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RESEARCH ARTICLE

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Above-ground plant metabolomic responses to plant–soil feedbacks and herbivory

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Abstract

1. Understanding the causes of variation in foliar plant metabolomes is essential for our understanding of ecological interactions between plants and other organisms. It is well-accepted that foliar herbivory alters metabolites in leaves. However, soil (micro)organisms can also induce such changes.
2. We generated plant-specific soil legacies by growing 12 plant species individually in a common starting soil. Then we planted all plant species in all soils and exposed a subset to foliar herbivory. We then used ^{1}H nuclear magnetic resonance to analyse the shoot metabolomes of all responding plants.
3. Above-ground herbivory and soil legacies altered shoot metabolomes. In most plant species, soil legacy more strongly affected shoot metabolomes than foliar herbivory.
4. *Synthesis*. Our results show that plant-induced changes in soil alter metabolomes of plants that grow later in those soils. Such below-ground legacy effects can have far-stretching consequences for above-ground multitrophic interactions as these often depend on the plant chemical composition. Recently, plant–soil feedbacks have received considerable attention in ecological studies, and our study now highlights that these feedbacks can be an important determinant of the often unexplained intraspecific variation in chemical composition among plants.

KEYWORDS
above–below-ground interactions, ecological omics, ecometabolomics, insect herbivory, nuclear magnetic resonance spectroscopy, plant–soil interactions, soil legacy effect

1 | INTRODUCTION

In natural systems, all organisms actively interact with other organisms and their surroundings. This is especially true for plants which are sessile and hence forced to cope and interact continuously with a range of biotic and abiotic challenges above- and below-ground. Chemical compounds play a key role in many of these biotic interactions, and one of the challenges in chemical ecology is to disentangle the drivers of variation in chemistry within and between plants and the role these compounds play in ecological interactions (Dyer et al., 2018). A large body of literature has shown that foliar herbivory by insects typically leads to changes in the composition or concentration of specialized compounds of plants, and that these changes generally increase the resistance of the plant to

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herbivory (Karban & Baldwin, 1997). In the soil, plant roots interact with soil-dwelling organisms such as microbes (e.g. pathogens and mycorrhizal fungi), nematodes and root feeding insects, and an increasing number of studies is showing that specific soil organisms such as insects or microbes can induce changes in the composition of plant-derived chemicals in the foliage (Bezemer & Van Dam, 2005; Etalo, Jeon, & Raaijmakers, 2018; Pieterse et al., 1998). Most of these studies focus on changes in one or a few specific groups of chemical compounds, but the plant metabolome consists of thousands of compounds. Several authors have argued that changes in the chemical composition within a plant may be more important for the interactions between plants and other organisms than changes in specific compounds (Berenbaum & Zangerl, 1993; Liu, Vrieling, & Klinkhamer, 2017; Nelson & Kursar, 1999). However, how plant metabolomes change in response to herbivory or interactions with below-ground organisms, and how common these responses are among plant species, is poorly understood.

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

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

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

Here we studied how soil legacies generated by six forb and six grass species altered the metabolomes of these forbs and grasses growing later in each soil. We used 12 species and each of these species was grown in all 12 conditioned soils. In addition, the response plants were either exposed to above-ground herbivory treatment by the polyphagous chewing herbivore *Mamestra brassicae*

(Lepidoptera: Noctuidae) or kept as no herbivory controls. We investigated the metabolomic changes in the shoots of the response plants that were growing in the different soils enabling us to detect changes in the metabolome due to soils and examine whether these patterns stay the same under herbivory or not. We hypothesized that: (a) soil-borne legacies created by different plant species will influence the metabolome of other plant species that grow later in the same soil, and that the response plants will respond differently to soils and herbivory. Furthermore, we hypothesized that: (b) grasses and forbs will have different metabolomic profiles, that grasses and forbs will differ in their response to soil conditioning and herbivory, and that this response will depend on the functional group of the species that conditioned the soil; and (c) soil conditioning will alter the metabolomic response of a plant to above-ground herbivory.

