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REGULAR ARTICLE

Timing of simulated aboveground herbivory influences population dynamics of root-feeding nematodes

Minggang Wang · Arjen Biere · Wim H. van der Putten · T. Martijn Bezemer · E. Pernilla Brinkman

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Abstract
Aims Plant damage inflicted by aboveground herbivores can occur at different stages of plant development and can induce plant responses that affect the growth of belowground herbivores. This study explores impacts of aboveground herbivory at different plant development stages on the population dynamics of root-feeding nematodes.
Methods We simulated aboveground herbivory by clipping the foliage of the grass species *Holcus lanatus*, and tested how plant defoliation at different times (1, 4 or 7 weeks after nematode inoculation) influenced the population of two root-feeding nematode species: the endoparasitic *Pratylenchus penetrans* and the ectoparasitic *Tylenchorhynchus dubius*.
Results Defoliation increased the total abundance of *P. penetrans* and the number per unit root mass (density) of both *P. penetrans* and *T. dubius*. Defoliation enhanced the density of *P. penetrans*, however, only when plants were defoliated early. Timing did not influence the density of *T. dubius,* although both abundance and density increased over time. Defoliation increased the nitrogen concentration of plant roots, but reduced root biomass. The strongest reduction of root biomass occurred after early defoliation.
Conclusions Our study indicates that plant responses to aboveground herbivory and their effects on belowground herbivores can be influenced by the time when plants are defoliated, as well as by the belowground herbivore species and their interactions.

Keywords Defoliation · *Holcus lanatus* · Plant-feeding nematodes · *Pratylenchus penetrans* · *Tylenchorhynchus dubius* · Above-belowground interactions

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Introduction

In grasslands, aboveground plant biomass is periodically removed by herbivore grazing or mowing, resulting into changes in plant nutrient content (Jaramillo and Detling et al. 1988) and productivity (McNaughton et al. 1998). Effects of defoliation on the availability of resources in roots and in the rhizosphere may also influence activities of decomposing microbes and root feeding soil fauna (Bardgett and Wardle 2003). Soil dwelling nematodes have frequently been used to test the response of soil biota to such aboveground disturbances (Bardgett et al. 1997, Frank et al. 2000; Wang et al. 2006; Veen et al. 2010). Several studies have

reported declines in soil nematode densities following aboveground grazing (e.g. Todd 1996), which is expected to be due to reduced availability of belowground resources (Bardgett et al. 1997). However, other studies have reported increases or non-significant effects of aboveground grazing by mammals or shoot clipping on the abundance of root-feeding nematodes (Freckman et al. 1979; Wall-Freckman and Huang 1998; Zolda 2006). The occurrence of positive effects can be explained by changes in the quality and quantity of root substrate or by the reduction in plant resistance as a result of defoliation (Stanton 1988).

The majority of studies that have examined effects of aboveground herbivory or grazing on soil-dwelling nematodes have focused on how defoliation affects root-feeding (also named plant-parasitic) nematodes. Root-feeding nematodes directly interact with their host plants and they can act as important belowground pests in agricultural systems (Abawi and Widmer 2000). Several mechanisms have been proposed for the effects of aboveground grazing on root-feeding nematodes. Ingham and Detling (1984) proposed that grazing by prairie dogs increased the abundance of root-feeding nematodes due to a grazing-induced improvement of microclimate. Further, grazing can increase plant root growth, which could also explain observed higher abundances of root-feeding nematodes under grazed plants (Schon et al. 2010). Finally, defoliation can also influence root-feeding nematode abundance through its impact on the nutritional quality of roots. For example, several studies have shown that aboveground defoliation can increase the nitrogen concentration of roots (Seastedt et al. 1988) or increase the carbon flux to roots, thereby influencing resources available to root-feeding nematodes and other soil organisms (Holland et al. 1996; Bazot et al. 2005). However, other studies have shown that defoliation can cause a decrease in nitrogen uptake of root feeders, possibly due to a decrease in plant root diameter (Mackie-Dawson 1999), or a decrease in nitrogen content in roots (Garcia and Rice 1994). This ultimately reduces the nutritional quality of the resources for root herbivores (Guitian and Bardgett 2000; Mikola et al. 2001, 2005; Lestienne et al. 2006). The ratio between carbon and nitrogen (C/N) in plant tissues is often considered an important measure of plant quality for herbivores (Mattson 1980; Seastedt et al. 1988; Masters et al. 1993), and an increase in the C/N ratio in plants exposed to defoliation has been shown to correlate with a reduction in root-feeding nematode numbers (Bazot et al. 2005).

The impact of defoliation on plant-associated belowground processes (Richards 1984; Hester et al. 2004) and subsequently on belowground organisms can also depend on its timing (Ilmarinen et al. 2005). So far, the majority of studies on effects of timing of defoliation on plant responses have predominantly focused on aboveground plant responses (Maschinski and Whitham 1989; Obeso and Grubb 1994; García and Ehrlén 2002; Akiyama and Ågren 2012), whereas information on the responses of plant roots is scant. Plants usually show seasonal changes in numerous properties, for example in traits related to resistance, levels of induced resistance (Karban and Baldwin 1997), plant chemistry (Bowers and Stamp 1993), or nutrient status (Mattson 1980). All these temporal changes may result in time-dependent responses of plant-associated organisms and this also applies to root-feeding nematodes (Ilmarinen et al. 2005).

