planzenfriesser (Herbivore) verglach. Des sënn doviir bekannt een staarken Afloss ob den Metabolome ze hunn. Dofir hunn mir een vollstännegen Versuuchsplang opgestallt. An dësem goufen 12 Planzenaarten ob Buedem wuessen gelooss. Des Biedem seen dunn benotzt gi vir an der zweeter Phase all 12 Planzenaarten ob allen Bieden wuessen ze loossen vir ze kucken wie een Effekt dat ob den Metabolome vun den Planzen huet. Zousätzlech guff gekuckt wie den

Metabolome ob een Frassfeind reagéiert. Vir ze evaluéieren wie een Effekt dëst ob den Metabolome hat gouffen all d´Gréngs vun den Planzen mat ^{1}H nuklear magnéitescher Resonanz (NMR) ënnersicht. Vir 7 vun 12 Planzenaarten huet Buedem méi vun der Varianz am Metabolome erkläert wei ob d´ Planzen mat engem Frassfeind trätereirt goufen oder net. Domat war et eis méiglech ze weisen dat den Afloss vun Buedem ob den Metabolome vun Planzen een generellen Phenomen bei vill verschidden Planzenaarten ass. Domat stellt den Buedem warscheinlech een vun deenen wichtegen Faktoren duer déi chemesch Variatioun teschend d´Planzen, och vun der selwechter Aart, kennen erklären.

Bakterien an Pilzen Communautéiten am Buedem hänken net nëmmen vun der Planzenaart déi am Buedem wiisst of mee si kennen sech och ob klengstem Raum vun Zentimeter bis Millimeter ënnerscheeden. An der Nature sënn des Communautéiten dovvir bekannt dat si Zesummestellung vun Planzen an d´Insekten déi mat dësen Planzen interagéieren kennen bestëmmen. Mee bis haut war onkloer ob des Ännerungen ob klengem Raum och een Effekt ob den Metabolome vun Planzen an soumat hir chemesch Zesummesetzung hunn. Vir des Fro ze adresséieren hunn ech een Experiment opgestallt an dem ech steriliséierten Buedem inoculeiert hunn mat Buedem den ech vun verschiddenen Felder ob verschiddenen Distanzen gesammelt hunn. An dësen Biedem hunn ech dunn genetesch identesch Klonen vun *Jacobea vulgaris* geplanzt. No dämms Planzen gewuess seen hunn ech se ofgeschnidden an d´Gewiicht bestëmmt mee och den Metabolome vun den Blieder mat ^{1}H NMR ennersicht. Den Metabolome vun Planzen déi ob Buedem gewuess seen den mat Buedem inoculleirt war den méi no beienee gesammelt gouf war sech am ännleschten. Awer just den Metabolome vun Planzen déi ob Buedem vun engem vun dënnen véier Felder gewuess seen huet den Metabolome signifikant zum 100% sterillem Buedem verännert. Dëst weist dat Biedem vun verschidden reimleschen Distanzen tatsächlech kënnen een ënnerschiddlechen Effekt ob den Metabolome hunn mee net all Inoculater hunn een gläich staarken Afloss ob den Metabolome.

Den Effekt vun Buedem Vermiechtnesser ënnerscheeden sech net nëmmen am Raum mee och Eegenschaften vum Buedem änneren sech mat der Zéit di eng Planz am Buedem gewuess ass. Vir des Variatioun vun Buedem Vermiechtnesser iwwert Zéit ze studéieren hunn mir iwwert een Joer ëmmer nees ënnersicht wei sech d´Biomass, Performance vun Herbivoren an den Metabolome vun genetesch identeschen *Jacobaea vulgaris* Planzen déi an Buedem gewuess seen ob dem virdrun Monokulturen vun aneren Planzen vir verschidden Zeiten gewuess seen geplanzt guewen, veränerd hunn. Wärend Biomass vun den Planzen an deenen ënnerschiddlechen Monokultur Biedem änlech iwwert Joer war, war den Effekt vum Buedem ob den Metabolome vun den Planzen staark ënnerschiddlech. Als Grond vir des Ännerungen hunn mir d´ Pilz an d´ Bakteriell Communautéiten am Buedem ënnersicht. D´ Variatioun am Metabolome vun Planzen konnt gréisstendeels duerch Ännerungen an den Bakterien Communautéiten am Buedem erkläert ginn. Wärend den Effekt ob d´ Biomass vun den Planzen warscheinlech gréisstendeels duerch Ännerungen an den Pilz Communautéiten vum Buedem kann erkläert ginn. Dësen Phenomen gouff an dëser Studie déi éischten Kéier gewisen an sollt an zukünftegen Studien getest ginn.

