

Direct and indirect root defences of milkweed (*Asclepias syriaca*): trophic cascades, trade-offs and novel methods for studying subterranean herbivory

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Summary

1. Entomopathogenic nematodes can function as indirect defence for plants that are attacked by root herbivores. By releasing volatile organic compounds (VOCs), plants signal the presence of host insects and thereby attract nematodes.
2. Nonetheless, how roots deploy indirect defences, how indirect defences relate to direct defences, and the ecological consequences of root defence allocation for herbivores and plant biomass are essentially unknown.
3. We investigate a natural below-ground tritrophic system, involving common milkweed, a specialist root-boring beetle and entomopathogenic nematodes, and asked whether there is a negative genetic correlation between direct defences (root cardenolides) and indirect defences (emission of volatiles in the roots and nematode attraction), and between constitutive and inducible defences.
4. Volatiles of roots were analysed using two distinct sampling methods. First, we collected emissions from living *Asclepias syriaca* roots by dynamic headspace sampling. This method showed that attacked *A. syriaca* plants emit five times higher levels of volatiles than control plants. Secondly, we used a solid phase micro-extraction (SPME) method to sample the full pool of volatiles in roots for genetic correlations of volatile biosynthesis.
5. Field experiments showed that entomopathogenic nematodes prevent the loss of biomass to root herbivory. Additionally, suppression of root herbivores was mediated directly by cardenolides and indirectly by the attraction of nematodes. Genetic families of plants with high cardenolides benefited less from nematodes compared to low-cardenolide families, suggesting that direct and indirect defences may be redundant. Although constitutive and induced root defences traded off within each strategy (for both direct and indirect defence, cardenolides and VOCs, respectively), we found no trade-off between the two strategies.
6. *Synthesis.* Constitutive expression and inducibility of defences may trade off because of resource limitation or because they are redundant. Direct and indirect defences do not trade off, likely because they may not share a limiting resource and because independently they may promote defence across the patchiness of herbivore attack and nematode presence in the field. Indeed, some redundancy in strategies may be necessary to increase effective defence, but for each strategy, an economy of deployment reduces overall costs.

Key-words: *Asclepias syriaca*, cardenolides, chemical ecology, common milkweed, *Heterorhabditis bacteriophora*, induced defence, plant defence theory, plant–herbivore interactions, root defences, *Tetraopes tetraophthalmus*.

Introduction

Plant resistance and trophic cascades provide alternative, but not mutually exclusive, explanations for how communities are structured and why most plants are not com-

pletely consumed by herbivores (Hairston, Smith & Slobodkin 1960; Murdoch 1966; Polis 1999). Indeed, the interplay between plant resistance and attraction of natural enemies of herbivores has been of long-standing interest (Price *et al.* 1980; Boethel & Eikenbary 1986; Thaler 1999; Heil 2008; Mooney *et al.* 2010). Plant defence against herbivores is characterized by two major strategies, each of

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which can be constitutively expressed or induced following damage (Karban & Baldwin 1997): the production of anti-herbivore traits (direct defence) and the attraction of natural enemies of herbivores (indirect defence); but only the latter may initiate a trophic cascade (Kessler & Baldwin 2001).

Theory has long predicted that the simultaneous expression of multiple defence traits is costly for the plant and thus will be minimized if redundancy in defence is not beneficial (Zangerl & Bazzaz 1992; Mauricio 1998; Koricheva 2002; Strauss *et al.* 2002; Rasmann & Agrawal 2009). Constraints on resource allocation are assumed to produce negative genetic correlations (trade-offs) between individual traits and between defence deployment strategies (e.g. constitutive vs. induced expression) (Brody & Karban 1992; Zangerl & Bazzaz 1992; Koricheva, Nykanen & Gianoli 2004). Trade-offs typically occur when adaptive traits compete for resources and thus cannot be simultaneously expressed (Agrawal, Conner & Rasmann 2010). Alternatively, traits may show negative correlations due to adaptation; that is, if traits are ecologically redundant and costly, it is predicted that they will evolve what appears to be a trade-off. For example, if two defensive traits or defence deployment strategies are redundant (i.e. no added fitness benefit of the second trait) and costly, they should show a negative correlation (Agrawal, Conner & Rasmann 2010). Nonetheless, trade-offs in defence are rarely found (Koricheva, Nykanen & Gianoli 2004) and simultaneous deployment of alternative strategies may provide redundancy or 'reinforcement' for enhanced protection (Agrawal & Fishbein 2006; Rasmann & Agrawal 2009).

Although the vast majority of studies testing for evidence of enemy-propagated trophic cascades has focused on predators in above-ground food webs (Rosenheim *et al.* 1995; Schmitz, Hamback & Beckerman 2000; Denno, Gruner & Kaplan 2008), root-feeding insects are remarkably common and are also confronted with toxic plant secondary metabolites as well as soil-dwelling predators (Strong *et al.* 1996, 1999; Preisser 2003; Ram *et al.* 2008; Rasmann & Agrawal 2008). Here we focus on below-ground defence and food web dynamics associated with common milkweed (*Asclepias syriaca*), a root-boring beetle *Tetraopes tetraophthalmus* (Agrawal 2004; Rasmann & Agrawal 2008; Rasmann *et al.* 2009), and a naturally occurring entomopathogenic nematode *Heterorhabditis bacteriophora* (Schroeder *et al.* 1994; Ferguson, Schroeder & Shields 1995; Shields *et al.* 1999). We conducted four complementary lab and field experiments in order to test (1) the hypothesis that root herbivory induces volatiles attractive to predatory nematodes, initiating an underground trophic cascade, and (2) classic predictions about trade-offs between direct and indirect defence, and their deployment as constitutive vs. induced strategies. To test these predictions, we conducted a genetic screen to establish high and low root defence (cardenolide) genotypes of milkweed and developed novel methods to quantify root volatiles.

