

Root herbivory indirectly affects above- and below-ground community members and directly reduces plant performance

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Summary

1. There is a widespread recognition that above- and below-ground organisms are linked through their interactions with host plants that span terrestrial subsystems. In addition to direct effects on plants, soil organisms such as root herbivores can indirectly alter interactions between plants and other community members, with potentially important effects on plant growth and fitness.

2. We manipulated root herbivory by *Acalymma vittatum* in *Cucumis sativus* to determine indirect effects on arbuscular mycorrhizal fungi, leaf herbivory, the leaf pathogen downy mildew and pollinators. We also manipulated pollen receipt by plants to determine whether root herbivory reduced plant reproduction through changes in pollinator visitation.

3. Overall, root herbivory had strong net negative effects on plant growth and fitness, with 34% reductions in both leaf and fruit production by high root damage levels relative to control, despite reduced infection by downy mildew. High root herbivory also reduced floral visitation by 39%, apparently due to lower flower production, as flower size and scent were unaffected. Above-ground herbivory was not affected by root herbivores.

4. Although root herbivory reduced pollinator visits, pollen receipt manipulations had no effect on fruit set, indicating that reduced pollinator service did not affect plant reproduction.

5. *Synthesis.* Root herbivory had indirect effects on a range of community members, including mutualists and antagonists both above- and below-ground. Although reduced pathogen infection associated with root herbivory would be expected to benefit plants, root herbivory had an overall strong negative effect on plant growth and reproduction, indicating that direct negative effects overrode any potential indirect benefits.

Key-words: *Acalymma vittatum*, arbuscular mycorrhizal fungi, *Cucumis sativus*, downy mildew, indirect effects, mutualism, plant–herbivore interactions, pollinator, volatile organic compounds

Introduction

There has been rapid advancement in ecologists' understanding of below-ground ecosystems over the last two decades, concurrent with wide recognition that organisms and processes in the soil are intimately linked to those above-ground (Masters 1995; Van der Putten *et al.* 2001; de Kroon &

Visser 2003; Wardle *et al.* 2004; Bezemer & van Dam 2005; Gehring & Bennett 2009; Bardgett & Wardle 2010; Bardgett & van der Putten 2014). Cross-domain indirect interactions between above- and below-ground organisms involve organisms as diverse as microbes, arthropods and vertebrates (Wardle 2006) and are often mediated by plants, which exist simultaneously on both sides of the soil surface (Bardgett & Wardle 2003; Bezemer & van Dam 2005; Soler *et al.* 2008). For example, tobacco (*Nicotiana rustica*) plants with roots colonized by an arbuscular mycorrhizal fungus attracted more parasitoid wasps that attacked whiteflies feeding on leaves

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(Wooley & Paine 2011). Thus, below-ground interactions can have surprisingly complex impacts on multispecies interactions above-ground.

Much research on herbivory effects across the soil surface has examined the effects of above-ground herbivores on below-ground organisms. Damage to leaves and other shoot tissues may affect root herbivores through induced resistance or changes in root nutrients (Johnson *et al.* 2012). Above-ground herbivory may also alter interactions among root symbionts like mycorrhizal fungi or N-fixing bacteria (Gehring & Bennett 2009; Barto & Rillig 2010; Heath & Lau 2011). However, the indirect effects of root herbivores in the soil on other community members, both above- and below-ground, are less understood. Root herbivory can modify floral characteristics, altering interactions with pollinators (Poveda *et al.* 2003, 2005; Barber & Soper Gordon 2014), and foliage traits, affecting herbivores, pathogens and parasitoids (Blossey & Hunt-Joshi 2003; Soler *et al.* 2008, 2012). In the soil, root damage may also shift associations with mycorrhizas (Currie, Murray & Gange 2006; Bennett *et al.* 2013) and other root antagonists (van Dam 2009).

By altering interactions with other organisms, root herbivores have the potential to influence plant growth and fitness indirectly (Wootton 1994), as well as through the direct effects of tissue damage (Ohgushi 2005). Although plant–herbivore interactions take place in this multitrophic community context (Strauss & Irwin 2004), studies of herbivore impacts on plants rarely assess multiple pathways of influence. This is in part due to the logistical constraints of manipulating a large number of interacting groups simultaneously, but such experiments are necessary to attribute herbivore effects to direct or indirect pathways. Plant–pollinator interactions illustrate this challenge. Although herbivory frequently affects floral traits (Adler 2007; Theis, Kesler & Adler 2009; Barber & Soper Gordon 2014) and pollinator behaviour (Strauss, Conner & Rush 1996; Lehtilä & Strauss 1997; Barber, Adler & Bernardo 2011; Kessler, Halitschke & Poveda 2011; Barber *et al.* 2012), changes in pollinator visitation alone do not demonstrate that altered pollination services are the cause of reduced plant fitness. Manipulations of pollen receipt in combination with herbivory manipulations can clarify the impacts of herbivores through the pollinator-mediated indirect pathway (Lehtilä & Syrjänen 1995; Strauss & Murch 2004).

