

RESEARCH ARTICLE

Foliar herbivory on plants creates soil legacy effects that impact future insect herbivore growth via changes in plant community biomass allocation

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ABSTRACT

1. Plants leave legacy effects in the soil they grow in, which can drive important vegetation processes, including productivity, community dynamics and species turnover. Plants at the same time also face continuous pressure posed by insect herbivores. Given the intimate interactions between plants and herbivores in ecosystems, plant identity and herbivory are likely to interactively shape soil legacies. However, the mechanisms that drive such legacy effects on future generations of plants and associated herbivores are little known.
2. In a greenhouse study, we exposed 10 common grasses and non-leguminous forbs individually to insect herbivory by two closely related noctuid caterpillars, *Mamestra brassicae* and *Trichoplusia ni* (Lepidoptera: Noctuidae) or kept them free of herbivores. We then used the soil legacies created by these plant individuals to grow a plant community composed of all 10 plant species in each soil and exposed these plant communities to *M. brassicae*. We measured conditioning plant biomass, soil respiration and chemistry of the conditioned soils, as well as individual plant, plant community and herbivore biomass responses.
3. At the end of the conditioning phase, soils with herbivore legacies had higher soil respiration, but only significantly so for *M. brassicae*. Herbivore legacies had minimal impacts on community productivity. However, path models reveal that herbivore-induced soil legacies affected responding herbivores through changes in plant community shoot: root ratios. Soil legacy effect patterns differed between functional groups. We found strong plant species and functional group-specific effects on soil respiration parameters, which in turn led to plant community shifts in grass: forb biomass ratios. Soil legacies were negative for the growth of plants of the same functional group.
4. *Synthesis.* We show that insect herbivory, plant species and their functional groups, all incur soil microbial responses that lead to subtle (herbivory) or strong

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(plants and their functional group) effects in response plant communities and associated polyphagous herbivores. Hence, even though typically ignored, our study emphasizes that legacies of previous insect herbivory in the soil can influence current soil–plant–insect community interactions.

KEYWORDS

above- and below-ground ecology, community ecology, grassland, herbivory, plant–insect interactions, plant–soil feedback, soil legacy effects

1 | INTRODUCTION

Plants modify the abiotic and biotic conditions in the soil and via these processes, they can create legacies in the soil. These typically are effects that persist in the soil after the biotic interaction that caused the effect ceases to exist (Wurst & Ohgushi, 2015). These soil legacies, in turn, can strongly impact the performance of plants that grow in the same soil (i.e. plant–soil feedbacks; Kulmatiski et al., 2008). Soil legacy effects are generally plant species-specific (e.g. Heinen, Biere, et al., 2020; Heinze et al., 2020; In 't Zandt et al., 2020) and vary between plants of different functional groups, such as grasses and forbs (Bezemer et al., 2006; Heinen, Hannula, et al., 2020; Kos et al., 2015; Kulmatiski et al., 2008). Plant-mediated soil legacies can affect various ecosystem processes, such as productivity, succession, and plant invasions (Thakur et al., 2021; Van der Putten et al., 2013). Plant responses to soil legacy effects depend on plant community composition (Heinen, Hannula, et al., 2020; Heinen, van der Sluijs, et al., 2018), as the effects that a legacy-specific soil community has on individual plant species within a responding plant community are often species-specific (Heinze et al., 2020). It has been shown in several studies that soil legacy effects – both on individual plants and plant communities—can further impact the growth and feeding of above-ground herbivores that feed on plants growing in soils with a specific legacy (Hannula et al., 2019; Heinen, Biere, et al., 2020; Heinen, van der Sluijs, et al., 2018; Howard et al., 2020; Kos et al., 2015). However, how interactions between herbivores and plants impact on soil legacy effects, is not fully understood, and thus warrants future study.

Insect herbivory is a common recurring threat to most plant species. Herbivory causes strong systemic defense responses, and cross-talk between below-ground and above-ground compartments is common in nature (e.g. reviewed in Biere & Goverse, 2016; Heinen, Biere, et al., 2018). As above-ground herbivory affects root processes, such as exudation, and soil respiration (Holland et al., 1996), it is likely that herbivory and plants interact in shaping soil legacies. Although the majority of plant–insect herbivore studies focus predominantly on the plant part where herbivory is occurring (e.g. only on the shoots or only on the roots), an increasing number of studies shows that insect herbivory in one part can also have consequences for plant interactions in other plant parts (Hol et al., 2004; Van Dam et al., 2003; Van der Putten et al., 2013). As reviewed in Biere and Goverse (2016), insect herbivory can suppress the performance of various below-ground bacteria, fungi and root-feeding nematodes via herbivore-induced plant defenses. For

instance, above-ground insect herbivory in a plant negatively affects mutualist associations between the plant roots and arbuscular mycorrhizal fungi in the soil (De Román et al., 2011). Similarly, above-ground herbivory on plants often negatively affects growth and survival of below-ground arthropods feeding on the same host plant (e.g. Hunt-Joshi & Blossey, 2005; Masters et al., 1993; Masters, 1995; Moran & Whitham, 1990; Salt et al., 1996; Tindall & Stout, 2001). There is some evidence that above-ground insect herbivory can leave biotic legacy effects in the soil, which can affect plants that grow later in the same soil (Heinze et al., 2020; Kostenko et al., 2012). For example, herbivory by the chewing herbivore *Mamestra brassicae* on *Jacobaea vulgaris* alters the structure of fungal communities in the soil in which their host plants grow, which negatively influences growth of plants that grow later in the same soil, but positively affects above-ground herbivores feeding on these plants (Bezemer et al., 2013; Kostenko et al., 2012). Whether herbivore-induced soil legacy effects are a widespread phenomenon in the plant kingdom, and whether effects differ between insect herbivore species is not well understood.

Responses of insect herbivores, particularly so for generalist herbivores, may also strongly differ between individual plants growing alone and plants growing in communities. For instance, in studies with individual plants, the importance of host plant preference of the herbivore is artificially minimized, as there is no choice for alternative hosts. In experimental plant communities, on the other hand, insect performance depends on the suitability of the host plants present in the plant community as food plants (Heinen, van der Sluijs, et al., 2018). Preference of host plants by herbivores may therefore play a key role in herbivore and plant responses to soil legacies within plant communities, as performance of even highly polyphagous herbivores such as noctuid caterpillars can strongly be determined by the host plant species (illustrated by Figure S1 in the Supplementary Information). In addition, soil legacy effects may affect host plant primary and secondary metabolism (Badri et al., 2013; Huberty et al., 2020; Ristok et al., 2019; Zhu et al., 2018). This may be one of the explanations why herbivores switch host plants within plant communities in soils with different legacies (Heinen, van der Sluijs, et al., 2018). Whether insect herbivores are affected by herbivore-induced soil legacy effects (as observed in Kostenko et al., 2012) when feeding in plant communities is not known, but this information is necessary to understand the role of insect herbivory in soil legacy effects in natural systems.

In this study, we assessed whether above-ground insect herbivory on 10 different plant species created legacy effects in the soil. Briefly, we exposed 10 grassland plant species (five grasses and five

forbs) individually to either 3 weeks of feeding by the cabbage moth, *M. brassicae*, or the cabbage looper, *Trichoplusia ni* (both Lepidoptera: Noctuidae), or to no herbivory. Then, we assessed whether herbivory left legacies in the soil, in terms of microbial respiration, and whether this, in turn, affected plant growth when all plant species were grown together in communities (i.e. the feedback phase). During the feedback phase, we further tested whether soil legacy effects would alter plant-herbivore interactions (i.e. the insect assay phase), for which we introduced four early instar *M. brassicae* caterpillars to each plant community.

We specifically hypothesized the following:

1. In line with the general suppressive effects of herbivory on below-ground organisms, herbivore feeding in the conditioning phase will generally suppress microbial activity in the soil.
2. Soil legacy effects created by different plant species and their functional group, as well as herbivory on these plant species, will alter the growth and composition of plant communities growing later in these soils.
3. Soil legacy effects will alter the performance of above-ground herbivores in the experimental plant communities and that the soil legacy effects will be plant- and herbivore-specific.

2 | MATERIALS AND METHODS

2.1 | Soil

The soil used in this study was obtained from a soil depot in Ede, The Netherlands. The soil originated from a pasture meadow at 'De Lange Dreef' in Driebergen, The Netherlands, that was excavated for building purposes. The soil was a sandy soil with 88% dry matter, 3% organic matter and 4% loam (Soil Qualification Report PJ Milieu BV, Nijkerk, The Netherlands).

