Effects of above- and belowground biodiversity on the metabolome of common grassland species in different environmental contexts

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"We are stories,

Beginnings, middles, ends.

Our power lies in living lives that touch a life or two."

(Stories by Lilli Furfaro)

Table of Contents

INTRODUCTION

Background

The current decline in biodiversity is unprecedented (IPBES, 2019) and in conjunction with global and climate change poses a severe threat to mankind and human well-being (Hooper et al., 2005; Sala et al., 2000). We are already observing negative effects caused by the loss of biodiversity on crucial ecosystem functions (Balvanera et al., 2006), ecosystem services (Cardinale et al., 2012; Isbell et al., 2017), and biotic interactions (Hooper et al., 2005). However, it is those biotic multitrophic interactions, *i.e.,* the interactions between producers, consumers, and decomposers, that are essential for ecosystem fitness, productivity, and resilience (Eisenhauer et al., 2019). More recent studies corroborated this mechanistic link between biodiversity and ecosystem functioning by integrating information within and across trophic levels (e.g. Barnes et al., 2020; Buzhdygan et al., 2020; Schuldt et al., 2019; Wan et al., 2020). Our chance to safeguard biodiversity and the derived ecosystem processes thus hinges on our understanding and knowledge of the mechanisms underlying these multitrophic interactions.

Multitrophic interactions are highly complex and associated organisms are connected by a multitude of pathways. Within those complex multitrophic networks, plants are primary producers as well as mediators between aboveground and belowground organisms and processes (Bezemer & van Dam, 2005; Scheu, 2001; Wardle et al., 2004). In this position, plants are not only affected by their interaction partners but are also able to trigger positive and negative responses in their above- and belowground partners (Gutbrodt et al., 2012; van Dam & Heil, 2011). In order to initiate and realize all these interactions, plants produce primary and secondary metabolites (van Dam, 2009). These plant metabolites are not only

perceived by all interaction partners, but plants also modulate the composition of their metabolome in response to biotic interactions. It is therefore that exploring the role of plant metabolites may be the key to understanding the underlying mechanisms of multitrophic interactions.

The plant's metabolome and (eco-)metabolomics

According to estimates, there are between 200,000 to 1,000,000 different metabolites in the plant kingdom, of which 57,919 (as listed in the KNApSAcK database 2021/07/09) have been found in higher plants (Peters et al., 2018; Saito & Matsuda, 2010; Weckwerth, 2003). These metabolites can be volatile or non-volatile, polar, semi-polar or nonpolar, and can vary in concentration from pmol to mmol per gram plant tissue (Macel et al., 2010). The entirety of all synthesized metabolites by an organism is called the metabolome (Fiehn, 2002; Oliver et al., 1998), while the measurement of hundreds of metabolites is called metabolite profiling and the measurement of all metabolites is called metabolomics (Kopka et al., 2004).

Metabolites may be separated into primary metabolites and secondary, or specialized, metabolites. Primary metabolites, such as amino acids, lipids, and carbohydrates, are considered essential to the survival of a plant as they are involved in homeostasis, growth, development, and reproduction (van Dam & van der Meijden, 2011). In addition, many primary metabolites are also precursors for secondary metabolites, *e.g.*, amino acids can be transformed into phenylpropanoids *via* the shikimate pathway (Buchanan et al., 2015). Secondary metabolites, on the other hand, help plants to cope with their ever changing abiotic and biotic environment (van Dam, 2009; van Dam & van der Meijden, 2011). In contrast to primary metabolites, which can be considered evolutionary more conserved, secondary metabolites display a sheer endless chemical diversity at every taxonomic level,

i.e., among individuals of the same species, individuals of different plant species, genera, and families (van Dam & van der Meijden, 2011). In particular phenolic secondary metabolites, and among them flavonoids and phenolic acids, are especially important, as they occur widely in all plants and are involved in many different biotic interactions (Whitehead et al., 2021).

Flavonoids can be divided into anthocyanidins, flavonols, flavones, flavanols, flavanones, chalcones, dihydrochalcones, and dihydroflavonols (Treutter, 2006). Not surprisingly, their functions in biotic interactions are as diverse as their sub-classes. Among other functions, plants employ flavonoids as allelochemicals in plant-plant interactions, as signals in plant-soil interactions, and as defensive compounds in plant-herbivore interactions (Treutter, 2006). Phenolic acids comprise metabolites such as caffeic acid, chlorogenic acid, coumaric acid, and ferulic acid (Vogt, 2010). Similar to flavonoids, phenolic acids fulfill multiple roles in biotic interactions, *e.g.,* as defensive metabolites in plant-herbivore interactions (e.g., Bennett & Wallsgrove, 1994; Erb et al., 2009; Usha Rani & Pratyusha, 2013). As a consequence of the diversity of secondary metabolites, authors have argued that metabolomics, *sensu stricto*, is currently not possible because no single analytical platform can measure all metabolites (Kopka et al., 2004). Hence, in this dissertation metabolomics and metabolite profiling are used as synonyms.

Recent technological and methodological advancements in analytical chemistry, bioinformatics, and the high-throughput measurement of samples have paved the way for metabolomics studies. Core to this development were the improvements made to measure a wide array of metabolites using untargeted approaches. Untargeted metabolomics, in contrast to targeted analyses, allows for exploration of the metabolome without prior

chemical knowledge (Peters et al., 2018). However, untargeted metabolomics comes with its own challenges. Among them is the choice of analytical platform that defines and limits the scope of any metabolomics study. In plant eco-metabolomics, liquid chromatography-mass spectrometry (LC-MS) is the most commonly used platform as it allows to measure the concentration and chemical diversity of most secondary metabolites (Peters et al., 2018; Sardans et al., 2020). Liquid chromatography is a technique in which metabolites are separated because of their physico-chemical characteristics. This separation is achieved by combining a hydrophobic stationary phase with a gradient of solvents, *e.g.,* starting with water and ending in acetonitrile (Viant & Sommer, 2013). Subsequent to the separation, the metabolites can be ionized, and their intensity detected in a mass spectrometer coupled to the liquid chromatograph. This approach produces a list of features for each sample with information on mass-to-charge ratios, retention times, and intensities. In other words, a dataset for each sample containing information on the chemical diversity of secondary metabolites. Following the necessary bioinformatics data processing, the thus generated metabolomics dataset can then be combined with experimental or environmental data as well as further trait information and analyzed under an eco-metabolomics context (Peters et al., 2018).

Eco-metabolomics employs metabolomics to illuminate the chemical mechanisms underpinning ecological and environmental processes (Peñuelas & Sardans, 2009; Peters et al., 2018; Sardans et al., 2011). One cornerstone of eco-metabolomics is the use of often complex experimental designs under controlled or natural conditions that focus on species interactions within and across trophic levels (Peters et al., 2018; Raguso et al., 2015). Another aspect of eco-metabolomics is the measurement and analysis of a multitude of metabolites in a singular approach (Peters et al., 2018; Raguso et al., 2015). Hence, many

eco-metabolomics studies often involve the analysis of chemical variation among non-model organisms under different environmental contexts (Peters et al., 2018). In the context of my dissertation, this approach allowed to discover overall changes in the plant's foliar and root metabolome composition as well as novel metabolites in response to plant-soil-herbivore interactions.

Biotic interactions among plants, soil biota, and herbivores

Biodiversity-ecosystem functioning (BEF) experiments expanded our knowledge on biotic interactions (Balvanera et al., 2006; Duffy, 2009; Jochum et al., 2020). Historically, BEF experiments started in climate chambers under controlled conditions (Naeem et al., 1994), but were shortly after also conducted in grassland communities (e.g., Roscher et al., 2004; Tilman et al., 1996). Grassland communities proved to be fertile ground for BEF research, as grassland ecosystems not only cover up to 26 % of the terrestrial land mass (Boval & Dixon, 2012), but are also defined by a high plant diversity at small spatial scales (Roscher et al., 2004). The past three decades of BEF research in grassland ecosystems have shown that plant diversity affects biomass production (e.g., Hector et al., 1999), nutrient cycling (e.g., Hooper & Vitousek, 1998), soil-borne pathogens (e.g., Latz et al., 2012), aboveground herbivores (e.g., Scherber et al., 2010), and many other ecosystem functions (Tilman et al., 2014). Hence, BEF research provided experimental evidence for the central role of plant diversity for ecosystem functioning, while also shedding light on the importance of soil biota diversity (Delgado-Baquerizo et al., 2016; van der Putten et al., 2013) and the aboveground herbivore community (Wan et al., 2020).

Positive plant diversity effects on ecosystem functioning can be attributed to selection or complementarity effects (Loreau & Hector, 2001). Selection effects occur when a plant

community becomes dominated by one or a few highly productive species, while complementarity effects occur when the performance of plant species in mixed communities is on average higher compared to their individual performance in monoculture communities (Fox, 2005; Loreau & Hector, 2001; Weisser et al., 2017). It is generally assumed that species-specific plant-plant and plant-soil interactions drive selection and complementarity effects (Eisenhauer, 2012; van der Putten et al., 2013; Weisser et al., 2017). On the one hand, plant-plant interactions control the composition of plant communities and affect the dynamics in other trophic levels through their effects on resource availability and habitat structure (Brooker, 2006; Callaway, 1995; Connell, 1983). These effects are the result of competition for resources, such as light, nutrients, space, pollinators, and water, and facilitative interactions (Brooker et al., 2008; Tilman, 1982). Facilitation among plants can protect them from herbivores and provide additional resources through soil microbial enhancements or mycorrhizal networks (Brooker et al., 2008). On the other hand, plant-soil interactions can affect soil properties by exudation of plant metabolites and biomass as well as through the creation and maintenance of habitats and resources for micro- and macroscopic organisms (Bardgett & van der Putten, 2014; van Dam, 2009). In fact, each plant species harbors a unique rhizosphere community (Bezemer et al., 2010). In addition, plant-induced changes in soil properties and organisms can feedback on the productivity and fitness of a plant (Bever, 1994; Ehrenfeld et al., 2005). These effects are called plant-soil feedbacks and are positive if the productivity and fitness of conspecific individuals is enhanced and thus the probability that this species is able to become dominant in a local habitat is increased (Bever, 1994; van der Putten et al., 2013). Plant growth-promoting bacteria and other mutualistic organisms, such as arbuscular mycorrhizal fungi, for instance, induce positive soil feedback effects by improving nutrient uptake and protection against antagonists (Bardgett & van der Putten, 2014; Latz et al., 2012; Wardle et al., 2004). In

contrast, plant-soil feedbacks are defined as negative if the induced changes in soil properties and organisms prevent dominance of conspecifics in a given community or reduce productivity (van der Putten et al., 2013). Negative plant-soil feedbacks can be the result of interactions with root parasites, pathogens, and herbivores that directly remove or damage root tissues and thus reduce root uptake capabilities (Bardgett & van der Putten, 2014).

Historically, most studies on plant and soil biodiversity effects focused on plant functional traits to explain the biodiversity-ecosystem functioning relationships (Funk et al., 2017; Lavorel & Garnier, 2002; van der Putten et al., 2013; Violle et al., 2007). A recent study, however, has shown that the most commonly used traits to predict ecosystem functioning are only able to explain 12.7% of the variation in ecosystem properties across years (van der Plas et al., 2020). In this light, one might argue that either plant functional traits fail to explain the link between plant and soil diversity and ecosystem properties, or that the mechanistic link may be found in a different set of so far unexplored traits. It is here that eco-metabolomics may provide answers to the underlying mechanistic links of biodiversity-ecosystem functioning (Peters et al., 2018). It has already been shown that plant-plant interactions alter the plant's metabolome through competition, inducing the production of volatile and non-volatile allelopathic compounds that may affect ecosystem functions, such as resistance to herbivores (Baldwin et al., 2006; Broz et al., 2010; Fernandez et al., 2016). Similarly, plant diversity effects have been attributed to induce shifts in foliar metabolomic profiles and change the concentration of over 100 metabolites in multiple grassland plant species (Scherling et al., 2010). The plant's metabolome is not only responding to these plant-plant interactions. In fact, these changes in the plant's chemistry feedback to influence plant-plant communication, *e.g., via* aboveground volatile emission (Baldwin et al., 2006) or belowground root exudation (van Dam & Bouwmeester, 2016). Similarly, there is ample

evidence that belowground organisms can modulate the root and shoot metabolome (e.g. Bezemer & van Dam, 2005; Huberty, Choi, et al., 2020). For instance, interactions with plant growth-promoting bacteria and fungi affect the hormonal regulation within plants by inducing systemic induced resistance, and thus improving herbivore resistance (Pieterse et al., 2014). Interactions with mycorrhizae can initially affect plant hormones and subsequently prime plant defenses against herbivore and pathogen attack (Ferlian et al., 2018). In addition, plant-soil feedback effects as a result of changes in soil fungal communities have been shown to change the concentration of amino acids and pyrrolizidine alkaloids (Kos et al., 2015). And lastly, soil microorganisms can increase the concentration of iridoid glycosides in roots, while nematodes affect the concentration in root exudates (Wurst et al., 2010). While there are many examples that reveal how the soil biota can change the concentration of primary and secondary metabolites in leaves and roots (e.g., Huberty, Choi, et al., 2020), the specific metabolomic response is generally species- and context-dependent as well as affecting subsequent biotic interactions, *e.g.,* with aboveground herbivores (Bezemer & van Dam, 2005; van Dam & Heil, 2011; van der Putten et al., 2013). It is therefore that the plant's metabolome may provide unique insights into the underlying mechanisms of plant-soil-herbivore interactions.

The interactions between plants, their soil biota, and aboveground insect herbivores belong to the most fundamental ecosystem processes. Indeed, insect herbivores consume substantial amounts of biomass in grasslands every season (Meyer et al., 2017; Seabloom et al., 2017). This herbivory can affect plant physiology and fitness (Karban & Strauss, 1993) as well as plant community composition (del-Val & Crawley, 2004). At the same time, plant diversity can influence herbivory rates as well as the abundance and diversity of insect herbivores (Ebeling, Meyer, et al., 2014; Haddad et al., 2001; Hertzog et al., 2016; Wan et al., 2020).

For instance, higher plant species richness increases the diversity and availability of plant resources and vegetational structure, thus supporting a higher abundance of insect herbivores (Haddad et al., 2001; Hertzog et al., 2016). In contrast, increased plant species richness may reduce herbivory through dilution effects because it may be more difficult for specialized insect herbivores to localize their host plant (Castagneyrol et al., 2014; Finch & Collier, 2000).

As a response to herbivory, plants have evolved multiple traits, such as thorns and toxic secondary metabolites, that reduce the ability of herbivores to find, choose, or consume a plant (Agrawal et al., 2006; Karban & Baldwin, 1997; Loranger et al., 2012). Plant defenses can be differentiated into direct and indirect defenses. Direct defenses are plant structures or metabolites that directly deter herbivores or pathogens, or reduce their performance (Bezemer & van Dam, 2005). Indirect defenses, on the other hand, affect carnivores or parasitoids of herbivores, *e.g., via* attraction by volatiles, thus reducing herbivore pressure (Bezemer & van Dam, 2005). In addition, plant defenses can further be differentiated into constitutive and induced defenses (Kaplan et al., 2008). Constitutive defenses are always expressed, while induced defenses are local or systemic responses to herbivore or pathogen attack (Bezemer & van Dam, 2005; van Dam & Heil, 2011). Moreover, recent work has shown that the induction of defense responses can persist, even after the attacker has been repelled, priming the plant for future attack (Conrath et al., 2006; Martinez-Medina et al., 2016). Given the multitude of these different ways for plants to respond to herbivory, as well as the plethora of secondary metabolites from diverse chemical classes, it is here again that eco-metabolomics may provide novel mechanistic insights into plant-herbivore interactions (van Dam & van der Meijden, 2011). Taken together, an eco-metabolomics approach to plant-soil-herbivore interactions will improve our understanding of the mechanisms that

govern multitrophic interactions.

The Trait-Based Experiment

The experiments of my dissertation were conducted within the wider framework of the Trait-Based Experiment (TBE; Ebeling, Pompe, et al., 2014). In 2010, the TBE was set up as a new diversity experiment within the Jena Experiment on a floodplain of the Saale river in Jena (Thuringia, Germany, 50°55'N, 11°35'E, 130 m a.s.l.) (Roscher et al., 2004; Weisser et al., 2017). Between 1980 – 2010, records show that for this region the mean annual precipitation was 610 mm and mean annual air temperature was 9.9°C (Hoffmann *et al.* 2014). The aim of the TBE was to test whether variation in functional traits related to spatial and temporal resource acquisition affects the functioning of plant communities (Ebeling, Pompe, et al., 2014; Weisser et al., 2017). To test this question, plant communities that varied in sown plant species richness and plant functional diversity were established in 138 plots of 3.5 x 3.5 m (Ebeling, Pompe, et al., 2014). The plant species of the TBE all belonged to the overall species pool of the Jena Experiment (N = 60 species) that represents Central European mesophilic grasslands (Roscher *et al.* 2004; Weisser *et al.* 2017). The final selection of species and assignment to plant species communities was based on a principle component analysis of the following six plant traits: plant height, leaf area, rooting depth, root length density, time of growth, and flowering onset (see Ebeling, Pompe, et al., 2014 for more details). In my dissertation, I sampled the following plant species: grasses: *Dactylis glomerata* L., *Holcus lanatus* L., *Phleum pratense* L., forbs: *Geranium pratense* L., *Leucanthemum vulgare* (Vaill.) Lam., *Plantago lanceolata* L., and *Ranunculus acris* L. (**Manuscript II**). In addition, soil from different plant communities of the TBE was used as inoculum to create soil biotic legacies (**Manuscript I + III**).

Objectives

The overall objective of my dissertation was to provide new mechanistic insights into plant-soil-herbivore interactions through the use of eco-metabolomics. The experimental framework included a combination of pot and field experiments to cover controlled and semi-natural conditions. Every study was set up in a way that plant and/or soil diversity was manipulated and the effect of changes in either type of biodiversity on the plant chemical diversity was measured. This experimental design allowed to test (1) if the plant diversity-induced soil biotic legacy affects the plant's metabolome; (2) if the plant diversity – herbivory relationship is modulated by soil biota diversity and the plant's metabolome; and (3) if plant diversity and soil biota diversity can independently affect the constitutive and herbivore-induced plant metabolome.

In **Manuscript I**, I investigated if plant diversity-driven soil biota legacy effects elicit changes in the composition and diversity of secondary metabolites. I further tested if the diversity in secondary metabolites correlated with shoot herbivory. In **Manuscript II**, I analyzed metabolomes in experimental plant communities to investigate if plant diversity-associated changes in plant metabolomes link to differences in soil communities, and how this relates to herbivory rates. In **Manuscript III**, I established two complementary microcosm experiments to investigate if plant diversity and soil legacy alter constitutive and herbivore-induced plant metabolomes independently. Finally, in the **Discussion**, I interpret my results in the light of other studies, provide examples how my dissertation advanced our understanding of plant-soil-herbivore interactions, and suggest avenues for future research.

MANUSCRIPT OVERVIEW

Manuscript I – Plant species richness elicits changes in the metabolome of grassland species *via* soil biotic legacy

Published in *Journal of Ecology* (2019) 107, 2240-2254

Christian Ristok, Yvonne Poeschl, Jan-Hendrik Dudenhöffer, Anne Ebeling, Nico Eisenhauer, Fredd Vergara, Cameron Wagg, Nicole M. van Dam, and Alexander Weinhold

In **Manuscript I**, I investigated if plant diversity-driven soil biotic legacy effects elicit changes in the shoot and root metabolome, and how this relates to aboveground herbivory. I analyzed individual plant metabolomes and shoot herbivory of four plant species that grew in sterile substrate inoculated with soil conditioned by different plant species communities. I found significant effects of soil biotic legacy on the overall shoot and root metabolome composition as well as on the richness of secondary metabolites. Moreover, I detected multiple metabolites that in combination could explain over 80% of the variation in shoot herbivory in two of the four plant species. Taken together, this study demonstrated that soil legacy effects can mechanistically link plant communities and aboveground herbivores *via* changes in the plant's metabolome.

JHD, AE and CW designed and conceptualized the experiment. CR, YP and AW designed and conceptualized the metabolomics analysis. CR, JHD, AE, FV and AW collected the data. CR and YP analyzed the data. CR, YP, NE, NMvD and AW interpreted the data. CR led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Manuscript II – Plant diversity effects on herbivory are mediated by soil biodiversity and plant chemistry

Under revision (21.07.2021) in *Ecology Letters*

Christian Ristok, Alexander Weinhold, Marcel Ciobanu, Yvonne Poeschl, Christiane Roscher, Fredd Vergara, Nico Eisenhauer, and Nicole M. van Dam

In **Manuscript II**, I investigated if the plant's metabolome directly, or indirectly *via* the soil biota, links plant diversity and aboveground herbivory. I analyzed individual plant metabolomes and herbivory rates of seven plant species growing in semi-natural experimental plant communities that varied in plant species richness and plant community resource acquisition strategies. I also assessed plant community-level soil microbial and nematode community composition. I found that individual plant herbivory rates decreased with increasing plant species richness, and that this relationship is likely mediated by changes in nematode community composition and plant metabolomes. Taken together, this study demonstrated that, by changing plant metabolomes, the soil community composition can reduce herbivory rates with increasing plant species richness in semi-natural plant communities.

CRi, AW, NE, and NMvD conceived the study. CRi, CRo, FV, and AW collected the data. MC identified and computed the nematode indices. CRi and YP analyzed the data. CRi, AW, NE, and NMvD interpreted the data. CRi wrote the manuscript under guidance of AW, NE, and NMvD. All authors contributed critically to the drafts and gave final approval for publication.

Manuscript III – Plant diversity and soil legacy independently affect the plant metabolome and induced responses following herbivory

Manuscript in preparation

Christian Ristok, Nico Eisenhauer, Alexander Weinhold, and Nicole M. van Dam

In **Manuscript III**, I investigated the independent effects of plant diversity and soil legacy on constitutive and herbivore-induced plant metabolomes. In two complementary microcosm experiments I grew plants either in sterile soil with different plant diversity levels, or single plant species on soil with different plant diversity-induced soil legacies. I assessed foliar and root metabolomes before and after herbivory by larvae of the generalist leaf-chewing herbivore *Spodoptera exigua*. I found that herbivore-induced metabolomes differed from those of control plants. Moreover, I detected over 100 metabolites that were significantly regulated by plant-plant and plant-soil interactions before or after herbivory. Taken together, this study demonstrated the independent effects of plant diversity and soil legacy on the plant's metabolome, and thereby the plant's defensive capability.

CR, NE, AW, and NMvD conceived the study. CR and AW collected the data. CR analyzed the data. CR, NE, AW, and NMvD interpreted the data. CR wrote the manuscript under guidance of NE, AW, and NMvD. All authors contributed critically to the drafts and gave final approval for publication.

MANUSCRIPT I

Plant species richness elicits changes in the metabolome of grassland species *via* soil biotic legacy

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Plant species richness elicits changes in the metabolome of grassland species via soil biotic legacy

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Abstract

1. Species-rich plant communities can induce unique soil biotic legacy effects through changing the abundance and composition of soil biota. These soil legacy effects can cause feedbacks to influence plant performance. In addition, soil biota can induce (defensive) secondary metabolites in shoots and roots and thus affect plant–herbivore interactions. We hypothesize that plant diversity-driven soil biotic legacy effects elicit changes in the shoot and root metabolome.
2. We tested this hypothesis by establishing an experiment with four plant species. We grew plants in a sterile substrate inoculated with soil conditioned by different plant species communities: (a) monocultures of either of the four species, (b) the four species in a mixture, (c) an eight species mixture including all four species or (d) a sterile inoculum. After at least 8 weeks in the field, we estimated shoot herbivory. At the same time, we took root and shoot samples for metabolomics analyses by liquid chromatography quadrupole time-of-flight mass spectrometry.
3. We found that shoot and root metabolomes of all plants grown in sterile soil differed significantly from those grown in living soil. The plant metabolomes in living soils differed by species and tissue. Across all species, shoots displayed a greater richness of secondary metabolites than roots. The richness of secondary metabolites differed by species and among living soils. The conditioning species richness significantly affected the Shannon diversity of secondary metabolites in *Centaurea jacea*. Shoot herbivory positively correlated with the richness and Shannon diversity of secondary metabolites in *Leucanthemum vulgare*. We detected multiple metabolites that together explained up to 88% of the variation in herbivory in the shoots of *C. jacea* and *Plantago lanceolata*.

4. *Synthesis*. Our findings suggest that plant diversity-driven shifts in soil biota elicit changes in the composition and diversity of shoot and root secondary metabolites. However, these plant responses and their effect on shoot herbivores are species-specific. Tracking changes in plant secondary chemistry in response to soil biotic legacy effects will help to understand the mechanisms that govern species-specific plant–plant and plant–herbivore interactions.

KEYWORDS
above-ground–below-ground interactions, biodiversity-ecosystem function, chemical diversity, eco-metabolomics, herbivory, Jena Experiment, metabolite profile

1 | INTRODUCTION

Each plant species harbours a unique rhizosphere community (Bezemer et al., 2010). In plant communities, each plant species thus contributes to the establishment of soil communities (Wardle et al., 2004). Relationships between plant community diversity and soil biota diversity have been reported to vary from neutral to positive over time in plant diversity experiments (Eisenhauer et al., 2010; Lange et al., 2015; Strecker, Macé, Scheu, & Eisenhauer, 2016). Such plant-induced changes in the soil community can remain operational over time and thus result in soil biotic legacy effects (Kardol, Cornips, van Kempen, Bakx-Schotman, & van der Putten, 2007).

Soil legacy effects can be either positive or negative, depending on whether the conditioned soil biota increase or reduce the performance of individual plants (Ehrenfeld, Ravit, & Elgersma, 2005; Kulmatiski, Beard, Stevens, & Cobbold, 2008; van der Putten et al., 2013). Negative intraspecific legacy effects can result from specialized soil pathogens. Soil pathogens can accumulate because of high and species-specific root exudation (Steinauer, Chatzinotas, & Eisenhauer, 2016; van de Voorde, van der Putten, & Bezemer, 2011). At the same time, the same root exudates may suppress root pathogens of neighbouring heterospecific plants, thus providing positive interspecific legacy effects (van de Voorde et al., 2011). Hence, chemical traits of individual plants can shape soil legacy effects through changing soil biota community composition and activity.

These altered soil communities, in turn, can also influence chemical traits of individual plants. For instance, the composition and concentration of defensive plant secondary metabolites, such as glucosinolates, iridoid glycosides or pyrrolizidine alkaloids, can change in response to soil microbial composition, nematodes and mycorrhizal fungi (Bezemer & van Dam, 2005; Hol et al., 2010; Kos, Tuijl, de Roo, Mulder, & Bezemer, 2015b; Wurst, Wagenaar, Biere, & Van der Putten, 2010). These interactions with plant growth facilitators and plant antagonists are thus likely to influence the diversity of secondary metabolites that a plant produces. This additionally implies that soil legacy effects may also affect the entire plant metabolome, that is the entirety of all metabolites synthesized by an organism (Oliver, Winson, Kell, & Baganz, 1998).

Changes in the composition and concentration of plant metabolites are known to affect important ecological functions, such as the resistance to above-ground herbivory. Herbivory can induce metabolite synthesis locally (induced defences) in the attacked plant tissue or systemically throughout the plant (Bezemer & van Dam, 2005). Systemic induction can also elicit changes at the concentration of shoot metabolites as a consequence of interactions with soil biota. This systemic induction can then affect the resistance to above-ground herbivores (van Dam & Heil, 2011). Such a response in above-ground herbivores thus constitutes indirect soil legacy effects. Indirect soil legacy effects can result in increased or reduced performance of specific plant species (Karban, Agrawal, Thaler, & Adler, 1999). This may ultimately affect the fitness of a species and their abundance in the plant community.

Here we analyse soil legacy effects as reflected in shifts in the individual plant metabolome at the end of a plant-soil feedback experiment (Dudenhöffer, Ebeling, Klein, & Wagg, 2018). We defined soil legacy effects on the metabolome as shifts in the composition or the diversity of secondary metabolites. Furthermore, we defined the strength of the legacy effects as the magnitude of the difference in the metabolite profile among different living soils. The plant-soil feedback experiment was designed to test how soils conditioned by different plant communities affect key plant life stages. In the context of the experiment, soil conditioning affected flower production and plant fitness with mostly neutral effects on plant biomass (Dudenhöffer et al., 2018).

Based on the previously reported effects, we hypothesized that (a) plants grown in living soil differ in their metabolome compared to plants grown in sterile soil, and that (b) plants grown in soil conditioned by plant communities that differ in their species richness display different shoot and root metabolomes. In addition, we hypothesized that (c) the individual diversity of secondary metabolites increases with an increasing species richness of the soil conditioning plant community, and that (d) the diversity of secondary metabolites correlates with shoot herbivory. In order to test these hypotheses, we grew four common grassland plant species in three living soils that differed in the diversity of the conditioning plant community. In addition, we added a control group grown in sterile soil.

2 | MATERIALS AND METHODS

2.1 | Experimental design

In summer 2014, we set up a soil legacy experiment with four common central European grassland herb species (*Centaurea jacea* L., *Knautia arvensis* (L.) Coult., *Leucanthemum vulgare* Lam., and *Plantago lanceolata* L.). We used 3 L pots filled with 2,700 g autoclaved (120°C for 20 min) sand-field soil mixture (50:50, v/v) taken from the 'Jena Experiment' (www.the-jena-experiment.de) field site (Thuringia, Germany; 50°55′N, 11°35′E, 130 m a.s.l, Roscher et al., 2004). In order to remove roots and coarse stones, we sieved the field soil through a 5 cm mesh. We added 100 g living or sterile soil inoculum to each pot and thoroughly mixed the inoculum with the sterile sand-field soil substrate. Finally, we added 200 g of the sterile background substrate on top, thus minimizing cross contamination (Dudenhöffer et al., 2018). For each pot, the inoculum comprised only 3.33% (w/w) of the entire substrate, with 96.66% (w/w) being the same sterile standard background substrate. This setup minimized the abiotic effect of any particular soil inoculum (Brinkman, Van der Putten, Bakker, & Verhoeven, 2010). However, this also meant that the autoclaved sterile background may contain pulsed nutrients and toxins (Alphei & Scheu, 1993; Trevors, 1996).

We established three living soil and one sterile soil treatments for each plant species. The living soil inocula had been conditioned by different plant species compositions, that is monocultures of either of the four species (CR1 – conditioning species richness 1), the four species in mixture (CR4) or an eight species mixture (CR8) including all four plant species supplemented by the four common grass species *Festuca rubra* L., *Helicotrichon pubescens* (Huds.) Domort., *Phleum pratense* L. and *Poa pratensis* L. All plant communities had been sown in plots of 3.5 m × 3.5 m in summer 2010 and are part of the 'Trait Based Experiment' (for more details see Ebeling et al., 2014). More specifically, soil collection for this study was conducted 4 years after establishment of the plots. We collected multiple soil cores from the upper 10 cm of each plot along a transect throughout the length of the plot to account for within-plot variability. We sieved each living soil inoculum through a 1 cm mesh and subsequently stored all soil inocula at 4°C for 24 hr prior to the experimental setup. The sterile soil inoculum was a mixture of equal parts of all living soil inocula sterilized by autoclaving at 120°C for 20 min. This created a common baseline for the sterile soil inoculum treatment.

The full experimental design resulted in 128 pots (Table S1). Each combination of plant species and corresponding soil inoculum treatment was replicated eight times, arranged in eight blocks (Dudenhöffer et al., 2018). The plants of six pots died during the experiment and were thus not available for further analyses; mortality was not related to any experimental treatment (Table S1). Initially, 20 (non-sterilized) seeds per pot were sown (Rieger-Hofmann GmbH, Blaufelden-Raboldshausen, Germany) at a depth of 1 cm and subsequently covered with clear plastic cellophane in order to keep humidity in the pots high to encourage germination and seedling establishment. We transferred all pots to two climate chambers (four blocks per chamber) equipped with artificial light (four Osram Powerstar HQI-T 1000/D, E40, 1,000 W, 80,000 lm lamps per chamber) with a photoperiod of 16 hr in light at 20°C and 8 hr in darkness at 16°C. We removed the plastic cellophane once seedlings had established. In mid-November 2014, after 11 weeks, we reduced the number of plants per pot to three individuals by cutting the other plants just below the shoot meristems. The corresponding roots remained in the soil to decompose. Decomposing roots can elicit negative as well as positive effects on plant biomass production (Zhang, Van der Putten, & Veen, 2016). In the context of this experiment, however, we detected mostly neutral effects on plant biomass (Dudenhöffer et al., 2018). We then transferred all pots to an unheated glasshouse located at the Botanical Garden in Jena, Germany. There, the plants were confronted with a natural winter photoperiod and 8°C. In early May 2015, we moved all pots to an open area at the field site of the Jena Experiment maintaining the original eight blocks (for more details see Dudenhöffer et al., 2018). During the field phase of the experiment, the lower half of each pot was covered in a bag that was closed to the bottom. This protected the pot against invasion of external soil biota and below-ground herbivores. At the same time, it allowed only the natural occurring above-ground herbivores and pollinators to interact with all plants. At the end of the experiment, we validated the sterile soil inoculum treatment by assessing the presence of mycorrhizal structures in roots (Dudenhöffer et al., 2018). Only three samples of the sterile treatment displayed the presence of mycorrhizal structures in roots. These samples, however, did not affect any of our consecutive analyses/results and were thus included.

2.2 | Sampling and sample processing

We harvested the shoot and root biomass of one plant per pot at the end of the flowering period, which occurred in July 2015 for *K. arvensis*, *L. vulgare* and *P. lanceolata*, and in September 2015 for *C. jacea*. We separated the shoot and root biomass by cutting the plants with scissors and removed all flowers from the shoot samples. We counted the total number of leaves and the number of leaves with herbivore damage. Herbivore damage included signs of sucking, chewing and mining on leaves. We washed the roots twice in tap water to remove soil particles, and then dried the samples with paper towels. This process took roughly 30 s, and samples were then immediately stored in paper bags on dry ice to stop further metabolism. In the laboratory, samples were stored in a −80°C freezer, and subsequently, freeze-dried (LABCONCO FreeZone Plus 12 Liter, Kansas City, USA) for 72 hr. Dried samples were stored in zip-lock bags filled with silica gel at room temperature until further processing. We measured the dry weight in milligram and ground each sample to a fine homogenous powder using a ball mill (Retsch mixer mill MM 400; Haan, Germany).

2.3 | Metabolome extraction and analysis

We extracted 20 mg dried ground plant tissue of each sample in 1 ml of extraction buffer (methanol/50 mM acetate buffer, pH 4.8; 50/50 [v/v]). The samples were homogenized for 5 min at 30 Hz using a ball mill (Retsch mixer mill MM 400), and subsequently centrifuged (25,155 *g*, 10 min, 4°C). The supernatant was collected in a 2 ml Eppendorf tube. We repeated the extraction procedure with the remaining pellet and combined the supernatant with the first one. We

centrifuged (25,155 *g*, 5 min, 4°C) all extracts, transferred 200 µl to an HPLC vial and added 800 µl extraction buffer, resulting in a 1:5 dilution.

We performed chromatographic separation of all diluted extracts by injecting 2 µl on a Thermo Scientific Dionex UltiMate 3000 (Thermo Scientific Dionex, Sunnyvale, CA, USA) UPLC unit, equipped with a C18 column (Acclaim RSLC 120 C18, 2.2 µm, 120 Å, 2.1 × 150 mm, Thermo Fisher Scientific). We applied the following binary elution gradient at a flow rate of 0.4 ml/min and a column temperature of 40°C: 0–2 min, 95% A (water and 0.05% formic acid), 5% B (acetonitrile and 0.05% formic acid); 2–12 min, 5%–50% B; 12–13 min, 50%–95% B; 13–15 min, 95% B; 15–16 min, 95%–5% B; 16–20 min, 5% B.

Metabolites were analysed on a liquid chromatography quadrupole time-of-flight mass spectrometer (LC-qToF-MS; Bruker maXis impact HD; Bruker Daltonik, Bremen, Germany) with an electrospray ionization source operated in negative mode. Instrument settings were as follows: capillary voltage, 2,500 V; nebulizer, 2.0 bar; dry gas temperature, 220°C; dry gas flow, 10 L/min; scan range, 50–1,000 m/z; acquisition rate, 1 Hz. We used sodium formate clusters (10 mM solution of NaOH in 50/50% [v/v] isopropanol/water containing 0.2% formic acid) to perform mass calibration.

2.4 | LC-MS data processing

We converted the LC-qToF-MS raw data to the mzXML format by using the CompassXport utility of the DataAnalysis vendor software. Subsequently, we trimmed each data file by excluding the same non-informative regions at the beginning and end of each run using the msconvert function of ProteoWizard v3.0.10095 (Chambers et al., 2012). We performed peak picking, feature alignment and feature group collapse in R v3.3.3 (R Core Team, 2017) using the Bioconductor (Huber et al., 2015) packages 'xcms' (Benton, Want, & Ebbels, 2010; Smith, Want, O'Maille, Abagyan, & Siuzdak, 2006; Tautenhahn, Böttcher, & Neumann, 2008) and 'CAMERA' (Kuhl, Tautenhahn, Böttcher, Larson, & Neumann, 2012). We performed simulation experiments to analyse the best set of parameters prior to data processing (Table S2). These parameters included, among others, the signal-to-noise ratio which determines the proportion of low-intensity metabolites. Based on our tests, we chose a low signal-to-noise ratio, that is the inclusion of low-intensity metabolites. We used the following 'xcms' parameters: peak picking method 'centWave' (snthr = 10; ppm = 10; peakwidth = 4, 10); peak grouping method 'density' (minfrac = 0.7; bw = 3; mzwid = 0.005); retention time correction method 'symmetric'. We used 'CAMERA' to annotate adducts, fragments and isotope peaks with the following parameters: extended rule set (https://gitlab.com/users/stanstrup/groups); perfwhm = 0.6; calcIso = TRUE; calcCaS = TRUE, graphMethod = lpc. Lastly, we collapsed each annotated feature group, hereafter referred to as 'metabolite' which is described by mass-to-charge ratio (m/z) and retention time (rt), using a maximum heuristic approach. This means in detail that the intensity values of the feature, which most often displayed the highest intensity across all samples represent the feature group. We performed pre-processing with 'xcms' and 'CAMERA' separately for each species and tissue type. We merged the four species-specific feature lists by m/z and rt values, allowing for a retention time window of 10 s and a mass deviation of 5 ppm. We tentatively identified metabolites through the comparison of LC-MS/MS data with literature references. We submitted high-resolution m/z values to the MassBank of North America (MoNA, http://mona.fiehnlab.ucdavis.edu/) spectral database for comparison using a mass tolerance of 0.5 D. In addition, we calculated low-resolution molecular weights, molecular formulae for putative molecular ions in neutral form, and particle weights for mass spectrometry generated fragments using ChemDraw Ultra 8.0 (www.cambridgesoft.com).

2.5 | Statistical analysis

We analysed our data in the statistical software R v3.3.3 using the packages 'mixOmics' (Cao et al., 2017), 'vegan' (Oksanen et al., 2017), 'lme4' (Bates, Mächler, Bolker, & Walker, 2015), 'lmerTest' (Kuznetsova, Brockhoff, & Christensen, 2016), 'car' (Fox & Weisberg, 2011) and 'multcomp' (Hothorn, Bretz, & Westfall, 2008).

In order to test our hypotheses and to accommodate the experimental design, we analysed the data only within species and tissue. We visualized the differences in metabolome composition between sterile soil and living soil and among the different living soil inoculums by performing Partial Least Squares - Discriminant Analyses (PLS-DAs). Differences in metabolome composition are not only based on the presence, absence, or identity of metabolites. The intensity of the corresponding signals in the mass spectrometer (which is proportional to the concentration of a particular metabolite) also contributes to the metabolome composition. Therefore, we ran pairwise multi-response permutation procedures (MRPP) on log + 1-transformed metabolite intensity data to test for significant differences in the metabolite profile between our different treatments. The MRPP dissimilarity matrix was Bray–Curtis and each analysis was permuted 10,000 times.

We calculated two metrics of metabolite diversity: (a) the number of metabolites within a plant individual (hereafter, richness of secondary metabolites) and (b) the abundance-weighed diversity of metabolites expressed as the Shannon–Weaver index (Hill, 1973) based on plant individual-level metabolite intensities (hereafter, Shannon diversity of secondary metabolites). We used Dunnett's test for single step comparison to compare the richness and Shannon diversity of secondary metabolites expressed by plants in either living soil against the expression in sterile soil. This analysis is similar to contrasts but corrects for the multiple comparison problem. In order to test if the richness of secondary metabolites increases with increasing conditioning species richness, we calculated a linear mixed effects model. We calculated a similar linear mixed effects model to test if the Shannon diversity of secondary metabolites increases with increasing conditioning species richness. In addition, we analysed if the richness and Shannon diversity of secondary metabolites correlates with above-ground herbivory

using linear mixed effects models. The linear mixed effects models were based on restricted maximum likelihood estimation and Type I analyses of variance (ANOVA) with Satterthwaite approximation for degrees of freedom. In the first two cases, the richness or Shannon diversity of secondary metabolites was the dependent variable. As explanatory variables, we fitted tissue, the conditioning species richness (CR) of the living soil inocula and the interaction of both. In order to account for the spatial arrangement and non-random design, we applied 'block' as the random effect. In the last case, the dependent variable was shoot herbivory (expressed as relative number of damaged leaves in percent). The explanatory variables were either the richness or the Shannon diversity of secondary metabolites, and random effects were the CR of the living soil inocula (random slope) and 'block' (random intercept).

In addition, we applied Least Absolute Shrinkage and Selection Operator (LASSO) regression (Bujak, Daghir-Wojtkowiak, Kaliszan, & Markuszewski, 2016; LeWitt, Li, Lu, Guo, & Auinger, 2017; Tibshirani, 1996), to identify the combination of metabolites that could best predict shoot herbivory patterns along the CR gradient. The LASSO algorithm assumes that the herbivory responses can be 'predicted' by a linear combination of metabolite intensities. LASSO estimates the coefficients of this linear combination by shrinking coefficients of predictors (here metabolites) using an *l1* penalty in order to minimize the mean squared error in the herbivory. Some coefficients are penalized to zero and non-zero coefficients of predictors (metabolites) indicate that these are important 'features' for predicting herbivory with the least error. We used the 'cv.glmnet' function, including a leave one out cross validation, provided by the 'glmnet' (Friedman, Hastie, & Tibshirani, 2010) package to determine the sets of metabolites for each species and tissue type that could explain the herbivory pattern. The cross-validation process returns the most parsimonious model that has a cross-validated error within one standard deviation of the minimum.

3 | RESULTS

3.1 | Soil biota effects on the composition and diversity of plant metabolomes

We compared the metabolomes of plants grown either in sterile or living soil, and observed significant differences across species, above- and below-ground (Figure 1). The metabolomes of plants grown in living soil were more similar to each other than to the metabolomes of plants grown in sterile soil. Based on the results of Dunnett's test, we found significant differences in the richness and Shannon diversity of secondary metabolites (Table S3) between plants growing in living and sterile soil. The richness of secondary metabolites in the roots of *L. vulgare* and *P. lanceolata* was consistently lower in sterile soil compared to living soil plants (Figure 2). When we compared sterile soil plants to plants grown in CR8 soil, we found a lower richness of secondary metabolites in the roots of *K. arvensis* and in the shoot of *C. jacea*, but a higher richness of secondary metabolites in the shoot of *L. vulgare*. The Shannon diversity of secondary metabolites was significantly lower in sterile soil

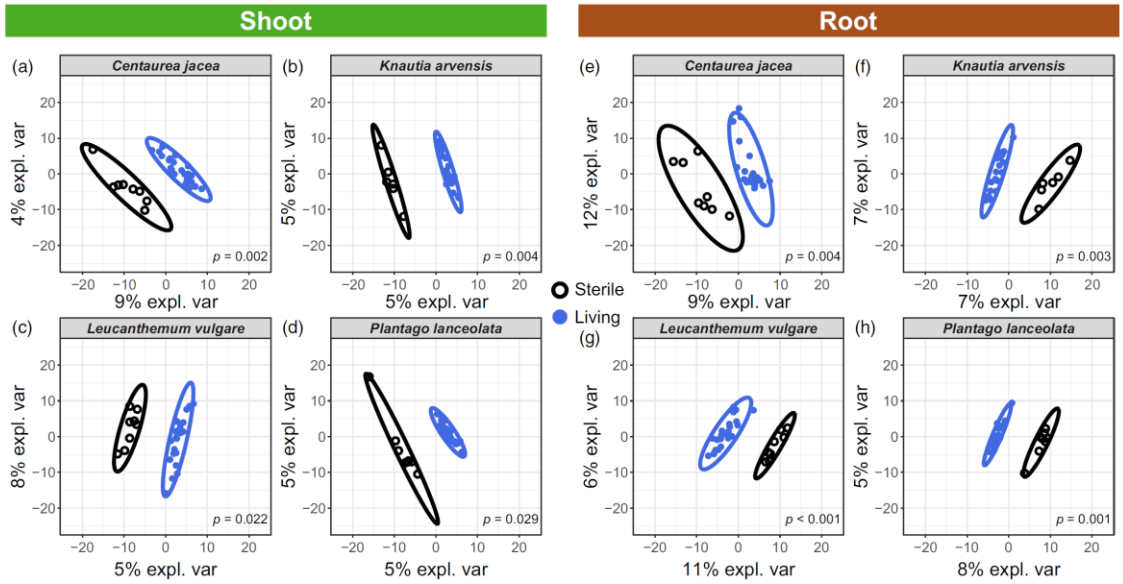

FIGURE 1 Per species Partial Least Squares - Discriminant Analysis plots of the metabolites found in shoot (a–d) and root metabolomes (e–h). Plants grew either in sterile soil (black, open circles, n = 6–8) or living soil (blue, closed circles, n = 22–24). Ellipses represent the 95% confidence interval. p-values are based on pairwise multi-response permutation procedures. The metabolite intensity matrix was log + 1 transformed for the purpose of data normalization. Abbreviations: expl. var = explained variance

compared to living soil in *L. vulgare* and *P. lanceolata* (with the exception of *P. lanceolata* plants grown in CR1 soil; Figure 2). Shoot herbivory did not significantly differ between plants grown in sterile soil and plants grown in living soil in either plant species (Table S3).

3.2 | Soil legacy effects on the composition of plant metabolomes

The three living soil inocula differed in their effect on shoot and root metabolomes across all plant species (Figure 3). Consecutive pairwise comparisons revealed that the differences in shoot metabolomes were more prevalent than in root metabolomes. The shoot metabolomes differed significantly between CR4 soil and CR8 soil across all species (Table 1). In *P. lanceolata*, the shoot metabolomes also differed significantly between all three living soil inocula. Root metabolomes differed between CR1 soil and CR4 soil in samples of *C. jacea* and *L. vulgare* (Table 1). In addition, root metabolomes differed between CR4 soil and CR8 soil in samples of *C. jacea*, *L. vulgare* and *P. lanceolata*. The root metabolome of *K. arvensis* was unaffected by the CR of the soil inoculum (Figure 3f).

3.3 | Soil legacy effects on the diversity of plant metabolomes

We found significant differences in the richness of secondary metabolites between shoots and roots across all species, with a higher richness of secondary metabolites in shoots than in roots (Table 2). In addition, we found significant differences in the Shannon diversity of secondary metabolites between shoot and roots in *K. arvensis*, *P. lanceolata* and marginally significant differences in *C. jacea* (Table 2). In contrast, the Shannon diversity of secondary metabolites between shoot and roots in *L. vulgare* did not differ (Table 2).

The richness of secondary metabolites significantly increased with increasing CR in *K. arvensis* and *P. lanceolata*. In contrast, the richness of secondary metabolites significantly decreased with increasing CR in *L. vulgare* (Figure 2; Table 2). We observed a tissue-specific response of the richness of secondary metabolites to increasing CR in *C. jacea* (Table 2). An increase in CR increased the richness of secondary metabolites in *C. jacea* shoots, but reduced the richness in *C. jacea* roots (Figure 2).

The Shannon diversity of secondary metabolites significantly responded to an increase in CR in *C. jacea*, only (Table 2). However, we observed a tissue-specific response with an increase in the Shannon diversity of secondary metabolites with increasing CR in *C. jacea* shoots, but a decrease in *C. jacea* roots (Figure 2).

