Root herbivory reduces growth and survival of the shoot feeding specialist *Pieris rapae* on *Brassica nigra*

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Abstract

Plants may respond to herbivore attacks by changing their chemical profile. Such induced responses occur both locally and systemically throughout the plant. In this paper we studied how *Brassica nigra* (L.) Koch (Brassicaceae) plants respond to two different root feeders, the endoparasitic nematode *Pratylenchus penetrans* Cobb (Tylenchida: Pratylenchidae) and the larvae of the cabbage root fly *Delia radicum* L. (Diptera: Anthomyiidae). We tested whether the activities of the root feeders affected the survival and development of the shoot feeding crucifer specialist *Pieris rapae* (L.) (Lepidoptera: Pieridae) via systemically induced changes in the shoots. Overall, *P. rapae* larvae grew slower and produced fewer pupae on plants that were infested with root feeders, especially on plants infested with *P. penetrans*. This effect could not be attributed to lower water or protein levels in these plants, as the percentage of water in the controls and root infested shoots was similar, and protein content was even higher in root infested plants. Both glucosinolate as well as phenolic levels were affected by root feeding. Initially, glucosinolate levels were the lowest in root infested plants, but on *P. penetrans* infested plants they increased more rapidly after *P. rapae* started feeding than in controls or *D. radicum* infested plants. Plants with *D. radicum* feeding on their roots had the highest phenolic levels at all harvest dates. Our results indicate that root feeding can significantly alter the nutritional quality of shoots by changes in secondary metabolite levels and hence the performance of a specialist shoot feeder.

Introduction

Green plants are the primary world-wide food source and are therefore heavily attacked by both root and shoot feeders. To defend themselves against this wide range of enemies, plants produce a variety of chemical compounds that are noxious or toxic to herbivores (Schoonhoven et al., 1998). Many of these chemical compounds are also inducible, i.e., their profile and levels change when plants are actually attacked by herbivores (Karban & Baldwin, 1997). Both the signalling hormones involved in the elicitation of induced responses, as well as the chemicals that are produced to defend the plant, may be transported throughout the plant and also cause the chemical profiles of undamaged plant parts to change (Stout et al., 1999). In the past, these systemic induced responses have often been shown to occur in above-ground plant parts, but recent studies have revealed that systemic induction also occurs between root- and shoot-induced responses.

The magnitude and direction of the systemic response between roots and shoots seems to depend on the plant species, the compounds that are produced, and the inducing agent. In cotton (*Gossypium herbaceum*) plants, both root feeding by click beetle larvae (*Agriotes lineatus*) and mechanical root damage increased terpenoid levels in shoots and roots. Conversely, shoot damage by a generalist caterpillar only increased shoot, but not root terpenoid levels (Bezemer et al., 2004). This contrasts with the pattern of pyrrolizidine alkaloid induction in ragwort (*Senecio jacobaea*), in which mechanical root damage significantly increased PA levels in the roots of all plants, but whether they increased in the shoot depended on the plant genotype. Shoot feeding by a generalist caterpillar even significantly decreased root PA levels in *S. jacobaea* (Hol et al., 2004). Finally, in two wild *Brassica* species it was found that the systemic induction of glucosinolates (GS) is unidirectional: root-induction with jasmonic acid (JA) did not

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significantly change root GS levels, but increased shoot levels 1.5–3-fold. Shoot JA-treatment only increased GS locally, and did not increase root GS levels (van Dam et al., 2004).

Root–shoot interactions mediated by induced plant chemistry may have profound effects on the performance of herbivores. Beet army worm (*Spodoptera exigua*) larvae that were forced to feed on cotton plants damaged by click-beetle larvae at their roots, showed reduced growth rates (Bezemer et al., 2003). Because differences in defence chemistry also negatively affect parasitoids and even hyperparasitoids (Harvey et al., 2003; Sznajder & Harvey, 2003), root–shoot interactions between induced responses may have profound consequences for higher trophic levels associated with plants (van der Putten et al., 2001; van Dam et al., 2003). The soil contains a wealth of macroscopic and microscopic root feeding organisms and their effects on plant defence have been poorly studied. We may expect that root-induced responses more commonly affect shoot quality than has been considered thus far.

As for above-ground feeders, there is a wide range of root feeders with very different feeding habits and host-plant ranges. It may be expected that, similar to shoot-induced responses, these differences in feeding habits will result in different and specific plant responses (Reymond & Farmer, 1998; Ozawa et al., 2000).

In this study we infested the roots of *Brassica nigra* (L.) Koch (Brassicaceae) plants with *Delia radicum* L. (Diptera: Anthomyiidae) (cabbage root fly) larvae or with the endoparasitic nematode *Pratylenchus penetrans* Cobb (Tylenchida: Pratylenchidae) (root lesion nematode). These are two very different types of root phytophages: *D. radicum* females deposit their eggs on the soil close to the root crown (Städler & Schoni, 1990). The maggots move down into the soil towards the upper, thicker, part of the roots, where they chew mines on the surface or into the root tissue (Gratwick, 1992). *Delia radicum* is a specialist on Brassicaceae, although it does not perform equally well on all species (Finch & Ackley, 1977). Apart from visible—economic—damage on crops such as turnip and swedes, they may also cause severe mortality amongst the seedlings of crops and wild species alike (Finch & Ackley, 1977; Gratwick, 1992). The closely related turnip root fly, *Delia floralis*, was found to increase the levels of shoot GS 1.2-fold in three cultivated *Brassica* species (Birch et al., 1992).

