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SPECIAL FEATURE

PLANT–SOIL FEEDBACKS IN A CHANGING WORLD

Above- and below-ground herbivory effects on below-ground plant–fungus interactions and plant–soil feedback responses

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Summary

1. Feeding by insect herbivores can affect plant growth and the concentration of defense compounds in plant tissues. Since plants provide resources for soil organisms, herbivory can also influence the composition of the soil community via its effects on the plant. Soil organisms, in turn, are important for plant growth. We tested whether insect herbivores, via their effects on the soil microbial community, can influence plant-soil feedbacks.

2. We first examined the effects of above-ground (AG) and below-ground (B) insect herbivory on the composition of pyrrolizidine alkaloids (PAs) in roots and on soil fungi in roots and rhizosphere soil of ragwort (*Jacobaea vulgaris*). The composition of fungal communities in roots and rhizosphere soil was affected by both AG and BG herbivory, but fungal composition also differed considerably between roots and rhizosphere soil. The composition of PAs in roots was affected only by BG herbivory.

3. Thirteen different fungal species were detected in roots and rhizosphere soil. The presence of the potentially pathogenic fungus *Fusarium oxysporum* decreased and that of *Phoma exigua* increased in presence of BG herbivory, but only in soil samples.

4. We then grew new plants in the soils conditioned by plants exposed to the herbivore treatments and in unconditioned soil. A subset of the new plants was exposed to foliar insect herbivory. Plant-soil feedback was strongly negative, but the feedback effect was least negative in soil conditioned by plants that had been exposed to BG herbivory. There was a negative direct effect of foliar herbivory on plant biomass during the feedback phase, but this effect was far less strong when the soil was conditioned by plants exposed to AG herbivory. AG herbivory during the conditioning phase also caused a soil feedback effect on the PA concentration in the foliage of ragwort.

5. *Synthesis*. Our results illustrate how insect herbivory can affect interactions between plants and soil organisms, and via these effects how herbivory can alter the performance of late-growing plants. Plant-soil feedback is emerging as an important theme in ecology and these results highlight that plant-soil feedback should be considered from a multitrophic AG and BG perspective.

Key-words: *Agriotes lineatus*, insect herbivory, *Jacobaea vulgaris*, *Mamestra brassicae*, plant-soil (below-ground) interactions, pyrrolizidine alkaloids, root herbivory, *Senecio jacobaea*, soil pathogens, T-RFLP

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Introduction

Plants differ in the amount and quality of resources that they add to the soil. Through these effects they can select for particular soil communities by affecting soil organisms that decompose dead organic matter or that are associated with living plant roots (Bardgett & Wardle 2010). Soil organisms, in turn, are important drivers of plant growth, as they can influence nutrient availability for the plant or directly affect plant roots via antagonistic or symbiotic interactions. Hence, the effects of a plant on the composition of the soil community can influence the performance of other plants, of the same or other species, that grow later in the soil (Bever, Westover & Antonovics 1997). These feedback effects between plants and soil organisms are often examined by comparing plant growth responses in soil previously conditioned by the same species and by different plant species (Kulmatiski *et al.* 2008). However, interactions between plants and soil organisms can also show great variation between individuals of a single plant species (e.g. Lankau *et al.* 2011). Here, we examine how herbivory influences plant–soil feedbacks within a single species by affecting the composition of the soil community.

Herbivores can affect soil organisms via changes in the quality and quantity of resources that enter the soil as frass, honeydew or dead roots. Moreover, herbivory can lead to changes in root biomass or the physiology of plant roots that can also influence soil biota (Ayres *et al.* 2007). For example, both above-ground (AG) and below-ground (BG) herbivory can cause increases in the rates of carbon and nitrogen exudation from roots which, in turn, enhances microbial activity and the composition and diversity of bacterial and fungal communities in the rhizosphere (Hamilton & Frank 2001; Broeckling *et al.* 2008). However, how AG or BG herbivory influence the composition of non-mycorrhizal fungi in roots and in soil and feeds back to plant growth remains poorly understood.

Above- and below-ground herbivory can also result in changes in the amount and quality of secondary plant compounds in root tissues (Bezemer & Van Dam 2005). These changes in secondary plant compounds can subsequently affect root-associated organisms such as root herbivores, pathogens or symbionts (Van Dam 2009). Secondary plant compounds can also negatively affect bacteria and fungi in the soil when they are released via root exudates or by leakages from damaged roots (Wu, Liu & Zhou 2010). Ragwort (*Jacobaea vulgaris* syn. *Senecio jacobaea*), for example, produces a variety of pyrrolizidine alkaloids (PAs) that are constitutively biosynthesized and present in roots and are transported to AG parts (Hartmann 1999). PAs can inhibit fungal growth and can play a significant role in shaping the soil fungal community of the rhizosphere (Kowalchuk, Hol & Van Veen 2006; Joosten & Van Veen 2011). Interestingly, Hol *et al.* (2004) showed that shoot herbivory decreased PA concentrations in *J. vulgaris* roots whereas artificial root damage resulted in increased root PA concentrations. These results suggest that AG and BG herbivory on *J. vulgaris* may lead to specific changes in the composition of the soil microbial community via changes in PA concentrations.