Plants also can show ontogeny-dependent growth responses to defoliation (McNaughton 1983; Boege 2005), which can influence root-feeding nematodes as well. For example, Ilmarinen et al. (2005) reported that defoliation reduced the root C/N ratio when plants were defoliated at an early growth stage, but increased the root C/N ratio when defoliation occurred at a later stage of plant development. In the same study, late defoliation promoted the abundance of herbivorous and predacious nematodes, whereas there was no such response by other trophic groups of nematodes (Ilmarinen et al. 2005). Other studies have reported that aboveground herbivory can reduce the performance of belowground herbivores, but only when the aboveground herbivores arrive on the plant prior to the belowground herbivores (Erb et al. 2011; Johnson et al. 2012). The physiological responses of roots may also be determined by the timing of aboveground herbivory, which in turn may influence the performance of root-feeding herbivores (Kaplan et al. 2008).

Here, we examined how timing of defoliation of the grass *Holcus lanatus* influenced root-feeding nematode population dynamics in a mixture of the migratory endoparasite *Pratylenchus penetrans* and the ectoparasite *Tylenchorhynchus dubius*. We also examined how the carbon and nitrogen concentration in roots changed following defoliation. *Holcus lanatus* is a common perennial grassland species that occurs on various soil types (Beddows 1961). We focused on *P. penetrans*, because it is commonly found in the roots of various grassland plant species (Thies et al. 1995) and it is an

economically important crop pathogen (Jones and Fosu-Nyarko 2014). The ectoparasite *T. dubius* is a common species that can develop high population densities and cause severe growth reduction to many plant species (Reynolds and Evans 1953; Sharma 1971). We tested the following hypotheses: 1) defoliation increases root quality (decreased C/N concentration) and numbers of root-feeding nematodes. 2) Earlier defoliation more strongly increases root quality and root-feeding nematode populations than later defoliation. 3) Due to their different feeding location on plant roots (Klinkenberg 1963), *P. penetrans* (feeding internally on cortical cells) will respond more strongly to changes in root quality following defoliation than *T. dubius* (feeding on epidermal cells).

Materials and methods

Soil, plant materials and inoculum

Soil was collected from a restored grassland (De Mossel, Ede, the Netherlands, 52.04 °N 5.44 °E) on former arable land. *Holcus lanatus* occurs abundantly in these restoration grasslands (Korthals et al. 2001). In the laboratory, the soil was sieved using 5 mm mesh, homogenized and gamma sterilized (> 25 KGray). Seeds of *H. lanatus* were obtained from a wild-seed supplier (Cruydt-hoeck, Nijeberkoop, The Netherlands). The seeds were surface sterilized with sodium hypochlorite (1%) for 1 min and rinsed 4 times with demineralized water, sown on moist glass beads and placed in an incubator (16 h light, 25/20 °C day/night temperature) until germination.

Nematodes were obtained from Applied Plant Research (PPO), Wageningen University & Research Center, who collected the soil from an agricultural field at Vredepeel experimental station, the Netherlands. The nematode community was dominated by two species of root-feeding nematodes: *P. penetrans* and *T. dubius*. *P. penetrans* is a migratory endoparasitic nematode species, mainly feeding on cell contents in the cortex of plant roots, while *T. dubius* is an ectoparasitic nematode species that prefers feeding on epidermal cells or root hair cells. The two species can simultaneously occur in natural areas and agricultural fields, where they can reach high densities and cause severe plant damage. These two species comprised 98.4% of the root-feeding nematode community, in which *P. penetrans* dominated due to its higher specialization with the cultivated crops in the field and higher resistance against agricultural practices. The ratio of *P. penetrans* to *T. dubius* in the field collected community was 10:1.

Experimental design

We filled 180 1 L pots each with 800 g soil (water content =12.3% *w*/w) and planted one one-week-old seedling of *H. lanatus* in each pot. Pots were randomly placed in a greenhouse with 16/8 h light/dark and 21/18 ± 2 °C day/night. Three weeks later, all pots were inoculated with 4 ml nematode suspension containing on average 100 (SE = ± 6.8) nematode individuals per 4 ml (91 *P. penetrans* and 9 *T. dubius*). At week 0, nematodes were inoculated into two 1-cm-deep holes (2 ml per hole), which were closed immediately using the surface layer of soil. The soil surface of each pot was then covered with a thin layer of fine sand to minimize evaporation.

Plants were watered three times per week. Once a week, the soil moisture content was adjusted to 12.3% (*w*/w) with demineralized water and all the pots were rotated within the greenhouse to limit effects of position. Nutrients were added once per week using Hoagland solution (Hewitt 1966). The nutrient dosage was gradually increased over time to meet plant growth demands (Van der Putten et al. 1988), based on earlier measurements of N concentration of *H. lanatus* over time (T.M. Bezemer, unpublished data). A quarter-strength Hoagland solution was added in weeks 1–4 (from 12.5 ml to 50 ml per week in steps of 12.5 ml), half-strength solution was added in weeks 5–9 (from 60 ml to 100 ml per week in steps of 10 ml), and full-strength solution was added in weeks 10–15 (From 60 to 100 ml per week in steps of 10 ml). The experiment was carried out in a greenhouse at 70% relative humidity and a 16 h light (21 °C) and 8 h dark (16 °C) photoperiod regime. The natural daylight was supplemented with 400-W metal halide lamps when needed to insure a minimum of 225 μmol m^{-2} s^{-1} photosynthetically active radiation.

