Legacy effects of aboveground-belowground interactions

LETTER

Legacy effects of aboveground–belowground interactions

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Abstract

Root herbivory can greatly affect the performance of aboveground insects via changes in plant chemistry. These interactions have been studied extensively in experiments where aboveground and belowground insects were feeding on the same plant. However, little is known about how aboveground and belowground organisms interact when they feed on plant individuals that grow after each other in the same soil. We show that feeding by aboveground and belowground insect herbivores on ragwort (*Jacobaea vulgaris*) plants exert unique soil legacy effects, via herbivore-induced changes in the composition of soil fungi. These changes in the soil biota induced by aboveground and belowground herbivores of preceding plants greatly influenced the pyrrolizidine alkaloid content, biomass and aboveground multitrophic interactions of succeeding plants. We conclude that plant-mediated interactions between aboveground and belowground insects are also important when they do not feed simultaneously on the same plant.

Keywords

Fungal community, herbivory, *Jacobaea vulgaris*, parasitoid, plant secondary chemistry, plant–soil interactions, plant–soil feedback.

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INTRODUCTION

A rapidly increasing number of studies is showing that belowground herbivory can influence plant growth and the composition and concentration of aboveground primary and secondary plant compounds (reviewed in Blossey & Hunt-Joshi 2003; Johnson *et al.* 2008; Van Dam 2009). Through these changes in host plant quantity and quality, root herbivores can affect the growth and survival of foliar feeding herbivores (e.g. Bezemer *et al.* 2003; Kaplan *et al.* 2008; Erb *et al.* 2011; Van Dam & Heil 2011), as well as the enemies of these herbivores (Bezemer *et al.* 2005; Soler *et al.* 2005). These studies have focused on interactions that occur simultaneously and on a shared host plant. Little is known about the temporal dynamics of belowground-aboveground interactions and their feedback effects (Bardgett *et al.* 2005; Van der Putten *et al.* 2009). Here, we show how aboveground and belowground herbivores can create soil legacy effects that affect the growth and nutritional quality of subsequent plants, as well as the aboveground multitrophic interactions occurring on those plants. These transferrable aboveground-belowground interaction effects due to induced legacy effects in the soil community have received little, if any, attention in ecology.

Ecological soil legacies can arise from effects on soil biota that subsequently affect the growth of plants colonising the soil at a later stage (Kardol *et al.* 2007; Van de Voorde *et al.* 2011). Both root and foliar herbivores can alter the composition of the soil microbial community (Bardgett & Wardle 2010; Bennett 2010). Belowground herbivores can directly interact with soil microorganisms through competition, facilitation or predation, and indirectly through their effects on the quality and quantity of root tissues, root exudates, and organic matter content in the soil (Anderson *et al.* 1983;

Bardgett *et al.* 1999; Van Dam 2009). Aboveground herbivores are physically separated from soil organisms. Nevertheless, they can influence soil microbial communities, for example by affecting the amount or quality of root exudates, or by altering the allocation or production of biomass, nutrients or allelochemicals to root tissues (Bardgett *et al.* 1998; Mikola *et al.* 2001; Soler *et al.* 2007; Hamilton *et al.* 2008).

A number of studies have shown that soil microorganisms can influence not only plant growth, but also aboveground plant nutritional quality (Gange 2007; Bonkowski *et al.* 2001; Bezemer *et al.* 2005; Eisenhauer *et al.* 2010; Hol *et al.* 2010). This can subsequently affect aboveground herbivores and their antagonists (Van Dam & Heil 2011). Thus, alteration of the soil microbial community by aboveground and belowground insect herbivory could potentially lead to soil legacy effects that impact the growth or nutritional quality of another plant individual, and its interactions with herbivores and carnivores. The aim of this study was to test whether such aboveground-belowground multitrophic interactions can occur.

We tested the hypothesis that aboveground and belowground herbivory will lead to legacy effects in soil that will subsequently affect the growth and chemistry of plants growing later in the same soil, and their interactions with aboveground herbivores and natural enemies. We further studied if legacy effects of belowground herbivory differ from those arising from aboveground herbivory. To test our hypothesis we performed a greenhouse experiment in two phases. In the first phase, plants were exposed to aboveground and belowground herbivory in a full factorial design. At the end of this phase the composition of the soil microbial community was determined. In the second phase, new plants were grown in the conditioned soils to assess whether the legacy effects of aboveground and belowground

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herbivory influenced plant growth, and primary and secondary plant compounds in the foliage. We then exposed these plants to aboveground insect herbivores and parasitoids, to determine the soil legacy effects on aboveground multitrophic interactions.

MATERIALS AND METHODS

Plant and insects

The study system consisted of ragwort plants, *Jacobaea vulgaris* Gaertn. ssp. *vulgaris* (synonym *Senecio jacobaea L.,* Asteraceae), wireworms as belowground herbivores (*Agriotes lineatus* L. Coleoptera: Elateridae), *Mamestra brassicae* L. (Lepidoptera: Noctuidae) caterpillars as aboveground herbivores, and *Microplitis mediator* Haliday (Hymenoptera: Braconidae) as parasitoid of the aboveground herbivore. Ragwort is a biennial monocarpic plant native to Europe and Asia, where it is widely distributed (Bezemer *et al.* 2006). It produces a variety of pyrrolizidine alkaloids (PAs), a group of plant defence compounds that are toxic to a wide range of generalist insects and soil organisms (Hol & Veen 2002; Thoden & Boppre 2010; Macel 2011). PAs are constitutively biosynthesised in roots as senecionine N-oxide, which is transformed into several related senecionine-type PAs. These PAs are transported to aboveground plant parts where additional diversification takes place (Hartmann 1999; Cheng *et al.* 2011). The concentration and composition of PAs can alter in response to abiotic factors, and to interactions of the plant with other aboveground and belowground organisms (Hol *et al.* 2004; Joosten *et al.* 2009).