Changes in the concentration or composition of secondary compounds in plant roots can lead to altered plant–soil feedback responses through changes in the composition of the soil community (Lankau *et al.* 2011). As AG and BG herbivory can affect the composition and functioning of soil communities directly or indirectly via changes in plant nutrients or defense compounds, this suggests that herbivory may affect plant–soil feedback responses. However, very few studies have examined this possibility (but see Sørensen *et al.* 2008; Mikola *et al.* 2005).

Plant-soil feedback studies typically report effects on plant growth (Kulmatiski *et al.* 2008). However, soil microorganisms can also induce physiological changes within plants that alter the nutritional quality or concentration of plant defense compounds in plant root and shoot tissues (Van Loon, Bakker & Pieterse 1998; Bennett, Bever & Bowers 2009; Wurst *et al.* 2010). PA composition in both shoot and root tissues of *J. vulgaris*, for example, is influenced by the type of soil microbial inoculum that is used (Joosten *et al.* 2009). Hence plant soil-feedback responses may also affect concentrations of primary or secondary compounds in plant tissues. These changes can subsequently affect processes such as decomposition or herbivory which, in turn, influence plant growth responses leading to complex AG and BG feedback interactions (Bardgett & Wardle 2010). So far, very few studies have examined how plant–soil feedback influences plant nutritional quality (but see Mikola *et al.* 2005).

Recently, we demonstrated for *J. vulgaris* that AG and BG insect herbivory in a greenhouse experiment differentially affected the composition of the soil fungal community. The composition of PAs in leaves of plants subsequently growing in that soil was also affected. This, in turn, influenced AG herbivore and parasitoid performance on plants grown subsequently on the same soil (Kostenko *et al.* 2012). Based on these results, we formulated a three-staged hypothesis about how herbivory affects plant–soil interactions in this system. First, as PAs are produced in the roots and the PA concentration depends on root biomass, we hypothesize that AG herbivory will cause an increase and BG herbivory a decrease in PA production in *J. vulgaris* roots. Secondly, we hypothesize that the PA concentration and composition in roots will influence the composition of fungi and presence of pathogenic fungi inside the roots and in the soil. Thirdly, we hypothesize that herbivore-induced changes in the soil fungal community will affect the growth and nutritional quality of plants that grow later in the soil. To test these hypotheses, we determined the composition of PAs and fungi in root material originating from the experiment of Kostenko *et al.* (2012). We also established pure cultures of fungi isolated from *J. vulgaris* roots and determined the AG and BG herbivory effects on the identified fungal species in roots and rhizosphere soil. Subsequently, we compared the direct and indirect effects of insect herbivory on plant–soil feedbacks, both in terms of plant biomass and PA composition. Finally, we used structural equation modelling (SEM) to explore how AG and BG herbivory affect plant–soil feedbacks.

Materials and methods

GREENHOUSE EXPERIMENT

The experimental details have been presented elsewhere (Kostenko *et al.* 2012). In brief, *J. vulgaris* plants were grown in a greenhouse (21/16 °C day/night, 16 h photoperiod) for two consecutive growth periods. During the first growth period (conditioning phase), plants were grown in 2 L pots filled with 2.2 kg sandy-loam soil collected from a natural grassland (Planken Wambuis, Ede, The Netherlands). In the laboratory, the soil was sieved through a 0.5 cm mesh and homogenized. Part of the sieved soil was stored at 4 °C to be used during the second growth phase. Five seedlings were transplanted into each pot. Four herbivory treatments were initiated simultaneously 7 weeks after transplanting, with 10 replicate pots per treatment: BG herbivory, AG herbivory, AG + BG herbivory and control (no herbivory). As AG herbivores we used the generalist leaf herbivore *Mamestra brassicae* L. (Lepidoptera: Noctuidae), and as BG herbivore we used the generalist root feeder *Agriotes lineatus* L. (Coleoptera: Elateridae). Three weeks after initiating the herbivory treatments, plants were harvested. A mixed and homogenized soil sample of 10 g and a small subsample of the fresh fine-root biomass (1 g) was stored at −80 °C for molecular and PA analyses (see below). Shoot and root biomass of each pot was oven-dried (70 °C) and weighed. The rest of the soil in each pot was homogenized and used for the second growth phase (feedback phase).

Soil from each individual pot was kept separately, divided into five equal parts, and each part was mixed (1:1 ratio) with sterilized field soil (gamma irradiation > 25 KGray; Isotron, Ede, The Netherlands). This was used in the feedback phase to fill five 1 L pots from each pot from the conditioning phase (200 pots with conditioned soil). In addition, 40 pots were filled with a mixture (1:1 ratio) of sieved (unconditioned) field soil and sterilized field soil. Soil was mixed with sterilized soil to avoid potential nutrient deficiencies after the first phase. Two seedlings were planted into each pot. Seven weeks after planting, the fifth youngest leaf of each plant was removed with a razor blade, immediately frozen (−20 °C), freeze-dried for 3 days, and ground for chemical analyses. All pots were then caged individually using fine-meshed cylindrical cages (70 cm height, 25 cm diameter) and two-second-instar larvae of *M. brassicae* were introduced into each of four replicates of each pot from the conditioning phase. The 40 unconditioned pots were also exposed to herbivory, as this experiment was originally set-up to study plant–soil feedback effects on AG insect herbivory. Half of the pots with herbivory during the feedback phase, received two non-parasitized larvae and the other half received two larvae parasitized by the solitary endoparasitoid *Microplitis mediator* Haliday. The fifth replicate pot with conditioned soil was kept without herbivory as a control. The results of herbivore and parasitoid performance have been presented elsewhere (Kostenko *et al.* 2012). In this study we focus on the effects of AG herbivory during the feedback phase on plant biomass production. Fourteen weeks after planting, root and shoot biomass was harvested, oven-dried at 70 °C and weighed. A small subsample of root biomass of each pot was used for PA analysis.

