

Heterodera schachtii Nematodes Interfere with Aphid-Plant Relations on *Brassica oleracea*

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Abstract Aboveground and belowground herbivore species modify plant defense responses differently. Simultaneous attack can lead to non-additive effects on primary and secondary metabolite composition in roots and shoots. We previously found that aphid (*Brevicoryne brassicae*) population growth on *Brassica oleracea* was reduced on plants that were infested with nematodes (*Heterodera schachtii*) prior (4 weeks) to aphid infestation. Here, we examined how infection with root-feeding nematodes affected primary and secondary metabolites in the host plant and whether this could explain the increase in aphid doubling time from 3.8 to 6.7 days. We hypothesized that the effects of herbivores on plant metabolites would depend on the presence of the other herbivore and that nematode-induced changes in primary metabolites would correlate with reduced aphid performance. Total glucosinolate concentration in the leaves was not affected by nematode presence, but the

composition of glucosinolates shifted, as gluconapin concentrations were reduced, while gluconapoleiferin concentrations increased in plants exposed to nematodes. Aphid presence increased 4-methoxyglucobrassicin concentrations in leaves, which correlated positively with the number of aphids per plant. Nematodes decreased amino acid and sugar concentrations in the phloem. Aphid population doubling time correlated negatively with amino acids and glucosinolate levels in leaves, whereas these correlations were non-significant when nematodes were present. In conclusion, the effects of an herbivore on plant metabolites were independent of the presence of another herbivore. Nematode presence reduced aphid population growth and disturbed feeding relations between plants and aphids.

Keywords *Heterodera* · *Brevicoryne* · *Brassica* · Glucosinolates · Phloem · EDTA

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Introduction

When a plant is attacked simultaneously by different species of the same feeding guild (e.g., chewers, miners, borers, or suckers), it is likely that these herbivores are competing for the same resources. The herbivores can affect each other via direct competition for primary compounds, if both parties tap from the same pool of solutes, or by systemically triggering the plant's defense system (Salt et al. 1996). This is well-studied for herbivores sharing the same shoot, and also increasingly so for spatially separated species such as aboveground and belowground herbivores (Bezemer and van Dam 2005; van Dam and Heil 2011). Interactions between plant-feeding nematodes and aphids are an interesting model to study multiple attackers on one plant since these organisms have remarkable similarities in feeding strategies and salivary composition (Carolan et al. 2011), are sensitive to the same plant resistance genes (Rossi et al. 1998), and often are economically important pests on the same host plant. However, effects of nematodes and aphids on each other might be asymmetric, as there is more evidence of nematodes affecting aphids (e.g., Bezemer et al. 2005; Kaplan et al. 2011; Wurst and van der Putten 2007) than on aphids affecting nematodes (Kutyniok and Müller 2012; Wardle et al. 2004).

The mechanism behind the asymmetric competition between aphids and nematodes may be found in changes in plant biomass or quality. Primary and secondary metabolites are more likely candidates to reduce aphid population growth than simple changes in plant biomass, since nematode presence also can reduce aphids without significantly affecting plant biomass (Vandegheuchte et al. 2010; Wurst and van der Putten 2007). The nature of these changes in plant quality remains unclear, but the involvement of both primary and secondary metabolites has been implied in many studies. Bezemer et al. (2005) found that nematode presence reduced foliar phenolics and amino acids in the phloem, and that nematode presence reduced aphid reproduction. Kaplan et al. (2011) explicitly tested whether the strength of aphid-induced sinks was dependent on the presence of the gall-inducing nematode *Meloidogyne incognita* (southern root-knot nematode), but found no evidence for competition for primary compounds.

Heterodera spp. are sedentary nematodes like *Meloidogyne* spp.: both species are vascular feeders inducing changes in plant tissue that redirect photoassimilates from the phloem into their feeding structures (Hofmann et al. 2009). There are few studies on *Heterodera*-aphid interactions, mostly done with soybean. In those studies, the presence of aphids and nematodes has additive effects on soybean yield (Hong et al. 2010, 2011; McCarville et al. 2012). Alate aphids preferred plants without nematodes over nematode-infested plants (Hong et al. 2010), but a mechanistic explanation is lacking. Hence, plant-mediated interactions between nematodes and aphids are quite common, but the chemical mechanisms are as

yet unknown. Only a subset of the nematode-aphid studies has investigated involvement of primary or secondary compounds (e.g. Kutyniok and Müller 2012), while it is known that nematode presence also can affect aboveground levels of secondary compounds (Kaplan et al. 2008; van Dam et al. 2005).