To examine effects of the timing of defoliation on nematode populations, four treatments were initiated and a subset of plants was harvested destructively every three weeks (Fig. 1). For each treatment and harvest time there were 10 replicate pots. The sampling scheme included: (1) Early defoliation: fifty plants were defoliated one week after inoculation (week 1), and 10

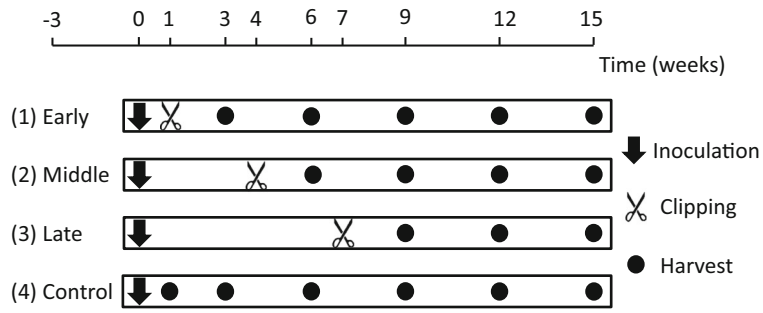

Fig. 1 Experimental design: nematode inocula were introduced at $T = 0$ (three weeks after the transplantation; $T = -3$). The arrows indicate the time point of inoculation, and scissor symbols indicate the time point of defoliation (1, 4 and 7 weeks after inoculation). The circles indicate the harvest time points of each treatment

randomly selected plants were harvested at each of the time points 3, 6, 9, 12 and 15 weeks. (2) Mid defoliation: forty plants were defoliated four weeks after inoculation (week 4), and 10 randomly selected plants were harvested at 6, 9, 12 and 15 weeks. (3) Late defoliation: thirty plants were defoliated seven weeks after inoculation (week 7) and 10 randomly selected plants were harvested at 9, 12 and 15 weeks after inoculation. (4) No defoliation: as a control, fifty plants were not defoliated and 10 randomly chosen plants were harvested at each of the time points at which plants of the early-defoliation treatment were harvested (Fig. 1). One week after nematode inoculation, 10 non-defoliated plants were harvested to determine the number of nematodes that were recovered after inoculation. Plants were defoliated by clipping all leaves at 4 cm above soil surface using alcohol-sterilized scissors.

Plant harvest and nematode extraction

At harvest, the soil was rinsed off the roots of each plant in a bucket with tap water to achieve a 4–5 l suspension. The shoot was then separated from the roots by scissors and aboveground tissues were dried for at least 5 days at 40 °C before weighing. For explicitly estimating total number of nematodes in each pot, nematodes were extracted from the soil and the roots separately. The suspension was stirred for 15 s and after letting the coarse soil particles settle for 30 s the water and suspended nematodes were decanted through a stack of one 1 mm, one 75 μm and three 45 μm sieves (Van der Stoel et al. 2002). The material from the 1 mm sieve was discarded and the material from the 75 and 45 μm sieves was transferred to a double cotton milk filter (Hygia rapid, Hartmann AG, Herdenheim, Germany) on a sieve in a dish with a layer of tap water (Oostenbrink 1960). The nematodes were allowed to pass through the filter during 48 h at 20 °C, which delivered clean suspensions for nematode counting.

The migratory endoparasites (*P. penetrans)* were extracted from the roots using the funnel-spray method (Oostenbrink 1960) for 96 h and counted separately from the nematodes extracted from soil. The suspensions were stored at 4 °C until the nematodes were determined and counted at 50–200× magnification under an inverted light microscope. We identified and counted all the nematodes in each sample for the early harvests (until 9 weeks after inoculation), while for the last two harvests (weeks 12 and 15) subsamples of the soil samples were counted depending on the number of nematodes in the suspension.

Plant carbon and nitrogen analysis

All the roots were oven-dried at 40 °C for a minimum of 5 days before weighing. Roots of 130 plants (10 replicates harvested at 3, 6, 9 and 12 weeks after nematode inoculation for the control and early defoliation treatments; 6, 9 and 12 weeks after inoculation for the mid defoliation treatment, and 9 and 12 weeks after inoculation for the late defoliation treatment) were ground to a powder and 1 mg was weighed into tin capsules. Carbon (C) and nitrogen (N) concentrations were measured using a C/N analyzer (Flash EA 1112, Interscience, Breda, NL).