Although the effects of root damage on above-ground damage have been comparatively well studied (Kaplan *et al.* 2008; Johnson *et al.* 2012), there have been very few investigations of root herbivore effects on pollinators and pollination services. Of three experiments, one found no effect (Hladun & Adler 2009), but the other two surprisingly demonstrated that root damage increased attractiveness to honeybees. Poveda *et al.* (2003, 2005) manipulated root herbivory in wild mustard (*Sinapis arvensis*; Brassicaceae), and honeybees visited plants with damaged roots more frequently. The mechanism behind this effect was unknown; root damage did not affect floral traits (Poveda *et al.* 2007). Barber, Adler & Bernardo (2011) showed that honeybees probed *Cucumis sativus*

(Cucurbitaceae) flowers for a longer time on plants that had suffered root herbivory. However, neither study documented a plant fitness increase as a result of increased attractiveness to honeybees.

We manipulated root-feeding herbivores to measure their effects on plant performance and interactions above- and below-ground with herbivores, pollinators, mycorrhizal fungi and a fungal pathogen in a field setting. In addition, we manipulated pollen receipt to determine whether indirect effects on pollinators translated to changes in plant reproduction. Thus, our experiment takes a comprehensive approach and is a response to calls to examine multiple pathways in ecological interactions and to study root herbivory effects under ‘more realistic’ conditions (Ohgushi 2005; van Dam & Heil 2011; Soler *et al.* 2012).

Materials and methods

STUDY SYSTEM

Cucumis sativus (cucumber) is a widely cultivated, monoecious annual. Throughout much of North America, it is attacked by the specialist herbivore *Acalymma vitatum* (Coleoptera: Chrysomelidae) (Capinera 2001), which accounts for almost all above-ground herbivory to *C. sativus* at the research site in Western Massachusetts, USA, where this study took place (Barber *et al.* 2012). *Acalymma vitatum* feed on above-ground tissues as adults and oviposit near the base of plants, where larvae hatch and move into the soil to feed on roots (Latin & Reed 1985; Necibi, Barrett & Johnson 1992). Above-ground feeding by *A. vitatum* has strong negative effects on *C. sativus* growth and fruit production (Barber *et al.* 2012). Members of Cucurbitaceae throughout the world are frequently attacked by the oomycete pathogen *Pseudoperonospora cubensis* (downy mildew, Peronosporaceae), a leaf pathogen that causes significant economic losses to cucurbit crops (Lebeda & Cohen 2011). Infection by downy mildew leads to leaf water loss, declines in photosynthetic rates of infected leaves and leaf necrosis (Lindenthal *et al.* 2005). *Cucumis sativus* requires pollinators to vector pollen between male and female flowers, and it is visited by a variety of insect pollinators including *Bombus* species and *Apis mellifera* (Barber, Adler & Bernardo 2011; Barber *et al.* 2012, 2013). Below-ground, *C. sativus* is commonly colonized by arbuscular mycorrhizal fungi (AMF) (Barber *et al.* 2012), which provide nutrients, such as phosphorus, in exchange for host carbon. In *C. sativus*, AMF can advance flowering and fruit production, increase photosynthesis rates and provide resistance against pathogens (Trimble & Knowles 1995; Valentine, Osborne & Mitchell 2001; Hao *et al.* 2005).

EXPERIMENTAL DESIGN

We manipulated root herbivory and pollen receipt in a 4 × 2 (herbivory × pollination) randomized block design. We germinated *C. sativus* seeds (Marketmore 76; Johnny’s Selected Seeds, Winslow, ME, USA) on Fafard Growing Mix 2 soil (Conrad Fafard Inc., Agawam, MA, USA) and transplanted 240 seedlings at the two-leaf stage to 7.5-L pots containing the same soil. We added 24-8-16 fertilizer (Peters Professional water-soluble fertilizer; Everris NA, Inc., Dublin, OH, USA) to each pot at transplanting. Pots were grouped in 30 blocks of eight plants each, with two plants in each block randomly assigned to one of four root herbivory treatments.

For root herbivory treatments, we collected *A. vittatum* adults from local farms and placed mating pairs in individual plastic cups. We collected eggs from each pair and stored them at 10 °C until inoculation. We suspended eggs in agar and inoculated each pot near the base of the plant at one of four egg densities: 0, 25, 50 or 75 eggs per plant. Inoculation occurred while plants were still in pots in the glasshouse, before transplanting to the field. We inoculated blocks as sufficient eggs were available: blocks 1–16 on 15–16 June, blocks 17–19 on 19 June, blocks 20–22 on 20 June and blocks 23–30 on 26 June. The timing of inoculation corresponds to the natural range of *A. vittatum* oviposition in the region (Capinera 2001), and the number of eggs applied is within the range we observed produced by females in the laboratory and in other studies (Eilers-Kirk & Fleischer 2006).

To verify that root herbivory treatments successfully manipulated the number of beetle larvae on plants, we sacrificed the plants in blocks 1–5 11 days after inoculation and placed the soil from each plant in a separate Berlese funnel. Nine to thirteen days after inoculation (22 June–9 July), we transplanted the remaining plants to a prepared plot (University of Massachusetts Center for Agriculture, South Deerfield, MA, USA, 42° 28.6' N, 72° 34.8' W). Oats were previously planted in the one-acre plot, which was disked and received 136 kg of 19-19-19 fertilizer (Crop Production Services, Loveland, CO, USA) prior to transplanting. Each block was arranged in a row, with 3-m spacing between rows and between plants in each row.