Here, we studied *Heterodera*-aphid interactions in *Brassica oleracea* (Brussels sprouts), a plant species with a relatively well-known defense system. Previously, we found that the presence of nematodes (*H. schachtii*, beet cyst nematode) in the soil severely hampered aphid population growth on *B. oleracea* (Hol et al. 2010). Those data were collected from an experiment where we manipulated soil microbial composition by inoculating a dilution series of soil samples to test the role of rare soil microbes on plant growth and how this could affect plant-herbivore interactions. The effects of variations in soil microbial communities have been published (Hol et al. 2010), while here we present changes in plant metabolites that might explain the effects of nematode presence on aphids. The best known defense compounds of the Brassicaceae are glucosinolates, which are expressed both constitutively and after induction (Hopkins et al. 2009; Textor and Gershenzon 2009). Glucosinolates in *Brassica* species can be induced by pathogens (Ludwig-Muller et al. 2002; Vierheilg et al. 2000), insects and nematodes (Lohmann et al. 2009; van Dam et al. 2005; Wurst et al. 2006). Nematodes can have species-specific effects on glucosinolate levels in *Sinapis alba* (white mustard) (Lohmann et al. 2009), with interactions between nematode and earthworm presence. In this study, *M. incognita* increased aromatic glucosinolates only when earthworms were present. Similar results were found by Wurst et al. (2006) showing that *Meloidogyne* infestation increases glucoraphanin concentrations but only in the presence of earthworms. Another interesting interaction between nematodes and other organisms was found for *Pratylenchus* sp. (root lesion nematode), which initially lowered glucosinolate concentrations, but primed plants to increase glucosinolate levels quickly after attack by aboveground caterpillars (van Dam et al. 2005). Thus, nematodes affect glucosinolate production in *Brassica* spp. shoots, and these effects can interact with those from other organisms. However, this will depend on the duration of infestation, as early nematode infection did not affect primary and secondary metabolic profile in *Arabidopsis* nor aphid population growth (Kutyniok and Müller 2012).

In the present study, we used the aphid *Brevicoryne brassicae* (cabbage aphid), a specialist that down-regulates production of jasmonic acid (Soler et al. 2012), a signaling molecule involved in inducing plant defense (Mueller et al. 1993). Glucosinolate biosynthesis can be induced by *B. brassicae*, with stronger effects on indole glucosinolates than on aliphatic glucosinolates (Bidart-Bouzat and Kliebenstein 2011; Khan et al. 2011). A positive correlation was found between aphid performance and concentration of aliphatic

glucosinolates in the phloem (Kos et al. 2012). The potential sensitivity of *B. brassicae* to glucosinolates (Kos et al. 2012) and the ability of nematodes to induce glucosinolates make changes in the levels of these secondary metabolites a plausible mechanism for explaining negative effects of nematode presence on aphids. However, nematode presence has been found to reduce aphid population growth in many different plant hosts that do not contain glucosinolates, and thus a universal mechanism involving primary metabolites may be involved as well. This could be amino acid concentrations in the leaves (van Emden and Bashford 1971) and phloem (Cole 1997), which are known to correlate with *B. brassicae* performance.

Here, both primary and secondary metabolites were investigated to identify changes in plant biomass and metabolites due to nematode presence, and to link these parameters to the reduction in aphid numbers on *B. oleracea*. Our first hypothesis was that the effects of both herbivores on plant parameters will be non-additive, i.e., effects of one herbivore will be magnified or reduced by the presence of the other. This is based on previous studies that found statistically significant interactions between the presence of nematodes and other organisms with respect to plant secondary metabolites (Lohmann et al. 2009; van Dam et al. 2005; Wurst et al. 2006). Our second hypothesis was that nematode-induced changes in primary metabolites correlate with reduced aphid performance. This is based on the fact that effects of nematode presence on aphids have been found in many plant species and a universal mechanism involving changes in primary metabolites seems more likely than species-specific changes in secondary metabolites.

Methods and Materials

Experimental Design The experimental setup was designed as a randomized complete block design with nematode (absence/presence) and aphid (absence/presence) treatments, 7 replicates and 3 soil treatments (Hol et al. 2010). The three soil treatments consisted of increasing dilutions of soil suspensions, which were added to sterilized soil to investigate the effects of bacterial species loss on plant growth (Hol et al. 2010). We used the data from that experiment to investigate plant parameters in relation to nematode presence and aphid performance. Pots filled with 550 g soil were each sown with 2 seeds of *Brassica oleracea* 'Maximus' (Syngenta Seeds, Enkhuizen, the Netherlands). Pots were placed randomly on a table in a greenhouse with controlled conditions (60 % RH; 16 h L, 8 h D, 21 °C/16 °C) and additional lighting. Plants were watered and fertilized regularly (Hol et al. 2010). After 6 week, seedlings were inoculated with 500 juveniles of *Heterodera schachtii*, suspended in water. This initial density of nematodes is low compared with the advised threshold for growing resistant cultivars in the field (Heijbroek et al. 2002) and compared with the thousands of eggs and juveniles

that can be found in 100 g infested soil (Hbirkou et al. 2011). The juveniles originated from a culture on sugar beets in the greenhouse. Non-nematode treatments received the same amount of water (5 ml), but without nematodes. Four weeks after nematode inoculation, five 4th instar *Brevicoryne brassicae* were placed on the *B. oleracea* plants that were allocated to aphid treatment. All pots were placed individually inside a fine-maze net cage. Numbers of aphids per plant were counted three times per week, while nematodes were counted only at the end of the experiment. Nematode cysts were extracted from 550 ml soil via stirring/decantation (Hol et al. 2010) and separated from organic debris using the acetone method (Den Ouden 1954). The number of cysts per sample was determined with image analysis software WINSEEDLE PRO v.7 (Regent Instruments, Canada). The average number of larvae and eggs inside the cysts was 195 ± 14 (min=20, max=396, $N=44$). Plants were harvested after a growing period of 16 week. All plants were still in the vegetative stage at harvest. The harvesting took 2 days, 4 blocks were harvested on day 1 and 3 blocks on day 2. Within blocks, the differences in harvest time for nematode absence/presence was on average less than 4 min, and for aphid absence/presence less than 7 min. Plant shoots and roots were weighed to determine fresh weight, freeze-dried, and weighed again to determine plant dry weight. From each plant, the first two young fully expanded leaves were flash-frozen in liquid nitrogen. The flash-frozen leaves were freeze-dried and ground in a ball mill (Retsch, Germany), and this material was used for analysis of carbon, nitrogen, amino acids, sugars, and glucosinolates.