Data analysis

We recorded the number of nematodes in plant roots and soil in each pot and calculated the total number and the density (total number of nematodes per gram

dry root biomass) per pot. This was done separately for *P. penetrans* and *T. dubius*. We randomly allocated subsets of the ten replicates of the non-defoliated plants that were harvested at 6, 9 and 12 weeks after inoculation to each of the defoliation treatments (early, mid and late), in order to obtain at least 3 non-defoliated control replicates for each time point and for each defoliation treatment. Thereafter, we performed a three-way ANOVA with defoliation (+/−), timing of defoliation (Timing: early, mid, and late), and weeks after defoliation (Weeks: 2, 5 and 8 weeks after defoliation) as fixed factors. We were particularly interested in testing the impact of factor "defoliation" and its interactions with the factor "Timing" and "Weeks". The interaction between "Defoliation" and "Timing" tests whether the impact of defoliation on nematode population depends on the timing of defoliation (early, mid and late). The interaction between "Defoliation" and "Weeks" tests whether the time-course of population development in the eight weeks following defoliation is different from that of non-defoliated plants. The interaction between "Defoliation", "Timing" and "Weeks" tests whether defoliation-induced changes in the time course of population development in the eight weeks following defoliation depend on when the plants were defoliated. To examine whether root quality was affected by defoliation and its timing, we analyzed root C and N concentration and C/N ratio using the three-way ANOVA previously described. As we did not measure the root resources of plants of the last harvest (15 weeks after nematode inoculation), only two time points (2 and 5 weeks after each defoliation) could be analyzed for plants that were defoliated late (seven weeks after inoculation). Therefore, in the three-way analyses of root C and N we used only two instead of three time points (2 and 5 weeks after defoliation). Root biomass of plants was also analyzed using the three-way ANOVA described above to test whether and how the timing of defoliation influenced plant biomass production. Tukey *post-hoc* tests ($P < 0.05$) were performed to compare treatment levels within each significant main factor.

Data of nematode numbers and density were Log_{10} (x + 1) transformed and root biomass was Log_{10} (x) transformed prior to analyses to meet the assumption of homogeneity of variances. All analyses were performed using the R statistical package, version 3.1.3 (R Core Team 2014).

Results

Nematodes

Density Defoliation increased the density of *P. penetrans* relative to the non-defoliated controls, but only when plants were defoliated early (Defoliation × Timing interaction, Table 1, Fig. 2a, b and c). The extent to which defoliation increased the *P. penetrans* density decreased over time (Weeks × Defoliation, Table 1, Fig. 2a, b and c). Defoliation also significantly increased the density of *T. dubius*, but this was independent of the timing of defoliation (main effect of "Defoliation", Table 1, Fig. 2d, e and f).

Total number The total numbers of *P. penetrans* per pot only marginally increased over time, but were significantly enhanced by defoliation during the eight-week-period following defoliation (main effect of "Defoliation", Table 1, Fig. 3a, b and c). By contrast, the total number of *T. dubius* strongly increased during the study period (main effect of "Weeks"), but their numbers were neither significantly affected by defoliation, nor by the timing of defoliation in the eight-week-period following defoliation (Table 1, Fig. 3d, e and f).

Proportion of P. Penetrans in roots The proportion of *P. penetrans* inside roots decreased over time within the examined timeframe (main effect of "Weeks"), but this was not influenced by defoliation (Table 2, Fig. 4).

Root biomass and C, N concentration

Root biomass was strongly reduced by defoliation during the timeframe analyzed. The extent of this reduction was smaller in the early and late defoliated plants than in the plants defoliated at the intermediate time point (significant Defoliation × Timing interaction); the extent varied with time after defoliation (significant Defoliation × Weeks interaction, Table 3, Fig. 5a, b and c). Root N concentrations were slightly enhanced by defoliation (main effect of "Defoliation", Table 3, Fig. 5d, e and f) but linearly decreased over time at defoliation (main effect of "Timing", Table 3, Fig. 5d, e, f). Neither root C concentration nor C/N ratio was affected by defoliation or its timing over the examined five weeks following defoliation (data not shown).

Table 1 ANOVA of density (individuals per gram root) and total number of *Pratylenchus penetrans* and *Tylenchorhynchus dubius* extracted from roots or in soil 2, 5 or 8 weeks after defoliation. The host plants *H. lanatus* was exposed to early, mid or late defoliation

Sources	*df*	*P. penetrans*				*T. dubius*			
		Density		Total no.		Density		Total no.	
		F	*p*	F	*p*	F	*p*	F	*p*
Weeks (W)	2	25.38	**<0.001**	1.88	0.157	10.90	**<0.001**	53.17	**<0.001**
Defoliation (D)	1	22.49	**<0.001**	6.65	**0.011**	7.30	**0.008**	1.47	0.227
Timing (T)	2	8.31	**<0.001**	2.56	0.082	5.80	**0.004**	35.28	**<0.001**
W × D	2	4.04	**0.020**	1.55	0.216	0.65	0.524	1.85	0.161
W × T	4	4.63	**0.002**	2.44	0.051	1.08	0.371	2.26	0.067
D × T	2	3.23	**0.043**	0.70	0.500	0.23	0.793	0.01	0.990
W × D × T	4	0.57	0.685	0.79	0.536	0.54	0.710	0.72	0.580
Error	121/119[a]								

Data were analyzed by 3-way ANOVA: Weeks after defoliation (Weeks, 2/5/8 weeks), defoliation (+/−) and the timing of defoliation (Timing, Early/mid/late) were the main factors. Bold values indicate the significances at level $P < 0.05$

[a] 121 and 119 indicate the *degree of freedom* of the error term for *P. penetrans* and *T. dubius* in a three-way ANOVA analysis, respectively

Discussion

General effects

Our study shows that defoliation increased both the population size and density of the migratory endoparasitic nematode species *P. penetrans* relative to the non-defoliated controls. However, in the case of the ectoparasitic nematode species *T. dubius* defoliation only increased its density on the roots and not the total population sizes in the pots. These differences show that root feeding nematodes may respond to defoliation in a species-specific manner. However, as both nematode species were introduced together in the pots and the initial densities of *P. penetrans* were much higher than those of *T. dubius* it is also possible that the different responses of the nematode species to aboveground clipping and the timing of clipping are caused by interspecific interactions.