ANALYSIS OF PYRROLIZIDINE ALKALOIDS

Root PA concentration was determined for all plants from the conditioning phase. For plants from the feedback phase, PAs were determined for root and foliar tissues from 10 pots with non-parasitized and 10 with parasitized *M. brassicae* larvae per herbivore treatment. PAs were determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS, Quattro Premier; Waters, Milford, MA, USA), following the procedure outlined by Cheng *et al.* (2011). In total 36 different PAs (including tertiary amine and corresponding N-oxide forms) were monitored and quantified using a set of reference standards. PAs were grouped into five types according to their structural characteristics and biosynthetic pathways (Cheng *et al.* 2011). Data were processed using Masslynx 4.1 software (Waters, Milford, MA, USA).

MOLECULAR IDENTIFICATION OF FUNGI IN ROOTS AND SOIL

The composition of the fungal community in soil and roots at the end of the conditioning phase was determined by terminal restriction fragment length polymorphism (T-RFLP) analysis. Total DNA was extracted from 0.5 g frozen soil with a Power Soil DNA isolation kit (MOBIO laboratories, Inc., Carlsbad, CA, USA) and from 0.1 g fresh weight of frozen root biomass using a Plant mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA quantity was checked using 1.5% agarose gel electrophoresis. For one soil sample DNA extraction failed. 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. PCR product presence and quality were verified on 1.5% agarose gels prior to restriction digestion. Two restriction enzymes, *Hha*I and *Taq*αI (New England Biolabs, Ipswich, MA, USA), were used to digest dual end-labeled DNA amplicons. A mixture containing 3.5 μL ddH_2O, 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 inactivated at 80 °C for 20 min. Restriction products were purified using ethanol precipitation. Fragment length polymorphism analysis was performed on an automated 3130 Genetic Analyser sequencer with GeneScan-500 LIZ (Applied Biosystems, Carlsbad, CA, USA) as a size standard. Samples which were over- (highest peak > 80 000 relative fluorescence units, rfu) or under loaded (highest peak < 1000 rfu) were re-run with an adjusted concentration. Peaks at sizes < 50 base pairs (bp) and > 500 bp were removed. Peaks were then aligned to terminal restriction fragments (TRFs) among the root and soil samples by applying a clustering threshold of 0.5 bp. Only peaks with higher rfu's than 0.3% of the sum of all rfu's in a sample were included.

To create a database of identified fungi from *J. vulgaris* roots, ragwort plants were grown in a greenhouse in field soil collected from ten restoration grasslands on former arable fields where ragwort was present (Van de Voorde, Van der Putten & Bezemer 2011). After 10 weeks of growth plants were harvested. Roots were surface sterilized (15 s in 1% bleach solution), and samples 1 cm in length were placed in Petri-dishes with Malt Agar or Water Agar (Merck, Darmstadt, Germany). All plates were incubated at 20 °C in the dark. Different cultures were inoculated separately on new plates and pure cultures were separated on morphological characteristics. From 34 cultures DNA was extracted from 100 mg fresh weight of fungal biomass using a Plant mini kit (QIAGEN). PCR amplification and primer specifications were as described above. The ITS1 and ITS4 region of the fungal rDNA were sequenced using a 3730XL DNA Sequencer (Macrogen, Amstelveen, the Netherlands). ITS1 and ITS4 contigs were aligned using SeqMan Pro (DNASTAR, Madison, WI, USA) and the sequences were compared with those of known species using the GenBank database (http://www.ncbi.nlm.nih.gov; Altschul *et al.* 1997). DNA of the 34 isolates was subsequently analysed using

T-RFLP as described above. The T-RFLP patterns obtained from root and soil samples were then screened for the presence of the isolated fungi using Database-TRFLP (Dickie & FitzJohn 2007). A fungal species was considered present in a root or soil sample when in all four enzyme-primer combinations, a TRF was present within a margin of ±0.5 bp of the TRF of the fungal species.