Organic carbon and total nitrogen were measured in 3 mg of the leaves and roots with a Flash EA 1112 NC analyzer (Interscience, Breda, The Netherlands). Nitrogen allocation to the shoot was calculated on the basis of dry weight as follows: (nitrogen concentration in shoot x shoot biomass)/(nitrogen concentration in shoot x shoot biomass + nitrogen concentration in root x root biomass). Extracts were made from 100 mg ground material to quantify amino acids, sugars, and glucosinolates with an HPLC. Methanol extracts were made according to van Dam et al. (2004), and this extract was used for sugar and amino acid measurements. The extract was diluted 1:100 for sugar and 1:50 for amino acids measurements as described in van Dam and Oomen (2008). Briefly, the diluted sugar extract was run on a Dionex HPLC using a 10 ppm solution containing sorbitol, manitol, trehalose, sucrose, melibiose, glucose, and fructose as references, detection limit 0.1 ppm. This reference solution was diluted to 7.5, 5 and 2.5 ppm to obtain a reference curve, and every 10 samples an additional reference sample was injected to check for deviations (van Dam and Oomen 2008). The analyses of amino acids also were done according to van Dam and Oomen (2008), and here the Sigma AA-S-18 amino acid standard (Sigma, St Louis, MO, USA) was used as reference and diluted for calibration with a detection limit of 0.1 μmol .

Glucosinolate detection was based on van Dam et al. (2004), using a single wavelength detector set to 226 nm. Sinigrin was used as external standard for the glucinolates, and correction factors from Buchner (1987) were used. Detection limits for glucosinolates were 2 μmol . A list of detected glucosinolates is given in Table 1. While the first two young fully expanded leaves were freeze-dried and used for glucosinolate analysis, the next fully expanded leaf was used for analysis of phloem by obtaining EDTA leachate (Bezemer et al. 2005): the fully expanded leaf was cut at the petiole and placed in an Eppendorf tube with ultrapure water for 5 min. In order to reduce the contamination with mesophyll, the leaf was transferred after 5 min. to an 2 ml Eppendorf tube with 1.5 ml EDTA 8 mM, pH=8. Later, this solution was analyzed as representing the phloem, but it might have been contaminated with leaf sap. Determination of phloem sap composition via stylectomy would have been more accurate, but exudation with EDTA can be used for rough analyses (Girousse et al. 1991). The leaf was incubated in the dark at room temperature for 2 h and afterwards the Eppendorf tubes were stored at $-20\text{ }^{\circ}\text{C}$. EDTA extracts were filtered (0.2 μm) and 100 μl were used for amino acids and sugar analysis on the HPLC. The total amount of EDTA solution in one Eppendorf was 1.5 ml, and the assumption was that the phloem contributed little to this total volume. Thus, the concentrations in the 100 μl were used to calculate the total amount of sugars and amino acids in the Eppendorf with 1.5 ml, and then divided by the leaf dry weight to correct for initial size differences. The remaining EDTA extract was used for glucosinolate analysis according to van Dam et al. (2004), but omitting the boiling step and directly starting with adding the extract to a DEAE-Sephadex A25 column. Since glucosinolate concentrations in the phloem are relatively low, we selected a subset of the EDTA extracts for analysis on basis of relatively high sugar and amino acid content (4 of the 7 blocks in total, $N=48$).

After obtaining the EDTA leachate, the leaf was freeze-dried and its weight was determined.

Statistical Analyses The experiment consisted of 84 pots, based on four herbivore treatments (no herbivores, nematodes only, aphids only, nematodes and aphids), seven replicates and a soil microbial treatment with three levels. The replicates were distributed randomly over 7 blocks (fully randomized block design) to account for spatial effects in the greenhouse. The three different soil microbial inoculum treatments where different dilutions of soil suspensions were added have been described previously (Hol et al. 2010). For all parameters presented here, we tested for 3-way interactions between nematodes, aphids, and microbes. The absence of significant 3-way interactions showed that there were no effects of microbial soil treatment on aphid-nematode interactions. Hence, the data shown here were pooled for all three soil treatments, resulting in $N=21$ per herbivore treatment. For the analysis of aphid and nematode effects on plant parameters, a *linear mixed effects model (lme)* was used with the soil microbial inoculum treatment nested within block as random factor and the presence of nematode and aphids as categorical factors. For all variables (except %water) presented in Table 2 that were significantly affected by either nematodes or aphids, we verified that there was no significant interaction with soil microbial treatment when this was included as fixed factor. Water percentages showed a significant interaction between nematodes and soil microbial treatment, but ignoring this interaction still gave overall significant main effects of nematodes on moisture. The aphid \times nematode interactions were not significant for any parameters in Table 2, and hence were removed from the model. Most variables were log-transformed to obtain normality of errors, which was assessed by visual inspection of q-q plots. When transformation did not result in sufficiently normal error distributions, non-parametric tests were performed to