Defoliation enhanced root N concentration, but reduced plant root biomass, and hence that the changes in population size and density of the root-feeding nematodes following defoliation may represent a response to both altered quality and quantity of roots. Whether these species-specific differences reflect differences related to the nematode feeding types would require additional work including multiple species of each feeding type. The effect of defoliation on the density of *P. penetrans* depended on the timing of defoliation. Effects of defoliation on root-feeding nematodes have been previously demonstrated (Russin et al. 1993; Kaplan et al. 2008, 2009). However, hitherto virtually no studies have examined how timing of defoliation affects the outcome of this type of above-belowground interaction.

Responses of root-feeding nematodes to timing of defoliation

Defoliation increased the abundance of *P. penetrans* relative to the non-defoliated controls, which supports part of our first hypothesis, that defoliation increases root quality and numbers of root feeding nematodes. The increase may have been caused by the higher root N concentration (or lower C/N ratio) as a result of defoliation (Todd 1996). It suggests that the population growth of *P. penetrans* in response to defoliation was modified by root quality. This result is in line with a study that also attributed changes in population growth of herbivorous nematodes to an altered root quality caused by defoliation in *Lolium perenne* (Bazot et al. 2005). However, in that study an increased root C/N ratio causing a decrease in root feeding nematode abundance was observed as a result of defoliation, illustrating that plant species may differ in their response to defoliation (Guitian and Bardgett 2000). In the current study, in contrast to *P. penetrans*, the abundance of *T. dubius* did not respond to defoliation, indicating that nematode