Agriotes lineatus is the generalist root-feeding larva of a click beetle, usually called wireworm. Wireworms are pests of many cultivated crops. They are also common in semi-natural grasslands where *J. vulgaris* occurs (T.M. Bezemer, personal observation). Wireworms were obtained commercially from Applied Plant Research Lelystad (WageningenUR), The Netherlands. *Mamestra brassicae* is a generalist leaf chewer that has been reported to feed on *J. vulgaris* (Hol *et al.* 2004). *Microplitis mediator* is a solitary larval endoparasitoid that attacks first to fourth instar larvae of *M. brassicae* and a few closely related hosts of the family Noctuidae (Gols *et al.* 2008). *M. brassicae* and *M. mediator* were obtained from an insect culture maintained at the Laboratory of Entomology of Wageningen University, The Netherlands.

Experimental setup

Phase 1: *J. vulgaris* seeds were collected from a single population from a restoration grassland at Planken Wambuis (Ede, the Netherlands). The seeds were surface sterilised (1 min in 0.1% sodium chloride solution and rinsed with water) and germinated on glass beads. Forty pots of 2 L (15cm diameter) were filled with 2.2 kg field soil (based on dry weight) collected from the restoration grassland at 5–20 cm below the soil surface. The soil was a sandy loam with particle size distribution: 3% < 2 µm, 17% 2–63 µm, 80% > 63 µm, with 4.5% organic matter. In the laboratory the soil was sieved through a 0.5 cm mesh and homogenised. During sieving, all insects were manually removed from the soil. Into each pot five seedlings were transplanted. Seedlings that died during the first week of the experiment were replaced. Pots were randomly located within a greenhouse (21/16 °C day/night, 16 h photoperiod). Natural daylight was supplemented by 400 W metal halide lamps

(225 µmol m^{-2} s^{-1} PAR). Plants were watered three times per week and randomly rearranged within the greenhouse once a week.

Seven weeks after transplanting, the pots were randomly allocated to one of the following treatments: belowground herbivory (B), aboveground herbivory (A), belowground and aboveground herbivory (AB) and undamaged control (C). All treatments were replicated 10 times. Four late-instar *A. lineatus* individuals were introduced into each pot assigned to one of the two root herbivory treatments. The larvae were placed into 1 cm deep holes and covered by soil. Similar holes were made for pots without root herbivory. Two third-instar larva of *M. brassicae* were placed individually in clip-on cages of 1.5 cm diameter on the youngest fully mature leaf of two plants in all A and AB treatment pots. Empty clip-on cages were attached to the same leaf of plants in pots that were not allocated to aboveground herbivory. The clip-on cages were kept on the leaf for a period of 2 days. During this period, the larva had consumed the entire leaf area available within the cage. Hereafter, the clip-on cages were moved to a similar-aged leaf of another plant within the same pot. Frass of *M. brassicae* larvae was removed from the clip cages every 2 days and it did not enter the soil. Plants were exposed to this treatment for 3 weeks. During this period, each plant within every pot was exposed to two bouts of herbivory. All herbivory treatments were initiated during the same day. Three weeks after initiating the herbivory treatments, shoots were clipped; roots were carefully removed from the soil and rinsed. Shoot and root biomass of each pot was oven-dried (70 °C for 3 days) and weighed. All wireworm larvae were recovered from the soil. A soil sample of 10 g was collected from each pot for molecular analysis (see below). The rest of the soil in each pot was homogenised, divided in five equal parts and used as inoculum in the second phase. Soil from each individual pot was kept separately during the entire following process.

Phase 2: Soil from each pot of the first phase was mixed with sterilised field soil (1 : 1 ratio) and used to fill five 1 L pots. This resulted in a total of 200 pots for Phase 2. The mixing of conditioned soil with sterilised soil minimised potential nutrient deficiencies after the first phase. Soil was sterilised by gamma irradiation (> 25 KGray) at Isotron, Ede, the Netherlands. Two *J. vulgaris* seedlings were planted into each pot. Plants were grown under the same conditions as during the first phase. Seven weeks after planting, the fifth youngest leaf of each plant was removed with a razor blade, immediately frozen at −20 °C, freeze-dried for 3 days under vacuum (−55 °C collector temperature, Labconco Free Zone 12 L Freeze Dry System, USA), and ground for chemical analysis (see below). The next day, all pots were caged individually using fine meshed cylindrical cages (70 cm height, 25 cm diameter). Two weighed second-instar larvae of *M. brassicae* were introduced to 160 pots. Eighty pots from each treatment received two non-parasitised (20 pots × 4 herbivory treatments) and the other 80 pots received two larvae parasitised by *M. mediator*. Larvae were introduced by carefully placing them with a small brush on the youngest fully mature leaf. The larvae had been parasitised individually in plastic vials using freshly mated female parasitoids. Parasitism was performed immediately prior to introducing the larvae into the cages. Within each cage, larvae could move freely on the plants. Insects were kept on the plants for 4 weeks. The remaining 40 pots (one replicate from each Phase 1 pot) were kept without insects and were used to measure plant growth (see Figure S1 in Supporting Information for a scheme of the experimental design). The weight of non-parasitised and parasitised larvae was recorded once a week