2 | MATERIALS AND METHODS

2.1 | Experimental design

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

2.2 | Plants

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

2.3 | Insects

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

2.4 | Experimental conditioning phase

Soil (0–10 cm) was collected from a natural grassland 'De Mossel' (Natuurmonumenten). Soil was sieved (sieve mesh Ø 1.0 cm) to remove stones, dead plant material, roots and most macroinvertebrates. More soil was collected from the same site from the 10 to 20-cm layer. This soil was also sieved and then sterilized by γ-irradiation (>25 KGray, Synergy Health). The soil was collected deeper since this layer contained fewer roots and we did not need to sample the layer containing most of the microbiome influenced by plants since this soil was sterilized. For each of the 12 plant species, 60 square 1-L pots (11 × 11 cm) were filled with 1,050 g live field soil (720 pots in total). One seedling was planted into each pot in a greenhouse (L:D 16:8, day temperature 21 ± 1°C, night temperature 16 ± 1°C). To avoid dehydration and assure establishment of the seedlings, the seedlings were covered with shade-cloth during the first 4 days. To minimize emerging weeds and prevent fungus gnats from laying eggs, a 1-cm layer of silver sand was added. Seedlings emerging from the seed bank present in the live soil were removed on a daily basis and the plants were watered three times per week. After 10 weeks, the above-ground biomass of all the pots was clipped and the roots were removed from the soil. The conditioned soil from each pot was kept at 4°C. A priori, the 60 individual pots per species were divided over five separate replicates so that each replicate contained all soil from 12 independently conditioned pots resulting in 60 soils.

2.5 | Experimental feedback phase

Each of the 60 soils was mixed with sterilized soil (1:2 conditioned: sterile v/v) to minimize potential differences in nutrient content among the conditioned soils and used to fill 24 pots (9 × 9 cm; 650 g). All 12 plant species were grown on each conditioned soil in double and randomly allocated to one of two herbivory levels (present, absent), resulting in 12 response species × 12 conditioning species × 5 replicates × 2 treatments = 1,440 pots. The plants were grown in a

greenhouse under the same conditions as the plants in the conditioning phase. After 4 weeks, all 1,440 pots were caged (9-cm diameter, 30-cm height) with a plastic tube made of transparent plastic with insect mesh fitted on top. In one of the two pots, a freshly hatched *M. brassicae* caterpillar was introduced. After 7 days of feeding, the caterpillars were removed and feeding marks were observed in all treated plants except for seven individuals. Those individuals were excluded from later metabolomics analysis. Upon harvest, all plants were at the vegetative state, except for *P. lanceolata*. For this species, only the leaves were sampled. The area that the caterpillars fed (damage) was recorded. All plants were then clipped, and fresh shoot biomass was recorded. The shoots were then immediately wrapped in aluminium foil and flash-frozen in liquid nitrogen and stored at −80°C until lyophilization. *Gnaphalium sylvaticum* grew much slower than the other species and therefore the herbivory treatment was applied exactly 1 week later than the other species and the shoots were harvested 1 week later.

2.6 | Metabolomics ^{1}H NMR analysis

^{1}H NMR was chosen for untargeted metabolomics due to its high reproducibility and ability to detect a large dynamic range of molecules. The samples were lyophilized for 5 days. Lyophilized material was ground with a Retsch Mixer Mill MM 400 for 4–5 min, depending on the structure of the plant material, at a frequency of 30 Hz. Dried powdered samples were weighed (20.21 ± 0.05 mg) and put in 1.5-ml microtubes. For the extraction, 300 µl of CH_3OH-d_4 (Sigma) was added to the samples followed by 300 µl of D_2O buffer with 0.01% Trisodium phosphate (TSP). The vials were sonicated for 10 min and then centrifuged for 10 min at 17,000 x *g* in a Heraeus Pico 17 Microcentrifuge. About 250 µl of the clear supernatant was then filled in a disposable 3-mm NMR tube (Bruker) using a glass pipette.