Table 1 Abbreviation and Full Names of the 11 Detected Glucosinolates

Abbreviation	Common name	Synonyms
IBE	Glucoiberin	3-methylsulfinylpropylglucosinolate
PRO	Progoitrin	2(S)-hydroxy-3-butenylglucosinolate
RAPH	Glucoraphanin	4-methylsulphinylbutylglucosinolate
SIN	Sinigrin	2-propenylglucosinolate
GNA	Gluconapin	3-butenylglucosinolate
GNL ^a	Gluconapoleiferin	2-hydroxy-4-pentenylglucosinolate
TROP	Glucotropaeolin	Benzylglucosinolate
4OH	4-hydroxyglucobrassicin	4-hydroxy-3-indolylmethylglucosinolate
GBC	Glucobrassicin	3-indolylmethylglucosinolate
4-MeOH GBC	4-methoxyglucobrassicin	4-methoxy-3-indolylmethylglucosinolate
NEO ^a	Neoglucobrassicin	1-methoxy-3-indolylmethylglucosinolate

^a glucosinolates which occurred at very low concentrations; not listed in Table 2

Table 2 Mean (\pm Se) of Plant Parameters from *Brassica oleracea* Plants without Herbivores (Control), with Aphids, Nematodes (Nema), or Both

	Control	Aphids	Nema	Aphids+Nema	<i>P</i> - Aphids ^a	<i>P</i> - Nema ^a
Shoot biomass ^b	3.79 \pm 0.13	3.49 \pm 0.17	3.07 \pm 0.13	3.02 \pm 0.11	0.15	<0.01
Root biomass ^b	1.84 \pm 0.11	1.70 \pm 0.07	1.73 \pm 0.11	1.90 \pm 0.14	0.94	0.88
Water % shoot ^c	79.9 \pm 0.3	80.4 \pm 0.4	78.5 \pm 0.3	79.0 \pm 0.4	0.18	<0.01
N % shoot	1.02 \pm 0.04	1.08 \pm 0.07	0.84 \pm 0.05	0.82 \pm 0.03	0.62	<0.01
N % root	1.26 \pm 0.07	1.25 \pm 0.06	1.43 \pm 0.07	1.47 \pm 0.07	0.77	0.01
N shoot allocation ^d	0.63 \pm 0.01	0.63 \pm 0.02	0.51 \pm 0.02	0.48 \pm 0.02	0.48	<0.01
Carbon shoot ^c	41.0 \pm 0.3	40.4 \pm 0.4	41.3 \pm 0.3	40.3 \pm 0.3	0.01	0.87
Carbon root ^c	31.0 \pm 1.65	31.2 \pm 1.54	31.7 \pm 1.56	31.5 \pm 1.7	0.99	0.72
C:N shoot	41.7 \pm 1.68	39.6 \pm 1.9	52.3 \pm 2.7	50.5 \pm 2.0	0.35	<0.01
C:N root	24.6 \pm 0.57	24.8 \pm 0.45	22.2 \pm 0.33	21.4 \pm 0.5	0.35	<0.01
<i>Sugars</i> ^e Leaf						
Total	46.1 \pm 3.2	43.3 \pm 2.9	49.8 \pm 2.0	50.4 \pm 2.0	0.57	<0.01
Glucose	23.6 \pm 2.0	21.3 \pm 1.6	25.7 \pm 1.1	26.2 \pm 1.1	0.42	<0.01
Fructose	16.8 \pm 1.1	16.1 \pm 1.1	18.4 \pm 0.9	18.3 \pm 0.8	0.62	0.02
Sucrose	5.7 \pm 0.4	6.0 \pm 0.5	5.7 \pm 0.3	5.8 \pm 0.3	0.51	0.77
<i>Sugars</i> ^e Phloem						
Total	1.63 \pm 0.18	1.81 \pm 0.25	1.31 \pm 0.21	1.15 \pm 0.13	0.66	<0.01
<i>Amino acids</i> ^e Leaf						
Total	92.2 \pm 5.9	89.8 \pm 5.8	95.3 \pm 4.7	94.2 \pm 4.2	0.63	0.33
Arginine	8.1 \pm 0.7	7.9 \pm 0.6	6.3 \pm 0.5	6.1 \pm 0.2	0.67	<0.01
Leucine	20. \pm 0 1.3	21.0 \pm 1.8	19.5 \pm 1.1	19.5 \pm 1.1	0.94	0.63
Threonine	31.2 \pm 2.5	28.7 \pm 2.1	33.8 \pm 1.6	33.6 \pm 1.5	0.40	0.02
Tryptophan	0.6 \pm 0.1	0.7 \pm 0.1	0.7 \pm 0.0	0.8 \pm 0.1	0.04	0.02
Valine	32.4 \pm 2.2	31.6 \pm 2.2	35.1 \pm 2.1	34.1 \pm 1.8	0.59	0.12
<i>Amino acids</i> ^e Phloem						
Total	2.49 \pm 0.31	2.89 \pm 0.46	1.96 \pm 0.34	1.86 \pm 0.23	0.42	<0.01
<i>Glucosinolates</i> ^{f,g} Leaf						
Total	1.91 \pm 0.34	1.89 \pm 0.25	1.66 \pm 0.37	1.42 \pm 0.17	0.74	0.21
IBE	0.60 \pm 0.12	0.56 \pm 0.10	0.44 \pm 0.11	0.38 \pm 0.07	0.93	0.06
PRO	0.14 \pm 0.04	0.15 \pm 0.03	0.12 \pm 0.05	0.09 \pm 0.02	0.94	0.15
RAPH	0.14 \pm 0.04	0.15 \pm 0.04	0.18 \pm 0.08	0.11 \pm 0.03	0.75	0.79
SIN	0.23 \pm 0.06	0.19 \pm 0.03	0.13 \pm 0.03	0.12 \pm 0.02	0.97	0.08
GNA	0.06 \pm 0.01	0.04 \pm 0.01	0.02 \pm 0.00	0.02 \pm 0.00	0.46	<0.01
TROP	0.05 \pm 0.01	0.06 \pm 0.01	0.06 \pm 0.00	0.06 \pm 0.00	0.49	0.57
4OH	0.12 \pm 0.01	0.14 \pm 0.01	0.14 \pm 0.02	0.13 \pm 0.01	0.34	0.58
GBC	0.49 \pm 0.09	0.43 \pm 0.06	0.44 \pm 0.08	0.39 \pm 0.05	0.60	0.73
4MeOH GBC	0.09 \pm 0.01	0.16 \pm 0.02	0.11 \pm 0.03	0.10 \pm 0.01	<0.01	0.41
<i>Glucosinolates</i> ^f Phloem						
Total	0.52 \pm 0.21	1.72 \pm 1.02	0.81 \pm 0.52	1.13 \pm 0.46	0.10	0.83