for 4 weeks starting 14 days after introduction. Mean relative growth rates and mortality were calculated. Cages with parasitised larvae were checked daily for egression of cocoons. Parasitoid cocoons were carefully collected from the plant and kept individually in Petri dishes until adult emergence. Cocoons were checked twice a day for adult parasitoid emergence. At emergence, the date of eclosion was recorded and parasitoids were sexed, and tibia length was recorded as a measure of adult size (Godfray 1994). Tibia length was measured using a calibrated slide under a stereomicroscope. Development time was calculated as days between parasitism and adult emergence. The percentage of parasitised larvae that emerged as adults was also calculated. Twelve weeks after planting, for each of the 40 pots that was not exposed to herbivory, all aboveground and belowground biomass was harvested, oven dried at 70 °C for 3 days, and weighed.

Chemical analysis of plants from Phase 2

Chemical analyses were carried out on 20 pots of each treatment (10 of these pots were assigned to receive non-parasitised larvae and 10 to receive parasitised larvae). Carbon (C) and Nitrogen (N) content were determined using a Flash EA1112 CN analyzer (Interscience, Breda, the Netherlands). PA analysis was carried out using liquid chromatography-tandem mass spectrometry (LC-MS/MS) following the procedure outlined by Cheng *et al.* (2011). In brief, 10 mg of freeze-dried ground plant material was extracted with 1.0 mL 2% formic acid solution containing heliotrine (1 μg mL^{-1}) as internal standard. After centrifugation and filtration, 25 μL of the extracted filtrate was diluted with 975 μL of 10 mM ammonium hydroxide solution and 10 μL was injected in a Waters Acquity ultra-performance chromatographic system coupled to a Waters Quattro Premier tandem mass spectrometer (Waters, Milford, MA, USA). Data were processed using Masslynx 4.1 software.

Molecular analysis of the soil fungal community at the end of Phase 1

The composition of the soil fungal community in each of the 40 pots at the end of Phase 1 was determined by Terminal restriction fragment length polymorphisms analyses (T-RFLP). Total DNA was extracted from 0.5 g frozen soil (−20 °C) with a Power Soil DNA isolation kit (MOBIO laboratories, Inc.) using a bead beating system. DNA quantity was checked using 1.5% agarose gel electrophoresis. The ITS region of the fungal rDNA was amplified by PCR using the primers ITS1F (White *et al.* 1990) and ITS4 (Gardes & Bruns 1993), which were labelled with FAM and NED respectively. The PCR reaction contained 13.8 μL Milli-Q, 2.5 μL 10× Fast Start High Fidelity Reaction Buffer (Roche Diagnostics), 2.5 μL DNTP Mix (2 mM each), 2.5 μL ITS−6FAM primer (10 μM), 2.5 μL ITS4-NED primer (0.2 μM), 0.2 μL Fast Start High Fidelity Enzym Blend (5 U μL^{-1}) (Roche Diagnostics) and 1 μL template DNA. PCR programme conditions were 5 min at 95 °C, 34 cycles of 30 s at 95 °C, 40 s at 55 °C and 1 min at 72 °C, followed by 10 min at 72 °C before cooling. PCR product presence and quality were verified on 1.5% agarose gels prior to restriction digestion. Two restriction enzymes, HhaI and TaqαI (New England Biolabs, Ipswich, MA, USA), were used to digest dual end-labelled DNA amplicons. A mixture containing 3.5 μL ddH$_2$O, 1 μL buffer, 0.1 μL Bovine Serume Albumin, 5 μL PCR product and 0.4 μL restriction enzyme

was incubated at 37 °C (HhaI) or at 65 °C (TaqαI) for 3 h, and inactivation at 80 °C for 20 min. Restriction products were purified using ethanol precipitation. Fragment length polymorphism analysis was performed on an automated 3130 Genetic Analyzer sequencer (Applied Biosystems) with GeneScan^{TM}−500 LIZ, Applied Biosystems as a size standard. Samples which were over- (highest peak > 80 000 rfu) or under-loaded (highest peak < 1000 rfu) were re-run with an adjusted concentration. Peaks were aligned to TRFs among the samples by applying a clustering threshold of 0.5 bp. Only peaks higher than 0.3% of the sum of all peaks in a sample were included.

Data analysis

All univariate analyses were performed using the R statistical language, version 2.10.1 (R Development Core Team 2010), and multivariate analysis using CANOCO version 4.55 (Ter Braak & Šmilauer 2002). Plant biomass data from both phases were analysed using two-way ANOVA. Other data from Phase 2 were analysed using two-way mixed effects ANOVA (restricted maximum likelihood method, Pinheiro & Bates 2000), with legacy effects of above- and belowground herbivory and their interaction as fixed factors. Individual pot identity during Phase 1 was used as a random factor. In all analyses, the interaction term was never significant, so that it was removed from the model. Sex was included as a fixed factor when differences in parasitoid performance were compared. Prior to analyses, plant biomass, foliar nitrogen concentration and herbivore relative growth rate were log transformed; C : N ratio and parasitoid tibia length were square-root transformed to fulfil assumptions of normality. Percentage data on herbivore mortality and parasitoid adult emergence were analysed using a logit model with aboveground and belowground herbivory as fixed factors. The Wald *z*-statistic was used to test the statistical significance of each coefficient in the logit model.