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

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

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

2.7 | Data analysis

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

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

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

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

response plant species separately, including as fixed factors 'conditioning plant identity' and 'herbivory'. For each response plant species, we also tested the effects of 'conditioning functional group' and 'herbivory' as well as 'self- and non-self-soil' as described above.

3 | RESULTS

3.1 | Overall comparisons of metabolomes

Above-ground metabolomes distinctly differed among the 12 plant species and between grasses and forbs (Figure 1; Table S1), and the profiles were strongly influenced by herbivory, soil legacies and the interactions between these two factors (Table S1). However, the metabolic responses to soil legacies were species-specific and did not differ consistently between soil legacies from grasses and forbs (Table S1; no significant soil conditioning effects in Models 2 and 4). Overall, the metabolic responses did not vary between plants grown in soil conditioned by the same plant species (self-soil) or in soils conditioned by other plant species (non-self; Supporting Information S1, Models 5 and 6). Herbivory strongly influenced the metabolome of the response plants for all species, but the area of leaf material consumed varied significantly between the plant species (Figure S5).

3.2 | Species-specific metabolomes

We subsequently analysed the effects of conditioning and herbivory for all plant species separately. Plant metabolic responses to soil legacies and herbivory largely differed (Figure 2). For most response species, soil legacies and herbivory significantly influenced the metabolome. However, the effect of soil legacies on the above-ground metabolome was stronger than the effect of herbivory for seven of the 12 species (Figure 3; Table S2). Moreover, the plant species that conditioned the soil explained a higher proportion of the variance in metabolic profiles than their functional group, that is, whether they were a grass or a forb (Tables S1 and S2; Figure S1). The conditioning plant species differed in the extent to which they influenced the metabolomes of the response species through the soil. Soil legacies of the grasses *A. odoratum* and *B. media* and of the forb *G. molle* most strongly influenced the metabolomic profiles across the tested plant species, while soil of the grass *A. capillaris* had the weakest effect (Figure S2). Only for the test plant *H. lanatus*, we observed a significant interaction between soil and herbivory, indicating that only for this species, the metabolic response to herbivory depended on the soil the plant was growing in (Table S2). The variation in metabolomes was also partly explained by the biomass of the shoots of the response plant for all species, except for *H. lanatus* and *G. sylvaticum* (Table S3). The metabolome of *A. capillaris*, *A. pratensis* and *T. officinale* varied significantly depending on whether the plants were grown in self-soil or non-self-soil (Table S2).

3.3 | NMR signals

Detailed inspection of the NMR signals shows that soil conditioning and herbivory influenced a large number of the signals (buckets, see Supporting Information S1: Methods) but that these responses were

