

LETTER

Physiological integration of roots and shoots in plant defense strategies links above- and belowground herbivory

Ian Kaplan,^{1*} Rayko Halitschke,²
 Andre Kessler,² Brian J. Rehill,³
 Sandra Sardanelli¹ and Robert F.
 Denno^{1†}

¹Department of Entomology,
 University of Maryland, College
 Park, MD, USA

²Department of Ecology and
 Evolutionary Biology, Cornell
 University, Ithaca, NY, USA

³Department of Chemistry,
 United States Naval Academy,
 Annapolis, MD, USA

*Correspondence: E-mail:
 ik223@cornell.edu

†Deceased.

Abstract

Roots play a critical, but largely unappreciated, role in aboveground anti-herbivore plant defense (e.g. resistance and tolerance) and root–leaf connections may therefore result in unexpected coupling between above- and belowground consumers. Using the tobacco (*Nicotiana tabacum*) system we highlight two examples of this phenomenon. First, the secondary metabolite nicotine is produced in roots, yet translocated aboveground for use as a foliar resistance trait. We demonstrate that nematode root herbivory interferes with foliar nicotine dynamics, resulting in positive effects on aboveground phytophagous insects. Notably, nematode-induced facilitation only occurred on nicotine-producing plants, and not on nicotine-deficient mutants. In the second case, we use stable isotope and invertase enzyme analyses to demonstrate that foliar herbivory elicits a putative tolerance response whereby aboveground nutritional reserves are allocated to roots, resulting in facilitation of phytoparasitic nematodes. Thus, plants integrate roots in resistance and tolerance mechanisms for leaf defense, and such root–leaf connections inherently link the dynamics of above- and belowground consumers.

Keywords

Indirect effects, phytoparasitic nematode, plant-mediated interactions, resistance, root herbivory, sink strength, stable isotope, tolerance.

Ecology Letters (2008) 11: 841–851

INTRODUCTION

The fundamental role of roots in terrestrial plants is to acquire growth-limiting water and nutrients (e.g. nitrogen) from the surrounding environment (Öpik & Rolfe 2005), although plants also utilize roots to cope with a more diverse array of biotic and abiotic challenges (McCully 1999; Bais *et al.* 2004; Schenk 2006). One such challenge that plants frequently encounter is attack from consumers at the second trophic level. Despite the fact that all plant tissues (i.e. leaves, roots, stems, flowers and fruits) are susceptible to herbivory, the vast majority of knowledge on plant–herbivore interactions derives from studies on leaf consumption (e.g. Johnson *et al.* 2006). Because leaves and roots are spatially separated from one another, often by great distances, the contribution of roots to leaf defense and patterns of aboveground herbivory is not widely recognized. Water stress and nutrient availability have well-documented effects on the preference and performance of leaf-feeding

insects (Hermes 2002; Huberty & Denno 2004), and consequently roots have been indirectly implicated in aboveground plant–herbivore dynamics. However, roots play a more direct, albeit less apparent, role in foliar plant defenses, and we argue that these leaf–root connections have important ecological consequences in linking above- and belowground consumers.

Because roots are surrounded by soil and thus inaccessible to most foliar herbivores, they provide the ideal storage site for resources used in aboveground defense. We highlight two examples of this phenomenon, one a resistance trait (Fig. 1a) and the other a putative tolerance mechanism (Fig. 1b). First, secondary plant compounds that protect leaves from consumers can be synthesized in root tissue, thus providing a direct link between roots and foliar resistance (Karban & Baldwin 1997; van der Putten *et al.* 2001). For example, plants in the genus *Nicotiana* employ alkaloids (e.g. nicotine) as a constitutive and inducible defense against leaf-chewing herbivores (Baldwin 1991;

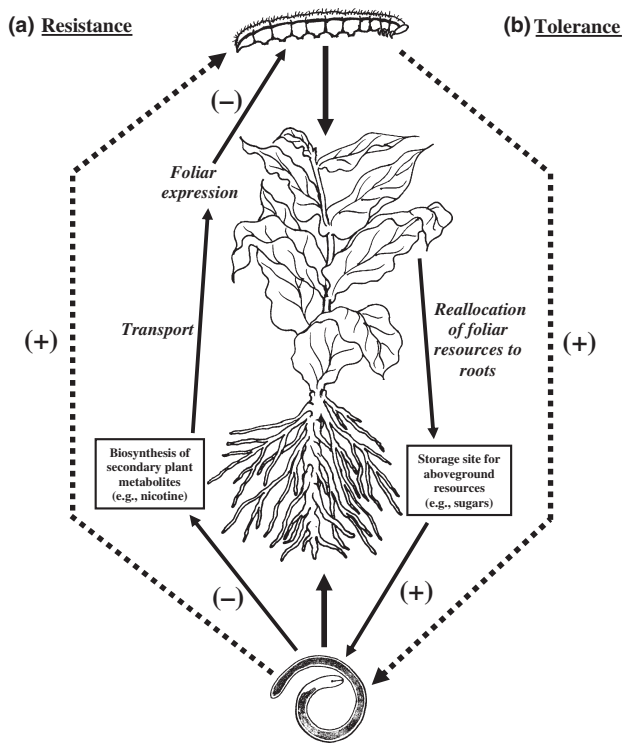


Figure 1 A mechanistic model depicting circumstances in which roots function in foliar plant defenses (A, resistance; B, tolerance), and thus promote indirect interactions between above- and belowground herbivores. (a) Because ecologically-important secondary plant metabolites can be synthesized in roots and are secondarily transported to leaves, root herbivory may interfere with the aboveground expression of allelochemistry and improve the performance of foliar herbivores. (b) Plants may tolerate foliar herbivory by temporarily storing valuable plant reserves in roots where they cannot be accessed by aboveground consumers, and in so doing improve the quality of root tissue for belowground consumers. Solid lines indicate direct effects, and dashed lines denote indirect effects. Illustration by Sarah Hughes.

Steppuhn *et al.* 2004). Notably, alkaloid biosynthetic sites are located belowground in tobacco roots, despite their well-established role in foliar defense (Dawson 1941; Baldwin 1988). Similar integration between roots and leaf resistance can be observed in other plant defense systems such as cotton (*Gossypium* sp.), which synthesizes terpenoid aldehydes (e.g. gossypol) in roots (Smith 1961; Heinstein *et al.* 1962), yet transports these compounds aboveground where they negatively affect foliar-feeding insects (Meisner *et al.* 1978; Parrott 1990; Agrawal & Karban 2000).

In addition to providing a 'safe haven' for the biosynthesis of secondary compounds, roots have recently been implicated in tolerance to foliar herbivores by providing a temporary storage site for primary metabolic products (e.g. photoassimilates) that would otherwise be vulnerable

to aboveground consumers (Babst *et al.* 2005; Schwachtje *et al.* 2006). In these cases, the sink strength of roots is elevated when leaves are attacked, thereby increasing belowground allocation of plant metabolites. Nutritional resources can then be re-allocated at a later time for aboveground growth and/or reproduction.

