

CONSTITUTIVE AND INDUCED DEFENSES TO HERBIVORY IN ABOVE- AND BELOWGROUND PLANT TISSUES

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Abstract. A recent surge in attention devoted to the ecology of soil biota has prompted interest in quantifying similarities and differences between interactions occurring in above- and belowground communities. Furthermore, linkages that interconnect the dynamics of these two spatially distinct ecosystems are increasingly documented. We use a similar approach in the context of understanding plant defenses to herbivory, including how they are allocated between leaves and roots (constitutive defenses), and potential cross-system linkages (induced defenses). To explore these issues we utilized three different empirical approaches. First, we manipulated foliar and root herbivory on tobacco (*Nicotiana tabacum*) and measured changes in the secondary chemistry of above- and belowground tissues. Second, we reviewed published studies that compared levels of secondary chemistry between leaves and roots to determine how plants distribute putative defense chemicals across the above- and belowground systems. Last, we used meta-analysis to quantify the impact of induced responses across plant tissue types.

In the tobacco system, leaf-chewing insects strongly induced higher levels of secondary metabolites in leaves but had no impact on root chemistry. Nematode root herbivores, however, elicited changes in both leaves and roots. Virtually all secondary chemicals measured were elevated in nematode-induced galls, whereas the impact of root herbivory on foliar chemistry was highly variable and depended on where chemicals were produced within the plant. Importantly, nematodes interfered with aboveground metabolites that have biosynthetic sites located in roots (e.g., nicotine) but had the opposite effect (i.e., nematodes elevated foliar expression) on chemicals produced in shoots (e.g., phenolics and terpenoids).

Results from our literature review suggest that, overall, constitutive defense levels are extremely similar when comparing leaves with roots, although certain chemical classes (e.g., alkaloids, glucosinolates) are differentially allocated between above- and belowground parts. Based on a meta-analysis of induced defense studies we conclude that: (1) foliar induction generates strong responses in leaves, but much weaker responses in roots, and (2) root induction elicits responses of equal magnitude in both leaves and roots. We discuss the importance of this asymmetry and the paradox of cross-system induction in relation to optimal defense theory and interactions between above- and belowground herbivory.

Key words: alkaloids; induced plant defenses; meta-analysis; nicotine; optimal defense theory; phenolics; phytoparasitic nematodes; priming; root herbivory; terpenoids.

INTRODUCTION

Optimal defense theory (ODT) was originally developed to explain the ecological and evolutionary forces that generate within-plant heterogeneity in the distribution of defenses against herbivory (McKey 1974, 1979, Rhoades 1979, Denno and McClure 1983). The basic premises of ODT are that defenses are costly to plants and thus should be preferentially allocated to parts that are of greatest value and risk of attack from consumers (Zangerl and Bazzaz 1992). Consequently, ODT can be used to predict the relative expression of defenses in different parts of the same plant. For example, young leaves are considered more valuable than old leaves and

often possess far greater concentrations of secondary metabolites (Krischik and Denno 1983, Raupp and Denno 1983, van Dam et al. 1996, de Boer 1999, Ohnmeiss and Baldwin 2000). Similarly, flowers and fruits are more difficult to replace than vegetative parts and frequently possess high levels of resistance to herbivores (Euler and Baldwin 1996, Zangerl and Rutledge 1996, Darrow and Bowers 1999, Kozukue et al. 2004).

An explicit consideration of how plant defenses are differentially allocated between leaves and roots, in the context of ODT, is more difficult to reconcile. Leaves and roots both serve essential functions, with leaves fixing carbon and roots providing water and nutrients. Therefore, leaves and roots, overall, must be considered of approximately equal value to the plant (Zangerl and Bazzaz 1992). However, it should be noted that the

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value, and thus defense, of above- and belowground parts will likely vary with growing environment. For instance, leaves may be of greater value to plants exposed to low light conditions, whereas roots might be more valuable when water or nutrients are the factors most limiting to plant growth. Additionally, leaves and roots are likely under comparable risk of attack from consumers (Zangerl and Bazzaz 1992; but see Zangerl and Rutledge 1996). Although aboveground herbivory is more widely documented, belowground herbivory is probably underestimated because of the difficulties associated with quantifying root damage (Brown and Gange 1990, Hunter 2001). Furthermore, a diverse assemblage of root-feeding organisms (e.g., nematodes, fungi) is well adapted for the soil environment (Coleman et al. 2004). Thus, because leaves and roots are equally valuable to plants, and experience similar risk of attack from consumers, ODT predicts that constitutive defenses, on average, should be similarly apportioned above and below ground.

Inducible plant defenses, however, provide a more intriguing scenario. In order to predict where plant defenses will be expressed in response to above- and belowground herbivory, ODT relies primarily on the risk of future attack (i.e., there is no a priori reason to assume that the relative value of leaves vs. roots will change greatly in the presence of herbivores). When an herbivore initiates feeding on a plant leaf, for example, defenses are often induced locally in the damaged leaf as well as systemically in undamaged leaves (Karban and Baldwin 1997). From the plant's perspective this allocation pattern may be adaptive, given that small amounts of leaf damage likely forecast additional leaf removal to herbivores in the near future. Consequently, defoliation places leaves under heightened risk of subsequent attack. The majority of foliar-feeding herbivores, however, do not simultaneously consume belowground tissues, and therefore, in most instances, defoliation should not greatly alter the risk of attack to plant roots (Brown and Gange 1990, Coleman et al. 2004). Accordingly, foliar herbivory should elicit increased defenses in leaves and little to no changes in the defensive phenotype of roots, and vice versa. In other words, ODT predicts elevated resistance in the subsystem under attack (e.g., roots in response to belowground herbivory) with no corresponding change in spatially separated plant parts that are not at risk of attack (e.g., leaves in response to belowground herbivory).

Despite the above predictions regarding defense induction between leaves and roots, we know from recent studies that cross-system induction is indeed possible (see reviews by Bezemer and van Dam 2005, van Dam and Bezemer 2006). For instance, beetle larvae feeding on roots of *Gossypium herbaceum* elicit a more than fourfold increase in the concentration of terpenoids in mature leaves (Bezemer et al. 2003, 2004). Superficially, this appears to violate one of the fundamental tenets of optimal defense. If plants are "smart investors"

(sensu van Dam et al. 1996), then why would costly defenses be expressed far from the site of attack where they seemingly serve no purpose?

Furthermore, many plants produce aboveground secondary chemicals in their roots (and vice versa), potentially as a means for spatially separating and thus protecting biosynthetic sites from their ultimate destination within the plant (Karban and Baldwin 1997, van der Putten et al. 2001, Bezemer et al. 2003, van Dam et al. 2003). For example, tobacco alkaloids are considered highly effective defenses against foliar herbivores, yet these compounds are synthesized entirely below ground (Dawson 1941, 1942, Baldwin 1991). Therefore, the magnitude and direction of induced responses to foliar and root herbivores may depend strongly on where plants produce secondary metabolites and the degree to which herbivores damage such tissues (i.e., root herbivores on tobacco may interfere with foliar alkaloid accumulation).