2.2 | Insects

Eggs of the cabbage moth, *M. brassicae* (Lepidoptera: Noctuidae) were obtained from the Department of Entomology at Wageningen University. The cabbage moth has been reared for many years on Brussel's Sprout, *Brassica oleracea* var. *gemmifera* cv. Cyrus. The larvae were originally collected from cabbage fields near the university. *M. brassicae* is a generalist herbivore known to feed on more than 70 plant species from at least 22 families (Rojas et al., 2000). *M. brassicae* is widely distributed across the Palaearctic region.

Eggs of the cabbage looper, *T. ni* (Lepidoptera: Noctuidae) were originally obtained from the Great Lakes Forestry Center (Ontario, Canada) and maintained in culture for several generations on Brussel's Sprout, *B. oleracea* var. *gemmifera* cv. Cyrus. The cabbage looper is a generalist herbivore that feeds on a wide range of plant species and is closely related to the cabbage moth (Akhtar & Isman, 2003). *Trichoplusia ni* is widely distributed across the northern hemisphere.

2.3 | Plants

Plant species were selected from a larger pool of 24 grassland species based on feeding preferences of the selected generalist herbivores. Herbivory on these plant species was assessed by combining (a) visual verification of herbivore consumption, (b) performance of the caterpillar in terms of gained biomass over 3 weeks and (c) by weighing the dry shoot biomass of herbivore-treated and control plants (see Figure S1). This combination was chosen due to difficulty in herbivory assessment related to complex composite leaf structures of some plants. Biomass responses are not always representative for herbivory levels as different species may differ in tolerance to—and compensation for—herbivory. The selected grassland species were five forbs *Achillea millefolium*, *Clinopodium vulgare*, *Tripleurospermum maritimum*, *Plantago lanceolata*, *Rumex acetosella* and five grasses *Anthoxanthum odoratum*, *Briza media*, *Festuca ovina*, *Phleum pratense* and *Trisetum flavescens*. All species regularly co-occur under natural conditions in grasslands in western Europe.

For germination, seeds were placed on sterile glass beads in a climate cabinet (light regime 16:8, L:D, day temperature 21°C, night temperature 15°C). After germination, the seedlings were stored at 4°C under the same light regime, for later use in experiments. Seeds were obtained from Cruydt-Hoeck (Nijberkoop, The Netherlands).

2.4 | Conditioning phase

For each plant species, 15 individuals were grown individually in 1,050 g of live soil in 11 × 11 cm square 1 L pots, totalling 150 pots. Pots were watered three times per week and weighed weekly, during which soils were brought to equal weights of 1,050 g (approximating ~15%–17% volumetric water content). The 15 individuals of the same plant species were randomly assigned to one of three treatments (*M. brassicae*/*T. ni*/No herbivore control). For 6 weeks, all plants were grown without herbivores. Then, each plant was individually caged using hanging mesh cages (100 × 30 cm; Heinen, van der Sluijs, et al., 2018) and two freshly emerged individuals of either *M. brassicae* or *T. ni*, or no herbivore were introduced into each respective treatment cage. For 3 weeks, the insects were left to feed undisturbed, after which the insects were removed and each plant was individually examined for visual damage.

Soils were harvested by shaking out the soil from each root system, keeping the individual replicate pots separate for further use in the feedback phase. From each soil, a subsample was taken and stored at 4°C for 2 weeks until subsequent soil respiration measurements (see below).

2.5 | Feedback phase

In the feedback phase, 600 g (~14%) of conditioned soil was added to 3600 g (~86%) of sterilized soil (sterilized by γ -irradiation; Synergy

Health, Ede, The Netherlands) and homogenized. This was done to minimize potential soil legacy effects in the form of nutrients. The homogenized 4200g soil was placed in 20 × 20 cm square 4 L pots. Pots were randomly placed in a greenhouse compartment, and left to acclimatize for 10 days, during which they were watered regularly to prevent dehydration of the soil. In each pot, one seedling of each of the 10 plant species was planted using the same pattern of species in each pot. Pots were weighed and watered three times per week and set to a total weight of 4,500 g per pot. The plant communities were left to grow for 30 days. Then, the plants were caged in hanging mesh cages (100 × 30 cm), and four newly hatched *M. brassicae* larvae were added to each cage. The caterpillars were left to feed for 21 days, after which they were collected and weighed. The above-ground biomass was individually clipped per plant species and oven-dried for at least 72 hr at 60°C. The root systems were washed to remove sand, stones and foreign material and first air-dried for 1 day and then oven-dried for at least 72 hr at 60°C.

2.6 | Soil respiration analysis

Basal respiration and substrate-induced respiration were measured using the method detailed in Bradford et al. (2008), with modifications specified below. Soil samples from the ~450 g of remaining soil from the conditioning phase were taken from cold storage and kept at 20°C for 48 hr in the dark to re-activate microbes. Then, 50-ml tubes with rubber injection-plug lids were filled with 5.0–5.5 g soil. The exact weight per tube was recorded. Samples were then capped and flushed with CO₂-free air to remove any CO₂ from the headspace. After incubation at 20°C for 24 hr, 12 ml of headspace was taken from the tubes and stored in gas-tight glass vial at 4°C under dark conditions until CO₂ measurements. The same sample tubes were used to measure substrate-induced respiration (Anderson & Domsch, 1986). To each tube, 2 ml of 75 mM D-glucose solution was added and shaken in a horizontal shaker for 1 hr. Next, the samples were capped and flushed with CO₂-free air and left in the incubator for 4 hr at 20°C, in which the present microbes were allowed to convert the added glucose into CO₂, which is a proxy for total microbial biomass. Again, a sample of 12 ml was taken from the headspace and stored in a gas-tight glass vial. CO₂ concentrations were measured on a Trace CG Ultra gas chromatograph (Thermo Fisher Scientific).

Gravimetric soil moisture was determined by drying the soil samples at 60°C for 24 hr to constant weight and calculating the difference in weight between fresh and dried soil.

2.7 | Soil characteristics

Soils were air dried at 40°C until dry and then sieved through a 2-mm sieve to remove coarse fragments. Three grams of soil was transferred to a 50-mL tube and 30 ml of 0.01 M CaCl₂ was added. The mixture was shaken for 2 hr on a shaker at 250 rpm. The samples were then centrifuged for 5 min at 3000 rpm. Then, 15 ml of the

supernatant was filtered through a Whatman Puradisc Aqua 30 syringe filter with cellulose acetate membrane. To measure Fe, K, Mg, P, S, Zn, 12.87 ml of the filtrate was transferred to a 15-ml tube with 130 µl of HNO₃ and vortexed. Extracts were analysed by inductively coupled plasma–optical emission spectrometer (ICP-OES, Thermo Scientific iCAP 6500 Duo Instrument with axial and radial view and CID detector microwave digestion system). The remaining part of the filtrate was used to measure (NO₂+NO₃) and NH₄ on a QuAAtro Autoanalyzer (Seal analytical).

Soil organic matter was determined by weighing soils after drying at 105°C for 24 hr, and then weighing again after burning at 450°C for 4 hr. The soil organic matter was calculated as the percentage of dry weight lost on ignition.

2.8 | Statistical analysis

All statistical analyses were performed in R Studio version 1.1.419 (RStudio, Inc.) using R version 3.3.1 (R Development Core team, 2020).

The effects of 'conditioning plant' (10 conditioning plant species), 'herbivore treatment' (legacy of herbivory by *M. brassicae*, *T. ni* or no herbivore) and their interactions on all parameters (soil moisture, basal respiration, substrate-induced respiration, community grass: forb ratio, community shoot:root ratio and caterpillar biomass) were analysed using two-way analysis of variance (ANOVA). As there were always four caterpillars per mesocosm in the feedback phase (and these are technically pseudo-replicates within the mesocosm), the caterpillar biomass was analysed using all individual caterpillars. Individual mesocosm was then included as a random intercept to account for the multiple measurements per mesocosm, in a mixed model. All mixed models were based on parameter estimations from restricted maximum likelihood approach using the `nlme` package (Pinheiro et al., 2018). We obtained *p*-values using the `ANOVA()` command from the `CAR` package, using type II Wald Chi-square tests (Fox & Weisberg, 2019).