The logic underlying the resistance and tolerance mechanisms described above presumes that roots are less susceptible to herbivory than leaves (Karban & Baldwin 1997; van der Putten *et al.* 2001). However, a large and diverse group of cryptic soil-dwelling consumers (e.g. nematodes, arthropods, mammals) subsist largely on plant roots (Brown & Gange 1990; Coleman *et al.* 2004). It is therefore likely the 'norm' under natural growing conditions that plants simultaneously host above- and belowground herbivores.

Recent studies demonstrate that plants can mediate competitive interactions between foliar and root herbivores via systemically-induced defenses (van Dam *et al.* 2003; Bezemer *et al.* 2003, 2004; Bezemer & van Dam 2005; Soler *et al.* 2005; van Dam *et al.* 2005; van Dam & Bezemer 2006; Kaplan & Denno 2007; Soler *et al.* 2007; Erb *et al.* 2008; but see Rasmann & Turlings 2007). In the present study, we demonstrate that physiological integration of roots and shoots can result in the opposite pattern, with positive interactions linking foliar and root consumers. When leaf defenses are synthesized belowground, root herbivory may interfere with the production or translocation of these compounds, and thus indirectly benefit foliar herbivores by reducing plant resistance (Fig. 1a). Similarly, if aboveground herbivores elicit a tolerance response whereby plants allocate valued nutritional resources belowground, this storage effect may benefit root herbivores (Fig. 1b). We present experimental evidence for such reciprocal positive interactions between foliar and root herbivory on tobacco (*Nicotiana tabacum*), a system in which roots are known to be involved in anti-herbivore resistance (Dawson 1941) and tolerance (Schwachtje *et al.* 2006). Thus, plant defense strategies that integrate discrete physiological units, such as leaves and roots, may be a common feature of how plants cope with consumers and provide novel opportunities for linking above- and belowground biota.

METHODS

Foliar insect performance on plants with nematode root herbivory

The impact of nematode root herbivory (*Meloidogyne incognita*) on foliar-feeding insect herbivores (*Trichoplusia ni* and *Manduca sexta*) of tobacco was tested, with the expectation that insects would perform better on plants with experimentally-imposed nematode herbivory via foliar nicotine

interference (Fig. 1a). The nematode, *M. incognita*, is a gall-forming species that is largely considered the dominant parasite of tobacco roots (Barker & Lucas 1984), whereas *T. ni* and *M. sexta* are generalist and specialist caterpillars, respectively, that defoliate tobacco leaves. The difference in host range of the two caterpillars is important to note because the specialist, *M. sexta*, can detoxify and excrete tobacco alkaloids (Wink & Theile 2002), and therefore is less sensitive to nicotine in its diet when compared with the generalist *T. ni* (Krischik *et al.* 1991). Thus, if nematode root herbivory benefits leaf-chewing insects via aboveground nicotine interference, then the effect should be relatively stronger for *T. ni* than for *M. sexta*.

Tobacco plants (var. MD 609) were propagated by seed in a greenhouse and seedlings (9 weeks of growth from seed to seedling stage) were subsequently transplanted singly into 4-gallon pots containing a sterilized growing medium [50% sand, 50% potting mix (SunGro LC1 and professional blend; sphagnum peat moss, bark, perlite, vermiculite and clay)]. Plants were supplemented with nutrients weekly using a soluble fertilizer (20 : 10 : 20 NPK). The roots from half of all seedlings were randomly assigned to root herbivory and inoculated at the time of transplant with *c.* 100 000 *M. incognita* eggs obtained from a laboratory culture. This egg density is well within the range documented for *M. incognita*-infested tobacco fields (Barker & Lucas 1984) and also corresponds with inoculum levels used in prior studies (e.g. Hanounik & Osborne 1975, 1977; Barker & Weeks 1991). The remaining plants that did not receive experimental addition of nematode eggs served as controls.

Three weeks after nematode-inoculation, caterpillars were added to the leaves of control and root herbivory plants. Hornworms (*M. sexta*) were obtained from a local colony (NC State University) and cabbage loopers (*T. ni*) from a biological supply company (Benzon Research, Inc., Carlisle, PA, USA). Second-instar larvae of each species were independently reared on plants, with each plant receiving a single caterpillar. We excluded any individuals that appeared unusually large or small before placing caterpillars on plants. Thus, all larvae were visually indistinguishable from one another and starting weights were assumed to be approximately equal. Moreover, caterpillars were randomly assigned to experimental plants to account for any potential bias associated with variation in starting weights. After 7 days of growth, caterpillars were removed from plants and weighed. The experiment involving *T. ni* included 42 replications each of control and root herbivory plants split over three dates. The experiment with *M. sexta* had 45 replications each of control and root herbivory plants split over four dates. New plants were cultivated and used for experiments on each date (i.e. plants were never reused from earlier trials).

In addition to measuring insect performance, the nicotine content of foliage was determined from samples of newly-expanding leaves. Tissue samples were immediately frozen in liquid nitrogen and extracted and analysed by HPLC (Keinänen *et al.* 2001) on a reversed phase C18 column (Gemini C18, 150 × 4.6 mm; Phenomenex, Torrance, CA, USA). Concentrations of foliar nicotine were quantified using calibration curves prepared from commercially available standards.

Differences in caterpillar weights (mg wet weight) and leaf nicotine content between control and nematode-inoculated plants were assessed using ANOVA, with date as a random effect (Proc Mixed; statistical analyses were performed using SAS, Version 9.1; SAS Institute, Inc., 2001). Data were square-root transformed prior to statistical analysis to meet assumptions of normality and homogeneity of variances.

The role of nicotine in nematode-induced effects on foliar insect herbivores

If nicotine is indeed the causal mechanism underlying nematode-induced effects on aboveground caterpillars, then nematode root herbivory should affect caterpillar performance on nicotine-producing plants, but not on nicotine-deficient plants. We tested this hypothesis using two near-isogenic lines of tobacco that differ in nicotine content. Burley 21 is a wild-type, nicotine-producing tobacco line, whereas LA Burley 21 is nicotine-deficient due to mutations at two nuclear loci (*nic1* and *nic2*) that control nicotine biosynthesis (Hibi *et al.* 1994). Seeds for the two lines were obtained through the USDA National Plant Germplasm System (NPGS) and cultivated in a greenhouse as described above. Plants that differed in nicotine content were fully-crossed in a 2 × 2 factorial design with nematode root herbivory (as described above), resulting in four treatment combinations (*n* = 38 replications split over two dates).

Second-instar larvae of the generalist caterpillar *Spodoptera exigua* were added singly to each plant 3 weeks after nematode-inoculations, and caterpillars were removed from plants and weighed after 7 days of growth. As in the above experiment, initial size of caterpillars was standardized and individuals were randomly assigned to plants to control for variation in pre-treatment weights. For logistical purposes we switched from *T. ni* to *S. exigua*, although we fully expect that the two caterpillar species would respond similarly to our experimental treatments. Both are polyphagous caterpillars that feed on tobacco and are highly sensitive to levels of nicotine in their diet (Krischik *et al.* 1991; Steppuhn *et al.* 2004). In addition to measuring caterpillar performance, we also assessed the quantity of leaf tissue damaged by caterpillars using an acetate grid to estimate defoliation (Agrawal 1999).