Materials and methods

NATURAL HISTORY AND ORGANISMS

Asclepias syriaca (Apocynaceae) is a native perennial plant commonly found throughout the central and eastern United States. It reproduces asexually via the production of new ramets from an underground stem and sexually via hermaphroditic flowers. Seeds from a single fruit of *A. syriaca* are full siblings due to the pollination system of milkweeds, i.e. a single pollen sac or pollinium sires all of the seeds from a flower (Gold & Shore 1995). The well-characterized arsenal of *A. syriaca* defences limits the herbivore community to about 10 species of specialist insects (Malcolm 1991; Agrawal & Malcolm 2002). In addition to exuding latex, *A. syriaca* produces cardenolides, bitter-tasting steroids that disrupt the sodium–potassium flux in animal cells and thus have a toxic effect (Malcolm 1991). Cardenolides occur in all milkweed tissues, including the roots (Rasmann & Agrawal 2008; Rasmann *et al.* 2009).

Cerambycid beetles in the genus *Tetraopes* share an evolutionary history with *Asclepias* spp. and the two groups appear to exhibit parallel phylogenies (Farrell & Mitter 1998). At our study site in Tompkins County, NY, USA, *T. tetraophthalmus* adults emerge from pupation in late June to early July and feed exclusively on *A. syriaca* leaves and flowers. Despite the feeding preferences of *T. tetraophthalmus* adults, females oviposit in the drying flowering stems of neighbouring thick-bodied grasses and occasionally in the stems of thin-bodied forbs (Matter 2001). After chewing a small hole in the stems, females will deposit clutches of 10–15 eggs. Ten to 12 days later, larvae hatch, drop to the soil and search underground for *A. syriaca* roots. Larvae consume roots and underground stems of the common milkweed and then overwinter as large prepupae in the surrounding soil.

For all of our experiments, *T. tetraophthalmus* adults were collected on naturally occurring milkweed patches around, Tompkins County, NY, USA, and kept in large ventilated containers (30 × 20 × 15 cm) in the laboratory. Male and female *T. tetraophthalmus* adults were provided with fresh milkweed leaves and oviposition sites (dried grass stems) daily. The oviposition substrate was removed from the rearing boxes every 3 days and incubated in the dark at 27 °C for 7–10 days. Newly hatched larvae were kept in large Petri dishes (10 cm diameter) on moist filter paper for a maximum of 24 h before the start of the experiments.

Entomopathogenic nematodes *Heterorhabditis bacteriophora* 'Oswego' strain (Schroeder *et al.* 1994; Ferguson, Schroeder & Shields 1995; Shields *et al.* 1999) were collected from field sites in Oswego County, NY, USA using, as a bait, last-instar *Galleria mellonella* (wax moth) and a culture medium (Woodring & Kaya 1988). Freshly collected third-instar infective juveniles were kept in tissue culture flasks (Merck) at room temperature in the dark. Nematodes used in the experiments were 7–10 days old.

TETRAOPES SURVIVAL EXPERIMENT

The first experiment was designed to assess the impact of nematodes on *T. tetraophthalmus* larval survival. *Asclepias syriaca* seeds were nicked, germinated in the dark on moist filter paper and grown for 4 weeks in a growth chamber at 14:10 D/N light, and 25:16 °C D/N temperature in plastic pots (10 cm diameter) in commercial potting soil (Metro-Mix, Sun Gro Horticulture, Canada CM Ltd). After 4 weeks, plants ($n = 22$) were transplanted to one side of a rectangular plastic tray (8 cm deep, 29 × 37 cm), 5 cm from the edge. Plants were watered daily to provide constant humidity to the soil (c. 10% relative

humidity). One week after transplanting, 10 *T. tetraophthalmus* larvae were placed near the roots of each plant, 1 cm deep in the soil. Three days later, each tray was randomly assigned to one of the following two treatments: (1) addition of a drop of water containing c. 10 000 *H. bacteriophora* nematodes to the centre of each tray, or (2) addition of one water drop without nematodes (control). Three days after nematode inoculation, the roots of all plants were carefully removed from the soil and checked for surviving beetle larvae. The soil was then placed in modified Berlese funnels (25 cm wide, 30 cm long and 8 cm deep, covered on the bottom with a 4 × 4 mm mesh size metal screen), and checked daily for additional surviving *T. tetraophthalmus* larvae until the soil was completely dry.

NEMATODE ATTRACTION EXPERIMENT

To investigate whether nematodes are more attracted to the roots of attacked plants than those of unattacked plants (i.e. indirect defence below ground), we measured the nematode infection rate of sentinel *G. mellonella* larvae near *T. tetraophthalmus*-damaged or undamaged *A. syriaca* roots. Plants were grown as described above for four weeks and, one week prior to the experiment, two plants were transplanted into opposite ends of a rectangular plastic tray (6 cm deep, 26 × 54 cm), each 5 cm from the edge. Three days before the experiment, five *T. tetraophthalmus* larvae were placed on one randomly chosen plant in each tray, 1 cm deep in the soil, and allowed to feed on the roots for three days. At the end of this induction period, five *G. mellonella* larvae were placed in a semi-circular shape around each plant and a drop of water including c. 10 000 nematodes was added to the middle of the tray. After 48 h, *G. mellonella* larvae were removed from the soil and placed individually in Petri dishes (5 cm diameter). Nematode infection was determined by observing colour changes (larva change from brown to red upon infection).