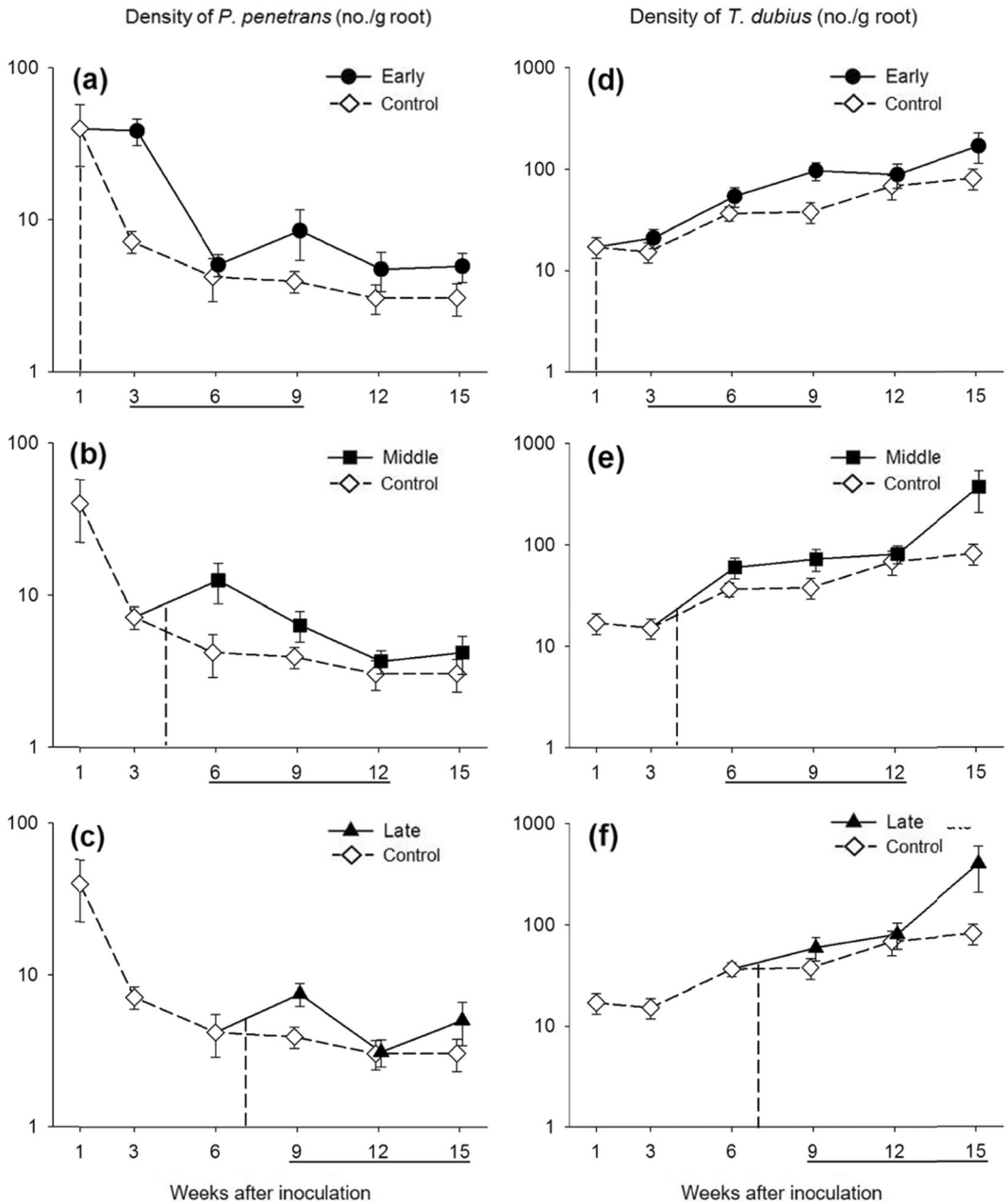

Fig. 2 Mean (±SE) density (number g dry root biomass^{-1}) of *Pratylenchus penetrans* and *Tylenchorhynchus dubius* in each pot when their host plant *Holcus lanatus* was defoliated (*filled symbols*) or not defoliated (*open diamond*). Plants defoliated at week 1 (*filled circle*), 4 (*filled square*) and 7 (*filled triangle*) after inoculation were regarded as early **a**, **d**, middle **b**, **e** and late **c**, **f** defoliation, respectively. The vertical dashed line indicates the time of defoliation. Data of harvest at underlined weeks were used for statistical analysis. See Table 1 for statistics

species can also differ in their responses to changes in plant quality. Possibly, our analysis of total N in the root material does not reflect changes in N concentration among feeding sites, which is mostly root cortex for *P. penetrans* and epidermal and root hair cells for *T. dubius* (Perry and Wright 1998). In addition, we noted that the abundance of *P. penetrans* was overall lower than that of *T. dubius* regardless of defoliation. Host plant suitability as well as interspecific interactions between these two nematode species could contribute to