To ensure that nicotine did not affect nematode root herbivory, and thus confound the two treatment effects, nematode performance on nicotine-producing and nicotine-deficient tobacco lines was also evaluated. After final caterpillar performance and defoliation measurements, plant roots were removed and nematode eggs harvested using a modified version of the Hussey & Barker (1973) extraction procedure. Briefly, galled roots were cut into 2 cm long segments, placed into a 250 mL flask containing 100 mL of a 0.6% NaOCl dilution, and subsequently poured through nested sieves (250- μ m pore sieve on top of a 25- μ m pore sieve). Performance was assessed by counting nematode eggs and fecundity was compared between wild-type and nicotine-deficient plants. Additionally, leaf tissue samples were harvested and analysed for secondary chemicals (e.g. nicotine, non-nicotine alkaloids, chlorogenic acid, caffeoyl putrescine) using the HPLC procedure described above (see Keinänen *et al.* 2001) to confirm that the two tobacco lines differed in nicotine content, but not in other compounds that potentially affect plant quality for herbivores. Last, because nicotine is a nitrogen-rich compound, we also measured % nitrogen in the leaves of both plant types using a CHN elemental analyser.

ANOVA was used to compare caterpillar weights (mg wet weight) and defoliation (cm^2 of leaf tissue removed) on control and root herbivory plants, with date as a random effect in the model (PROC MIXED). Separate analyses were conducted for wild-type and nicotine-deficient tobacco lines. We also compared nematode fecundity, secondary chemistry, and % nitrogen between plants that did and did not express nicotine using ANOVA (PROC MIXED). Caterpillar larval weights were log transformed prior to statistical analysis.

Nematode performance on plants with foliar insect herbivory

The reciprocal effect of aboveground insect herbivory on belowground nematode performance was assessed with the expectation that nematodes would perform better when co-occurring with foliar-feeding insects (Fig. 1b). Tobacco plants were cultivated and inoculated with nematode eggs (c. 10 000 eggs/plant) as described above. These plants were randomly assigned to one of four treatments: (i) undamaged control; (ii) *M. sexta* herbivory; (iii) *T. ni* herbivory and (iv) mechanical damage ($n = 12$ replications per treatment).

Plants in the *M. sexta* and *T. ni* herbivory treatments were defoliated by caterpillars at three times during the course of the experiment (2, 5 and 8 weeks after nematode inoculations). Bouts of defoliation were imposed every 3 weeks because many herbivore-induced plant responses are evident within the first several weeks after damage and the response wanes with time (Karban & Baldwin 1997). For

each defoliation bout, a single third-instar *M. sexta* larva or five fourth-instar *T. ni* larvae were added to each plant and removed after 4 days. Caterpillar leaf damage on each plant was estimated using an acetate grid (Agrawal 1999). Plants in the mechanical-damage treatment were defoliated each day using a hole-punch and the quantity and timing of tissue damage coincided with the caterpillar herbivory treatments. To do so, the amount of leaf tissue defoliated by caterpillars was visually estimated each day and the extent of hole-punching damage was then matched such that leaf area removal to caterpillars and mechanical damage was approximately equal.

Ten weeks after nematodes were added to plants, roots were collected and nematode performance was measured by harvesting and counting eggs as described above. ANOVA followed by Tukey–Kramer's test for means separation (PROC GLM) was used to assess the impact of defoliation on nematode performance. Pearson product–moment correlation was used to determine the relationship between overall levels of caterpillar feeding damage (cm^2 of leaf tissue removed) and nematode fecundity (PROC CORR).

Leaf–root carbon allocation on plants with above- and belowground herbivory

Foliar herbivory is predicted to affect root herbivores via changes in above- and belowground resource allocation, with an increase in root sink strength on plants with aboveground herbivory (Fig. 1b). To test this hypothesis, we manipulated foliar (*M. sexta*) and root (*M. incognita*) herbivores in a 2×2 factorial design ($n = 15$ replications per treatment combination), and quantified the effects of herbivory on within-plant carbon transport using stable isotope and invertase enzyme analyses.

Nematodes were inoculated on greenhouse-grown plants as described above and 3 weeks later a single second-instar *M. sexta* larva was added to those plants assigned to the foliar herbivory treatment. After 5 days of caterpillar damage (< 10% leaf area defoliated), all plants were labelled using the carbon isotope ^{13}C . Because c. 99% of carbon in terrestrial ecosystems occurs in the form ^{12}C , plants can be pulse-labelled using the heavier (^{13}C) isotope as a physiological tracer (Dawson *et al.* 2002). The first fully-expanded leaf on each plant was enclosed in an air-tight chamber and exposed to $^{13}\text{CO}_2$ by reacting 38 mg of sodium bicarbonate, $\text{NaH}^{13}\text{CO}_3$ (99 atom% ^{13}C ; Sigma-Aldrich, Milwaukee, WI, USA), with 500 μL DL-lactic acid for 90 min. Leaf chambers were removed and 48 h later all emerging leaves (i.e. those occurring above the source–sink transition leaf) and roots were harvested. Plant tissues were dried, ground and analysed for carbon isotopic signatures using an isotope ratio mass spectrometer (Cornell University Stable Isotope Laboratory, Ithaca, NY, USA). Two blocks of the

experiment (eight total plants) were not enriched to assess ambient levels of ^{12}C and ^{13}C . Average ambient levels of ^{13}C in non-enriched plants were subtracted from ^{13}C levels in labelled plants such that analysed and presented ^{13}C values represent actual quantities of carbon that were assimilated and translocated via the labelling process.

In addition to stable isotope analysis, we measured the activities of plant invertase enzymes in leaf and root tissues. Invertases are sugar-cleaving enzymes that hydrolyse the disaccharide sucrose into its constituent monosaccharides, glucose and fructose. Invertase enzymes are thought to regulate long-distance carbon transport by generating sucrose concentration gradients and thus levels of invertase enzyme activity serve as a reliable estimate of plant sink strength (Roitsch & González 2004).

Leaf and root samples were flash-frozen in liquid nitrogen and sample extraction and invertase assays were based on Rehill & Schultz (2003). Thirty to forty-five milligram of tissue samples were placed in microcentrifuge tubes, and 0.6 mL of 0 °C extraction buffer was added. The buffer contained: 50 mM MES pH 6.5, 20 mM DTT, 5 mM EDTA, 5% (v/v) PVPP (insoluble), 2% (v/v) Tween 20, 2% (v/v) glycerol, 2.5 mM benzamidine and 0.1 mM PMSF. Each sample was macerated with a TissueMaster 125 homogenizer (Omni International, Marietta, GA, USA) while the microcentrifuge tube was held on ice, and 1.0 mL of 0 °C extraction buffer was added after homogenization. The samples were sonicated for 30 min and then centrifuged at 21 000 *g* and 4 °C RCF for 20 min. The supernatant was removed and used for the assay of vacuolar invertase activity. Any remaining cell extract was removed from the sample residue by the addition of 1.6 mL of 0 °C of rinse buffer (identical with the extraction buffer except for the absence of PVPP), centrifuged as above, and the supernatant was removed. A total of three such rinses of the sample was performed. The remaining pellet was assayed for cell wall invertase activity.