3.4 | Linking richness, Shannon diversity and identity of secondary metabolites to herbivory

We analysed if the amount of above-ground herbivory relates to the richness of shoot secondary metabolites and their Shannon diversity.

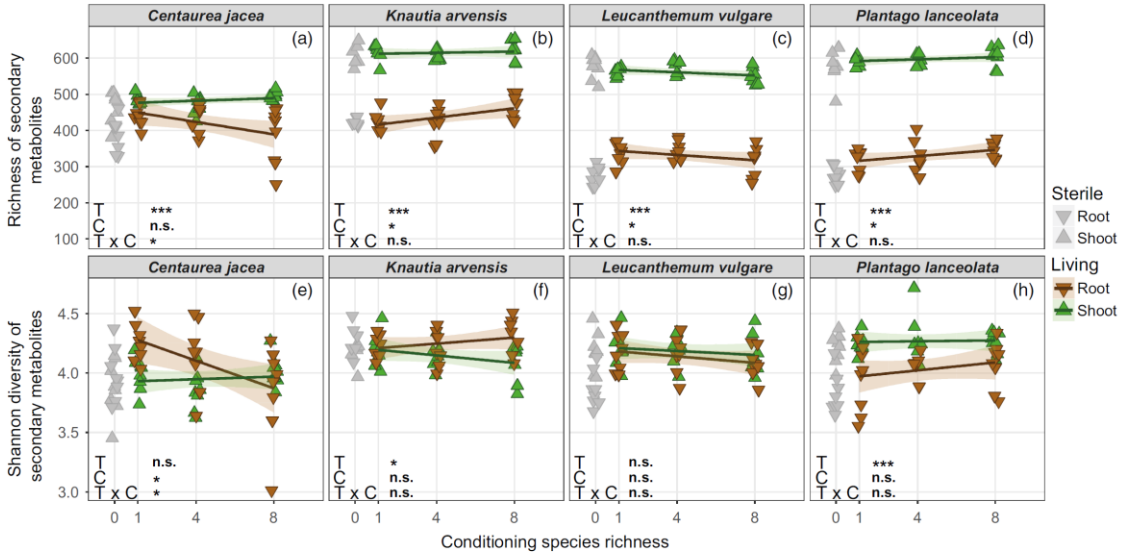

FIGURE 2 Richness (a–d) and Shannon diversity of secondary metabolites (e–h) per species in response to soil inocula that differ in the number of soil conditioning plant species richness. Shoot tissue-specific responses are displayed in green. Root tissue-specific responses are displayed in brown. Responses in sterile soil are displayed in grey but were excluded in the analyses. The shaded area surrounding the regression lines represent the 95% confidence band. The statistical significance of the responses is based on linear mixed effect models. Abbreviations: T, Tissue; C, Conditioning species richness; T × C, The interaction of tissue and conditioning species richness; n.s., not significant. The asterisks denote significance levels: ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

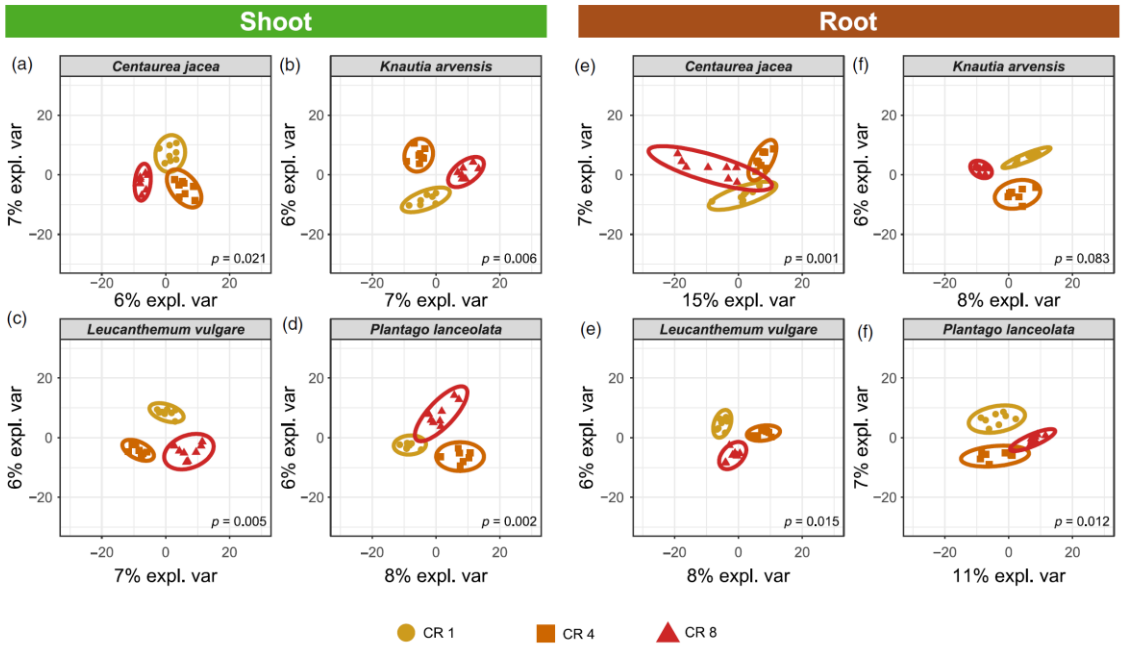

FIGURE 3 Per species Partial Least Squares - Discriminant Analysis plots of the metabolites found in shoot (a–d) and root metabolomes (e–h). Plants grew in soils conditioned by plant communities differing in species richness. Ellipses represent the 95% confidence interval. *p*-values are based on multi-response permutation procedures. The metabolite intensity matrix was log + 1 transformed for the purpose of data normalization. Abbreviations: CR 1 = conditioning species richness (CR) 1, that is monoculture (yellow circles, *n* = 7–8); CR 4 = 4-plant species mixture (orange squares, *n* = 7–8); CR 8 = 8-plant species mixture (red triangles, *n* = 7–8); expl. var = explained variance

TABLE 1 Differences in the species-specific shoot and root metabolomes of plants grown in three different living soil inocula. Represented are *p*-values, based on pairwise multi-response permutations procedures based on 10,000 permutations. Significant differences ($p < 0.05$) are given in bold

Soil conditioning plant species richness	*Centaurea jacea*	*Knautia arvensis*	*Leucanthemum vulgare*	*Plantago lanceolata*
Shoot				
1 versus 4	0.128	0.154	**0.004**	**0.005**
1 versus 8	**0.049**	**0.033**	0.203	**0.044**
4 versus 8	**0.037**	**0.015**	**0.006**	**0.011**
Root				
1 versus 4	**0.030**	–	**0.005**	0.221
1 versus 8	0.088	–	0.351	**0.023**
4 versus 8	**0.002**	–	**0.047**	**0.031**

We found significant positive relationships between above-ground herbivory and the richness ($F_{1,18} = 7.578$, $p = 0.013$) and Shannon diversity of secondary metabolites ($F_{1,19} = 17.390$, $p < 0.001$) in shoots of *L. vulgare* only (Figure 4; Table 3).

In addition, we related shoot herbivory to a combination of specific shoot secondary metabolites and found links between the identities of secondary metabolites and shoot herbivory. By using LASSO, we detected a combination of 15 metabolites in *C. jacea* shoot samples, which explained 88.8% of the total variation in shoot herbivory. Furthermore, we detected a combination of nine metabolites in *P. lanceolata* shoot samples that explained 86.1% of the total variation in shoot herbivory. In contrast, the LASSO regression found no congruent combination of metabolites in samples of *K. arvensis* and *L. vulgare* (Table 4). Because the full dataset contained on average 33.2% low-intensity metabolites, we repeated our analysis with a reduced dataset that only contained metabolites above median intensity. We did this as a sensitivity analysis of the results achieved for the full dataset. In the reduced

TABLE 2 Statistical parameters resulting from a Type 1 ANOVA on species-specific richness and Shannon diversity of secondary metabolites variables. The four plant species grew in soils conditioned by plant communities differing in species richness. We sampled both plant tissues, the shoot and root. Significant differences ($p < 0.05$) are given in bold

	Richness of secondary metabolite						Shannon diversity of secondary metabolites					
Plant species	NumDF	DenDF	SS	MS	*F*	*p*	NumDF	DenDF	SS	MS	*F*	*p*
Centaurea jacea												
Tissue	1	37	46,314	46,314	27.81	**<0.001**	1	37	0.216	0.216	4.00	0.053
CR	1	37	4,570	4,570	2.74	0.106	1	37	0.271	0.271	5.02	**0.031**
Tissue × CR	1	37	10,555	10,555	6.34	**0.016**	1	37	0.396	0.396	7.33	**0.010**
Knautia arvensis												
Tissue	1	40	346,569	346,569	379.78	**<0.001**	1	40	0.130	0.130	7.06	**0.011**
CR	1	40	4,569	4,569	5.01	**0.031**	1	40	0.001	0.001	0.04	0.833
Tissue × CR	1	40	2,748	2,748	3.01	0.090	1	40	0.069	0.069	3.76	0.060
Leucanthemum vulgare												
Tissue	1	42	603,301	603,301	785.78	**<0.001**	1	35	0.025	0.025	1.23	0.274
CR	1	42	3,376	3,376	4.40	**0.042**	1	35	0.047	0.047	2.32	0.137
Tissue × CR	1	42	220	220	0.29	0.595	1	35	0.003	0.003	0.14	0.706
Plantago lanceolata												
Tissue	1	35	817,956	817,956	1,317.62	**<0.001**	1	35	0.657	0.657	25.11	**<0.001**
CR	1	35	3,308	3,308	5.33	**0.027**	1	35	0.034	0.034	1.29	0.265
Tissue × CR	1	35	757	757	1.22	0.277	1	35	0.022	0.022	0.84	0.367

Abbreviations: CR, conditioning species richness; NumDF, numerator degrees of freedom; DenDF, denominator degrees of freedom; SS, sum of squares; MS, mean squares; F, F-value; *p*, *p*-value.

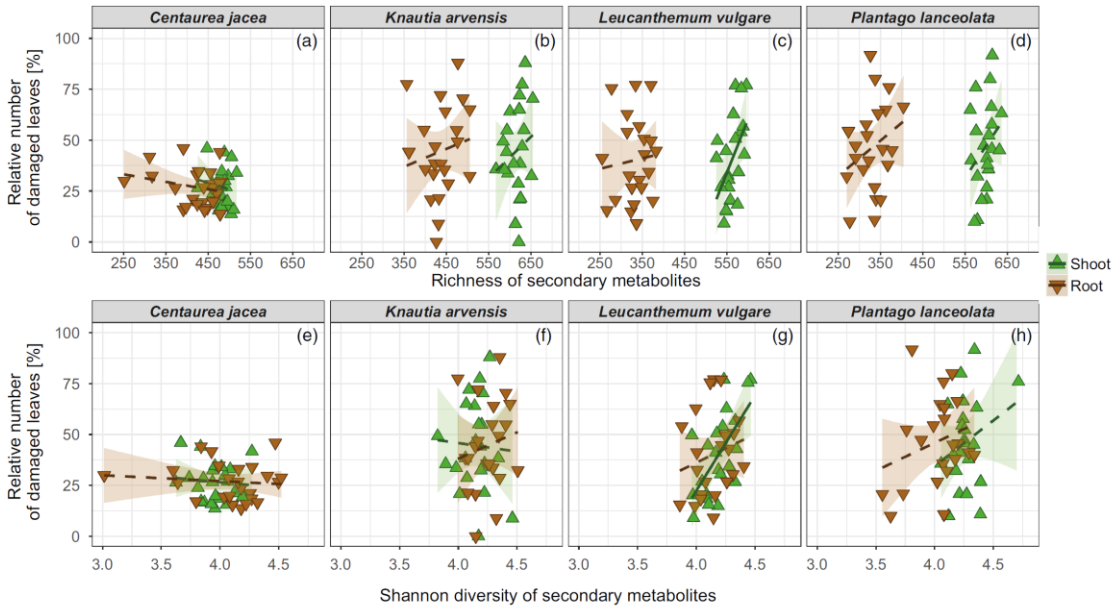

FIGURE 4 Relationship between shoot herbivory and the richness (a–d) and Shannon diversity of secondary metabolites (e–h) per species. Shoot tissue-specific responses are displayed in green. Root tissue-specific responses are displayed in brown. The shaded area surrounding the regression lines represent the 95% confidence band. Solid line – significant relationship; dashed line – non-significant relationship. The statistical significance of the responses is based on linear mixed effect models

dataset, we detected a combination of 11 metabolites in *C. jacea* shoot samples that explained 69.2% of the total variation in shoot herbivory. From the full dataset to the reduced dataset, LASSO retained six metabolites that could best predict herbivory. On the contrary, the set of selected metabolites from the reduced dataset contained five additional metabolites that were not included in the selected metabolite combination from the full dataset. In *P. lanceolata* shoot samples, we detected a combination of seven metabolites in the reduced dataset. These seven metabolites explained 64.2% of the total variation in shoot herbivory (Table 4). All seven metabolites that were part of the linear combination in the reduced dataset were part of the linear combination in the full dataset too.

We identified 10 putative metabolites in *C. jacea* samples and five putative metabolites in *P. lanceolata* samples (Table 5; Figure S1). In addition, we tentatively identified seven metabolites in *C. jacea* and *P. lanceolata* samples that were not part of any linear combination (Table S4; Figure S1).

4 | DISCUSSION

We demonstrated that both the root and shoot metabolome of four grassland species reacted to the presence of soil biota. Soils with a different legacy of plant species richness elicited shifts in the metabolite profiles. In addition, we detected combinations of metabolites that best explained the variation in shoot herbivory. Hence, our results point to soil legacy effects as a possible mechanism linking plant communities and above-ground herbivores through changes in secondary metabolites.

4.1 | Soil biota effects on the composition and diversity of plant metabolomes

The presence of soil biota had profound effects on shoot and root metabolomes across all four plant species in our study. Plants grown in living soils had substantially different metabolomes than plants grown in sterile soil. Hence, our results support the hypothesis (1) that plants grown in living soil differ in their metabolome compared to plants grown in sterile soil. Our results are in line with work on tomato and ragwort showing that plants grown in sterile soil had reduced phenolic and alkaloid concentrations, respectively, compared to plants grown in living soils (Chialva et al., 2018; Joosten, Mulder, Klinkhamer, & van Veen, 2009). These differences were attributed to the absence of arbuscular mycorrhizal fungi (AMF) in the sterile soil (Chialva et al., 2018; Rivero, Gamir, Aroca, Pozo, & Flors, 2015). AMF colonization can alter the levels of secondary metabolites, such as alkaloids and flavonoids, as well as primary metabolites, such as amino acids and sugars, through compound-specific up- or downregulation (Rivero et al., 2015). Notably, the effects of soil biota on the plant metabolome were reported to be stronger under suboptimal conditions, such as in relatively sandy soil and nutrient poor substrate (Kos, Tuijl, de Roo, Mulder, & Bezemer,

TABLE 3 Statistical parameters resulting from a Type 1 ANOVA on species-specific and tissue-specific shoot herbivory as a function of the richness and Shannon diversity of secondary metabolites variables. Significant differences ($p < 0.05$) are given in bold

Response variable Shoot herbivory	Richness of secondary metabolites						Shannon diversity of secondary metabolites					
	NumDF	DenDF	SS	MS	*F*	*p*	NumDF	DenDF	SS	MS	*F*	*p*
Shoot												
Centaurea jacea	1	17	72.36	72.36	1.35	0.261	1	16	40.54	40.54	0.70	0.414
Knautia arvensis	1	20	415.27	415.27	0.82	0.376	1	20	26.64	26.64	0.05	0.821
Leucanthemum vulgare	1	18	1,892.50	1,892.50	7.58	**0.013**	1	19	3,141.90	3,141.90	17.39	**<0.001**
Plantago lanceolata	1	15	452.88	452.88	2.94	0.108	1	11	425.97	425.97	1.96	0.189
Root												
Centaurea jacea	1	18	20.80	20.80	0.32	0.581	1	16	8.76	8.76	0.14	0.711
K. arvensis	1	18	251.88	251.88	0.50	0.489	1	17	356.24	356.24	0.76	0.396
L. vulgare	1	19	6.45	6.45	0.02	0.894	1	20	159.54	159.54	0.44	0.515
P. lanceolata	1	13	538.86	538.86	3.72	0.077	1	16	226.51	226.51	1.07	0.317

Abbreviations: NumDF, numerator degrees of freedom; DenDF, denominator degrees of freedom; SS, sum of squares; MS, mean squares; *F*, *F*-value; *p*, *p*-value.

2015a) like we used in our experiment. Our experimental setup also meant that all plants grew in a mostly sterilized substrate (96.66% of the total soil per pot). Sterilization by autoclaving can result in a pulse of nutrients and toxins (Alphei & Scheu, 1993; Trevors, 1996). It may be possible that the plants in our experiment have shown species-specific responses to the pulse in nutrient and toxins. But to address this possibility, we performed all tests within a species, rather than between species. Our results show that the presence of soil biota had a metabolome-wide impact on four different plant species. Hence, our results have strong implications for results obtained in experiments that solely use sterile soil when it comes to their extrapolation to natural systems.

4.2 | Soil legacy effects on the composition of plant metabolomes

We observed that the CR levels affected the shoot and root metabolomes across all plant species. However, the response to soils with different CR levels was species-specific and tissue-specific. These species-specific and tissue-specific responses support our hypothesis (2) that plant metabolomes change according to the plant diversity-driven soil legacy they encounter. It proved difficult to compare our results to similar studies, because research on plant diversity-driven effects on plant metabolomes is scarce (Peters et al., 2018). In one study, increasing plant diversity was linked to shifts in the above-ground metabolic profile of small-growing herbs but not of tall-growing herbs, with more than 100 detected metabolites that changed in concentration (Scherling, Roscher, Giavalisco, Schulze, & Weckwerth, 2010). In another case, metabolic fingerprinting revealed adaptation to monoculture or plant species mixture history (Zuppinger-Dingley, Flynn, Brandl, & Schmid, 2015). Here, the accumulation of soil pathogens in monocultures was suggested to drive shifts in certain metabolic groups. Our study now adds valuable insights by revealing that plant diversity-driven soil legacy effects induce shifts in the composition and diversity of secondary metabolites. Given the lack of further research on plant diversity-induced shifts in the metabolome, we compared our results to similar studies that focused on single species or single compound classes. These studies on single species or single compound classes confirm our interpretation of plant diversity-induced effects. For instance, bacterial or fungal root pathogens, and non-pathogenic soil bacteria, as well as mycorrhizal fungi caused changes in above-ground defence compounds (Bezemer et al., 2005; Hol et al., 2010; van Dam & Heil, 2011). These changes in above-ground defence compounds range from a decrease to an increase depending on the plant species and below-ground interaction partner (Bezemer & van Dam, 2005). Although shoot and roots can differ in their response to soil biota, shifts in single compound classes were also reported. For instance, in the roots of *P. lanceolata*, nematodes had no effect on iridoid glycoside concentration, whereas soil micro-organisms increased iridoid glycoside levels (Wurst et al., 2010).

A particular in our study was that within the living soil treatments, the soil legacy of CR 8 soils led to distinct metabolomes expressed across all species and tissues. We attribute this effect in part

TABLE 4 Comparison between the full metabolite dataset and the reduced metabolite dataset. The reduced metabolite dataset contains only metabolites above median intensity. Displayed are the total numbers of metabolites in both datasets, the number of metabolites selected using Least Absolute Shrinkage and Selection Operator (LASSO) algorithms, and the percentage of explained variance in shoot herbivory by the LASSO-picked metabolites. In addition, displayed are the number of similar metabolites picked by the LASSO algorithms in both the full metabolite dataset and the reduced metabolite dataset

	Full metabolite dataset			Reduced metabolite dataset			
Plant species	Number of metabolites	LASSO combination	Explained variance	Number of metabolites	LASSO combination	Explained variance	Overlapping metabolites
Centaurea jacea	608	15	88.8%	406	11	69.2%	6
Knautia arvensis	798	–	–	499	–	–	–
Leucanthemum vulgare	730	–	–	475	–	–	–
Plantago lanceolata	766	9	86.1%	490	7	64.2%	7

TABLE 5 Compounds tentatively assigned in shoot extracts of *Centaurea jacea* and *Plantago lanceolata* by LC-MS/MS. The metabolites were detected by LASSO and were part of a linear combination that explains shoot herbivory

Source	Rt [min]	$[M - H]^-$ [m/z]	Putative compound	Molecular formula	eV	MS/MS
C. jacea	1.02	191.0196	Quinic acid	$C_7H_{12}O_6$	35	
C. jacea	2.83	315.0714	Quinic acid octenoic ester	$C_{15}H_{24}O_7$	35	191
C. jacea	5.16	707.1817	Chlorogenic acid dimer 1	$C_{32}H_{36}O_{18}$	35	191, 163
C. jacea	5.26	515.1402	3,5-*O*-dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	35	337, 163
C. jacea	5.44	707.1818	Chlorogenic acid dimer 2	$C_{32}H_{36}O_{18}$	35	353, 191
C. jacea	5.50	707.1818	Chlorogenic acid dimer 3	$C_{32}H_{36}O_{18}$	35	353, 191, 163
C. jacea	6.64	367.1031	3-Feruloylquinic acid	$C_{17}H_{20}O_9$	35	191
C. jacea	6.74	675.1937	Chlorogenic acid dimer 4	$C_{32}H_{36}O_{16}$	35	337, 191
C. jacea	8.04	507.1150	Syringetin-3-*O*-galactoside	$C_{23}H_{24}O_{13}$	35	477
C. jacea	8.98	521.1297	Flavonoid glycoside	$C_{24}H_{26}O_{13}$	35	359
P. lanceolata	1.07	407.1190	Chlorogenic acid derivative	$C_{20}H_{24}O_9$	35	191
P. lanceolata	5.50	813.1378	Verbascoside 1	$C_{37}H_{50}O_{20}$	35	163
P. lanceolata	6.99	639.1935	Verbascoside 2	$C_{29}H_{36}O_{16}$	35	415
P. lanceolata	8.00	637.2141	Verbascoside 3	$C_{29}H_{34}O_{16}$	35	445
P. lanceolata	8.17	495.1509	Iridoid-*O*-glycoside	$C_{23}H_{28}O_{12}$	35	121

Abbreviations: eV, Fragmentation energy in electron volt; LASSO, Least Absolute Shrinkage and Selection Operator; MS/MS, mass spectrometry/mass spectrometry; Rt, Retention time in liquid chromatography in minutes.

to the higher conditioning species richness and to the presence of an additional plant functional group, that is grasses, in the conditioning phase. Our results contrast with those of Kos et al. (2015b). They found that the functional group of the conditioning plant species did not alter the concentration of pyrrolizidine alkaloids and amino acids in *Jacobaea vulgaris*. We believe that our approach of integrating a comprehensive part of the secondary metabolome in the analysis allowed for detection of plant functional group effects which went unnoticed so far.

4.3 | Soil legacy effects on the diversity of plant metabolomes

In addition to analysing the metabolite profile, we analysed the species-specific and tissue-specific richness and Shannon diversity of secondary metabolites. With regard to our hypothesis (3) stating that the individual diversity of secondary metabolites increases with conditioning species richness, we found inconsistent responses of the metabolome to the conditioning species richness treatment. In *K. arvensis* and *P. lanceolata*, we observed additional metabolites of low intensity with increasing CR level that did not contribute to the pool of dominating metabolites. This was indicated by an increase in metabolite richness whereas Shannon diversity indicators remained constant. *Leucanthemum vulgare* lost low-intensity metabolites with increasing CR level. This was indicated by a decrease in the richness at similar Shannon metabolite diversity. Only in *C. jacea* did we observe similar patterns of richness and Shannon diversity of secondary metabolites. This indicates that metabolites of higher intensity gained or lost intensity and thus changed the pool of dominant metabolites. This induced change in richness and Shannon diversity of secondary metabolites is indicative of the strength of the soil biotic legacy. The strength

of the soil biotic legacy varies with the composition and diversity in the conditioning plant species community. More abundant plant species generally experience stronger negative effects on plant performance than less abundant species (Kos, Veendrick, & Bezemer, 2013; Maron, Laney Smith, Ortega, Pearson, & Callaway, 2016; van de Voorde, van der Putten, & Bezemer, 2012). This phenomenon is most often attributed to an increase in species-specific soil pathogens with decreasing plant community richness (Kulmatiski et al., 2008; Zuppinger-Dingley et al., 2015).

We observed positive and negative intraspecific and interspecific soil legacy effects on the richness of secondary metabolites with increasing CR level. However, the richness and Shannon diversity of secondary metabolites did not reflect directional changes induced by the soil legacy. It is likely that the directional increase in conditioning species richness was not the only factor affecting soil biota. Induced non-directional legacy effects in the soil biota can be the result of shoot and root herbivory in the conditioning plant community (Kostenko, van de Voorde, Mulder, van der Putten, & Bezemer, 2012). Such effects can interfere with the directional soil legacy effect of increasing conditioning species richness.

Notably, given that the experimental setup minimized the effects of soil abiotic conditions by only adding 3.33% soil inoculum to common background substrate, we conclude that our results show that different plant communities can elicit unique metabolite profiles in individual plants via soil legacy effects. In addition, soil legacy effects of plant communities of increasing conditioning species richness induced directional to non-directional changes in the richness and Shannon diversity of secondary metabolites. We conclude that integrating richness and Shannon diversity with metabolite profiles provides a more holistic description of plant secondary chemistry than focusing on only one of these metrics. Hence, we advocate utilizing a comprehensive integration of the secondary plant metabolome rather than single compound classes, as interacting partners, for example herbivores, rarely encounter only a subset of compound classes but rather the full metabolome. Going forward, this knowledge can then help to understand the mechanisms that drive plant–herbivore interactions.

4.4 | Linking richness, Shannon diversity and identity of secondary metabolites to herbivory

We related the richness and Shannon diversity of secondary metabolites to the level of shoot herbivory in plants. Herbivory significantly related to the richness and diversity of secondary metabolites in the shoots of *L. vulgare*, only. Previous studies showed negative correlations between metabolite richness and herbivore damage in 12 species of Asteraceae (Macel, de Vos, Jansen, van der Putten, & van Dam, 2014). Hence, we expected to find similar correlations between the richness and Shannon diversity of secondary metabolites and plant herbivory across all species (hypothesis 4). However, our experiment provided limited support for this hypothesis, which we partly attribute to the design of the present study. In our study, we were unable to tease apart constitutive defences and their effect on plant herbivores from herbivory and its effect on plant secondary metabolites via herbivore-induced responses (Bezemer & van Dam, 2005; Macel et al., 2014). In addition, single one-dimensional variables, such as richness or Shannon diversity of secondary metabolites, might not be sufficient to describe the metabolome of a plant in plant–herbivore interactions. Hence, we applied LASSO to detect linear combinations of metabolites that may explain variance in shoot herbivory. We applied LASSO to a full and a truncated dataset to test the robustness of our findings. In both cases, LASSO identified a single linear combination of metabolites that explained variation in shoot herbivory in *C. jacea* and *P. lanceolata*, only. Differences in the linear combination between both datasets in *C. jacea* indicate that low-intensity metabolites contributed to the plant–herbivore interaction. In *P. lanceolata*, seven to nine high-intensity metabolites explained shoot herbivory. We tentatively identified the metabolites of the linear combinations as quinic acid/quinic acid derivatives, chlorogenic acid derivatives, flavonoid glycosides, verbascosides and iridoid glycosides in *C. jacea* and *P. lanceolata*. These metabolites are known for their significant role in plant–herbivore interactions (Bowers & Puttick, 1988; Erb et al., 2009; Leiss, Maltese, Choi, Verpoorte, & Klinkhamer, 2009; Sutter & Müller, 2011; Treutter, 2006). In addition, our LASSO outcome hints to two different strategies how plants can adjust their metabolome in plant–herbivore interactions. Either plants concentrate their energy on the synthesis of a few metabolites, in which case LASSO was able to detect a linear combination of metabolites that explains variation in herbivory, or plants spread their energy across the synthesis of many metabolites, in which case LASSO detected multiple equivalent combinations of metabolites. Both strategies may constitute viable approaches to act and react as plant–herbivore interactions unfold. Alternatively, LASSO may not have detected a single linear combination of metabolites in *K. arvensis* and *L. vulgare* because additional herbivore resistance factors exist. Additional resistance factors, such as physical defence, can result in trade-offs between these factors and chemical defence (Eichenberg, Purschke, Ristok, Wessjohann, & Bruelheide, 2015).

5 | CONCLUSIONS

Plant diversity-driven soil legacy affects the performance of plants, as well as the composition and concentration of certain secondary metabolites. We show that soil biota in general induced changes in the root and shoot metabolome of common grassland plant species. In addition, we provide evidence that plant community properties, that is differences in plant species richness, can translate into responses in the composition and diversity of secondary metabolites. We suggest two different strategies of plants to deal with shoot herbivory, based on the species-specific composition of secondary metabolites. Based on our results, we conclude that plant diversity-driven soil legacy can affect plant–herbivore interactions through changes in secondary metabolites. This field

of research may be a key to understand some of the mechanisms that govern species interactions including important ecosystem functions like herbivory.

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AUTHORS' CONTRIBUTIONS

J.H.D., A.E. and C.W. designed and conceptualized the experiment; C.R., Y.P. and A.W. designed and conceptualized the metabolomics analysis; C.R., J.H.D., A.E., F.V. and A.W. collected the data; C.R. and Y.P. analysed the data; C.R., Y.P., N.E., N.M.v.D. and A.W. interpreted the data; C.R. led the writing of the manuscript. All the authors contributed critically to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

The data are archived at MetaboLights: https://www.ebi.ac.uk/metabolights/MTBLS544 (Ristok et al., 2019).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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MANUSCRIPT II

Plant diversity effects on herbivory are mediated by soil biodiversity and plant chemistry

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Abstract

Insect herbivory is a key process in ecosystem functioning. While theory predicts that plant diversity modulates herbivory, the mechanistic links remain unclear. We postulated that the plant metabolome mechanistically links plant diversity and herbivory.

In late summer and in spring, we assessed individual plant aboveground herbivory rates and metabolomes of seven plant species in experimental plant communities varying in plant species and resource acquisition strategy diversity. In the same plots, we also measured plant individual biomass as well as soil microbial and nematode community composition.

Herbivory rates decreased with increasing plant species richness. Path modelling revealed that plant species richness and community resource acquisition strategy affected soil community composition. In particular, changes in nematode community composition affected plant metabolomes and thereby herbivory rates.

These results provide experimental evidence that soil community composition plays an important role in reducing herbivory rates with increasing plant diversity by changing plant metabolomes.

Keywords: Aboveground-belowground interactions, Biodiversity-ecosystem function, Chemical diversity, Eco-metabolomics, Herbivory, Jena Experiment, Metabolite profile

Introduction

Insect herbivory is an essential ecosystem process that can remove substantial amounts of biomass in grasslands within a single season (Meyer *et al.* 2017; Seabloom *et al.* 2017). Plant diversity can influence the abundance and diversity of insect herbivores (Haddad *et al.* 2001; Hertzog *et al.* 2016) as well as herbivory rates (Ebeling *et al.* 2014a; Wan *et al.* 2020). Plant traits, in particular those associated with resource acquisition and competition, are considered to provide mechanistic links between plant diversity and herbivory (Loranger *et al.* 2013). However, a recent study shows that the commonly used morphological and physiological traits only explain up to 12.7% of the variance in herbivory (van der Plas *et al.* 2020). Plant chemical composition may be a better predictor for individual herbivory, because many herbivores use plant metabolites to find their host (Agrawal & Weber 2015). By using the plant's metabolome, *i.e.,* the composition of all metabolites produced by an individual plant (Oliver *et al.* 1998), as an additional functional plant trait, we may gain deeper insights in the molecular mechanisms underlying differences in herbivory.

Several factors may explain differences in herbivory rates across plant diversity gradients. Higher levels of plant diversity may increase niche diversity by increasing spatial heterogeneity and the variety of food sources, thus, supporting more insect herbivores and increasing community-level herbivory rates (Ebeling *et al.* 2014a). At individual plant level, however, increased plant diversity may lead to dilution effects which decrease herbivory, as it will be more difficult for specialized insect herbivores to localize their host plant (Finch & Collier 2000; Castagneyrol *et al.* 2014). Indeed, in a previous study conducted in the Jena Experiment, individual herbivory decreased with increasing plant species richness (Scherber *et al.* 2006). Lastly, the abundance of predatory and parasitoid arthropods, which can reduce herbivore populations and thus plant community-level and individual herbivory *via* top-

down control, is commonly higher in more diverse plant communities (Haddad *et al.* 2009; Hines *et al.* 2015; Schuldt *et al.* 2019; Wan *et al.* 2020).

In addition, differences in herbivory across plant species richness gradients may also be explained through changes in plant chemistry. Plant metabolomes change in response to (a)biotic variation. This metabolomic response to environmental conditions co-determines the defensive status of a plant (van Dam & van der Meijden 2011). For instance, plants increase the synthesis of defensive metabolites following an attack by herbivores (Karban & Baldwin 1997; Bezemer & van Dam 2005). These induced responses can change defences both locally, *i.e.,* in the attacked tissue, and systemically, *i.e.,* throughout the plant (van Dam & Heil 2011). In addition to herbivory-induced changes, plant diversity itself can affect plant metabolomes. Plant-plant interactions can alter the metabolome through competition, which may induce the production of volatile (Baldwin *et al.* 2006) and non-volatile allelopathic compounds (Fernandez *et al.* 2016). Seen the broad biological activity spectrum of plant metabolites, these changes are likely to affect herbivory rates (Broz *et al.* 2010). Lastly, soil legacy effects, which may result from systemically induced changes triggered by soil biota, such as microbes and nematodes (van Dam & Heil 2011; Wondafrash *et al.* 2013) can also affect plant metabolomes (Ristok *et al.* 2019). Taken together, the plant metabolome both affects and reflects above- and belowground interactions with insect herbivores, other plants, and soil biota, in a species-specific and context-dependent way (Bezemer & van Dam 2005; Ristok *et al.* 2019). Hence, we argue that measuring plant metabolomes will provide unique mechanistic insights into the effects of plant diversity on herbivory.

Here, we analysed the metabolomes and individual plant herbivory of three grass and four forb species in experimental plant communities manipulated to vary in spatial or temporal resource acquisition traits (Ebeling *et al.* 2014b). We selected these species because they cover a range of functional traits related to resource acquisition (Ebeling *et al.* 2014b). We

included both grasses and forbs, because their metabolomes and response to the abiotic and biotic environment may differ (Dietz *et al.* 2019, 2020; Huberty *et al.* 2020).

All plants were grown in 34 experimental plant communities that varied in plant diversity, *i.e.,* species richness and functional trait diversity (Ebeling *et al.* 2014b). We tested if and how plant diversity alters the secondary metabolome and how this relates to herbivory. We hypothesized that (1) plant species richness and the resource acquisition strategy of the plant community affect individual plant herbivory. Moreover, we calculated partial-least-squares path models to explore if (2) plant species richness and plant community resource acquisition traits directly or indirectly, *via* the soil biota, relate to the plant's metabolome and thereby may explain variation in herbivory. Our hypotheses are based on observations that plant species richness affects soil community composition (Eisenhauer *et al.* 2010) and that differences in soil biota can affect the plant's metabolome and thereby herbivory (Ristok *et al.* 2019; Huberty *et al.* 2020). We also tested whether individual plant herbivory rates decrease with increasing plant species richness due to dilution effects (Scherber *et al.* 2006; Castagneyrol *et al.* 2014). We inferred similar paths for functional trait composition, but tested whether communities containing tall-statured species with large leaves and deep roots increase individual plant herbivory, as these species may provide more niches for insect herbivores (Loranger *et al.* 2012). We also tested whether growth and flowering time can affect herbivory as plant chemistry is known to change with ontogeny (Boege 2005; Barton & Koricheva 2010). Lastly, soil community composition can affect plant biomass, which in turn may affect herbivory, whereby larger plants may incur more herbivory (Windig 1993).

We show that increasing plant species richness reduces individual plant herbivory. Our analyses also indicate that plant diversity effects on herbivory can be mediated by soil biota,

in particular nematodes, and that these plant diversity-driven differences in soil communities can affect plant metabolomes.

Methods

Experimental design

The Trait-Based Experiment (Ebeling *et al.* 2014b) was established in 2010 within the 'Jena Experiment' (www.the-jena-experiment.de) field site, Thuringia, Germany; 50°55' N, 11°35' E, 130 m a.s.l. (Roscher *et al.* 2004; see **Appendix S1** for details). We sampled 34 plots that differed in plant species richness (1, 2, 4 and 8 species) and plant functional trait dissimilarity. The functional trait dissimilarity was based on traits that reflect spatial and temporal resource acquisition strategies. We chose plant height, leaf area, rooting depth, and root length density to reflect spatial resource acquisition. To reflect temporal resource acquisition, we chose growth starting date and flowering onset. All plots were arranged in three blocks, mown in June and September, and weeded three times per year.

Secondary metabolome sampling and sample processing

We sampled twice under different environmental conditions to account for seasonal variation in the plants' metabolomes. We sampled aboveground biomass of seven common central European grassland species (**Appendix S1**) on 24 – 25 August 2015 and 31 May – 1 June 2016 just before mowing. We sampled 444 plants across both sampling campaigns and all species, *i.e.*, three individuals per species and plot. One sample was lost during sample processing. We harvested the shoot biomass by cutting the plants ca. 1 cm above ground and removed all inflorescences. All samples were taken between 15.00 and 19.00 h each sampling day to minimize diurnal variation. All samples were processed, extracted, and

analysed according to Ristok et al. (2019) with slight changes (see **Appendix S1**).

LC-MS data processing and metabolite prediction

The LC-MS data are presented as a list of features described by mass-to-charge ratios, retention times, and intensities. We processed LC-MS data as in Ristok et al. (2019) with minor changes (see **Appendix S1**). We predicted metabolite structures through the comparison of LC-MS data with literature references. We submitted high-resolution mass-to-charge values to the MassBank of North America (MoNA, http://mona.fiehnlab.ucdavis.edu/) spectral database. We used a mass tolerance of 0.5 D for comparison. Furthermore, we calculated high-resolution molecular weights, molecular formulae for putative molecular ions in neutral form, and particle weights for mass spectrometry generated fragments using ChemDraw Ultra 8.0 (www.cambridgesoft.com).

Leaf herbivory rate assessment

For each plant, we counted the total number of leaves and the number of leaves with herbivore damage one day before we sampled aboveground biomass. We categorized herbivore damage for each damaged leaf that had signs of sucking, chewing, and mining. The damage categories were 1-10%, 10-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, 80-90%, and 90-100%. We multiplied the number of leaves in each category with their damage level (0.1 for 1-10%, 0.2 for 10-20%, etc.), and summed across all categories. Finally, we calculated relative herbivory rate for each sample by dividing the summed herbivory by the total number of leaves.

Soil sampling

In each plot, we took soil samples to a depth of 10 cm using a metal corer (diameter 2 cm)

on 27 August 2015 and 6 June 2016. We pooled and homogenized five subsamples per plot to account for spatial heterogeneity. We sieved soil samples to 2mm. We stored one part at – 20 °C for phospholipid fatty acid analysis and the other part at 4 °C for nematode extraction.

Phospholipid fatty acid analysis

We measured phospholipid fatty acids (PLFA) as a proxy for the soil microbial community composition, based on the abundance of functional groups (Wixon & Balser 2013). We extracted PLFAs following the Frostegård et al. (1991) protocol as described in Wagner et al. (2015). We analysed all samples on a gas chromatograph (see **Appendix S1**). We used the following PLFA-markers (ng g^{-1} dry weight soil) as bacterial markers: (a) gram-negative bacteria: cy17:0 and cy19:0; (b) gram-positive bacteria: i15:0, a15:0, i16:0, and i17:0; and (c) widespread in bacteria: 16:1ω5 and 16:1ω7. As fungal markers we used: (a) saprophytic fungi: 18:1ω9t, 18:2ω6t, and 18:2ω6c; and (b) arbuscular mycorrhizal fungi: 20:1 (Ruess & Chamberlain 2010; Wagner *et al.* 2015). We summed up all markers within each group of bacteria and fungi to receive a representative value.

Nematode extraction and identification

We extracted nematodes from 25 g fresh soil using a modified Baermann method (Ruess 1995; Wagner *et al.* 2015). We counted all nematodes at 100x magnification and identified at least 100 randomly chosen nematodes (if available) at 400x magnification using a Leica DMI 4000B light microscope. Nematodes were identified to genus or family level following Bongers (1994). We classified all nematodes into plant feeders, fungal feeders, bacterial feeders, predators, and omnivores. Moreover, we assigned all nematodes a c-p score (colonization-persistence gradient) that ranged from 1 to 5 (Bongers & Bongers 1998).

Finally, we combined the trophic group and c-p score to create functional nematode guilds as a proxy for nematode community structure (Ferris *et al.* 2001; Cesarz *et al.* 2015).

Statistical analysis

We analysed our data in R v3.5 (R Core Team 2017) (http://www.r-project.org) using the packages 'vegan' (Oksanen *et al.* 2017), 'pairwiseAdonis' (Arbizu 2017), 'lme4' (Bates *et al.* 2014), 'lmerTest' (Kuznetsova *et al.* 2016), 'effects' (Fox & Weisberg 2019), and 'plspm' (Sanchez *et al.* 2017).

Based on earlier studies in the same experiment (Steinauer *et al.* 2017; Beugnon *et al.* 2019), we calculated community mean scores (CMS) to represent resource acquisition strategy (spatial and temporal). We based our CMS calculations on the original PCA species scores calculated when the experiment was designed (Ebeling *et al.* 2014b; Fischer *et al.* 2016), and on the relative species-specific cover for each plant community recorded in August 2015 and May 2016, respectively. In short, the six functional traits plant height, leaf area, rooting depth, root length density, growth starting date, and flowering onset were analysed in a standardized PCA. The first PCA axis arranged species according to their spatial resource acquisition strategy. The second PCA axis arranged species according to their temporal resource acquisition strategy (Ebeling *et al.* 2014b). Plots with high community mean scores on the first PCA axis (CMS_PCA1) were mostly dominated by tall-statured species with deep roots and large leaves. In contrast, plots with negative community mean scores on the first PCA axis contained a high proportion of small-statured species with dense shallow roots and small leaves. Plots with high community mean scores on the second PCA axis (CMS_PCA2) contained mostly late growing and late flowering species (Fischer *et al.* 2016).

We tested our first hypothesis by calculating linear mixed effects models. We fitted herbivory rate (log-transformed) as the response variable. As predictor variables, we fitted sampling campaign (categorical; August 2015 or May 2016), plant functional group identity (categorical; grass or forb), and either plant species richness (metric; 1, 2, 4 or 8) or either CMS_PCA1 or CMS_PCA2 (metric), as well as the three-way interaction. We fitted plot nested in block and species identity as independent random effects. Model simplification was achieved by using the Akaike Information Criterion (AIC). All linear mixed effects models were based on restricted-maximum likelihood estimation and Type I analysis of variance with Satterthwaite approximation for degrees of freedom.

To test our second hypothesis, we calculated partial-least-squares path models (PLS-PM; see **Appendix S1**) (Sanchez 2013). We hypothesized direct links from the experimental design variables plant species richness and resource acquisition traits to microbial and nematode community composition (De Deyn & Van der Putten 2005; Strecker *et al.* 2016), as well as to plant individual biomass (Tilman *et al.* 2001), plant metabolome (Scherling *et al.* 2010), and individual plant herbivory (Scherber *et al.* 2006) (for details on latent variables see **Table 1**). In addition, we hypothesized links from the microbial and nematode community composition to plant biomass (van der Putten *et al.* 2013) and the composition of the plant metabolome (Ristok *et al.* 2019; Huberty *et al.* 2020), as well as from plant biomass to metabolome (de Jong 1995; Fernandez *et al.* 2016) and herbivory (Barnes *et al.* 2020). Finally, we hypothesized a link from plant metabolome to herbivory (van Dam & van der Meijden 2011) (**Fig. 2a**). We calculated three separate PLS-PMs: (a) the full model using all data of the seven plant species; (b) the grasses-only model using only data of the three grass species; and (c) the forbs-only model using only data of the four forb species. All data were scaled, and we used bootstrapping (n = 200) to calculate confidence intervals for the

effect sizes within the path models. We simplified all path models by reducing the number of outer model observable variables (so-called indicators), until the most parsimonious solution was achieved (Sanchez 2013). Indicators are always positively correlated with their latent variable; a low value of the latent variable relates to a low value in all respective indicators, and *vice versa*. Latent variables are estimated as a weighted linear combination of their indicators (Sanchez 2013). Across all three models, the latent variable resource acquisition traits was characterized by the community-weighted means of leaf area, rooting depth, root length density, growth starting date, and flowering start. The latent variable soil microbial community was characterized by gram-negative, gram-positive, general bacteria, saprophytic fungi, and arbuscular mycorrhizal fungi. The effects of the latent variable nematode community were mostly driven by bacterial feeders with a colonization-persistence score (c-p) of 1 and 3, predators (c-p 4 and 5), fungivores (c-p 4), omnivores (c-p 4), and plant feeders (c-p 2 and 3).

Results

The effects of plant species richness and resource acquisition strategy

Herbivory rates decreased significantly with plant species richness (**Table S1; Fig. 1a**) and were lower in May than in August (**Table S1; Fig. 1**). Together, plant species richness and sampling campaign explained 26% (marginal R^2 value, hereafter R^2_{marg}) of the total variation in herbivory rates.

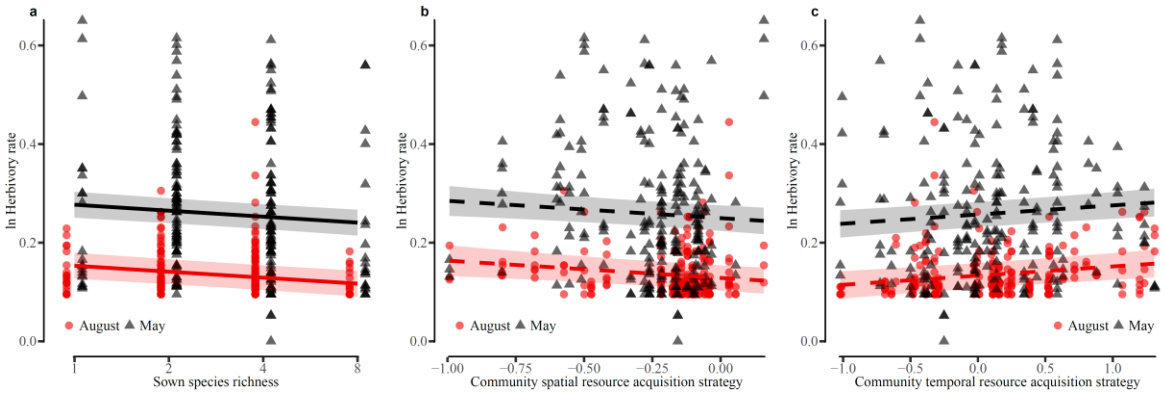

Figure 1 The plant-individual herbivory rate (log-transformed) in response to (a) sown species richness, (b) community spatial resource acquisition strategy, and (c) community temporal resource acquisition strategy of the Trait-Based Experiment. For clarity, the placement of the symbols corresponding to the sampling campaign in panel 'a' have been slightly shifted along the x-axis. The relationship in August is displayed in circles and red colour. The relationship in May is displayed in triangles and black colour. Significant relationships are displayed by solid lines. Non-significant relationships are displayed by dashed lines. Regression line estimates are based on linear mixed effect models with plot nested in block, and species identity as independent random terms (**Table S1**). The shaded band displays the standard error.

We found no significant effect of the community's spatial resource acquisition strategy on herbivory rates (**Table S1**). In other words, the relative abundance of tall-statured species with large leaves and deep roots did not significantly affect the plant-individual herbivory rates (**Fig. 1b**). However, when we tested for the effect of community spatial resource acquisition strategy within functional groups, we found a significant effect for grasses (**Table S2**). Grasses growing in communities that predominantly contained tall-statured plants with large leaves suffered lower herbivory rates (**Fig. S1a**; $R^2_{marg} = 0.35$). In contrast, we observed no significant effect of spatial resource acquisition strategy on individual herbivory in forbs (**Table S2; Fig. S2a**). When we tested for the effect of the temporal resource acquisition strategy of the plant communities on herbivory rates, we only found a marginally significant relationship (**Table S1**). More specifically, plant-individual herbivory tended to be greater in communities containing mostly later growing and flowering species

(**Fig. 1c**). Separate analyses for each functional group showed that this effect was significant for grasses (R^2_{marg} = 0.36; **Table S2; Fig. S1b**), but not for forbs (**Table S2; Fig. S2b**).

