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Oecologia

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https://doi.org/10.1007/s00442-014-2885-y

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Oecologia (2014) 175:187–198
DOI 10.1007/s00442-014-2885-y

PLANT-MICROBE-ANIMAL INTERACTIONS - ORIGINAL RESEARCH

Sequential effects of root and foliar herbivory on aboveground and belowground induced plant defense responses and insect performance

Minggang Wang · Arjen Biere · Wim H. Van der Putten · T. Martijn Bezemer

Received: 2 September 2013 / Accepted: 9 January 2014 / Published online: 22 January 2014

Abstract Plants are often simultaneously or sequentially attacked by multiple herbivores and changes in host plants induced by one herbivore can influence the performance of other herbivores. We examined how sequential feeding on the plant *Plantago lanceolata* by the aboveground herbivore *Spodoptera exigua* and the belowground herbivore *Agriotes lineatus* influences plant defense and the performance of both insects. Belowground herbivory caused a reduction in the food consumption by the aboveground herbivore independent of whether it was initiated before, at the same time, or after that of the aboveground herbivore. By contrast, aboveground herbivory did not significantly affect belowground herbivore performance, but significantly reduced the performance of later arriving aboveground conspecifics. Interestingly, belowground herbivores negated negative effects of aboveground herbivores on consumption efficiency of their later arriving conspecifics, but only if the belowground herbivores were introduced simultaneously with the early arriving aboveground herbivores. Aboveground–belowground interactions could only partly be explained by induced changes in an important class of defense compounds, iridoid glycosides (IGs). Belowground herbivory caused a reduction in IGs in roots without affecting shoot levels, while aboveground herbivory increased IG levels in roots in the short term (4 days) but only in the shoots in the longer term (17 days). We conclude that the sequence of aboveground and belowground herbivory is important in interactions between aboveground and belowground herbivores and that knowledge on the timing of exposure is essential to predict outcomes of aboveground–belowground interactions.

Keywords Aboveground–belowground interactions · Induced defense · Iridoid glycosides · Secondary plant compounds · Timing

Introduction

Virtually all plants in nature are exposed to herbivory by a variety of aboveground and belowground insect species. Insect herbivory can elicit morphological, physiological, and biochemical plant responses, which can depend greatly on the identity of the attacker (Karban and Baldwin 1997). Such insect-induced changes in plant defenses can subsequently influence the performance of the insect that causes the feeding damage on the plant. Moreover, via feeding-induced changes in the plant, an insect herbivore can also influence the performance of other insects that feed on the same plant (Kaplan and Denno 2007). These plant-mediated effects between insect herbivores can be positive and negative for one or both of the herbivores (Kaplan and Denno 2007).

Most studies that have examined inter- or intraspecific interactions between insect herbivores on a shared host plant have focused on aboveground insects. However, plants are also frequently attacked by belowground insects, and many studies have shown that aboveground and belowground insects can influence each other via changes in the

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Communicated by Julia Koricheva.

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shared host plant (Masters et al. 1993; Van der Putten et al. 2001; Bezemer et al. 2003; Wardle et al. 2004; Van Dam and Raaijmakers 2006; Kaplan et al. 2008). These effects can be mediated by changes in concentrations of primary compounds in the plant such as nitrogen or carbon (Masters et al. 1993). Moreover, root- and shoot-feeding insects on a plant can influence each other's performance via systemic induction of secondary (defense) compounds in the plant (Bezemer et al. 2003, 2004; Bezemer and Van Dam 2005; Kaplan et al. 2008).

Interactions between insect herbivores can occur when they feed simultaneously on a plant, but insects can also affect each other when they feed sequentially on the same plant (Maron 1998; Blossey and Hunt-Joshi 2003; Voelckel and Baldwin 2004). Interestingly, several studies have shown that concentrations of defense compounds in a plant may only change in response to the first attacker and may not be modified by later arrivers (Viswanathan et al. 2007; Poelman et al. 2008; Erb et al. 2011; Gomez et al. 2012). As the defense response of a plant strongly depends on the identity of the first attacker (Voelckel and Baldwin 2004; Viswanathan et al. 2007), the performance of an insect on a plant may depend both on the identity of the other herbivores on that plant, and on when they have been feeding on the plant (Poelman et al. 2008; Gomez et al. 2012).

Recently, Johnson et al. (2012) concluded in a meta-analysis of 35 studies that the interaction between aboveground and belowground insect herbivores also depends on the sequence of aboveground and belowground herbivory. Aboveground herbivory negatively influenced the performance of belowground insect herbivores, but only when aboveground herbivores were feeding first. In contrast, belowground herbivores appeared to facilitate the performance of aboveground herbivores, but only when they fed simultaneously; introduction before or after the aboveground herbivore did not result in facilitative effects of belowground herbivores (Johnson et al. 2012). In the meta-analysis sequential effects of aboveground and belowground herbivore additions were compiled from many different aboveground and belowground insect species that were feeding on a variety of different plant species. This leaves the following question unanswered: how may sequential feeding within single host-aboveground-belowground herbivore systems influence all individual components? However, such studies are rare. One exception is a study by Erb et al. (2011) who reported that the leaf chewer *Spodoptera frugiperda* negatively affected the performance of the root chewer *Diabrotica virgifera* on maize, but only when the aboveground herbivore started feeding on the plant before the belowground herbivore.

Several studies have suggested that in response to simultaneous aboveground and belowground herbivory, plant defense compounds may increase more in shoot than in root tissues (e.g., Bezemer et al. 2004; Rasmann et al. 2009; Erb et al. 2009). Similarly, simultaneous application of jasmonic acid to roots and shoots of Brassicaceous plants as a mimic of herbivory by chewing insect herbivores increased glucosinolate concentrations in shoots but not in roots (Van Dam et al. 2004). Via these effects on plant defenses, simultaneous aboveground and belowground herbivory may alter the performance of other herbivores in ways that may not be predictable from their effects when they differ in their time of appearance (Bezemer et al. 2003; Erb et al. 2009; Rasmann et al. 2009). We are not aware of any study that has examined how sequential feeding by both aboveground and belowground herbivores affects plant defenses and the performance of later feeding herbivores.