^a based on linear mixed effects model with main effects Aphids+Nema, no significant interactions, $n=21$ Significant values are indicated in bold (based on False Discovery Rate control, Benjamini and Hochberg 2000). Details of transformation and t -values are given in Online Resource 1

^b gram dry weight

^c percentage

^d (% N shoot x shoot biomass)/(% N shoot x shoot biomass+% N root x root biomass)

^e Sugars and amino acids concentrations in the leaf or in phloem ($\mu\text{mol g}^{-1}$ dw)

^f glucosinolate concentrations in the leaf ($\mu\text{mol g}^{-1}$ dw) and in phloem (nmol g^{-1} dw)

^g abbreviations of glucosinolates are listed in Table 1

verify the outcome of the linear mixed effects model. In all cases, results were congruent with the outcomes from non-parametric tests, and thus only results from the *linear mixed effects models (lme)* are shown. One phloem sample was lost during processing. Phloem data were analyzed with the same *linear mixed effects model (lme)* as above, but including harvesting time as covariate. For parameters significantly affected by aphids or nematodes the correlation between numbers per plant and response value was tested with Spearman rank correlation tests.

Aphid numbers per plant over time were used to fit an exponential growth curve and estimate population doubling times. *ANCOVAs* were used to test whether relations between plant parameters (%nitrogen in shoot, %water, and the individual sugars, amino acids, and glucosinolate concentrations) and aphid doubling times interacted with nematode presence. Plants with less than 10 aphid individuals (3 plants with nematodes, 6 plants without nematodes) were omitted from this analysis. To adjust *P*-values to correct for type 1 errors due to multiple testing, sharpened Benjamini and Hochberg (2000) false discovery rate control was performed (Verhoeven et al. 2005) for the *lmes* and for the *ANCOVAs*. Significance levels were determined based on the distribution of the *P*-values, and estimates of the true alternative cases and may, therefore, vary between datasets. In Table 2, individual *P*-values are shown, but only those that were significant after correcting for multiple tests are printed in bold ($P < 0.01$). The multivariate technique partial least squares regression (*PLSR*) was used to investigate the relation between aphid doubling times and all primary and secondary metabolites of the plant simultaneously. Data were log-transformed, scaled to unit variance, and were mean-centered before analyses. The analysis was done for all aphid doubling times and separately for those on plants with nematodes and those on plants without nematodes (3 *PLSR* in total). The optimal number of latent structures was determined on the basis of “leave one out” validation. Significance of the model was determined by comparing the explained variation in aphid doubling time with the explained variation of 1,000 models using permutations of the aphid doubling time data. A model explaining more variation than 95 % of 1,000 permutations was considered significant. All analyses were done in R 3.0.0 (R Development Core Team 2013) using the ‘nlme’ and ‘pls’ packages (Mevik and Wehrens 2007).

Results

Plant and Leaf Measurements Given the densities used and the duration of the infestation, it was no surprise that the impact of nematodes on plant biomass was more severe than that of aphids. Nematodes significantly decreased shoot biomass on average by 13 %, while the effects of aphids on shoot biomass were not significant (Table 2). Root biomass was not significantly affected

by either herbivore treatment (Table 2). Aphid population doubling time was significantly increased by nematode presence (Fig. 1a, *lme*, $df=13$, $P < 0.01$), while nematode numbers were not significantly affected by aphid presence (Fig. 1b). Water percentages in the shoot were significantly lower on plants with nematodes, although the differences were small (Table 2). Nematodes strongly decreased nitrogen allocation to the shoot, resulting in lower nitrogen concentrations (Table 2), while root nitrogen levels increased in plants with nematodes. The C:N ratio in shoots and roots showed the reversed pattern of the nitrogen concentrations, since carbon levels were not affected by nematodes (Table 2). Aphids had no significant effects on nitrogen concentrations or allocation.

Leaf sugar concentrations (glucose) increased in nematode-infested plants (Table 2). Nematodes decreased arginine, but the total concentration of amino acids was not significantly affected. Aphids did not affect sugar or amino acid levels in the leaves.