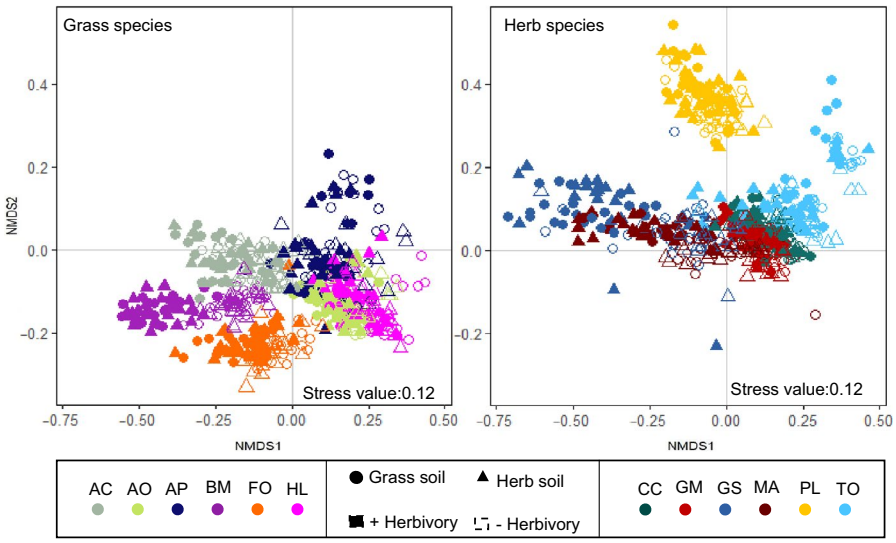

FIGURE 1 Metabolomic profiles of grasses and forbs grown in differently conditioned soils. Ordination using non-metric multidimensional scaling (NMDS) of metabolomes of the 12 different plant species (depicted in different colours) based on Bray–Curtis dissimilarities. The NMDS was conducted for all species growing in all soils but for clarity, only the data points for grasses (left) and forbs (right) are shown respectively. The plants were grown in soils conditioned by forbs (triangles) or grasses (circles) and subjected to herbivory (closed symbols, + herbivory, open symbols, − herbivory) by *Mamestra brassicae* caterpillars. The stress value is a measure of goodness-of-fit. Iterations were set at 31. The species used were *Agrostis capillaris* (AC), *Alopecurus pratensis* (AP), *Anthoxanthum odoratum* (AO), *Briza media* (BM), *Festuca ovina* (FO), *Holcus lanatus* (HL), *Crepis capillaris* (CC), *Geranium molle* (GM), *Gnaphalium sylvaticum* (GS), *Myosotis arvensis* (MA), *Plantago lanceolata* (PL) and *Taraxacum officinale* (TO)