In the present study, we examined how sequential feeding on ribwort plantain (*Plantago lanceolata* L., Plantaginaceae) by aboveground and belowground insect herbivores may influence aboveground and belowground plant biomass and defense responses, as well as insect herbivore performance. *P. lanceolata* is a short-lived perennial forb that produces a wide range of allelochemicals (Sutter and Müller 2011). Two important defense chemicals present in *P. lanceolata* are the iridoid glycosides (IGs) aucubin and catalpol. Numerous studies have shown that the concentrations of these compounds in shoots and roots can have strongly negative effects on the performance of generalist above- and belowground insect herbivores, but that they can be used as feeding cues and oviposition stimulants by specialist insect herbivores (e.g., Bowers and Puttick 1989; Nieminen et al. 2003; Wurst et al. 2008; Reudler Talsma et al. 2008, 2011). Moreover, these compounds affect the performance of both plant-beneficial and plant-pathogenic fungi (Marak et al. 2002b; Biere et al. 2004; De Deyn et al. 2009). Several studies have shown that IG concentrations can increase in response to damage by aboveground (e.g., Bowers and Stamp 1993; Darrow and Bowers 1999; Marak et al. 2002a) and belowground (e.g., Wurst et al. 2008) insect herbivores. Herbivory can cause both a local increase in IG concentrations in the damaged plant tissues, and a systemic induction in aboveground and belowground plant parts (Darrow and Bowers 1999; Marak et al. 2002a; Wurst et al. 2008), providing scope for mediation of interactions between aboveground and belowground organisms associated with the plant.

We tested the following hypotheses:

1. Aboveground herbivory will cause a systemic increase in IG concentrations in aboveground and belowground plant tissue and will reduce the performance of aboveground and belowground insect herbivores, but only when the aboveground herbivore is introduced first.

2. Belowground herbivory will positively affect aboveground insect herbivore performance, but only when these herbivores start feeding simultaneously.
3. When both an aboveground and a belowground herbivore are introduced prior to a later arriving aboveground herbivore, their effects on the later arriving herbivore will depend on their sequence of introduction.

Materials and methods

Plants and insects

Plantago lanceolata L. (Plantaginaceae) (ribwort, plantain) is a plant species with a world-wide distribution that serves as a model species in plant–insect interaction research (e.g., Bowers and Stamp 1992). *P. lanceolata* contains IGs, predominantly aucubin and catalpol, which are toxic or deterrent to generalist herbivores but act as feeding and oviposition cues for specialists. Seeds of *P. lanceolata* were purchased from a wild-seed supplier (Cruydt-hoeck, Nijeberkoop, the Netherlands). The seeds were surface sterilized with sodium hypochlorite (1 %), sown on glass beads and placed in an incubator (16 h light, 20/25 °C night/day temperature). Germinated seedlings were transplanted into 1.1-l pots (one plant per pot) filled with 1,100 g sandy loam mineral soil [particle size distribution with 3 % <2 μm, 17 % 2–63 μm, and 80 % >63 μm; relative humidity (RH) = 12.3 %] collected from a restoration grassland (De Mossel, Ede, the Netherlands) where *P. lanceolata* abundantly occurs. In the laboratory the soil was sieved through a 1-cm mesh, homogenized and gamma sterilized (>25 KGy). Pots were placed randomly in a greenhouse. Plants were watered three times per week and randomly redistributed within the greenhouse once a week. Natural daylight in the greenhouse was supplemented by 400-W metal halide lamps (225 μmol m^{-2} s^{-1} photosynthetically active radiation).

Wireworms are larvae of the click beetle *Agriotes lineatus* L. (Coleoptera: Elateridae). They are generalist root herbivores commonly found in grasslands and a pest of many cultivated crops (Parker and Howard 2001). Wireworms typically stay in the soil for 3–6 years as larvae before pupation (Parker and Howard 2001). Wireworms were purchased from Applied Plant Research (PPO-Wageningen University and Research Centre), Lelystad, the Netherlands. Before introduction, they were starved for 3 days in moist soil and weighed using a microbalance.

Spodoptera exigua H. (Lepidoptera: Noctuidae), the beet armyworm, is a generalist foliar herbivore that feeds on plants from more than 30 families (Merkx-Jacques et al. 2008). It originates from Southeast Asia, but nowadays has a world-wide distribution. The larvae go through five instars during development (Harvey et al. 2005). Beet armyworm eggs were obtained from the Laboratory of Entomology, Wageningen University, the Netherlands and reared until the third instar on an artificial diet (Singh et al. 1983). Rearing took place in a growth chamber (24 °C, 16:8-h light/dark photoperiod, 70 % RH) before being introduced on the plants.

Experiment 1: impact of the sequence of aboveground herbivore introduction on belowground herbivore performance

To determine the effects of the sequence of aboveground herbivore introduction on plant growth and defense and on the performance of a belowground herbivore, an experiment was set up with 66 pots. One seedling was planted into each pot. Twenty-eight days after planting ($t = -5$), all plants were caged using cylindrical mesh cages (height 1 m, diameter 35 cm). Six treatments were initiated (Fig. 1a), with 11 replicate pots per treatment. The treatments were: (1) the aboveground herbivore was introduced at $t = -5$, i.e., 5 days before the introduction of the belowground herbivores (A > B); (2) the simultaneous introduction of the aboveground and belowground herbivores at $t = 0$ (A = B); (3) the aboveground herbivore was introduced at $t = 5$, i.e., 5 days after the introduction of the belowground herbivores at $t = 0$ (B > A); (4) introduction of the belowground herbivores at $t = 0$ without the aboveground herbivores (B); (5) introduction of the aboveground herbivore at $t = 0$ without the belowground herbivores (A); and (6) control plants without the aboveground and belowground herbivores (Co).

As belowground herbivores, two pre-weighed late-instar (mean = 24.6 mg; SE = 0.7 mg) *A. lineatus* were placed into 1-cm-deep small holes made in the soil 33 days after transplantation ($t = 0$). All wireworms immediately burrowed into the soil. Similar holes were also made in the soil of pots without wireworms. The aboveground herbivore treatment consisted of one third-instar *S. exigua* per pot (mean = 49.2 mg; SE = 0.1 mg). The larva could move freely on the plant within the cage.