DATA ANALYSIS

Univariate data were analysed using Genstat for Windows 14th edition (Payne *et al.* 2011). The direct effects of AG and BG herbivory during the conditioning phase were analysed using two-way Analysis of Variance. Data from the feedback phase were first analysed by comparing conditioned and unconditioned soil using a one-way analysis of variance. Data from the conditioned soils were then analysed using mixed models (restricted maximum likelihood method) to test the soil feedback effects of AG and BG herbivory, and the direct effect of foliar herbivory as fixed factors and pot identity from the conditioning phase as a random factor. The effects of foliar herbivory by parasitized and non-parasitized *M. brassicae* larvae during the feedback phase on plant biomass and PA composition did not differ (data not shown). Therefore, data were analysed independent of herbivore status. TRFs in root and soil samples at the end of the conditioning phase were compared using Jaccard similarity. The effect of AG and BG herbivory on the composition of TRFs in root and soil samples was determined using multivariate canonical correspondence analysis (CCA; CANOCO version 4.55; Ter Braak & Šmilauer 2002). Differences between herbivore treatments in the presence of fungal species in soil or root samples were compared using a 2×4 Chi-square test. To explore the possible mechanisms of herbivore-mediated plant–soil feedbacks, we examined the effects of AG and BG herbivory during the conditioning phase on changes in plant and fungal characteristics using SEM. The conditioning phase and feedback phase were analysed separately. The conceptual model for the conditioning phase considered both direct and indirect effects of herbivory on fungal communities in roots and in the rhizosphere. All possible indirect effects of the herbivory on fungal communities either via changes in plant biomass or root PA compositions were included in the model. The conceptual model for the feedback phase included the direct and indirect effects of the composition of the fungal community in the rhizosphere. We also included the factors AG and BG herbivory during the conditioning phase as predictors to incorporate herbivore effects not mediated by changes in the soil fungal community. For multivariate data (relative concentration of the five PA types, root fungal TRF composition, soil fungal TRF composition) the first axis of a principle component analysis was used in the SEM analysis. Plant biomass was log-transformed prior to the SEM analysis. To select the model that best fitted our data we removed non-significant paths from our initial model. SEM was carried out using the SEM package in R (version 2.15.1, R Development Core Team 2012).

Results

In the conditioning phase, neither AG nor BG herbivory had a significant direct effect on root biomass. In the feedback phase, both roots ($F_{1,243} = 140.2$; $P < 0.001$) and shoots ($F_{1,243} = 23.6$; $P < 0.001$) weighed less in conditioned than in unconditioned soil, but roots showed a much stronger soil feedback effect (−48%) than shoots (−15%; Fig. 1). For plants growing in conditioned soil, foliar herbivory by *M. brassicae* during the feedback phase resulted in a reduction of both root and shoot biomass (Table 1). The direct effect of foliar herbivory by *M. brassicae* was stronger for root (−20%) than for shoot biomass (−9%). Root biomass was significantly higher in soil conditioned by plants with BG herbivory than in soil conditioned by plants that had not been

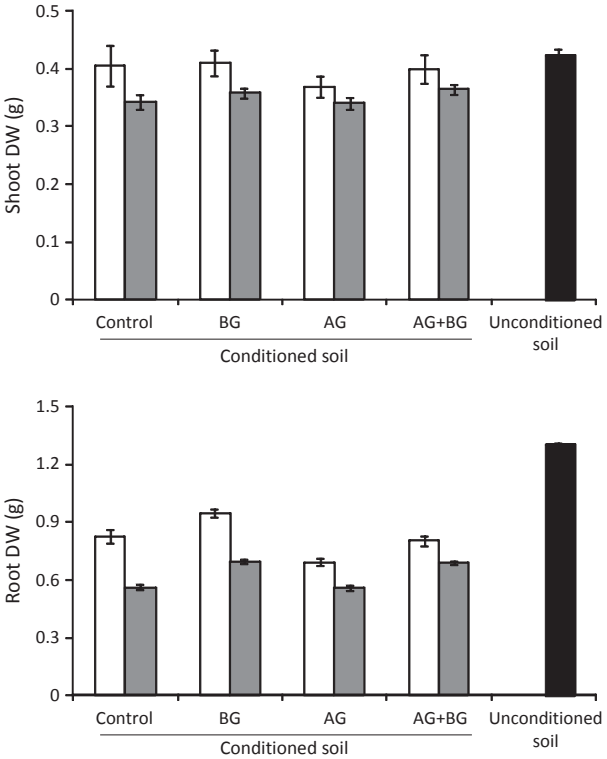

Fig. 1. Mean (±SE) shoot and root biomass of *Jacobaea vulgaris* that had been grown in conditioned and unconditioned soil during the feedback phase. Conditioned soil was collected from pots in which *J. vulgaris* plants were exposed to above-ground (AG) or below-ground (BG) herbivory, to both (AG + BG) or no herbivory (Control). During the feedback phase, plants were either exposed (grey bars) or not (white bars) to foliar herbivory. Root biomass data of plants growing in conditioned soil without exposure to AG herbivory (white bars) are from Kostenko *et al.* (2012).

Table 1. Results from linear mixed model analyses of the soil-mediated effects of above-ground (AG) and below-ground (BG) herbivory, and the immediate effects of foliar herbivory on root and shoot biomass of *Jacobaea vulgaris* during the feedback phase

	Root biomass		Shoot biomass	
	F value	*P*	*F* value	*P*
Foliar herbivory (H)	$F_{1,160} = 49.3$	**< 0.001**	$F_{1,160} = 20.3$	**< 0.001**
AG-soil	$F_{1,37} = 0.4$	0.53	$F_{1,37} = 0.04$	0.88
BG-soil	$F_{1,37} = 6.4$	**0.016**	$F_{1,37} = 1.75$	0.19
AG-soil × BG-soil	$F_{1,37} = 0.0$	0.96	$F_{1,37} = 0.2$	0.66
H × AG-soil	$F_{1,160} = 6.0$	**0.015**	$F_{1,160} = 1.58$	0.21
H × BG-soil	$F_{1,160} = 0.05$	0.82	$F_{1,160} = 0.01$	0.94
H × AG-soil × BG-soil	$F_{1,160} = 0.0$	0.97	$F_{1,160} = 0.17$	0.68

exposed to BG herbivory. In the absence of foliar herbivory by *M. brassicae* during the feedback phase, ragwort plants growing in soil conditioned by plants with AG herbivory had less root biomass than plants growing in soil that were conditioned by plants without AG herbivory. However, in soil conditioned by plants with AG herbivory, root biomass was far less reduced in plants exposed to foliar herbivory by *M. brassicae* during the feedback phase (−7%) than in plants growing in soil conditioned by plants without AG herbivory (−28%; Fig. 1). This resulted in a significant interaction between the direct effect of foliar herbivory during the feedback phase, and the soil-mediated effect of AG herbivory (Table 1).