When female flower production began (16 July), we added supplemental pollen to the stigmas of all female flowers in half the plants in each block (one plant at each egg density treatment). Supplemental pollen was added 5 days per week (excluding days of heavy rain, when pollinators were not active) using pollen collected from non-experimental donor plants planted at the field margins.

RESPONSES

To measure flower production, we counted male and female flowers on every plant 5 days per week beginning on 9 July, and we measured the length and width of a single petal on two male and two female flowers from each plant. To estimate plant growth, we counted the number of fully grown leaves per plant on 12 July, and 2–3 August. On these dates, we also estimated percentage herbivore damage to three recent fully expanded leaves. Starting 30 July, we collected all fruits 18 cm or longer; length and mass of each fruit were recorded, and we measured seed production from the first three fruits produced by each plant by slicing each fruit lengthwise and counting the number of developed seeds exposed. We calculated total seed production (average number of seeds per fruit × number of fruits) and fruit set (number of fruits/total number of female flowers). On 23 August, we scored downy mildew infection symptoms using a 0–5 scale based on the percentage of leaves infected (0 = 0%, 1 = 1–25%, 2 = 25–50%, 3 = 50–75%, and 4 = 75–100%).

We observed pollinator behaviour on 14 separate days for a total of 44.75 person-hours of observation (i.e., hours of observation × number of observers). Observations took place between 0945 and 1500 h, when pollinators were most active. We followed individual pollinators within the experimental plot and used hand-held digital voice recorders to record pollinator taxon, number of visits to each plant, proportion of flowers probed per visit and time spent per flower in seconds. Individual pollinators were followed as long as possible or until they left the plot. Because different pollinator species differ in probe time, we separately quantified this variable (as well as visits and proportion of flowers probed) for honeybees and *Pieris rapae* (Lepidoptera: Pieridae), the two most abundant pollinators at the site.

To measure AMF association, we took two soil cores (19 mm diameter, approximately 15 cm deep) 5 cm from the base of each plant between 1 and 9 August. Small root fragments were sorted, stained with trypan blue, mounted on microscope slides and quantified using the magnified gridline intersect method (McGonigle *et al.* 1990). We used arbuscule abundance as the response variable because it may better reflect AMF associations than mycorrhizal hyphae alone (García & Mendoza 2008; Johnson *et al.* 2010).

Beginning on 25 July, we collected floral volatile organic compound (VOC) emissions from 84 male flowers, all in the natural pollination treatment (not receiving supplemental pollen), using dynamic headspace sampling. We collected fragrance on a glass cartridge packed with 100 mg of poropak, later eluted with hexane. We added the internal standard anisole to samples, which were concentrated under nitrogen and analysed with GC-MS following methods of Theis *et al.* (2014). Peaks of each compound were identified based on mass spectra and retention times and quantified using the internal standard and standard curves.

STATISTICAL ANALYSES

We analysed data using generalized linear mixed models (GLMMs), treating block as a random factor and herbivory, supplemental pollination and their interaction as fixed factors. Number of leaves was surveyed twice during the season, so we included date as a fixed factor and plant as a random factor. All analyses were carried out in R (R Development Core Team 2012). For count data (larvae recovered from Berlese funnels, number of leaves and flowers, pollinator visits and number of fruits and seeds produced), we used Poisson errors and log link function with individual-level random effects to account for overdispersion (Agresti 2002). To analyse the proportion of flowers probed by pollinators, we used binomial errors with logit link function and individual-level random effects. These were weighed by flower number each plant, basing proportion on the number of observed probes and the total number of flowers open on days pollinator surveys took place. These analyses were carried out with the lme4 package (Bates, Maechler & Bolker 2012). For leaf damage, downy mildew symptoms, pollinator probe times (log-transformed), flower size (petal length × width) and fruit set, we used Gaussian errors and identity link function with the nlme package (Pinheiro *et al.* 2010). We evaluated fixed factors using likelihood ratio tests; when herbivory had a significant effect, we performed pairwise comparisons among the four treatment levels using Tukey's all-pairwise comparisons as implemented in the multcomp package (Hothorn, Bretz & Westfall 2008).

To analyse floral scent, we first examined herbivory treatment effects on total volatile production and production of two classes of volatiles, monoterpenoids and aromatic compounds, using GLMMs with Gaussian errors and identity link function. We then described scent blends using principal components analysis using the function `prcomp()` and analysed the effect of herbivory treatments on the first two principal components with GLMMs. Because adult *A. vittatum* are sometimes found in flowers, and prior work has demonstrated that their attraction can be explained in part by floral volatiles (Theis *et al.* 2014), we also examined Pearson correlations between leaf herbivory and floral VOC traits using the August leaf damage measurements (because few plants were in bloom during the July damage measurements).