To consider the paradox of cross-system induction and other issues (i.e., the importance of variation in biosynthetic sites for secondary metabolite induction) related to constitutive and induced defenses in an above/belowground context, we provide two different empirical approaches. First, we experimentally tested the impact of foliar and root herbivory on whole-plant secondary metabolite expression using tobacco (*Nicotiana tabacum*) as a model system. More specifically, we sought to determine if and how root-derived phytochemicals (e.g., alkaloids) differ from foliar-derived chemicals (e.g., phenolics) in their response to herbivore damage. Second, we conducted a review of the available literature comparing levels of constitutive and induced defenses in leaves and roots. Although several outstanding reviews have recently been published on herbivore-induced responses linking above- and belowground plant parts (van der Putten et al. 2001, Blossey and Hunt-Joshi 2003, van Dam et al. 2003, Bezemer and van Dam 2005, van Dam and Bezemer 2006), this is the first quantitative assessment of the topic. Specifically, we were interested in answering the following questions: (1) Do constitutive defenses differ between leaves and roots, and (2) how does the magnitude of induction compare between herbivory in the tissue being attacked (i.e., leaves in response to foliar herbivores) vs. the opposing tissue type (i.e., roots in response to foliar herbivores)?

METHODS

Study system

Tobacco (*Nicotiana tabacum*) is a perennial plant native to South America that is widely cultivated in the mid-Atlantic United States. In this study, we used an agricultural variety of tobacco, and consequently the expression of secondary metabolites may be influenced by the fact that tobacco has long been selected for various agronomic traits (e.g., foliar nicotine concentration).

Plants in the genus *Nicotiana* have been among the most well studied with regard to constitutive and induced defenses to herbivory (Karban and Baldwin 1997, Steppuhn et al. 2004). Several major classes of tobacco secondary chemicals are inducible and have been implicated in reducing the preference and/or performance of herbivores, including: alkaloids (e.g., nicotine [Baldwin 1988a, Krischik et al. 1991, Jackson et al. 2002, Steppuhn et al. 2004]); phenolics (e.g., chlorogenic acid, rutin [Krischik et al. 1991, Stamp et al. 1994, Pegard et al. 2005]); and terpenoids (e.g., diterpene glycosides [Snook et al. 1997, Jassbi et al. 2006]). The alkaloid nicotine, in particular, is a highly inducible compound that has been one of the most heavily studied chemicals in relation to herbivore resistance (Schmeltz 1971, Baldwin 1991, Steppuhn et al. 2004). Notably, nicotine biosynthetic sites are located entirely below ground in growing root tips, and nicotine is subsequently translocated above ground where it protects leaves from insect defoliation (Dawson 1941, 1942, Baldwin 1991). As a result, nicotine provides a potentially important link between the above- and belowground systems.

The foliar-feeding herbivores of tobacco include a diverse assemblage of leaf-chewing insects such as *Manduca sexta* and *Trichoplusia ni*. The tobacco hornworm, *M. sexta*, is an oligophagous caterpillar that specializes on solanaceous plants (particularly *Nicotiana* sp.) and therefore has physiological adaptations for tolerating alkaloids (Wink and Theile 2002). Moreover, *M. sexta* is considered a "stealthy" herbivore because it attenuates the induced nicotine response that occurs following leaf damage (McCloud and Baldwin 1997, Kahl et al. 2000, Winz and Baldwin 2001). Consequently, these caterpillars often have dramatic effects on their host and can defoliate entire plants. The cabbage looper, *T. ni*, is a polyphagous caterpillar that feeds from >25 different plant families (Soo Hoo et al. 1984), and tobacco secondary chemicals are known to strongly reduce the performance of this insect (Krischik et al. 1991).

The root-knot nematode, *Meloidogyne incognita*, is considered the dominant belowground plant parasite in tobacco-growing regions (Barker and Lucas 1984). This species is among the most economically important group of plant-parasitic nematodes worldwide, largely because of its extraordinarily broad host range (Trudgill and Blok 2001). *M. incognita*, along with other phytoparasitic nematodes, feed on cellular contents, and *Meloidogyne* sp. also induces the development of galls at feeding sites in root tissue.

Herbivore-induced foliar and root chemistry in Nicotiana tabacum

We tested the individual and combined effects of above- and belowground herbivory on secondary metabolite expression in tobacco leaves and roots. Foliar herbivory was manipulated by adding caterpillars

to leaves (three treatments: undamaged control, *M. sexta* defoliation, and *T. ni* defoliation), while root herbivory was manipulated by inoculating roots with nematodes (two treatments: undamaged control, and *M. incognita* inoculation). These treatments were randomly assigned to individual plants (the unit of replication) in a fully crossed block design (3 foliar \times 2 root = 6 treatments; $n = 10$ –15 replications per treatment combination).

Tobacco plants (var. MD 609) were propagated by seed in a greenhouse and seedlings (nine weeks of growth from seed to seedling stage) were subsequently transplanted into 15-L (four-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; Sun Gro Horticulture, Bellevue, Washington, USA]). Plants were supplemented with nutrients weekly using a soluble fertilizer (20:10:20 NPK). The roots of all seedlings assigned to root herbivory were inoculated at the time of transplant with 10^5 *M. incognita* eggs obtained from a laboratory culture (Sardanelli and Kenworthy 1997). This egg density is well within the range documented for *M. incognita*-infested tobacco fields (Barker and Lucas 1984), and also corresponds with inoculum levels used in prior studies (Hanounik and Osbourne 1975, 1977, Barker and Weeks 1991, Wheeler et al. 1991, Vovlas et al. 2004). Foliar herbivory was inflicted on nematode-infested and nematode-free plants four weeks after inoculation. This time period allowed *M. incognita* eggs to hatch, juveniles to penetrate roots and induce gall formation, yet plants remained in the rosette stage and thus were still inducible (Ohnmeiss and Baldwin 2000, van Dam et al. 2001). Caterpillars were placed in mesh sleeve cages (one second-instar *M. sexta* or six third-instar *T. ni*) on the two youngest fully expanded leaves, and remained until both leaves were entirely defoliated (eight days).

Four days after caterpillars were removed, coinciding with peak induction of foliar nicotine (Baldwin 1991), plants were harvested for chemical analyses. Leaf (first newly expanded [i.e., youngest] leaf) and root tissue samples were collected from all plants, while *M. incognita*-induced galls were harvested separately from nongalled root tissue on nematode-inoculated plants. Samples were immediately frozen in liquid nitrogen and extracted and analyzed by high-performance liquid chromatography (HPLC) (Keinänen et al. 2001) on a reversed-phase C18 column (Gemini C18, 150 \times 4.6 mm [Phenomenex, Torrance, California, USA]). Concentrations of identified alkaloids and phenolics were quantified using calibration curves prepared from commercially available standards. Concentrations of diterpene glycosides (DTGs) and two unknown caffeic acid-derived phenolics were calculated from peak areas at 210 nm (for DTGs) and 320 nm (for phenolics).

The impact of foliar and root herbivory on above- and belowground plant chemistry was first tested using a

three-way MANOVA on leaf, root, and gall tissues with nematodes, *M. sexta*, and *T. ni* as main effects (Proc GLM; statistical analyses were performed using SAS, Version 9.1 [SAS 2001]). Interactive effects were examined between nematodes and each individual caterpillar species. (The full model was not tested because our design did not include a treatment with damage by both caterpillar species on the same plant.) Next we used univariate ANOVAs (in the same pattern as just described, three-way with all three herbivores as fixed effects) for each chemical measured in each tissue type; block (i.e., spatial groupings of plants on benches) was considered as a random effect in the model (Proc Mixed). Data were transformed (square-root and log transformations) as needed to meet assumptions of normality and homogeneity of variances.