The nematode *P. penetrans*, on the other hand, is a generalist migratory endoparasite, which enters the root cortex using its stylet (Bongers, 1994; Siddiqi, 2000). *Pratylenchus penetrans* nematodes are able to dissolve the connections between cortical cell walls with enzymes produced by their salivary glands (Uehara et al., 2001). They complete their life cycle in about 4 weeks, depending on the temperature, and both juvenile and adult stages are infective (Bongers, 1994; Siddiqi, 2000). Even though the GS in Brassicaceae are found to reduce nematode numbers (Brown & Morra, 1997), *B. nigra* is a good host for the closely related nematode species, *Pratylenchus neglectus*: it supported 1.83-fold more of this nematode than a wheat control (Potter et al., 1999). There have been hardly any studies that reported whether *P. penetrans* or other nematodes affect shoot chemistry: the sedentary endoparasitic root-knot nematode *Meloidogyne incognita* may increase or decrease the levels of the toxin nicotine in tobacco, depending on the plant variety (Hanounik & Osborne, 1977; Barker & Weeks, 1991). However, sedentary endoparasitic nematodes are feeding specialists that form a feeding cell and therefore have a very different and more complicated interaction with their host-plant than migratory nematodes (Williamson & Gleason, 2003; Zinov'eva et al., 2004). To our knowledge there have been no studies examining the combined effects of root-feeding insects and nematodes on plant primary and secondary chemistry, and on the consequences for growth and development of above-ground herbivorous insects.

In this paper, we first addressed the question of whether root feeding by either *D. radicum*, *P. penetrans*, or a combination of both root feeders affected the performance of *Pieris rapae* (L.) (Lepidoptera: Pieridae) larvae. Even though *P. rapae* is a crucifer specialist, it still is negatively affected by high GS levels and other inducible defences, such as high trichome densities, which may alter in response to root feeding (Traw & Dawson, 2002b; Agrawal & Kurashige, 2003; van Dam et al., 2004). Secondly, we analyzed whether root feeding by *D. radicum* and *P. penetrans* induced changes in leaf quality that may explain differences in the performance of *P. rapae* larvae. We measured both primary and secondary shoot compounds, because both are known to affect insect growth and to alter in response to root damage (Berenbaum, 1995; Duffey & Stout, 1996; Schoonhoven et al., 1998; Hol et al., 2004). Because *P. rapae* feeding is also known to induce plant defences in *B. nigra* (Traw, 2002; Traw & Dawson, 2002a), we sampled the shoots of the plant at different times during *P. rapae* development to examine the dynamics of the different leaf quality measures over time.

Materials and methods

Plant rearing

Brassica nigra seeds, whose parent plants originated from a local wild population, were reared in a common garden in Heteren in 2002 and stored dry and in the dark at 10 °C until use. Seeds were germinated on glass beads in water in 10 × 10 cm plastic containers with a clear plastic lid. The

external standard. We used the response factors for detection at 229 nm from Buchner (1987) to calculate the concentrations of the different types of GS. As before, the allyl-glucosinolate sinigrin dominated the GS profile of the *B. nigra* leaves: over 99% of the profile consisted of this GS (van Dam et al., 2004). Because the different classes of GS in *B. nigra* shoots all responded similarly to the treatments, we added the amounts of individual GS per sample to obtain a total GS.

Proteins were extracted from 25 mg leaf powder with 2 ml 0.1 M NaOH following the procedure described in Jones et al. (1989). An aliquot of the extract was assayed using a colorimetric protein assay (Bio-Rad DC Protein Assay, Bio-Rad laboratories, Veenendaal, The Netherlands) which is a modified version of the Bradford method (Bradford, 1976) specifically designed to determine protein levels in NaOH solutions (Biorad Bulletin 1069 at http://www.biorad.com). Absorption was measured after 60 min at 750 nm. A series of Bovine Serum Albumin (Boehringer Mannheim, Germany) solutions ranging from 0.5 to 2.5 mg ml^{-1} was used as a reference.

To estimate total phenolic content, 50 mg of dry leaf powder was extracted twice with 50% MeOH at 90 °C (Allen, 1974). An aliquot of the extract was used to determine the phenolic content with Folin–Ciocalteu's Phenol reagent (Sigma). Absorbance was measured after 10 min at 595 nm. A series of chlorogenic acid (Sigma) solutions (0.27–2.7 mg ml^{-1}) was used as a reference. Even though this analysis has been criticized for being used to compare phenolic levels among plant species, it still is considered suitable and is being used to compare within-species samples for overall phenolic activities (Appel et al., 2001; Reddy et al., 2004).