We also tested for the effects of plant species richness on plant metabolome composition and on metabolite diversity, *i.e.*, richness or Shannon diversity of secondary metabolites (see **Appendix S1** for details on the calculation and analysis). We found a significant effect of plant species richness on the metabolome composition across most plant species in at least one sampling campaign, except for *Holcus lanatus* (**Table 2**). Consecutive pairwise comparisons revealed that the metabolome of plants grown in monocultures most frequently differed from the metabolome of plants grown in more diverse communities (**Table S3**).
We did not find an effect of plant species richness on the richness of secondary metabolites. Rather, we observed an effect of plant functional group identity on the richness of secondary metabolites ($F_{1,5}$ = 6.69; p = 0.049; **Table S4a**). Forb species had significantly more secondary metabolites (396 ± 8.1; mean ± SE) than grass species (311 ± 8.5; mean ± SE). Together with sampling campaign, plant functional group identity could explain 68% (R^2_{marg}) of the total variation in secondary metabolite richness. We also discovered that the effect of plant species richness on the Shannon diversity of secondary metabolites depended on the functional group identity ($F_{1,138}$ = 5.35; p = 0.022; **Table S4a**). Increasing plant species richness increased the Shannon diversity of secondary metabolites in grasses, while it reduced the Shannon diversity of secondary metabolites in forbs (**Fig. S3**). Sampling campaign, plant functional group identity, and plant species richness together explained 49% (R^2_{marg}) of the total variation in the Shannon diversity of secondary metabolites.

Moreover, we analysed the extent to which resource acquisition strategy affected metabolite diversity. We found that some traits associated with spatial resource acquisition strategy can

increase or decrease the richness of metabolites dependent on sampling campaign and functional group identity (**Table S4**). The community-weighted means of plant height, rooting depth, and root length density all reduced the richness of metabolites in grasses in May, but increased their richness in August (**Table S5; Fig. S4**). We observed a similar effect of plant height on the richness of metabolites in forbs. In contrast, rooting depth and root length density increased the richness of metabolites in forbs in May, but reduced their richness in August (**Table S5; Fig. S5**). In addition, we discovered that the community-weighted mean of leaf area, a trait associated with spatial resource acquisition, and the community-weighted mean of flowering onset, associated with temporal resource acquisition, had similar effects on the Shannon diversity of metabolites (**Table S4**). Both, leaf area and flowering onset, increased Shannon diversity of metabolites in grasses (**Table S5; Fig. S6**). In forbs, leaf area and flowering onset reduced the Shannon diversity in May, while they increased the Shannon diversity of metabolites in August (**Table S5; Fig. S7**).

The plant secondary metabolome links plant and soil diversity with aboveground herbivory

By analysing the significant paths in our full-model PLS-PM (**Fig. 2b**; Goodness-of-Fit (GoF) = 0.13), we found that nematode community composition was negatively related to plant species richness and resource acquisition traits, while the nematode community composition itself was positively related to plant metabolomes (for details of each latent variable see **Table 1**; for all direct, indirect, and total path coefficients see **Table S6**). Our most parsimonious model predicted 30% of the total variation in the secondary metabolome, and 23% of the total variation in individual herbivory. Plant species richness was negatively correlated with the relative abundance of predatory, omnivorous, and plant feeding nematodes (**Fig. S8**). The spatial resource acquisition trait leaf area was negatively correlated with the relative abundance of bacterial feeders, fungivores, omnivores, and plant feeders.

Rooting depth and root length density negatively correlated with predatory nematodes, but positively correlated with plant feeders. Conversely, the temporal resource acquisition traits growth starting date and flowering start were negatively correlated with the relative abundance of plant feeders (**Fig. S8**). In addition, we discovered that nematode community composition was positively related to plant metabolome composition, which itself was negatively correlated with plant individual herbivory. We extracted the 100 most important metabolite mass spectra that characterized the metabolome, *i.e.*, the metabolites with the strongest positive correlation with the latent variable 'metabolome'. We could assign molecular formulas and structures to 13 mass spectra (**Table S7; Fig. S9 – S21**). These metabolites were mainly phenolics, their precursors or their derivatives, which are all products of the shikimic acid pathway. Moreover, these compounds are known to respond to phytopathogenic nematode infection (Ohri & Pannu 2010) and play a role in plant-herbivore interactions (Whitehead *et al.* 2021). As part of the nematode community composition, the relative abundance of bacterial feeders, predators, omnivores, and plant feeders showed the strongest positive correlations with the concentration of the assigned metabolites. Especially sinapic acid, a flavonol, the chlorogenic acid dimers, and quinic acid, were negatively correlated with plant herbivory (**Fig. S22**).

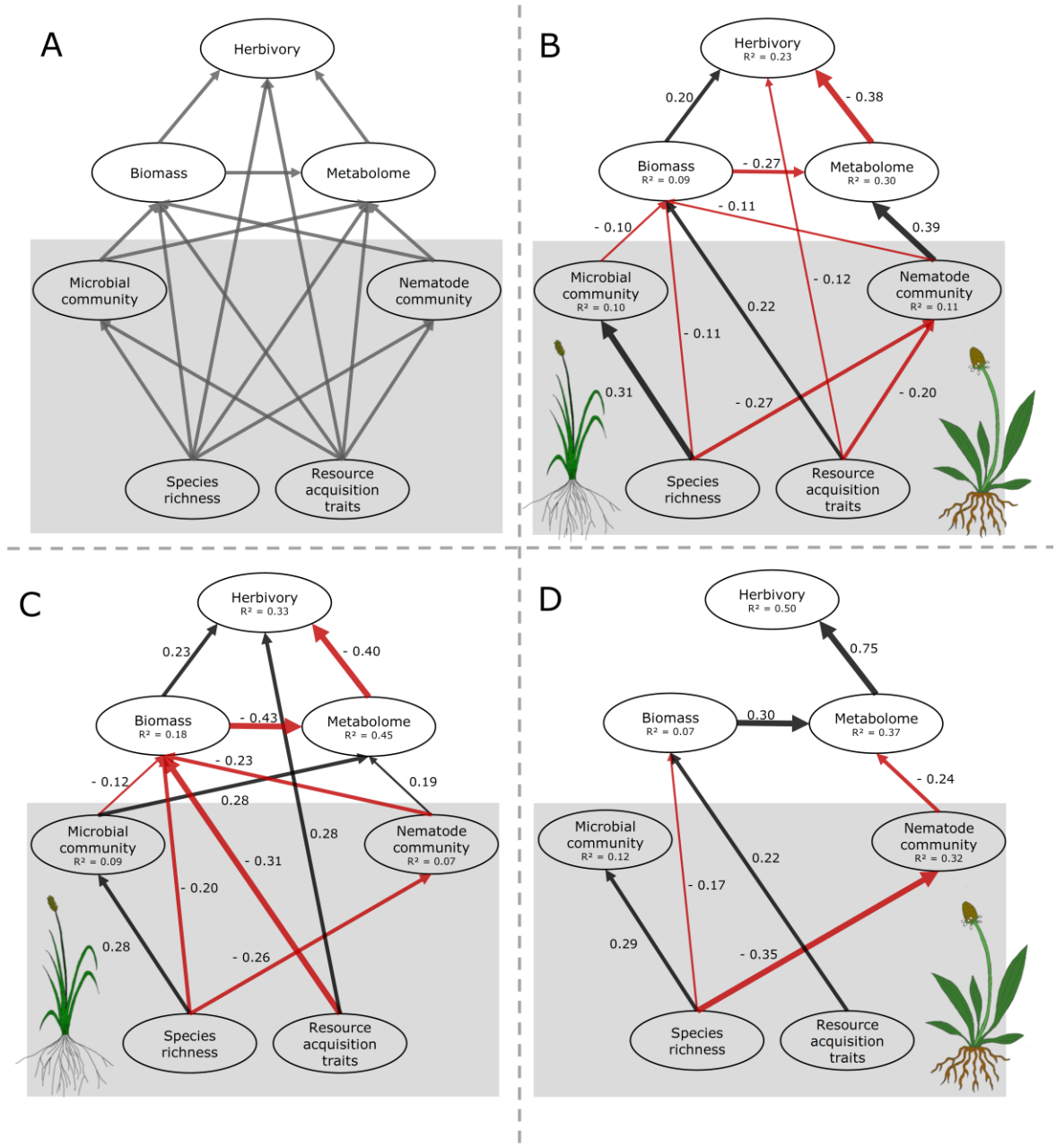

Figure 2 Hypothesis-based conceptual partial-least-squares path model (A) as well as path model including data across both sampling campaigns and all plant species (B), only across all grasses (C), and only across all herbs (D). Species richness represents the plot-level sown plant species richness. Resource acquisition traits represent the community-weighted mean traits maximum plant height, leaf area, rooting depth, root length density, growth starting date, and flowering start. Microbial community represents PLFA-based estimates on plot-level gram-negative, gram-positive, and undefined bacteria, as well as arbuscular mycorrhizal fungi and all other fungi abundance. Nematode community represents plot-level summed relative abundance of functional nematode guilds, *i.e.,* bacterial-feeding, carnivorous, fungal-feeding, omnivorous, and plant-feeding. Biomass represents plant-individual aboveground dry biomass. Metabolome represents plant-

individual secondary metabolite composition. Herbivory represents plant-individual herbivory rate expressed as the proportion of damaged leaves to the total number of leaves. All data is scaled. Variables taken at plot level are highlighted by a grey-shaded background. Variables taken at the plant-individual level are highlighted by a white-shaded background. Black arrows display significantly positive relationships. Red arrows display significantly negative relationships. Numbers on arrows are path coefficients. Numbers in the round boxes display the explained variation (R^2).

Our full-model PLS-PM also indicated that plant herbivory was negatively related to community-weighted resource acquisition traits and positively related to plant individual biomass (**Fig. 2b**). Leaf area, growth starting date, and flowering start were most strongly positively correlated with the latent variable 'resource acquisition traits'. Neither trait was individually correlated with plant herbivory (**Fig. S8**). However, our path model suggests a synergistic effect on plant herbivory, *i.e.*, plant communities of species with large leaves and late growth and flowering start may reduce plant individual herbivory. Plant biomass was positively related to resource acquisition traits and negatively related to plant metabolome, plant species richness, the nematode community composition, and the microbial community composition. Lastly, the microbial community composition was positively related to plant species richness. We calculated an alternative full-model PLS-PM (GoF = 0.12) that links herbivory to plant metabolome, which would account for herbivore-induced responses (**Fig. S23**). The Goodness-of-Fit of both models was similar, but the strength of the direct path between metabolome and herbivory was stronger in the original full-model PLS-PM (path coefficient of -0.38) than in the alternative model (path coefficient of -0.29). Moreover, the alternative model predicted only 11% of the total variation in individual plant herbivory, whereas the original model predicted 23%.

Based on our observations that functional group identity affects plant metabolomes and herbivory, we calculated two additional PLS-PMs: a grasses-only model (**Fig. 2c**; GoF = 0.19) and a forbs-only model (**Fig. 2d**; GoF = 0.19). In the grasses-only model both the

microbial and the nematode community composition were positively related to plant metabolome. In contrast to the full-model, the resource acquisition traits were not related to nematode community composition, but they were positively related to herbivory and negatively related to biomass (see **Table S6**). In the forbs-only model, the plant metabolome was negatively related to nematode community composition, but positively related to biomass and herbivory. Moreover, plant individual biomass was neither related to microbial and nematode community composition, nor to herbivory (see **Table S6**). Both functional group models, however, explained more variation in individual plant herbivory (grasses-only model 33%, forbs-only model 50%) than the full-model, suggesting that in grasses and forbs different mechanisms link plant diversity and soil community composition with plant metabolomes and herbivory.

Discussion

Our study highlights how different facets of biodiversity in plant communities jointly form a mechanistic explanation for reduced aboveground herbivory at high plant species richness. These results partially confirm our first hypothesis that plant species richness and the resource acquisition strategies of the plant community affect individual plant herbivory. Using partial-least-squares path-modelling, we revealed that these plant diversity effects on herbivory can be mediated by soil biota, in particular nematode community composition. These plant diversity-driven differences in soil communities affected plant metabolomes, thus supporting our second hypothesis. Compared to previous studies (Scherber *et al.* 2010; van Dam & Heil 2011) our study yields novel mechanistic insights by highlighting how belowground communities may shape plant metabolomes, thereby becoming a significant driver of aboveground herbivory.

The abundance, diversity, and community structure of soil biota are commonly determined by the species identity and traits of individual plants as well as the plant community diversity (Bezemer *et al.* 2010; Lange *et al.* 2015; Strecker *et al.* 2016). Accordingly, our path model showed that plant species richness and variation in resource acquisition-related functional traits can explain variation in soil microbial and nematode community composition. The effect of plant species richness and microbial community composition is likely due to an increased and more diverse influx of organic matter in the form of rhizodeposits (Lange *et al.* 2015; Steinauer *et al.* 2016; Eisenhauer *et al.* 2017). The observed negative relationship between resource acquisition traits and most functional nematode guilds was mainly driven by community-weighted leaf area, growth starting time, and flowering onset. This suggests that the abundance and seasonality of resource influx from the plant community into the soil determines nematode community structure (Yeates 1999). In contrast, rooting depth and root length density were positively correlated with phytophagous nematodes, suggesting that phytophagous nematodes are also affected by root architecture (Yeates 1999). Changes in soil community composition were related to significant changes in plant metabolomes. Specifically, the abundance of bacterial feeders, predators, and phytophagous nematodes positively correlated to the concentration of defence-related metabolites in individual plants. Bacterial-feeding nematodes contribute to the mineralization of nitrogen in the soil, which supports plant growth and potentially the synthesis of defence-related metabolites (Freckman & Caswell 1985). Predatory nematodes control plant parasitic nematodes, thus also indirectly supporting plant growth (Freckman & Caswell 1985). In contrast, phytophagous nematodes can induce systemic defence responses, which can explain the positive correlation between nematode abundance and defensive secondary metabolites in leaves (van Dam & Heil 2011; Wondafrash *et al.* 2013). Interestingly, we identified several phenolic compounds that are produced *via* the shikimic acid pathway. Salicylic acid, which

is involved in the plant's systemic response to root feeding nematodes, is a product of this pathway (Dempsey *et al.* 2011; Wondafrash *et al.* 2013). Our path models showed that plant diversity affected belowground community composition, which may have affected the shoot metabolome *via* the systemic induction of metabolites (van Dam & Heil 2011; Agrawal & Weber 2015). Such an induction of metabolites can affect herbivore resistance and may explain the significant link between nematode community composition, the composition of the plant metabolome, and herbivory in our model (van Dam & van der Meijden 2011). This is supported by earlier findings reporting effects of plant diversity on soil community composition, especially plant growth facilitators and plant antagonists, and on plant metabolomes and thereby herbivory (Bezemer & van Dam 2005; Hol *et al.* 2010; Wurst *et al.* 2010; Kos *et al.* 2015; Ristok *et al.* 2019). Similarly, also root herbivory may impact shoot metabolomes (Bezemer & van Dam 2005).

While the aim of our study was to test if the plant's metabolome can explain variation in herbivory, we could not disentangle potential effects of herbivory on the plant's metabolome. Because we analysed field plants, it is likely that the metabolomes result from several (a)biotic interactions. If anything, this adds realism to our results, as in nature aboveground herbivores often encounter plants induced by other interactors (van Dam & Heil 2011). In all models, the relationship between biomass and metabolome was maintained. The negative relationships found in the full and grasses model, may support the hypothesis that larger plants produced less defense, because they can tolerate biomass loss to herbivory and prioritize growth over defense production (de Jong 1995). In forbs, however, there was a positive relationship between biomass and metabolome. This might point to the fact that flowering forbs are commonly larger and produce more and different metabolites to protect their reproductive organs (McKey 1979).

We also discovered that the metabolite diversity in grasses and forbs varied differently to changes in resource acquisition-associated community-weighted traits. These contrasting responses are likely due to differences in defensive strategies. Grasses possess silica crystals providing mechanical protection from herbivory (Massey & Hartley 2009), while forbs invest in carbon-based defences, such as phenolics (Larson 1988; Cooke & Leishman 2012). Moreover, grasses and forbs differ in their associations with soil biota, such as the symbiosis with mycorrhizal fungi, which can contribute to diverging metabolomic responses (Chialva *et al.* 2018; Ristok *et al.* 2019). While the difference between grasses and forbs was not the focus of our study and we only analysed a small subset in each functional group, our results stress the importance of including functional group identity to improve predictive models analysing plant-herbivore interactions.

While the present experiment provides novel insights into the above-belowground controls of herbivory, additional experiments should disentangle the individual and interactive roles of plant and soil biodiversity in driving changes in plant metabolomes and herbivory rates (Peters *et al.* 2018). Such studies should be conducted in the presence and absence of aboveground herbivores (Seabloom *et al.* 2017), to assess if aboveground herbivory modulates plant and soil biodiversity effects on the metabolome, *e.g., via* induced responses (Peters *et al*. 2018). Preferably, these studies should include specialist and generalist herbivores as well as different feeding types (Mithöfer & Boland 2008).

Taken together, the present study shows that changes in plant species richness and community resource acquisition traits can alter belowground communities, thus driving changes in the shoot metabolome and herbivory rate. Our results suggest that the plant

metabolome is an important functional trait that can explain more variation (23%) in herbivory than commonly used morphological and physiological traits (on average 12.7%) (van der Plas *et al*. 2020). By including metabolomic analyses, we advanced our knowledge on the mechanisms linking plant diversity and herbivory rates *via* changes in plant metabolomes (Peters *et al*. 2018). It thereby expands our capability to better characterize the complex nature of multitrophic interactions above and below the ground.

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Data accessibility statement

The datasets generated during and/or analysed during the current study will be archived in Dryad, and the data DOI will be included at the end of the article upon acceptance of the manuscript.

Competing interests

The authors declare no competing interest.

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Table 1 Description of the latent and observed variables used in the PLS-PM models.

Latent Variable	Description
Resource acquisition traits	The latent variable represents the community-weighted mean (CWM) trait values of maximum plant height, leaf area, rooting depth, root length density, growth start, and flowering start. We based the calculations of the CWM trait values on the relative species-specific cover for each plant community. For instance, a positive relationship of *resource acquisition traits* with the latent variable *biomass* means higher values in the CWM traits correlated with a higher plant biomass.
Microbial community	The latent variable represents the microbial biomass for gram-negative bacteria, gram-positive bacteria, undefined bacteria, saprophytic fungi, and arbuscular mycorrhizal fungi. For instance, a negative relationship of *microbial community* with the latent variable *biomass* indicates that a higher biomass of soil bacteria and fungi correlated with a lower individual plant biomass.
Nematode community	The latent variable represents the relative abundance of functional nematode guilds. A functional nematode guild is the combined information of trophic guild and colonizer-persistence score. For instance, a positive relationship of *nematode community* with the latent variable *metabolome* means a greater relative abundance of certain functional guilds correlated with a higher concentration of metabolites.
Metabolome	The latent variable represents the abundance of secondary plant metabolites. For instance, a negative relationship of *metabolome* with the latent variable *herbivory* means that a higher abundance of metabolites is correlated with a lower herbivore damage on individual plants.

Observed Variable	Description
Species richness	The observed variable represents the plot level sown plant species richness from 1 to 8.
Biomass	The observed variable represents the aboveground dry biomass of individual plants.
Herbivory	The observed variable represents the herbivory rate on individual plants.

Table 2 Overall differences in the species-specific and sampling campaign-specific shoot metabolome composition of plants grown in plant communities of four different species richness levels. Statistical parameters resulting from permutational multivariate analyses of variance using distance matrices. We used Bray-Curtis dissimilarity matrices and 9999 permutations. Significant differences ($p < 0.05$) are given in bold. Abbreviations: F = pseudo-F-value; p = p-value.

Species	Sampling campaign					
	August 2015			May 2016		
	R^2	F	p	R^2	F	p
Dactylis glomerata	0.103	1.114	0.151	0.125	1.379	**0.012**
Holcus lanatus	0.111	1.080	0.259	0.102	0.986	0.523
Phleum pratense	0.120	1.182	0.098	0.141	1.532	**0.002**
Geranium pratense	0.172	1.727	**< 0.001**	0.079	0.740	0.456
Leucanthemum vulgare	0.115	1.260	**0.024**	0.091	0.968	0.576
Plantago lanceolata	0.108	1.294	**0.021**	0.093	1.093	0.192
Ranunculus acris	0.164	1.568	**0.001**	0.129	1.287	0.051

MANUSCRIPT III

Plant diversity and soil legacy independently affect the plant metabolome and induced responses following herbivory

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FORMULAR 1

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Abstract

Plant and soil biodiversity can have significant effects on herbivore resistance mediated by plant metabolites. Here, we disentangled the independent effects of plant diversity and soil legacy on constitutive and herbivore-induced plant metabolomes of three plant species in two complementary microcosm experiments.

First, we grew plants in sterile soil with three different plant diversity levels. Second, single plant species were grown on soil with different plant diversity-induced soil legacies. We infested a subset of all plants with *Spodoptera exigua* larvae, a generalist leaf-chewing herbivore, and assessed foliar and root metabolomes.

Neither plant diversity nor soil legacy had significant effects on overall foliar, root, or herbivore-induced metabolome composition. Herbivore-induced metabolomes, however, differed from those of control plants. We also detected 139 significantly regulated metabolites by comparing plants grown in monocultures with conspecifics growing in plant or soil legacy mixtures. Moreover, plant-plant and plant-soil interactions regulated 141 metabolites in herbivore-induced plants.

Taken together, plant diversity and soil legacy independently alter the concentration and induction of plant metabolites, thus affecting the plant's defensive capability. This is a first step towards disentangling plant and soil biodiversity effects on herbivore resistance, thereby improving our understanding of the mechanisms that govern ecosystem functioning.

Keywords: Aboveground-belowground interactions, Biodiversity-ecosystem function, Chemical diversity, Eco-metabolomics, Herbivory, Metabolite profile

Introduction

Plant and soil communities are linked via the plant and influence each other

In terrestrial ecosystems, aboveground and soil communities are inseparably linked *via* plants (Wardle *et al.* 2004). Such aboveground-belowground linkages determine plant diversity effects on ecosystem functioning (Eisenhauer 2012). Plant species often harbor unique rhizosphere communities and even influence the surrounding community composition of root-associated organisms through species-specific and context-dependent organic matter inputs (Bezemer *et al.* 2010; van der Putten *et al.* 2013). Similarly, each plant species has a specific herbivore community which can affect soil communities *via* herbivory, either directly *via* frass or indirectly *via* induced responses (Bardgett & Wardle 2010). Aboveground herbivory can, for instance, positively affect soil microbial activity by inducing the release of carbon into the rhizosphere, and change arbuscular mycorrhizal colonization by reducing the carbon allocation to roots (Gehring & Whitham 1994; Hamilton & Frank 2001). In turn, soil biota, especially root parasites, pathogens, and herbivores as well as mutualistic symbionts can influence plant community structure and functioning *via* soil feedback effects (Wardle *et al.* 2004; Van Der Heijden, Bardgett & Van Straalen 2008; van der Putten *et al.* 2013). Root parasites, pathogens, and herbivores generally induce a negative plant-soil feedback by directly removing or damaging root tissues and thus reducing root uptake capabilities. Mutualistic organisms, on the other hand, induce a positive soil feedback effect on plant growth by improving soil nutrient uptake (Wardle *et al.* 2004; Bardgett & van der Putten 2014), and protection against antagonists (Latz *et al.* 2012). The magnitude and direction of those plant-soil feedback effects, however, is not equal for all plant species and community contexts (Cortois, Schröder-Georgi, Weigelt, van der Putten & De Deyn 2016).

Plant diversity and soil legacy can affect the plant metabolome

Recently, research on the response of plants to plant-plant interactions and soil feedbacks has been expanded beyond the common morphological and physiological traits. The consideration of the plant metabolome, *i.e.,* the entirety of metabolites synthesized by a plant (Oliver, Winson, Kell & Baganz 1998) gave rise to a new discipline, eco-metabolomics, which uses metabolome analyses, or metabolomics, to illuminate the chemical mechanisms underpinning ecological and environmental processes (Peñuelas & Sardans 2009; Peters *et al.* 2018). Eco-metabolomics has been employed to investigate if plants respond on a molecular level to plant community composition and soil biota diversity (Scherling, Roscher, Giavalisco, Schulze & Weckwerth 2010; Ristok *et al.* 2019; Huberty, Choi, Heinen & Bezemer 2020).

Plant-plant interactions, for instance, can induce shifts in foliar metabolic profiles of multiple grassland plant species, with more than 100 metabolites changing in their concentration (Scherling *et al.* 2010). In addition, differential selection due to growing in monocultures or plant species mixtures can select for plants with distinct metabolomes (Zuppinger-Dingley, Flynn, Brandl & Schmid 2015). Similarly, in the presence of soil biota, plants produce species-specific shoot and root metabolomes that differ from those of plants grown in sterile conditions (Ristok *et al.* 2019). Furthermore, these plant-soil interactions often affect the diversity of a plant's metabolome and can exert stronger metabolomic shifts than foliar herbivory (Huberty *et al.* 2020). Root parasites, pathogens, and herbivores as well as mutualistic symbionts can change the concentration of primary and secondary metabolites in leaves and roots in multiple ways, *e.g.,* up- or down-regulation of specific metabolites (van Dam & Heil 2011; van der Putten *et al.* 2013). These responses are generally species-specific, context-dependent, and can affect subsequent biotic interactions (Bezemer & van Dam 2005; Ristok *et al.* 2019).

Herbivory-induced defenses can be altered by biotic interactions

One important interaction type is that between plants and herbivores. Plants have evolved a plethora of indirect and direct chemical defenses to deal with attackers (Karban & Baldwin 1997). Of special interest are induced defenses, *i.e.,* changes in the concentration of metabolites following an attack by parasites, pathogens or herbivores, or after interactions with beneficial microbes (Ferlian *et al.* 2018). Such induced responses can affect the plant metabolome locally or systemically (Bezemer & van Dam 2005). Both plant-plant interactions and plant-soil interactions can modulate the induction of defensive metabolites. Plant-plant interactions can affect induced defenses through plant competition, which forces the plant to either invest resources into growth or defense (*i.e.,* growth-defense trade off; van Dam & Baldwin 2001; Broz *et al.* 2010; Fernandez *et al.* 2016). In addition, volatile organic compounds can induce defensive responses immediately or prime for future attacks (Baldwin, Halitschke, Paschold, Dahl & Preston 2006). Plant-soil interactions with microbes, nematodes, and mycorrhizal fungi cannot just induce defenses locally in roots, but also systemically in foliar tissues (van Dam & Heil 2011). Either of these groups of soil biota can up- or down-regulate specific primary metabolites, such as amino acids and sugars, or secondary metabolites, such as glucosinolates and iridoid glycosides, in aboveground plant tissues (Hol *et al.* 2010; Wurst, Wagenaar, Biere & Van der Putten 2010; Rivero, Gamir, Aroca, Pozo & Flors 2015).

Taken together, both plant-plant interactions and plant-soil interactions play significant roles in modulating the plant's metabolome, thereby affecting resistance to aboveground herbivores (van Dam & Heil 2011; Ristok *et al.* 2019). Thus far, however, not much is known about the individual impact of plant-plant interactions or plant-soil interactions within plant communities. This is likely due to the fact that plant-plant and plant-soil interactions are tightly linked in natural communities. In addition, most microcosm studies only focus on

plant-soil interaction effects (see *e.g.,* Ristok *et al.* 2019; Huberty *et al.* 2020). Here, we explicitly investigate to which extent plant-plant-interactions (PPI) or plant-soil interactions (PSI) affect the metabolomes of three forb species in a similar microcosm set-up. Both the PPI and PSI experiment covered the same range of diversity levels and plant community compositions; either as assembled plant communities grown in sterile soil (PPI) or *via* the inoculation of sterile substrate with conditioned field soil of communities with similar plant diversity levels (PSI). In addition, a subset of all plants was infested with larvae of the generalist herbivore Spodoptera exigua to induce defense responses. We analyzed all samples using an untargeted metabolomics approach focusing on profiling plant secondary metabolites in leaves and roots. We hypothesized that (1) both plant diversity and soil legacy can alter the overall plant metabolome, as well as affect the regulation of specific metabolites. In addition, we hypothesized that (2) the induced defense to herbivory is differently affected by plant diversity and soil legacy.

Materials and methods

2.1 Experimental design

In summer 2017, we set up a plant-plant interaction (PPI) experiment and a plant-soil interaction (PSI) experiment with three common central European grassland forb species (*Geranium pratense* L., *Leucanthemum vulgare* (Vaill.) Lam., and *Ranunculus acris* L.). We chose these species based on their representation in the Trait-Based Experiment of the Jena Experiment (Ebeling *et al.* 2014), *i.e.,* monocultures of each species, all two-species mixtures, and the three-species mixture were established (see below). Prior to each experiment, we germinated seedlings of each species from non-sterilized seeds (Rieger-Hofmann GmbH, Blaufelden-Raboldshausen, Germany). To assure that we would use

similarly developed seedlings and to account for species-specific differences in germination, we treated the seeds as follows: all seeds of *Geranium pratense* were gently scarified with sandpaper, placed in a petri dish, and treated with 3 mL 1 g/L gibberellic acid for 24 h at 7°C. The same procedure was followed for *Ranunculus acris* seeds, but they were treated with 0.66 g/L gibberellic acid. No treatment was necessary for *Leucanthemum vulgare* seeds. Following the treatment, all seeds were transferred to plastic boxes half-filled with glass beads (50 seeds per box, only one species per box). Each box was covered with a transparent lid, and seeds were watered daily with tap water. All boxes were transferred to growth chambers (CLF Plant Climatics, Percival E-36L, Wertingen, Germany) with a photoperiod of 16 h light at 20°C and 8 h darkness at 12°C, and 50% relative humidity. Seeds of *Geranium pratense* and *Leucanthemum vulgare* were left in the growth chamber for 14 days, and those of *Ranunculus acris* for 28 days, until the seedlings reached similar sizes.

2.1.1 Plant-plant interaction experiment

We conducted the plant-plant interaction experiment in a greenhouse located at the Botanical Garden Leipzig, Germany, in May 2017. We recorded an average temperature of 22.6°C and an average relative humidity of 51.6% for the time of the experiment in the greenhouse. We used 2 L microcosms (rose pot 2.0 L, Hermann Meyer KG, Rellingen, Germany) filled with autoclaved (twice at 134°C for 20 min) 50:50 sand-peat (Floradur B Pot Clay Medium, Floragard, Oldenburg, Germany) mixture. We flushed each filled microcosms with water twice to remove pulsed nutrients and toxins prior to transplanting seedlings (Alphei & Scheu 1993; Trevors 1996). We established the following plant diversity levels and communities: (1) monocultures of each species, (2) the three possible two-species mixtures, and (3) the three-species mixture (**Supplementary Table S1**). We transplanted twelve similarly developed seedlings in each microcosm, and each plant community was replicated ten times

(total number of microcosms: 70). The relative proportion among species was equal, *i.e.,* six seedlings per species in the two-species mixture and four seedlings per species in the three-species mixture. In the two-species mixture, we transplanted the species in an alternating pattern, while we randomized the position of each seedling in the three-species mixture. All microcosms were randomly placed on tables in the greenhouse and covered with net cages to prevent unwanted herbivory. We watered all microcosms three times per week and randomized the position on the tables every 7 days. We fertilized all microcosms with 250 mL Hoagland solution after 5 weeks to counteract any loss of nutrients and ensure optimal growth.

After 7 weeks of growth, we harvested five microcosms per plant diversity level (see below). The next day, we infested two randomly selected plants per species and microcosm of the remaining microcosms with three 2nd instar *Spodoptera exigua* larvae each. We covered and closed each plant just above the soil with an organza net to ensure that the larvae could not escape. To ensure similar development of the larvae (eggs purchased from Entocare Biologische Gewasbescherming, Wageningen, the Netherlands), we maintained a laboratory colony on artificial diet in a growth chamber (25°C, 12 h light, 45% relative humidity). After 7 days of herbivory, we harvested the remaining microcosms (see below).

2.1.2 Plant-soil interaction experiment

We conducted the plant-soil interaction experiment in a greenhouse located at the Botanical Garden Leipzig, Germany, in July 2017. We recorded an average temperature of 23.5°C and an average relative humidity of 58.6% for the time of the experiment in the greenhouse. We used PVC tube microcosms (height 20 cm, diameter 10 cm, bottom closed with 250 µm mesh) filled with 1.6 L inoculated substrate and watered each microcosm twice. We prepared the inoculated substrate by mixing autoclaved (twice at 134°C for 20 min) 50:50 sand-peat

(Floradur B Pot Clay Medium, Floragard, Oldenburg, Germany) background substrate with liquid field soil inoculum 3 weeks prior to the establishment of the experiment. In June 2017 (*i.e.,* ~ 7 years after the establishment of the experiment), we collected field soil from plant communities established in 2010 as part of the Trait-Based Experiment (Ebeling *et al.* 2014). We collected and pooled six soil cores (2 cm x 10 cm) from each plant community accounting for within-plot heterogeneity. We sieved each field soil through a 4 mm mesh and subsequently dissolved 100 g field soil in 1 L demineralized water. We then added the liquid soil inoculum to our autoclaved background substrate (10 mL liquid inoculum per 1 kg background substrate) and stored each mixture in closed-lid plastic boxes at room temperature for 3 weeks. Each substrate-inoculum mixture was thoroughly mixed three times per week and stored with an open lid for 1 h once per week. We cleaned all used instruments, *i.e.,* sieves, boxes, beakers, mixer, before and after each step with distilled water and 70% ethanol to minimize cross contamination.

We established the following inoculated substrates (hereafter, soil legacy levels): (1) monocultures of each plant species, (2) the three possible two-species mixtures, and (3) the three-species mixture (**Supplementary Table S2**). Each soil legacy level represents the plot from the Trait-Based Experiment, we sampled the soil from. We transplanted four similarly developed seedlings per microcosm. Seedlings of plant species were only planted into soil legacy levels that also contained the respective species in the field experiment. This set-up resulted in twelve unique soil legacy level-planted species combinations. Each soil legacy level-planted species combination was replicated ten times (total number of microcosms: 120). All microcosms were randomly placed on tables in the greenhouse and covered with net cages to prevent unwanted herbivory. We watered all microcosms three times per week and randomized the position on the tables every 7 days. We fertilized all microcosms with 250 mL Hoagland solution after 5 weeks to counteract any loss of nutrients and ensure

optimal growth. After 7 weeks of growth, we harvested five microcosms per soil legacy level-planted species combination (see below). The next day, we infested two randomly selected plants per microcosms of the remaining microcosms with three 2nd instar *Spodoptera exigua* larvae each (see above). We covered and closed each plant just above the soil with an organza net to ensure that the larvae could not escape. After 7 days of herbivory, we harvested the remaining microcosms (see below).

2.2 Sampling and sample processing

After 7 weeks of growth, we harvested five microcosms per plant diversity level in the PPI experiment and five microcosms per soil legacy level-planted species combination in the PSI experiment (**Supplementary Table S1 & S2**). We separated the shoot and root biomass of one randomly selected plant individual per species and microcosm by cutting the plants with scissors. We washed the roots twice under tap water to remove soil particles, and then dried the samples with paper towels. This process took roughly 30 s. All shoot and root samples were then immediately stored in paper bags on dry ice to stop further metabolism. This resulted in a total of 20 shoot and 20 root samples per species and experiment.

After one additional week of herbivory (see above), we harvested the remaining five microcosms per diversity level in the PPI experiment and five microcosms per soil legacy level-planted species combination in the PSI experiment (**Supplementary Table S1 & S2**). We sampled the foliar tissue of one randomly selected control and one randomly selected induced plant individual per species and microcosm by cutting the plants ca. 1 cm above the ground. All samples were then immediately stored in paper bags on dry ice. This resulted in a total of 20 control and 20 induced samples per species and experiment.

In the lab, all samples were stored in a -80°C freezer, and subsequently, freeze-dried (LABCONCO FreeZone Plus 12 Liter, Kansas City, USA) for 72 h. Dried samples were

stored in zip-lock bags filled with silica gel at room temperature until we had ground each sample to a fine homogenous powder using a ball mill (Retsch mixer mill MM 400, Haan, Germany).

2.3 Metabolome extraction and analysis

We extracted and analyzed all samples according to Ristok *et al.* (2019) with slight changes. We extracted 20 mg dried and ground plant tissue of each sample in 1 mL of extraction buffer (methanol / 50 mM acetate buffer, pH 4.8; 50 / 50 [v/v]). All samples were homogenized for 5 min at 30 Hz using a Retsch mixer mill MM 400, and subsequently centrifuged for 10 min at 20,000 g and 4°C. We collected the supernatant in a 2 mL Eppendorf tube, repeated the extraction procedure with the remaining pellet, and combined both supernatants. Lastly, we centrifuged (20,000 g, 5 min, 4°C) all extracts, transferred 200 µL to an HPLC vial, and added 800 µL extraction buffer, resulting in a 1:5 dilution.

We performed chromatographic separation of all diluted extracts by injecting 2 µL on a Thermo Scientific Dionex UltiMate 3000 (Thermo Scientific Dionex, Sunnyvale, USA) UPLC unit, equipped with a C18 column (Acclaim RSLC 120 C18, 2.2 µm, 120 Å, 2.1 x 150 mm, Thermo Fisher Scientific). We applied the following binary elution gradient at a flow rate of 0.4 mL min^{-1} and a column temperature of 40°C: 0 – 2 min, 95% A (water and 0.05% formic acid), 5% B (acetonitrile and 0.05% formic acid); 2 – 12 min, 5 to 50% B; 12 – 13 min, 50 to 95% B; 13 – 15 min, 95% B; 15 – 16 min, 95 to 5% B; 16 – 20 min, 5% B.

Metabolites were analyzed on a liquid chromatography quadrupole time-of-flight mass spectrometer (LC-qToF-MS; Bruker maXis impact HD; Bruker Daltonik, Bremen, Germany) with an electrospray ionization source operated in negative mode. Instrument settings were as follows: capillary voltage, 2500 V; nebulizer, 2.5 bar; dry gas temperature,

220°C; dry gas flow, 11 L min^{-1}; scan range, 50 – 1400 m/z; acquisition rate, 3 Hz. We used sodium formate clusters (10 mM solution of NaOH in 50 / 50% [v/v] isopropanol / water containing 0.2% formic acid) to perform mass calibration.

2.4 LC-MS data processing

We followed the LC-MS data processing protocol described in Ristok *et al.* (2019) with minor changes. We converted the LC-qToF-MS raw data to the mzXML format by using the CompassXport utility of the DataAnalysis vendor software. We then trimmed each data file by excluding the same non-informative regions at the beginning and end of each run using the msconvert function of ProteoWizard v3.0.10095 (Chambers *et al.* 2012). We performed peak picking, feature alignment, and feature group collapse in R v3.3.3 (R Core Team 2020) using the Bioconductor (Huber *et al.* 2015) packages 'xcms' (Smith, Want, O'Maille, Abagyan & Siuzdak 2006; Tautenhahn, Böttcher & Neumann 2008; Benton, Want & Ebbels 2010) and 'CAMERA' (Kuhl, Tautenhahn, Böttcher, Larson & Neumann 2012). We used the following 'xcms' parameters: peak picking method "centWave" (snthr = 10; ppm = 5; peakwidth = 4, 10); peak grouping method "density" (minfrac = 0.75; bw = 6, 3; mzwid = 0.01); retention time correction method "symmetric". We used 'CAMERA' to annotate adducts, fragments, and isotope peaks with the following parameters: extended rule set (https://gitlab.com/R_packages/chemhelper/-/tree/master/inst/extdata); perfwhm = 0.6; calcIso = TRUE; calcCaS = TRUE, graphMethod = lpc. Finally, we collapsed each annotated feature group, hereafter referred to as 'metabolite' which is described by mass-to-charge ratio (m/z) and retention time (rt), using a maximum heuristic approach (Ristok *et al.* 2019). The intensity of each metabolite was subsequently normalized to the amount of dried ground plant tissue extracted. We processed all data separately for each experiment, species, and tissue.

2.5 Statistical analysis

We analyzed and plotted our data in the statistical software R v4.0.3 (R Core Team 2020) (http://www.r-project.org) using the packages 'DESeq2' (Love, Huber & Anders 2014), 'vegan' (Oksanen *et al.* 2020), 'mixOmics' (Rohart, Gautier, Singh & Le Cao 2017), and 'ggplot2' (Wickham 2016).

We tested for the overall differences in foliar, root, and induced metabolome composition among the plant diversity or soil legacy levels by calculating permutational multivariate analyses of variance using distance matrices. We log + 1 transformed the metabolite intensity data to achieve multivariate normality, and used Bray-Curtis dissimilarity to calculate the distance matrices. All analyses were permuted 9999 times. We used the same approach to test for the differences in the foliar metabolome composition between control and induced plants. We calculated each analysis separately for each species and experiment.

To test for the regulation of metabolites, we calculated differential expression analyses between the monoculture treatment level and each plant diversity or soil legacy mixture level. We used the 'DESeq' function provided by the 'DESeq2' package with default argument structure and values. We defined a metabolite to be significantly up-regulated when the log2 fold change was above 0.6 (1.5 x higher than in control) and the p-value below 0.05. Conversely, we defined a metabolite to be significantly down-regulated when the log2 fold change was below -0.6 (less than 0.66 x control) and the p-value below 0.05. We used the same approach to test for the regulation of metabolites between control and induced plants. We calculated each analysis separately for each species and experiment.

Subsequently, we assigned the putative molecular formula (https://www.chemcalc.org/mf-finder) and compound name (https://pubchem.ncbi.nlm.nih.gov) based on the high-resolution mass-to-charge values generated by liquid chromatography quadrupole time-of-flight mass spectrometry for 95 out of 362 up-or down regulated metabolites. In cases where our search query returned multiple candidate compounds, we limited the selection to compounds with a mass difference of less than 2 ppm and a verified description in at least one plant species.

Results

3.1 Plant diversity or soil legacy effects on plant metabolomes

Neither plant diversity nor soil legacy had a significant effect on overall foliar or root metabolome composition (**Table 1**). However, when we compared metabolomes of plants grown in monocultures with conspecifics growing in mixtures, we discovered a total of 139 significantly up- or down-regulated metabolites in both leaves and roots (**Fig. 1**). Across both experiments, we found that more foliar than root metabolites were regulated in response to heterospecific plant-plant and plant-soil interactions in *Leucanthemum vulgare* (25 *vs*. 12) and *Ranunculus acris* (36 *vs*. 2; **Fig. 1**). Only in *Geranium pratense* were the metabolites in leaves (31 regulated metabolites) and roots (33 regulated metabolites) similarly responsive to heterospecific plant-plant or plant-soil interactions. Overall, metabolites in the leaves of *R. acris* were most responsive, followed by roots and leaves of *G. pratense*, and leaves of *L. vulgare*. Plant-plant interactions generally up- and down-regulated metabolites across all species, while plant-soil interactions mostly down-regulated metabolites in leaves and roots of *G. pratense*, but up-regulated metabolites in leaves of *R. acris* (**Fig. 1**).

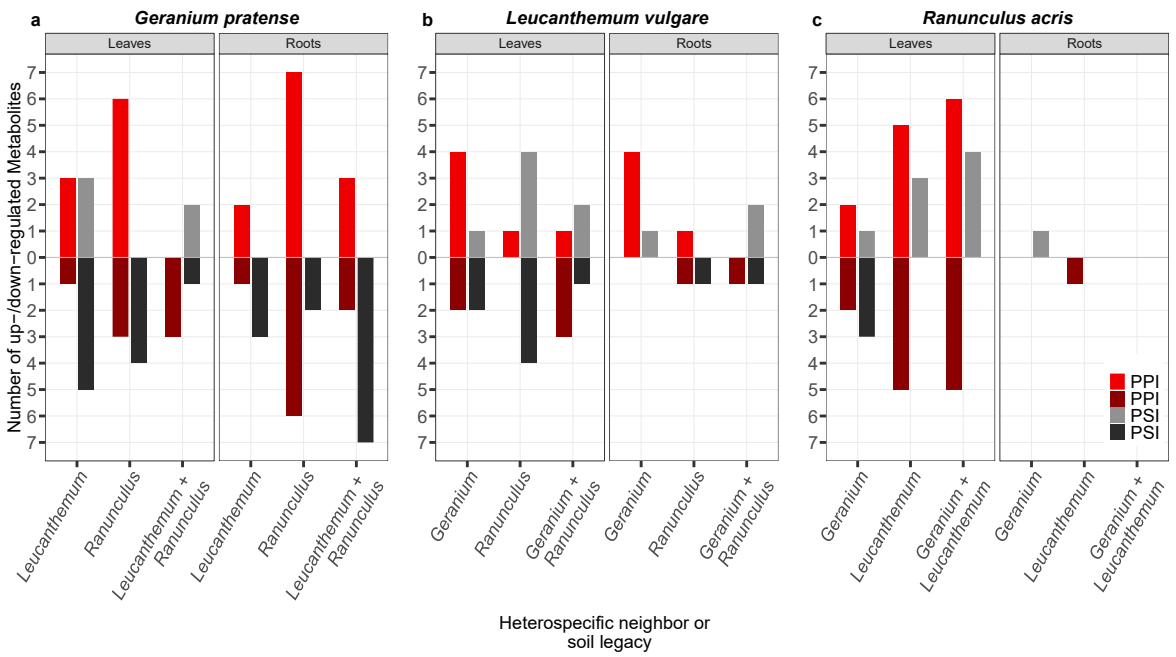

Figure 1. The total number of up- and down-regulated metabolites in leaves and roots of (a) *Geranium pratense*, (b) *Leucanthemum vulgare*, and (c) *Ranunculus acris* plants grown in microcosms with different neighbors (PPI) or different soil legacies (PSI). The number depicted is in comparison to the monoculture diversity/soil legacy level. Data collected as part of the plant-plant interaction (PPI) experiment are displayed in light red (up) and dark red (down). Data collected as part of the plant-soil interaction (PSI) experiment are displayed in grey (up) and black (down).

We found that most regulated metabolites were uniquely synthesized by a plant in response to either plant-plant or plant-soil interactions (**Fig. 2**). This pattern was true across leaves and roots, and across plant species. The only exceptions to this pattern occurred in leaves of *G. pratense* and *R. acris*. Here, we detected metabolites that were regulated in response to both plant-plant and plant-soil interactions (**Fig. 2**). Moreover, we observed that plants grown either in plant-plant or plant-soil interaction, synthesized and regulated unique metabolites in leaves and roots (**Supplementary Fig. S1**). The regulated metabolites that we could tentatively assign a molecular formula and compound class or name to, mostly belonged to phenolics, in particular flavonoids, their precursors, and derivatives (**Table 2**).