Differences in PA composition among treatments in Phase 2 were analysed using linear multivariate analyses [principle component analysis (PCA) and redundancy analysis (RDA)] as the longest gradient resulted from detrended correspondence analysis was < 3 (Lepš & Šmilauer 2003). Pot identities from Phase 1 were analysed as 'whole plots' and replicates pots in Phase 2 as 'split plots'. Whole plots were permuted freely and split plots were not permuted. The presence/absence matrix of T-RFLP fingerprints was analysed using distance-based redundancy analyses (db-RDA, Legendre & Anderson 1999), using the Jaccard coefficient of similarity. The calculation of distance matrix and principal coordinates analyses (PCoA) were carried out in PrCoord 1.0 (CANOCO). All eigenvalues were positive. Significances in multivariate analyses were inferred by Monte Carlo permutation tests (999 permutations). Non-metric multidimensional scaling (nMDS) was used to display variation in fungal communities among the four treatments. nMDs was performed in PAST (Hammer *et al.* 2001).
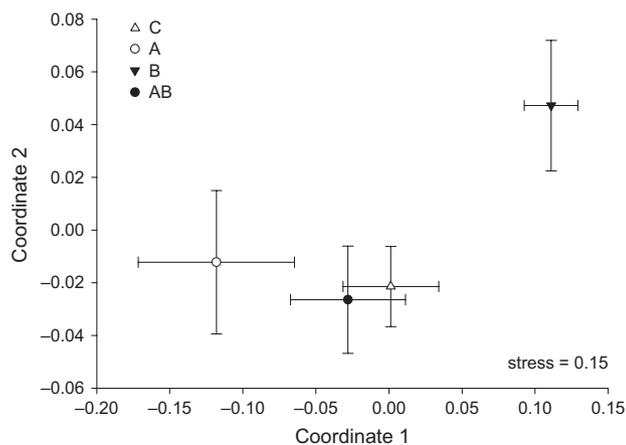
RESULTS

Phase 1

In soil with plants exposed to the different herbivore treatments 754 different fungal TRFs were detected; on average 59 TRFs per enzyme/dye combination per sample. Both belowground and aboveground herbivory significantly affected the composition of the

soil fungal community (B: $F = 1.41$, $P = 0.023$; A: $F = 1.902$, $P = 0.003$). Soil fungal communities belonging to the root herbivory treatment separated most distinctly from the other treatments (Fig. 1). Plant root biomass, at the end of Phase 1, did not significantly differ between herbivore treatments (B: $F_{1,38} = 0.54$, $P = 0.47$; A: $F_{1,38} = 0.015$; $P = 0.90$). Shoot biomass tended to decrease when plants were exposed to belowground herbivore but the effect was not significant (B: $F_{1,38} = 3.76$, $P = 0.060$; A: $F_{1,38} = 0.69$; $P = 0.41$).

Plant responses in Phase 2

In Phase 2, there was a significant negative legacy effect of aboveground herbivory on root biomass ($F_{1,38} = 4.87$, $P = 0.034$, Fig. 2a). The legacy effect of belowground herbivory on root biomass was not significant ($F_{1,38} = 2.83$, $P = 0.10$, Fig. 2a). Shoot biomass was not affected by herbivore legacy effects in the soil (B: $F_{1,38} = 0.65$, $P = 0.42$; A: $F_{1,38} = 0.90$, $P = 0.35$). The legacy effect of root herbivory tended to cause an increase in foliar nitrogen concentration and a decrease in C : N ratio. However, this effect was not significant (%N: $F_{1,38} = 2.63$, $P = 0.11$ and C : N ratio: $F_{1,38} = 3.14$, $P = 0.085$).

A total of 29 PAs were detected in the leaves of *J. vulgaris* (Table 1). Total PA concentration decreased in plants growing in soil with a legacy of aboveground herbivory ($F_{1,38} = 5.22$, $P = 0.028$, Fig. 2b), but not in soil with a legacy of belowground herbivory ($F_{1,38} = 0.24$, $P = 0.63$, Fig. 2b). However, the composition of PAs was significantly affected by a legacy effect of belowground herbivory (RDA: $F = 9.29$, $P = 0.001$, 19% explained variation). PCA analysis revealed that the unconstrained variation in PA composition on the second axis could be very well explained by whether plants were growing in soil with a legacy of root herbivory (Fig. 3). Acetylerucifoline, acetylerucifoline *N*-oxide, erucifoline, erucifoline *N*-oxide and jaconine *N*-oxide, contributed most to the separation of plants between treatments (Table 1).