Two additional unplanned comparisons not included in the above analyses were made to separate potential differences between the two caterpillar species (i.e., does the generalist have a different impact on plant chemistry than the specialist?) and galled vs. nongalled tissues (i.e., does the concentration of secondary chemicals in galls differ from adjacent nongalled root tissue?). With methods similar to the analyses just described, we used MANOVA followed by univariate ANOVAs to assess the impact of each factor. To avoid interactive effects with nematodes, we only used data from nematode-free plants to quantify the difference between generalist and specialist caterpillars. (Using the full data set does not qualitatively change the outcome.)

Because the chemicals measured in this study have biosynthetic sites that are spatially separated from one another, we predicted that this variation would explain the direction and magnitude of aboveground plant responses to root herbivores. Tobacco alkaloids are synthesized in the roots (Baldwin 1988b, 1989, Baldwin et al. 1994, Zhang and Baldwin 1997, Nugroho and Verpoorte 2002), whereas DTGs and phenolics are produced in the shoots (Keene and Wagner 1985, Shirley 1996, Harborne and Williams 2000, Tattini et al. 2000, Nugroho and Verpoorte 2002, Roda et al. 2003). Therefore, we used meta-analysis to quantify the impact of root vs. shoot production of defense chemistry on nematode-induced changes in aboveground defenses in tobacco (see the next section for details on meta-analysis procedures).

*Constitutive and inducible plant defenses
in leaves and roots: literature review*

The literature on constitutive (i.e., undamaged plants) and inducible defenses in leaves and roots was reviewed to: (1) compare constitutive plant defenses between foliar and root tissues; and (2) quantify the magnitude of induction in the plant subsystem under attack (e.g., roots in response to root herbivores, or leaves in response to foliar herbivores) vs. spatially separated parts (e.g., leaves in response to root herbivores, or roots in response to foliar herbivores). To find literature

relevant to these two issues we first surveyed the cited works from recently published reviews on leaf and root defenses (e.g., constitutive [Zangerl and Bazzaz 1992: Table 16.2]; induced [van Dam and Bezemer 2006: Table 1]). Additionally, we performed key word searches in the database *Web of Science* using various combinations of the following terms: herbivore, insect, nematode, pathogen, chemistry, defense, constitutive, induced, secondary plant metabolite, foliar, leaves, roots, aboveground and belowground.

The data set was restricted to studies that measured secondary plant metabolites in leaves and roots. Other plant resistance traits that are only expressed above or below ground (e.g., trichomes, extrafloral nectar) were not considered. Because the purpose of this analysis was to examine differential allocation patterns, we restricted our search to compounds that were shared between leaves and roots. In other words, if a chemical was detected in leaves, but not in roots (or vice versa) then it was excluded from the data set. This corrects for differences in the capacity to express compounds that are unrelated to allocation. For example, certain chemicals are only found in glandular trichomes on the leaf surface and thus cannot be expressed in roots (Roda et al. 2003).

For constitutive defense papers we gathered publications that quantified the concentration of secondary plant metabolites in both leaf and root tissues. We then used these data to calculate the proportion of secondary chemicals that are expressed above and below ground [i.e., proportion in leaves = (concentration in leaves)/(concentration in leaves + concentration in roots)]. In this case the proportion represents a quantitative estimate of the relative measure of defense when comparing leaves with roots; higher concentrations of secondary chemicals in leaves results in a lower proportion in the roots, and vice versa. As a result, we assume that one unit of defense compound is equally effective in roots and shoots.

Each unique chemical-plant species combination was considered an observation. In other words, if a study reported data on five different secondary chemicals in the leaves and roots from a single species of plant, these were considered separate observations in the data set. This unit of replication is justified given that above- and belowground allocation can vary drastically among different chemicals, even within the same plant and/or class of chemistry. We calculated average foliar and root concentrations from studies that varied plant genotype/variety or manipulated environmental factors (e.g., light, nutrients). The proportion of secondary chemicals in foliar vs. root tissues was tested against the null hypothesis of equal distribution (i.e., proportion in leaves and roots = 0.5) using a *t* test. This was assessed for the full data set (total secondary metabolites) and individually for classes of chemicals that were well replicated (more than five studies). Arcsine square-root

transformations were performed on proportional data prior to statistical analyses to satisfy normality.

Induced defenses within and across tissue types were investigated using meta-analysis. Therefore, our data set was restricted to studies that included means, variation (e.g., standard error, standard deviation), and sample size for the control and treatment groups. Ideally, we sought out studies that induced defenses in both leaves and roots and subsequently measured plant allelochemical responses in leaf and root tissues. However, this type of design has rarely been used. Most studies either manipulated induction in one tissue type (e.g., leaves) and measured the response in leaves and roots, or induced both tissue types (e.g., leaves and roots) and quantified the response in one of the tissues. We used defense induction in its broadest sense to include any organism that attacks plants, including herbivorous insects, pathogens, and nematodes. Additionally, we included studies that applied plant hormones (jasmonic or salicylic acid) known to elicit secondary metabolite responses similar to that observed following actual herbivory (e.g., Thaler et al. 1996). We did not include studies that used artificial (mechanical) damage because of the substantial differences in phytochemical responses that can occur when comparing artificial and actual herbivore damage (Karban and Baldwin 1997).

Three parameters defined what was considered a replicate in our data set: (1) the chemical, (2) the inducing agent, and (3) the tissue type. First, as with the constitutive defense data set, we considered each plant chemical measured as a replicate observation. Second, when studies elicited plant responses using multiple inducing agents (i.e., different species of herbivores) these were also considered separately. Last, if chemicals were measured from distinct tissue classes (e.g., old vs. young leaves, tap vs. fine roots) they remained as separate observations rather than combined. Many studies have now documented that these three factors are of paramount importance in explaining variation that underlies plant responses to attack from herbivores (Karban and Baldwin 1997). Therefore, we felt that averaging or otherwise removing this variation from the data set would ignore the considerable body of information that has accumulated on the dynamics of induced plant responses. In a similar manner, community ecologists typically split (rather than lump) species into separate observations when using meta-analysis to quantify organismal responses to environmental stimuli (Rosenberg et al. 2000).

Similar to most other ecological meta-analyses, we used Hedges' d to calculate effect sizes (Rosenberg et al. 2000). Noninduced plants were designated as the control, induced plants were considered the treatment, and plant secondary chemistry was used as the response variable. Thus, a positive effect size indicates that induction elevated plant allelochemistry, and a negative effect size demonstrates a decrease in chemistry. The meta-analytical program MetaWin 2.0 was used to score

effect sizes and conduct the overall analysis (Rosenberg et al. 2000). We used a mixed-effects categorical model to compare effect sizes in the plant tissue being induced vs. the opposing, spatially separated tissue (i.e., if the roots are induced, how do chemical responses compare in the roots vs. the leaves?). For each category a mean effect size (d_i) was calculated and reported with 95% bootstrap confidence intervals; effects are considered significant if confidence intervals do not bracket zero. Between-group heterogeneity (Q_B) was tested against a χ^2 distribution to determine if significant differences exist among groups of predictor variables.