INTERACTION BETWEEN DEFENCE STRATEGIES EXPERIMENT

To assess the relative effects of direct and indirect defences of *A. syriaca* roots in the field, we contrasted levels of root cardenolides (direct defence) while manipulating the presence of nematodes (indirect defence) and measured *T. tetraophthalmus* larval survival. To establish high and low root cardenolide lines of plants, we screened 20 full-sibling families ($n = 8$ plants per family), grown as described above. After four weeks of growth, roots were washed carefully and dried at 40 °C. We measured constitutive total cardenolide concentrations with a spectrophotometric assay (Rasmann *et al.* 2009) and selected the three lowest and three highest cardenolide-expressing genetic families. On average, the three high-cardenolide families had more than double the concentration of root cardenolides compared to the three low ones (data not shown).

Additional seeds of these six families were then germinated and sown, as above. After four weeks in the growth chamber (14:10 D/N light, and 25:16 °C D/N temperature), plants were relocated to a shade house for two weeks and then transplanted in circles in the field, where they grew for another two weeks prior to the start of the experiment. Before planting, the experimental field was deep-tilled four times to reduce native nematode populations (Millar & Barbercheck 2002). Indeed, following tilling, *G. mellonella* larvae incubated in 15 randomly chosen soil cores taken from the experimental field were not parasitized by nematodes. Each circle of six plants was created by randomly alternating plants of the three high and three low-cardenolide families; each plant was placed at the corner of a hexagon of a 1 m diameter circle ($n = 31$ circles total). 'Control' circles were left

untouched ($n = 6$). 'No nematode' circles received six freshly hatched *T. tetraophthalmus* larvae per plant ($n = 10$). 'Nematode' circles received six freshly hatched larvae per plant and, five days later, c. 30 000 *H. bacteriophora* nematodes, added to the centre of each circle in a drop of water ($n = 15$ circles). The number of *T. tetraophthalmus* larvae added was based on observations of natural loads on field-collected *A. syriaca* roots (Erwin, unpublished) and experimental data showing declining survival with increasing numbers of larvae initially added (Matter 2001). Nineteen days after *T. tetraophthalmus* application, all plants were collected by extracting a soil core of 20 cm diameter and 15 cm depth around the plant and placing all contents in modified Berlese funnels (as above). All funnels were placed in the shade over constantly humidified paper towels, which were checked regularly for larval presence until the soil was completely dry. Above-ground biomass was oven-dried for 3 days at 55 °C and weighed. Roots were carefully washed and frozen for chemical analysis (see below).

HEADSPACE COLLECTION OF ROOT VOLATILES

We used a modified dynamic headspace collection method (Kessler & Baldwin (2001) to characterize the volatile organic compound (VOC) emissions of roots of living *A. syriaca* plants. Twenty plants were germinated and grown in a growth chamber as described above, but in a 1:3 mixture vermiculate:potting soil to facilitate soil removal around roots. After four weeks of growth, half of the plants were each inoculated with four first-instar *T. tetraophthalmus* larvae, which were allowed to feed for four days before soil around the roots was gently removed by soaking roots in tap water. Excess water was removed with paper towels and plants were placed on a table with roots hanging through a 5-cm diameter hole. Root volatiles were collected by placing an open-top plastic cup (0.5 L volume) around the roots, and by pulling air through the chamber into a charcoal filter trap (Orbo-32; Supelco, Bellefonte, PA, USA) with a flow rate of 500 mL min⁻¹ for 4 h. Traps were eluted with 250 µL of dichloromethane after the addition of 448 ng of tetraline as internal standard, and 1 µL aliquots were analysed by gas chromatography mass spectrometry (GCMS, Varian Saturn 2200, Varian, Walnut Creek, CA, USA) equipped with a VF-5ms column (30 m, 0.25 mm internal diameter, 0.25 µm film thickness; Varian). Helium was used as carrier gas at a constant flow of 1 mL min⁻¹ and the following column temperature gradient: 40 °C for 5 min, increased to 180 °C at 10 °C min⁻¹, increased to 220 °C at 40 °C min⁻¹, followed by a final hold at 220 °C for 10 min. Peak areas were integrated from selected ion chromatograms specific to the individual compounds and normalized by the peak area of the internal standard. Peaks were identified where possible by comparison of retention times and mass spectra with standards. Volatile emissions are reported as micrograms tetraline equivalents.

We used dynamic headspace sampling procedure to characterize VOCs emitted from roots of living plants, and additionally the method following Rasmann *et al.* (2005) to characterize the total production and accumulation of all potential VOCs in the roots of *A. syriaca* genotypes (see below).

TOTAL ROOT VOLATILES AND CARDENOLIDES

To characterize volatile (indirect defence) and cardenolide (direct defence) concentrations in the roots, we sowed 11 additional randomly selected full-sib families of *A. syriaca*, as described above. Seedlings ($n = 10$ per family) were planted in cylindrical plastic containers (5 cm diameter, 20 cm deep) and grown in a growth chamber under controlled conditions (14:10 D/N light and 25:16 °C D/N tem-