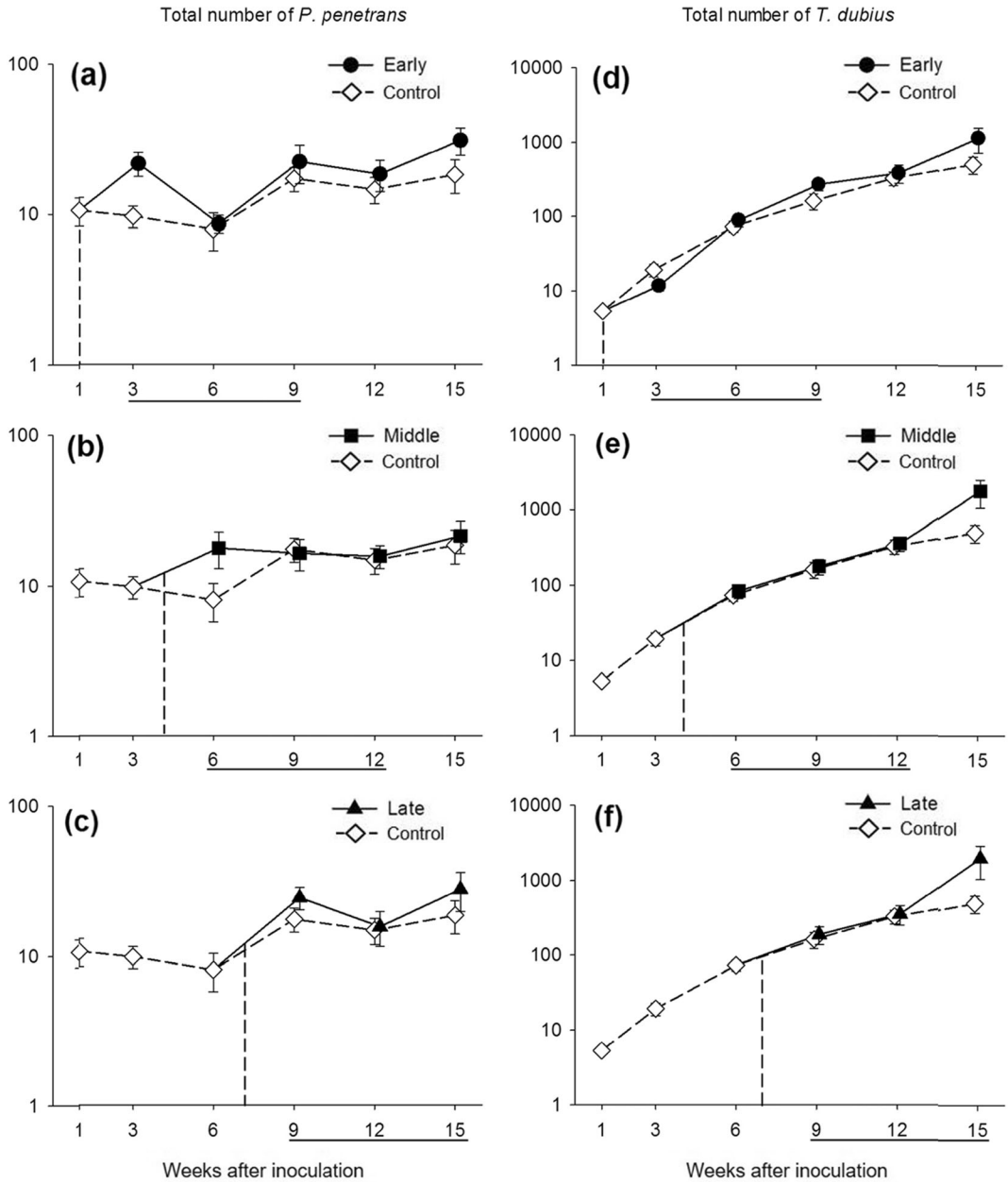

Fig. 3 Mean (±SE) total number of *Pratylenchus penetrans* and *Tylenchorhynchus dubius* in each pot when their host plant *Holcus lanatus* was defoliated (filled symbols) or not defoliated (*open diamond*). Plants defoliated at week 1 (*filled circle*), 4 (*filled square*) and 7 (*filled triangle*) after inoculation were regarded as early **a**, **d**, middle **b**, **e** and late **c**, **f** defoliation, respectively. The vertical dashed line indicates the time of defoliation. Data of harvest at underlined weeks were used for statistical analysis. See Table 1 for statistics

their population development over time (Eisenback 1993; Brinkman et al. 2005, 2008). Future studies should examine how changes in actual plant quality of the feeding sites may influence different nematode species and how this influences interspecific competition between nematodes.

In contrast to the total numbers of nematodes in a pot, the number of root-feeding nematodes per unit root mass (density of nematodes) was enhanced by defoliation for both nematode species. As the total abundance of *T. dubius* was not affected by defoliation, this shows that the enhanced density of *T. dubius* following

Table 2 ANOVA of proportion of total *P. penetrans* present in roots of host plant *H. lanatus* 2, 5 or 8 weeks after defoliation. Plants were exposed to early, middle or late defoliation

Sources	*df*	Proportion	
		F	*p*
Weeks (W)	2	6.50	**0.002**
Defoliation (D)	1	0.07	0.800
Timing (T)	2	2.30	0.105
W × D	2	1.12	0.330
W × T	4	2.76	**0.031**
D × T	2	0.39	0.677
W × D × T	4	1.97	0.103
Error	120		

Data were analyzed by 3-way ANOVA: Weeks after defoliation (Weeks, 2/5/8 weeks), defoliation (+/−) and the timing of defoliation (Timing, Early/mid/late) were the main factors. Bold values indicate the significances at level $P < 0.05$

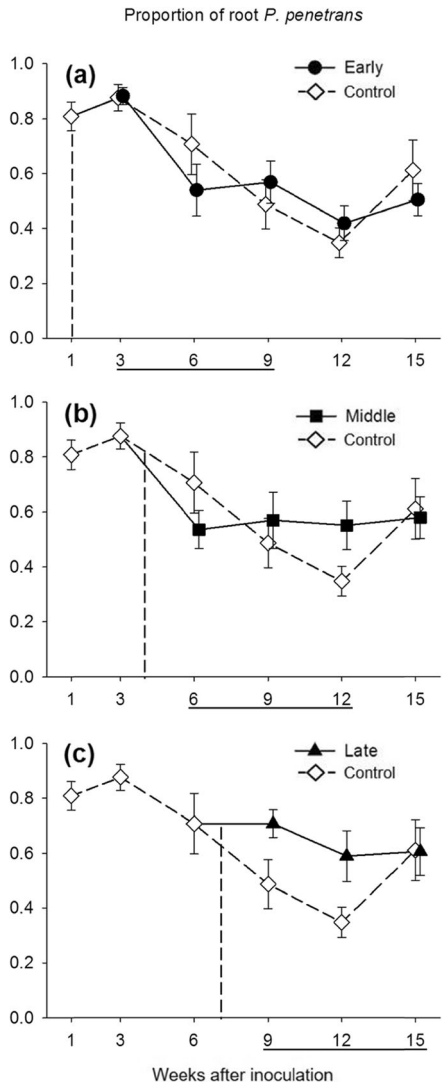

Fig. 4 Mean (±SE) proportion of *Pratylenchus penetrans* that was extracted from the roots of the total number in each pot with *Holcus lanatus* that was defoliated (*filled symbols*) or not defoliated (*open diamond*). Plants defoliated at week 1 (*filled circle*), 4 (*filled square*) and 7 (*filled triangle*) after inoculation were regarded as early **a, d**, middle **b, e** and late **c, f** defoliation, respectively. The vertical dashed line indicates the time of defoliation. Data of harvest at underlined weeks were used for statistical analysis. See Table 2 for statistics

defoliation can be explained by the less rapid increase in root biomass following defoliation. By contrast, the increased density of *P. penetrans* could have been caused both by an enhanced nematode abundance and by a less rapid increase in root biomass following defoliation. Moreover, the density of *T. dubius* steadily increased during the examined period while plant root biomass increased as well, again indicating that the growth rate of *T. dubius* population proceeded faster than the growth rate of root biomass.