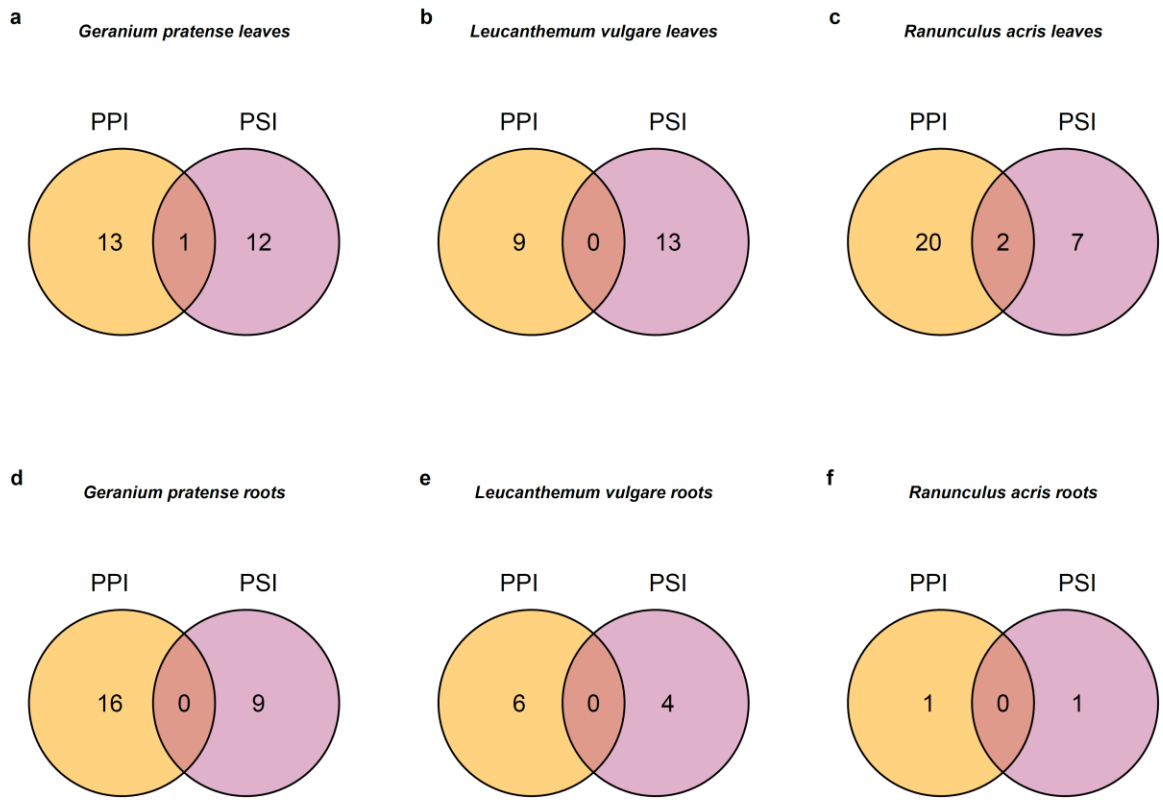

Figure 2. The total number of metabolites in (a – c) leaves or (d – f) roots that were uniquely up- and down-regulated in plants grown in microcosms with different neighbors (PPI) or different soil legacies (PSI). Metabolites uniquely regulated in the plant-plant interaction (PPI) experiment are depicted in orange. Metabolites uniquely regulated in plant-soil interaction (PSI) experiment are depicted in violet. Overlapping areas indicate the number of up- and down-regulated metabolites in both experiments. The number depicted is in comparison to the monoculture diversity/soil legacy level.

3.2 Plant diversity or soil legacy effects on herbivore-induced responses

Both in the PPI (**Fig 3a-c**) and the PSI (**Fig 3d-f**) experiment, we discovered significant differences in the foliar metabolome composition across all plant diversity levels and soil legacies between control and herbivore-induced plants in all plant species. When we tested for the regulation of metabolites between control and induced plants, we found that the total number of up-regulated metabolites was higher than the total number of down-regulated metabolites across all species (**Supplementary Fig S2**). Furthermore, we observed that the absolute number of regulated metabolites was highest when plants had grown in different soil legacies in the PSI experiment. This effect was strongest for *L. vulgare*, while *R. acris*

showed the overall strongest response in numbers of regulated metabolites in both the PPI and PSI experiment (**Supplementary Fig S2**).

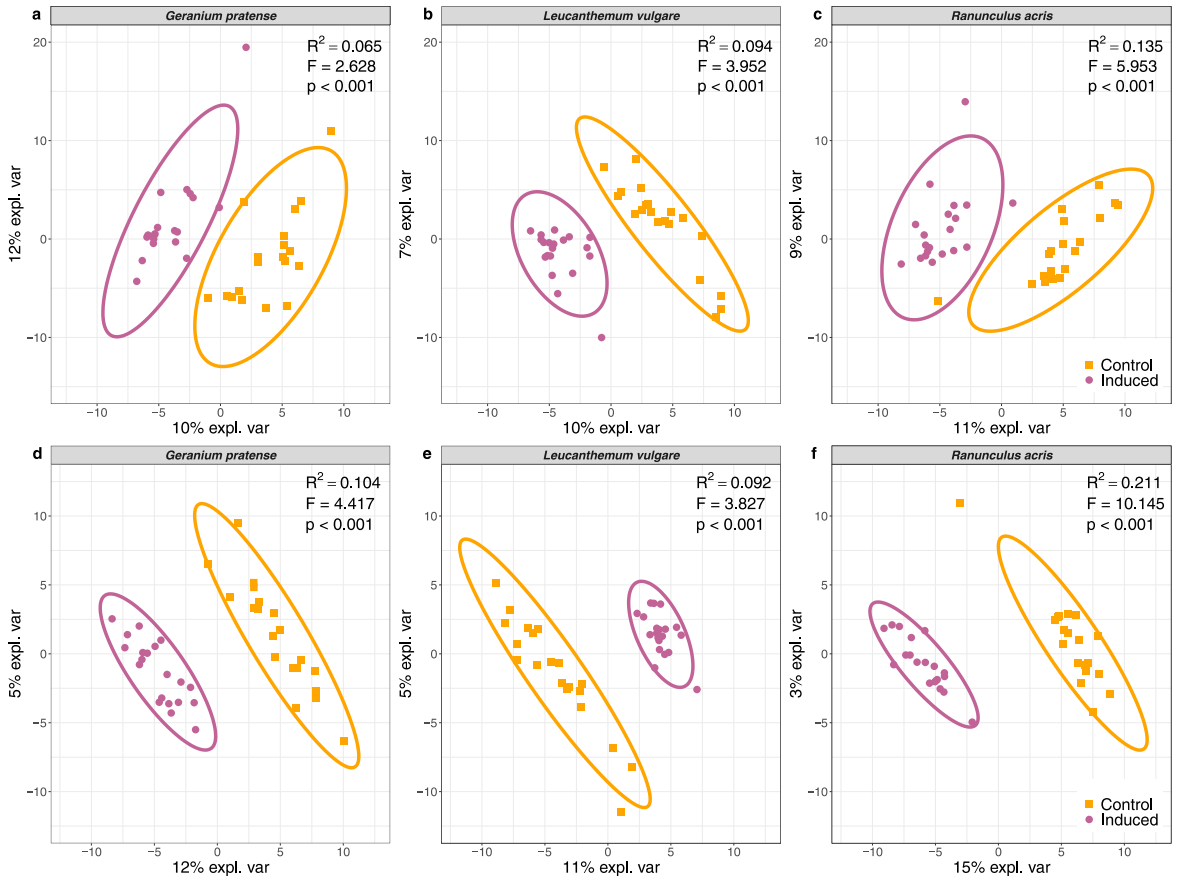

Figure 3. Per species Partial Least Squares – Discriminant Analysis plots of the metabolites found in the foliar metabolomes of *Geranium pratense*, *Leucanthemum vulgare*, and *Ranunculus acris* control or herbivore-induced plants as part of the (a – c) plant-plant interaction experiment and (d – f) plant-soil interaction experiment. Control plants are displayed in orange squares. Induced plants are displayed in violet circles. Ellipses represent the 95% confidence interval. The metabolite intensity matrix was log+1 transformed for the purpose of data normalization. Statistical parameters resulting from a permutational multivariate analysis of variance using distance matrices. Abbreviations: F = pseudo-F-value; p = p-value; expl. var = explained variance.

In contrast, we found no significant effect of plant diversity in the PPI experiment and of soil legacy in the PSI experiment on the induced metabolome in either species (**Table 1**). However, when we compared foliar metabolomes of herbivore-induced plants grown in monocultures with conspecifics growing in mixtures, we discovered a total of 141 significantly up- or down-regulated metabolites (**Fig. 4**). Both heterospecific plant-plant and plant-soil interactions affected the induction of metabolites compared to conspecific plant-plant or plant-soil interactions. Overall, heterospecific plant-plant interactions regulated more induced metabolites than plant-soil interactions in leaves of *L. vulgare* (26 *vs*. 14) and *R. acris* (40 *vs*. 24). In comparison, heterospecific plant-soil interactions had a stronger effect on the regulation of herbivore-induced metabolites in leaves of *G. pratense* than heterospecific plant-plant interactions (21 *vs*. 16; **Fig. 4**). In *R. acris*, we discovered that heterospecific plant-plant and plant-soil interactions had contrasting effects on the regulation of induced metabolites. Heterospecific plant-plant interactions strongly down-regulated the induction of metabolites, while plant-soil interactions strongly up-regulated the induction of metabolites (**Fig. 4**). In contrast, these modulating effects of heterospecific plant-plant or plant-soil interactions on the induction of metabolites were mostly similar or less pronounced in herbivore-induced plants of *G. pratense* or *L. vulgare* (**Fig. 4**). Across all species and both experiments, we found no de-novo regulated metabolites in herbivore-induced plants (**Supplementary Fig. S3**); all up- and down-regulated metabolites were present in control plants as well. Similar to the analysis of regulated metabolites in leaves and roots, the tentatively assigned metabolites in herbivore-induced plants mostly belonged to the family of phenolics, in particular flavonoids, their precursors, and derivatives. Besides, we tentatively assigned two metabolites in *L. vulgare* as an iridoid and an alkaloid glycoside (**Table 2**).

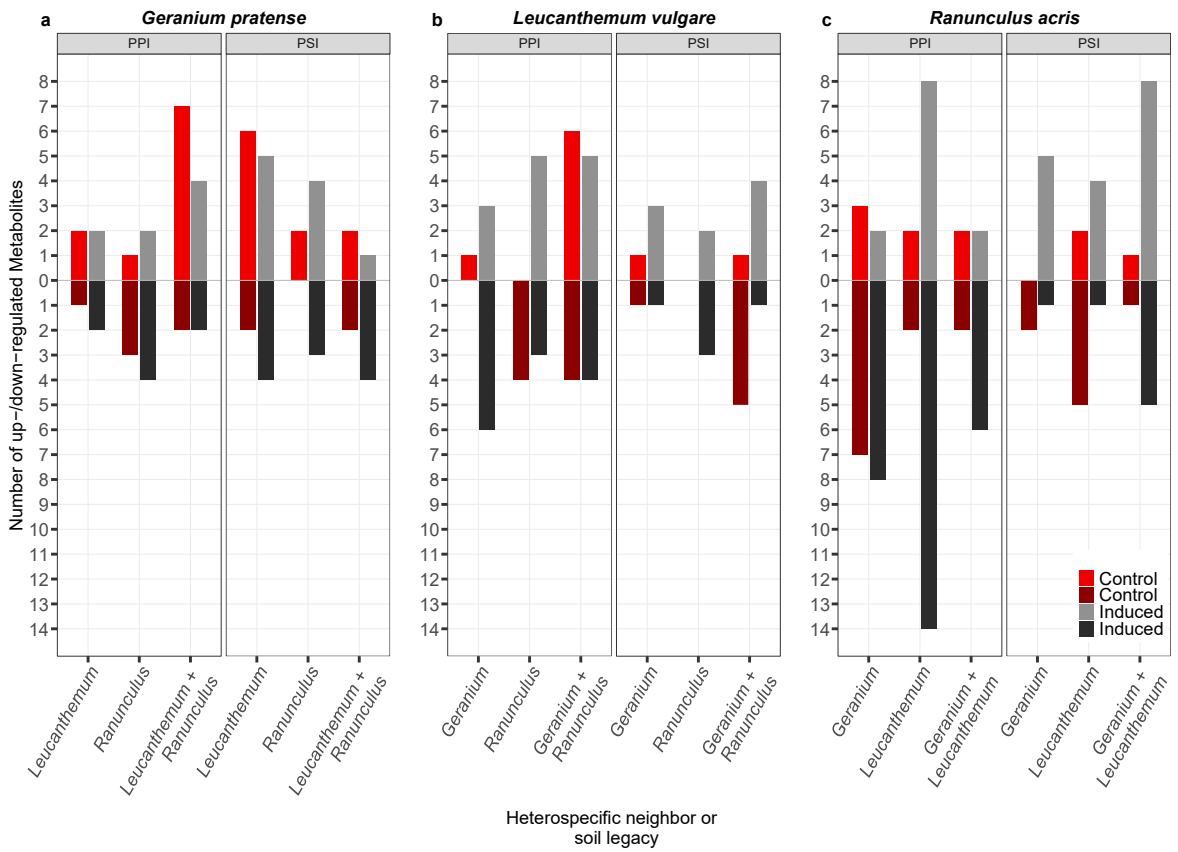

Figure 4. The total number of up- and down-regulated metabolites in leaves of (a) *Geranium pratense*, (b) *Leucanthemum vulgare*, and (c) *Ranunculus acris* control and herbivore-induced plants grown in microcosms with different neighbors (PPI) or different soil legacies (PSI). The number depicted is in comparison to the monoculture diversity/soil legacy level. Data collected in control plants are displayed in light red (up) and dark red (down). Data collected in induced plants are displayed in grey (up) and black (down). Induced plants were infested with *Spodoptera exigua* larvae for 7 days prior to sampling. Abbreviations: PPI – plant-plant interaction experiment; PSI – plant-soil interaction experiment.

Discussion

Our study highlights that both plant-plant interactions and plant-soil interactions can affect foliar and root metabolomic profiles *via* the regulation of specific metabolites. We showed that metabolites that were regulated in leaves differ from those in roots, and that for two of our three plant species the number of regulated metabolites in leaves was higher than in roots. These results partially confirm our first hypothesis that both plant diversity and soil legacy can alter the overall plant metabolome, as well as affect the regulation of specific

metabolites. Moreover, we revealed that the herbivore-induced metabolomic response is modulated by plant-plant and plant-soil interactions. This strongly suggests that the type and diversity of biotic interactions in the environment can alter induced responses to herbivores in plants. This confirms our second hypothesis that the induced defense to herbivory is differently affected by plant diversity and soil legacy. Compared to previous studies that focused on plant diversity effects in a field experiment (e.g., Scherling *et al.* 2010) or plant-soil feedback effects (e.g., Ristok *et al.* 2019; Huberty *et al.* 2020), our study provides new insights towards disentangling plant and soil diversity effects on plant metabolomes, and thus plant-herbivore interactions.

4.1 Plant diversity and soil legacy effects on plant metabolomes

While we did not find any overall changes in the foliar or root metabolome composition in response to plant diversity and soil legacy, we observed the unique regulation of 139 metabolites. This is in line with previous work showing that plant diversity or soil legacy can affect the regulation of foliar metabolites (Scherling *et al.* 2010; Huberty *et al.* 2020). Our study not only adds to this body of literature but also expands our knowledge by revealing that plant-plant and plant-soil interactions also affect the regulation of root metabolites.

Plant-plant and plant-soil interactions can range from positive, over neutral, to negative (Cortois *et al.* 2016; Barry *et al.* 2019). In particular negative plant-plant interactions, such as competition, can affect the regulation of metabolites. In our study, we detected 45 metabolites that were significantly up-regulated and 36 metabolites that were significantly down-regulated as a response to plant-plant interactions. This shift in regulation is potentially a consequence of competition for resources, such as light, nutrients, and water,

that can force the plant to either invest resources into growth or defense, as well as affect the production of allelopathic metabolites (Treutter 2006; Fernandez *et al.* 2016). Positive plant-soil interactions with mutualists, such as arbuscular mycorrhizal fungi and plant growth-promoting bacteria, that can improve nutrient uptake and protect against antagonists (Wardle *et al.* 2004; Latz *et al.* 2012; Bardgett & van der Putten 2014), can also affect the regulation of metabolites. In our study, we detected 24 metabolites that were significantly up-regulated and 34 metabolites that were significantly down-regulated as a response to plant-soil interactions. This shift in regulation may be a response to mycorrhization that, for instance, can affect phenyl alcohol and vitamin associated pathways (Rivero *et al.* 2015), and/or a response to negative plant-soil interactions with root parasites, pathogens, and herbivores that can reduce root uptake capabilities of resources (van der Putten *et al.* 2013; Bardgett & van der Putten 2014). The infection with nematodes, for instance, can affect the regulation of iridoid glycosides (Wurst *et al.* 2010), while the interaction among different types of soil organisms can further influence the plant metabolome and defense (Lohmann, Scheu & Müller 2009). In addition to these interaction-specific effects on foliar and root metabolomes, leaves and roots have different functions and are in different abiotic and biotic environments (van Dam 2009). These differences are the likely reason that certain metabolite classes in our study, such as alkaloids and phenolics, show different levels of concentration among leaves and roots (Kaplan, Halitschke, Kessler, Sardanelli & Denno 2008). Our study confirms that plant-plant and plant-soil interactions affect the regulation of metabolites in leaves and roots. Among the regulated metabolites, we tentatively identified some as flavonoids, iridoids, and alkaloid glycosides. Flavonoids are known as physiologically active compounds, playing important roles as signals in plant-soil biota interactions, as allelochemicals in plant-plant interactions, or as deterrents in plant-herbivore interactions (Treutter 2006). Iridoids and alkaloid glycosides are known for their significant roles in

plant-herbivore interactions (Bowers & Puttick 1988; Mithöfer & Boland 2008). Moreover, we also show for the first time that the nature of the regulated metabolites is unique to the tissue and type of biotic interaction. This strongly suggests that plants can adjust their constitutive metabolome and specifically react to their biological environment.

4.2 Plant diversity and soil legacy effects on herbivore-induced responses

We also observed alterations in the herbivore-induced metabolomic response due to plant diversity and soil legacy. Together, plant-plant and plant-soil interactions regulated 82 metabolites in control plants and 141 metabolites in herbivore-induced plants.

As shown above, plant-plant interactions can modulate growth-defense trade-offs that likely vary in strength with changes in plant diversity. In mixed communities, a combination of niche complementarity but increased competition for light, as well as a reduction of herbivory by specialized herbivores *via* dilution effects, may lead to a higher investment of resources into growth than defense compared to monocultures (Finch & Collier 2000; Castagneyrol, Jactel, Vacher, Brockerhoff & Koricheva 2014; van Moorsel *et al.* 2018; Eisenhauer *et al.* 2019). In fact, earlier work revealed that plants growing in mixed communities invested more resources into growth than defense-related metabolites compared to plants growing in monoculture (Broz *et al.* 2010), potentially reducing herbivore resistance. While we did not find differences in the overall metabolome composition of herbivore-induced plants in response to increasing plant diversity, we observed induced metabolite regulation in mixed communities. Our results suggest that the identity of the neighboring plant species determines the extent and direction of the plant-plant interaction. This has potential consequences for our understanding of plant-herbivore interactions in mixed communities, but further research is needed to confirm this hypothesis.

Plant-soil interactions, on the other hand, can prepare a plant for future attack, also called priming (Conrath *et al.* 2006). Systemic priming in plants can occur following interactions with soil microbes, nematodes, and mycorrhizal fungi, allowing the plant to better respond to subsequent herbivory (Kaplan *et al.* 2008; Martinez-Medina *et al.* 2016). While we have not explicitly tested for priming, it may explain why the absolute number of up-regulated metabolites in herbivore-induced plants (in comparison to control plants) was highest when plants had grown in different soil legacies. However, other possible mechanisms, such as systemic acquired resistance to microbial pathogens, exist that could also explain the patterns of metabolite regulation in our study (Ryals *et al.* 1996).

Finally, we observed differences in the regulation of herbivore-induced metabolites among our plant species. In *R. acris* plants, plant-plant interactions resulted in a strong down-regulation of induced metabolites, while plant-soil interactions resulted in a strong up-regulation of induced metabolites. The response to either type of biotic interaction was much more attenuated in *G. pratense* and *L. vulgare*, suggesting differences in the plant species-specific adaptability which requires future research before general assumptions can be made on the effects of plant diversity *versus* soil legacy on herbivore resistance.

While the present experiment provides novel insights into how metabolomic profiles, and thereby herbivore resistance, respond to changes in plant and soil biodiversity, it also calls for future studies. To allow for the comparison of plant-plant and plant-soil interactions in our study, we inoculated sterile substrate with liquid field soil inoculum from the Trait-Based Experiment (Ebeling *et al.* 2014) in the PSI experiment. This, however, meant that the soil biota communities were adapted and "linked" to the plot-specific plant communities. To fully disentangle plant from soil biodiversity effects on the plant metabolome, one would

need to expose plants to artificially constructed soil communities (see *e.g.,* de Souza, Armanhi & Arruda 2020), also including larger soil organisms (see *e.g.,* Lohmann et al. 2009). While this was not feasible in the scope of this study, it would also be important to explore the specific effects of pre-selected functional soil biota groups, such as nematodes (*e.g.,* Bezemer *et al.* 2005). Moreover, future studies should explore potential shifts in growth-defense trade-offs in more detail by exploring the performance of plants and herbivores.

Conclusion

Taken together, the present study shows that plant and soil biodiversity trigger unique responses in the plant's metabolomic profile that modulate the induced response to herbivory. By disentangling plant diversity from soil biodiversity effects, we advance our understanding of the mechanisms that shape plant metabolomes and thus, herbivore resistance.

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Data Accessibility

The datasets generated during and/or analyzed during the current study will be archived, and the data DOI will be included at the end of the article upon acceptance of the manuscript.

Competing interests

The authors declare no competing interest.

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Tables

Table 1. Differences in the species-specific foliar, root, and induced metabolome composition among the diversity/soil legacy levels. Statistical parameters resulting from a permutational multivariate analysis of variance using distance matrices. We used Bray-Curtis dissimilarity matrices and 9999 permutations. Abbreviations: F = pseudo-F-value; p = p-value.

Species	Plant-plant interaction experiment									Plant-soil interaction experiment								
	Foliar metabolome composition			Root metabolome composition			Induced metabolome composition			Foliar metabolome composition			Root metabolome composition			Induced metabolome composition		
	F	p	R^2	F	p	R^2	F	p	R^2	F	p	R^2	F	p	R^2	F	p	R^2
Geranium pratense	0.858	0.803	0.092	0.920	0.603	0.098	0.625	0.991	0.068	1.176	0.155	0.122	1.265	0.167	0.130	1.121	0.234	0.117
Leucanthemum vulgare	1.051	0.359	0.110	0.706	0.933	0.077	0.922	0.700	0.098	1.007	0.434	0.106	0.770	0.895	0.088	1.121	0.220	0.116
Ranunculus acris	0.995	0.486	0.105	0.901	0.634	0.096	1.167	0.216	0.121	0.916	0.662	0.097	0.710	0.766	0.077	0.903	0.703	0.096

Table 2. Up- and down-regulated metabolites tentatively assigned in leaves and roots of *Geranium pratense*, *Leucanthemum vulgare*, and *Ranunculus acris*. We assigned the molecular formula and the putative compound name based on the high-resolution mass-to-charge values generated by liquid chromatography quadrupole time-of-flight mass spectrometry. Abbreviations: Rt = Retention time in liquid chromatography in seconds; eV = Fragmentation energy in electron volt; MS = mass spectrometry; PPI = plant-plant interaction; PSI = plant-soil interaction.

Source	Rt [s]	Mass-to-charge [m/z]	Putative compound	Molecular formula	eV	MS fragment	Regulated by		Tissue		Modulated by herbivory
							PPI	PSI	Leaves	Roots	
Geranium pratense	64	173.045	Shikimic acid	$C_7H_9O_5$	35	93, 111, 137, 155		X	X		
Geranium pratense	120	169.014	Gallic acid	$C_7H_5O_5$	35	123, 141	X			X	
Geranium pratense	124	483.078	Di-Gallic acid glycoside	$C_{20}H_{19}O_{14}$	35		X			X	
Geranium pratense	191	483.078	Di-Gallic acid glycoside	$C_{20}H_{19}O_{14}$	35		X			X	
Geranium pratense	220	305.066	Flavonoid	$C_{15}H_{13}O_7$	35		X		X		
Geranium pratense	291	635.089	Flavonoid diglycoside	$C_{27}H_{23}O_{18}$	35		X	X	X		
Geranium pratense	297	609.145	Flavonoid diglycoside	$C_{27}H_{29}O_{16}$	35			X	X		

Geranium pratense	298	577.135	Flavonoid diglycoside	$C_{30}H_{25}O_{12}$	35		X			X		
Geranium pratense	308	299.077	Salicylate glycoside	$C_{13}H_{15}O_{8}$	35			X	X			
Geranium pratense	310	483.078	Di-Gallic acid glycoside	$C_{20}H_{19}O_{14}$	35		X			X		
Geranium pratense	315	577.134	Flavonoid diglycoside	$C_{30}H_{25}O_{12}$	35	343		X		X		
Geranium pratense	324	627.156	Flavonoid diglycoside	$C_{27}H_{31}O_{17}$	35	440		X	X		X	
Geranium pratense	328	289.072	Phenolic acid derivative	$C_{15}H_{13}O_{6}$	35	179, 245	X	X	X	X		
Geranium pratense	389	625.141	Flavonoid diglycoside	$C_{27}H_{29}O_{17}$	35		X		X			
Geranium pratense	393	477.104	Flavonoid glycoside	$C_{22}H_{21}O_{12}$	35	387	X		X		X	
Geranium pratense	400	507.114	Flavonoid glycoside	$C_{23}H_{23}O_{13}$	35		X		X		X	
Geranium pratense	404	479.083	Flavonoid glycoside	$C_{21}H_{19}O_{13}$	35			X	X			
Geranium pratense	409	667.151	Flavonoid diglycoside	$C_{29}H_{31}O_{18}$	35		X		X			
Geranium pratense	475	447.093	Flavonoid glycoside	$C_{21}H_{19}O_{11}$	35	183, 335	X		X			
Geranium pratense	492	417.082	Flavonoid glycoside	$C_{20}H_{17}O_{10}$	35			X	X		X	

Geranium pratense	520	431.097	Flavonoid glycoside	$C_{21}H_{19}O_{10}$	35		X	X	X			
Geranium pratense	532	459.092	Flavone glycoside	$C_{22}H_{19}O_{11}$	35			X	X			
Leucanthemum vulgare	185	315.072	Dihydroxybenzoic acid glucoside	$C_{13}H_{15}O_9$	35			X	X			
Leucanthemum vulgare	270	353.087	Caffeoylquinic acid	$C_{16}H_{17}O_9$	35	177, 191	X		X			
Leucanthemum vulgare	277	375.129	Iridoid	$C_{16}H_{23}O_{10}$	35		X	X	X			X
Leucanthemum vulgare	281	163.040	Phenolic acid derivative	$C_9H_7O_3$	35		X		X			
Leucanthemum vulgare	284	315.071	Dihydroxybenzoic acid glucoside	$C_{13}H_{15}O_9$	35			X	X			
Leucanthemum vulgare	307	137.024	Salicylate	$C_7H_5O_3$	35			X	X			
Leucanthemum vulgare	321	341.088	Caffeic acid glycoside	$C_{15}H_{17}O_9$	35			X		X		
Leucanthemum vulgare	333	163.040	Phenolic acid derivative	$C_9H_7O_3$	35		X		X			
Leucanthemum vulgare	336	353.087	Caffeoylquinic acid	$C_{16}H_{17}O_9$	35	173, 191, 319, 351	X	X	X			
Leucanthemum vulgare	383	551.104	Flavonoid glycoside	$C_{24}H_{23}O_{15}$	35	507	X		X			X
Leucanthemum vulgare	413	325.092	Phenolic acid glycoside	$C_{15}H_{17}O_8$	35		X		X			

Leucanthemum vulgare	420	535.109	Flavonoid glycoside	$C_{24}H_{23}O_{14}$	35	491	X		X		
Leucanthemum vulgare	461	223.061	Phenolic acid derivative	$C_{11}H_{11}O_5$	35			X	X		X
Leucanthemum vulgare	462	336.108	Alkaloid glycoside	$C_{16}H_{18}NO_7$	35	230		X	X		X
Leucanthemum vulgare	463	505.098	Anthocyanin glycoside	$C_{23}H_{21}O_{13}$	35			X	X		
Leucanthemum vulgare	465	591.172	Flavonoid diglycoside	$C_{28}H_{31}O_{14}$	35		X			X	
Leucanthemum vulgare	477	625.141	Flavonoid diglycoside	$C_{27}H_{29}O_{17}$	35	359, 415, 581	X		X		
Leucanthemum vulgare	484	515.119	Dicaffeoylquinate	$C_{25}H_{23}O_{12}$	35	353		X	X		
Leucanthemum vulgare	510	461.109	Flavonoid glycoside	$C_{22}H_{21}O_{11}$	35		X		X		X
Leucanthemum vulgare	536	193.050	Phenolic acid derivative	$C_{10}H_9O_4$	35			X	X	X	
Leucanthemum vulgare	540	693.167	Flavonoid diglycoside	$C_{31}H_{33}O_{18}$	35		X		X		X
Leucanthemum vulgare	573	163.076	Phenolic acid derivative	$C_{10}H_{11}O_2$	35		X		X		
Leucanthemum vulgare	577	655.188	Flavonoid diglycoside	$C_{29}H_{35}O_{17}$	35		X		X		X
Ranunculus acris	197	197.045	Phenolic acid derivative	$C_9H_9O_5$	35	135, 151, 179	X		X		X

Ranunculus acris	260	181.050	Phenolic acid derivative	$C_9H_9O_4$	35	122	X		X	X
Ranunculus acris	289	341.088	Caffeic acid glycoside	$C_{15}H_{17}O_9$	35	161, 179, 203	X		X	
Ranunculus acris	311	137.024	Salicylate	$C_7H_5O_3$	35		X	X	X	X
Ranunculus acris	319	465.103	Flavonoid glycoside	$C_{21}H_{21}O_{12}$	35	277	X		X	
Ranunculus acris	325	725.193	Flavonoid diglycoside	$C_{32}H_{37}O_{19}$	35		X		X	
Ranunculus acris	325	353.087	Caffeoylquinic acid	$C_{16}H_{17}O_9$	35		X		X	
Ranunculus acris	333	163.040	Phenolic acid derivative	$C_9H_7O_3$	35		X		X	
Ranunculus acris	335	325.093	Phenolic acid glycoside	$C_{15}H_{17}O_8$	35	145	X	X	X	
Ranunculus acris	341	623.160	Flavonoid diglycoside	$C_{28}H_{31}O_{16}$	35			X	X	X
Ranunculus acris	351	325.093	Phenolic acid glycoside	$C_{15}H_{17}O_8$	35	145	X	X	X	X
Ranunculus acris	355	179.035	Acetylsalicylate	$C_9H_7O_4$	35	135	X	X	X	
Ranunculus acris	361	695.183	Flavonoid diglycoside	$C_{31}H_{35}O_{18}$	35		X		X	
Ranunculus acris	385	317.066	Flavonoid	$C_{16}H_{13}O_7$	35	255	X		X	X

Ranunculus acris	392	325.092	Phenolic acid glycoside	$C_{15}H_{17}O_8$	35			X	X	X
Ranunculus acris	398	447.093	Flavonoid glycoside	$C_{21}H_{19}O_{11}$	35		X	X	X	
Ranunculus acris	407	699.178	Flavonoid diglycoside	$C_{30}H_{35}O_{19}$	35		X		X	
Ranunculus acris	419	577.155	Flavonoid diglycoside	$C_{27}H_{29}O_{14}$	35			X	X	X
Ranunculus acris	435	669.166	Flavonoid diglycoside	$C_{29}H_{33}O_{18}$	35		X	X	X	
Ranunculus acris	438	579.135	Flavonoid diglycoside	$C_{26}H_{27}O_{15}$	35		X		X	X
Ranunculus acris	443	595.166	Flavonoid diglycoside	$C_{27}H_{31}O_{15}$	35	529		X	X	X
Ranunculus acris	446	667.151	Flavonoid diglycoside	$C_{29}H_{31}O_{18}$	35	593		X	X	X
Ranunculus acris	446	449.108	Flavonoid glycoside	$C_{21}H_{21}O_{11}$	35	287		X	X	X
Ranunculus acris	448	193.050	Phenolic acid derivative	$C_{10}H_9O_4$	35			X	X	X
Ranunculus acris	449	447.092	Flavonoid glycoside	$C_{21}H_{19}O_{11}$	35			X	X	X
Ranunculus acris	449	289.072	Phenolic acid derivative	$C_{15}H_{13}O_6$	35		X		X	
Ranunculus acris	457	303.051	Flavonoid	$C_{15}H_{11}O_7$	35	125, 177, 259, 275, 285	X		X	

Ranunculus acris	465	451.124	Flavonoid glycoside	$C_{21}H_{23}O_{11}$	35	355	X	X	X
Ranunculus acris	477	577.156	Flavonoid diglycoside	$C_{27}H_{29}O_{14}$	35		X	X	
Ranunculus acris	497	331.082	Flavonoid	$C_{17}H_{15}O_7$	35		X	X	X
Ranunculus acris	507	165.055	Phenolic acid derivative	$C_9H_9O_3$	35	147	X	X	
Ranunculus acris	581	285.040	Flavonoid	$C_{15}H_9O_6$	35		X	X	X

DISCUSSION

Multitrophic interactions are essential to ecosystem functioning but are threatened by the current decline in biodiversity. In order for mankind to safeguard our ecosystems and the services they provide we need to understand how biotic multitrophic interactions operate. In terrestrial ecosystems, for instance, the interactions among plants, their soil biota, and aboveground herbivores drive many important ecosystem functions, such as biomass production (e.g., Eisenhauer et al., 2012; Kos et al., 2015; Maron et al., 2011; Seabloom et al., 2017; Yang et al., 2021) and nutrient cycling (Cherif & Loreau, 2013; Eisenhauer et al., 2012; Hooper & Vitousek, 1998). Hence, it is not surprising that researchers tried to understand the mechanisms that govern plant-soil-herbivore interactions (e.g., Bardgett & Wardle, 2003, 2010; Mbaluto et al., 2020, 2021; Tao et al., 2015; Wurst, 2013). While these efforts provided us with valuable insights into the close connection between plants and their soil biota community (e.g., van der Putten et al., 2013) or plants and their herbivore community (e.g., Barnes et al., 2020; Wan et al., 2020), we often are only able to predict around 12 % of the variation in ecosystem properties (Jesch et al., 2018; van der Plas et al., 2020). The aim of this dissertation was to help in those efforts to provide insights into the mechanisms underlying plant-soil-herbivore interactions *via* the application of eco-metabolomics.

In order to achieve this goal, I combined controlled pot experiments (**Manuscript I + III**) with a field experiment in a semi-natural grassland (**Manuscript II**). In **Manuscript I + III**, I analyzed the effect of soil biodiversity by inoculating sterile substrate with plant-diversity conditioned soil. In **Manuscript II + III**, I analyzed the effect of plant biodiversity by sampling in plots of the Trait-Based Experiment (Ebeling, Pompe, et al., 2014) that differed

in plant species richness (**Manuscript II**), or by planting communities that differed in plant species richness in a pot experiment (**Manuscript III**). In my experiments, I measured the foliar metabolome composition (**Manuscript I – III**), the root metabolome composition (**Manuscript I + III**) as well as the diversity of secondary metabolites (**Manuscript I + II**) across multiple forb and grass species. In addition, I assessed plant individual herbivory (**Manuscript I + II**) to test if changes in the plant's metabolome affect and can explain variation in aboveground plant individual herbivory. In **Manuscript III**, I let larvae of the generalist herbivore *Spodoptera exigua* feed on plants to test if plant diversity and soil legacy alter the regulation of induced secondary metabolites.

Across all three studies, I found two general patterns that I will discuss in more detail below: (1) soil biota, *i.e.,* their general presence, in particular of nematodes, as well as plant diversity-derived soil biotic legacies affect the plant's foliar and root metabolome; and (2) investigating the plant's metabolome and diversity of secondary metabolites provide valuable information in explaining plant diversity – herbivory relationships. Taken together, this dissertation is an example how eco-metabolomics can provide new insights about the mechanisms of plant-soil-herbivore interactions by incorporating the plant's metabolome as a new functional plant trait. Throughout this dissertation, the use of an eco-metabolomics approach to plant-soil-herbivore interactions also allowed for the detection and putative annotation of several plant metabolites (**Manuscript I - III**). While most of the tentatively annotated metabolites belonged to the class of phenolics, in particular flavonoids and phenolic acids, of which some have been described to play a role in plant-soil-herbivore interactions (e.g., Bennett & Wallsgrove, 1994; Erb et al., 2009; Treutter, 2006; Usha Rani & Pratyusha, 2013; Whitehead et al., 2021), new hypotheses of the bioactivity of so far undescribed metabolites can be generated and tested.

Soil biota affect the plant's metabolome

Throughout this dissertation, I could show that soil biota affect the plant's metabolome. In **Manuscript I**, I found that the foliar and root metabolome compositions of plants that grew in sterile soil differed from those grown in substrate inoculated with field soil. In addition, I observed that the plant diversity-specific history of the soil inoculum, *i.e.,* the soil biotic legacy, led to differences in the plant's metabolome and the richness of secondary metabolites. This suggests that even within the same plant species the plant's secondary metabolome is highly variable and reflects the interactions a plant has with its rhizosphere. This was further supported in **Manuscript II**, where I discovered that the soil nematode community composition was the strongest driver of the plant's metabolome composition. My analyses revealed that the abundance of most functional nematode guilds positively correlated with the concentration of defense-related metabolites, such as flavonoids and phenolic acids. This suggests that belowground interactions with nematodes can affect aboveground interactions with herbivores. At last, in **Manuscript III**, I found that the soil biotic legacy can affect the concentration of foliar and root metabolites. In the constitutive metabolome, I detected 24 metabolites that were significantly up-regulated and 34 metabolites that were significantly down-regulated as a response to heterospecific soil biotic legacy. Moreover, I could also show that the herbivore-induced induction of 59 metabolites was significantly affected by heterospecific soil biotic legacies. In line with **Manuscript II**, the results from **Manuscript III** suggest that belowground interactions alter the plant's constitutive metabolomic profile with potential consequences for aboveground interactions. In addition, I found that plant-soil interactions can modulate the induced response to aboveground herbivory.

Overall, my results are supported by other studies that focused on the effects of plant-soil interactions on the plant's metabolome. For instance, soil biotic legacies strongly influenced the foliar metabolome composition of twelve grass and forb species in a controlled pot experiment (Huberty, Choi, et al., 2020). Similar to my results, the plant's metabolomic response to different soil biotic legacies was species-specific (see **Manuscript I**), and depended on the species identity of the soil conditioning plant species (see **Manuscript III**) (Huberty, Choi, et al., 2020). Moreover, a study on genetically identical plants of *Jacobaea vulgaris* revealed differences in foliar metabolomic profiles between plants grown in sterile soil and those grown in inoculated soil (Huberty, Martis, et al., 2020). Together with my own data showing similar results (**Manuscript I**), this may have strong implications for the transferability of insights from experimental to natural communities. In fact, my data suggest that it is necessary to assess the metabolome of plants grown in living soil, rather than sterile soil, if one wants to study the role of the plant's metabolome in biotic interactions, such as plant-herbivore interactions. In addition, research on the effects of soil microorganisms and fungi on the concentration of specific metabolite classes, such as amino acids, pyrrolizidine alkaloids, and iridoid glycosides, has shown that the presence and composition of the soil community can regulate the concentration of secondary metabolites in leaves and roots (Joosten et al., 2009; Kos et al., 2015; Wurst et al., 2010). In line with results presented in **Manuscript III**, this supports the notion that the soil biota can modulate the defensive status of a plant by changing the concentration of metabolites. These changes in metabolite concentration may then further affect the outcome of plant-herbivore interactions, which may affect plant fitness and thus plant community composition (Strauss et al., 2002). Finally, it has been shown that feeding by phytophagous nematodes or infection by root-knot nematodes can interfere with the plant's salicylic acid signaling pathway, affect the gene expression of defense-related secondary metabolites, and further affect the concentration of

root exudates (Mbaluto et al., 2021; Wurst et al., 2010). These results support my conclusion that the soil nematode community is a key driver of the plant's metabolomic composition (**Manuscript II**), and potentially hint at the molecular mechanisms that link soil nematodes and plant secondary metabolites.

Regarding the possible mechanisms that link the soil biota to changes in the plant's metabolome, it was suggested that soil microorganisms can activate genes related to the primary and secondary metabolism (Chialva et al., 2018). Research on *Plantago lanceolata*, for instance, revealed that soil biotic legacy can influence defense-related genes, involved in jasmonic acid signaling (Zhu et al., 2018). The phytohormone jasmonic acid is, among others, involved in the coordination of signaling pathways related to plant-soil-herbivore interactions (Erb et al., 2012; Pieterse et al., 2012). In addition, interactions with plant growth-promoting bacteria and mycorrhizal fungi can initially affect plant hormonal regulation and subsequently prime plant defenses against herbivore and pathogen attack, thus improving overall resistance (Conrath et al., 2006; Ferlian et al., 2018; Martinez-Medina et al., 2016; Pieterse et al., 2014). While I have not explicitly tested for priming, it may explain the relationships between the plant's metabolome and herbivory, I observed in **Manuscript I + III**. In both studies, plants first interacted with different soil biotic legacies, which affected the plant's metabolome, and then later with aboveground herbivores. Based on these results, future research may test if soil biota diversity and the plant community-induced legacy affect priming and thus plant defenses. These studies, together with my results, highlight the important role that soil biota play in modulating the plant's metabolome, thereby potentially reducing plant herbivory, and provide evidence that novel mechanistic insights into plant-soil-herbivore interactions can be gained by including metabolomics analyses.

Plant-herbivore interactions and the role of the metabolome

Throughout this dissertation, I could establish a link between the plant's secondary metabolome and individual plant aboveground herbivory. In **Manuscript I**, I found that the richness and Shannon diversity of secondary metabolites was positively correlated with herbivory in one of four plant species. In addition, I detected that in two of four plant species a small set of up to 15 metabolites could explain over 80% of the variation in herbivory. This hints at potentially two different strategies how plants change their metabolome to deal with plant-herbivore interactions. Plants may either base their defense on a few highly effective metabolites or adjust the concentration of many metabolites to deter herbivores. In **Manuscript II**, I discovered that the species richness of the plant community can reduce individual plant herbivory, and that this may be related to changes in the plant's metabolome. Indeed, partial-least squares path model analyses indicated that the plant's metabolome may be an important functional trait that can explain more variation in individual plant aboveground herbivory than commonly used morphological and physiological plant traits. Moreover, I could also show that the interactions between plant community, soil biota, plant biomass, plant metabolomes, and herbivory can differ between grasses and forbs. While this may hint towards evolutionary conserved strategies among grasses and forbs, it also highlights the importance to include functional group information when trying to model plant-soil-herbivore interactions. At last, in **Manuscript III**, I found that the foliar metabolome composition of herbivore-induced plants significantly differed from that of control plants. I also discovered that the total number of up-regulated metabolites in response to herbivory was higher than the total number of down-regulated metabolites. These results may suggest that plants increase the concentration of defensive metabolites to deter herbivores, while herbivores simultaneously try to suppress the concentration of defensive

metabolites (Eisenring et al., 2018). In addition, this regulation of induced metabolites was modulated by heterospecific plant-plant and plant-soil interactions, suggesting that the type and diversity of biotic interactions can affect the defensive status of a plant.

Overall, my results that an increase in plant diversity can reduce individual plant herbivory (**Manuscript I + II**) and that herbivory can up- and down regulate secondary metabolites (**Manuscript III**) are supported by other studies. For instance, variation in chemical diversity related to intraspecific plant diversity has been shown to reduce individual plant herbivory (Bustos-Segura et al., 2017). Together with my results, this again supports the notion that the plant's metabolome is an important functional trait that can link plant diversity and herbivory. In addition, it has long been known that herbivory can induce plant responses, such as the regulation of secondary metabolite concentration (Bezemer & van Dam, 2005). This was again confirmed in my dissertation, but I could additionally show that the induction of secondary metabolites in response to herbivory is modulated by plant and soil biota diversity. In that regard, it was shown that experimentally manipulated individual herbivory increased the diversity and concentration of secondary metabolites, in particular flavonoids and phenolic acids, as well as led to changes in the metabolomic composition of twelve grass and forb species (Huberty, Choi, et al., 2020). Interestingly, the effect of herbivory on the metabolome composition was weaker than the effect of soil legacy on the metabolome composition (Huberty, Choi, et al., 2020). My findings (**Manuscript I – III**) support these results and highlight that plant diversity and soil biota diversity are important factors that determine the plant's metabolome. These results suggest that plant and soil biota diversity mechanistically drive ecosystem processes, such as herbivory, *via* their effect on the plant's metabolome. Consequently, one may speculate that a loss of plant and soil biota diversity may have more far reaching multitrophic consequences than a loss of herbivore

diversity.

Regarding the underlying mechanisms that led to the observable high diversity in secondary plant metabolites and the close relationship with herbivory, it was suggested that the co-evolution of plants and their herbivores (Agrawal & Weber, 2015; Ehrlich & Raven, 1964) or more general the large amount of simultaneous interactions with other organisms (van Dam, 2009; van Dam & Heil, 2011; Whitehead et al., 2021) posited a need for and drove the synthesis of new metabolites. Indeed, a study testing the hypothesis of plant-herbivore co-evolution using simulation models found that a higher diversity of defensive secondary metabolites increased plant fitness (Speed et al., 2015). This results was corroborated by a recent study that tested three competing hypotheses trying to explain chemical diversity in plants and found clear support for the interaction diversity hypothesis (Whitehead et al., 2021). In short, the interaction diversity hypothesis posits that several metabolites are necessary to protect plants from enemies and enable manipulation of mutualists because different interacting organisms can vary in their response and susceptibility to plant metabolite bioactivity (Whitehead et al., 2021). These studies and my dissertation highlight that above- and belowground biodiversity begets phytochemical diversity and provide evidence that the plant's metabolome is an integral part of plant-soil-herbivore interactions.

Synthesis

By nature, plant-soil-herbivore interactions are above-belowground interactions that are mediated by plant metabolites (Bezemer et al., 2003; Bezemer & van Dam, 2005; van Dam et al., 2003; van Dam & Heil, 2011). There is ample evidence that above-belowground interactions can shape aboveground herbivore communities (Huang et al., 2014; Johnson et al., 2012), multitrophic interactions with parasitoids (Bezemer et al., 2005), and plant

diversity-ecosystem functioning (Eisenhauer, 2012) *via* systemic induction of secondary metabolites (Hol et al., 2010; van Dam & Heil, 2011; Wondafrash et al., 2013). As seen in my dissertation, the interaction with soil biota, be it negative in the form of belowground herbivores such as phytophagous nematodes (**Manuscript II**) or positive in the form of mutualistic microbes and fungi such as arbuscular mycorrhiza (**Manuscript I + II**), can elicit metabolomic changes locally in the roots and systemically in the leaves (**Manuscript I + III**). In particular, systemic metabolomic changes in the leaves may then subsequently affect aboveground plant-herbivore interactions (Hol et al., 2010; van Dam & Heil, 2011; Wondafrash et al., 2013). While these plant-soil-herbivore interactions have been proven to generally occur in terrestrial ecosystems, it has also been shown that the outcome of any given plant-soil-herbivore interaction depends on the time of arrival of the interacting organisms (Bezemer et al., 2003; Erb et al., 2011; Johnson et al., 2012) as well as on the species identities of the plant, the soil biota, and the aboveground herbivores (Huang et al., 2014; van Dam & Heil, 2011; Wondafrash et al., 2013). My dissertation now adds to the body of literature and provides experimental evidence that the diversity of the plant community induces shifts in soil biota diversity that can affect the plant individual foliar and root metabolomic composition as well as the regulation and diversity of secondary metabolites. By including metabolomic analyses, we gained a better understanding for the importance of plant-soil interactions in shaping ecosystem processes, such as aboveground plant-herbivore interactions, *via* their effect on the plant's metabolome.

Outlook and future perspectives

The aim of my dissertation was to provide insights into the mechanisms underlying plant-soil-herbivore interactions *via* the application of eco-metabolomics. While my results certainly advanced our understanding of plant-soil-herbivore interactions, I only focused on

the foliar and root secondary metabolome. Future studies are needed that test my hypotheses for other parts of the metabolome, such as primary metabolites and volatile organic compounds, and thus further improve our understanding of plant-soil-herbivore interactions. Moreover, I conducted all my studies on grass and forb species, which allowed for insights into multitrophic interactions in grasslands but may not be transferable to other ecosystems such as forests. Hence, I suggest to test my hypotheses in established tree biodiversity experiments, such as BEF China (Bruelheide et al., 2014) and MyDiv (Ferlian et al., 2018), to search for general patterns that may occur in most or all terrestrial ecosystems. These experimental platforms would, for instance, allow to test if the species identity of the neighboring plant or the type and diversity of mycorrhization affect plant-soil-herbivore interactions. In addition, several secondary metabolites were putatively annotated and speculated to play a role in plant-soil-herbivore interactions. Additional research is needed to clearly identify the identity and structure of each metabolite and confirm their bioactivity. This was unfortunately beyond the scope of my dissertation. Metabolite identification is achieved either by comparison of MS data with reference standards, which often do not exist for non-model plant species, or by structural identification using nuclear magnetic resonance spectroscopy, which requires sufficient amounts of purified metabolites (Peters et al., 2018). Neither was achievable in my dissertation. Finally, my results provide experimental evidence that higher levels of plant species richness reduce individual plant herbivory, mediated by soil biota and the plant's metabolome. These results may be transferred to sustainable agriculture, where it would still be necessary to test if an increase in crop species diversity and/or an increase in soil biota diversity, *e.g.,* through inoculation, can improve plant resistance and thus crop yield.