During the conditioning phase, total PA concentration in roots tended to be higher in the presence of AG herbivory ($F_{1,37}$ = 3.61; P = 0.065). BG herbivory caused an increase in the relative concentration of seneciphylline-type PAs ($F_{1,37}$ = 13.86; P < 0.001) and a decrease in senecionine-type PAs ($F_{1,37}$ = 10.48; P = 0.002; Fig. 2). In the feedback phase, PAs in foliage differed significantly between plants growing in conditioned soil and in unconditioned soil. The relative concentrations of senecionine-type PAs ($F_{1,37}$ = 11.07; P = 0.001) and seneciphylline-type PAs ($F_{1,37}$ = 15.79; P < 0.001) were significantly higher, and the relative concentration of jacobine-type PAs lower ($F_{1,37}$ = 10.44; P = 0.002) in conditioned soil relative to unconditioned soil (Fig. 2). In root tissues, the relative concentration of jacobine-type PAs was also significantly lower in conditioned soil than in unconditioned soil ($F_{1,37}$ = 7.83; P = 0.007). In conditioned soil, the total PA concentration in roots tended to be higher than in unconditioned soil ($F_{1,37}$ = 3.24; P = 0.077) although this effect was not significant. The type of herbivory during soil conditioning did not affect root PA concentrations in the feedback phase. However, total PA concentration in foliage was significantly lower in soil conditioned by plants with AG herbivory ($F_{1,37}$ = 5.11; P = 0.029). In the foliage, the relative concentration of senecionine-type PAs increased ($F_{1,37}$ = 4.84; P = 0.034) and the relative concentration of jacobine-type PAs decreased ($F_{1,37}$ = 3.84; P = 0.057) in soil conditioned by plants exposed to AG herbivory.

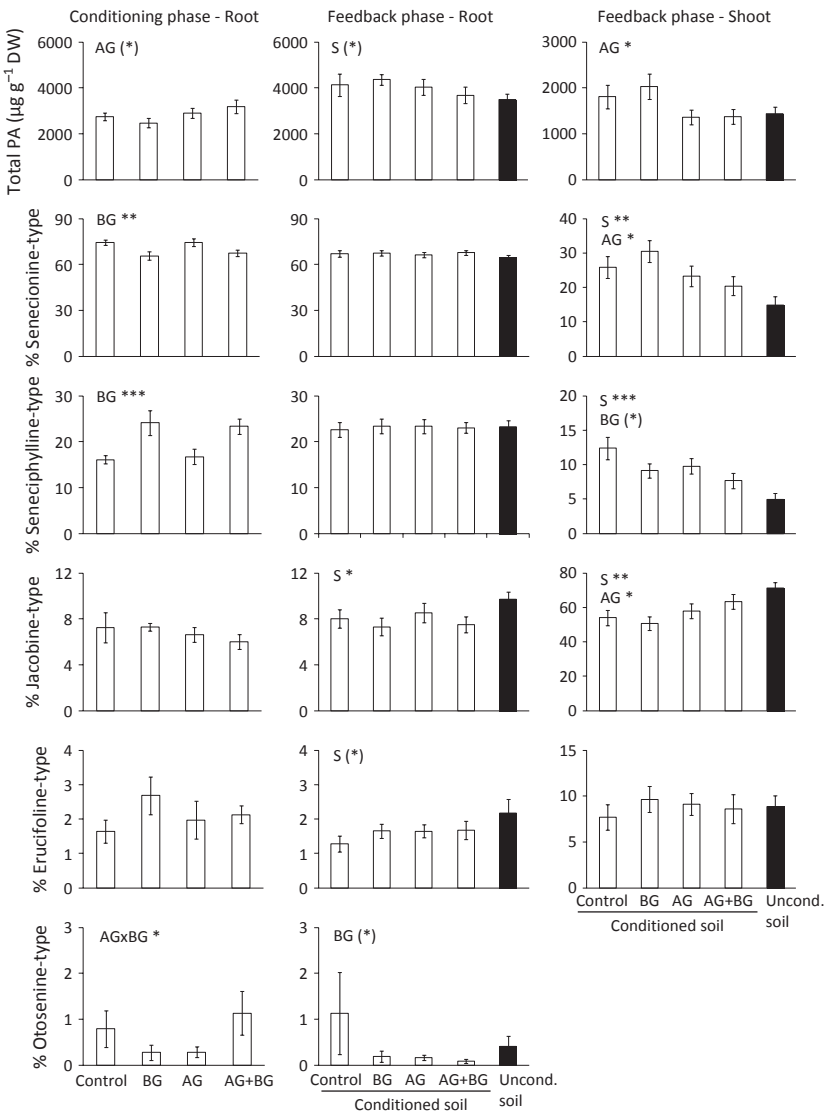

Fig. 2. Mean (±SE) total pyrrolizidine alkaloid (PA) and relative concentrations of senecionine-, seneciphylline-, jacobine-, erucifoline- and otosenine-type PAs of root tissues during the conditioning phase, and root and shoot tissues during the feedback phase. S indicates a significant difference between conditioned and unconditioned (Uncond.) soil during the feedback phase. BG and AG indicate significant direct (conditioning phase) or soil-mediated (feedback phase) effects of below-ground and above-ground herbivory respectively. ***P < 0.001; **P < 0.01; *P < 0.05; (*) P < 0.09. Results of shoot PAs in conditioned soil are based on data presented in Kostenko *et al.* (2012).