Statistical analyses

All statistical analyses were performed with STATISTICA 6.0 Software (Statsoft Inc., Tusla, OK) using a sigma-restricted Type VI sum of squares. Larval and pupal masses, as well as shoot and root masses, were log-transformed and all shoot quality measures (water, protein, phenolic, and GS content) were arcsine-square-root transformed to meet assumptions of normality and homogeneity of variances.

Larval mass on day 7 and day 13 were analysed with a repeated measures ANOVA. Because the pupal masses of individual larvae were measured on different days, namely the day the larva had pupated, these were not included in the repeated measures test, but analysed separately in a factorial ANOVA with presence of *P. penetrans* (0 or 1) and *D. radicum* (0 or 1) as main factors. Differences in developmental time (from neonate larva to pupa) between the four treatment groups were analysed with a non-parametric Kruskall–Wallis ANOVA. Differences in the frequency of successful larvae (i.e., those producing a living butterfly) between groups was analyzed by a G-test followed by Williams correction (Sokal & Rohlf, 1995; Dytham, 2003). The effects of root herbivory on plant quality measures were analysed by a factorial MANOVA with harvest date, *P. penetrans* and *D. radicum* as fixed factors. Because the MANOVA turned out to be significant, it was followed by univariate ANOVAs to analyse which of the dependent variables were affected by the different treatments.

Results

Larval growth and survival

The body masses of *P. rapae* larvae that were feeding on plants with a root feeder present increased more slowly during the first 13 days than larvae on uninfested control plants (Figure 1). Repeated measures analysis of the larval body masses on days 7 and 13 showed that this was especially true for larvae on plants infested with *P. penetrans* (Figure 1; *P. penetrans* effect: $F_{1,64} = 5.83$, $P = 0.018$). Moreover, we found a significant interaction between the repeated factor 'day' and *P. penetrans* treatment in the model ($F_{1,64} = 4.79$, $P = 0.032$). This was mainly due to the fact that the effect of *P. penetrans* infestation on body mass was the strongest on day 13 (Figure 1, lower panel; Univariate ANOVAs, *P. penetrans* effect: $F_{1,64} = 2.19$, $P = 0.14$ at day 7, and $F_{1,64} = 6.07$, $P = 0.016$ at day 13). *Delia radicum* infestation did not significantly reduce larval body mass increase (repeated measures ANOVA, *Delia* effect: $F_{1,64} = 0.82$, $P = 0.37$) although it significantly decreased larval body mass at day 7 (Figure 1, upper panel; univariate ANOVAs per day: $F_{1,64} = 4.55$, $P = 0.036$). We found no interactions between the effects of *P. penetrans* and *D. radicum* feeding on *P. rapae* growth in the ANOVA models.

Not only did *P. rapae* larvae grow slower on *P. penetrans* infested plants, they also had a significantly lower chance of producing a viable pupa than *P. rapae* on control plants (Table 2, G-test control vs. *P. penetrans* treatment: Williams adjusted $G_{adj} = 4.39$, $P = 0.039$). Larvae on the other root-treated plants showed a similar trend of producing fewer viable pupae, but these percentages did not significantly deviate from the control value. Despite the lower growth rates on root treated plants, eventually the development time from neonate larva to viable pupa of the surviving larvae was not significantly affected by root treatment (Table 2; Kruskall–Wallis analysis: $P = 0.58$), nor were pupal masses significantly affected by the presence of root feeders (Figure 2; Factorial ANOVA: all $P>0.11$). This was due to a higher mortality of the slowest growing larvae in these treatment groups between day 13 and before pupation.