Plants were harvested at $t = 17$ (17 days after introducing the belowground herbivores in treatments 1–5). At harvest the aboveground insects were removed from the plants, and the aboveground biomass was clipped at ground level. Wireworms were recovered from the soil, re-weighed and the weight gain of each wireworm was determined. The fifth youngest leaf and a subsample of roots of 11 randomly chosen plants assigned to each of the treatments A, B, B = A and Co were removed with a razor blade, immediately frozen at −20 °C, freeze-dried for 4 days under vacuum (−55 °C collector temperature; Labconco Free Zone 12 L Freeze Dry System, USA), weighed and ground.

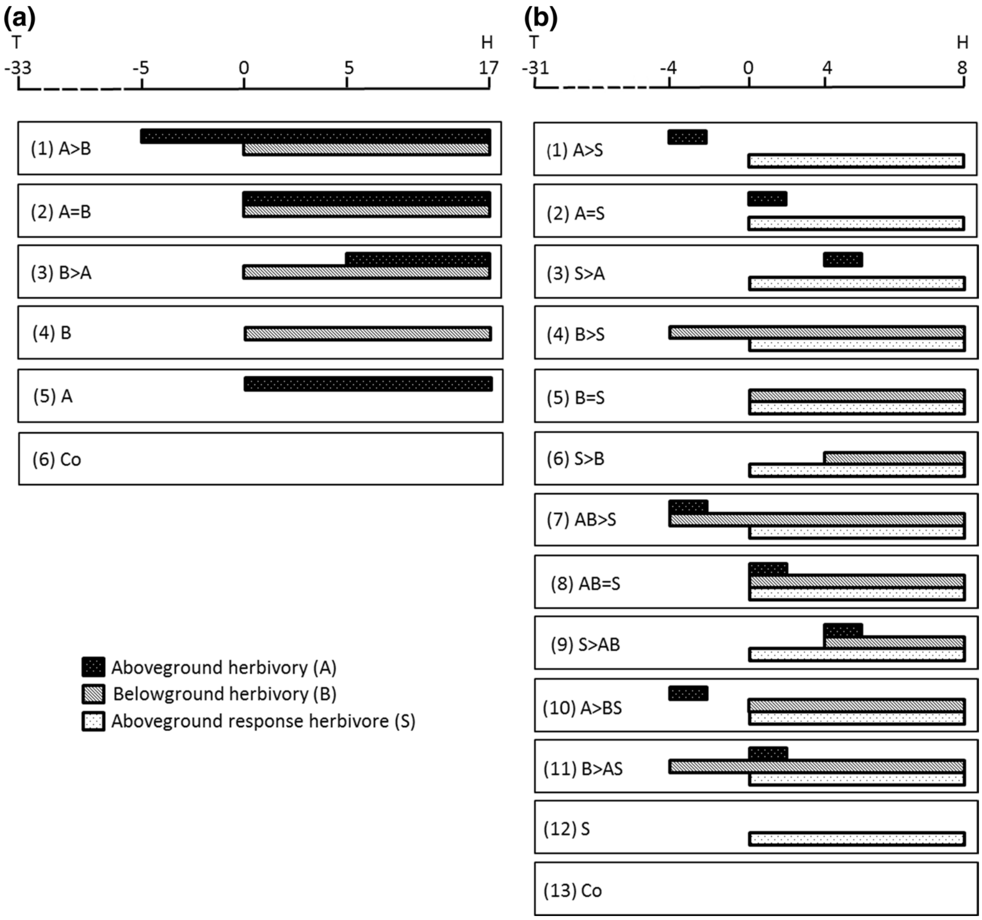

Fig. 1 Experimental design of **a** experiment 1 and **b** experiment 2. Response herbivores *Agriotes lineatus* (experiment 1) and *Spodoptera exigua* (experiment 2) were introduced at $t = 0$. The *horizontal bars* indicate when the aboveground and belowground herbivores were feeding, and the *length* of each *bar* represents the feeding duration. Day of transplantation (T) = −33 and day of harvest (H) = 17 (**a**); $T = -31$ and $H = 8$ (**b**)

Twenty-five mg of each sample was extracted overnight in 70 % methanol, then filtered and diluted ten times with ultrapure water. The concentrations of the IGs aucubin and catalpol were analyzed using high performance liquid chromatography as described by Marak et al. (2002b). Due to practical limitations, leaf and root chemistry could only be determined for a subset of the treatments. The remaining aboveground and belowground biomass of each plant was oven-dried at 70 °C and dry weight was determined.

The effect of aboveground herbivory on wireworm performance was first analyzed independent of sequence (treatments A > B, A = B, B > A vs. B) using one-way ANOVA. Subsequently, we carried out a one-way ANOVA in which we analyzed all four treatments, using a Dunnett post hoc test to compare treatment B with each of the treatments A > B, A = B, and B > A. The effect of the timing and duration of aboveground herbivory on wireworm performance was also analyzed using linear regression with the duration of aboveground herbivore feeding on plants as continuous variable (0 days for treatment B, 12 days for B > A, 17 days for A = B, and 22 days for A > B). Total plant biomass and shoot-to-root ratio were analyzed using one-way ANOVA for the effects of aboveground (A vs. Co), belowground (B vs. Co) and combined above- and belowground herbivory (A > B, A = B, B > A vs. Co). The latter was again followed by a Dunnett post hoc test in which the three combined herbivory treatments were each compared with control plants without herbivory. IG concentrations were analyzed using two-way ANOVA with presence or absence of aboveground herbivory and belowground herbivory as main factors. All data were checked for normality using a Kolmogorov–Smirnov one-sample test and for homogeneity of variance using a Levene test before analysis.

Experiment 2: impact of sequence of belowground and aboveground herbivore introduction on aboveground herbivore performance

To examine the effects of the introduction sequence of aboveground and belowground herbivores on aboveground herbivore performance, we set up an experiment with 221 pots. We used fourth instar *S. exigua* larvae (one per clip cage) as aboveground and wireworms (one per pot) as belowground ‘treatment’ herbivores and *S. exigua* larvae as aboveground response herbivores. To standardize the amount of damage caused by the aboveground treatment herbivores, one *S. exigua* larvae was introduced into a

2.0-cm diameter clip cage that was placed on the top area of a fully expanded mature leaf (one clip cage per plant). After 1 day, when the entire area within the clip cage was eaten, the clip cage was moved to another mature leaf so that on each plant there were two areas of 3.14 cm^2 consumed over a period of 2 days. At $t = 0$ (Fig. 1b; 31 days after transplanting), all plants were caged and in 204 cages (all treatments except the Co treatment, see below) one pre-weighed third-instar (mean = 27.2; SE = 0.4 mg) *S. exigua* was introduced. These larvae were considered aboveground response herbivores (S). The response herbivores could move freely on the plant. Eight days after introducing the aboveground response herbivores, they were collected from each cage, re-weighed and weight gain was calculated.