All Studien an dëser Aarbecht benotzen eng gemeinsam Metabolomics Technik – ^{1}H NMR. Des Technik ass gutt dodran eng breet Iwwersiicht iwwert den Metabolome ze ginn an Metaboliten die an héischen Concertatiounen vir kommen ze moossen. Mee d´ultimatiivt ziel vun Metabolomics ass all Metaboliten an engem Organismus ze extraheieren an ze detektéieren. Bis lo ass dëst een ambitionéiert Ziel wat net mat enger Technik erreecht kann ginn. An dem leschten Kapitel ginn ech een Iwwerbléck vun deenen Plattformen déi momentan zur Verfügung stinn vir Metabolomics. An engem spezifeschen Beispill vun *Taraxacum officinale* hunn mir eng Kombinatioun vun ^{1}H NMR an héisch performanter dünnschicht chromatography (HPTLC) an flessseg chromatography mat Tandem Massen Spektrometer (LC-MS-MS) benotzt vir ze ennersiichen wat vir eng Effekter Herbivory ob den Metabolome huet. Mir feieren den Konzept vun HPTLC an, an demonstréieren wei des kann benotzt ginn als zousätzlechen Schratt vir d´Transitioun zweschen ongezielter an gezielter Metabolomics. Mat HPTLC ass et eis gelongen een chemeschen Stoff, Campesterol, ze konzentréieren an ze identifizéieren den mir mat konventionellen Methoden net hätten kennen detektéieren an den awer signifikant ënnerschiddlech zweschend den Traitementer war.

A Conclusioun hunn ech an dëser Aarbecht gewisen dat ënnerierdesch Ännerungen am Buedem eng Auswierkung ob Sprooch vun den Planzen- den Metabolome iwwerierdesch kennen hunn. Desen Effekt ënnerscheed sech an Zäit a Raum. Domat bieden ech een Beweis dofir dat Biedem eng vun den Erklärungen vir déi oft ongekläert Variatioun an der Planzen Chimie an der Natur kennen sënn. Mee des Aarbecht representéiert just een éischten Schratt zou engem besseren Verständnis wei microbiell Communautéiten d´Chimie vun Planzen beaflossen an doduerch och d´ Insekten déi mat dësen Planzen interagéieren. Mir demonstréieren dat et eng héisch Variatioun an den Effekter vun Buedem ob Planzen Metabolome gëtt an mat dëser Aarbecht bidden ech d´ Grondlag vir zukünfteg Studien déi des Varianz ze ënnersichen an déi ënnerleiend Mechanismen. Dëst wäert hëllefen vir een bessert Verstees de mech vun Planzen Buedem Interaktiounen an der Natur ze kréien an raus ze fannen wei des Effekter kennen genotzt hin vir Ekologesch an Ëmwelt Problemer an Zukunft ze léisen.