perature). After four weeks, half of the plants were each inoculated with four first-instar *T. tetraophthalmus* larvae, which were allowed to feed for four days. Plants were then destructively harvested, and roots were carefully washed with water and ground to powder in liquid nitrogen. Half of the ground root material was dried at 45 °C for three days, and approximately 100 mg of this material was used to measure cardenolide concentrations using HPLC, as described below. The remaining powder of the frozen roots (50–200 mg dry weight) was placed in 10 mL headspace collection vials (2 cm diameter glass vials; National Scientific Company, USA). Tetraline (Sigma, CAS # 68412-24-8) was added as internal standard (172 ng), and vials were sealed with a septum lid for volatile trapping. A 100 µm polydimethylsiloxane (PDMS) SPME fiber (Supelco) was inserted through the septum and exposed to the sample headspace for 30 min at 40 °C. Volatiles were desorbed in the injector port (250 °C) of the gas chromatograph and analysed by GCMS (Varian Saturn 2200) on a polyethylene glycol column (AT-WAX, 30 m, 0.25 mm internal diameter, 0.25 µm film thickness; Alltech, USA). Helium was used as carrier gas at a constant flow of 1 mL min⁻¹ and the following column temperature gradient: 45 °C for 6 min, increased to 130 °C at 10 °C min⁻¹, increased to 180 °C at 5 °C min⁻¹, increased to 230 °C at 20 °C min⁻¹ with a 5 min hold at 230 °C, increased to 250 °C followed by a final hold at 250 °C for 5 min. Peak areas were integrated from selected ion chromatograms specific to the individual compounds and normalized by the peak area of the internal standard. Volatile emissions are reported as normalized peak area per mg dry mass for the unknown peaks, and are reported as nanograms per mg dry weight for those positively identified and quantified with standards calibration curves (see Table S1 in Supporting Information).

HPLC ANALYSIS OF ROOT CARDENOLIDES

Roots were dried at 45 °C for three days and then ground using a MM300 Retsch grinder (Retsch GmbH, Haan, Germany) in 10-mL steel grinding vessels at 27 Hz for 2 min. Fifty milligrams of fine powder was spiked with 20 µg of digitoxin (Sigma) as an internal standard and extracted with 1 mL methanol in a sonicating water bath at 55 °C for 20 min. After centrifugation and filtration with a 45-µm pore size Millex filter (Millipore, USA), HPLC analysis was conducted by injecting 15 µL of the supernatant into an Agilent 1100 series HPLC. Compounds were separated on a Gemini C18 reversed-phase column (3 µm, 150 × 4.6 mm, Phenomenex, Torrance, CA, USA). Cardenolides were eluted with a constant flow of 0.7 mL min⁻¹ and a 0.25% phosphoric acid in water–acetonitrile gradient as follows: 0–5 min 20% acetonitrile, 20 min 70% acetonitrile; 20–25 min 70% acetonitrile, 30 min 95% acetonitrile, 30–35 min 95% acetonitrile. Absorbance spectra were recorded from 200 to 400 nm with a diode array detector. Peaks with symmetrical absorption maxima between 216 and 222 nm were recorded as cardenolides (Malcolm & Zalucki 1996). Concentrations were calculated from peak areas at 218 nm standardized by the peak area of the digitoxin internal standard.

STATISTICAL ANALYSIS

All statistical analyses were conducted using JMP (Version 7, SAS Institute Inc., Cary, NC, USA). For the first two experiments (larval survival and nematode attraction), we performed one-way ANOVAS to test for treatment effects. Treatment differences between individual volatiles collected from the root headspace were assessed with a t-test. Field experiment data [log(larval survival + 1), plant biomass and cardenolide concentration] were analysed with mixed-model analyses

and included the following factors: treatment (control, plants plus *T. tetraophthalmus*, and plants plus *T. tetraophthalmus* and *H. bacteriophora* nematodes; fixed effect), root cardenolide levels (high vs. low; fixed effect), full-sib family nested within cardenolide level (random effect), block nested within treatment (planting circles; random effect), and the interaction between cardenolide level and treatment. Random effects were tested with a likelihood ratio test, which uses a chi-square comparison of the difference between the -2 log likelihoods of the full model and a model lacking each random factor. Because we expected proportional effects of cardenolides and nematodes on larval survival, we analysed log-transformed larval survival data to test for the multiplicative effects in the interaction between cardenolides and nematodes (Soluk & Collins 1988).

To assess genetic correlations (i.e. the potential for trade-offs) between constitutive vs. induced allocation to root cardenolides and volatiles among the 11 families of *A. syriaca*, we employed the test outlined by Morris, Traw & Bergelson (2006) and developed for MATLAB (version 7.5.0.342 – R2007b, MathWorks Inc., USA). This statistical approach accounts for several issues that have apparently confounded previous attempts to assess a trade-off between constitutive and induced resistance (Morris, Traw & Bergelson 2006). Specifically, this approach uses the difference in mean resistance between damaged and control plants as an optimal metric for induced resistance measurements, and uses a modified Monte Carlo procedure that takes into account sampling variation due to limited sample size, measurement error from environmental and genetic differences, and induced susceptibility, i.e. lower resistance in damaged than in undamaged plants (Morris, Traw & Bergelson 2006).

Additionally, we assessed treatment effects (herbivory by *T. tetraophthalmus*) on cardenolide and volatile production in the 11 families using a mixed-model analysis including treatment (fixed effect) and families (random effect), and their interaction (random effect). Random effects were tested as above. Finally, we used Pearson correlations between family means for root cardenolides and volatiles to assess the relationship between direct and indirect defensive traits.

Results

Heterorhabditis bacteriophora nematodes decreased *T. tetraophthalmus* larval survival by 80% ($F_{2,30} = 27.439$, $P < 0.0001$). In addition, *H. bacteriophora* were more attracted to *T. tetraophthalmus*-damaged plants than to healthy plants, and this was reflected in the 80% higher infection rate of sentinel *G. mellonella* larvae near *T. tetraophthalmus*-damaged plants compared to controls ($F_{1,24} = 7.41$, $P = 0.012$).