Herbivore performance in Phase 2

Mean relative growth rate (RGR) of *M. brassicae* larvae was significantly reduced on plants growing in soil with a legacy effect of root herbivory, and significantly increased on plants growing in soil with a legacy effect of aboveground herbivory (B: $F_{1,36} = 6.47$, $P = 0.015$; A: $F_{1,36} = 5.47$, $P = 0.025$; Fig. 4a). Larval mortality did not differ between treatments (B: $z = 0.53$, $P = 0.60$; A: $z = -0.60$, $P = 0.55$). RGR of *M. brassicae* was positively related to foliar nitrogen concentration ($R^2 = 0.19$, $P = 0.0087$). There was also a significant relationship between RGR and PA composition (RDA: $F = 3.02$, $P = 0.022$, 8.6% explained variation).
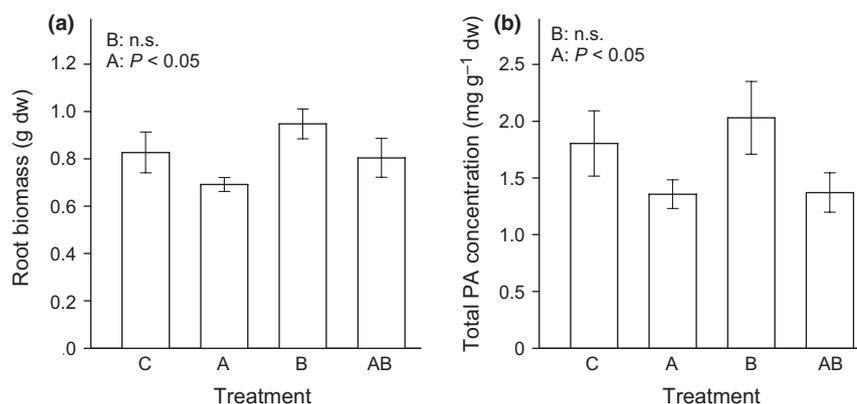
Parasitoid performance in Phase 2

Parasitoid adult size was reduced on plants growing in soil with a legacy effect of root herbivory but not when grown in soil with a legacy of aboveground herbivory (B: $F_{1,21} = 4.48$, $P = 0.046$; A: $F_{1,21} = 0.11$, $P = 0.74$; Fig. 4b). In contrast, more adults of *M. mediator* emerged on plants growing in soil with a legacy effect of aboveground herbivory (B: $z = 0.716$, $P = 0.47$; A: $z = 2.208$, $P = 0.027$, Fig. 4c). Development time of *M. mediator* was significantly longer for females than for males ($F_{1,7} = 11.12$, $P = 0.013$), and increased on plants growing in soil with a legacy of belowground herbivory (B: $F_{1,20} = 4.72$, $P = 0.045$; A: $F_{1,20} = 0.27$, $P = 0.61$, Fig. 4d).

DISCUSSION

This study demonstrates that aboveground multitrophic interactions can be affected by soil legacy effects created by aboveground and belowground herbivory on preceding plants. So far studies on aboveground-belowground interactions have mainly focused on interactions occurring simultaneously and on the same host plant (e.g. Bezemer *et al.* 2003; Soler *et al.* 2005; Kaplan *et al.* 2008; Erb *et al.* 2011). In this study, aboveground and belowground herbivory elicited specific effects on the soil fungal community, and, probably mediated by these effects on soil fungi, affected the growth and nutritional quality of plants growing later in the soil. These soil legacy effects also had multitrophic consequences aboveground, as they influenced the performance of aboveground herbivores and parasitoids on those plants. Other studies have already shown that herbivory can influence soil microbial communities (Bardgett *et al.* 1999; Mikola *et al.* 2001; Hamilton *et al.* 2008; Van Dam 2009), and that soil microbial communities can influence plant secondary chemistry aboveground (e.g. Joosten *et al.* 2009; Hol *et al.* 2010). However, these interactions were studied on the same plant and not on plant individuals that were growing in sequence. Therefore, the novelty of our results is that aboveground and belowground herbivore effects on microbial communities in the soil can be induced in one generation of plants and still influence multitrophic interactions on a subsequent generation of plants.

This study also adds a novel dimension to plant-soil feedback research. A large number of studies have shown that plants, through soil legacy or plant-soil feedback effects can affect the performance of plants that grow subsequently in the conditioned soil (Bever *et al.* 1997; Young *et al.* 2001; Van de Voorde *et al.* 2011). Our findings show that (1) both aboveground and belowground herbivory during the conditioning phase can affect soil conditioning; and (2) that plant-soil feedback effects go beyond affecting plant

Figure 1 Non-metric multidimensional scaling (nMDS) plot. Shown are mean sample scores (± SE) of the T-RFLP community composition in soil with undamaged plants (C), or in soil, in which plants had grown that were exposed to aboveground (A), belowground (B), or aboveground and belowground herbivory (AB).

Figure 2 Means (± SE) root biomass (a) and total shoot PA concentration (b) of *J. vulgaris* plants growing in soil with a legacy of undamaged plants (C), or with a legacy of aboveground (A), belowground (B), or aboveground and belowground herbivory (AB). Significance of the main effects of belowground and aboveground herbivory are based on linear mixed model analyses; n.s., non-significant. The interaction between A and B was never significant and therefore was excluded from the model.
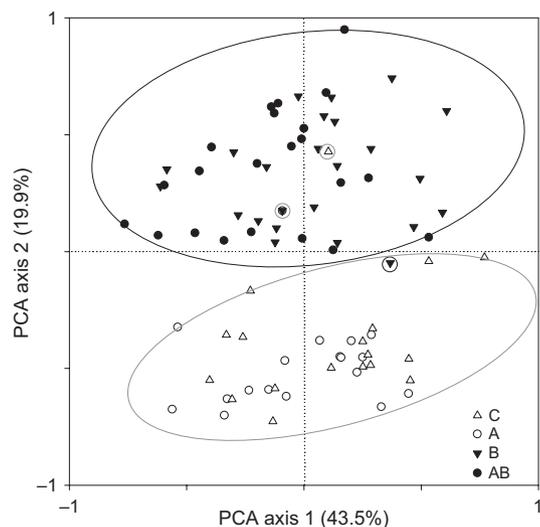
Figure 3 Ordination diagram of principal component analysis (PCA) of the shoot PA composition of *J. vulgaris*. Plants were grown in soil with a legacy of undamaged plants (C), or with a legacy of aboveground (A), belowground (B), or aboveground and belowground herbivory (AB). Percentages of total explained variation by PCA axes are given in parentheses.