SUMMARY

The loss of biodiversity is negatively affecting ecosystem functions, ecosystem services, and biotic interactions. However, it is those biotic interactions that are essential for ecosystem fitness, productivity, and resilience. In terrestrial ecosystems, for instance, the multitrophic above-belowground interactions among plants, their soil biota, and the aboveground herbivore community drive many important ecosystem processes. If we want to safeguard our ecosystems and the services they provide, we thus need to understand how plant-soil-herbivore interactions operate. While we know of the close connections between plants and their soil biota community or plants and their herbivore community, we still lack the mechanistic understanding underlying these biotic interactions. It is here that the newly emerging research area of ecological metabolomics (*eco-metabolomics*) may provide new insights by uncovering the molecular processes of plant-soil-herbivore interactions. My dissertation aimed to provide insights into the molecular mechanisms underlying plant-soil-herbivore interaction by combining the application of eco-metabolomics with biodiversity experiments. I combined controlled pot experiments with a field experiment in a semi-natural grassland to test whether different levels of plant diversity and soil biota diversity affect plant metabolomes. Furthermore, I tested if these changes in plant metabolomes and the diversity of secondary metabolites can be linked to aboveground herbivory to explain variation therein.

In a pot experiment with four different forb species, my research revealed that the foliar and root metabolome compositions of plants that grew in sterile soil differed from those grown in substrate inoculated with field soil. I also showed that different plant diversity-driven soil biotic legacies affected the plant's metabolome composition as well as the richness of secondary metabolites. These metabolomic insights may have strong implications for results

obtained in experiments that solely use sterile soil when it comes to their transferability to natural systems. Moreover, I detected multiple metabolites that in combination could explain over 80% of the variation in shoot herbivory in two of the four plant species, hinting at potentially two different strategies how plants change their metabolome to deal with plant-herbivore interactions. Plants may either base their defense on a few highly effective metabolites or adjust the concentration of many metabolites to deter herbivores. Taken together, this first pot experiment indicated that soil legacy effects can mechanistically link plant communities and aboveground herbivores *via* changes in the plant's metabolome.

To confirm the results from my first pot experiment and to test my hypotheses under semi-natural conditions, I sampled 34 plant communities of the Trait-Based Experiment of the Jena Experiment that varied in plant species richness and plant community resource acquisition strategies. I assessed individual herbivory and foliar metabolomes of seven grassland plant species as well as plant community-level soil microbial and nematode community composition. I found that individual plant herbivory rates decreased with increasing plant species richness, and that this relationship is likely mediated by changes in nematode community composition and plant metabolomes. I observed no direct effect of community-level resource acquisition strategy on individual plant herbivory but found some support that individual plant herbivory and the underlying mechanisms linking plant diversity and herbivory differ between grasses and forbs. Hence, this study confirmed the results from my first pot experiment that the plant diversity-driven soil biota can affect plant metabolomes and therefore aboveground herbivory. This study also showed that my results may be transferred to (semi-) natural ecosystems and highlights the importance to consider plant functional group identity when investigating plant diversity – herbivory relationships.

While both studies provided new and valuable insights into the molecular mechanisms underlying plant-soil-herbivore interactions, they could not disentangle the effect of plant

diversity from soil biota diversity. This is where my third and final study tried to provide new information. In a setup of two complementary microcosm experiments, I assessed the independent effects of plant diversity and soil legacy on constitutive and herbivore-induced plant metabolomes. I discovered that herbivore-induced metabolomes differed from those of control plants and detected over 100 metabolites whose concentrations were significantly changed by plant-plant and plant-soil interactions. These results indicated that both plant and soil biodiversity can independently induce unique responses in the plant's metabolomic profile, suggesting that the type and diversity of biotic interactions can affect the defensive status of a plant.

In conclusion, my dissertation provides experimental evidence that plant diversity can induce shifts in soil biota diversity that can affect the plant's metabolome as well as the concentration and diversity of secondary metabolites. In addition, my results suggest that the plant's metabolome is a novel and important functional trait that can link plant diversity and herbivory, and explain variation thereof. By including metabolomic analyses, we gained a better understanding for the importance of plant-soil interactions in shaping ecosystem processes, such as aboveground plant-herbivore interactions, *via* their effect on the plant's metabolome.

ZUSAMMENFASSUNG

Der Verlust der biologischen Vielfalt hat einen negativen Einfluss auf Ökosystemfunktionen, Ökosystemdienstleistungen und biotische Interaktionen. Es sind allerdings genau diese biotischen Interaktionen, die essenziell für Ökosystemfitness, Ökosystemproduktivität und Resilienz sind. In terrestrischen Ökosystemen, zum Beispiel, bestimmen multitrophische ober- und unterirdische Interaktionen zwischen Pflanzen, ihrer Bodenbiota und der oberirdischen Herbivorengemeinschaft viele wichtige Ökosystemprozesse. Wenn wir unsere Ökosysteme und deren Dienstleistungen bewahren wollen, müssen wir daher verstehen, wie Pflanze-Boden-Herbivoren-Interaktionen funktionieren. Obwohl uns die engen Zusammenhänge zwischen Pflanzen und ihrer Bodenbiota, oder Pflanzen und ihrer Herbivoren bekannt sind, fehlt uns noch das mechanistische Verständnis für die Prozesse dieser biotischen Interaktionen. Es ist diese Lücke in unserem Verständnis, die das neue Forschungsfeld Ökologische Metabolomanalysen (engl. *Eco-metabolomics*) versucht durch Aufklärung der molekularen Prozesse von Pflanze-Boden-Herbivoren-Interaktionen zu füllen. In diesem Zusammenhang hatte meine Dissertation das Ziel, durch die Kombination von Eco-metabolomics und Biodiversitätsexperimenten, neue Einsichten in die molekularen Prozesse von Pflanze-Boden-Herbivoren-Interaktionen zu liefern. Ich verwendete kontrollierte Topfexperimente und die Beprobung in einem semi-natürlichen Grasland-Feldexperiment, um zu untersuchen, ob unterschiedliche Grade pflanzlicher Diversität und Bodenbiodiversität einen Einfluss auf das pflanzliche Metabolom haben. Weiterhin habe ich untersucht, ob Änderungen im pflanzlichen Metabolom und der Diversität der sekundären Metaboliten in einen Zusammenhang mit oberirdischer Herbivorie gebracht werden können, und ob dadurch Variation in oberirdischer Herbivorie erklärt werden kann.

In einem Topfexperiment mir vier krautigen Pflanzenarten zeigte meine Forschung, dass die Zusammensetzung des Metaboloms in den Blättern und Wurzeln von Pflanzen, welche in sterilem Boden wuchsen, sich in der Zusammensetzung von Pflanzen unterschied, welche in inokuliertem Boden wuchsen. Weiterhin konnte ich zeigen, dass die Zusammensetzung des Metaboloms sowie die Vielfalt sekundärer Metabolite durch Pflanzendiversität-induzierte Änderungen in der Bodenbiota (engl. *soil biotic legacies*) beeinflusst wird. Diese neuen Einblicke in das pflanzliche Metabolom haben womöglich weitreichende Implikationen für Erkenntnisse, welche auf Experimenten basieren, die ausschließlich sterile Böden verwendet haben. Diese experimentellen Ergebnisse sind potenziell nicht auf natürliche Systeme übertragbar. Darüber hinaus habe ich in zwei von vier Pflanzenarten Metaboliten entdeckt, welche in Kombination über 80% der Variation in oberirdischer Herbivorie vorhersagen konnten. Dieses Ergebnis deutet auf zwei mögliche Strategien hin, mittels derer Pflanzen ihr Metabolom anpassen können, um auf Pflanze-Herbivoren-Interaktionen zu reagieren. Die Verteidigung von Pflanzen kann entweder auf wenigen, sehr effektiven Metaboliten basieren oder aber Pflanzen können die Konzentration vieler, weniger effektiver Metabolite anpassen, um so Herbivoren abzuschrecken. Zusammengefasst konnte ich mit diesem Topfexperiment zeigen, dass *soil biotic legacies* durch Änderungen des pflanzlichen Metaboloms mechanistisch Pflanzengesellschaften und oberirdische Herbivoren vernetzen.

Um die Ergebnisse aus meinem Topfexperiment zu bestätigen und um meine Hypothesen unter semi-natürlichen Bedingungen zu testen, habe ich im Folgenden 34 Pflanzengesellschaften beprobt. Diese Pflanzengesellschaften waren Teil des Trait-Based Experiments des Jena-Experiments und unterschieden sich in ihrer Pflanzendiversität sowie in den realisierten Strategien der Ressourcenakquise. Ich sammelte Daten zur individuellen Herbivorie und zur Zusammensetzung des Metaboloms in Blättern von sieben Grasland-Pflanzenarten. Zusätzlich untersuchte ich die Zusammensetzung der mikrobiellen und

Nematoden Gesellschaften im Boden. Ich konnte zeigen, dass die individuelle Herbivorie mit steigender Pflanzendiversität abnahm und dass dieser Zusammenhang höchstwahrscheinlich durch Änderungen in der Zusammensetzung der Nematodengesellschaft und Zusammensetzung des pflanzlichen Metaboloms bestimmt wurde. Ich fand keine direkten Effekte der Strategien der Ressourcenakquise auf die individuelle Herbivorie, konnte aber zeigen, dass die individuelle Herbivorie und die Mechanismen, welche Pflanzendiversität und Herbivorie verbinden, zwischen Gräsern und Kräuter unterschiedlich sind. Zusammengenommen konnte ich mit dieser Studie die Ergebnisse aus meinem Topfexperiment, dass *soil biotic legacies* Pflanzenmetabolome und damit oberirdische Herbivorie beeinflussen, bestätigen. Mit dieser Studie konnte ich ebenfalls zeigen, dass meine Ergebnisse auf (semi-)natürliche Ökosysteme übertragen werden können und dass die funktionelle Pflanzengruppe bedacht werden muss, wenn Pflanze-Herbivoren-Interaktionen studiert werden sollen.

Obwohl beide Studien neue und wertvolle Einblicke in die molekularen Mechanismen von Pflanze-Boden-Herbivoren-Interaktionen lieferten, erlaubten sie nicht die individuellen Effekte von Pflanzendiversität und Bodenbiodiversität zu trennen. Diese Lücke sollte mit meiner dritten und letzten Studie geschlossen werden. In einem Versuch mit zwei komplementären Topfexperimenten habe ich die individuellen Effekte von Pflanzendiversität und Bodenbiodiversität auf das pflanzliche Metabolom untersucht. Ich konnte zeigen, dass das Herbivorie-induzierte Pflanzenmetabolom signifikant unterschiedlich von dem Metabolom von Kontrollpflanzen war und dass die Konzentration von über 100 Metaboliten signifikant durch Pflanze-Pflanze- bzw. Pflanze-Boden-Interaktionen beeinflusst wurde. Diese Ergebnisse deuteten darauf hin, dass sowohl Pflanzendiversität als auch Bodenbiodiversität unabhängig voneinander einzigartige Effekte auf das pflanzliche Metabolom haben. Weiterhin zeigten meine Ergebnisse, dass die Art und

Diversität biotischer Interaktionen den defensiven Zustand einer Pflanze verändern kann. Abschließend lässt sich sagen, dass meine Dissertation experimentelle Beweise liefern konnte, dass Pflanzendiversität Änderungen in der Bodenbiodiversität hervorrufen kann und dass diese Änderungen das pflanzliche Metabolom sowie die Konzentration und Diversität sekundärer Metabolite beeinflussen. Weiterhin deuteten meine Ergebnisse daraufhin, dass es sich bei dem Pflanzenmetabolom um ein neues, wichtiges, funktionelles Merkmal handelt, welches Pflanzendiversität mit Herbivorie in Beziehung setzen und zeitgleich Variation erklären kann. Durch die Anwendung von ökologischen Metabolomanalysen erhielten wir ein besseres Verständnis für die Effekte von Pflanze-Boden-Interaktionen auf pflanzliche Metabolome sowie auf die Rolle und Bedeutung von Pflanze-Boden-Interaktionen in Bezug auf Ökosystemprozesse wie beispielsweise oberirdische Pflanze-Herbivoren-Interaktionen.

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EHRENWÖRTLICHE ERKLÄRUNG

Hiermit erkläre ich, Christian Ristok, dass mir die geltende Promotionsordnung bekannt ist, ich die Dissertation selbstständig und ohne unerlaubte Hilfe Dritter angefertigt habe, keine Textabschnitte Dritter oder eigener Prüfungsarbeiten ohne Kennzeichnung übernommen habe und alle benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in der Arbeit angegeben habe. Weiterhin erkläre ich, dass alle Stellen, die inhaltlich oder wörtlich aus Veröffentlichungen stammen, kenntlich gemacht wurden, dass ich keine Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen habe sowie dass keine Dritte weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Folgende Personen haben mich bei der Auswahl und Auswertung des Materials sowie bei der Herstellung der einzelnen Manuskripte unterstützt: Marcel Ciobanu, Jan-Hendrik Dudenhöffer, Anne Ebeling, Nico Eisenhauer, Yvonne Poeschl, Christiane Roscher, Nicole M. van Dam, Fredd Vergara, Cameron Wagg, Alexander Weinhold. Nähere Angaben sind den Manuskripten zu entnehmen.

Diese Dissertation lag noch nicht als staatliche oder andere wissenschaftliche Prüfungsarbeit einer Prüfungsbehörde vor und wurde bisher noch nicht veröffentlicht. Ich habe die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung bei keiner anderen Hochschule oder anderen Fakultät als Dissertation eingereicht.

Christian Ristok

Halle, den 01.03.2022

SUPPLEMENTARY INFORMATION

Supplementary information for Manuscript I

FORMULAR 2

Manuskript Nr. 1

Kurzreferenz Ristok et al (2019), J Ecol

Beitrag des Doktoranden / der Doktorandin

Beitrag des Doktoranden / der Doktorandin zu Abbildungen, die experimentelle Daten wiedergeben (nur für Originalartikel):

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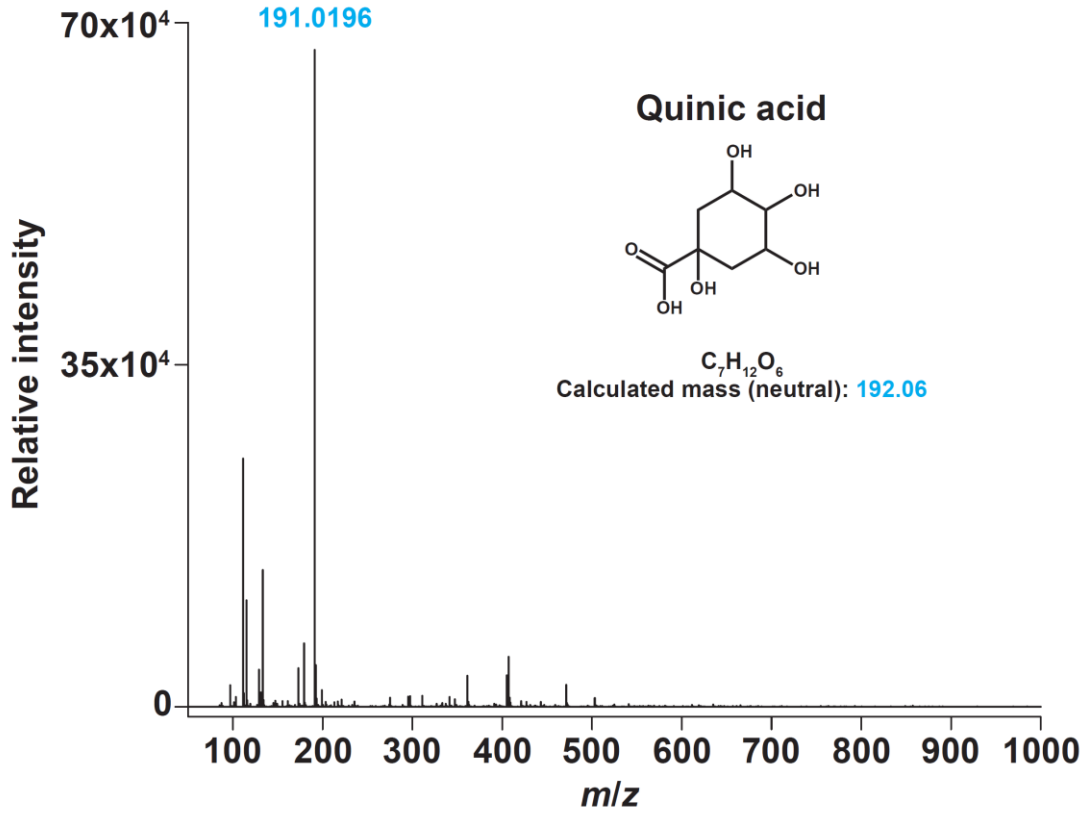
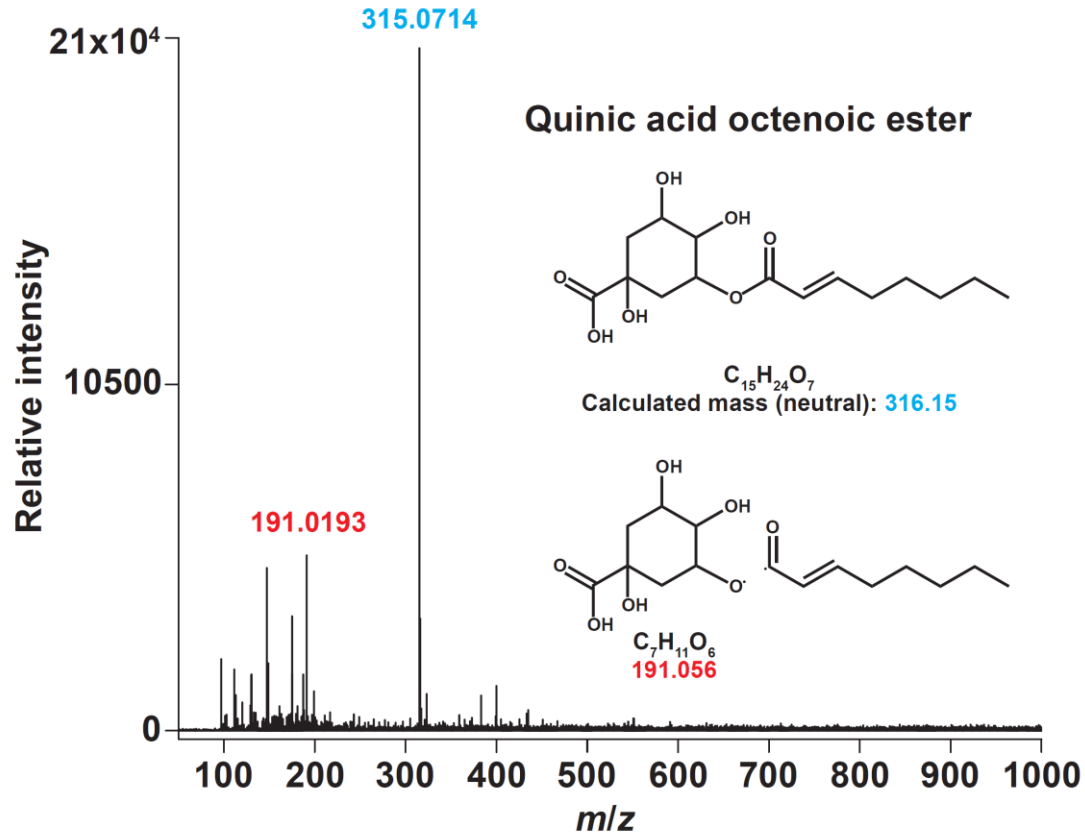
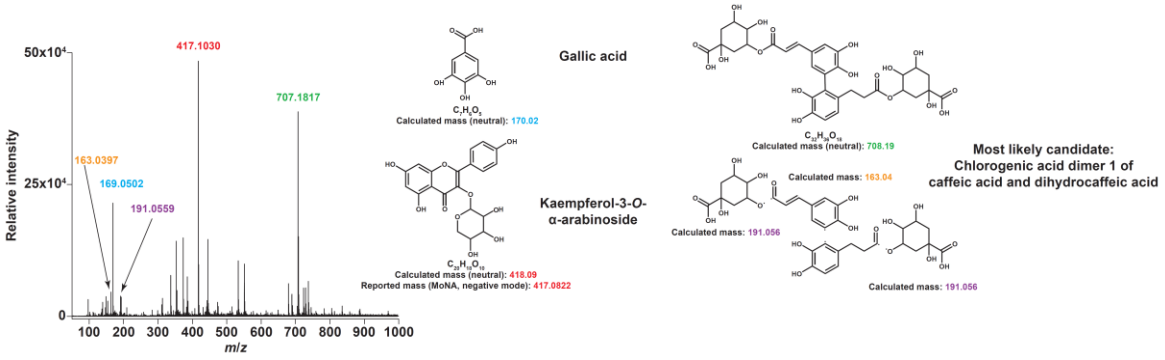
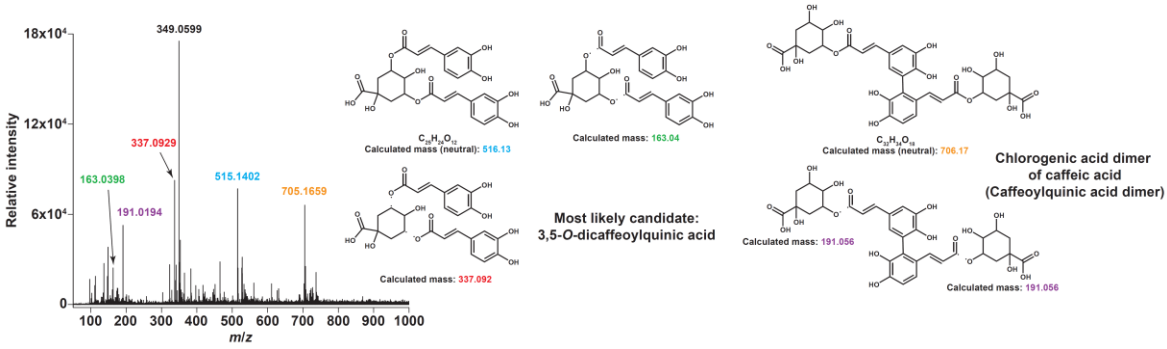
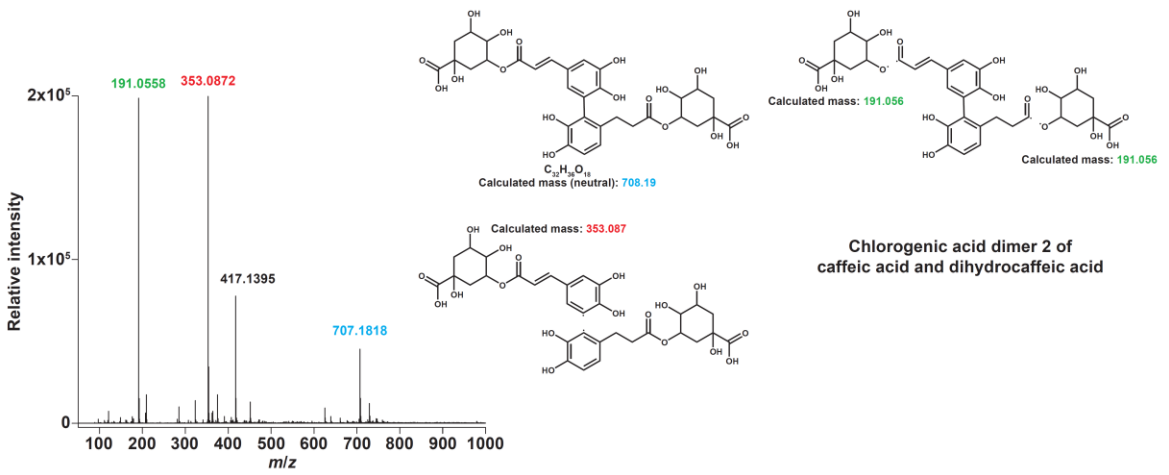
Supplementary Material Fig. S1. Tentative identification of candidate metabolites through the comparison of LC-MS/MS data with literature references. We submitted high-resolution m/z values to the MassBank of North America (MoNA, http://mona.fiehnlab.ucdavis.edu/) spectral database for comparison using a mass tolerance of 0.5 D. We calculated low-resolution molecular weights, molecular formulae for putative molecular ions in neutral form, and particle weights for mass spectrometry generated fragments using ChemDraw Ultra 8.0 (www.cambridgesoft.com).

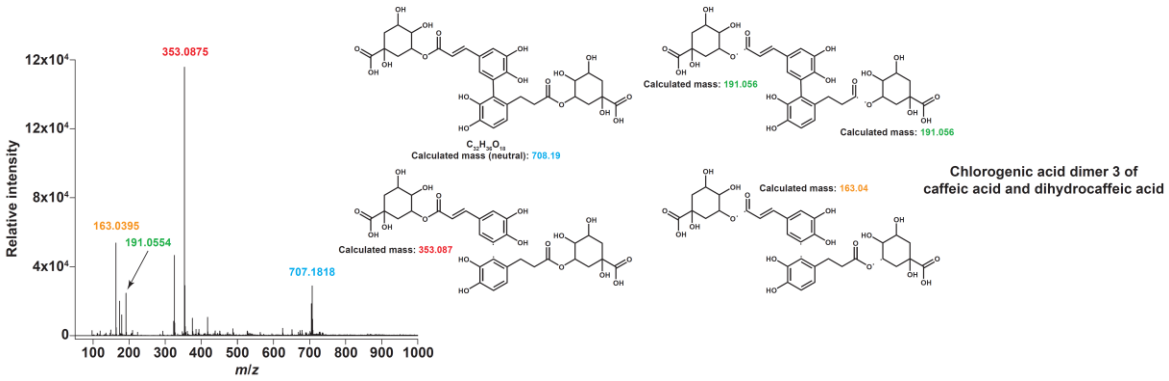


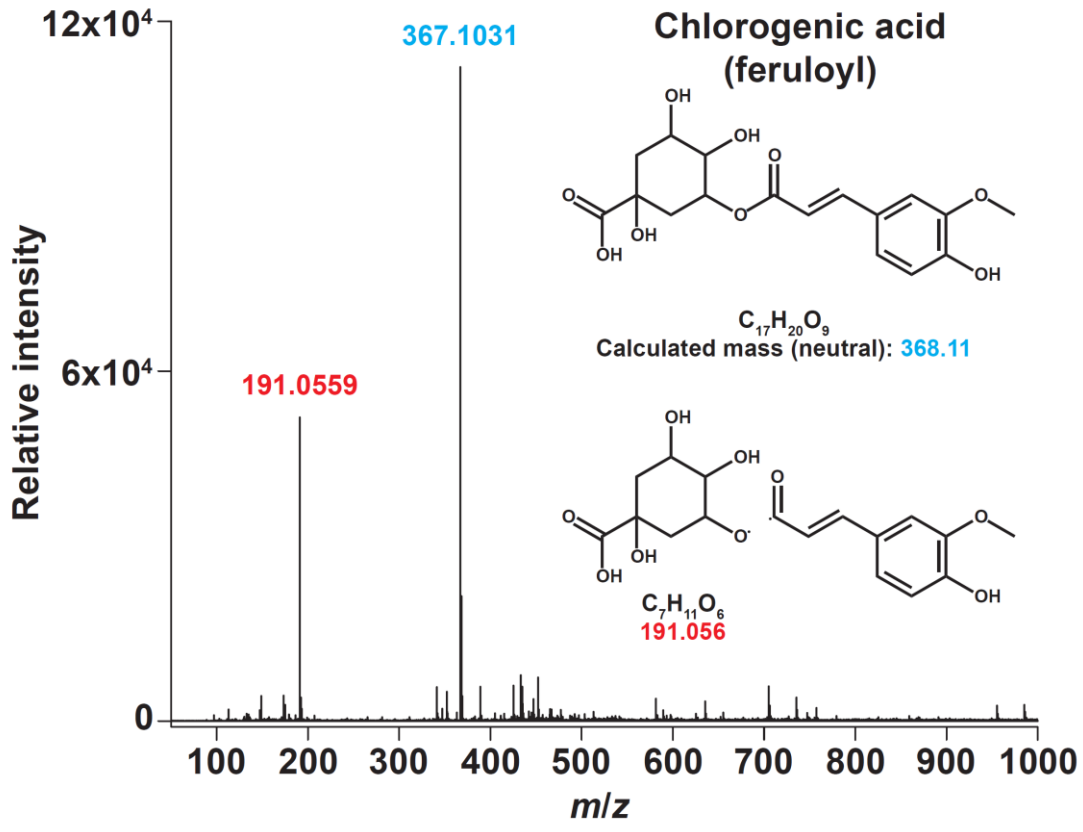

315.0714
21x10⁴
Quinic acid octenoic ester
Relative intensity
10500
191.0193
C15H24O7
Calculated mass (neutral): 316.15
C7H11O6
191.056
0
100 200 300 400 500 600 700 800 900 1000
m/z

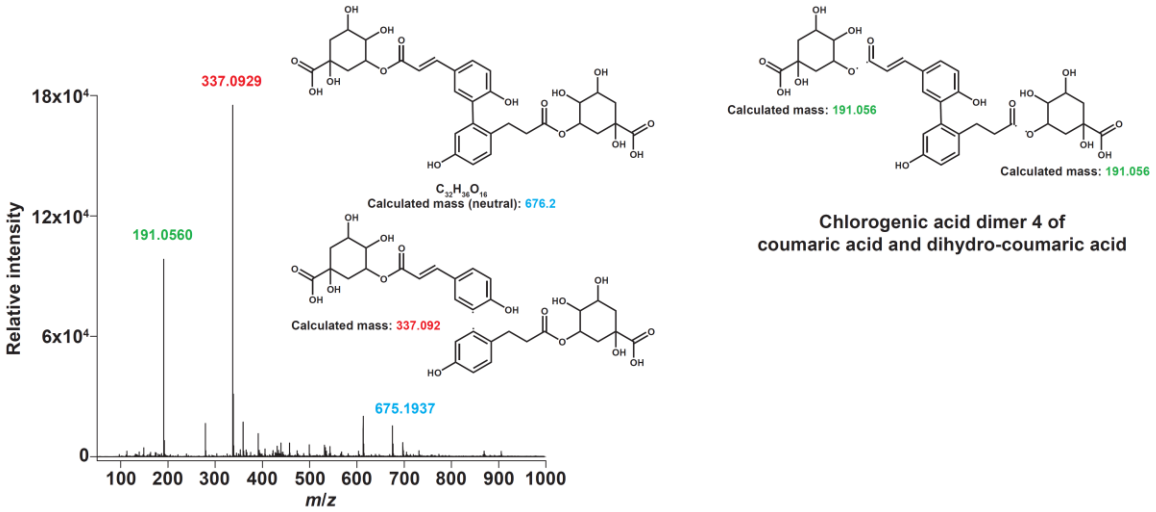

50x10⁴
417.1030
707.1817
163.0397
169.0502
191.0559
25x10⁴
Relative intensity
0
100 200 300 400 500 600 700 800 900 1000
m/z
Gallic acid
Calculated mass (neutral): 170.02
Kaempferol-3-O-α-arabinoside
Calculated mass (neutral): 418.09
Reported mass (MoNA, negative mode): 417.0822
Calculated mass (neutral): 708.19
Most likely candidate:
Chlorogenic acid dimer 1 of
caffeic acid and dihydrocaffeic acid
Calculated mass: 163.04
Calculated mass: 191.056
Calculated mass: 191.056

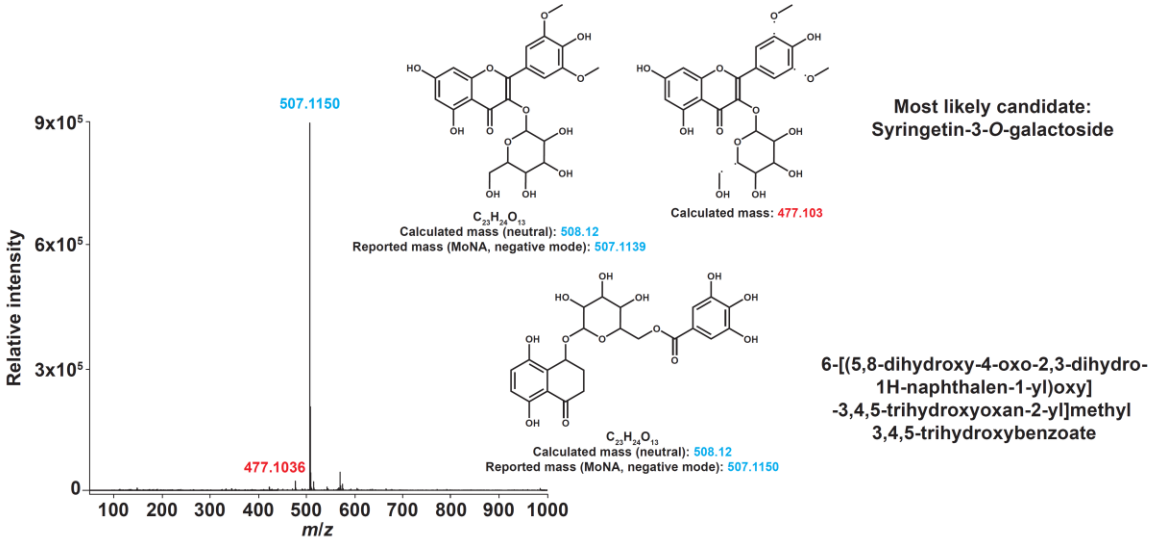

18x10⁴
349.0599
337.0929
163.0398
515.1402
705.1659
191.0194
12x10⁴
6x10⁴
Relative intensity
0
100 200 300 400 500 600 700 800 900 1000
m/z
Calculated mass (neutral): 516.13
Calculated mass: 163.04
Calculated mass: 337.092
Most likely candidate:
3,5-O-dicaffeoylquinic acid
Calculated mass (neutral): 706.17
Chlorogenic acid dimer
of caffeic acid
(Caffeoylquinic acid dimer)
Calculated mass: 191.056
Calculated mass: 191.056

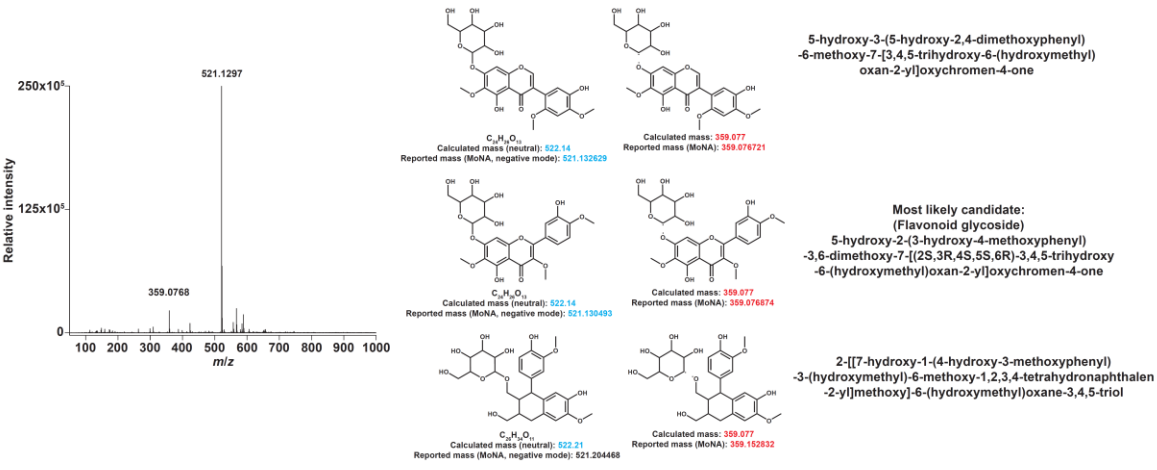

191.0558
353.0872
417.1395
707.1818
Relative intensity
m/z
Calculated mass (neutral): 708.19
Calculated mass: 353.087
Calculated mass: 191.056
Calculated mass: 191.056
Chlorogenic acid dimer 2 of
caffeic acid and dihydrocaffeic acid

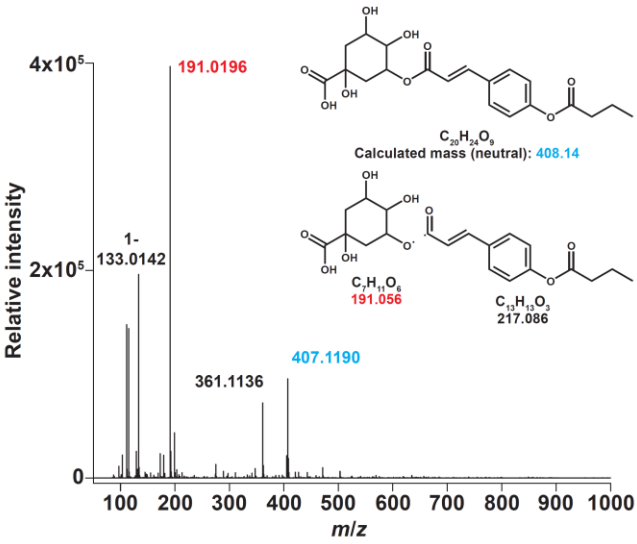

353.0875
163.0395
191.0554
707.1818
Relative intensity
m/z
Calculated mass (neutral): 708.19
Calculated mass: 353.087
Calculated mass: 191.056
Calculated mass: 191.056
Calculated mass: 163.04
Chlorogenic acid dimer 3 of
caffeic acid and dihydrocaffeic acid


367.1031
191.0559
Relative intensity
m/z
Chlorogenic acid
(feruloyl)
C17H20O9
Calculated mass (neutral): 368.11
C7H11O6
191.056


337.0929
191.0560
675.1937
Relative intensity
m/z
Calculated mass: 337.092
C32H36O16
Calculated mass (neutral): 676.2
Calculated mass: 191.056
Calculated mass: 191.056
Chlorogenic acid dimer 4 of
coumaric acid and dihydro-coumaric acid


507.1150
477.1036
Relative intensity
m/z
C23H24O13
Calculated mass (neutral): 508.12
Reported mass (MoNA, negative mode): 507.1139
Calculated mass: 477.103
Most likely candidate:
Syringetin-3-O-galactoside
C23H24O13
Calculated mass (neutral): 508.12
Reported mass (MoNA, negative mode): 507.1150
6-[(5,8-dihydroxy-4-oxo-2,3-dihydro-
1H-naphthalen-1-yl)oxy]
-3,4,5-trihydroxyoxan-2-yl]methyl
3,4,5-trihydroxybenzoate

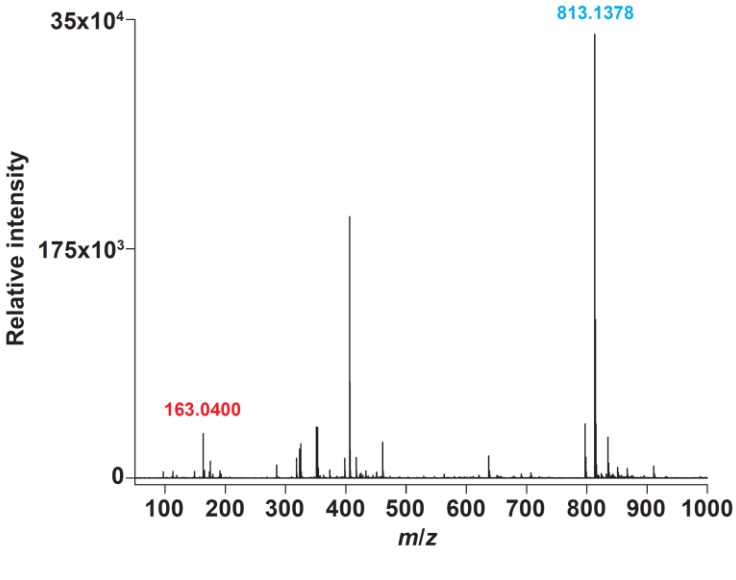

521.1297
359.0768
Relative intensity
m/z
C27H30O16
Calculated mass (neutral): 522.14
Reported mass (MoNA, negative mode): 521.132629
Calculated mass: 359.077
Reported mass (MoNA): 359.076721
5-hydroxy-3-(5-hydroxy-2,4-dimethoxyphenyl)
-6-methoxy-7-[3,4,5-trihydroxy-6-(hydroxymethyl)
oxan-2-yl]oxychromen-4-one
C27H30O16
Calculated mass (neutral): 522.14
Reported mass (MoNA, negative mode): 521.130493
Calculated mass: 359.077
Reported mass (MoNA): 359.076874
Most likely candidate:
(Flavonoid glycoside)
5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)
-3,6-dimethoxy-7-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy
-6-(hydroxymethyl)oxan-2-yl]oxychromen-4-one
C26H34O17
Calculated mass (neutral): 522.21
Reported mass (MoNA, negative mode): 521.204468
Calculated mass: 359.077
Reported mass (MoNA): 359.152832
2-[[7-hydroxy-1-(4-hydroxy-3-methoxyphenyl)
-3-(hydroxymethyl)-6-methoxy-1,2,3,4-tetrahydronaphthalen
-2-yl]methoxy]-6-(hydroxymethyl)oxane-3,4,5-triol

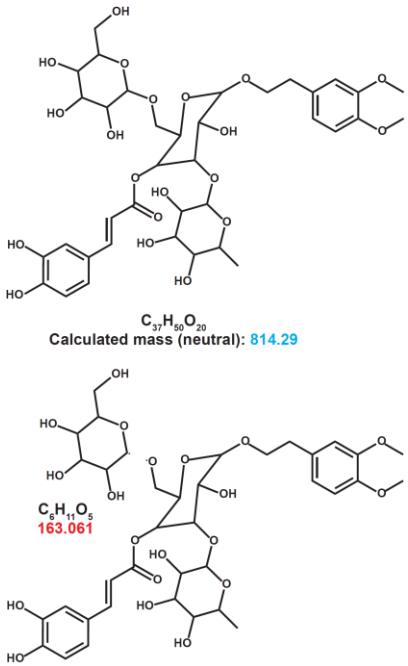


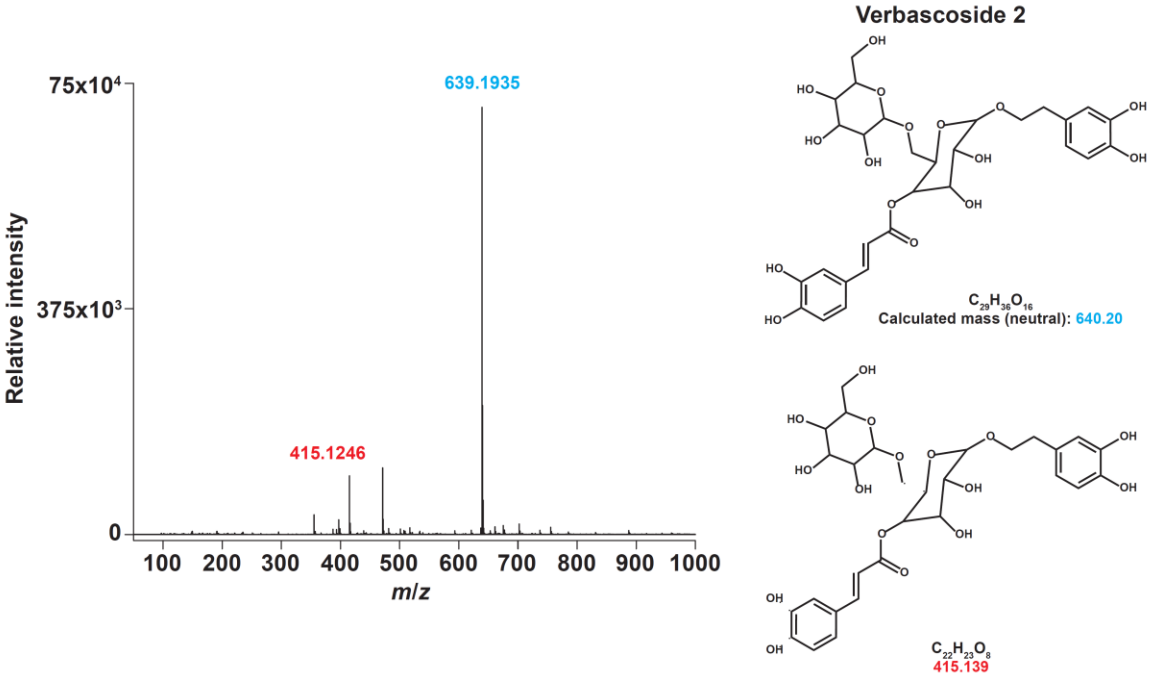
Chlorogenic acid derivative

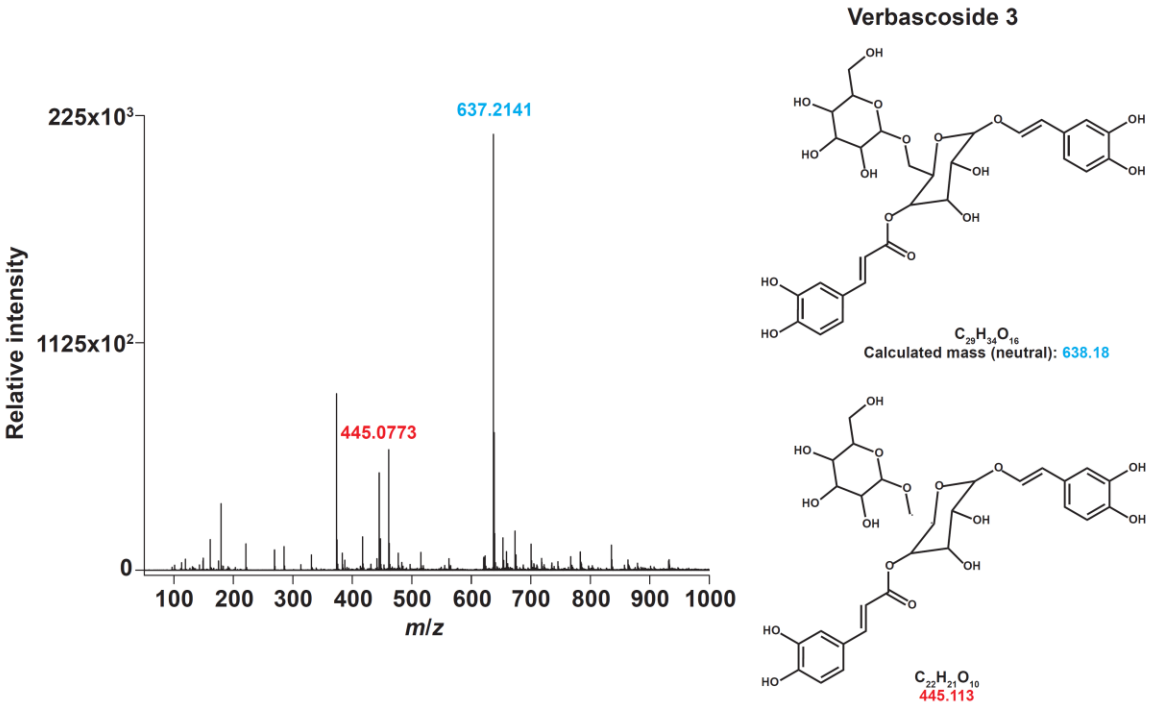
3-*O*-quinic acid [butanoic acid, 4-(2-carboxyethenyl)phenyl ester] ester