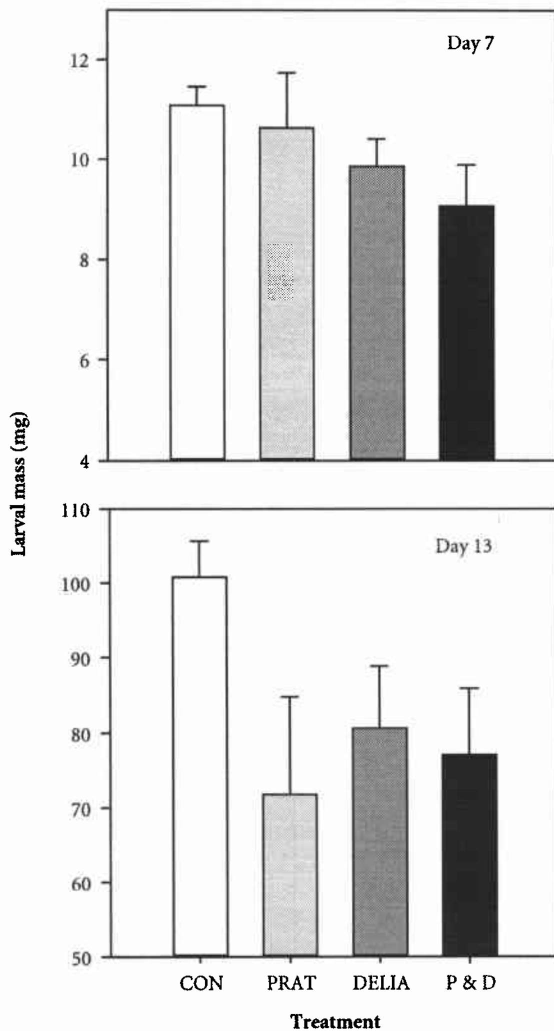

Figure 1 Larval body masses (+SEM) at 7 (upper panel) and 13 days (lower panel) of *Pieris rapae* larvae feeding on shoots of *Brassica nigra* plants, whose roots were not infested with root feeders (control; CON) or whose roots were infested with either *Pratylenchus penetrans* nematodes (PRAT), *Delia radicum* root fly larvae (DELIA), or both (P & D).

Plant quality measures

When all plant quality measures (Tables 3 and 4) were analysed in a full factorial MANOVA, there was no significant overall effect of *P. penetrans* or *D. radicum* infestation, although both the *D. radicum* as well as the *P. penetrans* × *D. radicum* effect showed P-values that were less than 0.1 (Table 5). The plant chemistry of *P. penetrans* infested plants, however, significantly interacted with harvest date (Table 5). Examination of the univariate ANOVAs revealed that this was because the dynamics of phenolics and GS levels in *P. penetrans* infested plants followed different

Table 2 The percentage of larvae that produced viable pupae and the average developmental time from neonate to pupae (days + SEM) in all treatment groups. The value in bold is significantly different (P<0.05) from the control percentage in the same column (see text for details)

Treatment	Viable pupae (% of larvae)	Development time (days)
Control	66.7%	18.0 (0.3)
P. penetrans	**37.5%**	18.0 (0.7)
D. radicum	56.0%	18.2 (0.3)
P. penetrans & *D. radicum*	54.2%	18.2 (0.2)

patterns than those in other treatments (Tables 3 and 4; Univariate ANOVAs; phenolics, Prat × Harvest date: $F_{2,131}$ = 3.45, P = 0.03; GS, interaction Prat × Harvest date: $F_{2,131}$ = 5.02, P = 0.05). Not surprisingly, harvest date greatly determined differences in shoot quality measures (Table 5). The harvest date effect comprises the added results of ontogenetic changes and induced changes caused by *P. rapae* feeding on all plants.

A closer examination of the shoot quality data shows that on all harvest dates, the water content of the shoots was quite similar between treatments (P>0.1 for all effects and interactions), and certainly not the lowest in the *P. penetrans* treatment group on which the *P. rapae* larvae performed the worst (Table 3). Protein levels were on average the highest in root infested plants, but it mattered which root herbivores were present. Plants with only *P. penetrans* infestation overall had higher protein levels than controls, but in the presence of *D. radicum*, nematode-infested plants had lower protein levels than

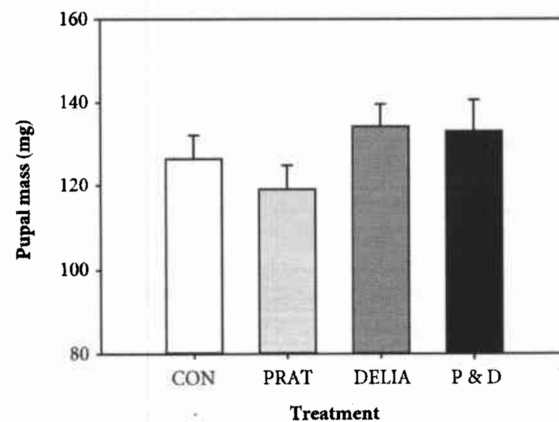

Figure 2 Pupal masses (+SEM) of viable pupae from *Pieris rapae* larvae that were feeding on shoots of *Brassica nigra* plants, whose roots were not infested by root feeders (control; CON) or whose roots were infested by either *Pratylenchus penetrans* nematodes (PRAT), *Delia radicum* root fly larvae (DELIA), or both (P & D).

Table 3 Primary compound measures (SEM) of *Brassica nigra* shoots from plants with roots not infested by root feeders (control) or roots infested by *Pratylenchus penetrans* nematodes, *Delia radicum* root fly larvae, or both. Samples were taken when the *Pieris rapae* larvae were put on the plant (0 days), 7 days later, and 13 days later. Note that data in the columns headed 0 days and 7 days are whole shoot data, whereas for 13 days sub-samples of two leaves were analyzed

	Water content (percentage of total fresh mass)			Total protein content (mg.g dry mass^{-1})		
	0 day	7 day	13 day	0 day	7 day	13 day
Control	85 (0.5)	83 (1.0)	81 (0.6)	83.4 (7.5)	87.7 (6.5)	97.6 (6.0)
P. penetrans	86 (0.6)	82 (1.1)	81 (0.4)	86.4 (5.7)	90.7 (4.4)	102.3 (5.3)
D. radicum	86 (0.3)	82 (1.1)	80 (0.5)	89.0 (5.3)	105.2 (5.7)	104.9 (5.0)
P. penetrans & *D. radicum*	86 (0.5)	80 (1.9)	81 (0.5)	86.0 (7.8)	82.4 (5.3)	101.7 (4.1)

plants with only root flies (Table 3; Univariate ANOVA, interaction Prat × Delia: $F_{1,131} = 5.02, P = 0.026$).