biomass, as they can also affect plant chemistry and aboveground multitrophic interactions.

In this study, soil legacy effects of aboveground and belowground herbivory influenced both the concentration and the composition of PAs in foliage. Remarkably, the effects differed greatly between the two types of herbivory. Aboveground herbivory caused a soil legacy effect that resulted in a reduction of the total shoot PA concentration. The overall PA composition was not affected, although the concentration of a number of individual PAs was significantly reduced in plants growing in soil with a legacy of aboveground herbivory. Legacy effects arising from root herbivory, however, resulted in changes in the composition of PAs but not in the total shoot PA concentration. These effects might depend on the species, or feeding guild acting as aboveground or belowground herbivore, but solving that question would require additional studies. Although this study does not provide a conclusive mechanism by which aboveground and belowground herbivory caused these specific soil

legacies that affected plant growth and defence compounds of subsequent plants, we propose that pathogenic fungi caused the observed effects. Aboveground and belowground herbivory can differentially affect the concentration of PAs in the roots (Hol *et al.* 2004). We suggest that these changes in PA concentrations influenced the abundance and composition of (pathogenic) fungi as we observed in this study. This, in turn, influenced the growth and chemical composition of plants that grew subsequently in the soil, and these changes in the plant then affected aboveground insect performance. Alternatively, it could have been possible that the observed effects in Phase 2 were the result of differences in nutrient availability. However, as plants were grown in conditioned soil mixed with 50% sterilised soil, this is unlikely. The short time span of the experiment makes it also unlikely that the effects were caused by differences in decomposition by fungi.

Data from other studies support our proposed mechanism. First, root pathogenic fungi are important antagonists of *J. vulgaris* and certain pathogens are sensitive to PAs (Hol & Veen 2002). In general, plant pathogens are often suppressed by plant defence compounds (Kowalchuk *et al.* 2006; Van Dam 2009). Second, *J. vulgaris* exhibits a strong negative plant-soil feedback caused by soil (pathogenic) fungi (Bezemer *et al.* 2006; Van de Voorde *et al.* 2011). Third, soil-borne microorganisms such as soil fungi can greatly affect the composition of PAs in *J. vulgaris* leaves (Joosten *et al.* 2009). Finally, aboveground herbivory by *M. brassicae* on *J. vulgaris* has been shown to cause a reduction in the concentration of PAs in root tissues (Hol *et al.* 2004). The results from this study therefore suggest that aboveground herbivory caused a decrease in PA concentration in the roots that led to an increase in soil fungal pathogens, which decreased root biomass of plants growing subsequently in that soil. Root herbivory could have caused a leakage of plant defence compounds from the roots into the rhizosphere, which may have negatively affected soil pathogens and changed the composition of other soil microorganisms. Inoculation trials with fungi that respond to the herbivory treatments are needed to verify whether changes in the abundance of these fungi indeed have caused the observed legacy effects.

We observed a significant relationship between herbivore performance and the concentration of primary and secondary compounds in the plant tissues during the second phase. As less than five percent of the foliage was consumed in all cages (O. Kostenko,

Table 1 Mean concentration (± SE, μg g^{-1} dw) and scores on the first two PCA axes of individual PAs of *J. vulgaris* plants growing in Phase 2 in soil with a legacy of undamaged plants (C), or with a legacy of aboveground (A), belowground (B), or aboveground and belowground herbivory (AB). Differences in concentrations of individual PAs between treatments are analysed using linear mixed model with aboveground (A) and belowground (B) herbivory as fixed factors and individual pot identity in Phase 1 as random factor. The interaction between A and B was never significant and therefore was excluded from the model; *P < 0.05; **P < 0.01; ***P < 0.001, the absence of asterisks denote no significant effects.