RESULTS

Herbivore-induced foliar and root chemistry in Nicotiana tabacum

Foliar herbivory by both caterpillar species had no impact on the secondary chemistry of tobacco roots independent of nematode presence (Fig. 1) (*Manduca sexta*, MANOVA, $F_{6,68} = 1.22$, $P = 0.3061$; *Trichoplusia ni*, MANOVA, $F_{6,68} = 1.21$, $P = 0.3119$), but induced higher levels of secondary chemicals in leaves (Fig. 2) (*M. sexta*, MANOVA, $F_{6,71} = 6.73$, $P < 0.0001$; *T. ni*, MANOVA, $F_{6,71} = 32.11$, $P < 0.0001$). (See Appendix A for mean concentrations, errors, and sample size of individual chemicals associated with each treatment combination and tissue type; see Appendix B for table of univariate ANOVAs.) All classes of foliar chemicals were responsive to caterpillar leaf damage, with alkaloids (Fig. 2A, B) and DTGs (Fig. 2F) significantly elevated and inconsistent responses among phenolics (Fig. 2C–E). Moreover, generalist (*T. ni*) and specialist (*M. sexta*) herbivores had differential effects on foliar chemistry in the absence of nematodes (MANOVA: $F_{6,20} = 8.55$, $P < 0.0001$); in every case the magnitude of induction elicited by the generalist exceeded that of the specialist.

Nematode herbivory affected the secondary chemistry of both nongalled (i.e., systemic) and galled (i.e., local) root tissue independent of caterpillar defoliation (Fig. 1) (MANOVA [nongalled root], $F_{6,68} = 10.74$, $P < 0.0001$; MANOVA [galled root], $F_{6,66} = 220.67$, $P < 0.0001$). In nongalled tissue the effects of nematodes were variable, ranging from positive (Fig. 1E, F) to negative (Fig. 1A, B). However, in virtually all instances galls accumulated higher concentrations of secondary chemicals than adjacent roots that were not galled (MANOVA, $F_{6,16} = 22.97$, $P < 0.0001$).

Leaves were similarly affected by root herbivory (Fig. 2) (MANOVA, $F_{6,71} = 20.74$, $P < 0.0001$). Again, the outcome of this interaction was variable, with nematodes strongly interfering with aboveground alkaloid expression independent of caterpillar damage (Fig. 2A, B), but elevating foliar concentrations of phenolics (Fig. 2C–E) and DTGs (Fig. 2F). For instance, the addition of nematodes resulted in a >400% increase in foliar chlorogenic acid on caterpillar-free plants. Furthermore, root herbivory modified the response of plants

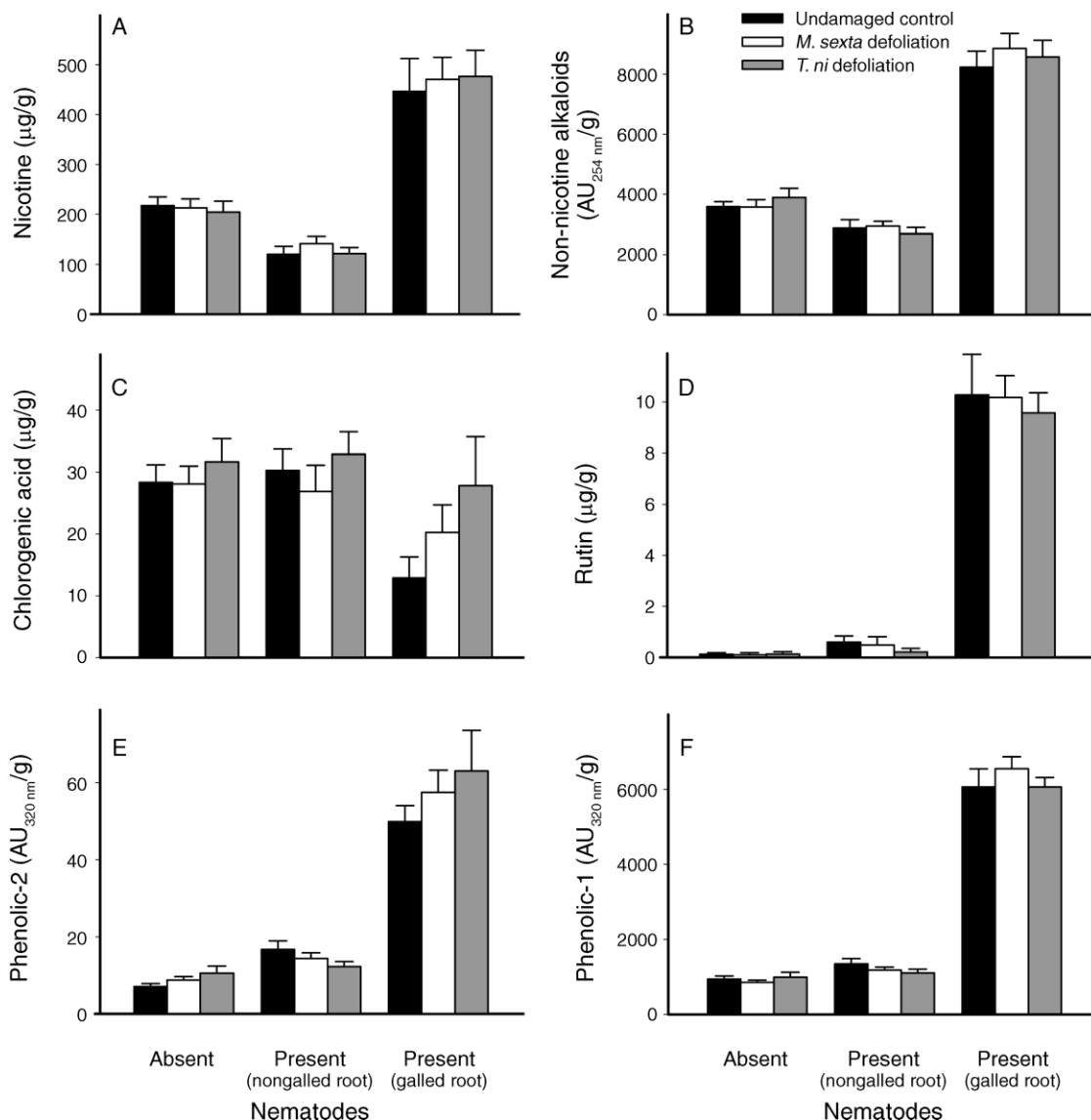


FIG. 1. The impact of aboveground herbivory (specialist caterpillar *Manduca sexta*, and generalist caterpillar *Trichoplusia ni*) and belowground herbivory (root-knot nematode *Meloidogyne incognita*) on secondary chemistry of tobacco roots (means + SE), including (A) nicotine, (B) non-nicotine alkaloids, (C) chlorogenic acid, (D) rutin, (E) phenolic-2, and (F) phenolic-1. All values are based on fresh mass. Concentrations of unidentified alkaloids and phenolic compounds are presented in arbitrary absorbance units (AU) at (B) 254 nm and (E and F) 320 nm, respectively. (See *Methods: Herbivore-induced foliar and root chemistry in Nicotiana tabacum*.)

to shoot herbivores. Most notably, nicotine was increased in response to defoliation by *T. ni* on nematode-free plants, but this inducible response was suppressed on plants that were experimentally inoculated with nematode root herbivores (Fig. 2A) (*Trichoplusia ni* \times *Meloidogyne incognita* interaction, $F_{1,63} = 7.35$, $P = 0.0087$).