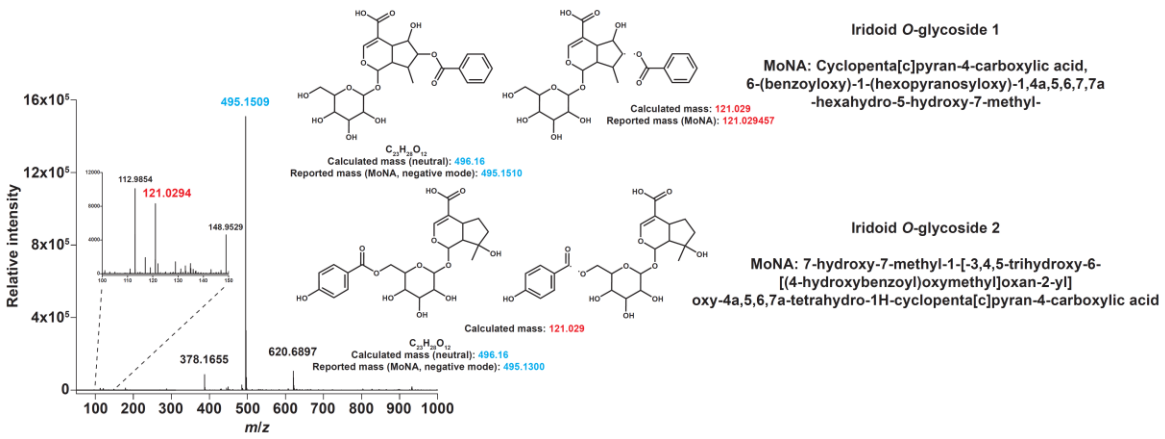
Verbascoside 1

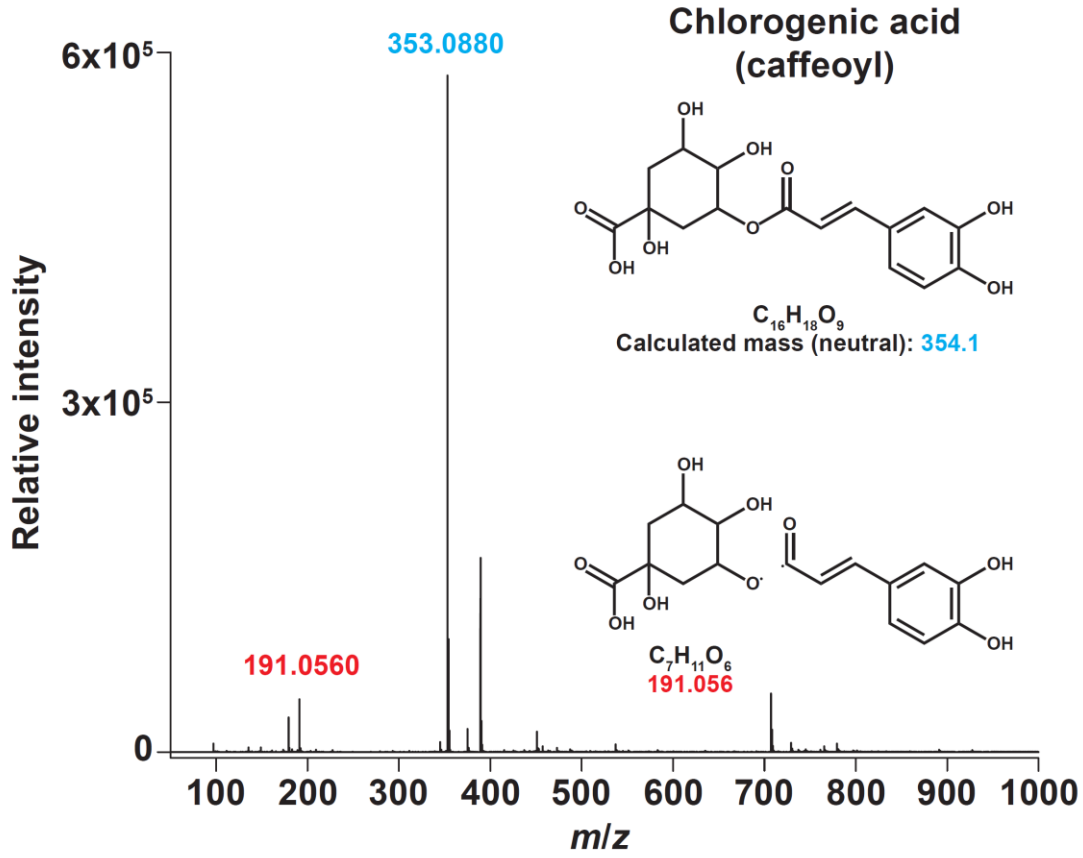


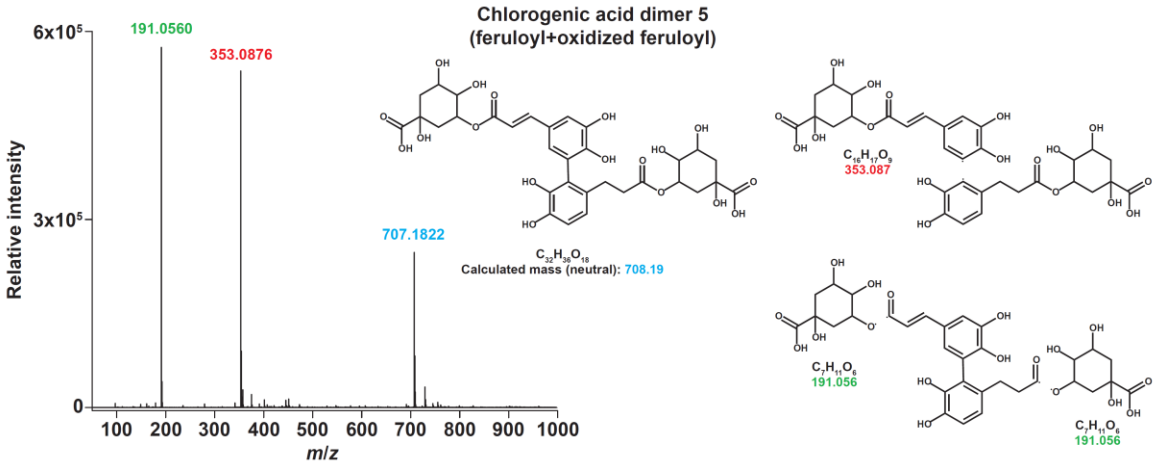


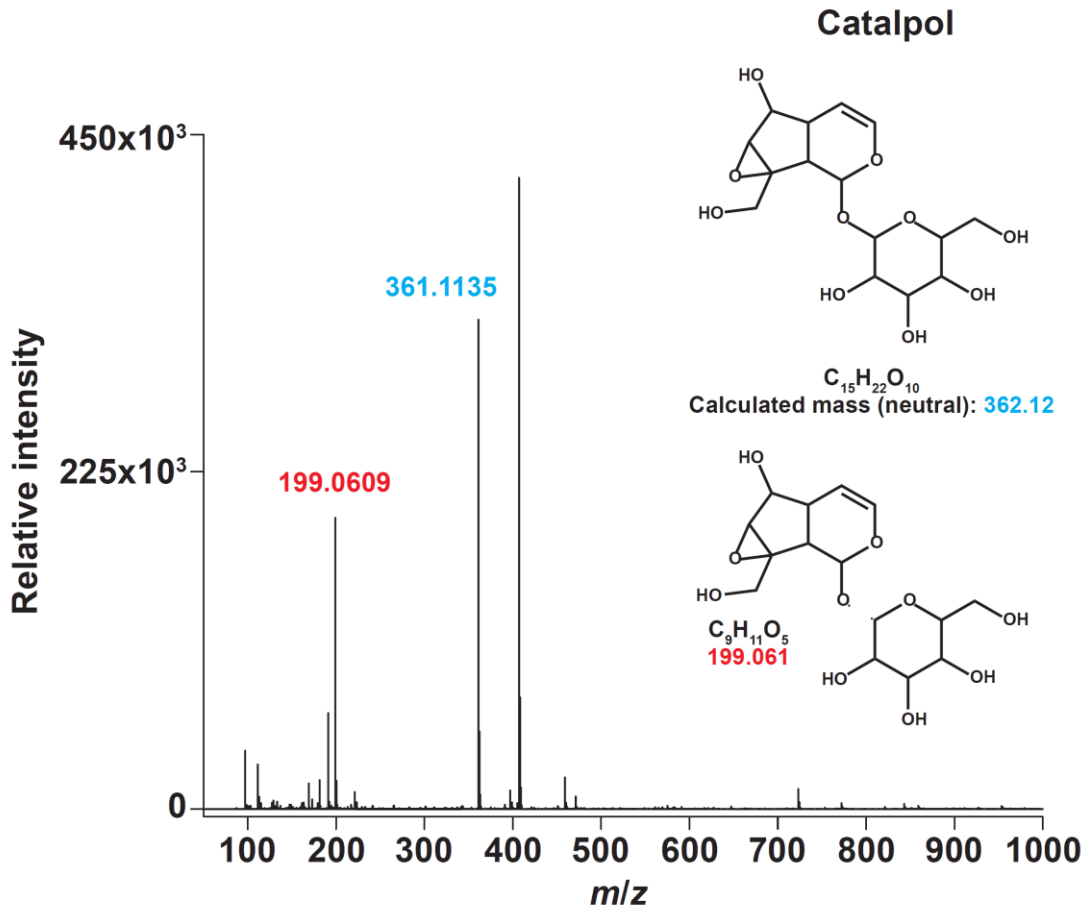

Verbascoside 2
75x10⁴
375x10³
0
Relative intensity
639.1935
415.1246
100 200 300 400 500 600 700 800 900 1000
m/z
C29H36O16
Calculated mass (neutral): 640.20
C22H23O8
415.139

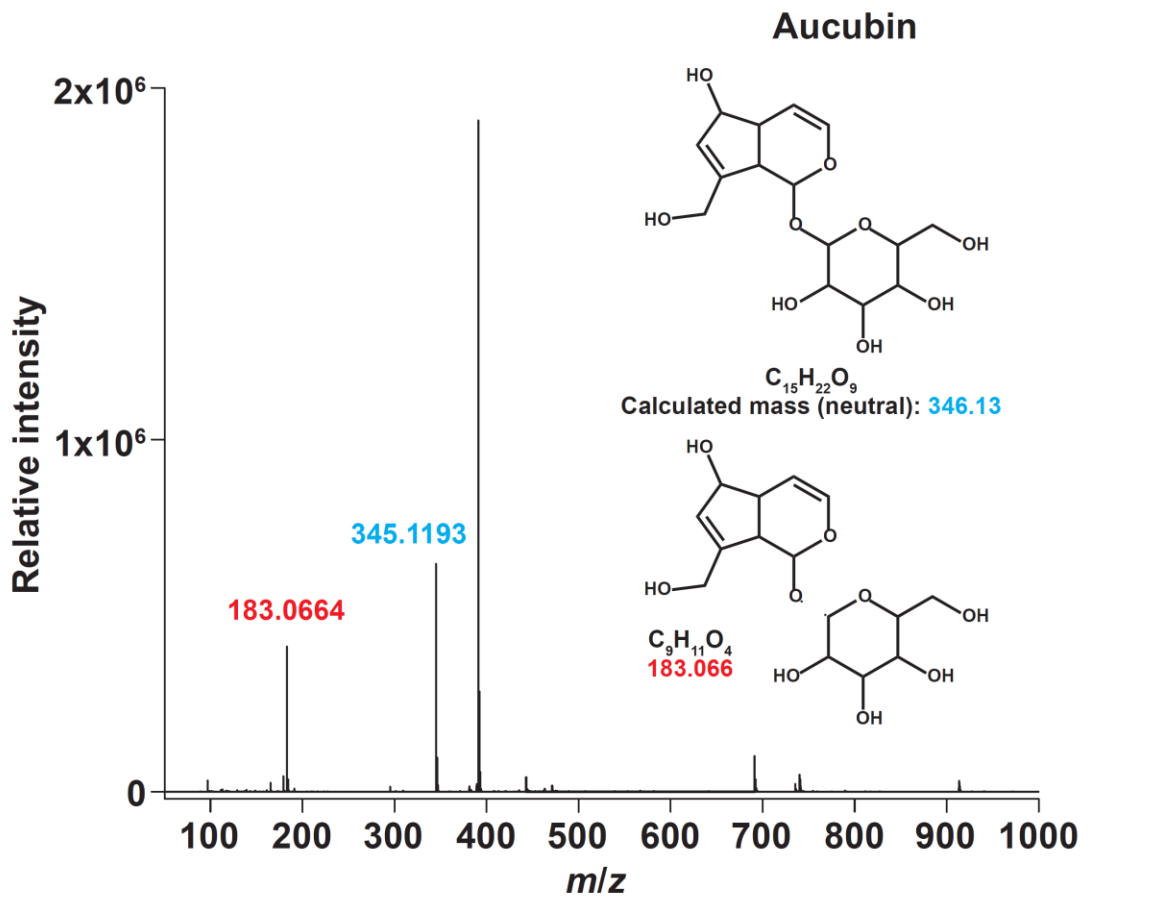

Verbascoside 3
225x10³
1125x10²
0
Relative intensity
637.2141
445.0773
100 200 300 400 500 600 700 800 900 1000
m/z
C29H34O16
Calculated mass (neutral): 638.18
C22H21O10
445.113


Iridoid O-glycoside 1
MoNA: Cyclopenta[c]pyran-4-carboxylic acid, 6-(benzoyloxy)-1-(hexopyranosyloxy)-1,4a,5,6,7,7a -hexahydro-5-hydroxy-7-methyl-
Calculated mass: 121.029
Reported mass (MoNA): 121.029457
C25H28O14
Calculated mass (neutral): 496.16
Reported mass (MoNA, negative mode): 495.1510
Iridoid O-glycoside 2
MoNA: 7-hydroxy-7-methyl-1-[-3,4,5-trihydroxy-6-[(4-hydroxybenzoyl)oxymethyl]oxan-2-yl] oxy-4a,5,6,7a-tetrahydro-1H-cyclopenta[c]pyran-4-carboxylic acid
Calculated mass: 121.029
C25H28O14
Calculated mass (neutral): 496.16
Reported mass (MoNA, negative mode): 495.1300
Relative intensity
m/z
495.1509
121.0294
112.9854
148.9529
378.1655
620.6897


Chlorogenic acid (caffeoyl)
353.0880
191.0560
C16H18O9
Calculated mass (neutral): 354.1
C7H11O6
191.056
Relative intensity
m/z


Chlorogenic acid dimer 5
(feruloyl+oxidized feruloyl)
6x10⁵
3x10⁵
0
Relative intensity
191.0560
353.0876
707.1822
100 200 300 400 500 600 700 800 900 1000
m/z
C32H36O18
Calculated mass (neutral): 708.19
C16H17O9
353.087
C7H11O6
191.056
C7H11O6
191.056


Catalpol
450x10³
225x10³
0
Relative intensity
361.1135
199.0609
100 200 300 400 500 600 700 800 900 1000
m/z
C15H22O10
Calculated mass (neutral): 362.12
C9H11O5
199.061


Aucubin
2x10⁶
1x10⁶
0
Relative intensity
345.1193
183.0664
HO
HO
O
O
O
OH
HO
OH
OH
C15H22O9
Calculated mass (neutral): 346.13
C9H11O4
183.066
100 200 300 400 500 600 700 800 900 1000
m/z

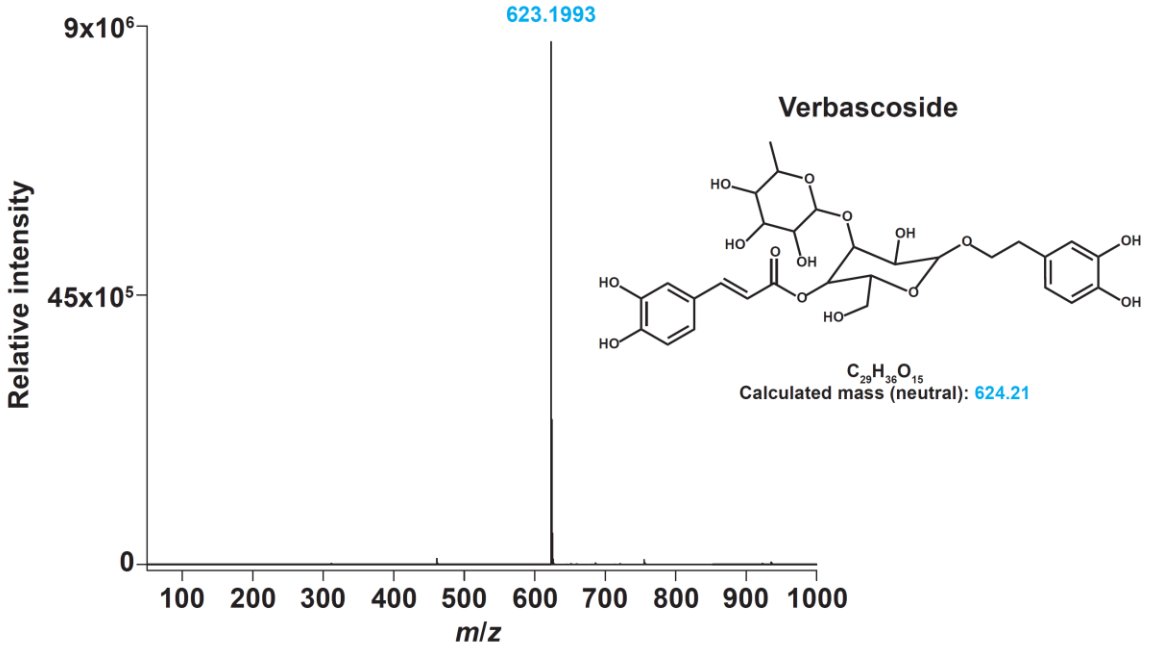

623.1993
9x10⁶
45x10⁵
0
Relative intensity
Verbascoside
HO
HO
O
OH
HO
OH
C29H36O15
Calculated mass (neutral): 624.21
100 200 300 400 500 600 700 800 900 1000
m/z

Table S1: Experimental design. Displayed are number of established pots for each realized combination of plant species and soil inoculum treatment. Number in brackets display number of pots at the end of the experiment. If no number in brackets is given, all pots have been harvested for further analyses.

Plant species	Soil inoculum treatment						
		Monoculture					
	Sterile	*C. jacea*	*K. arvensis*	*L. vulgare*	*P. lanceolata*	4 plant species richness	8 plant species richness
Centaurea jacea	8	8	0	0	0	8	8
Knautia arvensis	8 (6)	0	8 (7)	0	0	8	8 (7)
Leucanthemum vulgare	8	0	0	8	0	8 (7)	8
Plantago lanceolata	8	0	0	0	8	8 (7)	8

Table S2: Prior data processing simulation. Altered parameters and their respective values, snthr = signal to noise ratio, ppm = parts per million, minfrac = minimum fraction of samples necessary in a sample group to be valid, bw = bandwidth, mzwid = width of overlapping m/z slices for creating peak density chromatograms.

snthr	ppm	minfrac	bw	mzwid
10	5	0.7	3	0.005
20	10	0.9	5	0.01
50			10	0.015
100				
200				
300				

Table S3. Differences in the species-specific shoot and root richness of secondary metabolites, shannon diversity of secondary metabolites, and herbivory of plants grown in sterile or one of the three different living soil inocula. Represented are p-values, based on Dunnett's Test. Significant differences ($p < 0.05$) are given in bold.

Soil conditioning plant species richness	**Shoot**			
	Centaurea jacea	*Knautia arvensis*	*Leucanthemum vulgare*	*Plantago lanceolata*
	Richness of secondary metabolites			
0 vs 1	0.247	-	0.278	-
0 vs 4	0.985	-	0.983	-
0 vs 8	**0.030**	-	**0.009**	-
	Shannon diversity of secondary metabolites			
0 vs 1	-	-	-	-
0 vs 4	-	-	-	-
0 vs 8	-	-	-	-
	Herbivory			
0 vs 1	-	-	-	-
0 vs 4	-	-	-	-
0 vs 8	-	-	-	-
	Root			
	Richness of secondary metabolites			
0 vs 1	-	1.000	**<0.001**	**0.020**
0 vs 4	-	0.996	**<0.001**	**0.002**
0 vs 8	-	**0.034**	**0.023**	**<0.001**
	Shannon diversity of secondary metabolites			
0 vs 1	-	-	**<0.001**	0.400
0 vs 4	-	-	**<0.001**	**0.018**
0 vs 8	-	-	**0.011**	**0.019**

Table S4. Additional compounds tentatively assigned in shoot extracts of *Centaurea jacea* and *Plantago lanceolata* by LC-MS/MS. The metabolites were not detected by LASSO and thus were not part of a linear combination that explains shoot herbivory. Abbreviations: Rt = Retention time in liquid chromatography in minutes; eV = Fragmentation energy in electron volt; MS/MS = mass spectrometry / mass spectrometry.

Source	Rt [min]	$[M - H]^-$ [m/z]	Putative compound	Molecular formula	eV	MS/MS
Centaurea jacea	4.44	353.0880	Chlorogenic acid	$C_{16}H_{18}O_9$	35	191
Centaurea jacea	5.36	707.1822	Chlorogenic acid dimer 5	$C_{32}H_{36}O_{18}$	35	353, 191
Plantago lanceolata	1.34	361.1135	Catalpol	$C_{15}H_{22}O_{10}$	35	199
Plantago lanceolata	2.08	345.1193	Aucubin	$C_{15}H_{22}O_9$	35	183
Plantago lanceolata	4.44	353.0880	Chlorogenic acid	$C_{16}H_{18}O_9$	35	191
Plantago lanceolata	7.34	623.1993	Verbascoside	$C_{29}H_{36}O_{15}$	35	
Plantago lanceolata	7.70	623.1993	Isoverbascoside	$C_{29}H_{36}O_{15}$	35	

Supplementary information for Manuscript II

FORMULAR 2

Manuskript Nr. 2

Kurzreferenz Ristok et al (in review)

Beitrag des Doktoranden / der Doktorandin

Beitrag des Doktoranden / der Doktorandin zu Abbildungen, die experimentelle Daten wiedergeben (nur für Originalartikel):

Abbildung(en) # 1 – 2; S1 – S8; S22 – S23*	X	100% (die in dieser Abbildung wiedergegebenen Daten entstammen vollständig experimentellen Arbeiten, die der Kandidat/die Kandidatin durchgeführt hat)
	☐	0% (die in dieser Abbildung wiedergegebenen Daten basieren ausschließllich auf Arbeiten anderer Koautoren)
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**Kann sich auf mehrere Abb. beziehen, wenn die Antwort dieselbe ist*		

Abbildung(en) # S9 – S21*	☐	100% (die in dieser Abbildung wiedergegebenen Daten entstammen vollständig experimentellen Arbeiten, die der Kandidat/die Kandidatin durchgeführt hat)
	X	0% (die in dieser Abbildung wiedergegebenen Daten basieren ausschließllich auf Arbeiten anderer Koautoren)
	☐	Etwaiger Beitrag des Doktoranden / der Doktorandin zur Abbildung: _____% Kurzbeschreibung des Beitrages: *(z. B. „Abbildungsteile a, d und f" oder „Auswertung der Daten" etc)*
**Kann sich auf mehrere Abb. beziehen, wenn die Antwort dieselbe ist*		

_____ Unterschrift Kandidat/-in

_____ Unterschrift Betreuer/-in (Mitglied der Fakultät)

Appendix S1: Supporting Methods

Experimental design

According to a survey performed before the establishment of the Jena Experiment, the soil is a Eutric Fluvisol developed from up to 2 m-thick loamy fluvial sediments (Roscher *et al.* 2004). Soil pH varied between 7.1 and 8.4, in C_{org} concentration between 5 and 33 g C kg^{-1}, and N_{tot} concentration between 1.0 and 2.7 g N kg^{-1} (Roscher *et al.* 2004; Weisser *et al.* 2017). Mean annual precipitation is 610 mm and mean annual air temperature is 9.9°C (1980 – 2010) (Hoffmann *et al.* 2014). The plant communities in the Jena Experiment represent that of Central European mesophilic grasslands (Roscher *et al.* 2004; Weisser *et al.* 2017).

Secondary metabolome sampling and sample processing

We sampled aboveground biomass of seven common central European grassland species (grasses: *Dactylis glomerata* L., *Holcus lanatus* L., *Phleum pratense* L., forbs: *Geranium pratense* L., *Leucanthemum vulgare* (Vaill.) Lam., *Plantago lanceolata* L., and *Ranunculus acris* L.). All samples were processed, extracted, and analysed according to Ristok et al. (2019) with slight changes. We extracted 20 mg dried ground plant tissue of each sample in 1 mL of extraction buffer (methanol / 50 mM acetate buffer, pH 4.8; 50 / 50 [v/v]). The samples were homogenized for 5 min at 30 Hz using a ball mill (Retsch mixer mill MM 400), and subsequently centrifuged (20000 g, 10 min, 4°C). The supernatant was collected in a 2 mL Eppendorf tube. We repeated the extraction procedure with the remaining pellet and combined the supernatant with the first one. We centrifuged (20000 g, 5 min, 4°C) all extracts, transferred 200 µL to an HPLC vial and added 800 µL extraction buffer, resulting in a 1:5 dilution.

We performed chromatographic separation of all diluted extracts by injecting 2 µL on a Thermo Scientific Dionex UltiMate 3000 (Thermo Scientific Dionex, Sunnyvale, USA)

UPLC unit, equipped with a C18 column (Acclaim RSLC 120 C18, 2.2 µm, 120 Å, 2.1 x 150 mm, Thermo Fisher Scientific). We applied the following binary elution gradient at a flow rate of 0.4 mL min^{-1} and a column temperature of 40°C: 0 – 2 min, 95% A (water and 0.05% formic acid), 5% B (acetonitrile and 0.05% formic acid); 2 – 12 min, 5 to 50% B; 12 – 13 min, 50 to 95% B; 13 – 15 min, 95% B; 15 – 16 min, 95 to 5% B; 16 – 20 min, 5% B. Metabolites were analysed on a liquid chromatography quadrupole time-of-flight mass spectrometer (LC-qToF-MS; Bruker maXis impact HD; Bruker Daltonik, Bremen, Germany) with an electrospray ionization source operated in negative mode. Instrument settings were as follows: capillary voltage, 2500 V; nebulizer, 2.5 bar; dry gas temperature, 220°C; dry gas flow, 11 L min^{-1}; scan range, 50 – 1500 m/z; acquisition rate, 3 Hz. We used sodium formate clusters (10 mM solution of NaOH in 50 / 50% [v/v] isopropanol / water containing 0.2% formic acid) to perform mass calibration.

LC-MS data processing and metabolite prediction

We followed the LC-MS data processing protocol described in Ristok et al. (2019) with minor changes. We converted the LC-qToF-MS raw data to the mzXML format by using the CompassXport utility of the DataAnalysis vendor software. Subsequently, we trimmed each data file by excluding the same non-informative regions at the beginning and end of each run using the msconvert function of ProteoWizard v3.0.10095 (Chambers *et al.* 2012). We performed peak picking, feature alignment, and feature group collapse in R v3.3.3 (R Core Team 2017) using the Bioconductor (Huber *et al.* 2015) packages 'xcms' (Smith *et al.* 2006; Tautenhahn *et al.* 2008; Benton *et al.* 2010) and 'CAMERA' (Kuhl *et al.* 2012). We used the following 'xcms' parameters: peak picking method "centWave" (snthr = 10; ppm = 5; peakwidth = 4, 10); peak grouping method "density" (minfrac = 0.5; bw = 6, 3; mzwid = 0.01); retention time correction method "symmetric". We used 'CAMERA' to annotate

adducts, fragments, and isotope peaks with the following parameters: extended rule set (https://gitlab.com/R_packages/chemhelper/-/tree/master/inst/extdata); perfwhm = 0.6; calcIso = TRUE; calcCaS = TRUE, graphMethod = lpc. Lastly, we collapsed each annotated feature group, hereafter referred to as 'metabolite' which is described by mass-to-charge ratio (m/z) and retention time (rt), using a maximum heuristic approach. In detail, this means that the intensity values of the feature that most often displayed the highest intensity across all samples represents the feature group. The intensity of each metabolite was subsequently normalized to the amount of dried ground plant tissue extracted. We performed pre-processing with 'xcms' and 'CAMERA' (Kuhl *et al.* 2012) separately for each species and sampling campaign. We merged all created feature lists by retention time and mass-to-charge values. For each feature, we allowed for a retention time window of 10 seconds and a mass deviation of 5 ppm.

Phospholipid fatty acid analysis

We analysed all samples on a gas chromatograph (GC-FID; Clarus 680, Perkin Elmer) equipped with a SR-2560 column (0.25 mm x 100 m, 0.2µm, Sigma-Aldrich) and helium as carrier gas. We applied the following temperature gradient: 0-5 min, 100°C column temperature; 5-40 min, 100°C to 240°C; 40-50 min, 240°C. The dry gas flow was set to 1.4 ml min^{-1}.

Partial Least Squares (PLS) path modelling

PLS path modelling allows for the analyses of groups of observable variables, where each group can be summarized by a latent variable. Latent variables are hypothetical variables that can be regarded as a data reduction approach explaining the relationships between two or more observable variables. Observable variables, also called indicators or manifest

variables, contain information that reflect or indicate aspects of the latent variable (Sanchez 2013). PLS path models do not impose any distributional assumptions on the data and do not rely on causal-modelling interpretations. Rather, PLS path models analyse a system of linear relationships between multiple groups of variables with the aim to summarize how a set of dependent variables are explained by their sets of predictors (Sanchez 2013).

The PLS path model is comprised of a structural (inner model) and measurement model (outer model). The relationships of the inner model are treated as linear regressions, where the slope of the regression is the path coefficient among two latent variables. Similarly, the outer model relationships are also considered linear. In other words, latent variables are estimated as a weighted linear combination of their indicators (Sanchez 2013). These weight relations can then be used to simplify the model by removing indicators with a low weight. Lastly, through an iterative process, PLS path modelling estimates the linear combination of a latent variable taking into account the relationships in the inner and outer model (for more information on PLS path modelling, see Sanchez 2013).

Statistical analysis

We tested for the overall and pairwise differences in shoot metabolome composition among the different sown plant species richness levels by calculating permutational multivariate analyses of variance using distance matrices. We log + 1 transformed the metabolite intensity data to achieve multivariate normality, and used Bray-Curtis dissimilarity to calculate the distance matrices. All analyses were permuted 9999 times. Each analysis was species-specific and sampling campaign-specific. We were not able to calculate pairwise comparisons of the metabolome composition between plants grown in monoculture (lowest plant species richness level) and in the highest diversity plot (8 species mixture). This is due to the experimental design and its limitations. For each species, only one monoculture plot

was present. In addition, there was only one 8-species plot. This meant there were not enough replicates to run permutational multivariate analyses of variance and, as such, the pairwise comparisons between monoculture and the 8-species plot were excluded from the analyses.

We calculated two metrics of metabolite diversity: (a) the richness of secondary metabolites, *i.e.,* the number of metabolites within a plant individual; and (b) the Shannon diversity of secondary metabolites, *i.e.,* the abundance-weighed diversity of metabolites expressed as the exponential of the Shannon-Weaver index (Hill 1973) based on plant individual-level metabolite intensities.

Moreover, for each trait considered in the design of the Trait-Based-Experiment, we calculated community-weighted mean (CWM) trait values (Roscher *et al.* 2012). Here, we based the calculations on the relative species-specific cover for each plant community.

To test for the effect of sown plant species richness or CWM trait values on the richness or Shannon diversity of secondary metabolites, we calculated linear mixed effects models. We fitted either the richness or the Shannon diversity of secondary metabolites as response variables. As predictor variables, we fitted sampling campaign (categorical; August 2015 or May 2016), plant functional group identity (categorical; grass or forb), and either sown plant species richness (metric; 1, 2, 4 or 8) or each of the CWM traits separately (metric, scaled), as well as the three-way interaction. We fitted plot nested in block and species identity as independent random effects. Model simplification was achieved by model comparison using Akaike Information Criterion (AIC). All linear mixed effects models were based on restricted-maximum likelihood estimation and Type I analysis of variance with Satterthwaite approximation for degrees of freedom.

In order to ensure the robustness of our linear mixed effects models, we calculated a second set of models with our predictor variables fitted in the following order: either sown plant species richness or each of the CWM traits separately or either CMS_PCA1 or CMS_PCA2, plant functional group identity, and sampling campaign. Since the model outcomes were similar to our first set of models, we decided to present the result of our first set of models in the manuscript.

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Supporting Figures S1 – S23

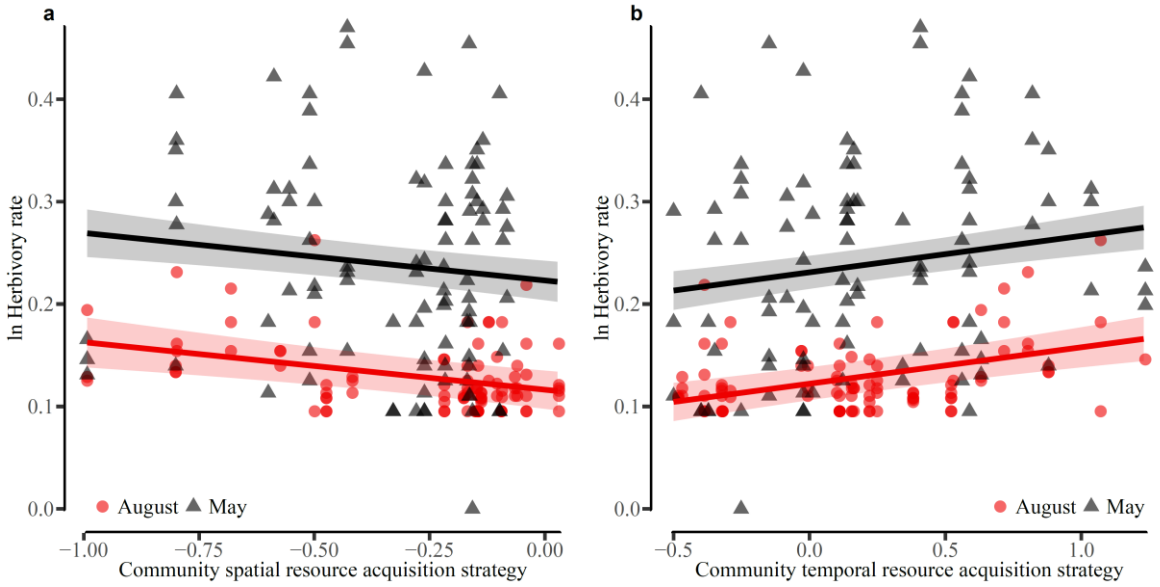

Figure S1 The plant-individual herbivory rate (log-transformed) on grasses only in response to (a) community spatial resource acquisition strategy ($F_{1,179} = 3.93$; $p = 0.049$) and (b) community temporal resource acquisition strategy ($F_{1,133} = 6.32$; $p = 0.013$) of the Trait-Based Experiment. The relationship in August is displayed in circles and red colour. The relationship in May is displayed in triangles and black colour. Regression line estimates are based on linear mixed effect models with plot nested in block, and species identity as independent random terms (**Table S2**). The shaded band displays the standard error.

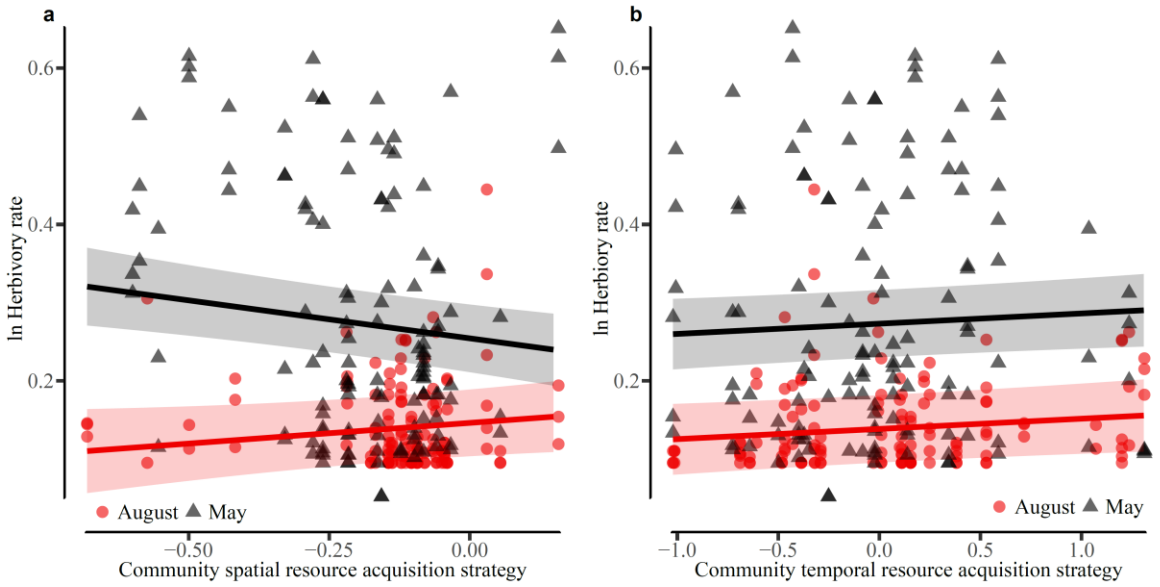

Figure S2 The plant-individual herbivory rate (log-transformed) on forbs only in response to (a) community spatial resource acquisition strategy ($F_{1,246} = 3.55$; $p = 0.061$) and (b) community temporal resource acquisition strategy ($F_{1,251} = 0.98$; $p = 0.324$) of the Trait-Based Experiment. The relationship in August is displayed in circles and red colour. The relationship in May is displayed in triangles and black colour. Regression line estimates are based on linear mixed effect models with plot nested in block, and species identity as independent random terms (**Table S2**). The shaded band displays the standard error.

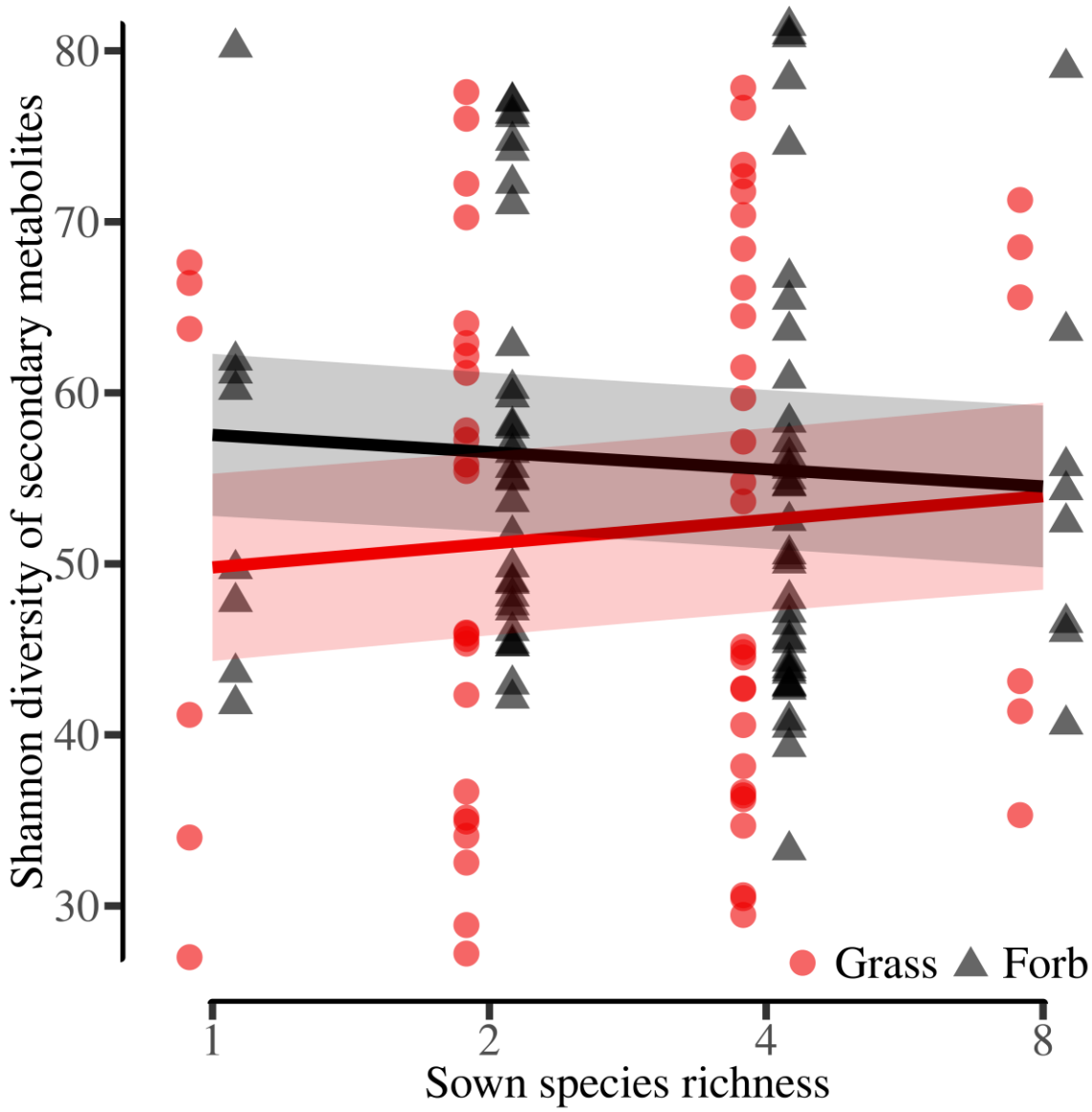

Figure S3 The Shannon diversity of secondary metabolites in response to sown species richness ($F_{1,138}$ = 5.35; p = 0.022) of the Trait-Based Experiment. For clarity, the placement of the symbols corresponding to the functional group identity have been slightly shifted along the x-axis. The relationship in grasses is displayed in circles and red colour. The relationship in forbs is displayed in triangles and black colour. Regression line estimates are based on linear mixed effect models with plot nested in block, and species identity as independent random terms (**Table S4**). The shaded band displays the standard error.

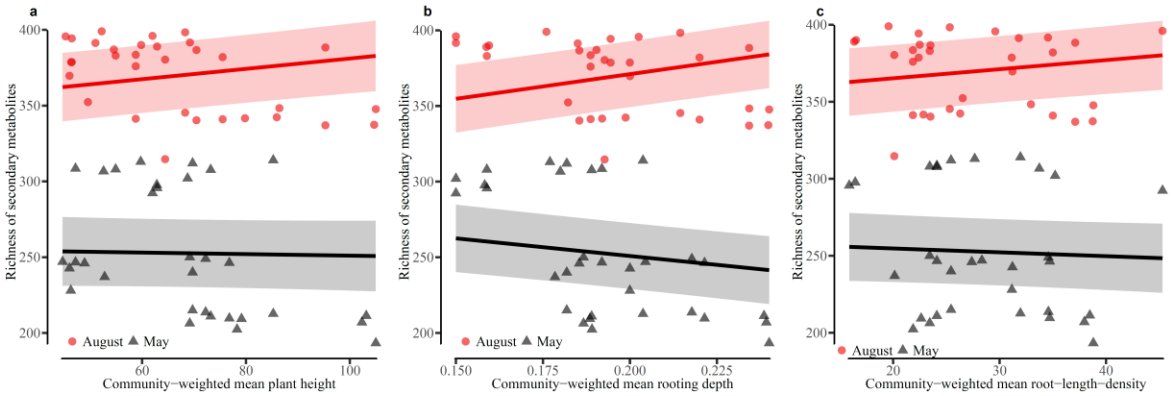

Figure S4 The richness of secondary metabolites in grasses only in response to (a) community-weighted mean plant height, (b) community-weighted mean rooting depth, and (c) community-weighted mean root-length-density of the Trait-Based Experiment. The relationship in August is displayed in circles and red colour. The relationship in May is displayed in triangles and black colour. Regression line estimates are based on linear mixed effect models with plot nested in block, and species identity as independent random terms (**Table S5**). The shaded band displays the standard error.

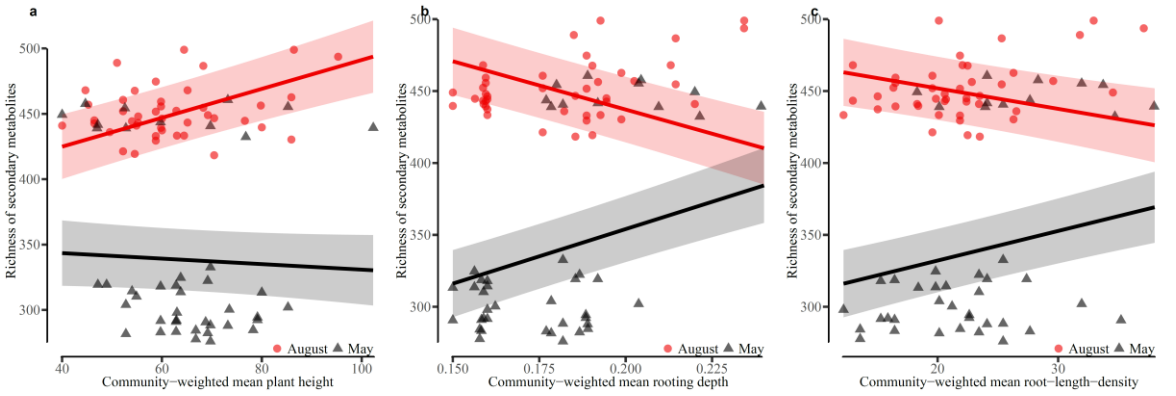

Figure S5 The richness of secondary metabolites in forbs only in response to (a) community-weighted mean plant height, (b) community-weighted mean rooting depth, and (c) community-weighted mean root-length-density of the Trait-Based Experiment. The relationship in August is displayed in circles and red colour. The relationship in May is displayed in triangles and black colour. Regression line estimates are based on linear mixed effect models with plot nested in block, and species identity as independent random terms (**Table S5**). The shaded band displays the standard error.

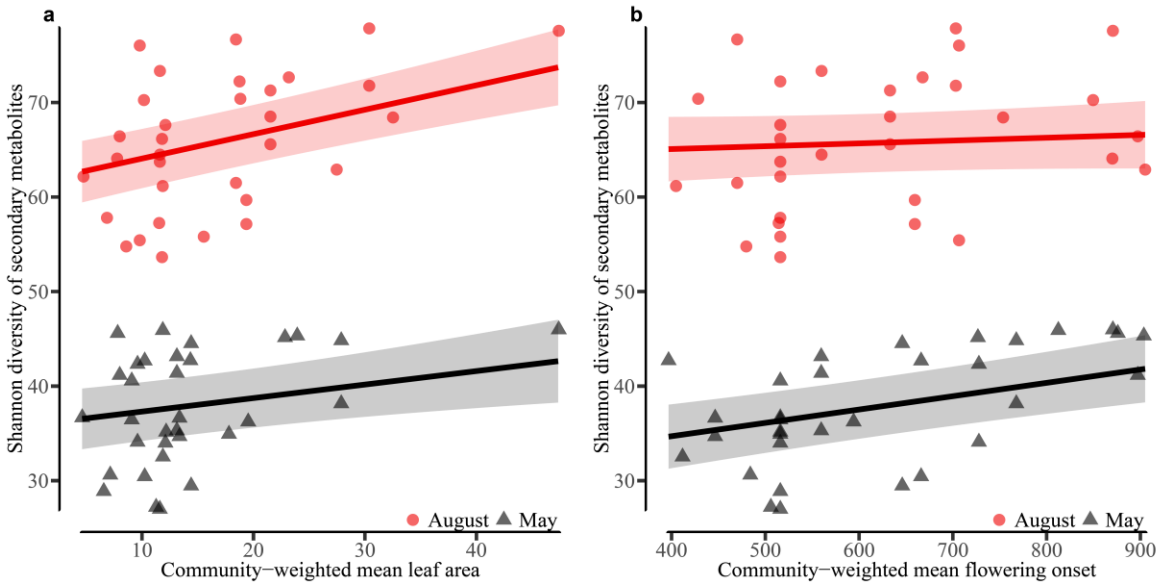

Figure S6 The Shannon diversity of secondary metabolites in grasses only in response to (a) community-weighted mean leaf area and (b) community-weighted mean flowering onset of the Trait-Based Experiment. The relationship in August is displayed in circles and red colour. The relationship in May is displayed in triangles and black colour. Regression line estimates are based on linear mixed effect models with plot nested in block, and species identity as independent random terms (**Table S5**). The shaded band displays the standard error.