| PA[†]	C	A	B	AB	$F_{1,38}$ A	B	PCA Score 1	Score 2
AcEr	3.8 ± 1.0	2.9 ± 0.6	0.24 ± 0.07	0.13 ± 0.04	0.55	38.53***	0.284	1.671
AcEr-ox	71.3 ± 13.3	78.9 ± 12.4	7.3 ± 1.9	3.5 ± 1.6	0.11	71.53***	0.601	−2.003
AcSp	0.2 ± 0.1	0.18 ± 0.05	0.16 ± 0.05	0.07 ± 0.03	1.94	4.80*	0.105	−0.595
AcSp-ox	3.2 ± 1.0	2.5 ± 0.6	4.7 ± 3.8	3.2 ± 1.9	0.24	0.20	0.568	−0.662
DHJn	0.19 ± 0.03	0.23 ± 0.06	0.04 ± 0.01	0.04 ± 0.01	0.42	33.65***	0.149	−1.057
Er	6.2 ± 3.4	2.7 ± 0.6	8.5 ± 1.4	9.4 ± 3.1	0.57	8.08**	0.380	0.947
Er-ox	30.3 ± 5.1	29.5 ± 5.1	164 ± 26	108 ± 19	3.07	30.11***	0.802	0.944
Ir	2.0 ± 1.1	0.7 ± 0.2	1.1 ± 0.3	1.4 ± 0.6	0.46	0.03	0.845	0.740
Ir-ox	93.8 ± 21.4	64.6 ± 10.2	119 ± 28	53.7 ± 11.1	5.91*	0.13	1.432	0.255
Jb	352 ± 39	348 ± 32	366 ± 48	358 ± 37	0.02	0.08	0.087	0.527
Jb-ox	446 ± 84	352 ± 70	467 ± 82	402 ± 68	1.08	0.21	0.780	0.126
Jl	23.5 ± 2.7	20.7 ± 1.7	23.2 ± 3.1	21.8 ± 2.2	0.59	0.02	0.084	0.414
Jl-ox	9.6 ± 1.5	7.6 ± 1.3	11.3 ± 1.9	10.9 ± 1.7	0.45	2.20	0.570	0.272
Jn	3.4 ± 0.8	3.0 ± 0.6	1.0 ± 0.2	1.3 ± 0.2	0.01	20.95***	0.401	−0.686
Jn-ox	1.0 ± 0.2	0.9 ± 0.2	0.08 ± 0.03	0.05 ± 0.01	0.17	63.85***	0.520	−1.540
Jz	2.3 ± 0.4	2.8 ± 0.3	2.3 ± 0.4	2.3 ± 0.2	0.53	0.50	0.188	0.210
Jz-ox	5.1 ± 1.3	3.9 ± 0.9	4.4 ± 0.8	3.3 ± 0.7	1.62	0.52	1.185	0.055
Rd	0.2 ± 0.1	0.06 ± 0.02	0.04 ± 0.01	0.04 ± 0.01	1.06	2.36	0.460	−0.157
Rd-ox	7.5 ± 1.1	6.7 ± 1.1	5.5 ± 1.1	3.2 ± 0.7	2.09	6.00*	1.296	−0.630
Rt	1.6 ± 1.1	0.4 ± 0.1	0.7 ± 0.2	0.7 ± 0.3	1.17	0.40	0.849	0.524
Rt-ox	30.0 ± 6.5	15.3 ± 2.0	31.5 ± 6.7	17.8 ± 3.7	7.42*	0.15	1.371	0.434
Sn	31.1 ± 19.7	7.8 ± 2.3	18.3 ± 6.1	16.9 ± 10.9	0.99	0.03	1.038	0.593
Sn-ox	413 ± 104	261 ± 43	585 ± 139	240 ± 62	6.67*	0.58	1.419	0.343
Sp	16.5 ± 8.4	4.4 ± 1.4	8.0 ± 2.6	7.6 ± 4.3	1.36	0.35	1.030	0.520
Sp-ox	239 ± 75	132 ± 24	193 ± 42	101 ± 21	5.17*	0.80	1.440	0.054
St	0.2 ± 0.1	0.08 ± 0.02	0.09 ± 0.02	0.14 ± 0.08	0.25	0.30	0.560	0.482
St-ox	7.5 ± 1.7	4.6 ± 0.8	3.7 ± 0.6	2.3 ± 0.5	4.69*	10.87**	1.315	−0.659
Us	0.2 ± 0.1	0.12 ± 0.02	0.19 ± 0.03	0.16 ± 0.03	1.37	0.64	0.734	0.500
Us-ox	4.9 ± 1.2	3.1 ± 0.6	4.2 ± 1.0	3.5 ± 0.7	1.89	0.02	1.179	0.242

AcEr, Acetylerucifoline; AcSp, Acetylseneciphylline; DHJn, Dehydrojaconine; Er, Erucifoline; Ir, Integerrimine; Jb, Jacobine; Jl, Jacoline; Jn, Jaconine; Jz, Jacozine; Rd, Riddelliine; Rt, Retrorsine; Sn, Senecionine; Sp, Seneciphylline; St, Spartioidine; Us, Usaramine; -ox, N-oxide form of the corresponding PA.

personal observation), we can assume that the legacy effects on herbivore performance were driven by changes in host plant quality and not by food quantity. The development and larval performance of parasitoids are strongly influenced by the size and the quality of their herbivorous hosts (Godfray 1994). Therefore, it appears that legacy effects, via changes in plant quality influenced herbivore and parasitoid performance in this study. Similar direct effects have been shown for root herbivory on aboveground herbivore-parasitoid interactions (Soler *et al.* 2005). Interestingly, in this study root herbivory exerted a negative soil legacy effect on herbivore and parasitoid performance while a soil legacy of foliar herbivory resulted in increased performance of herbivore. These results underline suggestions made in other studies that specific interactions between plants and insects can have far stretching consequences for other multitrophic interactions (Kaplan & Denno 2007; Soler *et al.* 2012).