Results

Our root herbivore manipulation successfully altered the number of *A. vittatum* larvae present on plants, as indicated

by larvae recovered in Berlese funnels (herbivory $\chi^2 = 42.72$, $P < 0.001$, Fig. 1a; pollination treatments not included in model). Root herbivory strongly reduced the number of leaves and female flowers (Table 1, Fig. 1b,c). Male flower number

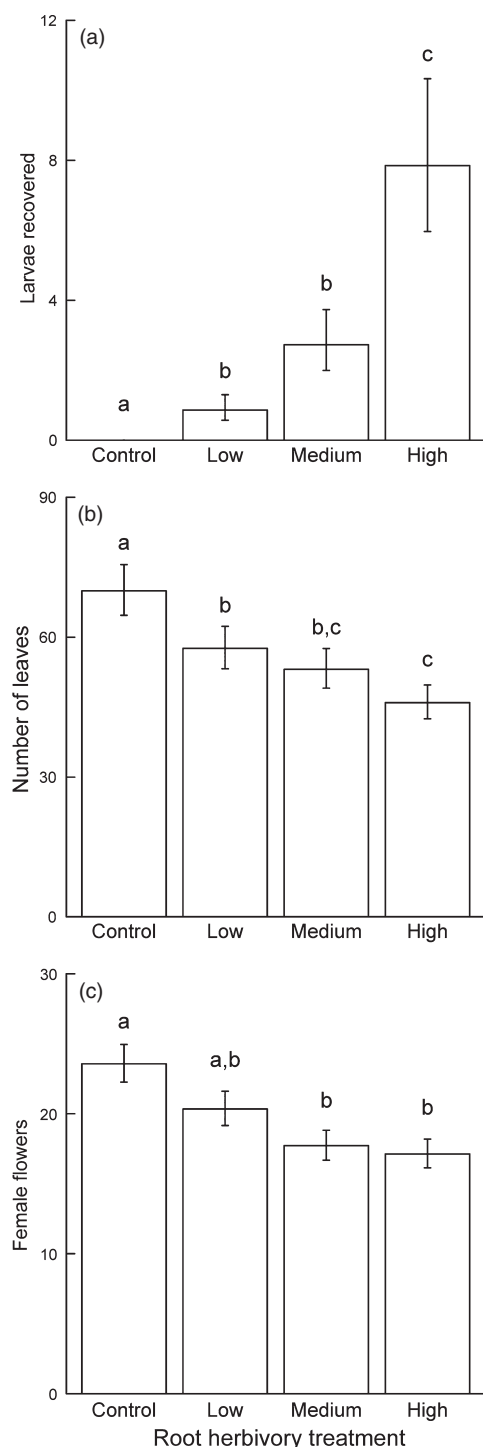


Fig. 1. Effects of root herbivory treatments on (a) larval recovery in Berlese funnels, verifying successful application of treatments, and (b) leaf and (c) female flower production in *Cucumis sativus*. Values are fitted model coefficients ± 1 SE. Letters above bars indicate significant differences at $P < 0.05$ based on Tukey's *post hoc* tests.

followed a similar pattern as female, with the highest flower production in the undamaged treatment, although this effect was not significant (Table 1). Flower size was unaffected by root herbivory (male and female, all $P > 0.15$), although female flowers were smaller on plants with supplemental hand pollination ($\chi^2 = 4.34$, $P = 0.037$) (Harder & Johnson 2005).

Treatments did not affect leaf herbivory (Table 2, Fig. 2a), but downy mildew symptoms were less severe as root damage increased (Table 2, Fig. 2b). Arbuscule abundance was also affected by root herbivores, with the highest level of colonization on high-damage plants and the lowest colonization on medium-damage plants (Table 2, Fig. 2c). We observed 1,825 pollinator visits to plants and 4386 flower probes. Visitation by pollinators in general (and honeybees and *Pieris* specifically) was reduced by the high root herbivore treatment (Table 3, Fig. 2d). However, neither the proportion of flowers probed nor time of probe duration were affected by treatments (Table 3).

Root herbivory treatments had a significant effect on fruit production, with the highest level of damage resulting in the fewest fruits (Table 4, Fig. 3a). Although there was a trend towards lower total seed production for all plants that received root herbivores, this was not significant (Table 4, Fig. 3b). Neither supplemental hand pollination nor root herbivory affected fruit set (Table 4, Fig. 3c).

Table 1. Results of generalized linear mixed models examining effects of root herbivory and pollination treatments on leaf and flower production of *Cucumis sativus*.

Response	Herbivory		Pollination		Herb \times Poll	
	χ^2	P	χ^2	P	χ^2	P
Leaf production	45.17	< 0.001	0.30	0.581	1.58	0.663
Flower production						
Female	24.75	< 0.001	1.32	0.250	7.59	0.055
Male	3.26	0.353	0.15	0.697	1.99	0.576

Leaf production model also includes date as a fixed factor because leaves were counted twice during the season; date was highly significant ($\chi^2 = 780.43$, $P < 0.001$). Bold values indicate results where $P < 0.05$.

Table 2. Results of generalized linear mixed models examining effects of root herbivory and pollination treatments on herbivory, downy mildew infection and AMF arbuscule abundance on *Cucumis sativus*.

Response	Herbivory		Pollination		Herb \times Poll	
	χ^2	P	χ^2	P	χ^2	P
Leaf damage	3.72	0.293	0.03	0.876	3.95	0.267
Downy mildew	10.73	0.013	0.14	0.711	0.48	0.924
AMF	8.87	0.031	4.52	0.034	0.97	0.809

Leaf damage model also includes date as a fixed factor because leaves were counted twice during the season. Bold values indicate results where $P < 0.05$.