Sample extracts were assayed in triplicate for vacuolar invertase as follows. Each reaction for leaf extracts contained 20 μL sample extract, 60 μL pH 4.5 50 mM sodium acetate buffer and 20 μL 1.0 M sucrose substrate in a total reaction volume of 100 μL (reactions with root extracts contained 40, 60 and 25 μL respectively). Each assay was incubated for 75 min at 37 °C (120 min for roots); enzyme kinetics were linear, and there was no substrate limitation. Reactions were stopped by addition of 100 μL of a modified Sumner's reagent that contained 1% DNSA, 0.5% phenol, 2.5% NaOH and 0.05% Na_2SO_3 . The reactions were heated at 100 °C for 10 min, 33 μL of 40% Rochelle salt solution was added to each reaction, and absorbance was read at 560 nm. Reducing sugar concentrations were computed using a standard curve of glucose in 0.2 M sucrose in pH 4.5 50 mM sodium acetate buffer.

Blanks consisted of complete reaction mixtures with the reaction stopped immediately.

Assays of cell wall invertase were performed directly on the prepared sample pellets. The pellet was suspended in 1.3 mL of pH 4.5 50 mM acetate buffer and the reaction was started by addition of 0.325 mL of 1.0 M sucrose substrate solution. Samples were reacted for 75 min at 37 °C, placed in boiling water for 5 min to denature the enzyme, and then centrifuged at 21 000 *g* RCF and 4 °C for 20 min. The supernatant was assayed for reducing sugars using Sumner's reagent and compared to a glucose standard containing 0.2 M sucrose in pH 4.5 50 mM acetate buffer. Enzyme activities are reported as units (U) per gram fresh weight. A unit equals 1 μmole sucrose hydrolysed per minute.

The impact of foliar and root herbivory on leaf-root ^{13}C allocation and invertase enzyme activity was assessed using two-way ANOVA, with insect and nematode herbivory as main effects (PROC MIXED). We independently analysed the impact of herbivory on ^{13}C allocation to leaves, roots and the root : leaf ratio; vacuolar and cell wall invertases were also independently assessed in leaf and root tissues. Spatial groupings of plants (i.e. blocks of each replicate) were considered as a random effect in the model. Data were log-transformed to improve normality.

RESULTS

Foliar insect performance on plants with nematode root herbivory

Belowground nematode feeding increased the larval weight of the generalist caterpillar *T. ni* by 29% ($F_{1,74} = 6.19$, $P = 0.0151$; Fig. 2a). However, root herbivory did not affect the performance of the specialist caterpillar *M. sexta* ($F_{1,80} = 2.19$, $P = 0.1425$; Fig. 2b), although there was a trend for greater weight gain on root herbivory plants. The concentration of leaf nicotine was > 2 \times higher on control than on root herbivory plants, regardless of whether *T. ni* ($F_{1,22} = 25.30$, $P < 0.0001$; Fig. 2c) or *M. sexta* ($F_{1,20} = 76.80$, $P < 0.0001$; Fig. 2d) was the aboveground herbivore.

The role of nicotine in nematode-induced effects on foliar insect herbivores

The foliar nicotine content of nicotine-expressing tobacco lines was > 5 \times higher than nicotine-deficient plants ($F_{1,18} = 52.06$, $P < 0.0001$). However, the two tobacco lines did not differ in nematode performance ($F_{1,16} = 0.02$, $P = 0.8998$), non-nicotine secondary chemistry (non-nicotine alkaloids: $F_{1,18} = 2.80$, $P = 0.1118$, chlorogenic acid: $F_{1,18} = 2.08$, $P = 0.1662$, caffeoyl putrescine: $F_{1,18} = 0.04$, $P = 0.8385$), or % foliar nitrogen ($F_{1,38} = 0.82$, $P = 0.3699$).

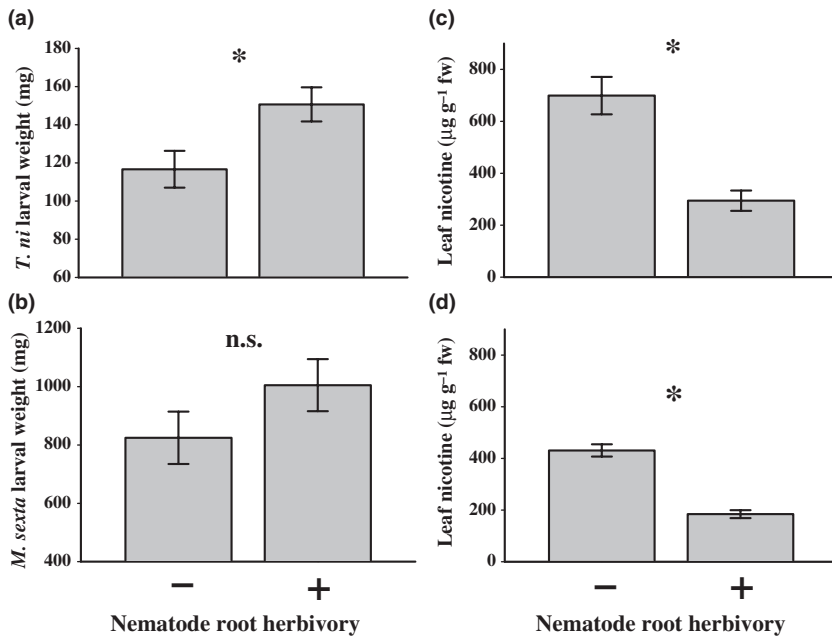


Figure 2 Impact of nematode root herbivory (+ or -) on the (a) performance (larval mass) of the generalist caterpillar *Trichoplusia ni*, (b) performance (larval mass) of the specialist caterpillar *Manduca sexta*, (c) leaf nicotine content of *T. ni*-damaged plants, and (d) leaf nicotine content of *M. sexta*-damaged plants (mean \pm SE). Asterisks denote significant differences between means ($P < 0.05$); n.s., non-significant.

Nematode root herbivory increased the weight gain of the caterpillar *S. exigua* on nicotine-expressing plants ($F_{1,75} = 6.10$, $P = 0.0158$), but had no impact on caterpillar performance on nicotine-deficient plants ($F_{1,71} = 0.00$, $P = 0.9902$; Fig. 3a). Similarly, caterpillars removed more leaf tissue on nicotine-producing plants with nematode root herbivory ($F_{1,21} = 7.43$, $P = 0.0126$), but nematode presence had no impact on caterpillar defoliation on nicotine-deficient plants ($F_{1,19} = 0.13$, $P = 0.7244$; Fig. 3b).

Nematode performance on plants with foliar insect herbivory

Aboveground insect herbivory elevated the fecundity of nematodes feeding belowground by 44%, an effect that was equally strong for the generalist and specialist caterpillar species ($F_{3,38} = 3.15$, $P = 0.0361$; Fig. 4a). Notably, mechanical damage using a hole-punch did not elicit the same positive response in nematodes as caterpillar defoliation. Overall, the level of leaf damage by aboveground herbivores was positively correlated with nematode egg production ($r = 0.5028$, $P = 0.0171$; Fig. 4b).