The headspace collection of root volatiles showed a blend of 19 compounds released by *A. syriaca* roots, of which 15 significantly increased after four days of *T. tetraophthalmus* damage (Table 1). Herbivore-induced root VOCs showed an average fivefold increase between constitutive and larva-induced emissions levels.

In the field experiment, *T. tetraophthalmus* larvae reduced above-ground biomass by 40% compared to unattacked plants, but this effect was almost completely attenuated in the presence of nematodes. This tritrophic interaction was independent of the cardenolide levels in the plant (Fig. 1, Table 2).

Nematode addition significantly reduced *T. tetraophthalmus* larval survival by 70% on low-cardenolide genotypes and by a

Table 1. Dynamic headspace collection analysis of *Asclepias syriaca* roots. Shown is the average amount (\pm SE) in μg of tetraline equivalents of volatile organic compounds collected from the headspace around intact (control) and *Tetraopes tetraophthalmus* damaged *A. syriaca* roots. Each compound is characterized by its retention time (RT) and its major ion

Compound	RT	Ion	Control	(\pm SE)	Damaged	(\pm SE)	<i>P</i>
Alpha-pinene (-)	9.65	93	10.166	1.395	33.007	7.035	0.023
Camphene	10.05	93	1.766	0.263	5.872	1.032	0.009
Benzene, propyl-	10.11	91	5.890	0.495	17.398	3.140	0.013
Unknown	10.64	81	60.301	9.116	226.081	41.715	0.008
Unknown	10.85	81	27.446	5.171	119.688	22.927	0.008
Unknown	10.92	81	72.521	10.686	260.618	47.273	0.008
Unknown sesquiterpene	11.41	93	0.600	0.093	3.643	0.908	0.019
Unknown	11.56	91	33.726	6.119	135.648	27.950	0.013
Limonene	11.64	93	74.048	12.321	297.305	61.336	0.013
1,8 cineol (eucalyptol)	11.71	93	4.302	0.168	22.538	5.614	0.022
Veratrol	12.24	138	10.416	1.403	34.702	5.952	0.007
Unknown	12.37	105	27.681	5.870	141.395	54.058	0.111
2-Methoxy-3-isopropyl pyrazine	12.68	137	4.140	1.567	31.598	11.133	0.068
Unknown monoterpene	12.85	57	33.657	12.583	136.499	32.447	0.029
Unknown	13.23	81	14.771	3.180	58.070	10.986	0.009
2-Hydroxyacetophenone	13.97	136	279.148	47.395	2084.121	743.738	0.071
2-Methoxyacetophenone	14.20	135	1.479	0.221	6.201	1.276	0.012
Unknown	14.70	168	3.716	0.425	31.599	8.261	0.018
2'-Hydroxy-4'-methoxy-acetophenone (paeonol)	18.04	151	3.169	0.540	38.699	14.429	0.067

Bold *P*-values indicate a significant difference between treatments ($P < 0.05$).

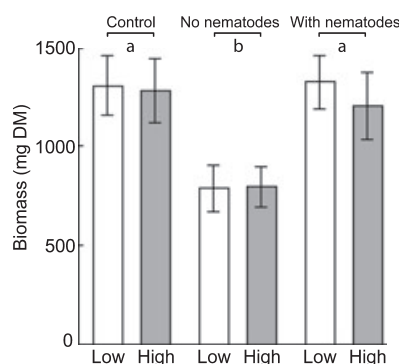


Fig. 1. Tritrophic interactions on high and low-root-cardenolide milkweeds. Mean (\pm SE) above-ground biomass of healthy *Asclepias syriaca* plants (Control), plants damaged with 6 *Tetraopes tetraophthalmus* larvae in the roots (No nematodes), and of plants that were inoculated with larvae on the roots and entomopathogenic nematodes (With nematodes). Open bars (Low) are means of three genetic families of *A. syriaca* with low constitutive levels of cardenolides in the roots, and shaded bars (High) are means of three families with high levels of cardenolides in the roots. Different letters above bars indicate significant difference across the three treatments ($P < 0.05$).

non-significant 30% on high-cardenolide genotypes (Fig. 2, see significant cardenolide \times nematode interaction in Table 2). Importantly, larval survival on the three genetic families within each cardenolide level was similar (see the non-significant genotype [cardenolide level] term in Table 2), indicating consistency among the high and low-cardenolide families.

We measured root cardenolide concentrations of healthy and attacked plants in the field and found a significant interaction between treatments and cardenolide levels. For high-cardenolide families, herbivory induced a 40% increase in

cardenolides, but this effect was attenuated by the presence of nematodes (Fig. 3; Table 2). Low-cardenolide families of *A. syriaca* showed little variation in cardenolide concentrations across the three treatments.

Root cardenolides of the 11 *A. syriaca* families showed more than twofold variation and were in the same range as cardenolide concentrations measured in the field (Fig. 4a). Families responded differently to herbivory by *T. tetraophthalmus* (family \times treatment effect: likelihood ratio (LR) = 3.39, $P = 0.033$). Across these families, inducibility (damage-control values) of cardenolides was negatively correlated with constitutive levels of cardenolides (Fig. 4a, $r = -0.653$; lower 5th percentile of the bootstrap distribution = 0, $P = 0.03$).

The analysis of volatiles from ground roots of *A. syriaca* revealed 21 peaks (Table S1) of which 16 were also found in the dynamic headspace collections of live roots (see Table 1). Two compounds (2-hydroxyacetophenone and paeonol) were present in all samples and represent the majority of the volatile compounds accumulated in the roots of *A. syriaca* (Table S1). For the analysis the genetic variability of VOCs, we therefore split the volatile data into two groups: major peaks (the sum of the two most abundant peaks) and minor peaks (the sum of the other 19 peaks). Across families, total volatile amounts showed more than twofold variation in expression in their constitutive and inducible levels (Fig. 4b).