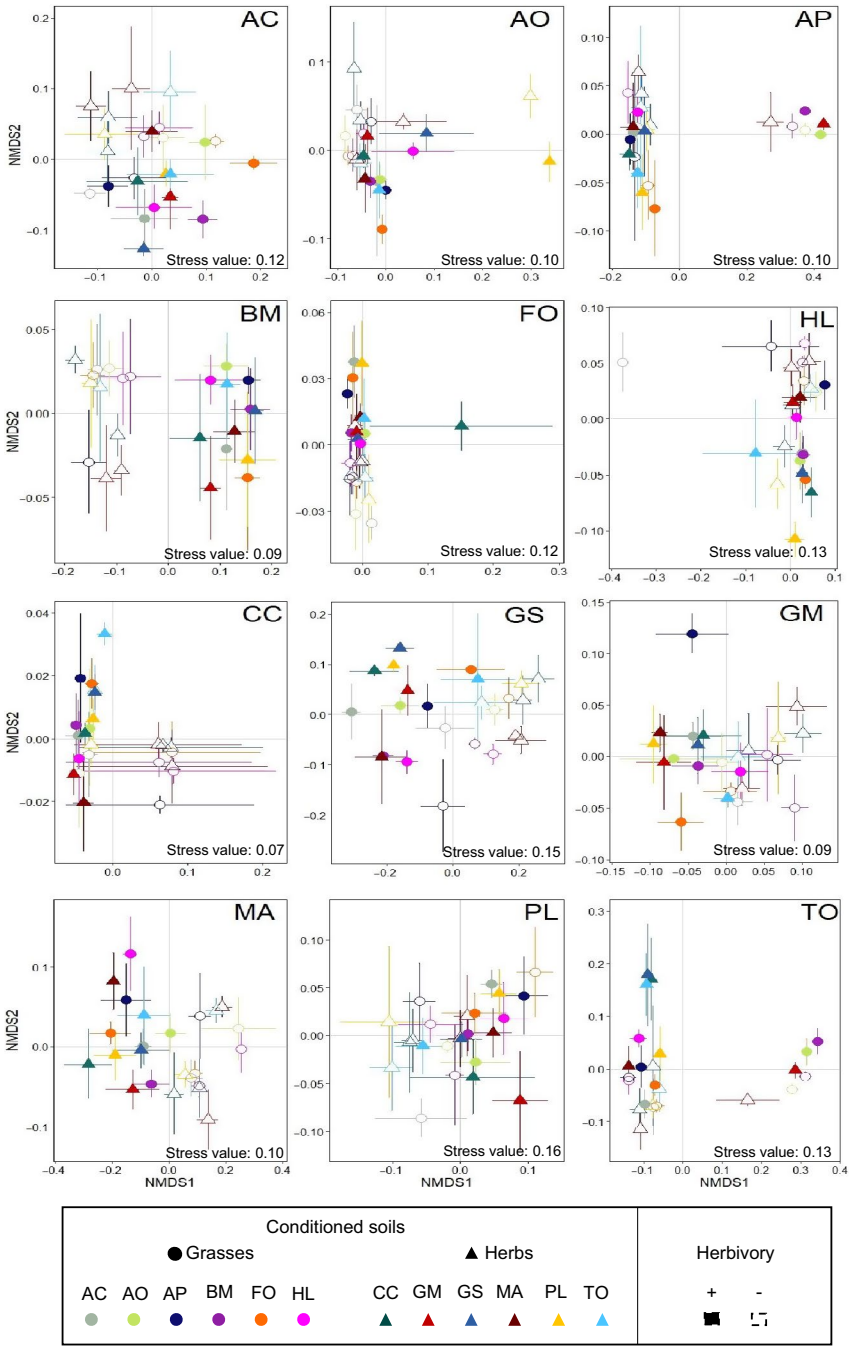

FIGURE 2 Responses of the metabolomes of the 12 test species to conditioned soil and herbivory. Ordination using non-metric multidimensional scaling (NMDS) of metabolomes of each response species (species abbreviations are in the right corner of each panel) based on Bray–Curtis dissimilarities. Depicted are the centroids ± *SE*. The top six panels are grass species and the lower panels are forbs. The plant species that conditioned the soil is depicted by colours and their functional group by symbols (triangles: forbs; circles: grasses). Open symbols depict plants that did not experience herbivory by *Mamestra brassicae* (H−) and filled circles ones that were subjected to herbivory (H+). The stress value is a measure of goodness-of-fit. The species used were *Agrostis capillaris* (AC), *Anthoxanthum odoratum* (AO), *Alopecurus pratensis* (AP), *Briza media* (BM), *Festuca ovina* (FO), *Holcus lanatus* (HL), *Crepis capillaris* (CC), *Gnaphalium sylvaticum* (GS), *Geranium molle* (GM), *Myosotis arvensis* (MA), *Plantago lanceolata* (PL) and *Taraxacum officinale* (TO)

species-specific (Table S5). NMR spectra are divided into 'buckets' of a specific size (0.04 ppm). The intensity within one bucket is the mean of all signals that fall into that bucket.

Of the total of 241 buckets, 83 buckets were associated to a specific metabolite or metabolite group. Overall, soil legacies mainly affected sugars (sucrose, glucose), sugar alcohols (inositol, mannitol), amino acids (glutamine, glutamate), other acids (fumaric acid, 3-caffeoylquinic acid and malate), flavonoids and lipids (Table S5). Herbivory mainly influenced the concentration of sugars and specialized compounds (flavonoids, chlorogenic acid and phenylpropanoids; Figure S3; Table S5). The variability of the metabolome of the response plants due to the treatments differed between response plant species (Figure S3). Chemical diversity calculated as Shannon index varied across the response species, herbivory treatments and also differed intraspecifically between response species grown on soils conditioned by the different plant species (Figure S4; Table S6). Diversity of the metabolome was higher in all plants which did receive a herbivore treatment. We further investigated the changes in particular specialized metabolites due to growth in the different soils and in the presence and absence of herbivory. Specialized metabolites changed most due to herbivory. In *P. lanceolata*, aucubin was affected by both the soil treatment and by herbivory. Its concentration was significantly higher in plants that experienced herbivory. In *H. lanatus*, concentrations of catechin were significantly higher upon herbivory treatment and the concentration of flavonoids was lower upon herbivory. In *F. ovina*, chlorogenic acid and phenylpropanoids