Total glucosinolate concentrations in the shoot were not different between treatments, but there were shifts in individual concentrations (Table 2). Gluconapin concentrations in the leaves were reduced by 58 % as a result of nematode infestation, while one minor glucosinolate, gluconapoleiferin, increased in plants with nematodes. In fact, in the absence of nematodes this glucosinolate was detected in only 1 out of 42 plants, while it was found in 41 of the nematode-infested

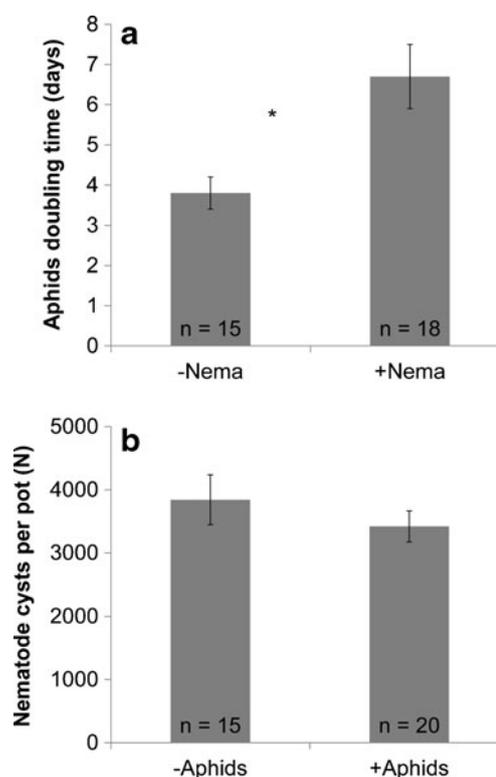


Fig. 1 The presence of nematodes *Heterodera schachtii* (Nema) increased the doubling time of the aphid *Brevicoryne brassicae* on *Brassica oleracea* (a) while the presence of aphids (Aphids) had no significant effect on the number of nematode cysts per pot (b)

plants. Gluconapoleiferin levels in the shoot were positively correlated with the number of cysts per plant (Fig. 2). Aphids had consistent effects on only one glucosinolate; 4-methoxyglucobrassicin, which was increased (+32 %) in the leaves of plants with aphids only. The number of aphids at the end of the experiment correlated positively with 4-methoxyglucobrassicin concentrations in the leaves (Fig. 3).

Phloem Measurements Concentrations of compounds in phloem sap varied strongly depending on the harvest time and day (Online Resource 2). This had a large impact on the absolute amounts of the compounds, but not on the detection of treatment effects. The nematode treatments reduced total sugar concentration in the phloem sap by 20 % (Table 2), with similar decreases in all individual sugars (data not shown). Four amino acids were detected in all phloem samples: arginine, threonine, leucine, and tyrosine. Aphids alone did not affect total or individual amino acid concentrations (Table 2) in the phloem compared to the control plants, while nematodes reduced total amino acid concentrations by 21 % (Table 2). The individual amino acids showed the same pattern. The concentrations of glucosinolates in the phloem varied widely, from 0.03 to 12.7 nmol g⁻¹ dw. The highest concentration was found in the plant with the highest number of aphids. 4-Methoxyglucobrassicin was found in all phloem samples analyzed, while it was usually one of the less abundant components in the leaves (Table 2). Five other glucosinolates (sinigrin, glucobrassicin, raphanin, gluconapin, and 4-hydroxyglucobrassicin) were found occasionally, in 2–15 % of the phloem samples. There were no significant effects of aphids or nematodes treatments on glucosinolate concentrations in phloem samples.

Linking Aphid Growth Rate to Metabolites Significant effects on aphid population doubling times were found for eight plant parameters. The relation between those plant parameters and

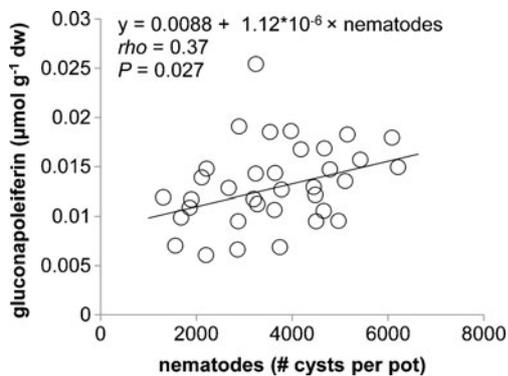


Fig. 2 Spearman Rank Correlation between the number of *Heterodera schachtii* (cysts per plant) and the concentrations of gluconapoleiferin in the shoot of *Brassica oleracea*; except for one plant gluconapoleiferin was not detected in the absence of nematodes

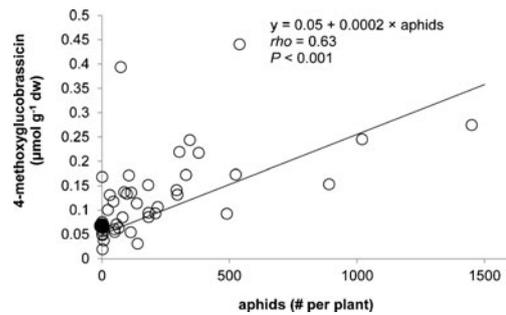


Fig. 3 Spearman Rank Correlation between the number of *Brevicoryne brassicae* (number of aphids per plant) and the concentrations of 4-methoxyglucobrassicin in the shoot of *Brassica oleracea*; the closed circle denotes the median 4-methoxyglucobrassicin in plants without aphids

aphid population doubling time depended on the presence of nematodes: in the absence of nematodes, aphid population doubling time correlated negatively with both primary and secondary metabolites in the leaves. Three amino acids (arginine, valine, threonine) and five glucosinolates (all aliphatic glucosinolates) correlated negatively with aphid doubling times (Fig. 4a and b, Online Resource 3). However, on plants with nematodes, no significant correlations were found. Similar results were found when exploring the relationship between aphid doubling times and plant parameters with the multivariate partial least squares regression (*PLSR*). When separate *PLSR* models were made for the datasets in absence and presence of nematodes, no significant model was found for the aphid doubling times on plants with nematodes. In contrast, in absence of nematodes, 77 % of the variation in aphid doubling times was explained (Online Resource 4) by