The experiment was set up with 13 treatments and 17 replicate pots per treatment. In all treatments except treatment 13, the response herbivore (S) was introduced at $t = 0$. The treatments were (Fig. 1b): (1) the AG treatment herbivore was introduced 4 days before (A > S), (2) at the same time (A = S), and (3) 4 days after the response herbivore (S > A). The BG treatment herbivores were introduced (4) 4 days before (B > S), (5) at the same time (B = S), and (6) 4 days after the response herbivore (S > B). The AG and BG treatment herbivores were both introduced (7) 4 days before (AB > S), (8) at same time (AB = S), and (9) 4 days after the response herbivore (S > AB). To determine how the relative sequence of prior aboveground and belowground herbivory influenced the performance of the response herbivore, the aboveground treatment herbivore was introduced (10) 4 days before the belowground treatment herbivore and the aboveground response herbivore (A > BS), (11) the belowground herbivore was introduced 4 days before the aboveground treatment herbivore and the response herbivore (B > AS). Finally, (12) the response herbivore was introduced without other herbivores (S), and there was a control (13) without aboveground and belowground herbivory (Co).

Forty extra plants were grown to determine the effects of aboveground and belowground herbivory on IG concentrations in the plant at the time that the response herbivore was introduced on the plant. Treatments included 4 days of aboveground (A), belowground (B), or aboveground and belowground herbivory (AB), and no herbivory (Co) with ten replicate plants per treatment. All herbivory treatments for these extra plants were initiated at $t = -4$ and the plants were harvested at $t = 0$. The fifth leaf and a subsample of roots was freeze-dried and analyzed as described for experiment 1. All other plants were harvested at $t = 8$, i.e., 8 days after the response herbivore was introduced. Roots were removed carefully from the soil and rinsed. All leaves of each plant were scanned using a photo scanner (Perfection 4990; Epson, Japan) and the leaf area consumed by the response herbivore was determined using the software WinFOLIA (Regent Instruments, Sainte-Foy, Canada). Consumption efficiency was calculated as weight gain of *S. exigua* per consumed square centimeter leaf area. As the *S. exigua* were reared on a moist artificial diet until introduction on the plant, the initial weight of the larvae was adjusted to compensate for the 30 % moisture loss after introduction (Boldt et al. 1975). Shoot and root biomass was then oven-dried and total biomass was determined.

To determine the influence of belowground herbivory on the weight gain, leaf area consumption, and food consumption efficiency of the response herbivore *S. exigua*, we performed a one-way ANOVA testing effects of belowground herbivory (treatments 4–6) against the treatment with the response herbivore only (treatment 12). As in experiment 1, a one-way ANOVA was followed by a Dunnett post hoc test in which each of the treatments B > S, B = S, S > B were contrasted with S, to examine whether the significance of the effect of belowground herbivores depended on their introduction sequence. Similar analyses were performed for the effects of aboveground herbivory (treatments 1–3 vs. 12) and simultaneous above- and belowground herbivory (treatments 7–9 vs. 12). Effects of the

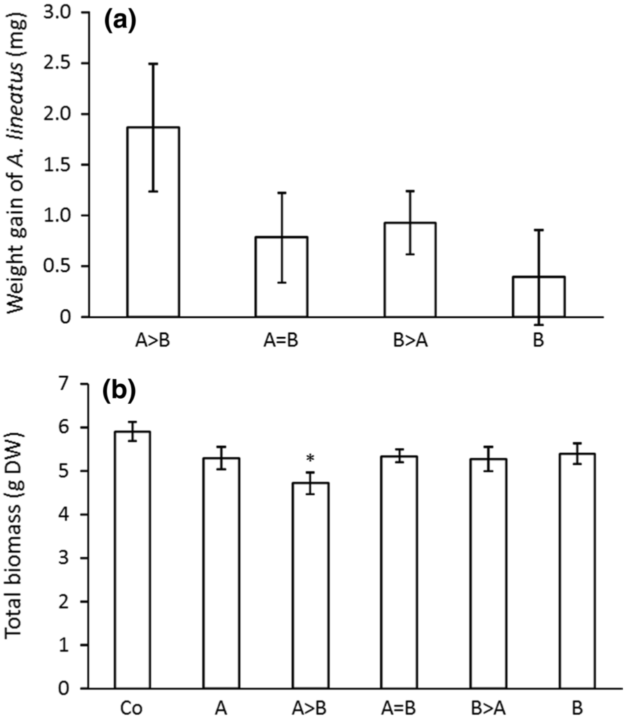

Fig. 2 Effects of the timing of aboveground herbivore introduction on the performance of the belowground herbivore *A. lineatus* and on the host plant *Plantago lanceolata*. Shown are mean (±SE) **a** weight gain of *A. lineatus* and **b** total plant biomass. Plants were exposed to aboveground (*A*) or belowground (*B*) or both A and B herbivory in different sequences (see Fig. 1) or left undamaged (*Co*). *Asterisks* in Fig. 2b denote treatments significantly different from the Co based on a Dunnett post hoc test ($P < 0.05$)

joint herbivory by response caterpillars and aboveground, belowground, or aboveground plus belowground herbivores on plant biomass and shoot-to-root ratio were also analyzed using one-way ANOVAs using undamaged plants (treatment 13) as a control.

To determine the effect of the sequential introduction of aboveground and belowground treatment herbivores on the aboveground response herbivore, the treatments 7, 8, 10 and 11 were analyzed using two-way ANOVA, with the sequence of aboveground herbivory (before and simultaneous) and belowground herbivory (before and simultaneous) as main factors. IG concentrations in root and shoot tissues were analyzed using two-way ANOVA with presence or absence of aboveground and belowground herbivory as main factors. All data were checked for normality and homogeneity of variance before analysis.