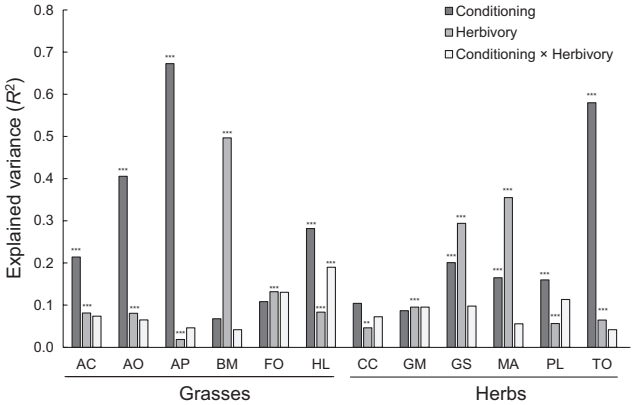

FIGURE 3 Variation in shoot metabolic profiles explained by soil conditioning and foliar herbivory. Variance explained by soil (conditioning; dark grey), herbivory by *Mamestra brassicae* (present or absent; light grey) and their interaction (white) for each of the 12 plant species grown on 12 different soils. Variance explained was obtained as R^2 from PERMANOVA models. *, **, *** indicate significant effects in the PERMANOVA tests at $p < 0.05$, $p < 0.01$, $p < 0.001$ respectively. The species used were *Agrostis capillaris* (AC), *Anthoxanthum odoratum* (AO), *Alopecurus pratensis* (AP), *Briza media* (BM), *Festuca ovina* (FO), *Holcus lanatus* (HL), *Crepis capillaris* (CC), *Geranium molle* (GM), *Gnaphalium sylvaticum* (GS), *Myosotis arvensis* (MA), *Plantago lanceolata* (PL) and *Taraxacum officinale* (TO)

concentrations were significantly higher upon herbivory. More details can be found in Table S5.

4 | DISCUSSION

Our study shows that the composition of the metabolome of eight of the 12 response species depended on the soil the plant was growing in. Hence, we provide strong evidence that PSFs alter shoot metabolomes. The effects were better explained by the species identity of the plant that conditioned the soil, than by the functional group of the plant that conditioned the soil. This indicates that the effect that a plant has on the metabolome of another plant via its effect on the soil is species-specific. Although it is well known that foliar herbivory causes considerable changes in plant metabolomes (Marti et al., 2013; Widarto et al., 2006), remarkably, we observed here that for most response plant species, soil conditioning explained more of the variation in the plant metabolome than did above-ground herbivory. We note that the herbivory and soil treatments differed in duration and that we cannot exclude that this has influenced the outcome of our experiment. However, as the intensity of the treatments also differs, similar durations would not resolve this issue and the comparison between the two treatments in our study should be done cautiously. These findings show that the foliar chemistry of plants greatly depends on the soil that the plant grows in. Our study adds a new perspective to the rapidly growing field of PSFs. We now show that these feedbacks do not only alter plant biomass, but also can alter entire plant metabolomes, with potential consequences for above-ground plant–insect interactions. Previously, effects of PSFs have been shown for specific plant compounds in studies that tested the effects of PSFs on specific plant species (e.g. Bezemer, Harvey, Kowalchuk, Korpershoek, & Van der Putten, 2006; Kos, Bukovinszky, Mulder, & Bezemer, 2015; Kostenko, Van de Voorde, Mulder, Van der Putten, & Bezemer, 2012; Ma et al., 2017; Zhu et al., 2018). To our knowledge, our study is the first to test entire metabolic profiles for a larger number of plant species growing on a range of soil legacies. This enables us to make broader conclusions about PSF effects on plant chemistry. Interestingly, our metabolomics approach now shows that the influence of soil conditioning on plant chemistry is not limited to specialized compounds, but instead, that these soil effects predominantly lead to changes in primary compounds such as sugars. This result highlights the importance of metabolomics approaches to study above-ground–below-ground interactions and PSFs. With the method that we used it is possible to identify specialized compounds; however, since primary compounds are more common among all plant species, the probability to assign these to a particular compound is higher.