Interestingly, we found that on day 0, 3 weeks after plants had been infested with *P. penetrans* and 1 week after *D. radicum* was added, the GS levels in plants infested with root feeders overall were lower than in control plants, whereas phenolic levels were higher in the same plants (Table 4). The low GS values at the beginning, however, did not prohibit a local induction of shoot GS by *P. rapae* feeding, because on days 7 and 13, the shoot levels of root infested plants were higher than in controls, especially in *P. penetrans* infested plants (Table 4; Univariate ANOVA, interaction Prat × Harvest date: $F_{2,131} = 5.02, P = 0.05$). Total phenolic levels overall were significantly higher in *D. radicum* infested plants (Univariate ANOVA, effect Delia: $F_{1,131} = 5.41, P = 0.02$). *Pratylenchus penetrans* infested plants had reduced phenolic levels on day 7 and increased levels on day 13 compared to controls or *D. radicum* infested plants (Table 4; interaction Prat × Harvest date: $F_{2,131} = 3.45, P = 0.03$).

Discussion

Here, for the first time, we have shown that root feeding by the generalist migratory endoparasitic nematode *P. penetrans* significantly decreases the performance of a specialist above-ground herbivore. Larvae of *P. rapae* on plants infested with the root-mining larvae of *D. radicum* also tended to grow more slowly and to produce fewer viable pupae. The decrease in food quality for the shoot herbivore was not due to water stress nor to a decrease in shoot protein levels. This refutes the assumption that root feeders generally impair root function and that the negative effects they have on shoot feeders are due to a reduced primary nutritional quality of the shoot per se (Brown & Gange, 1990; Masters & Brown, 1992).

Additionally, we found evidence that the feeding of *P. penetrans* and *D. radicum* at the roots significantly affected the secondary compound profile of shoots, which in turn may have caused the reduced performance of *P. rapae* on these plants. Even though *P. rapae* is a specialist herbivore whose feeding is stimulated by GS (Schoonhoven et al., 1998) and that produces a special nitrile-specifier protein to disarm the GS-myrosinase defence system (Wittstock et al., 2004), it has not completely overcome its host-plant's defences. Sinigrin, and especially the isothiocynates (ITC) that are formed upon leaf damage, reduce both the growth and survival of *P. rapae* larvae (Agrawal & Kurashige, 2003). It is possible that the production of nitrile-specifier

Table 4 Secondary compound levels (SEM) of *Brassica nigra* shoots from plants with roots not infested by root feeders (control) or roots infested by *Pratylenchus penetrans* nematodes, *Delia radicum* root fly larvae, or both. Samples were taken when the *Pieris rapae* larvae were put on the plant (0 days), 7 days later, and 13 days later. Note that data in the columns headed 0 days and 7 days are whole shoot data, whereas for 13 days sub-samples of two leaves were analyzed

	Total glucosinolates (μmol.g dry mass^{-1})			Total phenolics (mg.g dry mass^{-1})		
	0 day	7 day	13 day	0 day	7 day	13 day
Control	32.9 (2.1)	28.8 (2.8)	42.3 (2.7)	16.5 (0.8)	15.6 (0.6)	16.1 (0.7)
P. penetrans	21.7 (2.0)	29.4 (2.4)	44.8 (3.8)	17.3 (0.8)	15.2 (0.4)	16.5 (0.9)
D. radicum	23.2 (2.3)	32.2 (2.2)	38.4 (4.0)	17.1 (0.7)	17.0 (0.9)	16.9 (0.5)
P. penetrans & *D. radicum*	24.9 (2.4)	29.0 (3.9)	44.9 (3.6)	18.5 (1.2)	14.9 (0.8)	17.8 (0.7)

Table 5 Results of a factorial MANOVA of all plant quality measures in Tables 3 and 4, testing the effects of *Pratylenchus penetrans* (Prat) and *Delia radicum* (Delia) feeding. P-values<0.05 are in bold, P<0.1 are in italics

Factor	F	Effect d.f.	Error d.f.	P
Prat	0.61	4	128	0.653
Delia	2.17	4	128	*0.076*
Harvest date	23.94	8	256	**0.000**
Prat × Delia	2.29	4	128	*0.064*
Prat × Harvest date	2.10	8	256	**0.037**
Delia × Harvest date	0.85	8	256	0.558
Prat × Delia × Harvest date	0.87	8	256	0.541

protein is metabolically costly, as has been shown for the production of nicotine-detoxifying enzymes in the tobacco hornworm, *Manduca sexta* (Appel & Martin, 1992; Snyder et al., 1994). Since sinigrin is the major GS in *B. nigra* (> 99%) and plants infested by *P. penetrans* showed the largest increase in GS after *P. rapae* began feeding, the metabolic costs of detoxifying sinigrin may have contributed to the decreased performance of *P. rapae* on these plants. These costs may be more prominent when *P. rapae* larvae are confronted with high sinigrin levels from the first instar onwards (Agrawal & Kurashige, 2003; this study), because last instar larvae are not affected by high sinigrin levels in their food (Blau et al., 1978).