Leaf-root carbon allocation on plants with above- and belowground herbivory

Neither caterpillars ($F_{1,43} = 1.70$, $P = 0.1991$) nor nematodes ($F_{1,43} = 2.59$, $P = 0.1149$) affected the allocation of ^{13}C to developing tobacco leaves (Fig. 5a). However, aboveground caterpillar herbivory increased ^{13}C allocation to roots ($F_{1,42} = 4.17$, $P = 0.0474$), whereas nematode root

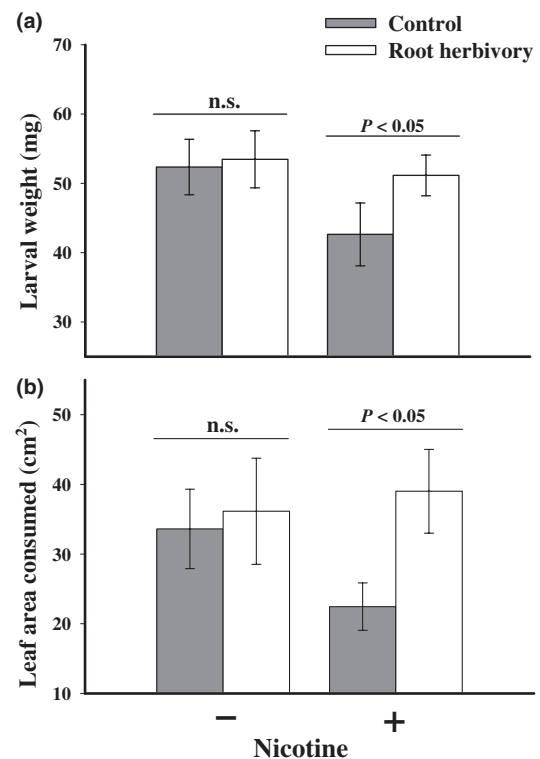


Figure 3 Interactive effects of nematode root herbivory and nicotine on (a) caterpillar performance (larval weight), and (b) caterpillar leaf damage (mean \pm SE). Nicotine (+) = nicotine-expressing tobacco line and nicotine (-) = nicotine-deficient tobacco line; n.s., non-significant.

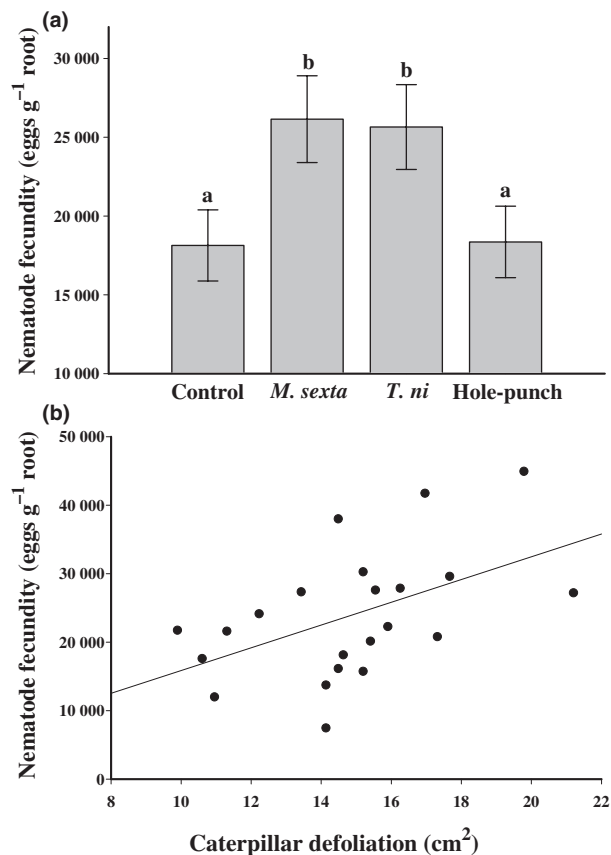


Figure 4 Consequences of foliar herbivory for the performance (fecundity) of root-feeding nematodes. (a) Nematode egg production on plants with caterpillar herbivory (the specialist *Manduca sexta* and the generalist *Trichoplusia ni*) and mechanical damage (hole punch). Means (\pm SE) with different letters are significantly different ($P < 0.05$). (b) Relationship between quantity of leaf tissue damaged by caterpillars and nematode fecundity.

herbivory had no impact ($F_{1,42} = 2.60$, $P = 0.1142$; Fig. 5b). Similarly, the ¹³C root : leaf ratio was higher (i.e. greater belowground allocation) on plants with foliar insect herbivory ($F_{1,42} = 4.97$, $P = 0.0312$), whereas nematodes did not affect the root : leaf ratio ($F_{1,42} = 0.01$, $P = 0.9306$; Fig. 5c). Interactions between foliar and root herbivory were non-significant for all response variables.

Overall, responses of invertase enzymes were broadly similar to that of stable isotopes. Leaves were unaffected by either aboveground consumers (vacuolar: $F_{1,69} = 0.42$, $P = 0.5200$, Fig. 6a; cell wall: $F_{1,77} = 0.00$, $P = 0.9785$, Fig. 6b) or belowground consumers (vacuolar: $F_{1,69} = 0.25$, $P = 0.6221$; cell wall: $F_{1,77} = 1.76$, $P = 0.1887$). However, vacuolar invertase enzyme activity was higher in the roots of plants incurring aboveground caterpillar herbivory (caterpillar effect: $F_{1,51} = 5.03$, $P = 0.0294$, nematode effect: $F_{1,51} = 0.00$, $P = 0.9629$; Fig. 6c). Cell wall invertases exhibited the opposite pattern with higher activity in the

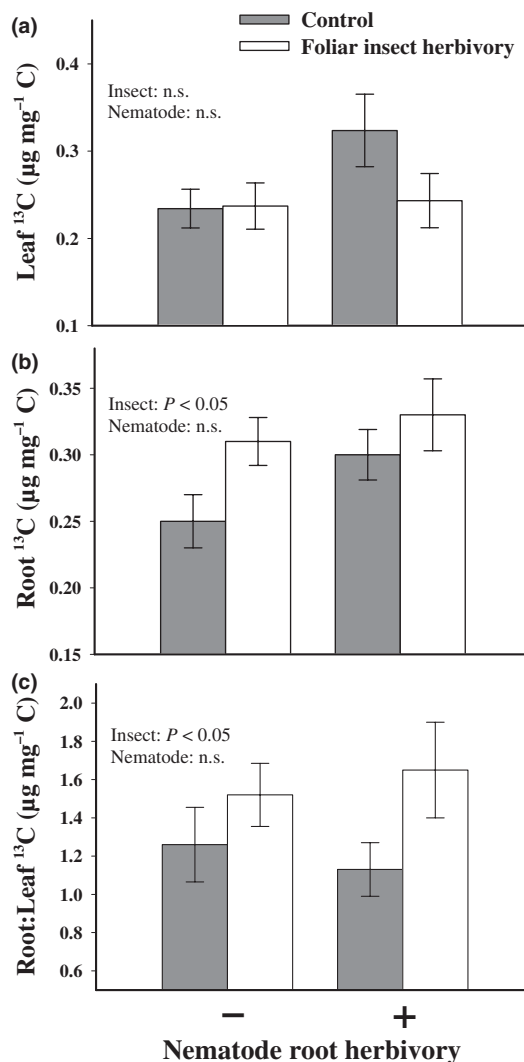


Figure 5 Effects of foliar and root herbivory on above- and belowground carbon allocation. Plants were pulse-labelled with ¹³C and stable isotope analysis was used to measure the impact of leaf and root herbivory on whole-plant carbon partitioning, including (a) leaves, (b) roots and (c) root : leaf ratio (mean \pm SE). Significance of the main effects of foliar insect and root nematode herbivory are indicated for each response variable; n.s., non-significant.

roots of nematode-inoculated plants ($F_{1,74} = 5.43$, $P = 0.0225$; Fig. 6d), but no response to caterpillars ($F_{1,74} = 0.28$, $P = 0.6011$).