The location of biosynthetic sites for each secondary chemical quantified in this study strongly determined whether root herbivory interfered with or elevated aboveground expression (Fig. 3). Nematodes induced a

decrease in foliar chemistry when considering compounds that are produced in the roots, but elicited higher foliar concentrations of chemicals synthesized in shoots (Appendix C; without aboveground herbivory: $Q_B = 24.22$, $df = 1$, $P < 0.0001$; with aboveground herbivory: $Q_B = 68.26$, $df = 1$, $P < 0.0001$).

Constitutive and inducible plant defenses in leaves and roots: literature review

For the database comparing constitutive above- and belowground defenses, we accumulated a total of 230

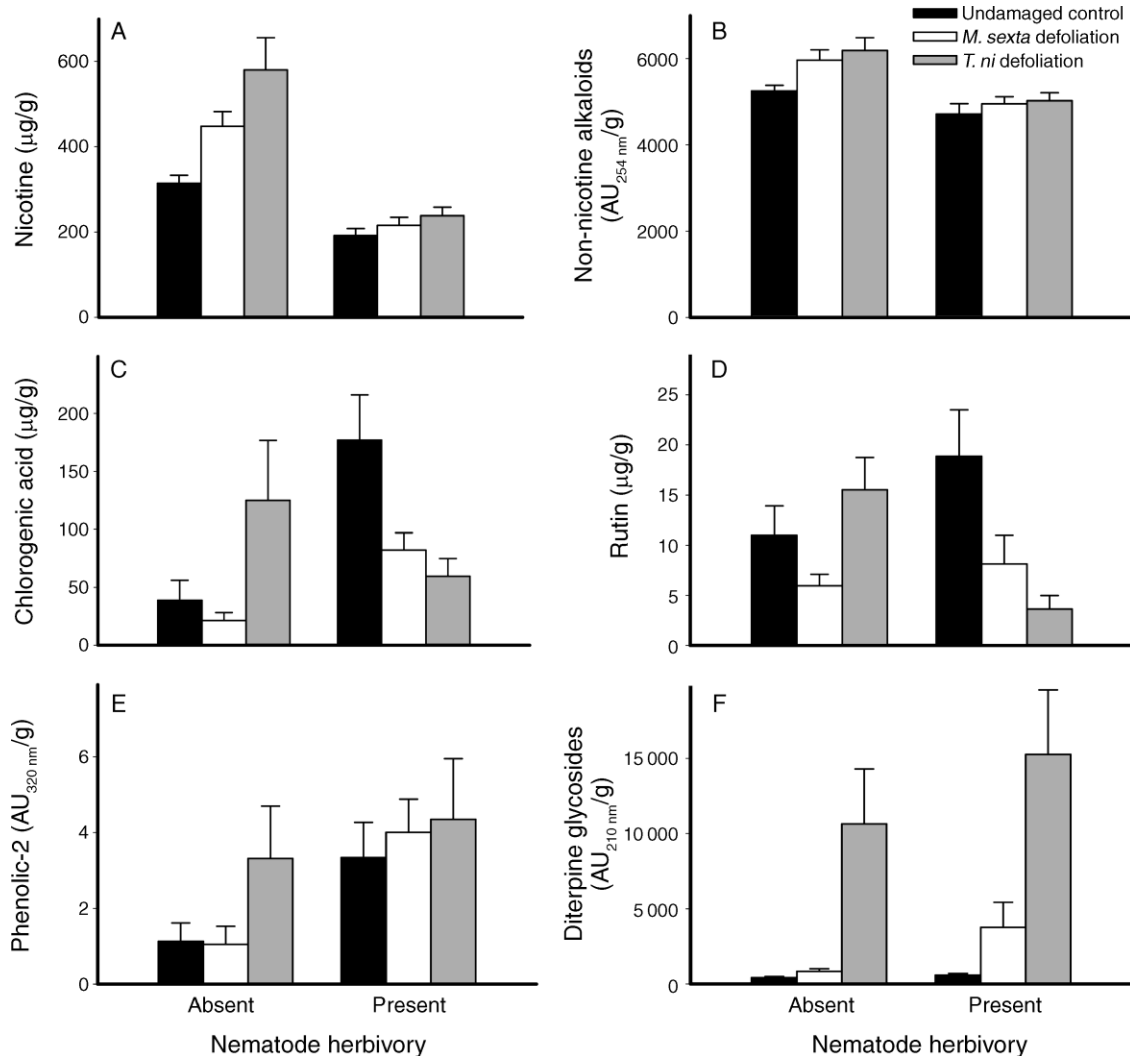


FIG. 2. The impact of aboveground herbivory (specialist caterpillar *Manduca sexta* and generalist caterpillar *Trichoplusia ni*) and belowground herbivory (root-knot nematode *Meloidogyne incognita*) on secondary chemistry of tobacco leaves (means \pm SE), including: (A) nicotine, (B) non-nicotine alkaloids, (C) chlorogenic acid, (D) rutin, (E) phenolic-2, and (F) diterpene glycosides. All values are based on fresh mass. Concentrations of unidentified alkaloids, phenolic, and diterpene glycoside compounds are presented in arbitrary absorbance units (AU) at (B) 254 nm, (E) 320 nm, and (F) 210 nm, respectively. (See *Methods: Herbivore-induced foliar and root chemistry in Nicotiana tabacum*.)

observations obtained from 74 different studies (Appendices D and F). Overall, there was no difference in the proportion of secondary metabolites allocated between leaves and roots (Fig. 4) ($t = 0.95$, $df = 229$, $P = 0.3452$). However, we detected differential above- and belowground expression for two of the four classes of chemicals that were investigated in greater detail. Alkaloids occurred at higher concentrations in leaves than roots ($t = 2.98$, $df = 48$, $P = 0.0045$), whereas glucosinolates demonstrated the opposite pattern with higher levels in roots compared with leaves ($t = 3.24$, $df = 68$, $P = 0.0019$). Phenolics ($t = 0.96$, $df = 33$, $P = 0.3430$) and terpenoids ($t = 0.12$, $df = 22$, $P = 0.9082$) were allocated similarly between above- and belowground plant parts.

In our meta-analysis of induced defenses from leaf and root herbivory we scored effect sizes from 22 studies that provided a total of 315 observations (Appendices E and F). Regardless of where plants were induced or where the responses were measured, induction elevated the expression of secondary plant chemistry (Fig. 5) (None of the 95% confidence intervals bracket zero.) There were differences, however, in the spatial extent of induction depending on where the response was elicited. Foliar induction resulted in a stronger response in leaves than roots (Fig. 5A) ($Q_B = 5.14$, $P = 0.0234$). Root induction elicited similar effects when comparing the magnitude of expression between leaves and roots (Fig. 5B) ($Q_B = 0.004$, $P = 0.9509$).

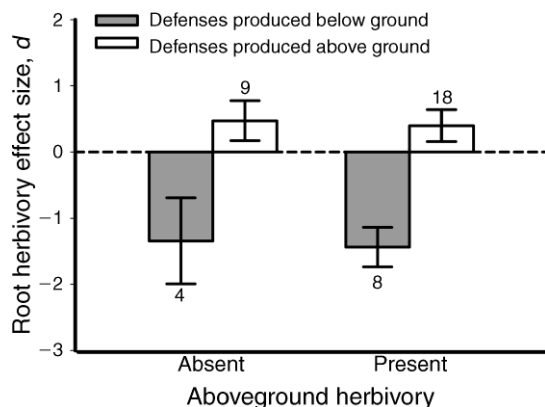


FIG. 3. Effect sizes, d (Rosenberg et al. 2000), comparing the impact of nematode root herbivory on tobacco foliar defenses that are synthesized either above or below ground. Numbers above or below error bars represent the number of observations per class. Error bars represent 95% bootstrap confidence intervals.