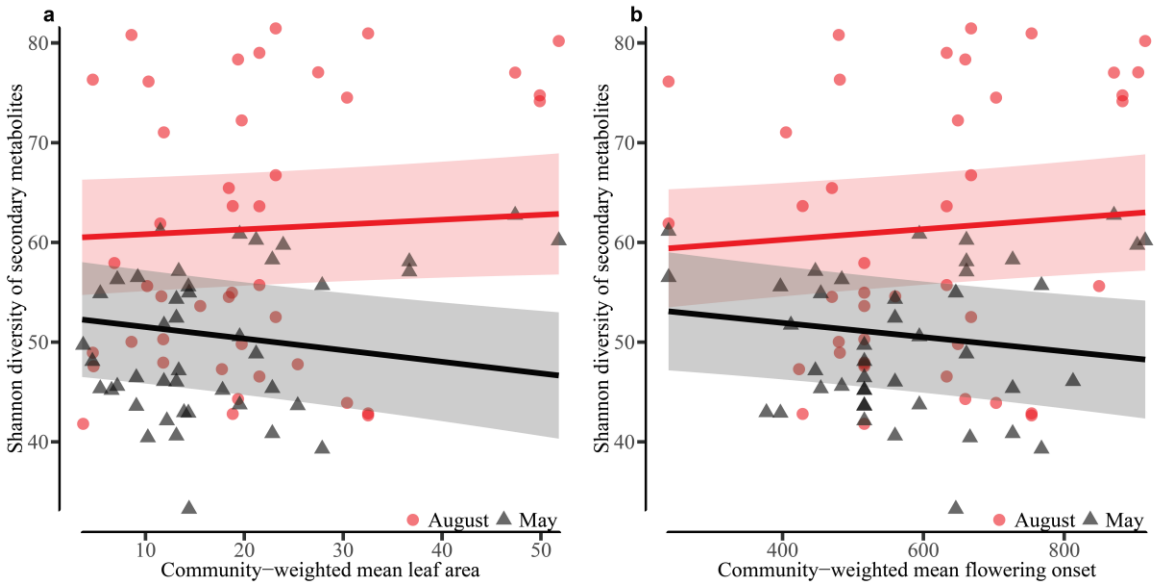

Figure S7 The Shannon diversity of secondary metabolites in forbs only in response to (a) community-weighted mean leaf area and (b) community-weighted mean flowering onset of the Trait-Based Experiment. The relationship in August is displayed in circles and red colour. The relationship in May is displayed in triangles and black colour. Regression line estimates are based on linear mixed effect models with plot nested in block, and species identity as independent random terms (**Table S5**). The shaded band displays the standard error.

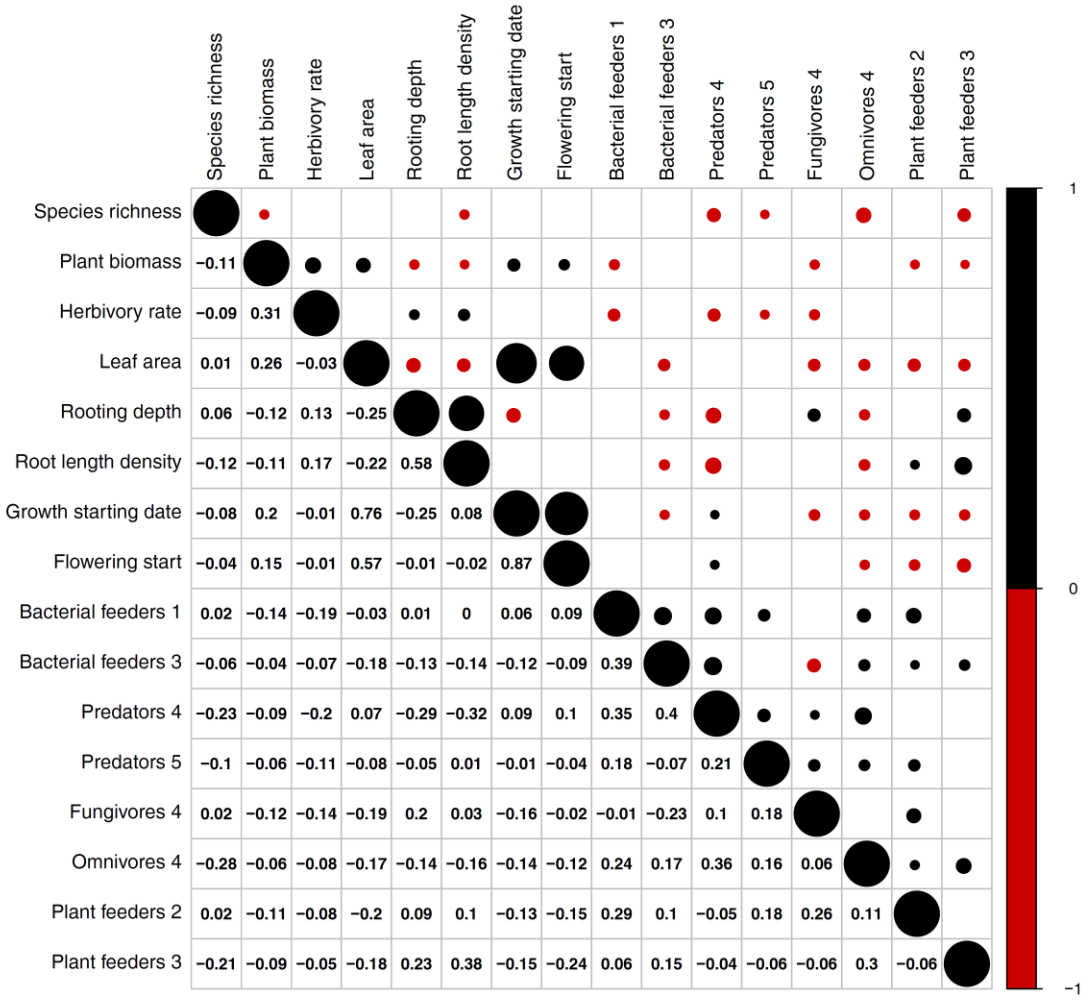

Figure S8 Correlation between plant species richness, plant individual biomass, herbivory rate, selected community-weighted mean traits and functional nematode guilds of the Trait-Based Experiment. The selection is based on the important indicators in the most parsimonious *full-model* PLS-PM. The upper triangle displays positive Pearson correlations in black circles and negative Pearson correlations in red circles. The size of the circle corresponds to the strength of the correlation. Empty cells display non-significant correlations. The lower triangle displays the Pearson correlation coefficients. The number at the end of each functional nematode guild represents their c-p score on the colonization-persistence gradient.

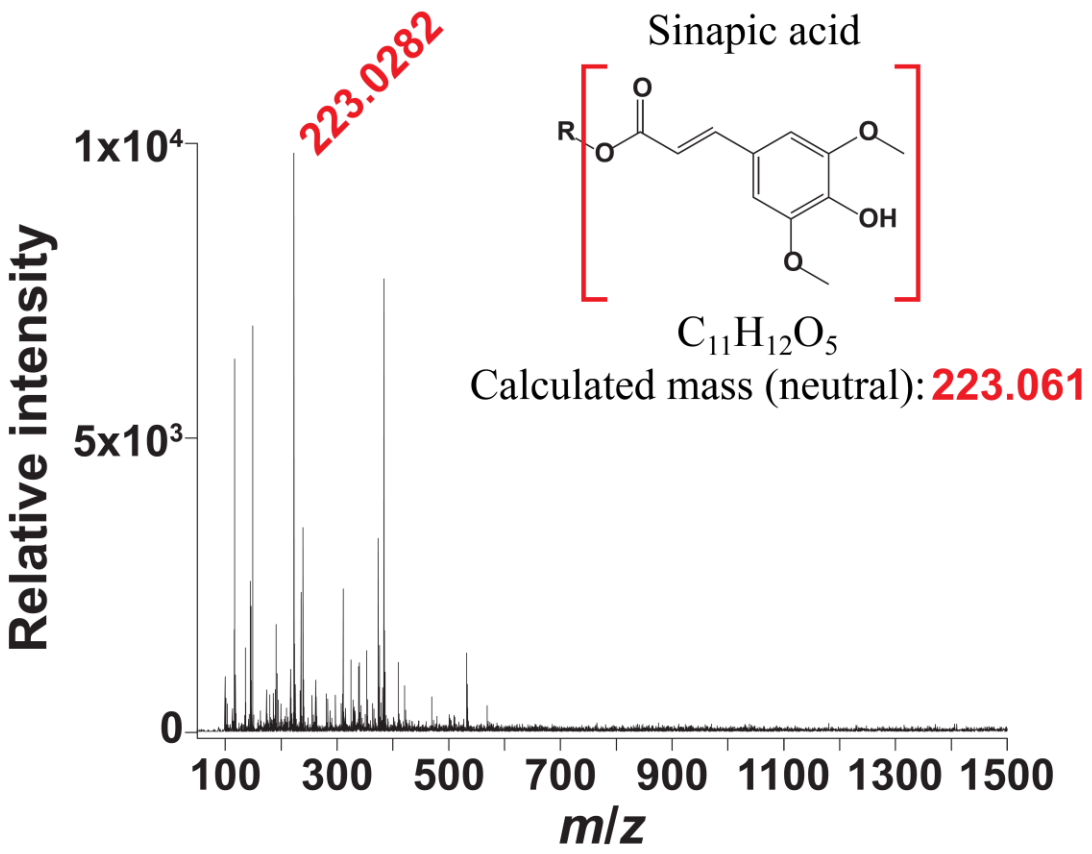

Figure S9 Predicted metabolite structure of sinapic acid extracted from *Dactylis glomerata* through the comparison of LC-MS data with literature references. We submitted high-resolution mass-to-charge values to the MassBank of North America (MoNA, http://mona.fiehnlab.ucdavis.edu/) spectral database. We used a mass tolerance of 0.5 D for comparison. In addition, we calculated high-resolution molecular weights, molecular formulae for putative molecular ions in neutral form, and particle weights for mass spectrometry generated fragments using ChemDraw Ultra 8.0 (www.cambridgesoft.com). For more details see Supplementary Table S7.

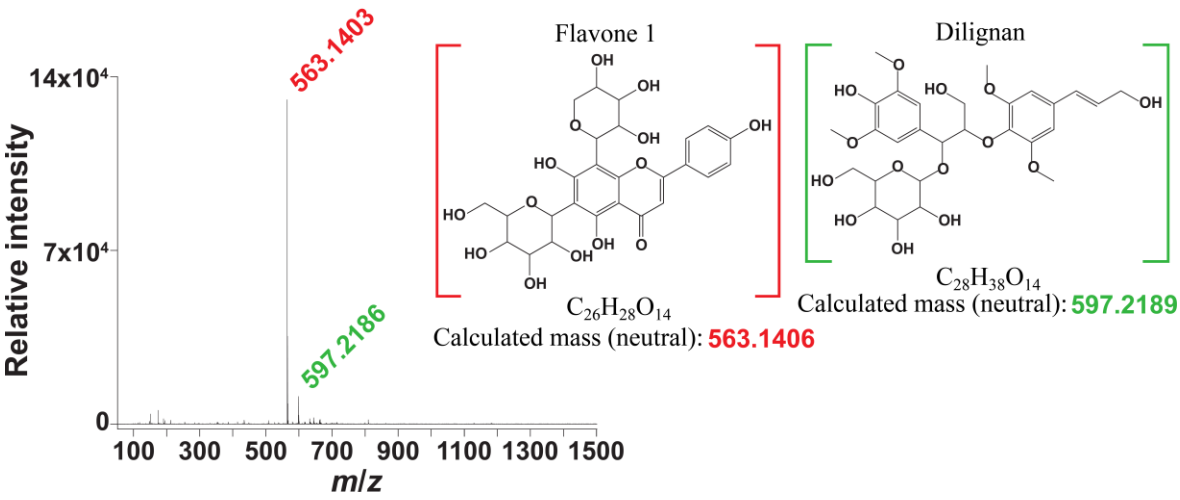

Figure S10 Predicted metabolite structures of flavone 1 and dilignan extracted from *Dactylis glomerata* through the comparison of LC-MS data with literature references.

We submitted high-resolution mass-to-charge values to the MassBank of North America (MoNA, http://mona.fiehnlab.ucdavis.edu/) spectral database. We used a mass tolerance of 0.5 D for comparison. In addition, we calculated high-resolution molecular weights, molecular formulae for putative molecular ions in neutral form, and particle weights for mass spectrometry generated fragments using ChemDraw Ultra 8.0 (www.cambridgesoft.com). For more details see Supplementary Table S7.

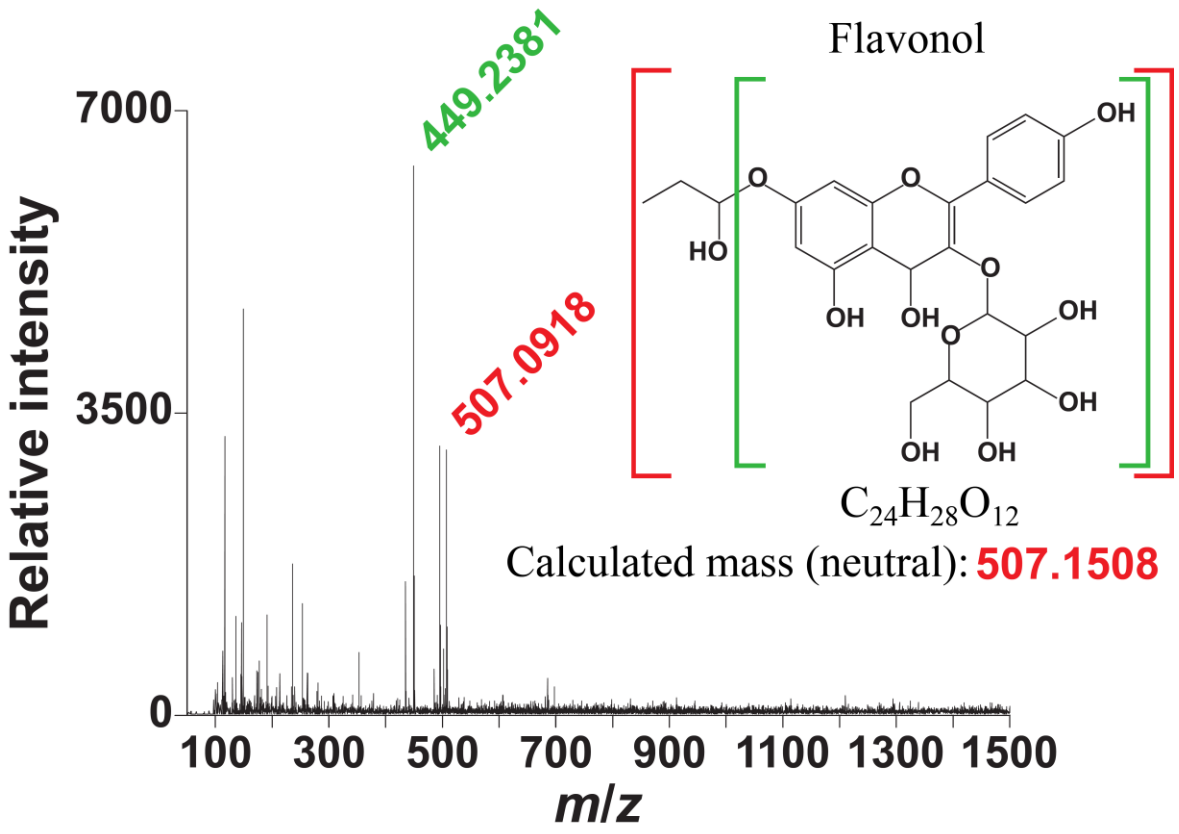

Figure S11 Predicted metabolite structure of flavonol extracted from *Dactylis glomerata* through the comparison of LC-MS data with literature references. We submitted high-resolution mass-to-charge values to the MassBank of North America (MoNA, http://mona.fiehnlab.ucdavis.edu/) spectral database. We used a mass tolerance of 0.5 D for comparison. In addition, we calculated high-resolution molecular weights, molecular formulae for putative molecular ions in neutral form, and particle weights for mass spectrometry generated fragments using ChemDraw Ultra 8.0 (www.cambridgesoft.com). For more details see Supplementary Table S7.

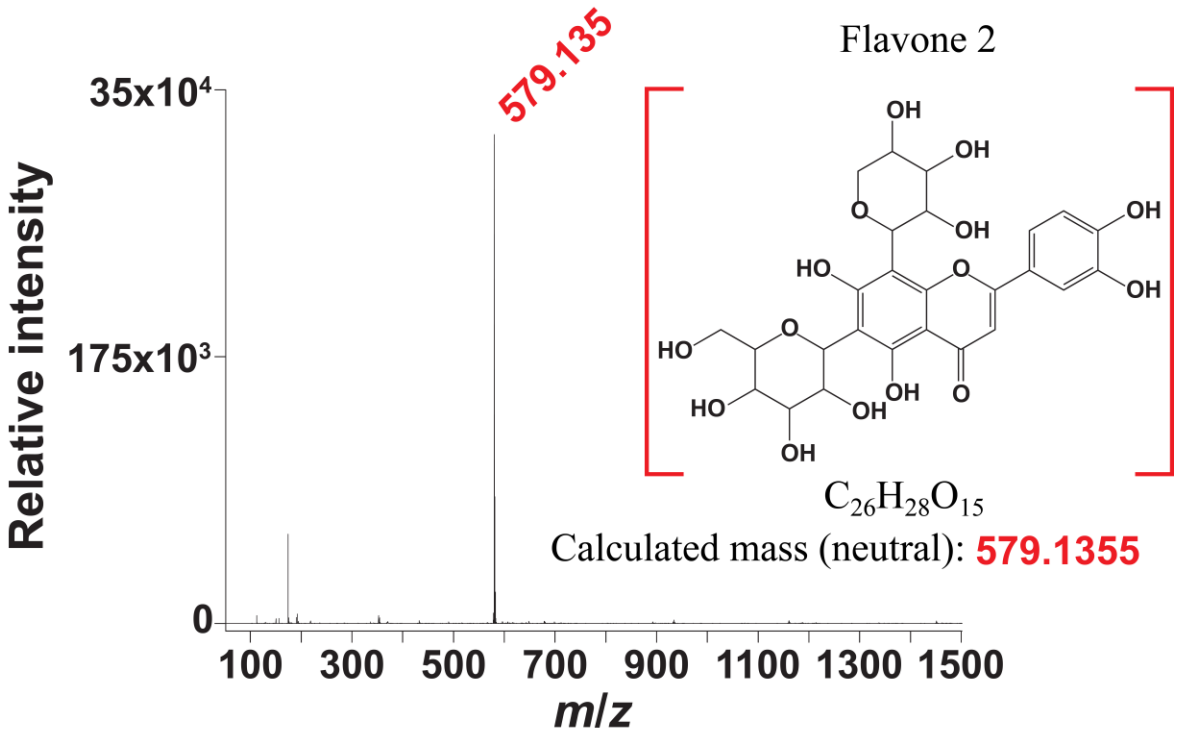

Figure S12 Predicted metabolite structure of flavone 2 extracted from *Dactylis glomerata* through the comparison of LC-MS data with literature references. We submitted high-resolution mass-to-charge values to the MassBank of North America (MoNA, http://mona.fiehnlab.ucdavis.edu/) spectral database. We used a mass tolerance of 0.5 D for comparison. In addition, we calculated high-resolution molecular weights, molecular formulae for putative molecular ions in neutral form, and particle weights for mass spectrometry generated fragments using ChemDraw Ultra 8.0 (www.cambridgesoft.com). For more details see Supplementary Table S7.

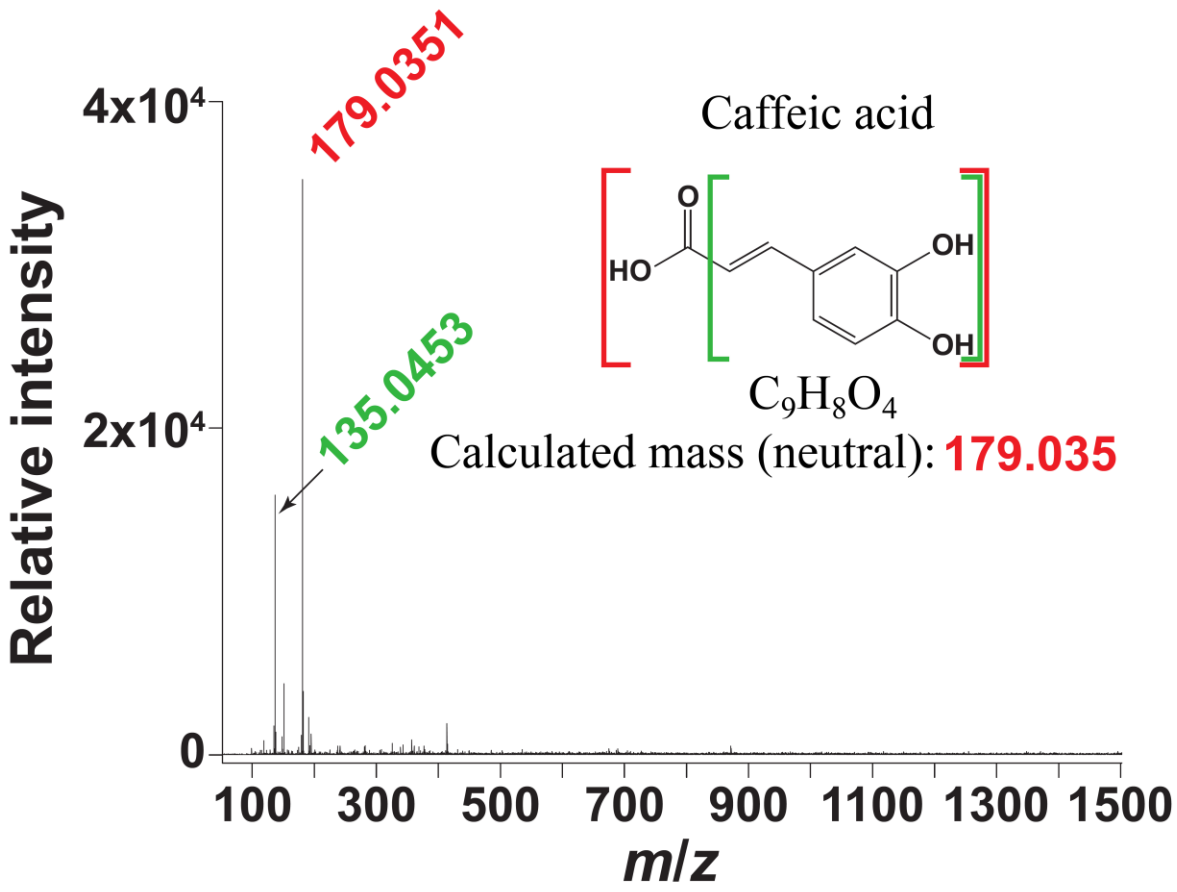

Figure S13 Predicted metabolite structure of caffeic acid extracted from *Dactylis glomerata* through the comparison of LC-MS data with literature references. We submitted high-resolution mass-to-charge values to the MassBank of North America (MoNA, http://mona.fiehnlab.ucdavis.edu/) spectral database. We used a mass tolerance of 0.5 D for comparison. In addition, we calculated high-resolution molecular weights, molecular formulae for putative molecular ions in neutral form, and particle weights for mass spectrometry generated fragments using ChemDraw Ultra 8.0 (www.cambridgesoft.com). For more details see Supplementary Table S7.

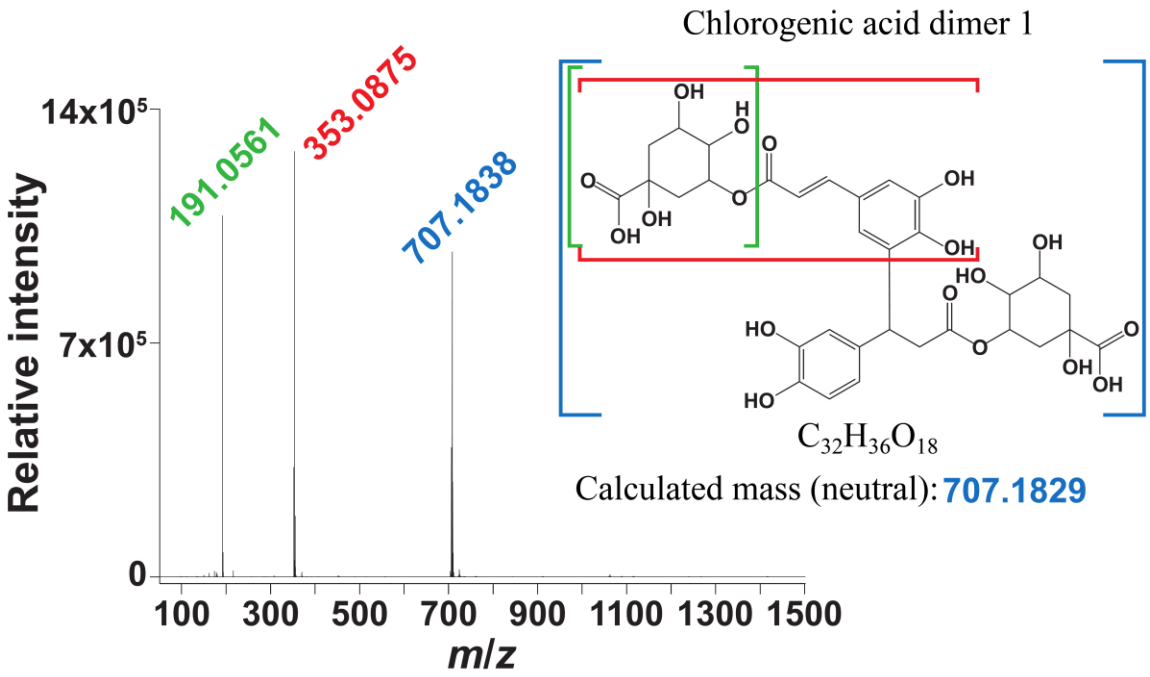

Figure S14 Predicted metabolite structure of chlorogenic acid 1 extracted from *Leucanthemum vulgare* through the comparison of LC-MS data with literature references. We submitted high-resolution mass-to-charge values to the MassBank of North America (MoNA, http://mona.fiehnlab.ucdavis.edu/) spectral database. We used a mass tolerance of 0.5 D for comparison. In addition, we calculated high-resolution molecular weights, molecular formulae for putative molecular ions in neutral form, and particle weights for mass spectrometry generated fragments using ChemDraw Ultra 8.0 (www.cambridgesoft.com). For more details see Supplementary Table S7.

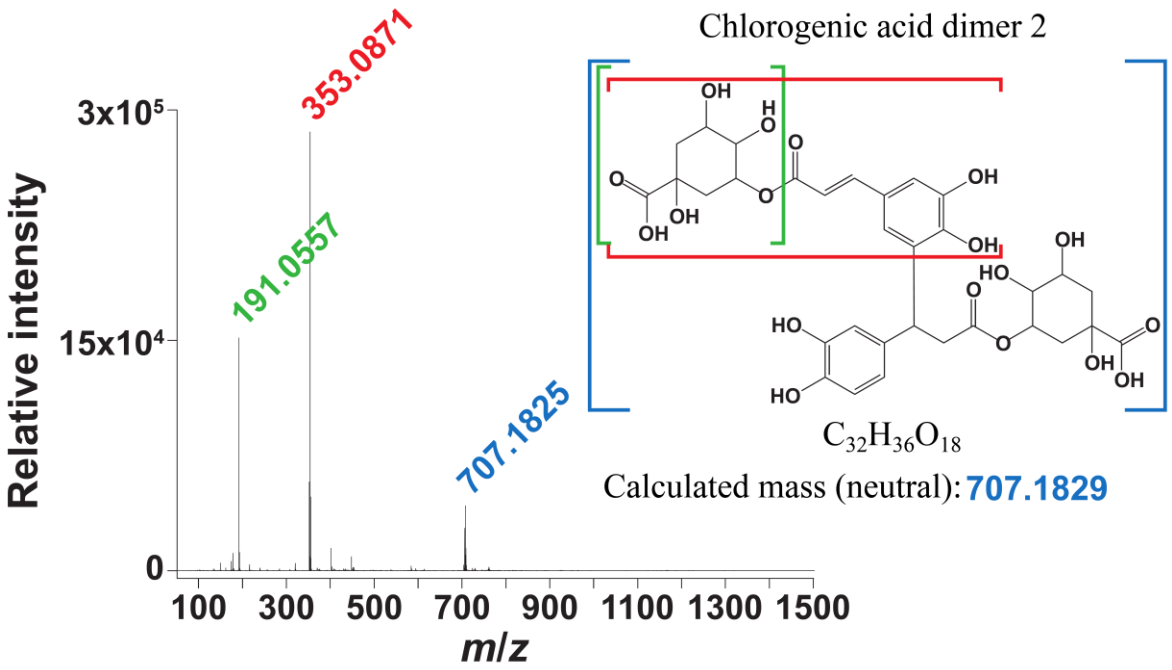

Figure S15 Predicted metabolite structure of chlorogenic acid 2 extracted from *Leucanthemum vulgare* through the comparison of LC-MS data with literature references. We submitted high-resolution mass-to-charge values to the MassBank of North America (MoNA, http://mona.fiehnlab.ucdavis.edu/) spectral database. We used a mass tolerance of 0.5 D for comparison. In addition, we calculated high-resolution molecular weights, molecular formulae for putative molecular ions in neutral form, and particle weights for mass spectrometry generated fragments using ChemDraw Ultra 8.0 (www.cambridgesoft.com). For more details see Supplementary Table S7.

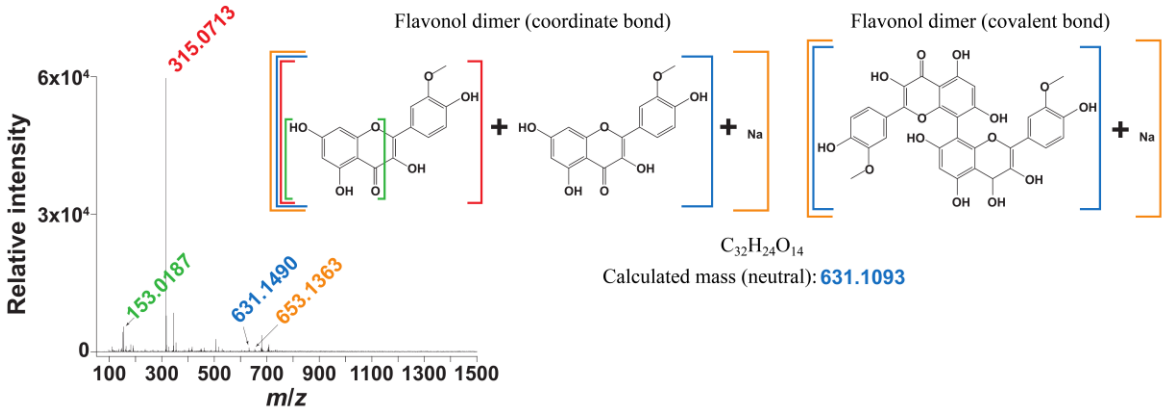

Figure S16 Predicted metabolite structure of flavonol dimer extracted from *Leucanthemum vulgare* through the comparison of LC-MS data with literature references. We submitted high-resolution mass-to-charge values to the MassBank of North America (MoNA, http://mona.fiehnlab.ucdavis.edu/) spectral database. We used a mass tolerance of 0.5 D for comparison. In addition, we calculated high-resolution molecular weights, molecular formulae for putative molecular ions in neutral form, and particle weights for mass spectrometry generated fragments using ChemDraw Ultra 8.0 (www.cambridgesoft.com). For more details see Supplementary Table S7.

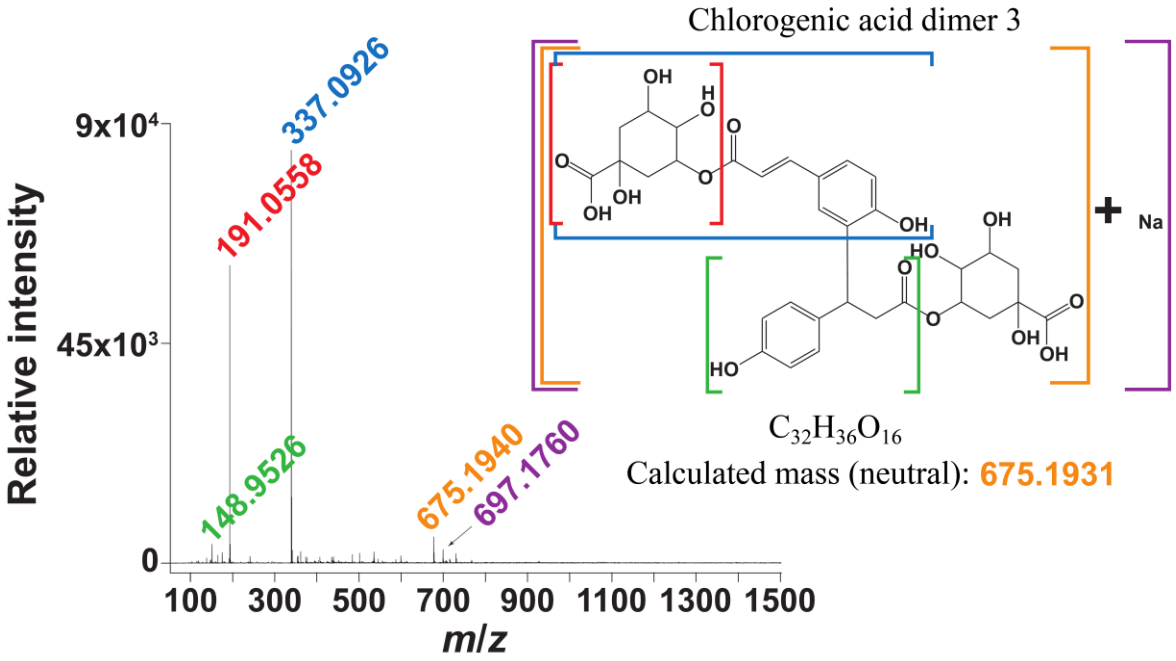

Figure S17 Predicted metabolite structure of chlorogenic acid dimer 3 extracted from *Leucanthemum vulgare* through the comparison of LC-MS data with literature references. We submitted high-resolution mass-to-charge values to the MassBank of North America (MoNA, http://mona.fiehnlab.ucdavis.edu/) spectral database. We used a mass tolerance of 0.5 D for comparison. In addition, we calculated high-resolution molecular weights, molecular formulae for putative molecular ions in neutral form, and particle weights for mass spectrometry generated fragments using ChemDraw Ultra 8.0 (www.cambridgesoft.com). For more details see Supplementary Table S7.

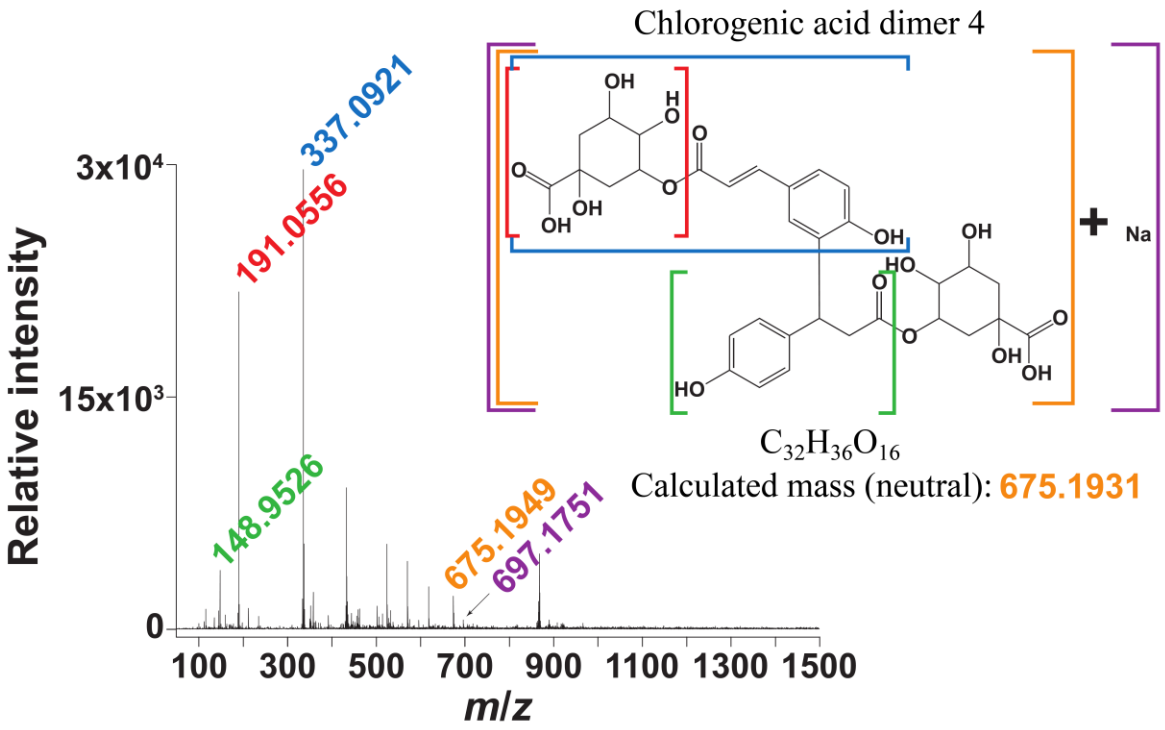

Figure S18 Predicted metabolite structure of chlorogenic acid dimer 4 extracted from *Leucanthemum vulgare* through the comparison of LC-MS data with literature references. We submitted high-resolution mass-to-charge values to the MassBank of North America (MoNA, http://mona.fiehnlab.ucdavis.edu/) spectral database. We used a mass tolerance of 0.5 D for comparison. In addition, we calculated high-resolution molecular weights, molecular formulae for putative molecular ions in neutral form, and particle weights for mass spectrometry generated fragments using ChemDraw Ultra 8.0 (www.cambridgesoft.com). For more details see Supplementary Table S7.

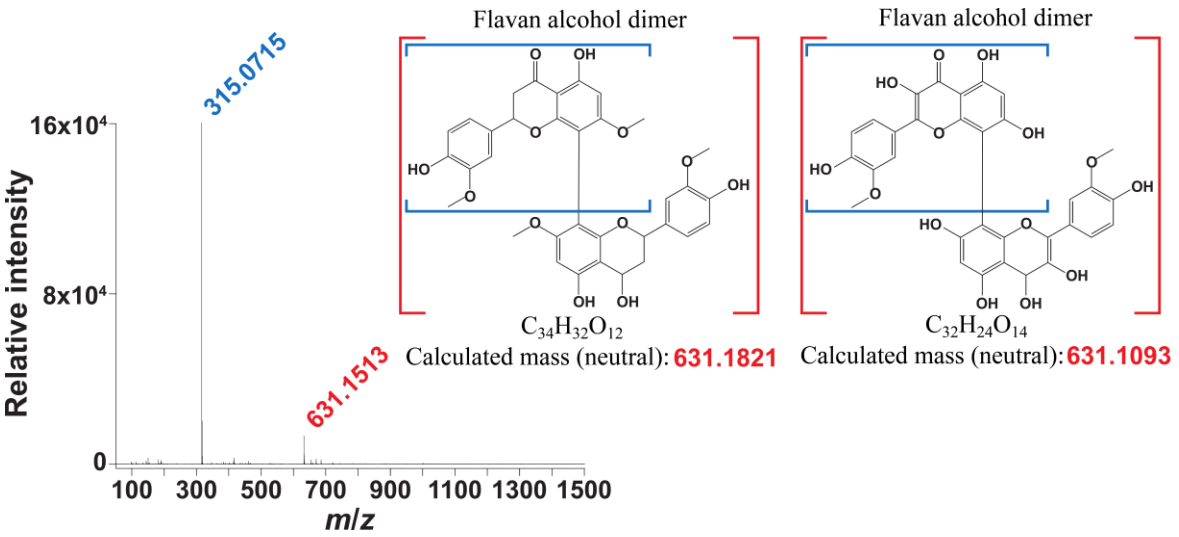

Figure S19 Predicted metabolite structure of flavan alcohol dimer extracted from *Leucanthemum vulgare* through the comparison of LC-MS data with literature references. We submitted high-resolution mass-to-charge values to the MassBank of North America (MoNA, http://mona.fiehnlab.ucdavis.edu/) spectral database. We used a mass tolerance of 0.5 D for comparison. In addition, we calculated high-resolution molecular weights, molecular formulae for putative molecular ions in neutral form, and particle weights for mass spectrometry generated fragments using ChemDraw Ultra 8.0 (www.cambridgesoft.com). For more details see Supplementary Table S7.

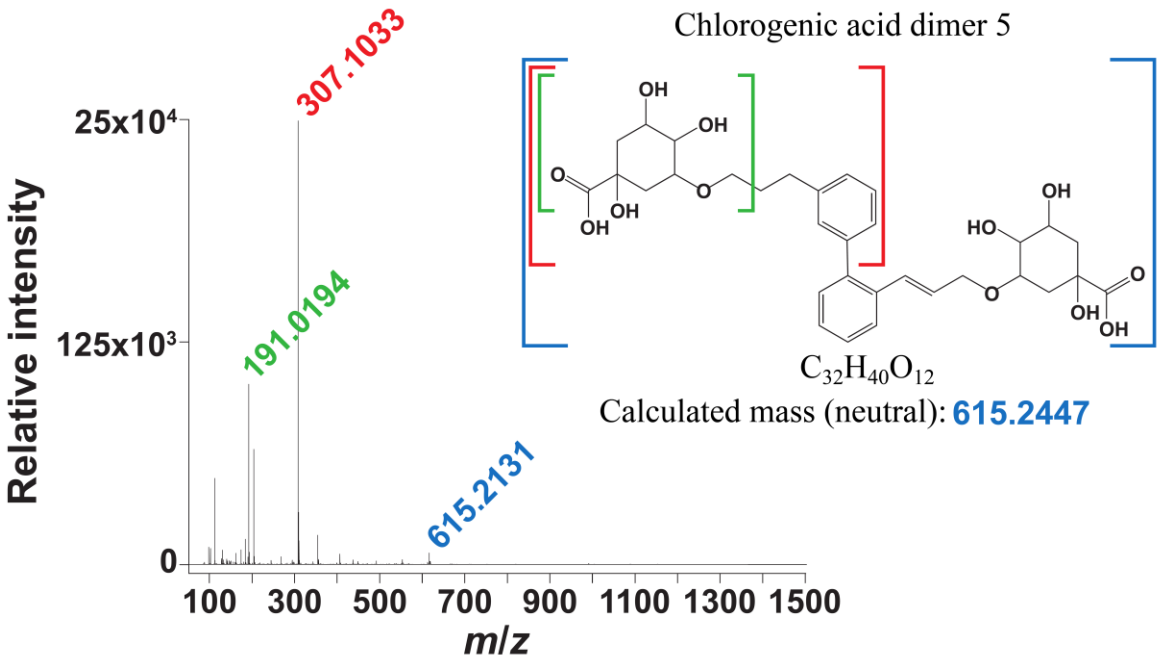

Figure S20 Predicted metabolite structure of chlorogenic acid dimer 5 extracted from *Leucanthemum vulgare* through the comparison of LC-MS data with literature references. We submitted high-resolution mass-to-charge values to the MassBank of North America (MoNA, http://mona.fiehnlab.ucdavis.edu/) spectral database. We used a mass tolerance of 0.5 D for comparison. In addition, we calculated high-resolution molecular weights, molecular formulae for putative molecular ions in neutral form, and particle weights for mass spectrometry generated fragments using ChemDraw Ultra 8.0 (www.cambridgesoft.com). For more details see Supplementary Table S7.

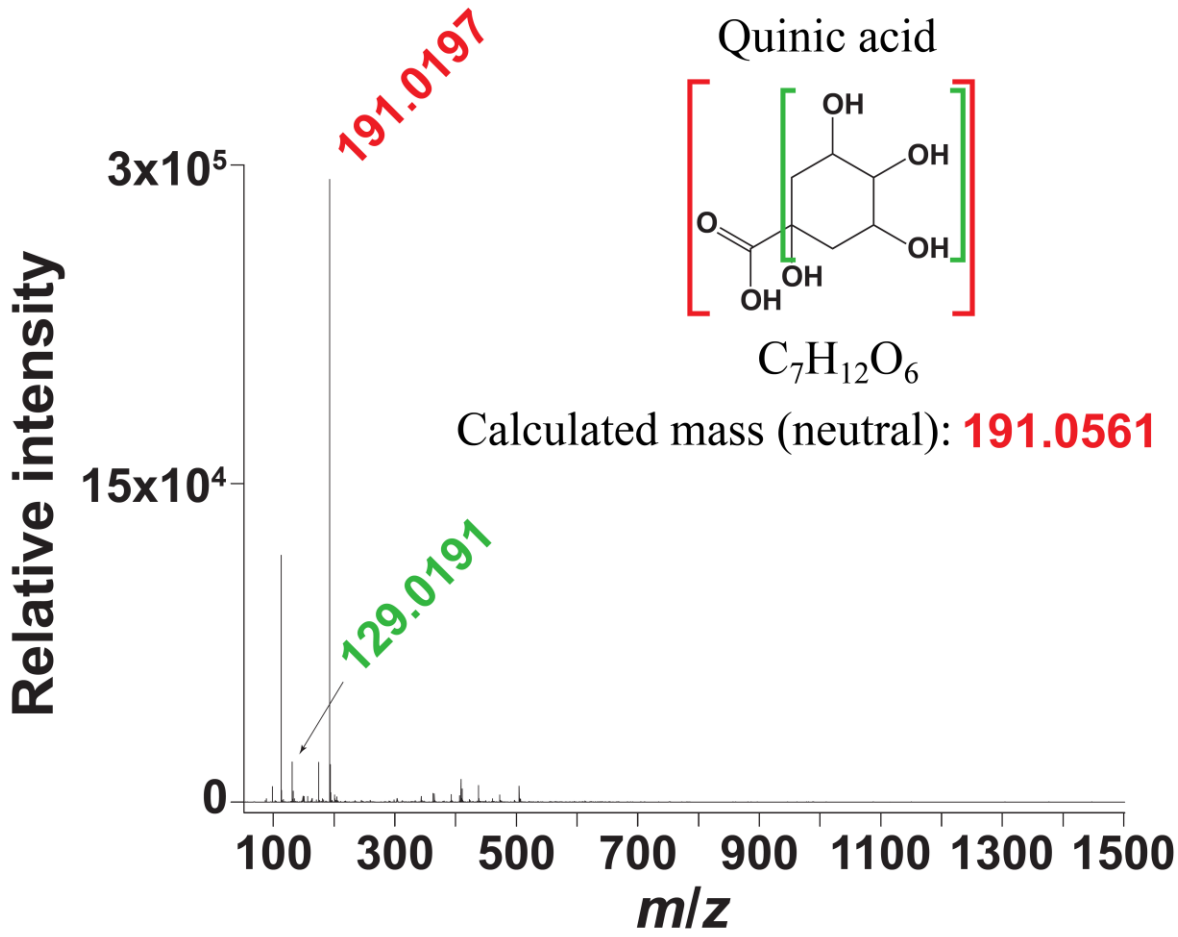

Figure S21 Predicted metabolite structure of quinic acid extracted from *Plantago lanceolata* through the comparison of LC-MS data with literature references. We submitted high-resolution mass-to-charge values to the MassBank of North America (MoNA, http://mona.fiehnlab.ucdavis.edu/) spectral database. We used a mass tolerance of 0.5 D for comparison. In addition, we calculated high-resolution molecular weights, molecular formulae for putative molecular ions in neutral form, and particle weights for mass spectrometry generated fragments using ChemDraw Ultra 8.0 (www.cambridgesoft.com). For more details see Supplementary Table S7.