A total of 1067 TRFs were detected in root and rhizosphere soil samples: 521 were found both in root and soil samples, 338 only in root samples and 208 only in soil samples. The similarity in TRF composition between root and soil samples originating from the same pot was low (17%). For both root and soil samples, the number of TRFs per sample did not differ between the four treatments ($P > 0.2$). TRF patterns varied more between root samples (29% similarity) than between soil samples (52% similarity), and the average number of TRFs was significantly lower ($F_{1,36} = 76.1$; $P < 0.001$) in root samples (168 ± 6) than in soil samples (221 ± 6). TRF patterns of a root and soil sample originating from the same pot were not more similar than TRF patterns originated from different pots ($F_{1,72} = 0.027$; $P = 0.87$). The TRF composition was mainly determined by whether samples originated from roots or soil (CCA, 14.5% explained variation first axis alone, $F = 12.93$; $P = 0.001$; 22% total explained variation, $F = 2.95$; $P = 0.001$, Fig. 3). However, when sample origin (root/soil) was included as a covariable, the effects of AG or BG herbivory were still significant for both root and soil samples ($P < 0.04$ in all cases). When the composition of TRFs in root and soil samples was analysed separately, the composition of TRFs in soil samples was significantly affected by the herbivory treatments (CCA, $P = 0.001$; 9.7% explained variation) but there were no significant effects on the composition of TRFs in root samples (CCA, $P = 0.12$).

Thirteen different fungal taxa (referred to below as species) were detected in root and soil samples. There were, on average, three per root sample and two per soil sample. The species *Leptodontidium orchidicola*, *Fusarium oxysporum*, *Ilyonectria crassa* and *Ilyonectria rufa* were dominant in root samples (Table 2). In soil samples, *I. crassa* was detected only in two samples, but in contrast to root samples, the species *Phoma exigua* and *Trichosporon dulcitum* were more frequently detected in soil than in root samples. There were no significant effects of the herbivory treatments on any of the fungal species in root samples. However, the species *P. exigua* was detected in 70% of soil samples from the BG herbivory treatment, whereas it was absent or rare in the other three treatments resulting in a significant treatment effect ($\chi^2_3 = 16.3$; $P = 0.001$; see Table S1). *Fusarium oxysporum* was detected in more than 90% of samples of the treatment with both AG and BG herbivory and in the control treatment, but only in 40% of samples from the treatments with only AG or only BG herbivory ($\chi^2_3 = 11.3$; $P = 0.01$).

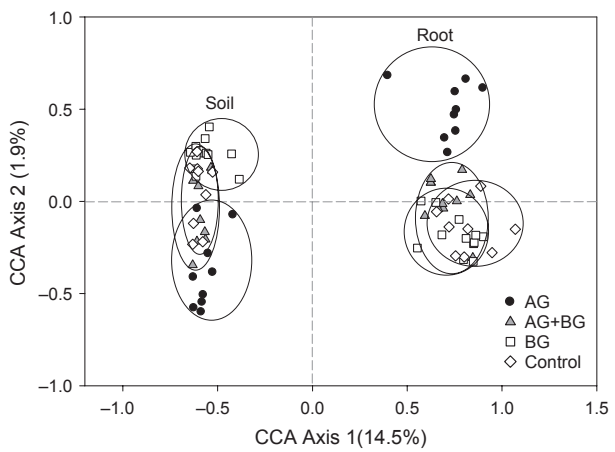

Fig. 3. Canonical correspondence analysis of fungal communities in root and soil samples of *Jacobaea vulgaris* plants exposed to above-ground herbivory (AG), below-ground herbivory (BG), above-ground and below-ground herbivory (AG + BG) or no herbivory (Control) during the conditioning phase. Percentages in parentheses refer to the amount of variation explained by each axis.

Both structural equation models provided good fits to the data (conditioning phase: $\chi^2_9 = 3.18$, $P = 0.96$; feedback phase: $\chi^2_7 = 3.23$, $P = 0.86$). In the final model for the conditioning phase, both AG and BG herbivory directly affected soil fungal communities, but the effects were opposite in direction (positive and negative coefficient respectively; Fig. 4). There was also a direct effect of AG herbivory on the composition of root fungi. BG herbivory had an indirect negative effect on root fungi through changes in the composition of root PAs. The direct effect of AG herbivory on root fungi was much stronger than the indirect effect of BG herbivory (Table S2). In the final model for the feedback phase, the composition of soil fungi was not related to plant biomass or PA composition. The soil feedback effect of BG herbivory was positively related to plant biomass. There was also a significant negative indirect effect of BG herbivory on shoot PA composition through changes in plant biomass. The soil feedback effect of AG herbivory had a negative direct effect on shoot PA composition.