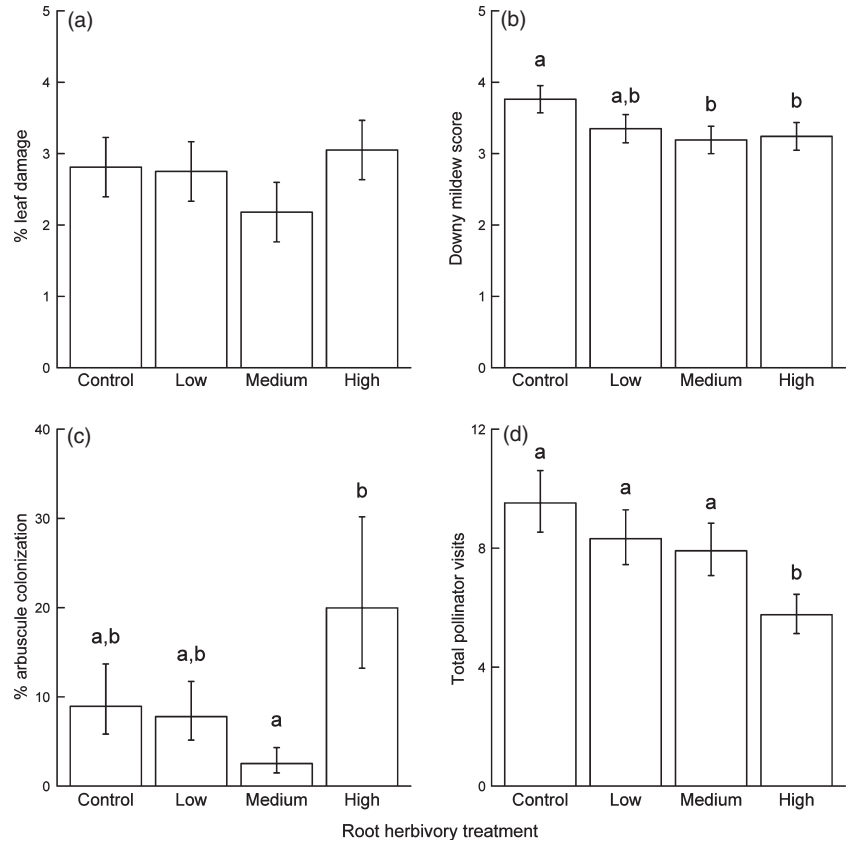


Fig. 2. Effects of root herbivory treatments on (a) leaf herbivory, (b) downy mildew infection, (c) arbuscule abundance and (d) total pollinator visits in *Cucumis sativus*. Downy mildew symptom severity was visually scored on a 0–5 scale. Values are fitted model coefficients ± 1 SE. Letters above bars indicate significant differences at $P < 0.05$ based on Tukey's *post hoc* tests.

Table 3. Results of generalized linear mixed models examining effects of root herbivory and pollination treatments on pollinator visitation behaviours on *Cucumis sativus*. A visit occurs when an individual insect probes at least one flower on a plant; proportion probed is the number of flowers observed probed divided by total flowers produced; probe time is the average time a pollinator spent in contact with flower reproductive parts.

Response	Herbivory		Pollination		Herb \times Poll	
	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>
Total visits	20.98	< 0.001	0.28	0.597	1.30	0.730
Honeybee visits	16.79	< 0.001	0.85	0.356	0.97	0.808
<i>Pieris</i> visits	11.40	0.010	0.07	0.793	1.07	0.784
Proportion probed	4.31	0.230	0.51	0.475	1.73	0.629
Honeybee probed	4.69	0.196	0.72	0.396	1.68	0.641
<i>Pieris</i> probed	2.17	0.537	0.29	0.591	2.58	0.462
Honeybee probe time	0.15	0.985	0.05	0.831	2.98	0.395
<i>Pieris</i> probe time	5.27	0.153	0.50	0.480	4.29	0.232

Bold values indicate results where $P < 0.05$.

Floral volatiles were unaffected by herbivory treatments. Production of total volatiles, as well as production of monoterpenoids and aromatic compounds, did not differ with root damage treatments (Tables S1 and S2). The first principal component, which explained 88.6% of variation, reflected production of benzyl alcohol, the most abundant compound in floral blends. The second principal component (7.7% of variation) reflected a subset of plants with higher β -pinene produc-

Table 4. Results of generalized linear mixed models examining effects of root herbivory and pollination treatments on fruit production, seed production and fruit set of *Cucumis sativus*.

Response	Herbivory		Pollination		Herb \times Poll	
	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>
Fruit production	12.00	0.007	0.29	0.588	4.30	0.231
Seed production	6.63	0.085	0.01	0.924	0.61	0.894
Fruit set	6.98	0.072	0.01	0.911	2.75	0.433

Bold values indicate results where $P < 0.05$.

tion. However, neither of these principal components were affected by root herbivory (Table S1). Leaf herbivory was significantly positively correlated with total VOC production, but this relationship was driven by aromatic compounds (reflected in principal component 1; Table 5), especially benzyl alcohol (Pearson's $r = 0.335$, $P = 0.002$).

The effects of root herbivory on other community interactions and on plant variables are summarized in Fig. 4.