DISCUSSION

Above- and belowground induction in Nicotiana tabacum

Caterpillars induced defenses in leaves, but had no measurable impact on tobacco root chemistry (Figs. 1 and 2). Additionally, the generalist *Trichoplusia ni* tended to elicit stronger responses than the specialist *Manduca sexta* (nicotine, 42% increase from *M. sexta* vs. 85% increase from *T. ni*; DTGs, 95% increase from *M. sexta* vs. 2400% increase from *T. ni*). Hornworms (*M. sexta*) are known to suppress damage-induced nicotine accumulation in wild tobacco (McCloud and Baldwin 1997, Kahl et al. 2000, Winz and Baldwin 2001), although their impact on DTG expression has not previously been quantified. Nicotine is considered to be a suboptimal defense against *M. sexta* because the larvae can detoxify and excrete alkaloids (Wink and Theile 2002), perhaps explaining why tobacco plants induce a weak nicotine response to hornworms. Similarly, *M. sexta* is relatively tolerant of diterpenes in their diet (Jassbi et al. 2006), particularly when compared with generalist caterpillars (Snook et al. 1997). Thus, in a manner analogous to nicotine, tobacco may suppress diterpene accumulation in response to *M. sexta* damage because it is an ineffective resistance mechanism against specialists. Interestingly, DTGs are only mildly increased in leaf tissue after methyl jasmonate application to wild tobacco (1.5- to 1.9-fold increase; Keinänen et al. 2001) suggesting that *T. ni* may greatly amplify the jasmonate-mediated induction. Although specialist herbivores are known to be more tolerant of secondary plant chemistry than generalists, our results add to a growing body of evidence that generalists and specialists elicit very different responses in their host plant (Hartley and Lawton 1987, Bowers and Stamp 1993, Sirvent et al. 2003, Voelckel and Baldwin 2004, Mewis et al. 2006; but see Agrawal 2000, Reymond et al. 2004).

Nematode-induced galls strongly accumulated all classes of chemicals (Fig. 1). The concentration of rutin, for example, was >8000% as high in galled compared with nongalled roots. These results are in accordance with several studies showing that herbivore-induced galls possess extraordinarily high quantities of secondary chemicals (Abrahamson et al. 1991, Hartley 1998, Allison and Schultz 2005, Motta et al. 2005). Nematode herbivory, however, did not always result in systemic elevation of root chemistry. In fact, alkaloid concentrations were significantly lower in nongalled roots of nematode-inoculated plants (Fig. 1A, B), indicating that allocation of alkaloids to galls may come at the expense of nongalled root tissue.

In marked contrast with caterpillars, nematode root herbivory affected secondary chemistry not only at their feeding sites (i.e., galls) but also above ground in tobacco leaves (Fig. 2). We differentiate the impact of root herbivory on foliar chemistry as (1) *direct effects*, nematodes alter aboveground chemistry, independent of leaf damage by caterpillars; and (2) *indirect effects*, nematodes influence the magnitude of induction by caterpillars (i.e., statistical interaction between root and shoot herbivory). Both direct and indirect effects of root herbivory were evident, depending on the chemicals measured. Nematodes had negative direct and indirect effects on foliar alkaloids (Fig. 2A, B; Hanounik and Osborne 1975, 1977, Barker and Weeks 1991). Aboveground nicotine concentrations, for example, were ~40% lower on plants with root herbivory. Similarly, nicotine was highly inducible by caterpillar defoliation

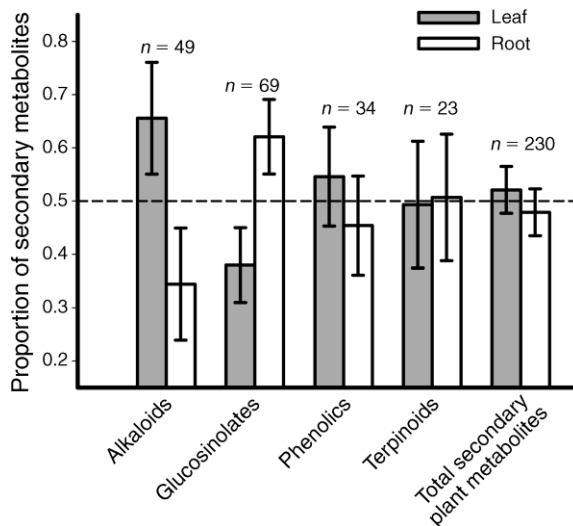


FIG. 4. Comparison of constitutive plant defenses expressed in foliar vs. root tissues. Data were obtained from a literature review of studies quantifying the concentrations of secondary plant chemicals above and below ground. The variable n represents the number of unique chemical-plant species combinations; sample sizes are identical for leaves and roots. The dashed line indicates the level at which secondary plant metabolites are allocated equally between leaves and roots. Error bars (based on t test) represent 95% confidence intervals.

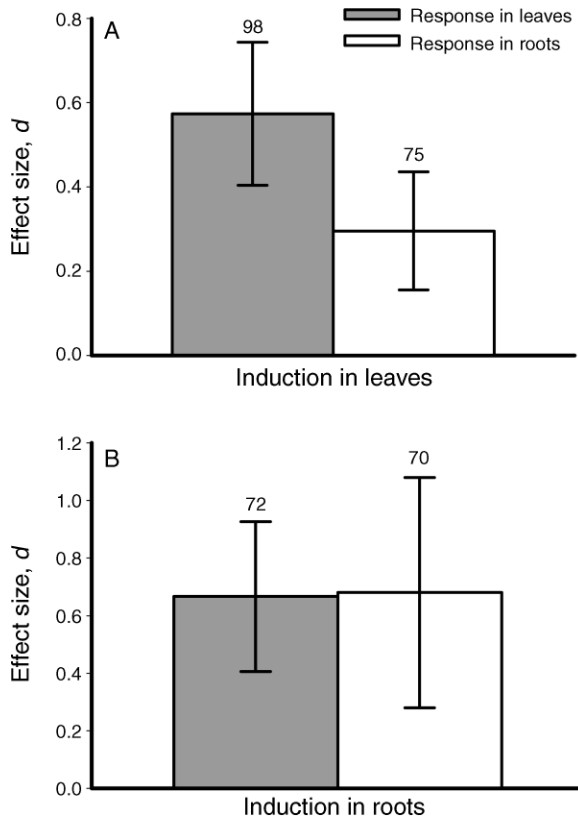


FIG. 5. Induced changes in above- and belowground plant defenses for responses (effect sizes, d [Rosenberg et al. 2000]) that are elicited in (A) leaves and (B) roots. A meta-analysis was conducted using published studies that quantified induced plant defenses in an above/belowground context. Numbers above error bars represent the number of observations per class. Error bars represent 95% bootstrap confidence intervals.

on nematode-free plants, yet became almost entirely noninducible when nematodes were present (*M. sexta*, 42% increase without root herbivory vs. 12% increase with root herbivory; *T. ni*, 85% increase without root herbivory vs. 24% increase with root herbivory).