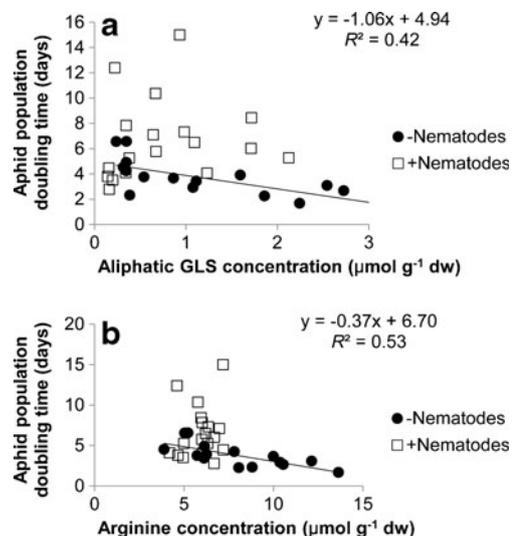


Fig. 4 Pearson Correlation between the doubling time of *Brevicoryne brassicae* population and a. concentrations of aliphatic glucosinolates or b. arginine in the leaves of *Brassica oleracea*; closed circles are plants without nematodes, open squares are plants with nematodes. The correlations were only significant for plants without nematodes (closed symbols) and those correlations are indicated here with trendlines

the same compounds as above: the amino acids arginine, valine, threonine, and the glucosinolates sinigrin, glucoiberin, progoiterin, glucoraphanin, and gluconapin were all negatively correlated with aphid doubling time.

Discussion

In this study, inoculation with nematodes altered a number of plant parameters, but there was no evidence that those effects were magnified or reduced by the presence of aphids. Based on these results, we reject our first hypothesis that the shared feeding guild of nematodes and aphids leads to non-additive effects on plant parameters. This result is in strong contrast with other studies where the presence of nematodes had different effects on plant parameters when other herbivores or decomposers were present. Wurst et al. (2006) and Lohmann et al. (2009) observed that nematodes increased glucosinolate levels in the plant, but only when earthworms were present. Van Dam et al. (2005) found that aboveground herbivores induced glucosinolates faster on plants with nematodes than on plants without nematodes. Possibly the infestation levels of aphids in our experiment were not sufficient to induce defense mechanisms and/or affect induction by nematodes via crosstalk. However, a recent study (Soler et al. 2012) found that even lower numbers of *B. brassicae* reduced jasmonic acid levels tenfold in *Brassica oleracea* within 144 h after attack. Similarly, Kutyniok and Müller (2012) demonstrated effects of ten adult aphids on secondary metabolites in *Arabidopsis thaliana* already 3 days after infestation. Moreover, Kuśnierczyk et al. (2008) found changes in *Arabidopsis thaliana* glucosinolate levels already two days after infestation with *B. brassicae*. The responses observed in the latter two studies, however, may have been short-term effects caused by the immediate stress of infesting a relatively small plant (<0.5 g fresh weight) with ten or more aphids at once. In our experiment, only five aphids were used to start the populations, which developed in a more natural way over 6 weeks' time. Eventually, the infestation levels should have been sufficient to induce plant defense responses, yet plant biomass and primary metabolites were hardly affected by aphid presence. Possibly, plants can compensate for the effects of aphid infestations when populations start with a few individuals. Another possible explanation for the difference in effects between different experiments stems from the order of attack. In our experiment, nematodes were inoculated 4 weeks earlier than the aphids. Timing and order of attackers has been shown to affect defense levels as well as effects on herbivores (Erb et al. 2008, 2011). In a study where *Heterodera schachtii* and *Brevicoryne brassicae* were added at the same time to *Arabidopsis thaliana*, aphids showed strong effects on plant parameters, and their presence reduced nematode numbers (Kutyniok and Müller 2012).

Both shoot and root herbivores induced changes in glucosinolate composition: aphids increased 4-methoxyglucobrassicin and nematodes gluconapoleiferin. This corroborates earlier findings that root induction increases aliphatic, and shoot induction indole glucosinolates (van Dam and Oomen 2008). The positive correlation between aphid numbers and 4-methoxyglucobrassicin levels in the shoot does not reveal, however, which factor is driving the other. The overall increase in 4-methoxyglucobrassicin concentrations compared to plants without aphids suggests that aphids are inducing 4-methoxyglucobrassicin production, as has been found earlier for *B. oleracea* infested with *B. brassicae* (Khan et al. 2011). An earlier study showed that *Myzus persicae* also induced 4-methoxyglucobrassicin levels in *A. thaliana*, which enhanced resistance to this generalist aphid species (Kim and Jander 2007). It is not known whether 4-methoxyglucobrassicin or its breakdown products (Agerbirk et al. 2009) could affect this specialist aphid, but in a multivariate analysis, Kos et al. (2012) found a positive correlation between 4-methoxyglucobrassicin and aphid adult weight as well as number of offspring.