Discussion

We manipulated root herbivores to investigate their effects on other community members as well as growth and reproduction of the focal host plant. Our results demonstrate that root herbivory has both direct negative effects on host plants as well as indirect effects on other community members

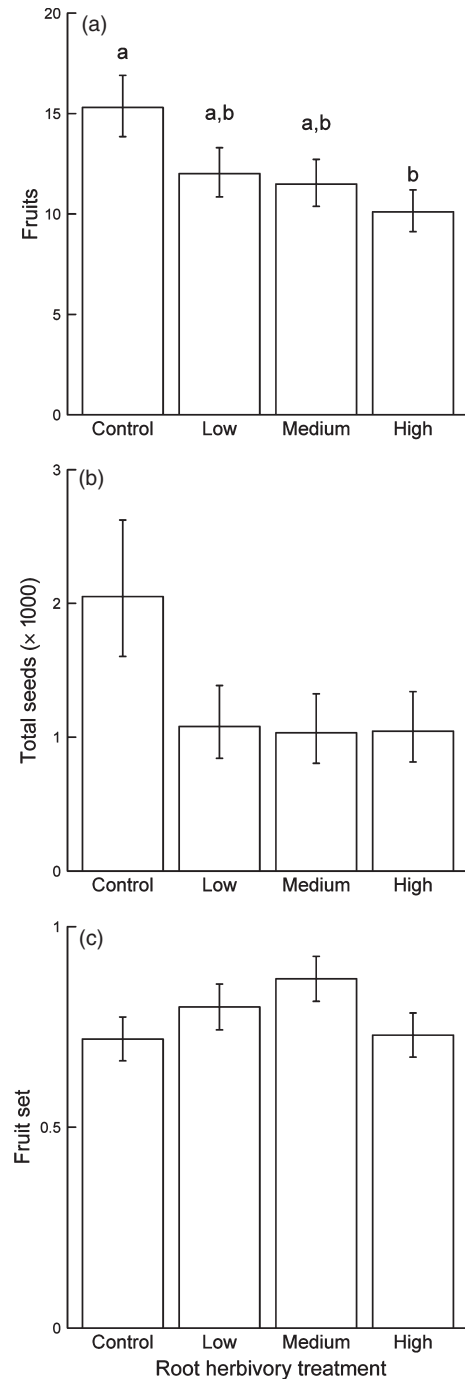


Fig. 3. Effects of root herbivory treatments on (a) fruit production, (b) estimated total seed production and (c) proportion fruit set (fruits/female flowers) in *Cucumis sativus*. Values are fitted model coefficients ± 1 SE. Letters above bars indicate significant differences at $P < 0.05$ based on Tukey's *post hoc* tests.

above- and below-ground. However, the indirect effects on other organisms appear to be much less important for plant growth and reproduction. Plants with high root herbivory had increased resistance to a pathogen but still had significantly reduced fruit production. Further, the strong reduction in pollinator visitation due to root herbivory was not responsible for this reduced fruit production. Plants were not pollen limited, as reproduction did not differ between open and sup-

Table 5. Correlations between August leaf herbivory and floral VOC variables.

Response	Pearson's r	P
Total VOC production	0.315	0.004
Monoterpenoid production	0.178	0.105
Aromatic compound production	0.336	0.002
VOC principal component 1	-0.335	0.002
VOC principal component 2	-0.085	0.439

Bold values indicate results where $P < 0.05$.

plemental pollination treatments. Thus, the direct effect of root herbivory on plants is the most likely driving force behind reduction in plant performance. Below, we discuss these indirect and direct impacts and their consequences for plants.

INTERACTIONS

We found that root herbivory altered interactions with other plant-associated community members, both above- and below-ground, including AMF, downy mildew, and pollinators, but not leaf herbivores. The effects of root damage on above-ground antagonists were mixed: leaf herbivores were unaffected (Fig. 2a), but high levels of root herbivory conferred some resistance against the oomycete pathogen, downy mildew (Fig. 2b). Other root herbivore studies have documented induced resistance in above-ground plant tissues, including changes in defensive chemistry (Kaplan *et al.* 2008; Kostenko, Mulder & Bezemer 2013), reduced herbivore damage or performance (Anderson, Sadek & Wäckers 2011; Kutyniok, Persicke & Müller 2014) and changes in the recruitment or effectiveness of parasitoids (A'Bear, Johnson & Jones 2014). However, consistent with our results here, previous work in this system did not find an effect of root herbivory on subsequent folivory (Barber, Adler & Bernardo 2011), although the opposite effect, reduced root herbivore preference following leaf damage, occurs (Barber *et al.* 2012; Milano, Barber & Adler 2015). Thus, cross-system induction between leaf and root herbivores appears to be asymmetric in *C. sativus*. The apparent protection against a leaf pathogen induced by root damage is somewhat unexpected, although Hladun & Adler (2009) found that leaf herbivory reduced infection by a fungal pathogen in another cucurbit, *Cucurbita moschata*. However, root damage had no effect on infection in that study, and root herbivory experiments in maize show no impact on levels of salicylic acid (SA) (Erb *et al.* 2009, 2011), the plant hormone which often mediates plant resistance to pathogens. The complex interactions between jasmonic acid and SA pathways that regulate plant responses to both herbivores and pathogens (Stout *et al.* 1999; Felton & Korth 2000) clearly require further study in the context of below-ground herbivory.