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

The concept of PSF assumes that a plant changes the abiotic and/or biotic properties of the soil it grows in and that these changes then influence the growth of another plant that grows later in the soil. While we detected clear differences among the soils in how they influenced the metabolomes, we do not know the causal agents of these changes in the soil. Previous work in our laboratory using the same plant species growing in similar soil (but collected from the same field site several months earlier) showed that the composition of the bacterial and fungal communities depended on the species that conditioned the soil, and that there were clear differences between grass- and forb-conditioned soils (Heinen et al., 2018). Whether the impact of the soils on plant

metabolomes was due to, for example, pathogens, mycorrhiza or plant growth-promoting rhizobacteria remains to be tested. Other studies have shown, for example, that mycorrhiza (Schweiger et al., 2014) and beneficial soil bacteria can alter plant metabolomes (Zhou, Huang, Guo, dos-Santos, & Vivanco, 2018). We mixed the conditioned soil with sterilized soil to diminish the potential differences in conditioned soils in, for example, soil nutrient availability. However, we cannot be sure that the effects of soil conditioning are only due to the differences in the microbial communities in the conditioned soils. For example, allelopathic compounds present in the conditioned soils or extracellular DNA could also influence on the performance of the plant that grows later in the soil (Mazzoleni et al., 2015; Van de Voorde, Ruijten, Van der Putten, & Bezemer, 2012).

In our study, conditioned soil mainly influenced the concentration of primary plant compounds, such as organic acids and sugars. Similarly, Badri et al. (2013) showed that amino acids, phenolics, sugars and sugar alcohols changed in the metabolome of *Arabidopsis thaliana*, if the soil the plant was growing in was inoculated with soil slurries from various sources. A common function of sugars is that they are used as building blocks for cell walls and therefore they play a vital role in cell wall biosynthesis (Loewus & Murthy, 2000). The composition and quantity of primary compounds in a plant may be directly related to changes in assimilation patterns in the plant but can also influence interactions between the plant and its environment. For example, variation in the primary compounds can be important in defence against oligophagous herbivores (Berenbaum, 1995). Furthermore, below-ground infestations with pathogens, which may have accumulated in conditioned soils, can result in increased concentrations of defence compounds in the roots and this can result from reallocation of defence compounds from above- to below-ground tissues, altering concentrations of primary and specialized compounds in the foliage (Bezemer & Van Dam, 2005; Biere & Goverse, 2016). In our study, the reduced concentration of sugars in plants grown on particular soils suggests that there were pathogens present in those soils, but this needs to be tested in further studies. From a plant-herbivore or plant-pathogen perspective, it is interesting that one of the sugar alcohol for which the signal varied among soils is inositol which can act as a feeding stimulant for insects (Thorsteinson & Nayar, 1963). Moreover, inositol as well as glucose are known to activate genes in the pathogen *Pseudomonas syringae* that are related to toxin production (Li, Starratt, & Cuppels, 1998).

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

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

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AUTHORS' CONTRIBUTIONS

T.M.B. and R.H. designed and planned the research and performed the experiment; M.H. prepared the samples for metabolomics analysis and M.H. and Y.H.C. carried out the metabolomics analysis; M.H., T.M.B. and Y.H.C. analysed the data and M.H. and T.M.B. led the writing of the manuscript. All authors contributed critically to the manuscript and read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

Data of this study are available from Figshare: https://doi.org/10.6084/m9.figshare.8834537 (Huberty, Choi, Heinen, & Bezemer, 2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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