An important question is whether our experiment, which was performed under controlled conditions in a greenhouse, represents a process that also plays a role under natural conditions where interactions are more complex. We argue that these soil legacy effects can significantly affect plant population dynamics and insect communities in systems that comply with three rules. First, the plant

species should grow sequentially at the same location. In Europe, semi-natural grasslands can be severely dominated by *J. vulgaris* for a number of years, during which the plant goes through several plant generations and new rosettes often appear close to flowering plants. Second, in the field, there should be effects of soil biota on plant growth and aboveground insect performance and abundance. A recent study by Reidinger *et al.* (2012) showed that interactions with arbuscular mycorrhizal fungi can affect aboveground insects in *J. vulgaris* plants grown in such semi-natural grasslands, and that these effects may have been mediated by changes in PA composition. Third, there must be differences among plants in aboveground and belowground herbivory. It is well documented that in the field insect abundances vary greatly among individual plants, and recently we showed that this is also the case for *J. vulgaris* (Kostenko *et al.* 2012). Therefore, we conclude that these soil legacy effects can influence the growth of individual ragwort plants as well as the abundance and performance of insects on those plants in nature. Field studies are needed to understand how long this legacy effects would last, how widespread they are in natural communities, and how important they are for insect performance relative to abiotic effects such as changes in temperature or rainfall. Interestingly, we
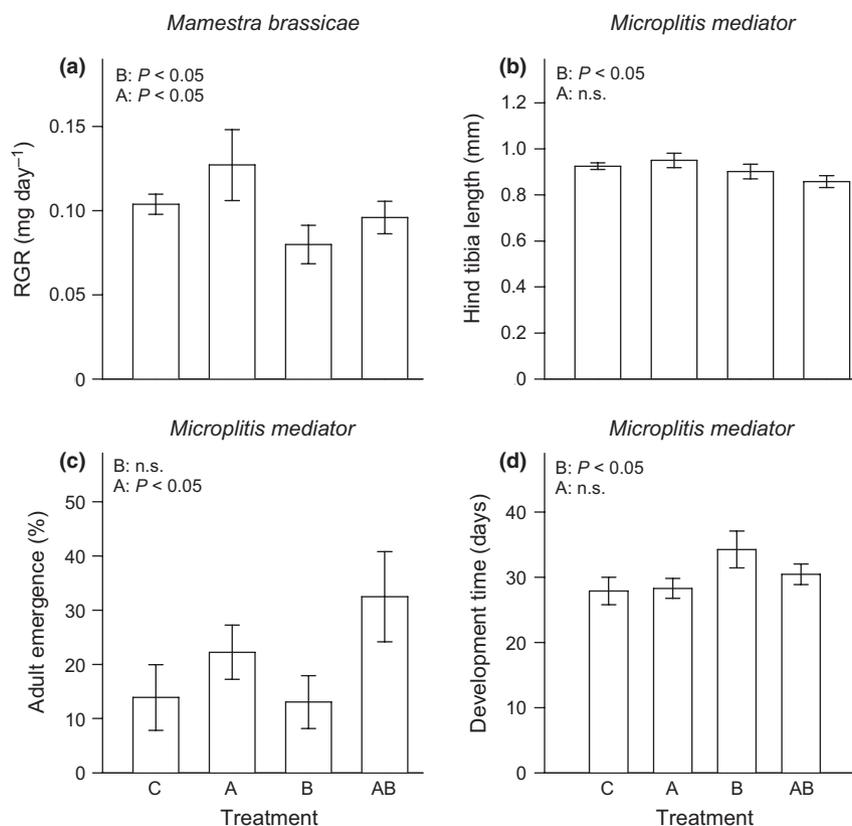
Figure 4 Performance of the herbivore *M. brassicae* and the parasitoid *M. mediator* on *J. vulgaris* plants in Phase 2, growing in soil with a legacy of undamaged plants (C), or with a legacy of aboveground (A), belowground (B), or aboveground and belowground herbivory (AB). Means (± SE) of (a) larval relative growth rate of the herbivore, and (b) hind tibia length, (c) adult emergence and (d) development time of the parasitoid. Significance of the main effects of below- and aboveground herbivory are based on linear mixed model analyses; n.s., non-significant. The interaction between A and B was never significant and therefore was excluded from the model.

observed that aboveground herbivory had a negative effect on subsequent plant growth and quality but positively affected aboveground insect performance. This positive aboveground-belowground feedback effect could be an alternative mechanism that can explain the decline of *J. vulgaris* in natural populations.

We conclude that herbivore-induced soil legacy effects can mediate interactions between spatially and temporally separated organisms. This study shows that specific interactions between plants and insects can even extend beyond a single growth period of a plant, emphasising the complexity of ways by which plants and insects interact, and that the insect community present at any stage of ecosystem development may reflect insect-plant interactions from the past. The observed soil legacies of aboveground and belowground insect herbivores can play a role in the field, but tests under more complex conditions are required. Isolation and inoculation studies to determine which soil organisms are involved are also needed. The implication of this study is that there are connections between the concepts of plant-soil feedback and aboveground–belowground multitrophic interactions, and this opens up new avenues for research in these areas.

AUTHORSHIP

OK, TMB, TFJV and WHP designed the study. OK and TMB performed the experiment. PPJM carried out secondary metabolites analysis. OK analysed plant and insect data. TFJV and OK analysed T-RFLP data. OK and TMB wrote the first draft of the manuscript. All authors contributed substantially to revising the manuscript.

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