Below-ground herbivory also had a strong effect on an above-ground mutualism, pollinator visitation. However, the direction of the effect was opposite to that of previous experiments, in which root damage increased attractiveness of

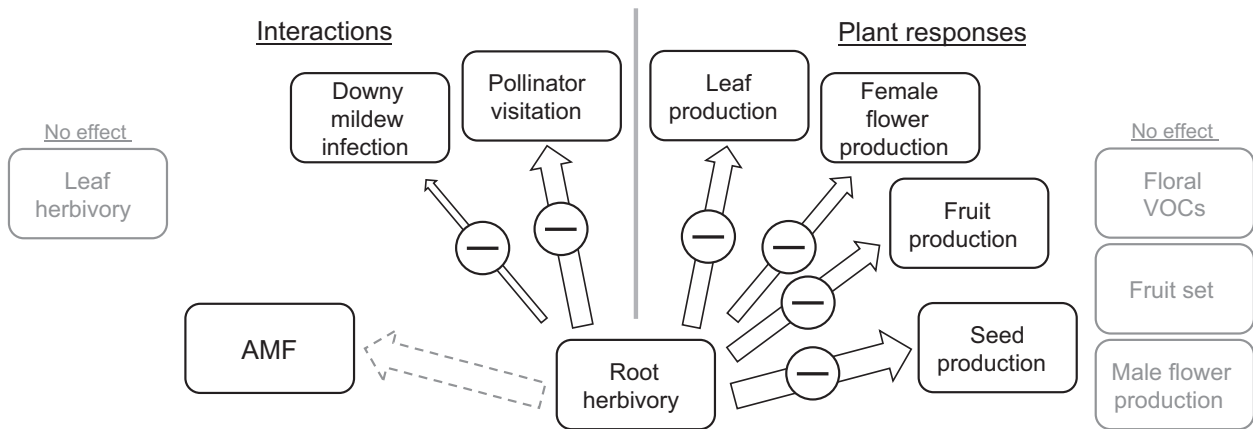


Fig. 4. Summary of net effects of root herbivory, separated into interactions and plant response variables. All effects are negative except AMF, where there was not a clear directional effect of increasing root damage (i.e. colonization did not linearly increase or decrease with root damage). Arrow width of negative effects is proportional to effect size of high-damage levels relative to undamaged control plants. Variables without arrows ('No effect') were not significantly affected by root damage, except seed production, which was marginally significant.

flowers to honeybees (Poveda *et al.* 2003, 2005; Barber, Adler & Bernardo 2011) or had no effect (Hladun & Adler 2009). We found a consistent decline in pollinator visitation as root damage levels increased (Fig. 2d). There were no differences in the display traits of individual flowers; petal size and floral VOCs were unaffected by herbivory treatments. Although other floral traits that we did not measure (e.g., nectar production or composition) could explain the visitation patterns, it seems most likely that reduced visitation is due to reduced flower production following root damage (Fig. 1c). This mechanism would be consistent with our finding that root-damaged plants had fewer pollinator visits (i.e. initiation of foraging) per plant, but no change in the proportion of flowers probed per visit; a change in flower quality would be more likely to reduce this latter measure of pollinator behaviour. Our result mirrors the effects of leaf herbivory on pollinator visitation in this system (Barber *et al.* 2012), where herbivory significantly reduced flower number (but not VOCs), and per-plant pollinator visitation declined as well, but the proportion of flowers probed per visit did not. It is not clear why honeybee flower probe time results differed from previous work in *C. sativus*, where bees spent more time probing flowers on damaged plants (Barber, Adler & Bernardo 2011). The number of eggs added (and application technique) in that study was identical to our medium treatment level here, but the study was conducted at a different research farm approximately 26 km away. The differences between studies suggest that the effect of root damage on honeybee behaviour may be sensitive to abiotic or biotic variation due to location or interannual variation.

While root herbivory by larval *A. vittatum* did not affect floral VOCs, floral scent was positively correlated with leaf damage by adult *A. vittatum*. Because we did not manipulate leaf damage, it is not possible to disentangle cause and effect, but plants that produced greater quantities of aromatics (particularly benzyl alcohol, the dominant compound in the blend) also suffered greater leaf herbivory. One possibility is that beetles choose host plants based on floral scent.

A comparison of twenty cucurbit species and varieties found increased *A. vittatum* visitation to plants with larger flowers that produced more sesquiterpenoids in their floral scent (Theis *et al.* 2014). In that study, sesquiterpenoids were the dominant class of compounds in the most fragrant varieties. Manipulation of the dominant compound of *Cucurbita pepo* subsp. *texana*, 1,4-dimethoxybenzene also increased *A. vittatum* visits to squash flowers (Theis & Adler 2012). Beetles may simply be attracted to plants that produce large quantities of volatiles and thus be more likely to feed on leaves of these plants when they visit. Alternatively, although we did not find effects of leaf damage by *A. vittatum* on *C. sativus* volatiles in a previous study (Barber *et al.* 2012), beetle damage may have caused the increase in floral volatiles. A growing number of studies have found that leaf damage induces changes in floral chemical traits (Adler *et al.* 2006; Theis, Kesler & Adler 2009; McArt *et al.* 2013; Schiestl *et al.* 2014), and leaf damage in wild tomato induced floral volatiles that deterred pollinators (Kessler, Halitschke & Poveda 2011). Further work in multiple systems should focus on the conditions under which leaf damage influences floral traits and interactions important for plant reproduction.