Nematodes did not affect 4-methoxyglucobrassicin, but changed the levels of several other glucosinolates in the shoots. The increase in gluconapoleiferin was remarkable, since it was essentially not detected in plants without nematodes. The induction of gluconapoleiferin by nematodes was at least 2.5-fold if we postulate that the concentrations of gluconapoleiferin in plants without nematodes was just below the detection limit. After induction, the levels were still low (min 0.005 max 0.025 $\mu\text{mol g}^{-1}$ dw), but it was found consistently in plants with nematodes. We can speculate about the mechanism behind this specific induction. Gluconapoleiferin is the only aliphatic glucosinolate in *B. oleracea* plant tissues that has five carbon atoms in its side-chain. Sinigrin and glucoiberin have only three (C3), whereas glucoraphanin, gluconapin, and progoitrin have four (C4). Gluconapoleiferin is the hydroxylated product of glucobrassicinapin, the alkenyl product of glucoalyssin, a side chain modification of glucobetteroin (Sun et al. 2011). The precursors could be produced by one more cycle of side chain elongation (Padilla et al. 2007), and the side chain modifications could be performed by the same enzyme converting C3 and C4 glucosinolates. However, none of these precursors were found in our samples, and thus the simplest pathway to gluconapoleiferin would be a chain elongation of progoitrin. The biological relevance of the increased levels of gluconapoleiferin also remains unclear. It has been reported to increase susceptibility to the fungus *Sclerotinia sclerotiorum* in *Brassica napus* (Fan et al. 2008), but nothing is known about effects on aphids. Similarly, it is unclear how the glucosinolate gluconapin, which was decreased by nematodes, affects aphid populations. Other studies that addressed effects of nematodes on leaf glucosinolates (Kabouw et al. 2011; Kutyniok and Müller 2012; Lohmann et al. 2009; Van Dam et al. 2005; Wurst et al. 2006) showed no clear pattern, but we can conclude

that there was no increase in total glucosinolates after nematode infestation. In terms of specific glucosinolate classes, increases (Lohmann et al. 2009), decreases (van Dam et al. 2005; Wurst et al. 2006), or no effects (Kutyniok and Müller 2012) have been found in aliphatic compounds. Changes in indole glucosinolate concentrations after nematode infestation have not yet been reported.

Our hypothesis that primary metabolites correlate with aphid performance could not be supported. Nematodes affected both primary and secondary metabolites, but we could not correlate those to aphid performance. Decreases in arginine and threonine concentrations also were observed by Bezemer et al. (2005) who found that nematodes decreased amino acid levels in the phloem of grasses. Based on the relationship between arginine concentration and aphid doubling time (Fig. 4b), the average reduction of arginine concentration by nematodes from 8 to 6.2 $\mu\text{mol g}^{-1}$ dw in our study could explain an increase in aphid population doubling time from 3.8 to 4.4 days. However, other factors will have played a role as well since aphid population doubling time actually increased to 6.7 days in the presence of nematodes. The negative effects of nematode presence on aphids could be caused by a combination of primary and secondary metabolites as well as by mechanical changes, and hence the precise mechanism may be difficult to reveal. Alternatively, the plant parameter responsible for the effects might not have been included in our measurements, or was measured at an inappropriate time. Induced defense might change over time. Phloem chemistry was expected to correlate with aphid growth rate, but the analysis was hampered by fluctuations in concentrations during the day and between days in this study. Diurnal fluctuations in phloem are somewhat controversial due to methodological issues (Kallarackal et al. 2012), and the present experiment was not aimed at detecting those fluctuations. Regardless of the lack of correlation between measured chemical parameters and aphid numbers, the delay in aphid population growth by nematode presence could be caused by mechanical factors, such as potential changes in cuticular waxes (Kuhlmann and Müller 2010), leaf toughness, or water content. The slight decrease in water content of the shoots caused by nematodes may have reduced aphid population growth, as continuous water stress in general impacts negatively on phloem feeding insects (Huberty and Denno 2004).

In Summary, we showed that nematodes interfere with the interactions between aphids and their host plants, and that this correlates with chemical changes in the host plants. However, the correlations between aphid population doubling time and amino acids or glucosinolates disappear when nematodes are present, and this was confirmed by the multivariate analysis. This shows some similarity with the study from Kutyniok and Müller (2012), who found that early *Brevicoryne brassicae* infestation had a significant impact on the metabolic profile of *Arabidopsis thaliana* compared to non-infested control plants, but this was not significant when plants were infested with

both *Brevicoryne brassicae* and *Heterodera schachtii*. In our study, correlations between aphid performance and plant metabolites were found for metabolites that were significantly affected by nematodes (arginine, threonine, gluconapin) as well as for metabolites not significantly affected by nematodes (valine, glucoiberin, progoitrin, glucoraphanin, sinigrin). The compounds themselves may not be the mechanism by which nematode presence affects aphids, but rather a marker for other chemical changes interfering with aphid metabolism. Future research should not only focus on the concentration of compounds that are significantly changed by nematodes, but also consider the correlation between aphids and (non)-chemical plant parameters. Additionally, the roles of primary and secondary metabolites in aphid performance need to be verified in experiments using manipulated plants (Kim and Jander 2007) or diets (Macel et al. 2005) to test whether specific compounds are responsible for reduced aphid performance. While such studies are crucial to prove mechanisms, it is also crucial to use ecological and evolutionary relevant study systems, such as wild cabbages, which are much better defended than cabbage cultivars (Harvey et al. 2011).

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