The effects of 'conditioning plant' (10 conditioning plant species), 'herbivore treatment' (legacy of herbivory by *M. brassicae*, *T. ni* or no herbivore) and their interactions on the (multivariate) structure of the plant community (above-ground individual biomass of all 10 species per mesocosm) and (multivariate) structure of soil abiotic parameters (Fe, K, Mg, P, S, Zn, (NO₂+NO₃), NH₄ and soil organic matter) were further analysed using nonparametric permutational analysis of variances (PERMANOVAs) on distance matrices. Distance matrices were obtained using the `vegdist()` command using Euclidean distances, and PERMANOVAs were performed using 999 permutations using the `adonis()` command in the `VEGAN` package (Oksanen et al., 2007). Ordinations were visualized using nonmetric multidimensional scaling (NMDS) by calculating centroids and showing individual samples as spider graphs using the `GGPLOT2` package (Wickham & Wickham, 2007).

To specifically test for the differences between grasses and forbs, all analyses detailed above were also performed using 'conditioning plant functional group' (instead of 'conditioning plant') as a fixed

factor. In these mixed models, 'conditioning plant' was specified as a random intercept. This way, the conditioning plant species were used as the true replicates ($n = 5$) for each functional group in this analysis.

We finally ran path models to examine the contribution of specific mechanistic pathways to explain the effects of herbivore presence during the conditioning phase on herbivore performance during the feedback phase. As predictors in the full path model, we included herbivore presence/absence (conditioning phase), individual plant biomass (conditioning phase), soil moisture (conditioning phase), soil basal respiration (conditioning phase), substrate-induced respiration (conditioning phase), plant community shoot: root ratio (feedback phase) and caterpillar biomass (feedback phase). We excluded soil nutritional parameters, as these were not affected by herbivory. A conceptual figure of the experimental overview and full model structure (with predicted direction of paths) is given in Figure 1. Path models were created separately, for grasses and forbs, as the two functional groups generally generate different (responses to) soil legacy effects (e.g. Heinze et al., 2020). Model structure in path models were the same as of the mixed-effect models earlier implemented using the NLME package (Pinheiro et al., 2018), and were run with the PIECEWISESEM package (Lefcheck et al., 2016). Shipley's test of d-separation (Fisher's C statistic, Chi-square distributed, Shipley, 2009) indicated that the full models did not meet the criteria of global goodness of fit for both grasses (Fisher's C = 120.80, $p < 0.001$) and forbs (Fisher's C = 151.46, $p < 0.001$). We have accordingly presented the results of path models with global goodness of fit based on d-separation by excluding some of the nonsignificant paths.

3 | RESULTS

3.1 | Effects of soil conditioning and above-ground herbivory on soil parameters

Conditioning plant species and herbivore treatment both had significant main effects on soil moisture (Table 1a), but the effect of each herbivore depended on the plant species that it was feeding on, indicated by a significant interaction between the two (Table 1a; Figure 2A). At the end of the conditioning phase, forb soil moisture content was 40.3% higher than that of grasses, resulting in a significant effect of functional group on soil moisture (Table 1b; Figure 2A).

Conditioning plant species had a significant effect on soil basal respiration rates (Table 1a; Figure 2B). Moreover, forb soils had 13.4% higher basal respiration rates than grass soils, indicated by a significant effect of conditioning plant functional group (Table 1b; Figure 2B). Soil basal respiration rates increased when herbivores were present on the conditioning plant (Table 1a,b; Figure 2B). However, only plants exposed to *M. brassicae* had soil basal respiration rates that were significantly (+10%) higher than controls, whereas basal respiration rates in those exposed to *T. ni*, although also being higher (+2.4%), did not differ significantly from controls (Table 1a,b; Figure 2B).

Substrate-induced respiration rate was not affected by conditioning plant species nor by herbivore treatment (Table 1a). However, it was

affected by functional group of the conditioning plant species, being higher (+7.7%) in forb soils than in grass soils (Table 1b; Figure 2C).

Conditioning plant species, and functional group significantly explained 47% and 22% of the variation in soil abiotic parameters (species: Table 2a, functional group: Table 2b; Figure S2). Herbivore legacies had no significant impact on soil abiotic characteristics.

3.2 | Effects of soil conditioning and above-ground herbivory on feedback plant community parameters

The biomass distribution within the plant community was significantly affected by conditioning plant species, although it explained only 9% of the variation (Table 3a; Figure 3A). Furthermore, the functional group of the conditioning plant species marginally affected the biomass distribution within the plant community, but this explained only 1% of the variation (Table 3b; Figure 3A). Herbivore legacies had no significant impact on plant community structure during the feedback phase (Table 3a,b).

Conditioning plant species, or functional group, did not significantly affect shoot: root ratio of the feedback plant community (Table 1a,b). Herbivore legacies tended to decrease the shoot: root ratio, but the effect was only marginally significant ($p = 0.099$ and $p = 0.092$, in the two models, respectively, Table 1a,b; Figure 3B). However, when we grouped both herbivores in one category, we observed that the legacy of herbivory significantly decreased shoot: root ratios ($\chi^2_{1,146} = 4.67$, $p = 0.032$).

Conditioning plant species and functional group both significantly affected the grass: forb ratio in the feedback plant community ($F_{9,120} = 4.47$, $p < 0.000$; and $\chi^2_{1,2} = 6.15$, $p = 0.013$ for conditioning species and functional groups, respectively), whereas herbivory had no effect on grass: forb ratio in either model. Grass: forb ratios in the response communities were higher on forb soils than on grass soils, and these patterns were strongest for *P. lanceolata* and *A. millefolium* soils for forbs, and on *P. pratense* soils for grasses (Figure 3C).

3.3 | Effects of soil conditioning and above-ground herbivory in the conditioning phase on above-ground herbivores in the feedback community

In both the species-specific and functional group-specific models, the herbivore legacy affected caterpillar biomass in the feedback phase. Specifically, on soils with a herbivore legacy of *T. ni*, *M. brassicae* caterpillars in the feedback phase were 8% smaller compared with the no herbivore legacy. On soils with a legacy of *M. brassicae*, in the feedback phase caterpillars were 6.1% larger compared with the no herbivore legacy, indicated by marginally significant (Table 4a), and significant effect (Table 4b) of herbivore legacy on caterpillar biomass in the feedback community. Although the biomass of the *M. brassicae* caterpillars significantly differed between the two herbivore treatments, none differed significantly from the control treatment (Figure 4A).