The changes in arbuscule abundance by AMF did not follow a simple linear pattern of increasing or decreasing colonization with root herbivory intensity (Fig. 2c) and thus cannot easily be attributed to root damage. Rather, plants with the highest level of damage had the highest arbuscule abundance, while medium-damage plants were the lowest. Control plants and plants with low root herbivory had intermediate colonization levels. In the few previous studies of root herbivore-AMF interactions, root herbivory has reduced (Bennett *et al.* 2013), increased (Currie, Murray & Gange 2006, 2011) or had no effect on (Gange, Brown & Sinclair 1994; Gange 2001) AMF colonization (reviewed in Johnson & Rasmann 2015). Studies that only examine a single level of root damage may miss complex fungal responses, and more detailed studies of plant resource allo-

cation patterns in response to different levels of root herbivory are warranted (Bennett *et al.* 2013). Further, Johnson & Rasmann (2015) emphasize that herbivory effects on AMF colonization may differ when studying a single fungal species compared to a multispecies AMF community, as was presumably present in the field where our study took place. This review also documented generally negative impacts on root herbivores by mycorrhizas, such as reduced herbivore body mass or survival. Perhaps plants under severe attack recruit AMF to bolster resistance against below-ground damage.

PLANT GROWTH AND FITNESS

Overall, root herbivory treatments had strong net negative effects on plant growth and reproduction. Increased root damage reduced total leaf number (Fig. 1b), an estimator of plant size for a vining plant like *C. sativus*. These smaller plants also produced fewer female flowers (Fig. 1c), and thus fewer fruits (Fig. 3a), with a trend towards fewer total seeds produced (Fig. 3b). These reductions in plant performance due to root herbivory occurred despite some positive indirect interactions, such as reduced pathogen infection and increased mycorrhizal colonization of high-damage plants. Ultimately, positive indirect effects were not strong enough to overcome the direct impacts of root damage. For example, downy mildew infections occurred in the second half of the growing season, and plant growth was already significantly reduced by root herbivory at this point (Fig. 1b). Although reduced pathogen infection might have benefited fruit production during the second half of the season, it appears that this potential benefit was outweighed by the direct negative consequences of root damage. Similarly, potential benefits of increased AMF colonization were not reflected in plant growth or fitness responses.

Pollinator visitation patterns, however, did closely resemble fruit production results, with a clear decline in total visitation as root damage increased. However, this decline in pollinator visitation was not responsible for the lower fruit production. Hand-pollination treatments, which should replace lost insect pollination services, had no effect on fruit or seed production. Similarly, fruit set (the proportion of female flowers that successfully develop into fruit) was unaffected by herbivory and pollination manipulations. Even high-damage plants, with the fewest pollinator visits, still received sufficient pollen to fertilize female flowers. The reduced fruit production was instead probably a result of limited resource availability by the plant to grow female flowers and allocate resources to their successful development into fruits. This result underscores the importance of pollination manipulations to establish whether reduced pollinator visitation is the cause of reduced reproduction, or a consequence of other factors, such as root damage, that are the underlying cause of lower reproduction. Similarly, in a previous study of the effects of leaf herbivory on these same interactions, pollinator visitation and fruit production were also significantly reduced, but pollen addition had no effect (Barber *et al.* 2012). These and other studies in the

C. sativus system (Barber, Adler & Bernardo 2011) provide strong evidence that, although both above- and below-ground herbivory can alter interactions between the plant and other community members, changes in these interactions are of much less importance than the direct negative effect of root or leaf damage on the plants.

Conclusion

The growth of research on indirect multitrophic effects (Miller & Travis 1996; Strauss & Irwin 2004), combined with wider appreciation for below-ground ecology, has led to a deeper understanding of above-ground–below-ground linkages (Bezemer & van Dam 2005; Bardgett & Wardle 2010; Bardgett & van der Putten 2014). Indirect effects, and specifically root herbivory, can have important consequences for communities and plant reproduction (Blossey & Hunt-Joshi 2003). However, as our study illustrates, the presence of indirect effects does not necessarily mean that those effects have important consequences for the host plant, particularly when compared to strong negative effects of direct interactions with herbivore antagonists. Heil (2011) emphasized the importance of reporting ‘negative’ results of above-ground–below-ground experiments, where no indirect effects are detected, to advance our understanding of the ecological and evolutionary consequences of these interactions. Similarly, we argue that clarifying the significance of indirect effects is necessary to understanding plants’ evolutionary adaptations and the forces structuring terrestrial communities on both sides of the soil surface.

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Data accessibility

Data available from the Dryad Digital Repository: <http://datadryad.org/resource/doi:10.5061/dryad.cg4c7> (Barber *et al.* 2015).

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Supporting Information

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

Table S1. Results of GLMMs analysing effects of root herbivory treatments on floral volatile organic compounds (VOCs).

Table S2. Floral VOC production under each root herbivory treatment level and loadings for the first and second principal components.