DISCUSSION

Because roots are concealed belowground and spatially separated from leaves, their contribution to aboveground anti-herbivore plant defense strategies is not generally acknowledged. Here, we experimentally demonstrate that

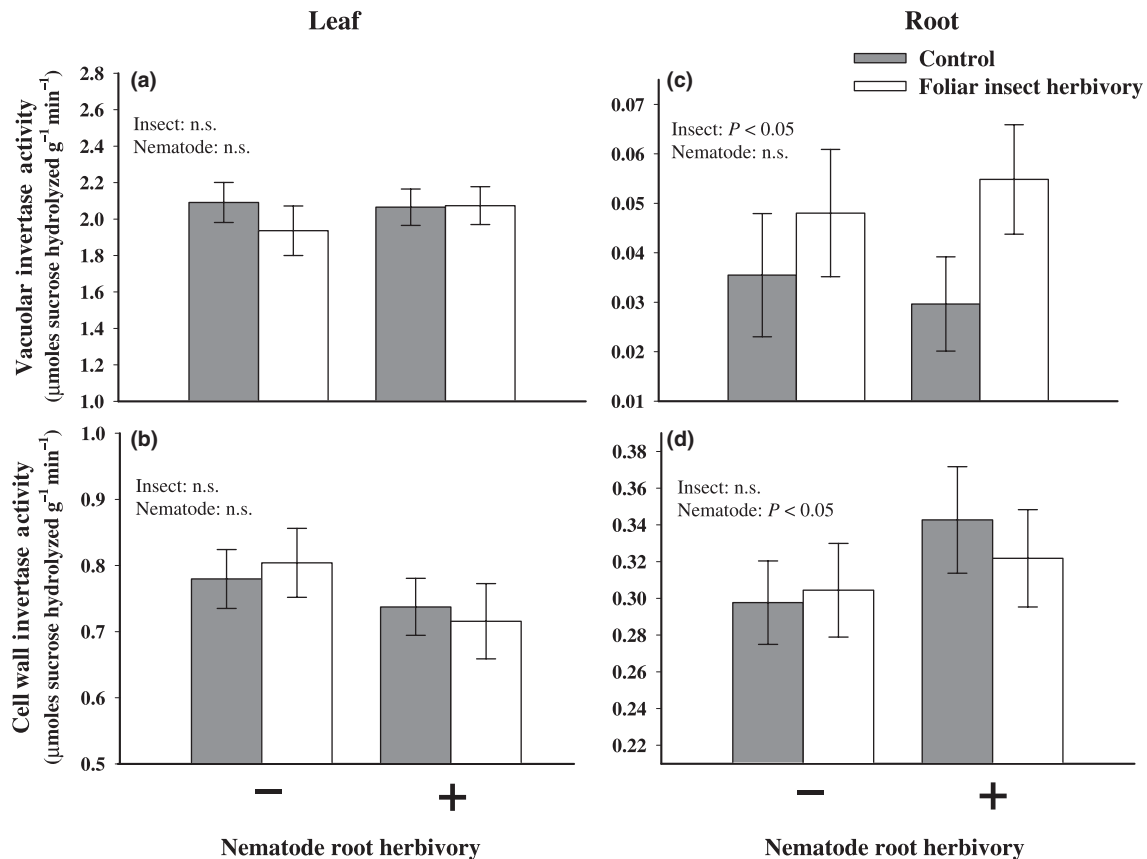


Figure 6 Effects of foliar and root herbivory on above- and belowground invertase enzyme activities, including (a and b) leaves, and (c and d) roots (mean \pm SE). Significance of the main effects of foliar insect and root nematode herbivory are indicated for each response variable; n.s., non-significant.

roots are intimately involved with leaf defense against phytophagous insects and that such leaf–root connections mediate indirect interactions between above- and belowground consumers.

In the case of foliar resistance, nicotine provides one of the best examples of leaf–root integration (Fig. 1a). Not only is nicotine root biosynthesis well-established (Dawson 1941; Baldwin 1988), but the ecological relevance of nicotine as an aboveground defense is also well-documented (Baldwin 1991; Steppuhn *et al.* 2004). Nematode root herbivory is known to suppress the constitutive and inducible foliar expression of nicotine in tobacco (Hanounik & Osborne 1975; Hanounik & Osborne 1977; Barker & Weeks 1991; Kaplan *et al.* 2008), and therefore we predicted that root-feeding herbivores would induce susceptibility to leaf-feeding insects by interfering with aboveground nicotine dynamics. Indeed, caterpillars performed better when co-occurring on plants with nematode root herbivores (Fig. 2a,b). Several lines of evidence implicate nicotine as the causal mechanism underlying this effect. First, nematodes had a relatively greater impact on the performance of

the nicotine-sensitive generalist caterpillar *T. ni* than on the nicotine-tolerant specialist *M. sexta*. Second, leaf nicotine content was $> 50\%$ lower on plants incurring root herbivory compared with nematode-free control plants (Fig. 2c,d). Last, nematodes only affected caterpillar performance (Fig. 3a) and defoliation (Fig. 3b) on nicotine-expressing tobacco lines, whereas no interaction occurred on nicotine-deficient mutant plants.

Thus, despite the emphasis on induced defenses linking leaf and root herbivores (Bezemer *et al.* 2003, 2004; van Dam *et al.* 2003, 2005; Bezemer & van Dam 2005; Soler *et al.* 2005, 2007; van Dam & Bezemer 2006; Kaplan & Denno 2007; Erb *et al.* 2008), the opposite effect can occur in systems where aboveground allelochemicals are produced belowground. In a recent study, root herbivory was found to interfere with the foliar expression of root-derived secondary chemicals, but elevate the foliar expression of compounds that are synthesized in leaves (Kaplan *et al.* 2008). As a result, the location of biosynthetic sites for ecologically-important phytochemicals may ultimately dictate whether root herbivory leads to net positive or negative effects on

foliar consumers. Additionally, the amount of damage inflicted on roots will likely affect the outcome of the interaction, with stronger aboveground interference occurring on plants with more intense root herbivory (Preisser *et al.* 2007; Kaplan *et al.* 2008). Last, we suspect that herbivore feeding guild may be a contributing factor. The focal root herbivore in our study is a gall-forming nematode whose feeding style and effects on root morphology and physiology are entirely different than those of chewing insects, which to date are vastly over-represented in the literature on plant-mediated interactions (Bezemer & van Dam 2005; van Dam & Bezemer 2006; Kaplan & Denno 2007; Erb *et al.* 2008).