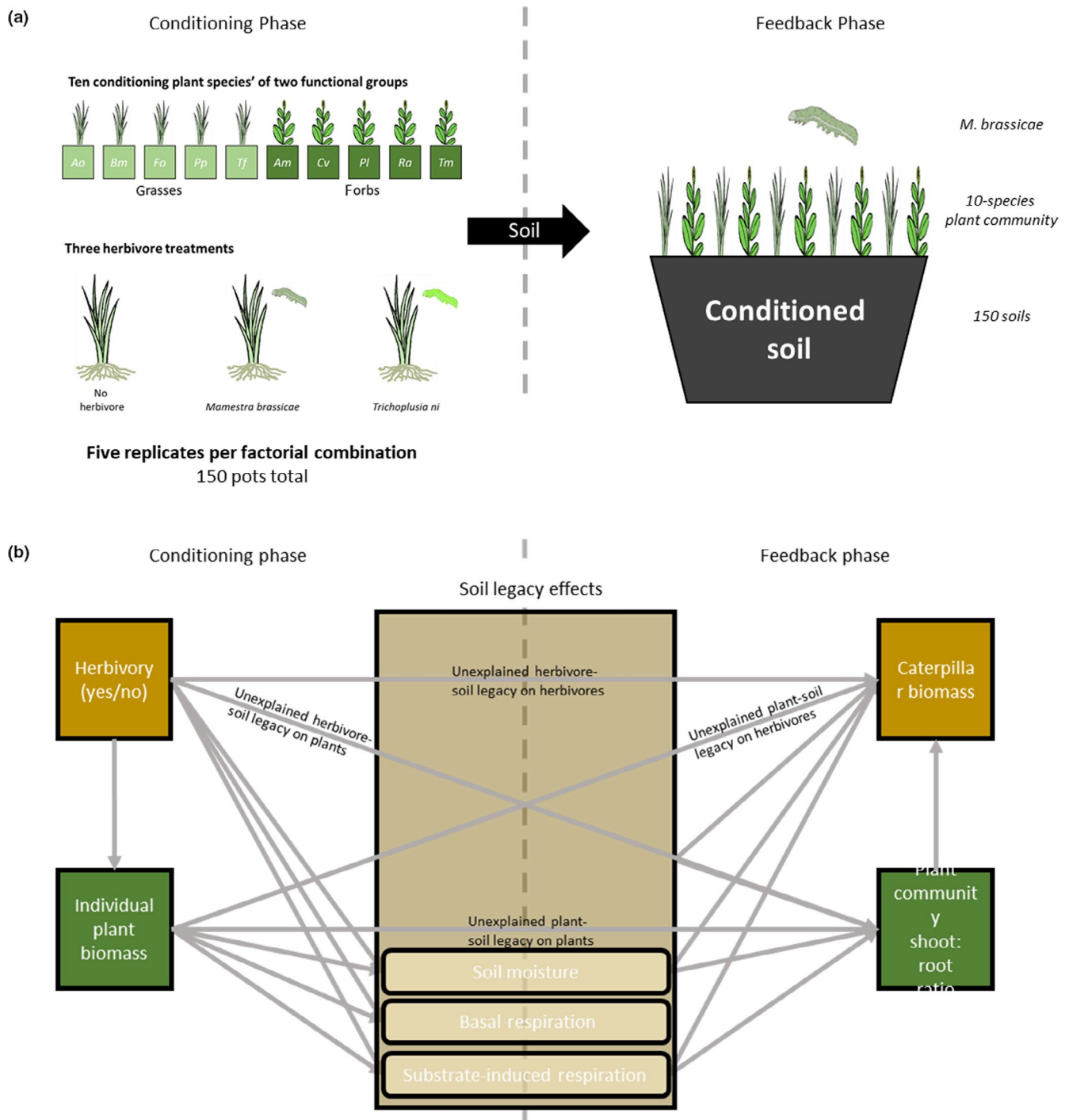


FIGURE 1 (A) Conceptual overview of the experimental design. The experimental setup consisted of two phases, a conditioning phase and a feedback phase. During the conditioning phase, individual plants were grown in live soil, and either exposed to herbivory or kept free of herbivores. At the end of the conditioning phase, soil was collected individually, and three parameters, soil moisture, soil basal respiration and substrate-induced respiration were measured. These soils were then used in the feedback phase to grow plant communities composed of all species from the conditioning phase. Finally, four individuals of an insect herbivore, *Mamestra brassicae*, were introduced, and weighed after 3 weeks of feeding, at the final harvest. (B) Conceptual representation of our path model. The figure includes all potential investigated paths included in our full path models. Final models and model output are presented in Figure 5

Overall, shoot: root ratio of the feedback plant community negatively correlated with individual *M. brassicae* caterpillar biomass in the feedback phase ($R^2 = 0.016$; $F_{1,403} = 6.47$; $p = 0.011$, Figure 4B) indicating that a higher investment of the plant community in shoot

relative to root biomass was associated with smaller caterpillars. Because of this observed correlation, we further tested whether shoot: root ratio in feedback phase and conditioning treatments interactively explained the variation in caterpillar biomass in our two

TABLE 1 (a) Model 1: Output of linear (mixed) models with 'conditioning plant species' (10 conditioning plant species), 'herbivore treatment' (*Mamestra brassicae*/*Trichoplusia ni*/no herbivore control) and interactions as factors. (b) Model 2: Output of linear mixed models with 'conditioning plant functional group' (grass or forb), 'herbivore treatment' (*M. brassicae*/*T. ni*/no herbivore control), and interactions as factors, with 'conditioning plant species' as random factor to use species as true replicates. Both models were used to analyse the response variables soil moisture, basal respiration, substrate-induced respiration after the conditioning phase, and plant community shoot and root biomass, shoot:root ratio and grass:forb ratio, at the end of the feedback phase. Values presented are *F*-values followed by *p*-values in parentheses (for the linear models), or χ^2 values followed by *p*-values in parentheses (for the mixed models). Statistically significant ($p < 0.05$) values are given in bold, and marginally significant trends ($0.05 < p < 0.10$) are presented in italic

		Soil moisture	Basal respiration	Substrate-induced respiration	Shoot biomass	Root biomass	Shoot:root ratio	Grass:forb ratio
(a) Model 1	<i>df</i> 1, <i>df</i> 2	<i>F</i> (<i>p</i>)	<i>F</i> (<i>p</i>)	<i>F</i> (<i>p</i>)	<i>F</i> (<i>p</i>)	<i>F</i> (<i>p</i>)	<i>F</i> (<i>p</i>)	<i>F</i> (<i>p</i>)
Conditioning plant species	9, 149	17.0 (<0.001)	4.9 (<0.001)	1.6 (0.134)	1.6 (0.136)	1.6 (0.110)	0.7 (0.744)	4.7 (<0.001)
Herbivore treatment	2, 149	3.4 (0.037)	4.5 (0.013)	1.7 (0.180)	0.6 (0.574)	2.7 (0.071)	2.4 (0.092)	0.6 (0.529)
Cond × herb	18, 149	1.7 (0.049)	1.1 (0.374)	1.1 (0.315)	0.6 (0.907)	1.7 (0.054)	1.4 (0.126)	1.5 (0.101)
(b) Model 2		χ^2 (<i>p</i>)	χ^2 (<i>p</i>)	χ^2 (<i>p</i>)	χ^2 (<i>p</i>)	χ^2 (<i>p</i>)	χ^2 (<i>p</i>)	χ^2 (<i>p</i>)
Conditioning plant functional group	1, 8	14.6 (<0.001)	7.8 (0.005)	5.8 (0.016)	1.1(0.294)	0.3 (0.592)	0.5 (0.483)	6.2 (0.013)
Herbivore treatment	2, 136	6.2 (0.044)	8.8 (0.012)	3.3 (0.194)	1.2 (0.557)	4.9 (0.085)	4.6 (0.099)	1.2 (0.550)
Funct × herb	2, 136	2.2 (0.336)	0.2 (0.904)	1.5 (0.464)	0.8 (0.672)	0.8 (0.683)	0.3 (0.882)	1.1 (0.571)

separate models (species-specific or functional group-specific models; Table 4a,b). The main effect of shoot:root ratio on caterpillar biomass as described above, disappeared in the species-specific model (Table 4a), as the effect differed between conditioning plant species, indicated by a significant interaction between shoot: root ratio and conditioning plant species (Table 4a). Specifically, negative relationships between shoot: root ratio and caterpillar biomass were observed on soils conditioned by *Plantago* and *Rumex* (two forbs), and *Anthoxanthum*, *Festuca* and *Trisetum* (three grasses). In contrast, the relationship between shoot: root ratio and caterpillar biomass was positive on soils conditioned by *Clinopodium* (forb) and *Briza* (grass), and relationships were absent in the remaining soils (Figure 4C). In the functional group-specific model (Table 4b), the significant effect of shoot:root ratio on caterpillar biomass was still present, and did not differ statistically between grasses and forbs (Table 4b).

3.4 | Path models

The path models revealed that variation in herbivore biomass in the feedback phase could be partly explained by legacy effects of herbivore presence in the conditioning phase, and by conditioning plant biomass, but that the pathways differed between grasses and forbs. In general, herbivore presence in the conditioning phase significantly explained the negative effect on individual plant biomass in both grasses (R^2 : 0.66) and forbs (R^2 : 0.93). We measured soil respiration parameters as explanatory variables in our study, but different variables offered an optimal model fit in grasses and forbs. In conditioning grasses, herbivory positively affected substrate-induced

respiration, and individual plant biomass had a marginally positive effect on substrate-induced respiration. Together these explained a rather low amount (R^2 : 0.07) of the variation in substrate-induced respiration. In forbs, a marginally positive effect of herbivory was observed on basal respiration, whereas basal respiration was strongly negatively impacted by individual plant biomass. Together, herbivory and plant biomass explained a large amount (R^2 : 0.58) of the variation in basal respiration (Figure 5). In conditioning grasses and forbs, substrate-induced respiration and basal respiration, respectively, both positively affected shoot: root ratios in the feedback phase, which in turn had a negative effect on caterpillar biomass in the feedback phase, but this was only significant in grasses. In forbs, basal respiration had a marginal negative effect on caterpillar biomass in the feedback phase.