Across all families of *A. syriaca*, inducibility of total volatiles was significantly negatively correlated with constitutive levels (Fig. 4b, $r = -0.725$, lower 5th percentile of the bootstrap distribution = 0, $P = 0.024$; this held true for minor peaks $r = -0.714$, lower 5th percentile = -0.653 , $P = 0.039$, and for major peaks $r = -0.725$, lower 5th percentile = -0.609 , $P = 0.022$).

Table 2. Overall mixed-model ANOVA for effects of treatment and cardenolide levels on *Tetraopes tetraophthalmus* larval survival, plant biomass and root cardenolide concentration. Treatments are the presence or absence of entomopathogenic nematodes *Heterorhabditis bacteriophora* in the circles of six different genotypes of *Asclepias syriaca*. Additionally, control plants (without herbivores or nematodes) were included in the plant biomass and cardenolide data analyses

Response	Factor	d.f.	F value or LR*	P
Log(survival + 1)	Treatment (T)	1,23	6.723	0.016
	Cardenolides levels (CL)	1,4.18	6.354	0.063
	T × CL	1,119	6.564	0.012
	Circles[T]*	1	3.336	0.068
	Genotypes[CL]*	1	0.939	0.333
Plant biomass	T	2,28.2	3.450	0.046
	C	1,4.82	0.125	0.739
	T × CL	2,144.8	0.136	0.873
	Circles[T]*	1	11.287	0.001
	Genotypes[CL]*	1	0.197	0.657
Cardenolides	T	2,27.83	1.532	0.234
	C	1,4.72	47.835	0.001
	T × CL	2,132.6	6.158	0.003
	Circles[T]*	1	5.584	0.018
	Genotypes[CL]*	1	0.046	0.830

*Random effects tested by likelihood ratio (LR) test. Brackets [] indicate nested effects. Bold numbers highlight significant effects ($P < 0.05$).

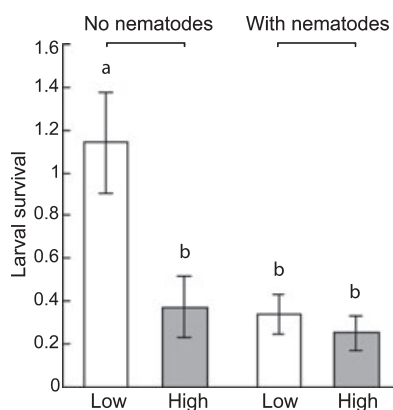


Fig. 2. Impacts of direct and indirect root defense on larval survival. Mean (\pm SE) *Tetraopes tetraophthalmus* survival out of six larvae in the absence (No nematodes) or in the presence (With nematodes) of entomopathogenic nematodes *Heterorhabditis bacteriophora*. Open bars (Low) are means of three genetic families of *A. syriaca* with low constitutive levels of cardenolides in the roots, and shaded bars (High) are means of three families with high levels of cardenolides in the roots. Different letters above bars indicate significant differences across the four treatments ($P < 0.05$).

Finally, we tested for a relationship between levels of volatiles and cardenolides produced in the roots of *A. syriaca*. Constitutive root cardenolides were not genetically correlated with the constitutive production of volatiles (for total volatiles, $n = 11$, $r = 0.085$, $P = 0.803$; for major peaks, $r = 0.088$, $P = 0.798$; for minor peaks, $r = 0.135$, $P = 0.693$, and none of the phenotypic correlations were significant). Cardenolides and VOCs were also not correlated in the induced state (data not shown). However, the relationship between inducibility of volatiles and cardenolides was positive for total and major peaks, but not for minor peaks (Fig. 4c, total volatiles,

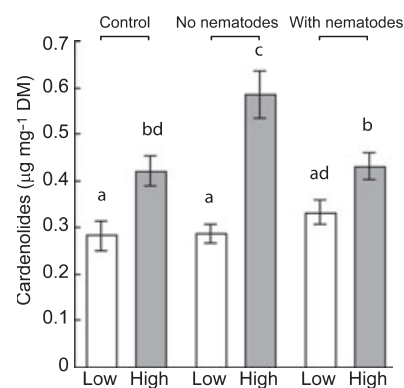


Fig. 3. Impacts of root herbivores and root herbivores plus predatory nematodes on root cardenolide concentrations. Mean (\pm SE) root cardenolide (μ g of ditoxin equivalents per mg dry root mass) levels of *Asclepias syriaca* healthy plants (control), plants damaged with 6 *Tetraopes tetraophthalmus* larvae in the roots (No nematodes), and plants that were inoculated with larvae on the roots and entomopathogenic nematodes (With nematodes). Empty bars (Low) are means of three genetic families of *A. syriaca* with low constitutive levels of cardenolides in the roots, and shaded bars (High) are means of three families with high levels of cardenolides in the roots. Different letters above bars indicate significant differences across the six treatment combinations ($P < 0.05$).

$n = 11$, $r = 0.606$, $P = 0.048$; for major peaks, $r = 0.607$, $P = 0.048$; for minor peaks, $r = 0.318$, $P = 0.341$).