The second means by which roots are integrated with aboveground defense is through plant tolerance responses whereby foliar nutritional resources are preferentially allocated to roots (Fig. 1b; Babst *et al.* 2005; Schwachtje *et al.* 2006). Unlike the above-described resistance trait which resulted in unidirectional plant-mediated effects of nematodes on insects, herbivore-induced tolerance responses appear to mediate the reciprocal effect of insects back onto nematodes. In this case, we found that nematode root herbivores benefited from sharing plants with leaf-feeding insects (Fig. 4), and this positive response was associated with an increase in root sink strength for photoassimilates elicited by caterpillar feeding (Figs 5b,c and 6c). Because sink strength is known to affect the success of herbivores, especially vascular tissue feeders, aboveground caterpillar defoliation likely facilitated root-galling nematodes by redirecting assimilate flow within the plant (Rehill & Schultz 2003; Denno & Kaplan 2007). Moreover, in prior experimental work we found no evidence that caterpillar leaf herbivory alters the secondary chemistry (e.g. alkaloids, phenolics) of tobacco roots, and thus it is unlikely that caterpillars benefited nematodes by inducing changes in belowground defense patterns (Kaplan *et al.* 2008).

Surprisingly, nematodes did not elicit direct or interactive effects on within-plant carbon partitioning, which would be expected given that gall-forming herbivores are often strong sinks for plant resources. However, there was a trend in the data suggesting a potential interaction between root and shoot herbivory on belowground resource allocation (Fig. 5b), although this interaction was non-significant ($F_{1,41} = 0.65$, $P = 0.4257$). Because the sink activity of galls often changes drastically with development (Rehill & Schultz 2003), it is also possible that our isotope labelling simply occurred when nematode-induced galls were not at their maximum developmental stage.

Notably, the positive effect of defoliation on nematode fecundity was only elicited in response to actual herbivory and not in response to simple mechanical damage (i.e. hole-punch). This finding suggests that some feature unique to caterpillar feeding (e.g. an elicitor in saliva) caused this effect

to occur, as has been shown for other systems (e.g. Turlings & Tumlinson 1992; Halitschke *et al.* 2003). A recent study documenting similar tolerance responses found an increase in root sink strength when plants were treated with caterpillar (*M. sexta*) regurgitant and not in response to simple tissue damage (Schwachtje *et al.* 2006).

CONCLUSIONS

Although the defensive phenotype of plant leaves against consumers is influenced by several ecological and evolutionary factors (e.g. resource availability, genotype; Herms & Mattson 1992), the within-plant contribution of roots is not generally considered, despite the well-known physiological integration of plants (Öpik & Rolfe 2005). However, we offer two distinct examples of how roots contribute to aboveground plant defenses (although it should be noted that, unlike nicotine, the putative tolerance response documented in this study necessitates far more research to elucidate its potential defensive value in plants). Importantly, the mechanisms by which these two defenses function are extremely different; one a neurotoxic chemical that poisons consumers, and the other a means for re-allocating and thus protecting valued nutritional reserves. Yet in both cases roots are directly involved in the process. Also, each example of leaf–root integration results in ecological consequences when plants simultaneously host above- and belowground consumers. Because root-feeding nematodes and other belowground phytoparasites are ubiquitous components of the soil environment (Brown & Gange 1990; Coleman *et al.* 2004), herbivore-induced facilitation may indeed be an important feature that links the dynamics of above- and belowground communities.

REFERENCES

- Agrawal, A.A. (1999). Induced responses to herbivory in wild radish: effects on several herbivores and plant fitness. *Ecology*, 80, 1713–1723.
- Agrawal, A.A. & Karban, R. (2000). Specificity of constitutive and induced resistance: pigment glands influence mites and caterpillars on cotton plants. *Entomol. Exp. Appl.*, 96, 39–49.
- Babst, B.A., Ferrieri, R.A., Gray, D.W., Lerdau, M., Schlyer, D.J., Schueller, M. *et al.* (2005). Jasmonic acid induces rapid changes in carbon transport and partitioning in *Populus*. *New Phytol.*, 167, 63–72.
- Bais, H.P., Park, S.W., Weir, T.L., Callaway, R.M. & Vivanco, J.M. (2004). How plants communicate using the underground information superhighway. *Trends Plant Sci.*, 9, 26–32.
- Baldwin, I.T. (1988). Damage-induced alkaloids in tobacco: pot-bound plants are not inducible. *J. Chem. Ecol.*, 14, 1113–1121.
- Baldwin, I.T. (1991). Damage-induced alkaloids in wild tobacco. In: *Phytochemical Induction by Herbivores* (eds Tallamy, D.W. & Raupp, M.J.). John Wiley & Sons, New York, pp. 47–69.