Various 'direct' paths further connected the conditioning and feedback phases, both in grasses and forbs, and these should be interpreted as indirect and soil-mediated processes, as soil was the only medium connecting the two phases. Specifically, for grasses, herbivory during the conditioning phase had a negative effect on shoot: root ratio of the feedback community, whereas individual plant biomass had a positive effect. Furthermore, herbivory in the conditioning phase had a marginally negative effect on caterpillar biomass in the feedback phase. For forbs, on the other hand, both herbivory and individual plant biomass had negative effects on shoot: root ratio of the feedback phase community. The variance explained in shoot: root ratio and caterpillar biomass in the feedback communities was similar for grasses and forbs (shoot: root: R^2 : 0.12 and R^2 : 0.10; caterpillar biomass: R^2 : 0.30 and R^2 : 0.31 for grasses and forbs, respectively; Figure 5).

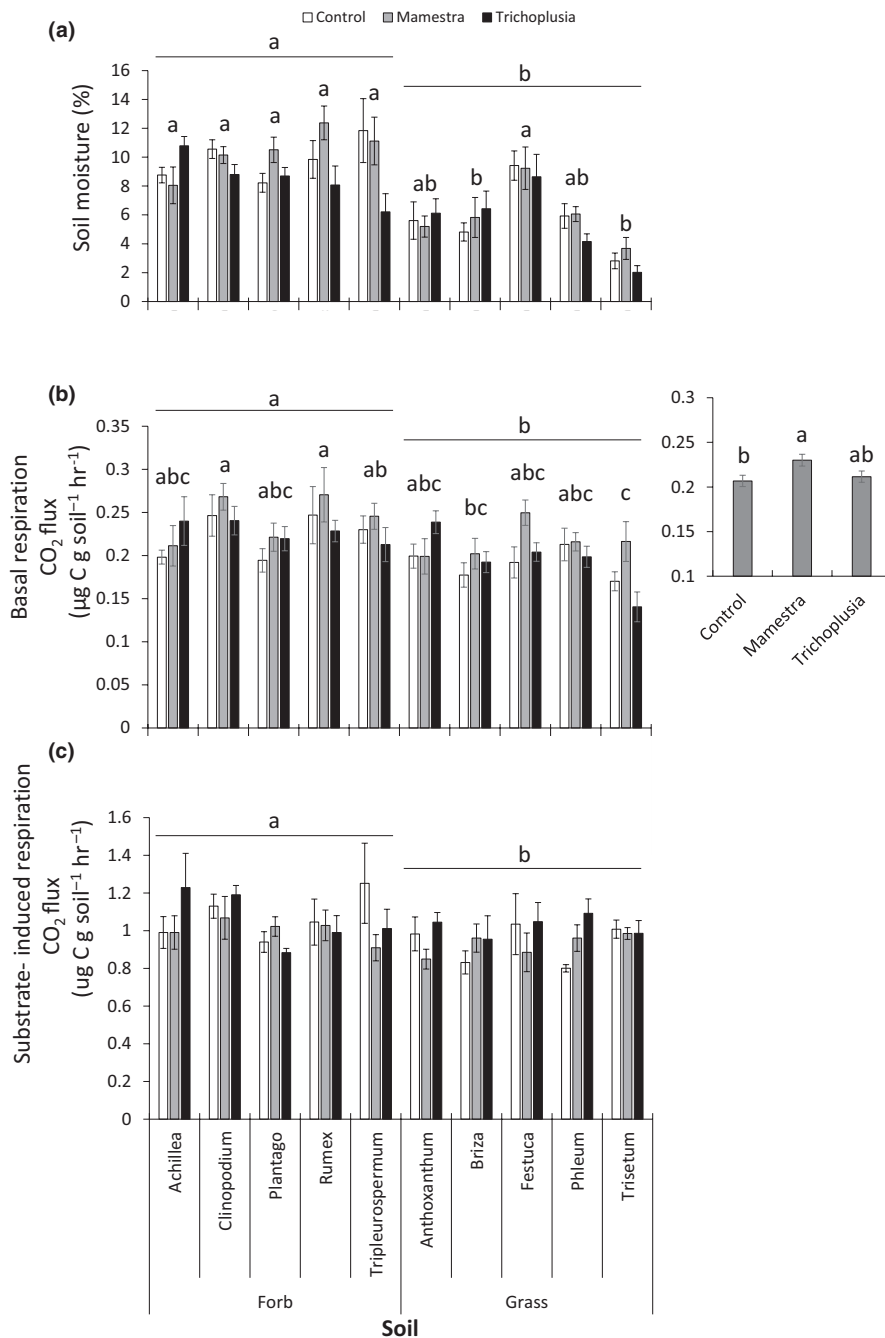


FIGURE 2 The effects of conditioning plant species, plant functional group and above-ground herbivore treatment on (A) soil moisture, (B) soil basal respiration (right hand panel visualizes the main effect of above-ground herbivory) and (C) substrate-induced respiration. Bars represent means, with error bars representing standard errors ($n = 5$). White bars represent no-herbivore treatment, grey bars represent *Mamestra brassicae* treatment and black bars represent *Trichoplusia ni* treatment

4 | DISCUSSION

In this study, we tested whether foliar herbivory by insect herbivores alters plant-mediated soil legacy effects on subsequent plant communities and on the insect herbivores that feed on those communities. We show that conditioning plant species and herbivore treatments both left measurable legacy effects in the soil that affected the responding plant community, as well as herbivores feeding on the plants. Specifically, conditioning plants affected soil microbial activity patterns, and in turn shifted the community structure of the responding plant community. The legacy of the herbivore treatment negatively affected shoot: root ratios of the response community, which in turn, negatively affected caterpillar biomass.

Importantly, the effects differed between conditioning grasses and forbs. By taking a community approach and using a wide range of grassland plant species, our study expands on earlier findings that herbivory on ragwort, *J. vulgaris*, changes microbial legacies in the soil, that in turn affected later plant-insect interactions (Bezemer et al., 2013; Kostenko et al., 2012).

Contrary to our hypothesis, we found that the soil legacy of the herbivory treatment led to a significant increase, not a decrease, in soil basal respiration (a proxy for baseline microbial activity), but it did not alter substrate-induced respiration (a proxy for total microbial biomass). Previous studies have indicated that plant-mediated soil legacy effects on plant-insect interactions may be microbially mediated (e.g. Hannula et al., 2019; Howard et al., 2020), and it is

TABLE 2 (a) Model 1: Output of permutational ANOVAs for the effects on soil abiotic characteristics (Fe, K, Mg, P, S, Zn, (NO₂+NO₃), NH₄ and soil organic matter) of 'conditioning plant species' (10 conditioning plant species), 'herbivore treatment' (*Mamestra brassicae*/*Trichoplusia ni*/no herbivore control), and interactions. (b) Model 2: Output of permutational ANOVAs with 'conditioning plant functional group' (grass or forb), 'herbivore treatment' (*M. brassicae*/*T. ni*/no herbivore control) and interactions as factors, with 'conditioning plant species' as random factor. *p*-Values are based on 999 permutations. Presented are explained variance (*R*²), *F*-values, followed by *p*-values in parentheses. Statistically significant (*p* < 0.05) values are given in bold, and marginally significant trends (0.05 < *p* < 0.10) are presented in italics

(a) Model 1	df1, df2	Soil abiotic characteristics	
		<i>R</i> ²	<i>F</i> (<i>p</i>)
Conditioning plant species	9, 149	0.47	13.6 (0.001)
Herbivore treatment	2, 149	0.01	1.6 (0.147)
Cond × herb	18, 149	0.06	0.9 (0.609)
(b) Model 2			
Conditioning plant functional group	1, 149	0.22	42.5 (0.001)
Herbivore treatment	2, 149	0.01	1.2 (0.327)
Funct × herb	2, 149	0.01	1.0 (0.371)

thought that the soil legacy effects of herbivores are also, at least partly, mediated by microorganisms (Bezemer et al., 2013; Kostenko et al., 2012). Our results suggest that foliar herbivory can stimulate the baseline activity of soil organisms, but not their potential activity. This is interesting, as above-ground herbivory often has antagonistic effects on various individual taxa in the soil, including mycorrhizal fungi and soil arthropods (e.g. De Román et al., 2011; Erb et al., 2011; Johnson et al., 2012), mediated by the herbivore-induced systemic upregulation of plant defenses (Bezemer & van Dam, 2005; Huang et al., 2014; Li et al., 2016). These studies generally assess individual groups. It is likely that an assessment of activity of the entire soil community leads to different results. Given that herbivory changes rhizodeposition in the soil upon herbivory (Holland et al., 1996), it is likely that soil communities respond to this, and this may explain why we find an upregulation of activity in the soil. Several studies have shown that plants can recruit beneficial organisms in their rhizosphere upon attack by above-ground antagonists such as pathogens (Berendsen et al., 2018), and it is plausible that an attraction or stimulation of (beneficial) organisms, for instance via increases in rhizodeposits (Holland et al., 1996), may explain our findings. It is important to note that in our study soil respiration was measured in undiluted conditioned soil, whereas the plant community test phase was performed on 1:6 dilution of soils and hence our observations in the feedback phase may underestimate the microbial effects on response plant communities. Obviously, there are key differences between measuring individual taxa or soil communities, and soil community respiration data. Respiration rates provide an indication that parts of the soil community become more active, but do not