Interestingly, the initial shoot GS levels before the *P. rapae* started feeding were lower in all root-herbivore treatment groups than in control groups. Previous studies have shown that *D. floralis* infestation can increase GS levels 1.2-fold in the shoots of three cultivated *Brassica* species, but this was observed following a much longer period (8 weeks) after the plants were infested (Birch et al., 1992). The lower starting levels did not reflect a disruption of GS production or an induction in the shoot by *P. rapae* feeding (Traw & Dawson, 2002a), because 7 and 13 days after *P. rapae* was added the GS levels of root-infested plants were on average higher in root-infested plants than in the controls. This indicates that, especially in experiments in which root- and shoot-feeders are present on the plants simultaneously, it is important to track the dynamics of plant chemical profiles over time.

Phenolics, on the other hand, are generally not as acutely toxic as ITC, but are known to act as anti-feedants or to reduce the digestibility of proteins in the food (Duffey & Stout, 1996; Schoonhoven et al., 1998). It is possible, that the higher phenolic levels in plants with *D. radicum* at their roots have contributed to the reduced growth rates of larvae on the shoots of these plants. We found, however, no additive or synergistic effects of increased GS and total phenolic levels on *P. rapae*, because *P. rapae* survived better on plants with both *D. radicum* and *P. penetrans* than with *P. penetrans* alone. Our results thus suggest that the increased diversity of root feeders does not necessarily result in enhanced negative effects of induction to the above-ground herbivore.

We realize that, next to GS and phenolics, there is a plethora of other primary and secondary compounds that were affected differently by root herbivores and that may have contributed to the reduced *P. rapae* performance on root-infested plants. Instead of analysing only a subset of all the primary and secondary compounds in plants, it would be preferable to analyze as many compounds as possible without a bias towards specific compound classes (Fiehn, 2002; Choi et al., 2004). These so-called metabolomic approaches are still underdeveloped for ecological purposes, but eventually will provide a better view on the total dynamics of plant chemical profiles. For example, a metabolomic approach would have allowed us to observe whether there were shifts in the production of specific phenolic products that may have contributed to the observed differences in growth and survival rates of the larvae (Appel & Martin, 1992).

The ecological implications of our finding, that root feeders, and especially phytophagous nematodes, co-determine the chemical profiles of plant shoots are far-reaching. Similar to bacteria and fungi, nematodes are omnipresent in soils and plant roots, and also in *B. nigra*. Of the 15 *B. nigra* plants that we collected at three different locations near Heteren, The Netherlands, in 2004 we found that the roots of 11 individuals contained between 11 and 1023 individuals of *Pratylenchus* spp. (average number of individuals 220 ± 344 (±SEM); C.E. Raaijmakers, H. Duyts and N.M. van Dam, unpubl.). Next to *Pratylenchus* spp., several of these roots were also infested by sedentary endoparasitic root-knot (*Meloidogyne* spp.) and cyst (*Heterodera* spp.) nematodes, as well as by ectoparasitic nematodes (*Paratylenchus* spp.). These nematodes all contain specific sets of digestive enzymes to suit their feeding type and hence elicit different signal interactions with their host plant (Williamson & Gleason, 2003). As a result, the chemical profiles of the above-ground plant parts may differ, depending on which types of nematodes are feeding on the roots.

Our results add to the evidence that the presence of soil organisms, next to genetic variation and nutrient availability, are important determinants of plant quality to above-ground herbivores. Non-pathogenic soil bacteria, for example, are known to induce systemic resistance (ISR) against leaf pathogens as well as resistance against specific leaf herbivores (van Loon et al., 1998). Similarly, the symbiotic association of plant roots with mycorrhiza are

Table 1 Overview and time-line of the experiment. Day 0 = the day that neonate *Pieris rapae* were placed on the shoots of the plants

Day	Event
−48	*B. nigra* seeds sown on glass pearls
−40	*B. nigra* seedlings transferred to pots
−19	*P. penetrans* infestation
−5	*D. radicum* added
0	*P. rapae* larvae placed on plant in clipcages First whole plant harvest (n = 10)
7	*P. rapae* larvae weighed (d7) Second whole plant harvest (n = 10)
13	*P. rapae* larvae weighed (d13) 2-leaf subsample of remaining plants
14	*P. rapae* in sleeve-cages
16	First pupa *P. rapae*
23	Last pupae (three larvae still small, ended)

containers were placed on a table in a greenhouse (21 °C/ 16 °C; L16:D8 photoperiod). Eight days later (Table 1), the seedlings were transferred to 1 l pots containing 1200 g (dry mass) fine river sand that had been sterilized by gamma irradiation (25 kGray). Before the seedlings were planted, three batches of sand were weighed, dried for 12 h at 110 °C, and weighed again to determine the initial water content of the sand. After transferring the seedlings, the pots were supplied with nutrient solution (see below) so that the total water content in them amounted to 14% of the dry sand mass. The sand in the pots was covered with aluminium foil to reduce evaporation. The pots with seedlings were placed in a glasshouse kept at 21 °C during the day and 16 °C at night, under ambient light conditions that were supplied with sodium lamps to maintain the minimum photosynthetical active radiation at 225 μmol m^{-2} s^{-1} for 16 h per day.