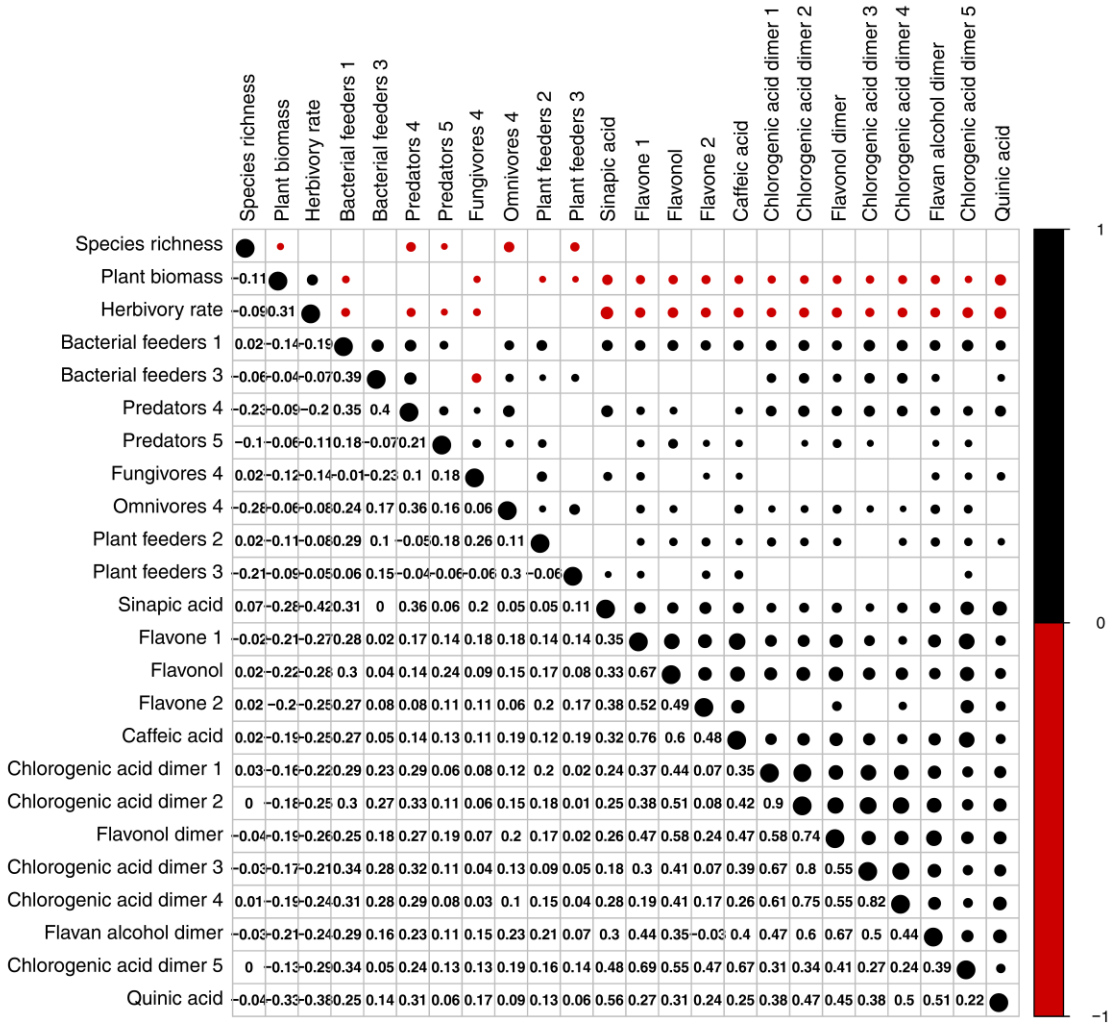

Figure S22 Correlation between plant species richness, plant individual biomass, herbivory rate, selected functional nematode guilds and assigned plant compounds of the Trait-Based Experiment. The selection is based on the important indicators in the most parsimonious *full-model* PLS-PM. The upper triangle displays positive Pearson correlations in black circles and negative Pearson correlations in red circles. The size of the circle corresponds to the strength of the correlation. Empty cells display non-significant correlations. The lower triangle displays the Pearson correlation coefficients. The number at the end of each functional nematode guild represents their c-p score on the colonization-persistence gradient.

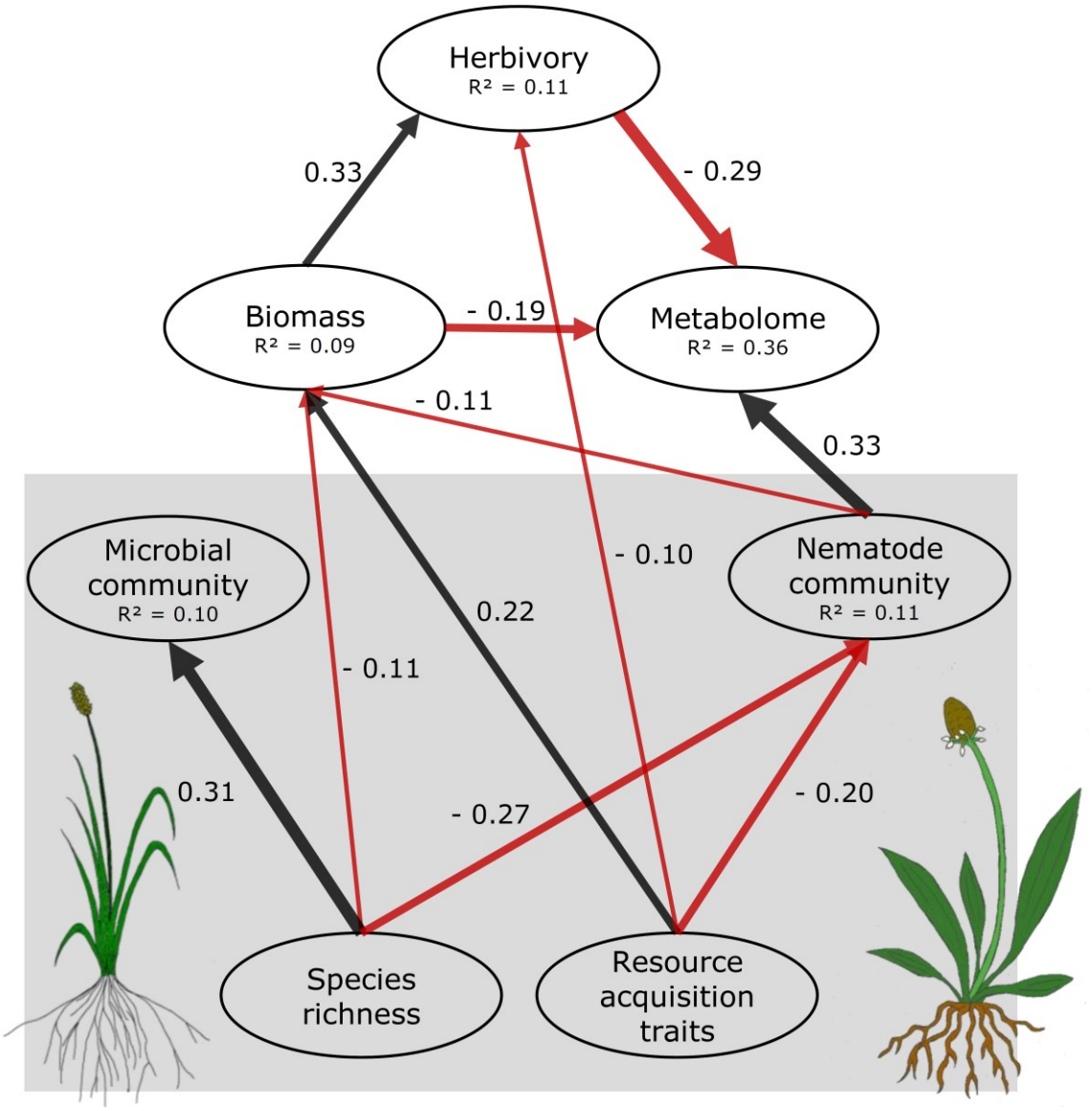

Figure S23 Alternative partial-least-squares path model with a link from herbivory to metabolome including data across both sampling campaigns and all plant species. Species richness represents the plot-level sown plant species richness. Resource acquisition traits represent the community-weighted mean traits leaf area, growth starting date, and flowering start. Microbial community represents PLFA-based estimates on plot-level gram-negative, gram-positive, and undefined bacteria, as well as arbuscular mycorrhizal fungi and all other fungi abundance. Nematode community represents plot-level summed relative abundance of functional nematode guilds, i.e., bacterial-feeding, carnivorous, fungal-feeding, omnivorous, and plant-feeding. Biomass represents plant-individual aboveground

dry biomass. Metabolome represents plant-individual secondary metabolite composition. Herbivory represents plant-individual herbivory rate expressed as the proportion of damaged leaves to the total number of leaves. All data is scaled. Variables taken at plot level are highlighted by a grey-shaded background. Variables taken at the plant-individual level are highlighted by a white-shaded background. Black arrows display significantly positive relationships. Red arrows display significantly negative relationships. Number on arrows are path coefficients.

Supporting Tables S1 – S7

Table S1 Statistical parameters resulting from a Type 1 ANOVA on the individual plant herbivory rates as a function of sampling campaign, plant functional group identity, and either sown plant species richness or each Principle Component Axis, based on the community-weighted mean traits, separately, as well as the three-way interactions. We fitted plot nested in block and species identity as independent random effects. Represented are the most parsimonious models based on Akaike Information Criterion (AIC) comparisons. Variables removed in the most parsimonious model are represented by a dash. Significant differences ($p < 0.05$) are given in bold. PCA 1 represents the spatial resource acquisition strategy based on the plant traits plant height, leaf area, rooting depth, and root length density. PCA 2 represents the temporal resource acquisition strategy based on the plant traits growth starting date and flowering onset. Abbreviations: PFG = plant functional group; CWM = community-weighted mean; NumDF = numerator degrees of freedom; DenDF = denominator degrees of freedom; SS = sum of squares; F = F-value; p = p-value.

Parameter	Individual plant herbivory rate				
	NumDF	SS	denDF	F	p
a) Model *sown species richness*					
sampling campaign	1	1.70	432.01	229.39	**< 0.001**
plant functional group	1	-	-	-	-
sown species richness	1	0.04	425.61	5.24	**0.023**
campaign x PFC	1	-	-	-	-
campaign x richness	1	-	-	-	-
PFC x richness	1	-	-	-	-
campaign x PFC x richness	1	-	-	-	-
Explained Variation		$R^2_{marg} = 0.257$ / $R^2_{cond} = 0.517$			

b) Model Community s*patial resource acquisition strategy*

sampling campaign	1	1.70	413.83	229.39	**< 0.001**
plant functional group	1	-	-	-	-
CMS PCA 1	1	0.02	103.30	2.35	0.128
campaign x PFC	1	-	-	-	-
campaign x PCA 1	1	-	-	-	-
PFC x PCA 1	1	-	-	-	-
campaign x PFC x PCA 1	1	-	-	-	-
Explained Variation		R^2_{marg} = 0.254 / R^2_{cond} = 0.516			

c) Model *Community temporal resource acquisition strategy*

sampling campaign	1	1.70	413.98	228.99	**< 0.001**
plant functional group	1	-	-	-	-
CMS PCA 2	1	0.03	107.55	3.50	0.064
campaign x PFC	1	-	-	-	-
campaign x PCA 2	1	-	-	-	-
PFC x PCA 2	1	-	-	-	-
campaign x PFC x PCA 2	1	-	-	-	-
Explained Variation		R^2_{marg} = 0.255 / R^2_{cond} = 0.520			

Table S2 Statistical parameters resulting from a Type 1 ANOVA on the individual plant herbivory rates within grasses only or forbs only as a function of sampling campaign, and either sown plant species richness or each Principle Component Axis, based on the community-weighted mean traits, separately, as well as the three-way interactions. We fitted plot nested in block and species identity as independent random effects. Represented are the most parsimonious models based on Akaike Information Criterion (AIC) comparisons. Variables removed in the most parsimonious model are represented by a dash. Significant differences ($p < 0.05$) are given in bold. PCA 1 represents the spatial resource acquisition strategy based on the plant traits plant height, leaf area, rooting depth, and root length density. PCA 2 represents the temporal resource acquisition strategy based on the plant traits growth starting date and flowering onset. Abbreviations: CMS = community mean scores; NumDF = numerator degrees of freedom; DenDF = denominator degrees of freedom; SS = sum of squares; F = F-value; p = p-value.

Parameter		Individual plant herbivory rate within grasses				Individual plant herbivory rate within forbs			
	NumDF	SS	denDF	F	p	SS	denDF	F	p
a) Model *sown species richness*									
sampling campaign	1	0.57	181.04	110.09	**< 0.001**	1.14	247.00	126.02	**< 0.001**
sown species richness	1	0.02	178.93	3.20	0.075	0.03	217.07	3.32	0.070
campaign x richness	1	-	-	-	-	-	-	-	-
Explained Variation		$R^2_{marg} = 0.348$ / $R^2_{cond} = 0.427$				$R^2_{marg} = 0.223$ / $R^2_{cond} = 0.561$			
b) Model *Community spatial resource acquisition strategy*									
sampling campaign	1	0.57	181.08	110.63	**< 0.001**	1.14	226.84	126.98	**< 0.001**

CMS PCA 1	1	0.02	179.70	3.93	**0.049**	0.005	62.073	0.523	0.472
campaign x PCA 1	1	-	-	-	-	0.032	245.781	3.548	0.061
Explained Variation		R^2_{marg} = 0.350 / R^2_{cond} = 0.433				R^2_{marg} = 0.228 / R^2_{cond} = 0.559			

c) Model *Community temporal resource acquisition strategy*

sampling campaign	1	0.57	181.13	111.43	**< 0.001**	1.14	247.06	125.25	**< 0.001**
CMS PCA 2	1	0.03	133.91	6.32	**0.013**	0.009	251.440	0.977	0.324
campaign x PCA 2	1	-	-	-	-	-	-	-	-
Explained Variation		R^2_{marg} = 0.365 / R^2_{cond} = 0.431				R^2_{marg} = 0.219 / R^2_{cond} = 0.561			

Table S3 Differences in the species-specific and sampling campaign-specific shoot metabolome composition of plants grown in plant communities of four different species richness levels. Statistical parameters resulting from permutational multivariate analyses of variance using distance matrices. We used Bray-Curtis dissimilarity matrices and 9999 permutations. P-values are adjusted for multiple testing following the Benjamini-Hochberg procedure. Significant differences ($p < 0.05$) are given in bold. Abbreviations: F = pseudo-F-value; p = p-value.

Sown plant species richness	*Dactylis glomerata*					
	August 2015			May 2016		
	R^2	F	p	R^2	F	p
Global	0.103	1.114	0.151	0.125	1.379	**0.012**
1 versus 2	-	-	-	0.119	1.764	**0.030**
1 versus 4	-	-	-	0.111	2.000	**0.006**
2 versus 4	-	-	-	0.048	1.253	0.185
2 versus 8	-	-	-	0.065	0.903	0.786
4 versus 8	-	-	-	0.047	0.794	0.810

Sown plant species richness	*Geranium pratense*					
	August 2015			May 2016		
	R^2	F	p	R^2	F	p
Global	0.172	1.727	**< 0.001**	0.079	0.740	0.456
1 versus 2	0.099	1.321	0.120	-	-	-
1 versus 4	0.164	2.542	**0.009**	-	-	-
2 versus 4	0.086	1.973	**0.006**	-	-	-
2 versus 8	0.118	1.606	0.072	-	-	-
4 versus 8	0.072	1.006	0.448	-	-	-

Sown plant species richness	*Holcus lanatus*					
	August 2015			May 2016		
	R^2	F	p	R^2	F	p
Global	0.111	1.080	0.259	0.102	0.986	0.523
1 versus 2	-	-	-	-	-	-
1 versus 4	-	-	-	-	-	-

Sown plant species richness	*Leucanthemum vulgare*					
	August 2015			May 2016		
	R^2	F	p	R^2	F	p
Global	0.115	1.260	**0.024**	0.091	0.968	0.576
1 versus 2	0.117	1.723	0.066	-	-	-
1 versus 4	0.068	1.164	0.270	-	-	-

2 versus 4	-	-	-	-	-	-
2 versus 8	-	-	-	-	-	-
4 versus 8	-	-	-	-	-	-

Sown plant species richness	*Phleum pratense*					
	August 2015			May 2016		
	R^2	F	p	R^2	F	p
Global	0.120	1.182	0.098	0.141	1.532	**0.002**
1 versus 2	-	-	-	0.077	1.085	0.286
1 versus 4	-	-	-	0.099	1.656	0.081
2 versus 4	-	-	-	0.053	1.338	0.118
2 versus 8	-	-	-	0.141	2.142	**0.006**
4 versus 8	-	-	-	0.076	1.229	0.209

2 versus 4	0.044	1.140	0.270	-	-	-
2 versus 8	0.093	1.330	0.200	-	-	-
4 versus 8	0.065	1.112	0.278	-	-	-

Sown plant species richness	*Plantago lanceolata*					
	August 2015			May 2016		
	R^2	F	p	R^2	F	p
Global	0.108	1.294	**0.021**	0.093	1.093	0.192
1 versus 2	0.119	1.749	0.054	-	-	-
1 versus 4	0.082	1.696	0.063	-	-	-
2 versus 4	0.041	1.195	0.236	-	-	-
2 versus 8	0.076	1.063	0.353	-	-	-
4 versus 8	0.042	0.836	0.794	-	-	-

Sown plant species richness	*Ranunculus acris*					
	August 2015			May 2016		
	R^2	F	p	R^2	F	p
Global	0.164	1.568	**0.001**	0.129	1.287	0.051
1 versus 2	0.118	1.600	**0.039**	-	-	-
1 versus 4	0.112	1.644	0.065	-	-	-
2 versus 4	0.062	1.391	0.065	-	-	-
2 versus 8	0.154	2.007	**0.039**	-	-	-
4 versus 8	0.100	1.331	0.137	-	-	-

Table S4 Statistical parameters resulting from a Type 1 ANOVA on richness and Shannon diversity of secondary metabolites variables as a function of sampling campaign, plant functional group identity, and either sown plant species richness or each of the community-weighted mean traits separately, as well as the three-way interactions. We fitted plot nested in block and species identity as independent random effects. Represented are the most parsimonious models based on Akaike Information Criterion (AIC) comparisons. Variables removed in the most parsimonious model are represented by a dash. Significant differences ($p < 0.05$) are given in bold. Abbreviations: PFG = plant functional group; CWM = community-weighted mean; NumDF = numerator degrees of freedom; DenDF = denominator degrees of freedom; SS = sum of squares; F = F-value; p = p-value.

Parameter		Richness of secondary Metabolites				Shannon diversity of secondary Metabolites			
	NumDF	SS	denDF	F	p	SS	denDF	F	p
a) Model *sown species richness*									
sampling campaign	1	483387	142.01	811.40	**< 0.001**	12205	136.80	512.92	**< 0.001**
plant functional group	1	3986	5.01	6.69	**0.049**	8	5.00	0.33	0.590
sown species richness	1	-	-	-	-	0	90.51	0.00	0.972
campaign x PFG	1	-	-	-	-	2741	136.80	115.18	**< 0.001**
campaign x richness	1	-	-	-	-	-	-	-	-
PFG x richness	1	-	-	-	-	127	138.24	5.35	**0.022**
campaign x PFG x richness	1	-	-	-	-	-	-	-	-
Explained variation		$R^2_{marg} = 0.680$ / $R^2_{cond} = 0.918$				$R^2_{marg} = 0.492$ / $R^2_{cond} = 0.889$			

b) Model *CWM maximum plant height*

sampling campaign	1	483387	140.00	873.67	**< 0.001**	12205	138.66	502.52	**< 0.001**
plant functional group	1	3398	4.99	6.14	0.056	8	5.00	0.33	0.591
CWM max height	1	1938	141.50	3.50	0.063	-	-	-	-
campaign x PFG	1	-	-	-	-	2741	138.66	112.84	**< 0.001**
campaign x max height	1	4904	140.01	8.86	**0.003**	-	-	-	-
PFG x max height	1	-	-	-	-	-	-	-	-
campaign x PFG x max height	1	-	-	-	-	-	-	-	-
Explained variation		R^2_{marg} = 0.672 / R^2_{cond} = 0.925				R^2_{marg} = 0.487 / R^2_{cond} = 0.887			

c) Model *CWM leaf area*

sampling campaign	1	483387	142.01	811.40	**< 0.001**	12205	135.81	539.49	**< 0.001**
plant functional group	1	3986	5.01	6.69	**0.049**	8	4.97	0.33	0.591
CWM leaf area	1	-	-	-	-	21	138.78	0.93	0.337
campaign x PFG	1	-	-	-	-	2753	135.82	121.69	**< 0.001**
campaign x leaf area	1	-	-	-	-	83	136.02	3.66	0.058
PFG x leaf area	1	-	-	-	-	151	137.33	6.67	**0.011**
campaign x PFG x leaf area	1	-	-	-	-	-	-	-	-
Explained variation		R^2_{marg} = 0.680 / R^2_{cond} = 0.918				R^2_{marg} = 0.496 / R^2_{cond} = 0.895			

d) Model *CWM rooting depth*

sampling campaign	1	483387	136.97	1085.26	**< 0.001**	12205	138.66	502.52	**< 0.001**
plant functional group	1	2962	4.94	6.65	**0.050**	8	5.00	0.33	0.591
CWM rooting depth	1	13	138.72	0.03	0.864	-	-	-	-

campaign x PFG	1	350	136.97	0.79	0.377	2741	138.66	112.84	**< 0.001**
campaign x rooting depth	1	4056	136.98	9.11	**0.003**	-	-	-	-
PFG x rooting depth	1	40	138.73	0.09	0.765	-	-	-	-
campaign x PFG x rooting depth	1	19067	136.98	42.81	**< 0.001**	-	-	-	-
Explained variation		R^2_{marg} = 0.699 / R^2_{cond} = 0.939				R^2_{marg} = 0.487 / R^2_{cond} = 0.887			

e) Model *CWM root length density*

sampling campaign	1	483387	136.99	888.50	**< 0.001**	12205	138.66	502.52	**< 0.001**
plant functional group	1	3789	4.98	6.97	**0.046**	8	5.00	0.33	0.591
CWM root length density	1	576	137.66	1.06	0.305	-	-	-	-
campaign x PFG	1	351	136.99	0.65	0.423	2741	138.66	112.84	**< 0.001**
campaign x root length density	1	2299	137.01	4.23	**0.042**	-	-	-	-
PFG x root length density	1	49	137.69	0.09	0.764	-	-	-	-
campaign x PFG x root length density	1	6970	137.00	12.81	**< 0.001**	-	-	-	-
Explained variation		R^2_{marg} = 0.694 / R^2_{cond} = 0.924				R^2_{marg} = 0.487 / R^2_{cond} = 0.887			

f) Model *CWM growth start*

sampling campaign	1	483387	142.01	811.40	**< 0.001**	12205	136.77	510.07	**< 0.001**
plant functional group	1	3986	5.01	6.69	**0.049**	8	4.97	0.34	0.586
CWM growth start	1	-	-	-	-	5	139.31	0.21	0.651
campaign x PFG	1	-	-	-	-	2752	136.78	115.01	**< 0.001**
campaign x growth start	1	-	-	-	-	79	137.04	3.28	0.072
PFG x growth start	1	-	-	-	-	-	-	-	-
campaign x PFG x growth start	1	-	-	-	-	-	-	-	-

Explained variation		R^2_{marg} = 0.680 / R^2_{cond} = 0.918				R^2_{marg} = 0.495 / R^2_{cond} = 0.887			
g) Model *CWM flowering start*									
sampling campaign	1	483387	142.01	811.40	**< 0.001**	12205	134.68	514.78	**< 0.001**
plant functional group	1	3986	5.01	6.69	**0.049**	8	4.98	0.34	0.583
CWM flowering start	1	-	-	-	-	8	137.42	0.35	0.553
campaign x PFG	1	-	-	-	-	2756	134.69	116.23	**< 0.001**
campaign x flowering start	1	-	-	-	-	8	134.98	0.35	0.555
PFG x flowering start	1	-	-	-	-	42	137.18	1.77	0.186
campaign x PFG x flowering start	1	-	-	-	-	112	134.76	4.74	**0.031**
Explained variation		R^2_{marg} = 0.680 / R^2_{cond} = 0.918				R^2_{marg} = 0.498 / R^2_{cond} = 0.889			

Table S5 Statistical parameters resulting from a Type 1 ANOVA on richness and Shannon diversity of secondary metabolites variables within grasses only or forbs only as a function of sampling campaign, and each of the significant community-weighted mean traits separately, as well as the two-way interactions. We fitted plot nested in block and species identity as independent random effects. Represented are the most parsimonious models based on Akaike Information Criterion (AIC) comparisons. Significant differences ($p < 0.05$) are given in bold. Abbreviations: CWM = community-weighted mean; NumDF = numerator degrees of freedom; DenDF = denominator degrees of freedom; SS = sum of squares; F = F-value; p = p-value.

Parameter		Grasses								Forbs							
		Richness of secondary Metabolites				Shannon diversity of secondary Metabolites				Richness of secondary Metabolites				Shannon diversity of secondary Metabolites			
	NumDF	SS	denDF	F	p	SS	denDF	F	p	SS	denDF	F	p	SS	denDF	F	p
a) Model *CWM maximum plant height*																	
sampling campaign	1	219336	58.00	1040.62	**<0.001**					264402	79.01	331.41	**<0.001**				
CWM max height	1	238	58.53	1.13	0.292					2185	79.44	2.74	0.102				
campaign x max height	1	665	58.01	3.15	0.081					5709	79.01	7.16	**0.009**				
Explained Variation		$R^2_{marg} = 0.676$ / $R^2_{cond} = 0.959$								$R^2_{marg} = 0.525$ / $R^2_{cond} = 0.869$							
b) Model *CWM leaf area*																	
sampling campaign	1					12500	41.34	698.30	**<0.001**					2446	76.75	91.48	**< 0.001**

CWM leaf area	1					181	34.30	10.13	**0.003**					4	78.56	0.14	0.708
campaign x leaf area	1					16	43.85	0.87	0.355					74	76.95	2.76	0.101
Explained Variation						R^2_{marg} = 0.819 / R^2_{cond} = 0.927								R^2_{marg} = 0.164 / R^2_{cond} = 0.852			
c) Model *CWM rooting depth*																	
sampling campaign	1	219336	57.99	1255.84	**<0.001**					264402	78.98	410.43	**<0.001**				
CWM rooting depth	1	77	58.39	0.44	0.511					3	80.20	0.004	0.947				
campaign x rooting depth	1	2947	58.00	16.87	**<0.001**					20162	78.98	31.30	**<0.001**				
Explained Variation		R^2_{marg} = 0.691 / R^2_{cond} = 0.966								R^2_{marg} = 0.562 / R^2_{cond} = 0.892							
d) Model *CWM root length density*																	
sampling campaign	1	219336	58.00	1016.51	**<0.001**					264402	78.99	336.78	**<0.001**				
CWM root length density	1	116	58.03	0.54	0.466					569	79.79	0.72	0.397				
campaign x root length density	1	552	58.00	2.56	0.115					8657	78.99	11.03	**0.001**				
Explained Variation		R^2_{marg} = 0.688 / R^2_{cond} = 0.957								R^2_{marg} = 0.348 / R^2_{cond} = 0.427							

e) Model *CWM flowering start*

sampling campaign	1	12500	39.93	701.91	**<0.001**	2446	76.79	91.88	**< 0.001**
CWM flowering start	1	53	47.01	3.00	0.090	0.42	78.49	0.02	0.900
campaign x flowering start	1	41	41.36	2.29	0.138	84	77.08	3.14	0.080
Explained Variation		R^2_{marg} = 0.813 / R^2_{cond} = 0.928				R^2_{marg} = 0.167 / R^2_{cond} = 0.850			

Table S6 Direct, indirect, and total path coefficients among all latent variables for the *full-model* PLS-PM, *grasses-only-model* PLS-PM, and *forbs-only-model* PLS-PM. Significant direct and total path coefficients (based on confidence intervals) are given in bold.

Relationships	*full-model* PLS-PM			*grasses-only-model* PLS-PM			*forbs-only-model* PLS-PM		
	direct	**indirect**	**total**	**direct**	**indirect**	**total**	**direct**	**indirect**	**total**
Species richness -> Resource acquisition traits	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Species richness -> Microbial community	**0.31**	0.00	**0.31**	**0.28**	0.00	**0.28**	**0.29**	0.00	**0.29**
Species richness -> Nematode community	**-0.27**	0.00	**-0.27**	**-0.26**	0.00	**-0.26**	**-0.35**	0.00	**-0.35**
Species richness -> Biomass	**-0.11**	0.00	**-0.11**	**-0.20**	0.03	**-0.17**	**-0.17**	-0.01	**-0.17**
Species richness -> Metabolome	0.03	-0.05	-0.02	-0.10	0.11	0.01	-0.06	0.03	-0.03
Species richness -> Herbivory	-0.07	-0.02	-0.09	-0.06	-0.04	-0.10	-0.09	-0.03	-0.12
Resource acquisition traits -> Microbial community	0.08	0.00	0.08	-0.07	0.00	-0.07	0.14	0.00	0.14
Resource acquisition traits -> Nematode community	**-0.20**	0.00	**-0.20**	-0.11	0.00	-0.11	-0.38	0.00	-0.38
Resource acquisition traits -> Biomass	**0.22**	0.01	**0.23**	**-0.31**	0.03	**-0.28**	**0.22**	0.01	**0.22**
Resource acquisition traits -> Metabolome	-0.01	-0.13	**-0.14**	0.13	0.08	0.21	0.33	0.16	0.49
Resource acquisition traits -> Herbivory	**-0.12**	0.10	-0.02	**0.28**	-0.15	0.14	-0.12	0.37	0.25
Microbial community -> Nematode community	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Microbial community -> Biomass	**-0.10**	0.00	**-0.10**	**-0.12**	0.00	**-0.12**	-0.08	0.00	-0.08
Microbial community -> Metabolome	0.09	0.03	0.12	**0.28**	0.05	**0.33**	-0.01	-0.02	-0.04
Microbial community -> Herbivory	0.00	-0.06	-0.06	0.00	-0.16	**-0.16**	0.00	-0.03	-0.03

Nematode community -> Biomass	**-0.11**	0.00	**-0.11**	**-0.23**	0.00	**-0.23**	-0.05	0.00	-0.05
Nematode community -> Metabolome	**0.39**	0.03	**0.42**	**0.19**	0.10	**0.29**	**-0.24**	-0.01	**-0.26**
Nematode community -> Herbivory	0.00	-0.18	**-0.18**	0.00	-0.17	**-0.17**	0.00	-0.19	**-0.19**
Biomass -> Metabolome	**-0.27**	0.00	**-0.27**	**-0.43**	0.00	**-0.43**	**0.30**	0.00	**0.30**
Biomass -> Herbivory	**0.20**	0.10	**0.30**	**0.23**	0.17	**0.40**	0.01	0.22	**0.23**
Metabolome -> Herbivory	**-0.38**	0.00	**-0.38**	**-0.40**	0.00	**-0.40**	**0.75**	0.00	**0.75**

Table S7 Metabolite predictions assigned in shoot extracts of *Dactylis glomerata, Geranium pratense, Leucanthemum vulgare, Plantago lanceolata,* and *Ranunculus acris* by LC-MS. The metabolites were part of the 100 most important 'Metabolome' moderators in the final full PLS-PM model. The source name corresponds to the molecular structure displayed in the Supplementary Fig. S9 – S21. The source name also corresponds to the species the compound was detected in. We used the letters *a* and *b* in the source name when our predictions of the LC-MS data resulted in two equally likely compounds. Abbreviations: Rt = Retention time in liquid chromatography in seconds; eV = Fragmentation energy in electron volt; MS = mass spectrometry.

Source	Rt [s]	$[M - H]^-$ [m/z]	Predicted compound	Molecular formula [M]	eV	MS fragments	MS adducts
D. glomerata 1	774	*223.0282	Sinapic acid* or ester of sinapic acid	*$C_{11}H_{12}O_5$	35		
D. glomerata 2a	394	563.1403	Flavone 1	$C_{26}H_{28}O_{14}$	35		
D. glomerata 2b	394	597.2186	Dilignan	$C_{28}H_{38}O_{14}$	35		
D. glomerata 3	578	507.0918	Flavonol	$C_{24}H_{28}O_{12}$	35	449	
D. glomerata 4	370	579.1350	Flavone 2	$C_{26}H_{28}O_{15}$	35		
D. glomerata 5	357	*179.0351	Caffeic acid* or ester of caffeic acid	*$C_9H_8O_4$	35	135	
L. vulgare 6	327	707.1838	Chlorogenic acid dimer 1	$C_{32}H_{36}O_{18}$	35	353, 191	
L. vulgare 7	368	707.1838	Chlorogenic acid dimer 2	$C_{32}H_{36}O_{18}$	35	353, 191	
L. vulgare 8a	285	631.1490	Flavonol dimer (coordinate bond)	$C_{32}H_{24}O_{14}$	35	315, 153	653
L. vulgare 8b	285	631.1490	Flavonol dimer (covalent bond)	$C_{32}H_{24}O_{14}$	35	315, 153	653
L. vulgare 9	375	675.1940	Chlorogenic acid dimer 3	$C_{32}H_{36}O_{16}$	35	337, 191, 148	697
L. vulgare 10	411	675.1940	Chlorogenic acid dimer 4	$C_{32}H_{36}O_{16}$	35	337, 191, 148	697
L. vulgare 11a	186	631.1513	Flavan alcohol dimer	$C_{34}H_{32}O_{12}$	35	315	

L. vulgare 11b	186	631.1513	Flavan alcohol dimer	$C_{32}H_{24}O_{14}$	35	315
L. vulgare 12	81	615.2131	Chlorogenic acid dimer 5	$C_{32}H_{40}O_{12}$	35	307, 191
P. lanceolata 13	82	*191.0197	Quinic acid* or ester of quinic acid	*$C_7H_{12}O_6$	35	129

Supplementary information for Manuscript III

FORMULAR 2

Manuskript Nr. 3

Kurzreferenz Ristok et al (in prep)

Beitrag des Doktoranden / der Doktorandin

Beitrag des Doktoranden / der Doktorandin zu Abbildungen, die experimentelle Daten wiedergeben (nur für Originalartikel):

Abbildung(en) # 1-4; S1 – S3*	X	100% (die in dieser Abbildung wiedergegebenen Daten entstammen vollständig experimentellen Arbeiten, die der Kandidat/die Kandidatin durchgeführt hat)
	☐	0% (die in dieser Abbildung wiedergegebenen Daten basieren ausschließllich auf Arbeiten anderer Koautoren)
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**Kann sich auf mehrere Abb. beziehen, wenn die Antwort dieselbe ist*		

_____ Unterschrift Kandidat/-in

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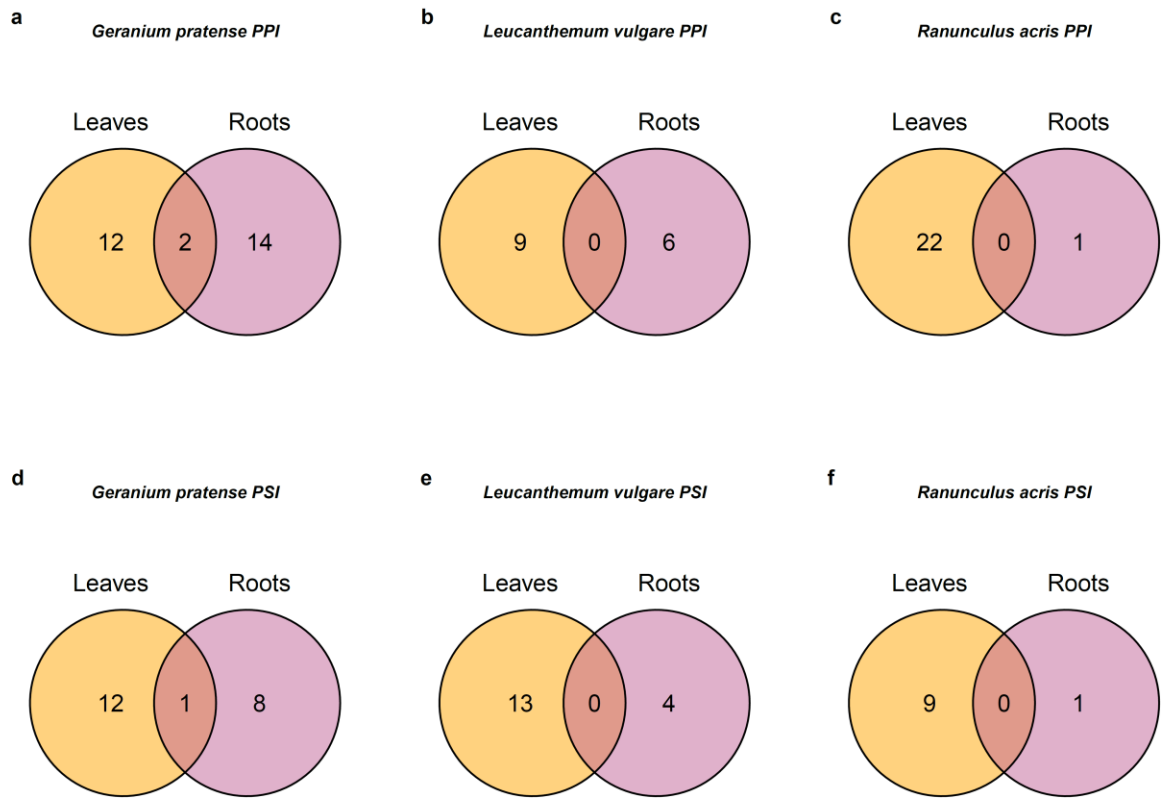

Figure S1. The total number of up- and down-regulated metabolites in plants grown in microcosms with (a – c) different neighbors (PPI) or (d – f) different soil legacies (PSI). Metabolites uniquely regulated in leaves are depicted in orange. Metabolites uniquely regulated in roots are depicted in violet. Overlapping areas indicate the number of up- and down-regulated metabolites in both tissues. The number depicted is in comparison to the monoculture diversity/soil legacy level. Abbreviations: PPI – plant-plant interaction experiment; PSI – plant-soil interaction experiment.

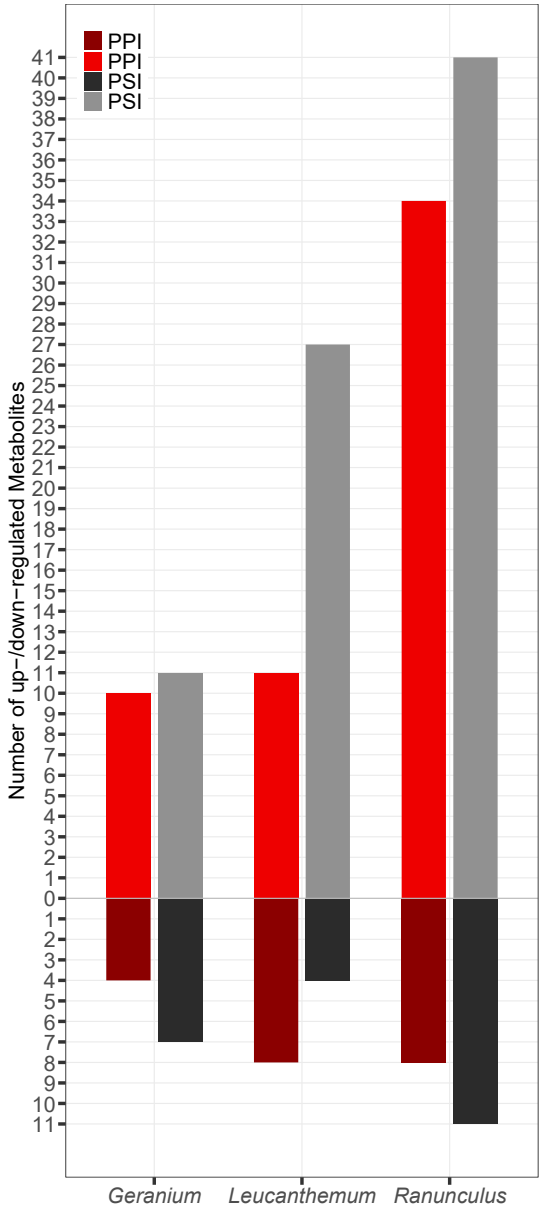

Figure S2. The total number of up- and down-regulated metabolites in leaves of herbivore-induced plants. The plants were subjected to 7 days of frass by three 2nd instar *Spodoptera exigua* larvae each. The number depicted is in comparison to control plants grown in similar soil or plant diversity levels, but without herbivore damage. Data collected as part of the plant-plant interaction (PPI) experiment are displayed in light red (up) and dark red (down). Data collected as part of the plant-soil interaction (PSI) experiment are displayed in grey (up) and black (down). Abbreviations: *Geranium* = *Geranium pratense*; *Leucanthemum* = *Leucanthemum vulgare*; *Ranunculus* = *Ranunculus acris*.

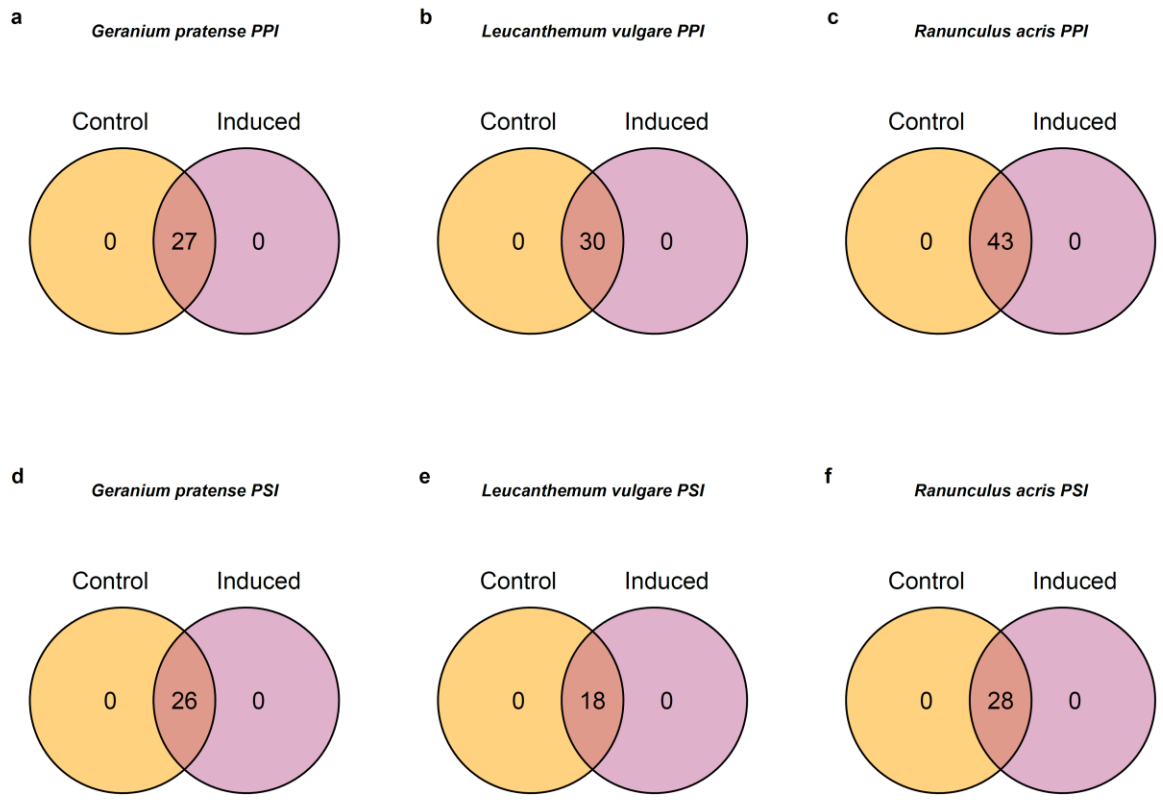

Figure S3. The total number of up- and down-regulated metabolites in leaves of control and herbivore-induced plants grown in microcosms with (a – c) different neighbors (PPI) or (d – f) different soil legacies (PSI). Metabolites uniquely regulated in control plants are depicted in orange. Metabolites uniquely regulated in herbivore-induced plants are depicted in violet. Overlapping areas indicate the number of up- and down-regulated metabolites in both control and herbivore-induced plants. The number depicted is in comparison to the monoculture diversity/soil legacy level. Abbreviations: PPI – plant-plant interaction experiment; PSI – plant-soil interaction experiment.

Table S1: Overview of the experimental design of the plant-plant-interaction experiment.

Experimental design plant-plant interaction experiment							
Species pool	*Geranium pratense* (G) – *Leucanthemum vulgare* (L) – *Ranunculus acris* (R)						
Diversity levels	G	L	R	G + L	G + R	L + R	G + L + R
Nr. of plants / microcosm	12	12	12	6 + 6	6 + 6	6 + 6	4 + 4 + 4
Nr. of microcosms	5	5	5	5	5	5	5
Herbivory treatment	x2	x2	x2	x2	x2	x2	x2
Total Nr. of microcosms	10	10	10	10	10	10	10
1st Harvest after 7 weeks of growth							
Nr. of microcosms	5	5	5	5	5	5	5
Nr. of plants sampled/microcosm	1	1	1	1 + 1	1 + 1	1 + 1	1 + 1 + 1
Total Nr. of samples (shoot / root)	5 / 5	5 / 5	5 / 5	G: 5 / 5 L: 5 / 5	G: 5 / 5 R: 5 / 5	L: 5 / 5 R: 5 / 5	G: 5 / 5 L: 5 / 5 R: 5 / 5
2nd Harvest after 1 additional week of herbivory							
Nr. of microcosms	5	5	5	5	5	5	5
Nr. of plants / microcosm (control / induced)	10/2	10/2	10/2	G: 4 / 2 L: 4 / 2	G: 4 / 2 R: 4 / 2	L: 4 / 2 R: 4 / 2	G: 2 / 2 L: 2 / 2 R: 2 / 2
Nr. of plants sampled/microcosm (control / induced)	1 / 1	1 / 1	1 / 1	G: 1 / 1 L: 1 / 1	G: 1 / 1 R: 1 / 1	L: 1 / 1 R: 1 / 1	G: 1 / 1 L: 1 / 1 R: 1 / 1
Total Nr. of samples (control / induced)	5 / 5	5 / 5	5 / 5	G: 5 / 5 L: 5 / 5	G: 5 / 5 R: 5 / 5	L: 5 / 5 R: 5 / 5	G: 5 / 5 L: 5 / 5 R: 5 / 5

Table S2: Overview of the experimental design of the plant-soil-interaction experiment.

Experimental design plant-soil interaction experiment							
Species pool	*Geranium pratense* (G) – *Leucanthemum vulgare* (L) – *Ranunculus acris* (R)						
Soil legacy levels	G	L	R	G + L	G + R	L + R	G + L + R
Planted species & nr. of plants / microcosm	G:4	L:4	R:4	G: 4 L: 4	G: 4 R: 4	L: 4 R: 4	G: 4 L: 4 R: 4
Nr. of microcosms	5	5	5	G: 5 L: 5	G: 5 R: 5	L: 5 R: 5	G: 5 L: 5 R: 5
Herbivory treatment	x2	x2	x2	x2	x2	x2	x2
Total Nr. of microcosms	10	10	10	G: 10 L: 10	G: 10 R: 10	L: 10 R: 10	G: 10 L: 10 R: 10
1st Harvest after 7 weeks of growth							
Nr. of microcosms	5	5	5	G: 5 L: 5	G: 5 R: 5	L: 5 R: 5	G: 5 L: 5 R: 5
Nr. of plants sampled / microcosm	1	1	1	G: 1 L: 1	G: 1 R: 1	L: 1 R: 1	G: 1 L: 1 R: 1
Total Nr. of samples (shoot / root)	5 / 5	5 / 5	5 / 5	G: 5 / 5 L: 5 / 5	G: 5 / 5 R: 5 / 5	L: 5 / 5 R: 5 / 5	G: 5 / 5 L: 5 / 5 R: 5 / 5
2nd Harvest after 1 additional week of herbivory							
Nr. of microcosms	5	5	5	G: 5 L: 5	G: 5 R: 5	L: 5 R: 5	G: 5 L: 5 R: 5
Nr. of plants / microcosm (control / induced)	2 / 2	2 / 2	2 / 2	G: 2 / 2 L: 2 / 2	G: 2 / 2 R: 2 / 2	L: 2 / 2 R: 2 / 2	G: 2 / 2 L: 2 / 2 R: 2 / 2
Nr. of plants sampled/microcosm (control / induced)	1 / 1	1 / 1	1 / 1	G: 1 / 1 L: 1 / 1	G: 1 / 1 R: 1 / 1	L: 1 / 1 R: 1 / 1	G: 1 / 1 L: 1 / 1 R: 1 / 1
Total Nr. of samples (control / induced)	5 / 5	5 / 5	5 / 5	G: 5 / 5 L: 5 / 5	G: 5 / 5 R: 5 / 5	L: 5 / 5 R: 5 / 5	G: 5 / 5 L: 5 / 5 R: 5 / 5