Unlike alkaloids, root herbivory induced positive direct and indirect effects on tobacco phenolics and terpenoids. Nematodes tended to have positive direct effects on phenolics (i.e., higher foliar concentrations on plants with nematodes alone), but inconsistent and unpredictable indirect effects (Fig. 2C–E). This result is not entirely surprising given the diverse role that many phenolic compounds play in the biology of plants (Shirley 1996, Harborne and Williams 2000, Roda et al. 2003). In contrast, nematodes had weak direct effects on foliar DTGs, but positive indirect effects (Fig. 2F). For instance, on nematode-free plants, *M. sexta* damage elicited a 95% increase in DTGs, but on plants with nematodes *M. sexta* herbivory resulted in a >500% increase in DTGs. Thus, nematodes alone have weak effects on foliar DTGs, but prime plants for much stronger responses to aboveground caterpillar feeding.

This priming phenomenon is known to occur in plant–pathogen interactions (Conrath et al. 2002, 2006), and recent work suggests that herbivore-induced plant volatiles can prime neighboring plants (Engelberth et al. 2004, Heil and Kost 2006, Kessler et al. 2006, Ton et al. 2006). Additionally, van Dam et al. (2005) demonstrated that the phytoparasitic nematode *Pratylenchus penetrans* primes plants for more rapid phenolic responses to caterpillar herbivory.

Although several studies provide evidence that root herbivores elevate defenses in foliar tissues (see reviews by Bezemer and van Dam 2005, van Dam and Bezemer 2006), our data suggest highly divergent effects of belowground herbivory on aboveground secondary metabolite expression in the tobacco system. Nematodes interfered with certain classes of compounds (e.g., alkaloids), but seemed to induce higher levels of defense when assessing other groups of chemicals (e.g., phenolics and DTGs). What factor(s) are responsible for generating this variation? We hypothesize that the location of biosynthetic sites for these diverse phytochemicals is an extremely important consideration. Tobacco alkaloids are known to be synthesized entirely below ground in growing root tips, whereas the phenolics and terpenoids that we measured are produced in leaf tissue. As a result, root herbivory interfered with foliar chemicals that are synthesized in roots and elevated foliar chemicals that are produced above ground (Fig. 3).

The importance of this phenomenon was predicted by Karban and Baldwin (1997) who proposed that since “nicotine biosynthesis is located in the roots... a plant’s ability to respond would rapidly diminish with increasing amounts of root herbivory.” The presumed advantage of producing alkaloids in roots is to protect the machinery for launching a sustainable defense reaction in the face of intense defoliation (Baldwin and Schmelz 1994, Karban and Baldwin 1997). However, this also suggests that the alkaloid-based defense system of tobacco is extremely susceptible to root herbivory. Interestingly, nematodes increased foliar nicotine concentrations on nematode-resistant tobacco varieties (Hanounik and Osborne 1977). Similarly, Preisser et al. (2007) inoculated tobacco roots with low nematode densities and found no impact on leaf nicotine. Thus the magnitude of damage incurred by roots may ultimately dictate the degree of interference from belowground consumers.

Several recent studies have emphasized the importance of roots as biosynthetic sites for aboveground chemicals (e.g., terpenoids in *Gossypium* sp., furanocoumarins in the Apiaceae) with known defensive properties (Karbon and Baldwin 1997, van der Putten et al. 2001, Bezemer et al. 2003, van Dam et al. 2003). As a result, we do not consider our findings to describe an idiosyncratic response that is unique to plants in the genus *Nicotiana*. Rather, our results likely represent a phenomenon with broad implications for plant defense

and linkages between above- and belowground communities.

Constitutive plant defenses in foliar vs. root tissues

Constitutive defenses, overall, were equally allocated between leaves and roots (Fig. 4). This outcome is precisely what ODT would predict given that above- and belowground parts are considered to be similar both in value and risk of attack from consumers (Zangerl and Bazzaz 1992). However, it should be noted that despite the remarkable similarity of overall secondary metabolite allocation between leaves and roots (52% and 48%, respectively) we found large amounts of variation in our data set (see Appendix D). The class of chemical measured partially explained this variation, with alkaloids exhibiting higher foliar expression and glucosinolates demonstrating the opposite pattern with greater root expression.

We presume that these differences are not necessarily associated with biochemical constraints on where alkaloids/glucosinolates can be stored within the plant. Rather, we suspect that these differences may be driven by selective pressures imposed on certain plant groups that predominantly exploit alkaloids or glucosinolates as defenses. For instance, glucosinolates are largely restricted to plants in the Brassicaceae, and not surprisingly, the vast majority of studies in our data set that quantified leaf and root glucosinolates comprised plants in this family. Consequently, the observation that roots contained higher concentrations of glucosinolates than leaves may simply reflect the fact that brassicaceous plants evolved in an environment that promoted this allocation pattern. This would be expected if root herbivores were relatively more common or damaging than foliar herbivores (greater risk), or if soil resources (e.g., water and nutrients) were more limiting than light availability (greater value) (Zangerl and Bazzaz 1992).

Herbivore-induced plant defenses in foliar vs. root tissues

If induced defenses to above- and belowground herbivores are allocated primarily on the basis of risk from future attack, then plants should induce stronger responses in the tissue type that is damaged compared with undamaged tissues (i.e., leaves should be more responsive than roots to foliar herbivores). In support of this prediction, we found that the magnitude of induction to aboveground herbivory was more than twice as great in leaves than roots (Fig. 5A). However, plant responses to belowground herbivory did not follow this pattern. Responses elicited in roots were equally expressed in foliar and root tissues (Fig. 5B). Thus, induced changes to leaf- and root-feeders are asymmetrical; root herbivory has systemic effects that propagate throughout the plant, and leaf herbivory results in more localized changes in secondary plant chemistry. The ecological ramifications of these findings are that root herbivores may have stronger plant-mediated effects on foliar herbivores than the reverse

situation. This asymmetry may be amplified further by the timing of attack to above- and belowground tissues, with roots exposed to consumers well in advance of leaf expansion for many plant species (Bezemer and van Dam 2005). Yet the limited data on reciprocal plant-mediated interactions linking foliar and root herbivores do not entirely support the supposition that root-feeders are competitively dominant over foliar-feeders (Denno et al. 1995, van Dam et al. 2003, Denno and Kaplan 2007).

It remains unclear why such a discrepancy occurs in the systemic nature of plant responses to root and shoot herbivores. Clearly roots have the capacity to respond to herbivory as indicated by their strong induction to root herbivores (Fig. 5B). One possibility might involve inherent differences in how plants translocate materials between above- and belowground tissues. Although bidirectional movement of solutes through the vascular system has been documented, materials, in general, move upward from the roots through the xylem and downward from the shoots in the phloem (Hopkins and Hüner 2004). For example, in the inducible nicotine system of tobacco, defoliation is thought to elicit defense signals that move from damaged leaf to roots via the phloem, triggering an increase in nicotine production which is translocated from biosynthetic sites in the roots to aboveground foliage via the xylem (Baldwin 1989, Baldwin et al. 1994, Zhang and Baldwin 1997). Therefore, differential translocation of defense signals/compounds in vascular tissues may place physiological constraints on whole-plant induction (Davis et al. 1991, Shulaev et al. 1995, Arnold and Schultz 2002). In a similar manner, vascular architecture is known to strongly influence the spatial distribution of induced secondary compounds in plant leaves (Oriens et al. 2000, Schittko and Baldwin 2003, Viswanathan and Thaler 2004).