Every 2–3 days, five randomly chosen pots were weighed to determine the volume of solution needed to maintain the water content in the pots at 14%. The plants received a 0.5 Hoagland solution with elevated levels of KH_2PO_4 to avoid symptoms of phosphorus deficiency (van Dam et al., 2004). During the entire 9-week experiment, the *B. nigra* plants each received 1100 ml 0.5 Hoagland solution, containing 2 mmol P.

At 19 days before the *P. rapae* larvae were added, the plants were randomly distributed over four root treatment groups with 50 plants each: control (no root herbivores), *P. penetrans* added to roots, *D. radicum* added to roots, and *P. penetrans* and *D. radicum* added to roots.

Addition of the root herbivores

Pratylenchus penetrans nematodes were mass reared in the greenhouse on roots of *Brassica oleracea* plants, grown from seeds collected from a wild *B. oleracea* population near Heteren (Harvey et al., 2003). The plants were grown in 10 l pots, filled with sterilized sand, and fed with Hoagland solution. Nematodes were extracted with tap water by decanting from roots and sand in soil cores drawn from these pots (Brinkman et al., 2004). After 2 days, the *P. penetrans* extracted from the soil and the roots were pooled, and an aliquot was counted using a microscope to determine the nematode density. Eventually, we added 400 *P. penetrans* in 5 ml of tap water per pot to the plants in the *P. penetrans* and *P. penetrans* and *D. radicum* groups, 19 days before the plants were to receive *P. rapae* on their shoots (Table 1). Previous experiments have shown that shoot GS levels decreased significantly 14–28 days after infesting *B. nigra* plants with 350 *P. penetrans* nematodes (N.M. van Dam, C.E. Raaijmakers and W.H. van der Putten, unpubl.). To facilitate infestation of the roots with *P. penetrans*, we added the nematodes through an opening in the sand, created by a 3 cm long piece of straw that was inserted in the soil at the time the seedlings were transplanted. Plants that were not to be infested with *P. penetrans* were treated similarly, but received 5 ml of tap water only.

Delia radicum eggs were kindly provided by Sandrine Gouinguené and Erich Städler, Eidgenossische Forschungsanstalt für Obst-, Wein, und Gartenbau, Wädenswil, Switzerland. For every plant in the *D. radicum* and *P. penetrans* and *D. radicum* groups, ten 2-day-old eggs were placed on a wedge of heavyweight filter paper (300 g m^{-2}) saturated with water. The tip of wedge was inserted into the sand next to the plant at ~1 cm from the root–stem interface (Finch & Ackley, 1977). Plants that were not to be infested with *D. radicum* received a similar wedge without the eggs. After 2 days the wedges were removed. Previous experiments had shown that this method causes visible root damage in >90% of the *B. nigra* plants, and that after 12 days on average 4–5 *D. radicum* larvae are retrieved per plant (N.M. van Dam and C.E. Raaijmakers, unpubl). The plants were infested with *D. radicum* 5 days before the *P. rapae* larvae were placed on the shoot (Table 1).

Pieris rapae growth and survival

Neonate *P. rapae* larvae from a colony reared on Brussels sprout plants were kindly provided by Rieta Gols, Laboratory of Entomology, Wageningen University. At 19 days after infestation with *P. penetrans* and 5 days after the *D. radicum* was added, each plant received one neonate *P. rapae* larvae (Table 1). At the same time, 10 plants per group were harvested to determine the chemical profiles of the shoots just before the *P. rapae* started feeding (procedures see below). The larvae were placed in clip cages [2.5 cm^2 diameter, as in Bezemer et al. (2003)] on a fully expanded leaf at the 3rd or 4th node from the lowest

leaf on the plant. The cages were supported by small bamboo sticks inserted into the soil to prevent their weight from damaging the leaf. Because we removed several plants that had suffered from drought stress from the groups before the larvae were placed on the leaves, the control group contained 36 plants, the *P. penetrans* treatment 32, the *D. radicum* treatment 33, and the *P. penetrans* and *D. radicum* group 34.

The clip cages were checked for larval survival daily. Larvae that had died within the first 2 days were recorded and replaced with larvae from the original batch maintained on Brussels sprouts. These new larvae were added to the total number of larvae that were included in the treatment group, thus the control group eventually contained 37 larvae (1 larva replaced), the *P. penetrans* group 34 (2 replaced), the *D. radicum* group 35 (2 replaced), and the *P. penetrans* and *D. radicum* group 34 (0 replaced). Larvae that died after day 3 were not replaced. During the first 7 days, the cages were moved to a different place on the same leaf before the amount of leaf area available became depleted to ensure that the larvae always had access to ample food.