Discussion

Our study provides evidence that insect herbivory can influence plant–soil feedback effects, and illustrates two ways by which insect herbivores can affect such plant–soil feedback responses: by affecting soil biota and by altering the plant's response to soil biota. Herbivory during the conditioning phase influenced the strength of the plant–soil feedback. As plants in the feedback phase were grown in conditioned soil these effects of herbivory must have been caused by changes in biotic or abiotic soil conditions. All soils used in the feedback phase were mixed with 50% sterilized soil and it is therefore unlikely that the effects were caused by differences in nutrient availability. Hence, we propose that herbivory led to changes in the composition of the soil biotic community. It is well known that AG herbivory, via changes in the plant, can affect soil biota (Bardgett & Wardle 2010). However, very few studies have examined whether this can also affect the performance of subsequently grown plants (Mikola *et al.* 2005). Our results provide evidence that herbivory indeed can affect plant–soil feedback responses.

Interestingly, the effects of herbivory also depended on whether the insects were feeding on AG or BG plant parts during the conditioning phase. BG herbivory during conditioning caused a less negative feedback whereas AG herbivory increased the strength of the negative feedback. As we examined the effects of only one AG and one BG herbivore it is important to note that we could not distinguish between

Table 2. Details of fungi that were isolated from *Jacobaea vulgaris* roots and that were detected using T-RFLP in root and soil samples of plants exposed to below-ground herbivory, above-ground herbivory, below-ground and above-ground herbivory or kept without herbivores

					Root		Soil	
Phyla	Order	Closest species	Accession	% Identity	Number	χ^2	Number	χ^2
Ascomycota	Helotiales	*Leptodontidium orchidicola*	AF486133.1	99	34	0.4	14	2.5
Ascomycota	Hypocreales	*Fusarium oxysporum*	EU219559.1	99	30	2.0	28	11.3**
Ascomycota	Hypocreales	*Ilyonectria crassa*	JF735275.1	99	23	0.8	2	2.3
Ascomycota	Hypocreales	*Ilyonectria rufa*	JF735278.1	100	19	1.3	18	3.8
Ascomycota	Pleosporales	*Tetracladium furcatum*	EU883432.1	100	4	2.9	0	NA
Basidiomycota	Tremellales	*Trichosporon dulcitum*	AF44428.1	100	2	2.3	13	0.9
Ascomycota	Hypocreales	*Trichoderma sprirale*	JF439515.1	99	2	7.5	2	2.3
Ascomycota	Pleosporales	*Altenaria alternata*	GQ121322.2	100	2	7.5	2	2.0
Ascomycota	Pleosporales	*Phoma exigua*	EU343173.1	100	1	2.5	10	16.3***
Ascomycota	Hypocreales	*Gibberella avenacea*	EU255795.1	99	1	2.8	0	NA
Ascomycota	Hypocreales	*Trichoderma harzianum*	GU566243.1	100	1	3.6	0	NA
Ascomycota	Sordariales	*Chaetomium globosum*	EU301640.1	99	0	NA	2	1.8
Ascomycota	Eurotiales	*Penicilium ochrochloron*	AY213675.1	99	0	NA	1	3.1

For each isolated fungus, the closest species match, the% similarity in DNA identity with that species and accession code from a blast analysis are presented. The total number of root and soil samples in which the fungus was detected and the results of a Chi-square test comparing the four treatments are also presented.
NA, not available.
$^{**}P < 0.01$; $^{***}P < 0.001$.

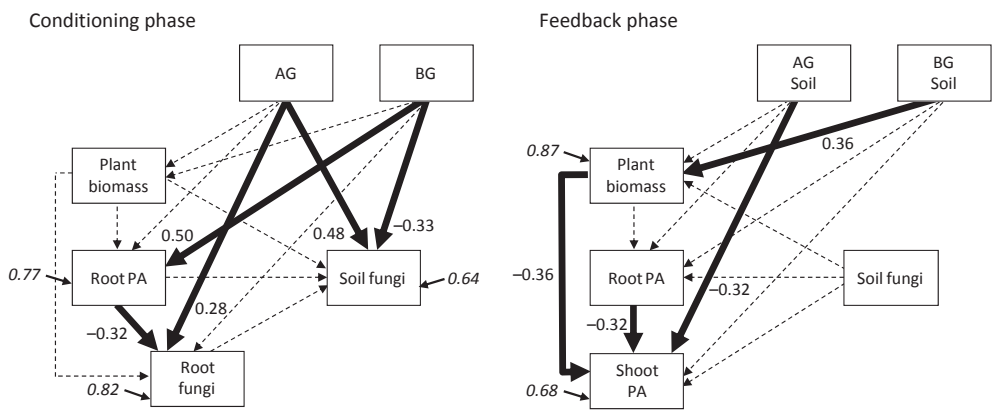

Fig. 4. Structural equation models of the relationships between herbivory, plant biomass, pyrrolizidine alkaloid composition and fungal community composition for the conditioning and feedback phase. Solid arrows depict significant effects ($P < 0.05$). Standardized path coefficients are also presented for solid arrows. Dashed arrows depict non-significant effects. Values in italics next to small arrows represent the proportion of unexplained variation for the variable ($1 - R^2$).

species-specific effects of herbivores and between differences in AG and BG herbivore effects. Other studies have shown that AG herbivore effects on soil biota can even differ between species within a single feeding guild (e.g. Wardle *et al.* 2004). Our results emphasize that generalizations about the effects of herbivory on plant–soil interactions should be made with caution.