Results

Experiment 1: impact of the sequence of aboveground herbivore introduction on belowground herbivore performance

There was no overall significant effect of the aboveground herbivore treatments on the mean weight gain of wireworms ($F_{1,39} = 2.16$; $P = 0.150$; Fig. 2a). Wireworms on average gained more weight when *S. exigua* had been introduced before the wireworms, and with increasing duration of aboveground herbivore feeding, but these effects were not statistically significant (Dunnett test, $P = 0.086$, and $F_{1,41} = 3.83$; $P = 0.057$, respectively; Fig. 2a). Total plant biomass was significantly reduced by combined aboveground and belowground herbivory ($F_{1,40} = 8.40$; $P = 0.006$; Fig. 2b), but this effect was only significant for the longest feeding duration of *S. exigua* (22 days; Dunnett test; $P = 0.002$). Total plant biomass was not significantly affected by a 17-day period of either aboveground or belowground herbivory alone ($F_{1,19} = 3.27$; $P = 0.086$, and $F_{1,19} = 2.45$; $P = 0.134$, respectively) and shoot-to-root biomass ratio was not affected by any of the treatments (all $P > 0.10$). The concentrations of IGs in shoots were significantly higher in plants exposed to aboveground herbivory than in control plants ($F_{1,36} = 8.74$; $P = 0.005$; Fig. 3a), while the concentration of IGs in roots were significantly lower in plants exposed to belowground herbivory than in control plants ($F_{1,36} = 7.23$; $P = 0.011$; Fig. 3b). The ratio of catalpol to aucubin was not affected by any of the treatments.

Experiment 2: effects of sequence of introduction of belowground and aboveground herbivores on aboveground herbivore performance

Weight gain of the response caterpillars was significantly reduced by aboveground herbivory ($F_{1,61} = 5.17$; $P = 0.027$), but only when feeding occurred before introduction of the response caterpillars (Dunnett test; $P = 0.009$) and not when feeding occurred later ($P > 0.10$) (Fig. 4a). Belowground herbivory alone only marginally reduced weight gain of response caterpillars ($F_{1,63} = 3.79$; $P = 0.056$). The leaf area consumed by response caterpillars was significantly reduced by previous aboveground herbivory, both in the case of aboveground herbivory

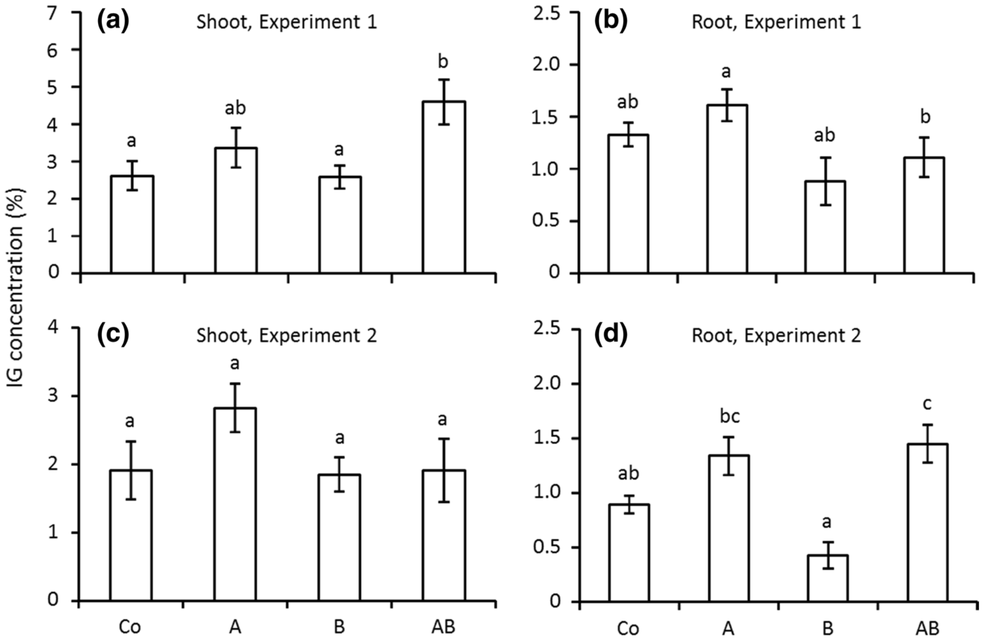

Fig. 3 Mean (±SE) iridoid glycoside (*IG*) concentration (% dry weight) in shoot and root tissues of *P. lanceolata* exposed to no herbivory (*Co*), only aboveground herbivory by *S. exigua* (*A*), only belowground herbivory by *A. lineatus* (*B*), and both A and B herbivory (*AB*) in experiment 1 (**a**, **b**) and experiment 2 (**c**, **d**). Plants were exposed to the herbivore treatments for 17 days in experiment 1 and for 4 days in experiment 2. For illustrative purposes, *different letters* have been assigned to treatments that are significantly different from each other based on a Tukey post hoc test following a one-way ANOVA ($P < 0.05$). For statistical analyses, see text