TABLE 3 (a) Model 1: Output of permutational ANOVAs with 'conditioning plant species' (10 conditioning plant species), 'herbivore treatment' (*Mamestra brassicae*/*Trichoplusia ni*/no herbivore control), and interactions as factors. (b) Model 2: Output of permutational ANOVAs with 'conditioning plant functional group' (grass or forb), 'herbivore treatment' (*M. brassicae*/*T. ni*/no herbivore control) and interactions as factors, with 'conditioning plant species' as random factor to use species as true replicates. Both models were used to analyse plant community structure (using Euclidean distance matrices calculated on individual biomass of the plant species in the community) with 999 permutations. Presented are explained variance (*R*²), *F*-values, followed by *p*-values in parentheses. Statistically significant (*p* < 0.05) values are given in bold, and marginally significant trends (0.05 < *p* < 0.10) are presented in italics

(a) Model 1	df1, df2	Plant community structure	
		<i>R</i> ²	<i>F</i> (<i>p</i>)
Conditioning plant species	9, 149	0.09	1.5 (0.031)
Herbivore treatment	2, 149	0.01	1.0 (0.470)
Cond × herb	18, 149	0.12	1.0 (0.499)
(b) Model 2			
Conditioning plant functional group	1, 149	0.014	2.1 (0.064)
Herbivore treatment	2, 149	0.012	0.9 (0.508)
Funct × herb	2, 149	0	0.28 (0.985)

provide any information on the taxa that are responsible for this increase in activity. We may speculate that an increased recruitment by the plant of beneficial soil organisms on above-ground herbivore attack could explain the increased microbial activity in our study. However, future work including more focused approaches, for example, sequencing, enzyme activity assays or analysis of the soil transcriptome, to determine what kind of soil functionality is up-regulated in the soil when plants experience foliar herbivory, are needed to test such hypotheses.

Conditioning soils with different plant species led to measurable effects in soil respiration, which in turn affected the plant community that developed later in the same soil. However, neither total shoot biomass (i.e. the productivity) of the plant community, nor the individual biomasses of the feedback plant species were affected by any of our herbivory treatments. We did observe strong functional group-level responses, indicated by conditioning effects on grass: forb ratios in the feedback communities. Grass: forb ratios were higher on forb soils than on grass soils, indicating a negative (functional group) plant-soil feedback effect (de Kroon et al., 2012; Heinen, van der Sluijs, et al., 2018; Heinen, Hannula, et al., 2020; Petermann et al., 2008). The conditioning plant species, as well as their functional group (albeit marginally significant), also altered plant community structure. This shows that the distribution of biomass across plant species within the communities differed between soils. It may suggest that soil legacies can alter competitive balances between plants within communities. Previous work by our group has shown similar effects of conditioning plants in simpler feedback

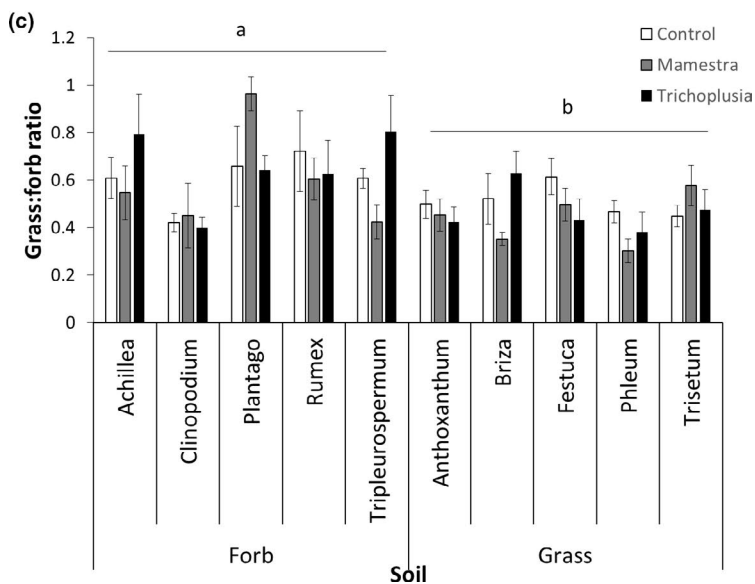
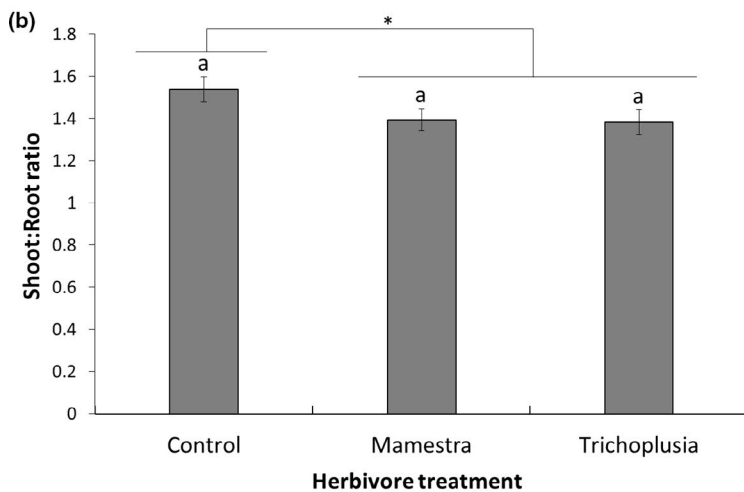
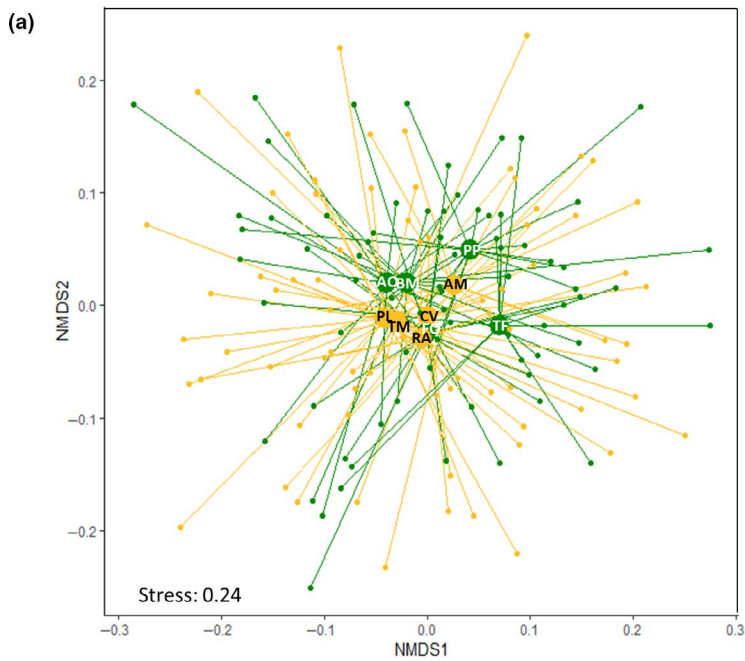


FIGURE 3 Soil conditioning effects on the feedback plant community. In (A) the ordination plot visualizes the effects of conditioning plant species on the structure of the response plant community, using nonmetric multidimensional scaling based on a Bray–Curtis dissimilarity matrix. Stress value = 0.24. Shown are spider plots with centroids, including all samples of the herbivory treatment (control, *Mamestra brassicae* and *Trichoplusia ni*) for each of the conditioning plant species, as main effects of herbivory treatment on plant community structure were absent (see Table 2). Green dots indicate grass species, and yellow dots indicate forb species. Panel (B) shows the effects of the herbivory treatment on the shoot:root ratio of the response plant community. Bars represent means with error bars. The three herbivory levels did not significantly differ, indicated by the same letters derived from posthoc Tukey tests. Herbivory (measured as absence/presence) significantly lowered plant community shoot:root ratios, indicated by the asterisk