On day 7, all surviving larvae were weighed. Per group, 10 randomly chosen plants were harvested. The larvae on these plants were removed from the experiment and killed by freezing at −20 °C. The remaining larvae were checked for survival as before, but now the cages were also moved to younger leaves if older leaves became depleted. At day 13, all the remaining larvae were re-weighed. Because we did not have sufficient extra plants to take whole plant samples again, we took two young leaves from each plant to determine shoot chemistry. At day 15, the larvae were removed from their clip cages and enclosed in a sleeve-cage that included several leaves on the plant to ensure that the larvae had continuous access to food.

From day 16 onwards, the larvae started to pupate. At day 23, all but three larvae had either pupated or died. The three remaining larvae were still so small that they were unlikely to pupate in the next few days, if at all, so they were weighed and the experiment was ended.

The pupae were carefully removed from the plant as soon as their chrysalis had formed. They were weighed and placed individually in glass containers covered with perforated parafilm. The pupae were kept in the same greenhouse as the plants until they hatched. The butterflies were frozen at −20 °C for 2 days, then sexed and weighed. The body mass of pupae and butterflies were closely and significantly correlated [regression of log(pupal mass) on log(butterfly mass): $R^2 = 0.71$, $F_{48} = 122.4$, $P<0.001$]. Here, we will use pupal mass as a proxy for adult body mass, because several butterflies that had hatched successfully were damaged during eclosion, for example because their wings stuck to the glass before they were dry. For this reason, we could not determine butterfly mass for all individuals as accurately as pupal mass. Both dates of pupation and emergence were recorded. Only larvae that eventually turned into a butterfly were counted as 'successful'; larvae yielding distorted or dead pupae were counted as 'failures'.

Plant harvest and analysis

Sand was removed from the roots by flushing with ample water. At the same time, roots that were infested by *D. radicum* were checked for the presence of feeding damage. All the *D. radicum* infested plants that we harvested throughout the experiment showed visible feeding damage on the roots, with the exception of two plants at day 0 in the *D. radicum*, and *D. radicum* and *P. penetrans* groups, respectively. These two plants were assumed not to be infested and were excluded from further analysis. Similarly, we checked for the successful infestation of plants by the *P. penetrans* nematodes. At the end of the experiment, nematodes were extracted from five *P. penetrans* and five *P. penetrans* and *D. radicum* plants. Both the soil (by decanting) and the roots (by funnel-extraction) were extracted, as described in Brinkman et al. (2004). The extracts were combined, and the numbers of *P. penetrans* individuals were counted by microscope. All the plants that were examined contained *P. penetrans* nematodes; on average we retrieved 44 (±7 SE) *P. penetrans* per pot. There was no significant difference between the average numbers of nematodes in the *P. penetrans* or *P. penetrans* and *D. radicum* pots (t-test: $t_8 = 0.07$, $P = 0.94$).

The roots and shoots were separated, and the roots were dried with a paper tissue. Both plant parts were weighed to determine fresh mass and placed in separate paper bags to be frozen and stored at −20 °C until they were freeze-dried to a constant weight. After the dry masses had been determined, the water content of the shoots and leaves was calculated as: (total fresh mass − total dry mass)/total fresh mass. The dried plant parts were ground to a fine powder in a ball mill (Retsch, type MM301, Retsch GmbH and Co., Haan, Germany) and stored dry and in the dark until extraction.

For GS extraction, a 50 mg aliquot was weighed in a 15 ml tube and extracted with 4 ml of boiling 70% methanol solution, desulphatased with arylsulphatase (Sigma, St Louis, IL) on a DEAE-Sephadex A25 column (EC, 1990), and separated on a reversed phase C-18 column (Alltima C-18, 150 × 4.6 mm, 3 µm; Alltech, Breda, The Netherlands) on HPLC with an acetonitrile–water gradient (2–65% acetonitrile from 0 to 30 min; flow 0.75 ml min^{-1}). Detection was performed with a photodiode array detector and peaks were integrated at 229 nm. Sinigrin (sinigrin monohydrate, ACROS, New Jersey, USA) was used as an

thought to affect above-ground insect specialization (Gange et al., 2002). Because the distribution of soil organisms is much patchier than that of above-ground organisms (van der Putten et al., 2001), differences in resistance and chemical profiles between plants in natural populations may very well be co-determined by the identity of the root-feeders to which the roots are exposed. It is, however, still an open question whether above-ground herbivores or parasitoids are able to discriminate among plants with different communities of nematodes in their roots.

Thus far, entomologists and chemical ecologists alike have treated the soil mainly as a black box. Now that evidence is accumulating that soil organisms affect shoot quality as well as shoot herbivore performance, we argue that it is time to include the impact of root feeders in our experiments as well as our theories on the evolution of plant–insect interactions.

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