Despite the apparent lack of symmetry in the magnitude of cross-system induction, herbivory elicited higher levels of secondary chemicals independent of which tissue was induced or where the response was measured (Fig. 5; none of the error bars [95% CI] overlap zero). The mere fact that such putative defenses were elevated at all in undamaged tissue is puzzling and seems to be suboptimal from the plant's perspective (Wäckers and Bezemer 2003). Yet it is clear that this phenomenon occurs in an above/belowground context, as well as between other combinations of spatially distinct plant tissues (e.g., vegetative and reproductive parts [Euler and Baldwin 1996, Adler et al. 2006, McCall and Karban 2006]). Below we outline a series of hypotheses that attempt to explain the paradox of cross-system induction (also see Wäckers and Bezemer 2003).

1. *Inducible secondary chemicals serve alternative functions.*—The function of secondary plant chemicals has long been debated (Fraenkel 1959, Ehrlich and Raven 1964, Levin 1971, Whittaker and Feeny 1971,

Seigler and Price 1976, Jones 1979), but most ecologists now consider them to serve a defensive role involving interactions with herbivores. It has also become apparent that secondary chemicals (e.g., phenolics) are multifunctional in nature and thus have likely evolved under diffuse selective pressures imposed by numerous biotic and abiotic challenges (Berenbaum 1995, Matsuki 1996, Seigler 1996). Although ODT predicts heterogeneity in plant defenses entirely on the basis of herbivore deterrence, this may underestimate the diversity of threats encountered by plants (Coleman and Jones 1991). Thus it is difficult to reject the possibility that systemically induced changes in plant chemistry are not always allocated for the sole purpose of defense against herbivory.

2. *Physiological constraints on defense expression.*—

An implicit assumption of ODT is that plants are unconstrained in how they distribute defenses. In other words, any allocation pattern is possible presuming that it minimizes costs and maximizes the efficacy of defenses. Yet detailed studies of intraplant variation in induced aboveground defenses clearly demonstrate that expression of secondary chemicals is often mediated by vascular connections linking damaged with undamaged leaves (Davis et al. 1991, Shulaev et al. 1995, Orians et al. 2000, Arnold and Schultz 2002, Schittko and Baldwin 2003, Viswanathan and Thaler 2004). As a result, allocation must be viewed within the constraints imposed by plant physiology, which is not always consistent with the ideal strategy for herbivore deterrence. We know little about potential physiological constraints that shape patterns of induction between leaves and roots, but such constraints may partially explain why plants protect leaves when roots are attacked. Despite this possibility, empirical evidence increasingly provides support for the ODT principle that defenses are costly to plants (Zangerl and Bazzaz 1992, Purrington 2000, Heil and Baldwin 2002, Strauss et al. 2002). As a result, we presume that selective pressures imposed from these costs would reduce inefficient and wasteful use of resources, making the documented cases of cross-system induction all the more puzzling.

3. *Synergistic effects of multiple enemies on plant fitness.*—Many plants have evolved the ability to tolerate herbivore damage (van der Meijden et al. 1988, Strauss and Agrawal 1999). However, herbivory by one consumer may alter the level of tolerance expressed to a second herbivore, resulting in nonadditive effects of multiple enemies on plant fitness (Strauss and Irwin 2004). For instance, several studies have found that the combined impact of two herbivores on plant performance is greater than the sum of each herbivore occurring individually (i.e., synergism [Strauss 1991, Pilson 1996, Wise and Sacchi 1996, Hufbauer and Root 2002, Eubanks et al. 2005]). Under these circumstances it might be optimal to protect all parts following herbivory regardless of where the initial damage occurs,

thus avoiding the more severe fitness penalties incurred by additional damage.

4. *Anticipation of future attack.*—Foliar defenses induced from root herbivory may be an adaptive response by the plant in anticipation of future aboveground attack. The logic underlying this hypothesis is based on the fact that several root herbivores implicated in cross-system induction are holometabolous insects; they undergo ontogenetic shifts whereby larvae consume roots and adults subsequently emerge above ground to attack leaves (e.g., chrysomelid beetles in the subfamily Alticinae). Therefore, plants may preempt foliar herbivory by inducing aboveground defenses early and circumvent the time lag associated with inducing defenses after leaves are already damaged. This possibility is problematic in that many foliar and root herbivores are restricted to feeding on either above- or belowground tissues, but not both (Brown and Gange 1990, Coleman et al. 2004). Furthermore, these types of consumers can nonetheless elicit cross-system induction. For example, we found higher concentrations of foliar phenolics and terpenoids in response to nematodes that are obligate root-feeders (Fig. 3).

5. *Secondary invaders that act systemically.*—Plants are subject to attack by a diverse array of microorganisms (e.g., bacteria, fungi), which often cannot infiltrate the peripheral defenses of healthy, undamaged plants (Agrios 2005). Herbivores, however, may act as “Trojan horses” by opening wounds and thus facilitating entry by secondary invaders (Karban and Baldwin 1997). Phytoparasitic nematodes, for example, penetrate roots to access feeding sites, and in the process, create openings that are exploited by pathogens (Webster 1985, Sikora and Carter 1987, Abawi and Chen 1998). In many instances these secondary effects of nematodes via pathogens have a far greater impact on plant performance than the direct effects of nematode herbivory. If such pathogenic microorganisms are disseminated throughout the plant, then it would be misleading to expect that defenses are only induced at the site of entry. For instance, the fungal pathogen *Sclerospora graminicola* infects root tissue, but most of the pathogen is subsequently localized in the shoot region (Sharada et al. 1995). Therefore, when assessing optimal defenses of plants to herbivory, one must consider the possibility that the response measured may not necessarily be elicited by the herbivore.

Conclusions

The importance of root herbivory is increasingly recognized in ecological studies, yet root herbivores remain minor contributors to our overall understanding of plant–herbivore interactions (van der Putten et al. 2001, van Dam et al. 2003, Bezemer and van Dam 2005, Stevens and Jones 2006, Preisser et al. 2007). Given that plant defenses often span above- and belowground systems, they represent a potentially important link that connects root herbivores with the foliar-based commu-

nity (and vice versa). This is particularly true in light of induced defenses that are systemically expressed. Moreover, identifying potential similarities and differences in how plants interact with consumers of leaves and roots is instructive in assessing how ecological theory that has largely been developed above ground (i.e., ODT) corresponds with interactions occurring below ground. Exploring these issues is timely given the recent and continuing interest among ecologists of interconnections between spatially separated communities (Wardle 2002, Wardle et al. 2004).

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APPENDIX A

A table showing means and error per treatment and tissue type in *Nicotiana tabacum* damaged by foliar and root herbivores (*Ecological Archives* E089-021-A1).

APPENDIX B

MANOVA table for the effects of root herbivory (nematodes), shoot herbivory (generalist and specialist caterpillars), and their interaction on above- and belowground secondary chemistry in *Nicotiana tabacum* (*Ecological Archives* E089-021-A2).

APPENDIX C

A table showing effect sizes and variances used in meta-analysis of root herbivory on aboveground secondary chemistry of *Nicotiana tabacum* by biosynthetic site (*Ecological Archives* E089-021-A3).

APPENDIX D

A table showing plants and secondary chemicals used for comparison of constitutive defenses between leaves and roots (*Ecological Archives* E089-021-A4).

APPENDIX E

A table showing effect sizes and variances used in meta-analysis of induced defenses elicited and expressed in leaves and roots (*Ecological Archives* E089-021-A5).

APPENDIX F

Publications used for analyses of constitutive and induced defenses (*Ecological Archives* E089-021-A6).