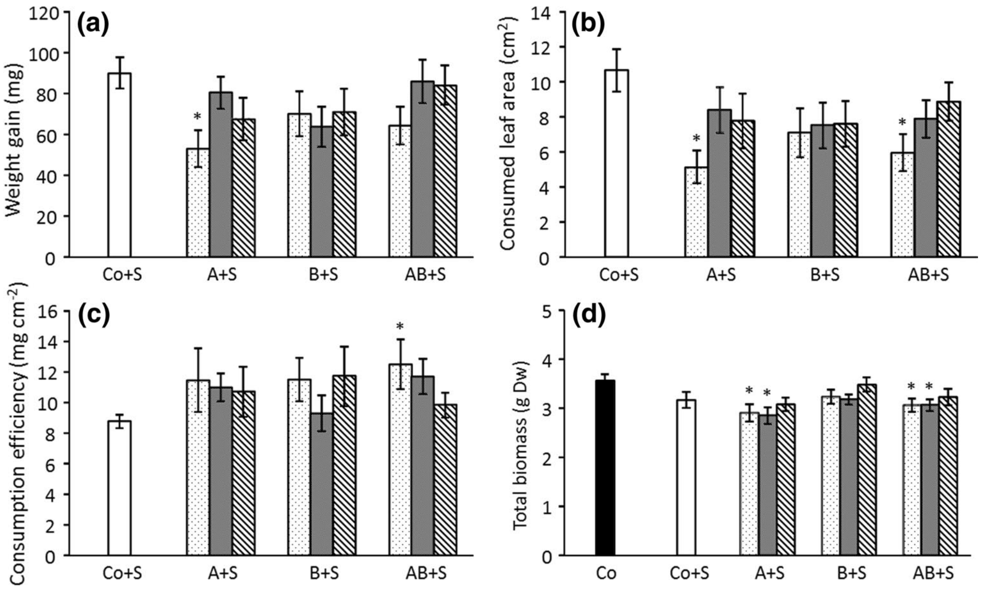

Fig. 4 Effects of aboveground and belowground herbivores introduced before (*dotted bars*), at the same time as (*grey bars*), or after (*dashed bars*) the aboveground response herbivore on the performance of the response herbivore *S. exigua* and on the host plant *P. lanceolata*. Shown are mean (±SE) **a** weight gain, **b** consumed leaf area, **c** food consumption efficiency of *S. exigua*, and **d** plant total biomass. Plants were exposed to aboveground herbivory by *S. exigua* response larvae (*S*), and in addition to aboveground (*A*) or belowground (*B*) treatment herbivores, or not exposed to treatment herbivory (*Co*). *Asterisks* denote treatments significantly different from the control [Co + S for the herbivore traits (**a**–**c**), Co for the plant trait (**d**)] based on a Dunnett post hoc test ($P < 0.05$)

alone ($F_{1,61} = 6.32$; $P = 0.015$), and in combination with belowground herbivory ($F_{1,62} = 5.79$; $P = 0.019$; Fig. 4b). Effects of the introduced herbivores on the leaf area consumption by response caterpillars were strongly dependent on the timing of their feeding. When the 2-day feeding period by *S. exigua* occurred before the response caterpillars were put on the plants, the leaf area consumption by response caterpillars was reduced, both in the aboveground treatment (Dunnett test; $P = 0.006$) and in the combined aboveground and belowground treatment (Dunnett test; $P = 0.011$). However, when the treatment feeding started at the same time, or after introduction of the response caterpillars, the leaf area consumption of response caterpillars was not significantly affected (all $P > 0.10$). By contrast, belowground herbivory consistently reduced the leaf area consumption of response caterpillars ($F_{1,61} = 4.96$; $P = 0.030$), independent of the timing of their introduction (Fig. 4b). The consumption efficiency of response caterpillars was only affected by the combined aboveground and belowground herbivory treatments ($F_{1,62} = 4.34$; $P = 0.041$; Fig. 4c). Combined aboveground and belowground herbivory slightly increased the consumption efficiency of response caterpillars relative to that of insects on plants that were not exposed to 'treatment' herbivory, but only when the treatment caterpillars started feeding prior to the response caterpillars (Dunnett test; $P = 0.045$).

Total plant biomass was only marginally reduced by the 8-day period of feeding by the response caterpillars alone compared to the control with no herbivory (Co + S vs. Co) ($F_{1,32} = 3.44$; $P = 0.073$; Fig. 4d). However, total plant biomass was significantly reduced in combination with the aboveground herbivore treatment due to the effects of the additional 2-day feeding period by *S. exigua,* both in the absence of belowground herbivores (A + S vs. Co) ($F_{1,64} = 12.02$; $P < 0.001$) and in their presence (AB + S vs. Co) ($F_{1,64} = 7.72$; $P = 0.007$; Fig. 4d). Root herbivory reduced root biomass ($F_{1,66} = 4.81$; $P = 0.032$), but it did not affect total plant biomass ($F_{1,66} = 2.78$; $P = 0.100$). None of the treatments significantly affected the shoot-to-root biomass ratio of the plants (all $P > 0.30$).

In the treatments involving combined effects of aboveground and belowground herbivores on response caterpillars presented above, so far we only considered the cases in which these aboveground and belowground herbivores were introduced simultaneously. Below we present results of how the sequence of their introduction further affects their impact on response caterpillars. As observed above, simultaneous introduction of aboveground and belowground herbivores before the response caterpillars (AB > S) reduced the weight gain of response caterpillars compared to when they were introduced at the same time as the response caterpillars (AB = S; Fig. 5a). This reduction was also observed when the aboveground herbivore was introduced before, but the belowground herbivore simultaneously with the response caterpillar (A > BS; Fig. 5a), but not when the belowground herbivore was

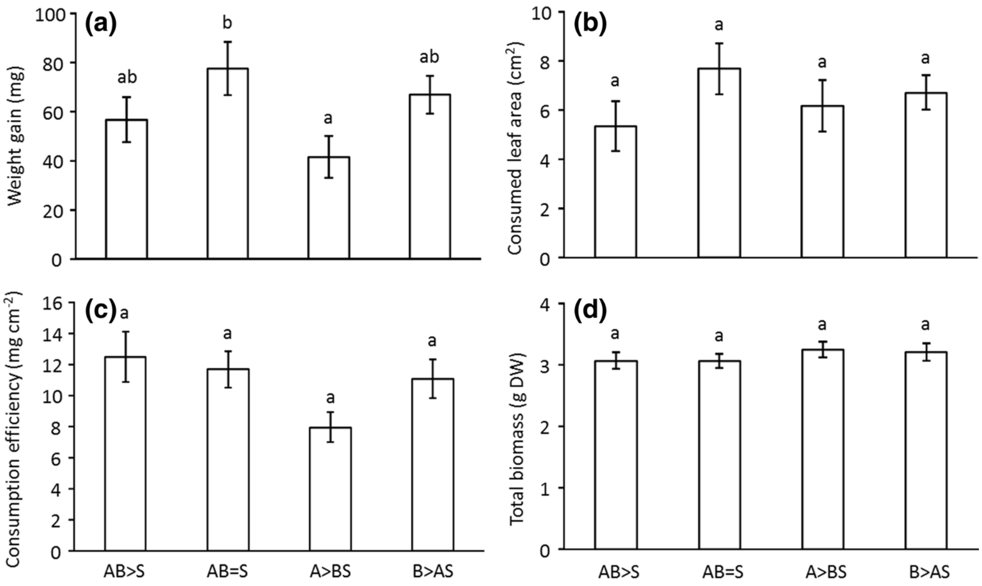

Fig. 5 Effects of the sequence of aboveground and belowground herbivory on the performance of the aboveground response herbivore *S. exigua* and on the host plant. Shown are mean (±SE) **a** weight gain, **b** consumed leaf area, **c** food consumption efficiency of *S. exigua*, and **d** plant total biomass. Treatments represent four different introduction sequences of the aboveground (*A*), and belowground (*B*) treatment herbivores and the response herbivore (*S*): A and B introduced before S (*AB* > *S*), only A introduced before S (*A* > *BS*), only B introduced before S (*B* > *AS*), or all introduced simultaneously (*AB* = *S*). For illustrative purposes, *different letter*s have been assigned to treatments that are significantly different from each other based on a Tukey post hoc test following a one-way ANOVA ($P < 0.05$). For statistical analyses, see text