During the feedback phase, there was an interaction between the soil-mediated effects of AG herbivory during conditioning and the direct effects of foliar herbivory on ragwort biomass. When plants were growing in soil conditioned by plants that were exposed to AG herbivory, their biomass was less reduced by foliar herbivory than when plants were growing in other soils. Remarkably, although plant biomass was less reduced by AG herbivory, the foliar herbivores gained significantly more weight on these plants (Kostenko *et al.* 2012). Our results therefore show that herbivore effects on plant–soil feedback responses go beyond plant growth responses but can also influence higher trophic levels. This is important because plant–soil feedback is often examined without herbivory (but see Mangan *et al.* 2010). Matthews & Clay (2001) showed that fungal endophyte infection in the plant can affect plant–soil feedback responses. Apart from plant–soil feedbacks, abiotic factors such as soil nutrient availability and biotic factors such as AG herbivory or competition can also greatly affect the performance, abundance and persistence of plants in the field (Casper & Castelli 2007). More studies are needed to examine how plant–soil feedbacks operate in a multitrophic AG and BG context, and the role of plant–soil feedback relative to other environmental factors that can influence plant performance.

In our study, we also examined the immediate effects of AG and BG herbivory on root chemistry and plant-fungal interactions. Foliar herbivory caused a weak increase in total PA concentration in roots, whereas root herbivory significantly altered the composition of PAs in root tissues. PAs act as deterrents against polyphagous AG insects, but can also have a negative effect on soil organisms such as nematodes and fungi (Hol & Van Veen 2002; Thoden, Boppre & Hallmann 2009). The composition of the fungal community in the rhizosphere can also be affected by PAs (Kowalchuk, Hol & Van Veen 2006). In our study, the fungal community composition in roots and in the soil was significantly influenced by AG and BG herbivory. However, in contrast to what we expected, there was no significant relationship between the fungal composition in roots and in soil. Moreover, the fungal composition in the soil could not be explained by PAs in the roots. Clearly, herbivory can affect the composition of the soil community through various mechanisms such as changes in the amount or quality of root exudates (e.g. Holland, Cheng & Crossley 1996) or changes in the supply of resources to the soil community (Bardgett & Wardle 2010).

Fungal pathogens can be important enemies of *Senecio* (*Jacobaea*) species but most studies, so far, have focused on AG pathogens (Hawkes, Douglas & Fitter 2010; Hol 2011). We isolated 13 different fungal taxa from roots of ragwort plants. Some of these fungi may play a role in the negative plant–soil feedback in *J. vulgaris*, but future studies should address their pathogenicity. In our study, the occurrence of two of these potentially pathogenic soil fungi, *P. exigua* and *F. oxysporum* differed significantly between herbivore treatments. Both genera contain many plant pathogens (Van Loon, Bakker & Pieterse 1998; Aveskamp, De Gruyter & Crous 2008), and it is possible that one or both of these fungi mediated the herbivore-induced plant–soil feedback effects in our study. Unfortunately, we do not have information about the pathogenicity of the isolated fungal strains for ragwort, but *F. oxysporum* is a known pathogen of ragwort (Hol & Van Veen 2002). In our study *F. oxysporum* was detected in roots of most plants independent of the herbivory treatment. Fungal TRFs were detected qualitatively (presence/absence), so it is possible that there were differences in the abundance of *F. oxysporum* in roots of plants between the different herbivory treatments. Quantitative molecular techniques such as Q-PCR are needed to determine whether this is the case. *Phoma exigua*, in contrast, was rarely detected in roots in our study even though it was abundant in soil samples. However, several *Phoma* species have been isolated from ragwort roots (De Gruyter *et al.* 2012). DNA from root and soil samples was extracted using two different methods. Hence, it is possible that the discrepancy between presence in root and in soil samples in our study is due to technical limitations of the DNA extraction or T-RFLP technique.

Our study adds a novel dimension to the rapidly growing body of literature demonstrating the importance of plant–microbe interactions for plant performance and feedback effects. It illustrates the important role of insect herbivory in plant–soil feedbacks. We used SEM to explore the pathways by which AG and BG insect herbivores influence PAs, soil fungi and plant–soil feedbacks. We hypothesized that effects of herbivory on fungi would be mediated by root PAs. While this was true for BG herbivore effects on root fungi, the effects of AG and BG herbivory on soil fungi were direct. This indicated that factors that we did not measure in this study such as root exudates mediated the herbivore effects. Moreover, as we detected soil-mediated herbivory effects in the feedback phase, the structural equation model indicated that these effects were not driven by changes in soil fungi. We were not able to pinpoint the mechanism by which insect herbivory influenced plant–soil feedbacks. However, the models clearly showed that AG and BG effects were mediated by different pathways, and that the mechanisms of AG and BG herbivory effects on PA and soil fungi differed. Further experiments that examine the interactions between foliar and root feeding insect herbivores and root pathogenic fungi in more detail and other possible mechanism of herbivore-mediated effects on plant soil feedback such as changes in root exudates are needed. We conclude that to understand the role of plant–soil interactions in influencing plant performance and plant community dynamics, plant–soil feedbacks should be considered from a multitrophic AG and BG perspective.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Frequency of detection of fungal species in root and soil samples of plants exposed to different herbivory treatments.

Table S2. Direct and indirect effects of above-ground and below-ground herbivory on the composition of root fungi and shoot PAs.