TABLE 4 (a) Model 1: Output of a linear mixed model with 'conditioning plant species' (10 conditioning plant species), 'herbivore treatment' (*Mamestra brassicae*/*Trichoplusia ni*/no herbivore control) and interactions as factors and 'mesocosm' as random factor to account for multiple caterpillars per mesocosm. (b) Model 2: Output of a linear mixed model with 'conditioning plant functional group' (grass or forb), 'herbivore treatment' (*M. brassicae*/*T. ni*/no herbivore control), and interactions as factors, with 'conditioning plant species' as random factor to use species as true replicates and 'mesocosm' as random factor to account for multiple caterpillars per mesocosm. Both models were used to analyse caterpillar biomass at the end of the feedback phase. Log(x) transformations were used to meet model assumptions. Values presented are *F*-values followed by *p*-values in parentheses (for the linear models) or χ^2 values followed by *p*-values in parentheses (for the mixed models). Statistically significant ($p < 0.05$) values are given in bold, and marginally significant trends ($0.05 < p < 0.10$) are presented in italics

		Caterpillar biomass
(a) Model 1	<i>df1, df2</i>	χ^2 (<i>p</i>)
Shoot:root ratio	1, 90	1.82 (0.177)
Conditioning plant species	9, 90	6.02 (0.738)
Herbivore treatment	2, 90	3.87 (0.144)
S:R × cond	9, 90	20.95 (0.013)
S:R × herb	2, 90	4.64 (0.098)
Cond × herb	18, 90	24.97 (0.126)
S:R × cond × herb	18, 90	11.97 (0.849)
(b) Model 2		χ^2 (<i>p</i>)
Shoot:root ratio	1	7.08 (0.008)
Conditioning plant functional group	1	1.23 (0.267)
Herbivore treatment	2	4.79 (0.091)
S:R × func	1	1.70 (0.191)
S:R × herb	2	4.11 (0.128)
Func × herb	2	2.18 (0.337)
S:R × func × herb	2	0.07 (0.966)

plant communities, composed of four species (Heinen, van der Sluijs, et al., 2018), as well as multi-species communities (commonly >30 species) in the field (Heinen, Hannula, et al., 2020), and the consistency in these findings across different soils and growth conditions by various studies and by different research groups (Bezemer et al., 2005; Bukowski et al., 2018; Cortois et al., 2016; Geisen et al., 2022; Kulmatiski et al., 2008; Petermann et al., 2008), indicates predictable patterns in plant–soil interactions in grasses and forbs, with conditioning by either of the two groups suppressing growth of plants of their own functional group, and promoting the other.

In accordance with our hypothesis, the soil legacy of the herbivory treatment significantly affected biomass of *M. brassicae* caterpillars feeding on the above-ground tissues in the responding plant communities. Remarkably, a legacy of conspecific herbivory (*M. brassicae*) positively affected herbivores on the responding plant communities,

whereas heterospecific feeding during the conditioning phase by a closely related noctuid, *Trichoplusia ni*, created soil legacy effects that negatively affected *M. brassicae* caterpillars. These results suggest that different herbivores can impose distinct legacy effects and, that even closely related herbivores differ in how they induce defense responses in plants, and in how this changes soil conditions. This challenges the classical idea that phylogenetically closely related organisms elicit similar defense responses in plants (Karban & Baldwin, 2007).

The soil legacy of the herbivory treatment altered the responding plants via shifting the allocation of plant biomass from shoots to roots (i.e. lowering the shoot: root ratio). This might be a potential mechanism explaining the observed herbivore responses in the feedback community. As indicated by our path models, in soils with a legacy of above-ground herbivory, plant communities allocate more biomass to below-ground than to above-ground parts. Plants commonly increase their biomass allocation to roots in direct response to above-ground herbivores (e.g. Dong et al., 2018; Schwachtje et al., 2006; Strauss & Agrawal, 1999). Importantly, our results indicate that a legacy of herbivory in the soil can also cause a similar—but indirect—response in plant communities. Herbivore treatments created soil legacy effects that altered plant biomass allocation patterns, and these in turn affected the interaction between plants and above-ground herbivores in the feedback phase. Indeed, in our study, caterpillar biomass in the feedback phase was negatively affected by plant community shoot: root ratio. Various mechanistic explanations have been put forward for soil–plant–insect interactions, such as soil-mediated phytohormonal and secondary defense responses in plants (Badri et al., 2013; Howard et al., 2020; Huberty et al., 2020; Joosten et al., 2009; Kos et al., 2015; Ristok et al., 2019; Zhu et al., 2018), as well as direct effects of soil microbes on insect herbivore performance (Hannula et al., 2019). At least in grasses, we could show that shifts in (substrate-induced) soil microbial respiration caused by herbivore presence during the conditioning phase play an important role in determining biomass allocation in the feedback phase with subsequent effects for feedback phase herbivores. Our results suggest that soil legacies can also determine future plant–insect interactions, mediated by alterations to plant resource allocation patterns, with detrimental effects to the herbivores. This is important, as it suggests that plants may be able to sense and respond to indicators of previous above-ground herbivore presence by investing in below-ground plant parts that are not accessible to future above-ground herbivores.

Lastly, we observe that a large part of herbivore and plant soil legacies is not explained by the soil respiration parameters that we recorded in our study, but yet, that both herbivory and plant species drive a large proportion of 'unexplained' variation in the plants and insect herbivores in the response phase. This suggests that there are other variables at play, that may not be visualized by a rather coarse method, such as soil respiration. Herbivores, for instance, may influence the soil below them, through the frass they deposit, which may affect various soil parameters of biotic and abiotic origin. For conditioning plants, it seems likely that quantity and quality of rhizodeposits may play an important role in influencing the soil. It