introduced before and the aboveground herbivore simultaneously with the response caterpillar (B > AS; Fig. 5a). Statistical analysis confirmed that there was a negative effect of prior aboveground herbivory ($F_{1,58} = 6.46$; $P = 0.014$), independent of when the belowground herbivore was introduced ($F_{1,58} = 0.043$; $P = 0.84$). By contrast, effects of combined aboveground and belowground herbivory on leaf area consumption by response caterpillars were unaffected by the sequence in which aboveground and belowground herbivory was initiated (Fig. 5b). Interestingly, the consumption efficiency of response caterpillars was reduced when the aboveground treatment herbivore was introduced before the response herbivore, but only when the root herbivore was introduced simultaneously with the response herbivore (A > BS; Fig. 5c) and not when the root herbivore was also introduced before the response herbivore (AB > S; Fig. 5c) (interaction between prior aboveground and belowground herbivory; $F_{1,58} = 4.15$; $P = 0.046$; Fig. 5c). Effects on plant biomass were also independent of the onset of aboveground and belowground herbivory in the combined above- and belowground herbivory treatments (Fig. 5d).

Shoot IG concentrations were not significantly affected by aboveground ($F_{1,36} = 1.65$; $P = 0.208$) or belowground herbivory ($F_{1,36} = 1.69$; $P = 0.20$; Fig. 3c). Root IG concentrations were significantly increased by aboveground herbivory ($F_{1,36} = 22.98$; $P < 0.001$; Fig. 3d), while the effects of belowground herbivory on root IG concentrations depended on aboveground herbivory (interaction between above- and belowground herbivory; $F_{1,36} = 4.40$; $P = 0.043$; Fig. 3d). In the absence of aboveground herbivory, root herbivory decreased root IG concentrations compared to control plants. However, in the presence of aboveground herbivory, root herbivory did not affect root IG concentrations compared to aboveground herbivory alone (Fig. 3d). Similarly, root herbivory increased the ratio of catalpol to aucubin in the roots in the absence, but not in the presence, of aboveground herbivores (interaction between above- and belowground herbivory; $F_{1,36} = 6.12$; $P = 0.018$).

Discussion

Our study indicates the importance of both the presence and the timing and sequence of arrival of aboveground and belowground herbivores for the performance of these organisms on their shared host plant. Importantly, we provide evidence that the timing of prior aboveground and belowground herbivory can affect the performance of later arriving aboveground herbivores. Thus, we stress the importance of considering arrival sequence in order to understand the outcome of more complex aboveground-belowground interactions.

Arrival sequence and aboveground interactions

Our study shows that the sequence in which aboveground herbivores arrive on *P. lanceolata* is an important determinant of their intraspecific interactions. Prior aboveground herbivory by *S. exigua* significantly reduced the leaf area consumption and weight gain of response caterpillars of *S. exigua,* whereas neither simultaneous arriving nor later arriving conspecifics affected the consumption or weight gain of the response caterpillars. It is unclear to what extent the induction of leaf IGs by earlier arriving conspecifics contributed to the reduced performance of the later arriving *S. exigua*. In agreement with findings from previous studies in which *P. lanceolata* was exposed to generalist (Wurst and Van der Putten 2007) and specialist (e.g., Darrow and Bowers 1999) leaf chewers, a prolonged period of aboveground herbivory (17 days, experiment 1) resulted in a significant increase in the levels of leaf aucubin and catalpol (experiment 1). However, this induction was not yet observed 4 days after the initiation of the 2-day period of leaf herbivory in experiment 2, when the response caterpillars of *S. exigua* were introduced. Therefore, if induction or priming of IGs by previous herbivory played any roles in the reduced performance of later arriving *S. exigua,* these chemical changes were not expressed until after the response herbivores had been introduced. Alternatively, the reduced performance may have been due to the induction or priming of other compounds or traits than IGs. More detailed studies on the precise time pattern of induction and decay of the IG response for each of the sequences of arrival are needed to assess the role of IGs in this response. Earlier studies using this model system have shown that such patterns can be complex (Fuchs and Bowers 2004).

Arrival sequence and aboveground–belowground interactions

In our study, the foliar generalist, *S. exigua,* did not significantly affect the performance of the root herbivore (experiment 1). On average, wireworm performance was enhanced when the aboveground herbivore was introduced prior to the wireworms and with increased feeding duration, but these effects were not significant ($P < 0.06$). The absence of a significant effect of *S. exigua* on the performance of the belowground herbivore contrasts with the pattern revealed in the meta-analysis by Johnson et al. (2012) that leaf-chewing insects, when introduced prior to root feeders, generally have a negative impact on root-feeding insects (see e.g., Bezemer et al. 2003; Soler et al. 2007; Erb et al. 2011). This pattern is thought to be due to the systemic induction or priming of defense compounds in the roots that takes time and requires sustained feeding of the aboveground herbivores (Erb et al. 2011; Johnson et al. 2012). In our system, however, aboveground herbivory only resulted in a transient increase in IGs in roots (4 days after initiation of a 48-h feeding bout, experiment 2), but we did not observe enhanced levels of root IGs after sustained feeding by *S. exigua* for 17 days (experiment 1). This pattern corresponds with earlier findings in this system. Induction of root IGs by aboveground herbivory was observed in a study that allowed aboveground herbivores to feed for a short period of time (72 h) (Darrow and Bowers 1999), but not in a study in which aboveground herbivores were allowed to feed for sustained periods (Quintero and Bowers 2011). Interestingly, the transient increase in root IGs induced by *S. exigua* completely counterbalanced the reduction of root IGs caused by feeding of the wireworms. Wireworms alone strongly reduced the levels of IGs in roots and increased the ratio of the more toxic compound catalpol relative to aucubin. The latter effect has been observed for other root herbivores as well (Bennett et al. 2013). The reduction in root IG levels was initially counterbalanced by the increase in root IG levels caused by simultaneously introduced *S. exigua*. However, after sustained *S. exigua* feeding, wireworms were able to reduce root IGs even in the presence of the aboveground herbivore. Since wireworm performance is negatively affected by root IGs (J. Huang, unpublished results) this may be a mechanism by which wireworms can enhance their own performance. It should be noted that the absence of an effect of aboveground herbivory on the performance of the belowground herbivore in our study should be interpreted with caution. While *P. lanceolata* in temperate grasslands commonly interacts with wireworms, it may not naturally encounter *S. exigua*. The latter species was used as a model for a generalist chewing insect herbivore, but we cannot rule out that *P. lanceolata* may have evolved different responses to generalist chewers that it more often encounters in the field, resulting in a different set of consequences of such encounters for interactions with belowground herbivores.