Discussion

We have shown a complex interaction between below-ground direct and indirect defences that resulted in reduced herbivore performance below ground and, subsequently, benefits to above-ground plant growth. Direct and indirect

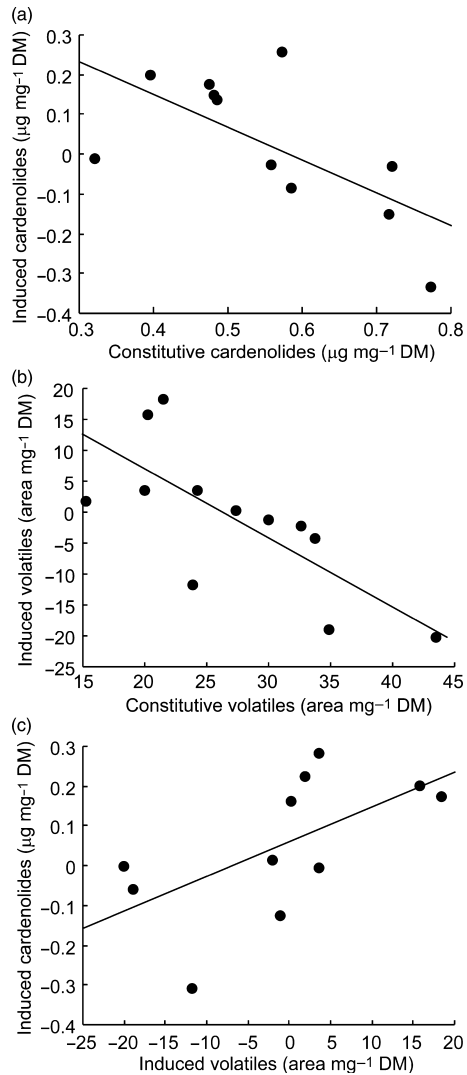


Fig. 4. Raw genetic correlations ($n = 11$ full sib families) indicating a trade-off between constitutive and induced allocation to root cardenolides (a) and total root volatiles (b). Inducibility is measured as the difference between damaged and control values for each trait. Data analysis of panels (a) and (b) were conducted with a bias-corrected Monte Carlo procedure (Morris, Traw & Bergelson 2006). Panel (c) shows the relationship between inducibility of total volatiles and cardenolides.

defences were not negatively correlated, countering the widespread notion that these defence strategies may be redundant and costly, and therefore should trade off. Nonetheless, plasticity of the responses (i.e. induced defence after attack) showed a negative correlation with constitutive investment within both direct and indirect defence traits. Thus, most milkweed genotypes deploy multiple defences below ground, but employ economy in each, by favouring either a constitutive or induced strategy.

INTERACTIVE EFFECT OF DIRECT AND INDIRECT DEFENCES

Root-boring *T. tetraophthalmus* larvae reduced *A. syriaca* above-ground plant biomass by 40%, but this loss was not

observed when entomopathogenic nematodes (*H. bacteriophora*) were present. Evidence of nematodes having negative impacts on herbivore performance is more common than evidence of nematodes having a positive impact on plant biomass, with most work conducted in agricultural systems (Denno, Gruner & Kaplan 2008). A notable exception involves the nematode *Heterorhabditis marelatus* and its host, the ghost moth *Hepialus californicus*, which bores into the roots of bush lupine (*Lupinus arboreus*) in sand-dune habitats of coastal California (Strong *et al.* 1996, 1999; Preisser 2003; Ram *et al.* 2008). In this natural system, soil moisture promotes nematode survival, which inflicts widespread mortality on the root borer and, in turn, increases plant survival.

In our study, both the production of high levels of cardenolides and the presence of soil-dwelling nematodes resulted in the low survival of *T. tetraophthalmus* larvae, but the combined effects did not surpass each effect individually. This pattern suggests redundancy between the two types of defences, at least for the numbers of larvae and nematodes present in our field experiment. We propose four non-mutually exclusive explanations for this pattern of redundancy. First, our result may be driven by the presence of few *T. tetraophthalmus* larvae, impeding nematodes from having an additional effect in the presence of high-cardenolide roots. Second, low *T. tetraophthalmus* larval survival on high-cardenolide roots might diminish the success of nematodes foraging for host larvae due to a negative density-dependent function between host abundance in the soil and nematode foraging success (Fenton *et al.* 2000). A density-dependent reduction in host finding could be due to a limited emission of volatile cues by the attacked plants, but this has not been tested for root-herbivore interactions.

A third possible explanation for the pattern of redundancy in direct and indirect defence is that high levels of cardenolides interfere with the behaviour or survival of predatory nematodes. This study, and data showing that *T. tetraophthalmus* larval survival negatively correlates with root cardenolides across 18 species of *Asclepias* (Rasmann & Agrawal, unpublished), suggest that cardenolides are indeed toxic to *T. tetraophthalmus*. Nonetheless, Isman, Duffey & Scudder (1977) showed that *T. tetraophthalmus* adults can store cardenolides during feeding, and it is plausible that larvae do as well. Little evidence exists on the effects of host plant defences on entomopathogenic nematodes, but two studies showed that predatory nematodes had reduced fecundity on squash varieties with cucurbitacins (bitter) compared to those without (sweet) cucurbitacins (Barbercheck, Wang & Hirsh 1995; Eben & Barbercheck 1997).

The fourth, somewhat implicit hypothesis for the maintenance of different plant defences is that local heterogeneity in the abundance of both herbivores and nematodes promotes the maintenance of different strategies. Because of limited dispersal and constraints imposed by variation in soil, both herbivores and predators are often heterogeneously clustered in soil patches (Andersen 1987; Stuart & Gaugler 1994).