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Curriculum vitae

Martine was born on the 27 of November in Ettelbrück, Luxembourg. She finished school with an apprenticeship as medical technical technician in Luxembourg city and then decided that she wanted to understand natural processes better. She started to study biology in Tübingen (Germany) in 2009 and focused on plant ecology in her bachelor thesis. Inspired by her work during the bachelor she decided to pursue a master in ecology and evolution also at the University of Tübingen. In her master thesis she explored the chemical defences of a range expanding plant and tested different invasion theories. Although she really enjoyed working with this established hypothesis, she wanted to get a broader answer to research questions in ecology and understand the role of soil in above belowground interactions. Furthermore, she wanted to explore the whole picture of chemistry of plants and learn about metabolomics. Therefore, she moved to the Netherlands and started a PhD, in 2016, on the effect of plant soil feedbacks on metabolomics of plants. In the Netherlands she accomplished her work for the PhD at the NIOO-KNAW in Wageningen and at the Institute of Biology (IBL) of Leiden University.

Publications

Huberty, M*., B. Martis, J. van Kampen, Y.H. Choi, K. Vrieling, P.G.L. Klinkhamer, T.M. Bezemer (2020) Soil inoculation alters leaf metabolic profiles in genetically identical plants, Journal of Chemical Ecology, https://doi.org/10.1007/s10886-020-01156-8

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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)

The C.T. De Wit
Graduate School
PE&RC
PRODUCTION
ECOLOGY
& RESOURCE
CONSERVATION

Review of literature (4.5 ECTS)

- Belowground plant interactions in a plant soil feedback context

Writing of project proposal (4.5 ECTS)

- From soil legacies to chemical legacies-understanding plant-microbe interactions belowground

Post-graduate courses (7.7 ECTS)

- Introduction to statistics in R; PE&RC (2016)
- Metabolomics for ecologist; iDiv, Leipzig (2017)
- General research skills for PhDs and R/RStudio course; R/RStudio (2017)
- Multivariate analysis of ecological data Canoco; University of South Bohemia (2018)

Competence strengthening / skills courses (7.3 ECTS)

- Time management, self-management; HRM Learning and Development Leiden University (2017)
- Effective communication; HRM Learning and Development Leiden University (2017)
- Scientific communication; HRM Learning and Development Leiden University (2018)
- Communication in science, writing skills; HRM Learning and Development Leiden University (2018)
- Improve your memory for PhD's; HRM Learning and Development Leiden University (2018)
- Job orientation for PhDs; HRM Learning and Development Leiden University (2020)

Scientific integrity / ethics in science activity (0.6 ECTS)

- Workshop: the Netherlands code of conduct for academic practice and principles of good academic research; NIOO Research Integrity Board (2016)
- Scientific conduct; HRM Learning and Development Leiden University (2017)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.2 ECTS)

- Plant-soil-microbe interaction workshop (2016)
- PE&RC First year weekend (2017)

Discussion groups / local seminars / other scientific meetings (8.4 ECTS)

- 11th Plant insect interactions; EPS, Leiden (2016)
- Plant-soil -microbe interactions for crop & pest management; Wageningen (2016)
- Metabolomics workshop; Leiden University (2017)
- Discussion group: spectrometric identification of organic compounds; Leiden (2017)
- One health symposium; Bern, Switzerland (2019)

International symposia, workshops and conferences (9.6 ECTS)

- Workshop COST-Action: plant mediated communication between above and belowground foodwebs; poster presentation; Leipzig, Germany (2016)
- Workshop metabolomics in chemical ecology; oral presentation; Wageningen, the Netherlands (2016)
- Joint annual meeting BES, GFÖ, NECOV and EEF: ecology across borders poster presentation; Gent, Belgium (2017)
- ISCE 35th International society of Chemical Ecology; oral presentation; Atlanta, USA (2019)

Lecturing / supervision of practicals / tutorials (0.3 ECTS)

- Exploitation of natural products (2018)

Supervision of students (9 ECTS)

- The effect of spatial dispersion in the plant-soil legacies on Senecio within four different ex-agricultural grassland fields
- Disentangling the effect of root and soil exudates on plant-soil feedback in Chrysanthemum indicum
- Metabolomic analysis of plant-soil feedbacks in *Senecio Jacobaea* with 1H-NMR spectroscopy
- The influence of different depths in the soil in relation to plant soil feedback
- The influence of plant root extracts on the growth of bacteria
- Effects of plant extracts on bacterial growth rates