Root herbivory by wireworms significantly reduced the leaf area consumption and marginally reduced the weight gain of the shoot herbivore *S. exigua*, independent of whether wireworms were introduced before, simultaneously with, or after the aboveground herbivore (experiment 2). Johnson et al. (2012) speculated that positive effects of root herbivores on shoot herbivores may arise if root feeders can reduce the resistance or increase the nutritional status of aboveground tissues. Wireworms did not appear to cause such effects in our host-herbivore system. Previous studies in *P. lanceolata* have shown that sustained wireworm feeding for 5 (Wurst and Van der Putten 2007) or 8 weeks (Wurst et al. 2008) does not affect leaf nitrogen or glucose concentrations, whereas effects on leaf IGs are either absent (Wurst and Van der Putten 2007) or dependent on plant genotype (Wurst et al.

2008). Interestingly, although wireworms did not affect leaf IGs in our experiment, they did reduce leaf area consumption by the aboveground herbivore, indicating that these effects were mediated by induced plant responses other than changes in IGs.

Arrival sequence and more complex above–belowground interactions

One of the novelties of our study is that the setup also allowed us to investigate what happens in more complex above–belowground interactions. In particular: how is the performance of aboveground response herbivores affected by the sequence of arrival of both conspecifics and belowground herbivores? One of the most intriguing findings was that whereas early arriving *S. exigua* were able to reduce the weight gain and consumption efficiency of their later arriving conspecifics when they also arrived prior to the root herbivores (A > BS; Fig. 5a, c), their negative effect on consumption efficiency completely disappeared when wireworms were introduced simultaneously with the early arriving *S. exigua* (AB > S; Fig. 5c). This suggests that wireworms either repress the induction of the defenses by *S. exigua* that are responsible for the lower consumption efficiency of their later arriving conspecifics, or that they induce compounds that compensate for the induced lower consumption efficiency. Since there were no indications that wireworms suppressed the induction of shoot IGs by *S. exigua* when they were introduced simultaneously, we speculate that this modulation may have been mediated by other compounds than IGs. Despite the alleviating effects of wireworms on the induction of traits lowering the consumption efficiency of the aboveground herbivore, later arriving *S. exigua* still suffered a lower weight gain on plants previously exposed to their conspecifics, probably due to effects of previous herbivory on other components of the relative growth rate of later arriving conspecifics. The impact of such, more complex, interactions between aboveground and belowground herbivores on their performance stresses the importance of getting more insight into the actual patterns of the sequence and timing of arrival of above- and belowground herbivores in the field (Bezemer and Van Dam 2005). Currently we lack such information in our study system.

Effects of the interactions between aboveground and belowground herbivores on plant biomass and shoot-to-root biomass ratio in our experiments were relatively small. In experiment 2, up to 8 days of wireworm feeding reduced root biomass but not total biomass, while in experiment 1, seventeen days of wireworm feeding on its own did not exert significant effects on either root or total biomass, only in combination with 17 or more days of aboveground feeding by *S. exigua.* In similar experiments using this system in which wireworms were allowed to feed for 5 weeks (Wurst and Van der Putten 2007), wireworms reduced root biomass and induced compensatory growth of shoot tissue, whereas a feeding duration of 8 weeks did not affect root biomass but enhanced shoot biomass (Wurst et al. 2008). Most probably, the feeding durations in our experiments were too short to exert such effects. Conversely, in combination with the 2-day period of aboveground herbivory, aboveground response caterpillars did reduce shoot and root biomass in experiment 2, both in the presence and absence of root herbivores.

In conclusion, our study shows that the timing and sequence of appearance of aboveground and belowground herbivores can be important in mediating the outcomes of interactions between aboveground and belowground herbivores. In contrast to patterns from a meta-analysis synthesized from many different systems (Johnson et al. 2012), aboveground herbivory tended to enhance the performance of belowground herbivores when they arrived earlier, and belowground herbivory reduced leaf consumption by aboveground herbivores, irrespective of whether they arrived earlier, simultaneously or later. While our results may just reflect an exception to the general pattern, it is also possible that the predicted patterns are partly biased by the different host-enemy systems in which the different interaction sequences that formed the basis of the meta-analysis had been studied (Johnson et al. 2012). Thus, more studies are required that examine the effects of different sequences of aboveground and belowground herbivore encounters within a single system. Furthermore, our study included more complex types of sequential encounters in belowground–aboveground interactions. We showed that belowground herbivores can disrupt the induction of resistance to aboveground herbivory by prior conspecific herbivores, but only if they arrived simultaneously with the inducing aboveground herbivores. This illustrates that in a dynamic system, where aboveground herbivores may encounter prior aboveground, as well as belowground herbivory, we need to know the history of encounters in order to understand the outcomes of the above–belowground interactions.

Acknowledgments We thank Ciska Raaijmakers, Roel Wagenaar, Jinghua Huang, Minghui Fei and Jingying Jing for the technical help and two anonymous reviewers for their helpful comments on a previous version of the manuscript. This work was funded by a grant from the China Scholarship Council (no. 2011630083 to M. G. W.). This is publication no. 5567 of the Netherlands Institute of Ecology (NIOO-KNAW).

Conflict of interest The authors acknowledge that there are no conflicts of interest and that the experiments comply with the current laws of the Netherlands where the experiments were performed.

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