- Barker, K.R. & Lucas, G.B. (1984). Nematode parasites of tobacco. In: *Plant and Insect Nematodes* (ed. Nickle, W.R.). Marcel Dekker, Inc., New York, pp. 213–242.
- Barker, K.R. & Weeks, W.W. (1991). Relationships between soil and levels of *Meloidogyne incognita* and tobacco yield and quality. *J. Nematol.*, 23, 82–90.
- Bezemer, T.M. & van Dam, N.M. (2005). Linking aboveground and belowground interactions via induced plant defenses. *Trends Ecol. Evol.*, 20, 617–624.
- Bezemer, T.M., Wagenaar, R., van Dam, N.M. & Wäckers, F.L. (2003). Interactions between above- and belowground insect herbivores as mediated by the plant defense system. *Oikos*, 101, 555–562.
- Bezemer, T.M., Wagenaar, R., van Dam, N.M., van der Putten, W.H. & Wäckers, F.L. (2004). Above- and below-ground terpenoid aldehyde induction in cotton, *Gossypium herbaceum*, following root and leaf injury. *J. Chem. Ecol.*, 30, 53–67.
- Brown, V.K. & Gange, A.C. (1990). Insect herbivory belowground. *Adv. Ecol. Res.*, 20, 1–58.
- Coleman, D.C., Crossley, D.A. & Hendrix, P.F. (2004). *Fundamentals of Soil Ecology*, 2nd edn. Academic Press, New York.
- van Dam, N.M. & Bezemer, T.M. (2006). Chemical communication between roots and shoots: towards an integration of aboveground and belowground induced responses in plants. In: *Chemical Ecology: From Gene to Ecosystem* (eds Dicke, M. & Takken, W.). Springer, The Netherlands, pp. 127–143.
- van Dam, N.M., Harvey, J.A., Wäckers, F.L., Bezemer, T.M., van der Putten, W.H. & Vet, L.E.M. (2003). Interactions between aboveground and belowground induced responses against phytophages. *Basic Appl. Ecol.*, 4, 63–77.
- van Dam, N.M., Raaijmakers, C.E. & van der Putten, W.H. (2005). Root herbivory reduces growth and survival of the shoot feeding specialist *Pieris rapae* on *Brassica nigra*. *Entomol. Exp. Appl.*, 115, 161–170.
- Dawson, R.F. (1941). The localization of the nicotine synthetic mechanism in the tobacco plant. *Science*, 94, 396–397.
- Dawson, T.E., Mambelli, S., Plamboeck, A.H., Templer, P.H. & Tu, K.P. (2002). Stable isotopes in plant ecology. *Ann. Rev. Ecol. System.*, 33, 507–559.
- Denno, R.F. & Kaplan, I. (2007). Plant-mediated interactions in herbivorous insects: mechanisms, symmetry, and challenging the paradigms of competition past. In: *Ecological Communities: Plant Mediation in Indirect Interaction Webs* (eds Ohgushi, T., Craig, T.P. & Price, P.W.). Cambridge University Press, Cambridge, pp. 19–50.
- Erb, M., Ton, J., Degenhardt, J. & Turlings, T.C.J. (2008). Interactions between arthropod-induced aboveground and belowground defenses in plants. *Plant Phys.*, 146, 867–874.
- Halitschke, R., Gase, K., Hui, D.Q., Schmidt, D.D. & Baldwin, I.T. (2003). Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. VI. Microarray analysis reveals that most herbivore-specific transcriptional changes are mediated by fatty acid-amino acid conjugates. *Plant Physiol.*, 131, 1894–1902.
- Hanounik, S.B. & Osborne, W.W. (1975). Influence of *Meloidogyne incognita* on the content of amino acids and nicotine in tobacco grown under gnotobiotic conditions. *J. Nematol.*, 7, 332–336.
- Hanounik, S.B. & Osborne, W.W. (1977). The relationship between population density of *Meloidogyne incognita* and nicotine content of tobacco. *Nematologica*, 23, 147–152.
- Heinstein, P.F., Smith, F.H. & Tove, S.B. (1962). Biosynthesis of C¹⁴-labeled gossypol. *J. Biol. Chem.*, 237, 2643–2646.
- Hermes, D.A. (2002). Effects of fertilization on insect resistance of woody ornamental plants: reassessing an entrenched paradigm. *Environ. Entomol.*, 31, 923–933.
- Hermes, D.A. & Mattson, W.J. (1992). The dilemma of plants: to grow or defend. *Quart. Rev. Biol.*, 67, 283–335.
- Hibi, N., Higashiguchi, S., Hashimoto, T. & Yamada, Y. (1994). Gene expression in tobacco low-nicotine mutants. *Plant Cell*, 6, 723–735.
- Huberty, A.F. & Denno, R.F. (2004). Plant water stress and its consequences for herbivorous insects: a new synthesis. *Ecology*, 85, 1383–1398.
- Hussey, R.S. & Barker, K.R. (1973). A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. *Plant Disease Rep.*, 57, 1025–1028.
- Johnson, S.N., Birch, A.N.E., Gregory, P.J. & Murray, P.J. (2006). The ‘mother knows best’ principle: should soil insects be included in the preference–performance debate? *Ecol. Entomol.*, 31, 395–401.
- Kaplan, I. & Denno, R.F. (2007). Interspecific interactions in phytophagous insects revisited: a quantitative assessment of competition theory. *Ecol. Lett.*, 10, 977–994.
- Kaplan, I., Halitschke, R., Kessler, A., Sardanelli, S. & Denno, R.F. (2008). Constitutive and induced defenses to herbivory in above- and belowground plant tissues. *Ecology*, 89, 392–406.
- Karban, R. & Baldwin, I.T. (1997). *Induced Responses to Herbivory*. University of Chicago Press, Chicago, IL.
- Keinänen, M., Oldham, N.J. & Baldwin, I.T. (2001). Rapid HPLC screening of jasmonate-induced increases in tobacco alkaloids, phenolics, and diterpene glycosides in *Nicotiana attenuata*. *J. Agric. Food Chem.*, 49, 3553–3558.
- Krischik, V.A., Goth, R.W. & Barbosa, P. (1991). Generalized plant defense: effects on multiple species. *Oecologia*, 85, 562–571.
- McCully, M.E. (1999). Roots in soil: unearthing the complexities of roots and their rhizospheres. *Ann. Rev. Plant Phys. Plant Mol. Biol.*, 50, 695–718.
- Meisner, J., Ishaaya, I., Ascher, K.R.S. & Zur, M. (1978). Gossypol inhibits protease and amylase activity of *Spodoptera littoralis* larvae. *Ann. Entomol. Soc. Am.*, 71, 5–8.
- Öpik, H. & Rolfe, S. (2005). *The Physiology of Flowering Plants*, 4th edn. Cambridge University Press, New York.
- Parrott, W.L. (1990). Plant resistance to insects in cotton. *Fla. Entomol.*, 73, 392–396.
- Preisser, E.L., Gibson, S.E., Adler, L.S. & Lewis, E.E. (2007). Underground herbivory and the costs of constitutive defense in tobacco. *Acta Oecol.*, 31, 210–215.
- van der Putten, W.H., Vet, L.E.M., Harvey, J.A. & Wäckers, F.L. (2001). Linking above- and belowground multitrophic interactions of plants, herbivores, pathogens, and their antagonists. *Trends Ecol. Evol.*, 16, 547–554.
- Rasmann, S. & Turlings, T.C.J. (2007). Simultaneous feeding by aboveground and belowground herbivores attenuates plant-mediated attraction of their respective natural enemies. *Ecol. Lett.*, 10, 926–936.
- Rehill, B.J. & Schultz, J.C. (2003). Enhanced invertase activities in the galls of *Hormaphis hamamelidis*. *J. Chem. Ecol.*, 29, 2703–2720.
- Roitsch, T. & González, M.C. (2004). Function and regulation of plant invertases: sweet sensations. *Trends Plant Sci.*, 9, 606–613.

- SAS. (2001). SAS for Windows, Version 9.1. SAS Institute, Cary, North Carolina, USA.
- Schenk, H.J. (2006). Root competition: beyond resource depletion. *J. Ecol.*, 94, 725–739.
- Schwachtje, J., Minchin, P.E.H., Jahnke, S., van Dongen, J.T., Schittko, U. & Baldwin, I.T. (2006). SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots. *Proc. Natl. Acad. Sci. U S A.*, 103, 12935–12940.
- Smith, F.H. (1961). Biosynthesis of gossypol by excised cotton roots. *Nature*, 192, 888–889.
- Soler, R., Bezemer, T.M., van der Putten, W.H., Vet, L.E.M. & Harvey, J.A. (2005). Root herbivore effects on above-ground herbivore, parasitoid and hyperparasitoid performance via changes in plant quality. *J. Anim. Ecol.*, 74, 1121–1130.
- Soler, R., Bezemer, T.M., Cortesero, A.M., van der Putten, W.H., Vet, L.E.M. & Harvey, J.A. (2007). Impact of foliar herbivory on the development of a root-feeding insect and its parasitoid. *Oecologia*, 152, 257–264.
- Steppuhn, A., Gase, K., Krock, B., Halitschke, R. & Baldwin, I.T. (2004). Nicotine's defensive function in nature. *PLoS Biol.*, 2, 1074–1080.
- Turlings, T.C.J. & Tumlinson, J.H. (1992). Systemic release of chemical signals by herbivore-injured corn. *Proc. Natl. Acad. Sci. USA.*, 89, 8399–8402.
- Wink, M. & Theile, V. (2002). Alkaloid tolerance in *Manduca sexta* and phylogenetically related sphingids (Lepidoptera: Sphingidae). *Chemoecology*, 12, 29–46.

Editor, Michael Hochberg

Manuscript received 13 February 2008

First decision made 18 March 2008

Manuscript accepted 11 April 2008