Whatever the exact reasons for such a redundancy in plant defences, we speculate that the benefit of redundancy may outweigh the costs, especially if the effectiveness of direct and indirect defences varies over space and time. For example, in the current study, nematodes had a much stronger effect on plant biomass than cardenolides, even though both strategies equally reduced herbivore numbers. Additionally, cardenolides might be primarily effective at killing early life stages of herbivores (Zalucki, Clarke & Malcolm 2002) and the effectiveness of nematodes as an indirect defence may only be strong in certain soil types (Campbell *et al.* 1996; Alekseev, Glazer & Samish 2006; Hiltbold & Turlings 2008), or when larvae are in a later developmental stage.

INDIRECT DEFENCES BELOW GROUND

Our attraction experiment showed that nematodes are indeed more attracted to insect-damaged roots than undamaged roots. We assume that plant-induced volatiles are driving this behaviour, as was previously shown for *H. megidis* nematodes that were attracted to volatiles emitted by damaged corn roots (Rasmann *et al.* 2005; Degenhardt *et al.* 2009). In corn, only one volatile was emitted by the roots, the sesquiterpene (*E*)- β -caryophyllene, a compound whose physical and biochemical properties result in a low cost of production and a high rate of diffusion through the soil (Hiltbold & Turlings 2008). Recently, Ali, Alborn & Stelinski (2010) similarly demonstrated that citrus roots attacked by the larvae of the weevil *Diaprepes abbreviatus* were attractive to an entomopathogenic nematode, *Steinernema diaprepesi*. This was associated with the induction of at least four terpene compounds in damaged roots. *Asclepias syriaca*, on the other hand, produces a more complex blend of root volatiles (21 peaks), of which 80% showed increased production after herbivory. This general increase of volatile production seems to explain nematode attraction, but further studies are needed to assess the individual role of each compound on nematode behaviour. Although we identified only half of the VOCs found in the roots, the two methods of volatile collection yielded very similar qualitative patterns of volatile emissions. Ali, Alborn & Stelinski (2010) proposed the collection of volatiles on super-Q traps by pushing air through the soil surrounding plant roots, but the collection efficiency of this type of analysis would be highly dependent on the type of growing medium used. Our methods for collecting and analyzing volatiles from living root tissue are an additional means of characterizing volatile signals involved in below-ground plant–herbivore interactions.

GENETIC CORRELATIONS IN DEFENCE STRATEGIES

By measuring volatiles and cardenolides in the same set of roots, with and without herbivory, we found a negative genetic correlation between constitutive levels and inducibility for both types of defence. Thus, milkweed genotypes appear to have a maximal expression of root cardenolides and VOCs and these are allocated either constitutively, following attack,

or in some balance between the two. Such trade-offs between constitutive and induced responses suggest (1) that the traits are costly or otherwise constrained, or (2) that there is simply no benefit to additional defence beyond some upper threshold (Agrawal, Conner & Rasmann 2010).

We did not find a negative genetic relationship between allocation to direct and indirect defences; on the contrary, we found some evidence for a positive relationship in their inducibility (for total volatiles and cardenolides). Trade-offs between direct and indirect defence are a widely assumed (Rudgers, Strauss & Wendel 2004; Ballhorn *et al.* 2008; Ballhorn, Pietrowski & Lieberei 2010), but scantily tested. Agrawal *et al.* (2002) found that infested sweet cucumber plants (lacking cucurbitacins) attracted 37% more predatory mites (via volatile cues) than did infested bitter plants (that produce constitutive and inducible cucurbitacins). Ballhorn *et al.* (2008) showed a negative correlation between expression of toxic hydrogen cyanide and the release of herbivore-induced VOCs in 15 lines of lima beans. Nonetheless, several other studies have failed to demonstrate such trade-offs (Steward & Keeler 1988; Letourneau & Barbosa 1999; Dyer *et al.* 2001; Heil *et al.* 2002). Such discrepancies in the literature are difficult to explain as long as we lack a mechanistic understanding of how trade-offs arise (Agrawal 2007; Agrawal, Conner & Rasmann 2010). Variable production of direct and indirect defences can be triggered by insect-derived elicitors (Halitschke *et al.* 2003), plant hormones (Harfouche *et al.* 2006) or herbivore-induced volatile organic compounds (Frost *et al.* 2007; Ton *et al.* 2007).

The positive genetic correlation between inducibility of volatiles and of cardenolides (i.e. milkweed genotypes that induce high levels of cardenolides also induce high levels of volatiles) indicates that direct and indirect resistance traits may function independently, but that herbivore attack triggers both in a similar way. Therefore, both types of defence may be mediated by a common signalling pathway (Chehab *et al.* 2008; Howe & Jander 2008). Shared signalling between direct and indirect defence is common (Thaler 1999; Halitschke *et al.* 2000), and this may indicate that these strategies are not truly redundant, but beneficially overlapping. For example, delayed growth of the herbivore due to direct defences may allow for greater foraging success of predators and parasitoids (Benrey & Denno 1997).

Conclusion

Trade-offs occur where multiple traits are favoured by natural selection, but compete for shared resources (Agrawal, Conner & Rasmann 2010). This is a plausible explanation for the negative relationship between constitutive levels of defence and their inducibility.

This is unlikely, however, for the relationship between direct and indirect defences: they may not share a specific limiting resource and may independently promote herbivore resistance across the patchiness of herbivore attack and nematode presence in the field. Indeed, redundancy or ‘reinforcement’ of different strategies may be necessary for effective defence, but for

each strategy, we found an economy of deployment which may reduce overall costs.

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Supporting information

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

Table S1. Two-way ANOVAS on families of *A. syriaca*, treatments (healthy plants or plants attacked by *T. tetraophthalmus* larvae feeding on the roots), and their interaction for: total amount, major peaks, minor peaks, the ratio between minor and major peaks, and each of the 21 compounds analyzed alone.

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