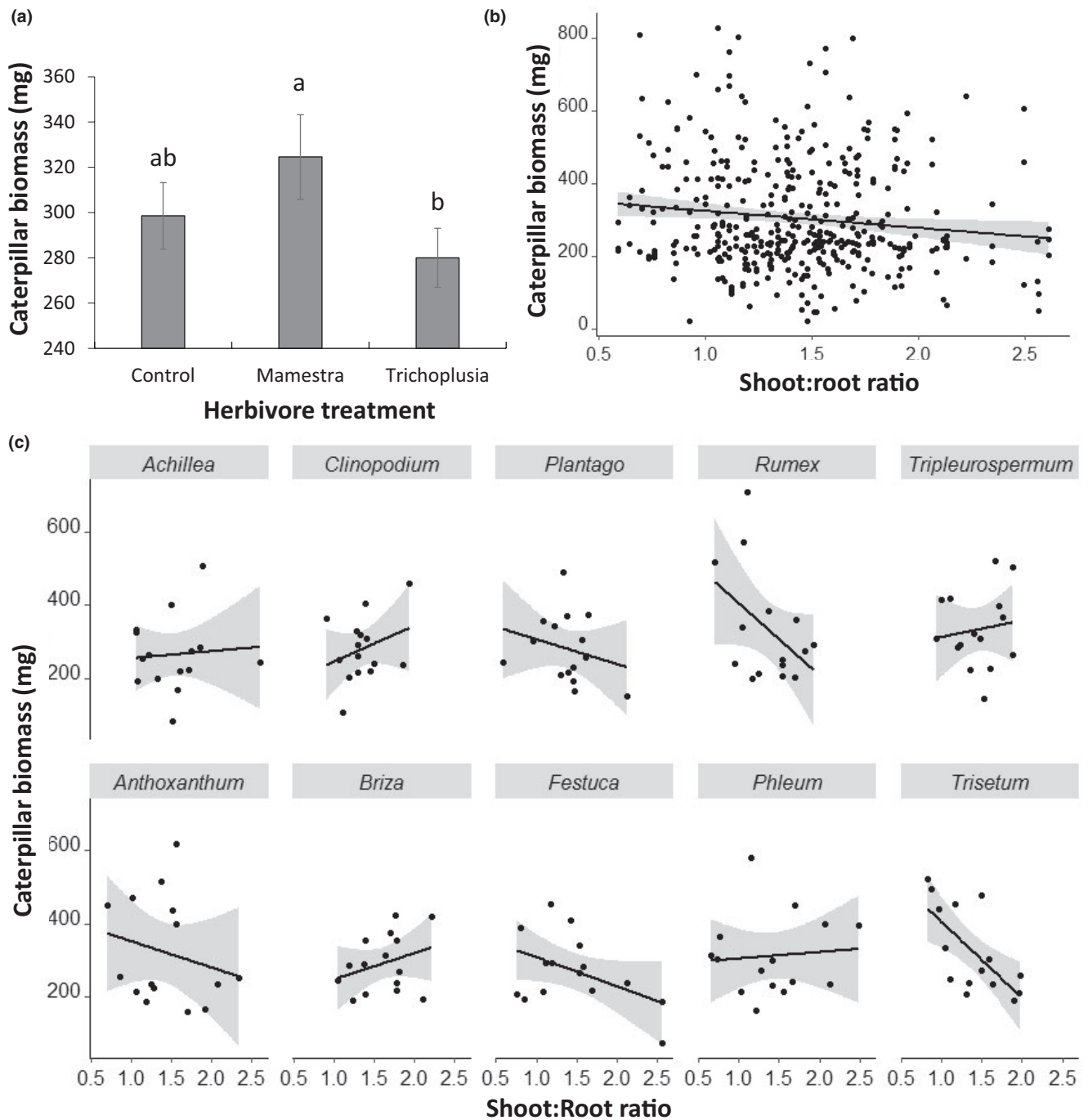


FIGURE 4 (A) The effects of the herbivory treatment during conditioning on the performance of *Mamestra brassicae* in the feedback plant community. (B) There was a significant correlation between plant community shoot:root ratio and *M. brassicae* caterpillar biomass in the feedback plant community, but only in Model 2 (Table 4). (C) The correlation between plant community shoot:root ratio and caterpillar biomass in the feedback plant community depended strongly on conditioning plant species in Model 1 (Table 4). For complete statistical output see Table 4

is likely that the relative abundances of mutualistic or antagonistic organisms in the soil are affected by both plant and herbivore conditioning. Disentangling the effects that herbivores and their host plants may have on these soil parameters, and the impacts they may have on subsequent plant growth and quality, and either directly, or indirectly via the plant, on the associated herbivores, could help explain this large proportion of unexplained variation.

5 | CONCLUSIONS

In this study, we show that the presence of herbivory on plants can leave a lasting legacy in the soil, in addition to the legacies that plants themselves leave in the soil. Plant communities respond to such herbivore legacies accordingly, by allocating more biomass to root, rather than to shoot. These findings indicate a

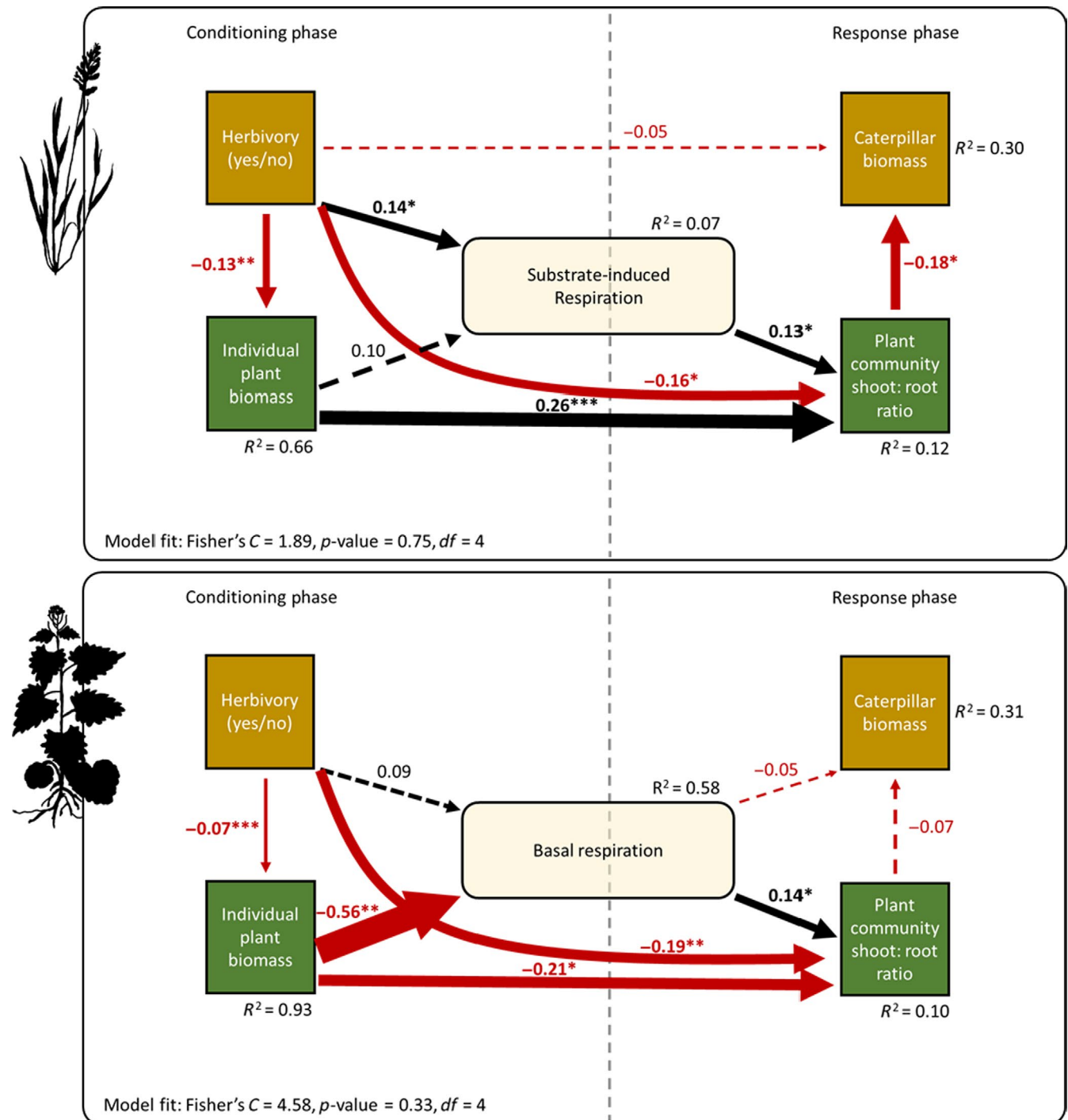


FIGURE 5 Path models indicating the effects of direct and indirect paths from herbivory treatment in the conditioning phase, via conditioning plant biomass, soil basal respiration or substrate-induced respiration, plant community shoot: root ratio, to caterpillar biomass in the feedback plant community. Path models were performed with herbivory treatment included as absence/presence (as path models do not allow three-level factors) and were performed separately for conditioning grasses (top panel) and forbs (bottom panel). These separated path models indicate that the legacy of herbivory consistently lowers plant community shoot: root ratio, and that this negatively affects *Mamestra brassicae* biomass in the feedback plant community, but more strongly so in grass than in forb-conditioned soils. Arrow thickness represents effect sizes. Red arrows indicate negative path effects, and black arrows indicate positive path effects. Thick full arrows represent significant paths, and dashed arrows indicate marginally significant paths. The R^2 are conditional R^2 that accounts for both fixed and random effects

novel way via which plants may sense and anticipate herbivore presence. Moreover, we show that two closely related herbivores create significantly different and contrasting legacy effects. Our

results confirm that soil legacy effects induced by previous insect herbivores on subsequent herbivores, as has been observed in individual plant species (Bezemer et al., 2013; Kafle & Wurst, 2019;

Kostenko et al., 2012), also play a role in a plant community setting. This begs the important question whether herbivore-driven soil legacy effects are present in natural ecosystems, and warrants future research in this area. Our results highlight that the presence of herbivory does not only directly impact the host plant but can also leave lasting effects in the soil, influencing later generations of plants, as well as future herbivores (Stam et al., 2019; Wurst & Ohgushi, 2015).

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CONFLICT OF INTEREST

The authors declare no conflict of interest. Madhav P. Thakur is an Associate Editor of Functional Ecology but took no part in the peer review and decision-making processes for this paper.

AUTHORS' CONTRIBUTIONS

R.H. designed the experiment; T.M.B., M.P.T., J.R.H.D.F. and K.S. provided additional ideas and comments that improved the experimental design and analyses; R.H. and J.R.H.D.F. performed the feedback experiment; J.R.H.D.F. and K.S. performed the soil respiration analyses; R.J. performed the soil chemistry analyses; R.H. and M.P.T. performed statistical analyses; R.H. led the writing of the manuscript. All authors contributed critically to the final version of the manuscript and approved it for publication.

DATA AVAILABILITY STATEMENT

Data associated with the publication are archived in Dryad Digital Repository <https://doi.org/10.5061/dryad.8kpr4xq9> (Heinen et al., 2022).

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