We hypothesized that the changes in total population sizes of root-feeding nematodes following defoliation would depend on the timing of defoliation. In contrast to this hypothesis, we did not observe changes in total population size of either nematode species in response to the timing of defoliation. However, we did observe an increase in the density of one of the two nematode species, *P. penetrans*, which only occurred following early defoliation, suggesting a dependence of defoliation effects on plant development stage. We hypothesized that effects of the timing of defoliation would be caused by growth stage-specific changes in plant quality following defoliation as suggested by Ilmarinen et al. (2005). Unexpectedly, overall root quality, as indicated by N concentration, was not influenced by the timing of defoliation in our study, suggesting that at least this aspect of root quality did not change with time at which defoliations occurred. Alternatively, another widely recognized indicator of plant quality, the concentration or composition of secondary compounds in plant tissues, may also be influenced by the timing of defoliation. However, these compounds are usually considered more important in forbs than grasses, in which silica may be more appreciated in plant defense (McNaughton et al. 1985; Massey et al. 2007; Frew et al. 2016), and thus were not analyzed in the current study. Furthermore, since

Table 3 ANOVA of plant root biomass (g, DW) and nitrogen (N) concentration (%) in the roots of *H. lanatus* 2, 5 or 8 (only for biomass) weeks after defoliation. Plants were exposed to early, middle or late defoliation

Sources	Root biomass			N concentration		
	df	F	*p*	*df*	F	*p*
Weeks (W)	2	297.18	**<0.001**	1	2.38	0.126
Defoliation (D)	1	48.89	**<0.001**	1	11.62	**<0.001**
Timing (T)	2	219.93	**<0.001**	2	5.28	**0.006**
W × D	2	7.11	**0.001**	1	0.16	0.691
W × T	4	17.80	**<0.001**	2	1.27	0.285
D × T	2	6.89	**0.001**	2	0.88	0.420
W × D × T	4	2.34	0.060	2	1.06	0.350
Error	122			88		

Data were analyzed by 3-way ANOVA: Weeks after defoliation (Weeks, 2/5/8 weeks for root biomass but 2/5 weeks for N content), defoliation (+/−) and the timing of defoliation (Timing, Early/mid/late) were the main factors. Bold values indicate the significances at level $P < 0.05$

no effect of the timing of defoliation on the total abundance of *P. penetrans* was observed, it is also likely that the effect of timing of defoliation on the density (number per unit root mass) of *P. penetrans* was mediated by a less strong reduction in root biomass following early than late defoliation, even though the strongest reduction seemed to occur at the mid defoliation. Therefore, we suggest that the timing of defoliation may also determine the nematode response to defoliation because the proportion of nematodes per unit root mass increases due to retardation of root growth during the recovery from defoliation. By contrast, neither abundance nor density of *T. dubius* was influenced by the timing of defoliation. This is consistent with our hypothesis that ectoparasitic nematode species are less sensitive to changes in root quality and/or quantity than endoparasites. However, this hypothesis needs further testing by future studies examining multiple nematode species per feeding guild, in order to be conclusive about whether the observed effects are due to feeding type differences, or species differences.

Pratylenchus penetrans is a migratory endoparasitic nematode that can occur either inside or outside root tissues, and it has been proposed that location may (in)directly reflect the conditions inside the root tissues (Zunke 1990). Alternatively, location can be due to the stage of development of the nematodes. Although defoliation did not affect the proportion of *P. penetrans* in roots, the location preferences of *P. penetrans* appeared to change during plant development. A relatively high proportion of *P. penetrans* was extracted from root tissues of plants immediately after early defoliation, but this proportion declined with time. This pattern appears to correspond with changed N concentration in roots after defoliation. Therefore, over time the proportion of *P. penetrans* inside roots decreased as root quality decreased. However, since variation in the proportion of *P. penetrans* inside roots overall could not be explained by variation in root N concentration across time points ($R^2 = 0.04$, $P = 0.012$), our data suggest that other attributes of plant roots changed during plant development, leading to the decreased proportion of *P. penetrans* inside roots. For instance, this could be due to secondary chemicals that may accumulate when roots age (Elger et al. 2009; Quintero and Bowers 2012).

Responses of root biomass to timing of defoliation

Plants tend to direct assimilates to shoots for regrowth after defoliation, which usually results in a decrease of root biomass (Hokka et al. 2004; Ilmarinen et al. 2005). In this study, we indeed observed a lower root biomass induced by defoliation which may constrain plant photosynthesis and decrease plant growth, further resulting in less growth in root biomass. In addition, plants typically change their priority of resource allocation to vegetative growth compared to storage or reproductive demands during development (Boege and Marquis 2005), which may also contribute to plant biomass responses to defoliation over time. However, in the current study, we used a perennial grass species and the defoliation treatments were applied during the vegetative growth phase of the plant. Thus, the possibility that the root biomass response to defoliation was the result of a priority switch towards allocation to reproduction can be excluded (Lubbers and Lechowicz 1989; Miyazaki et al. 2002). The reduced root biomass and unaltered C concentration in roots of the defoliated *H. lanatus* plants suggests that regrowth of shoot biomass may have been prioritized in terms of resource allocation rather than resource storage in roots in this study.

In line with previous studies (Seastedt et al. 1988; Jaramillo and Detling 1988; Hokka et al. 2004), plant N concentration in the roots was increased by defoliation,

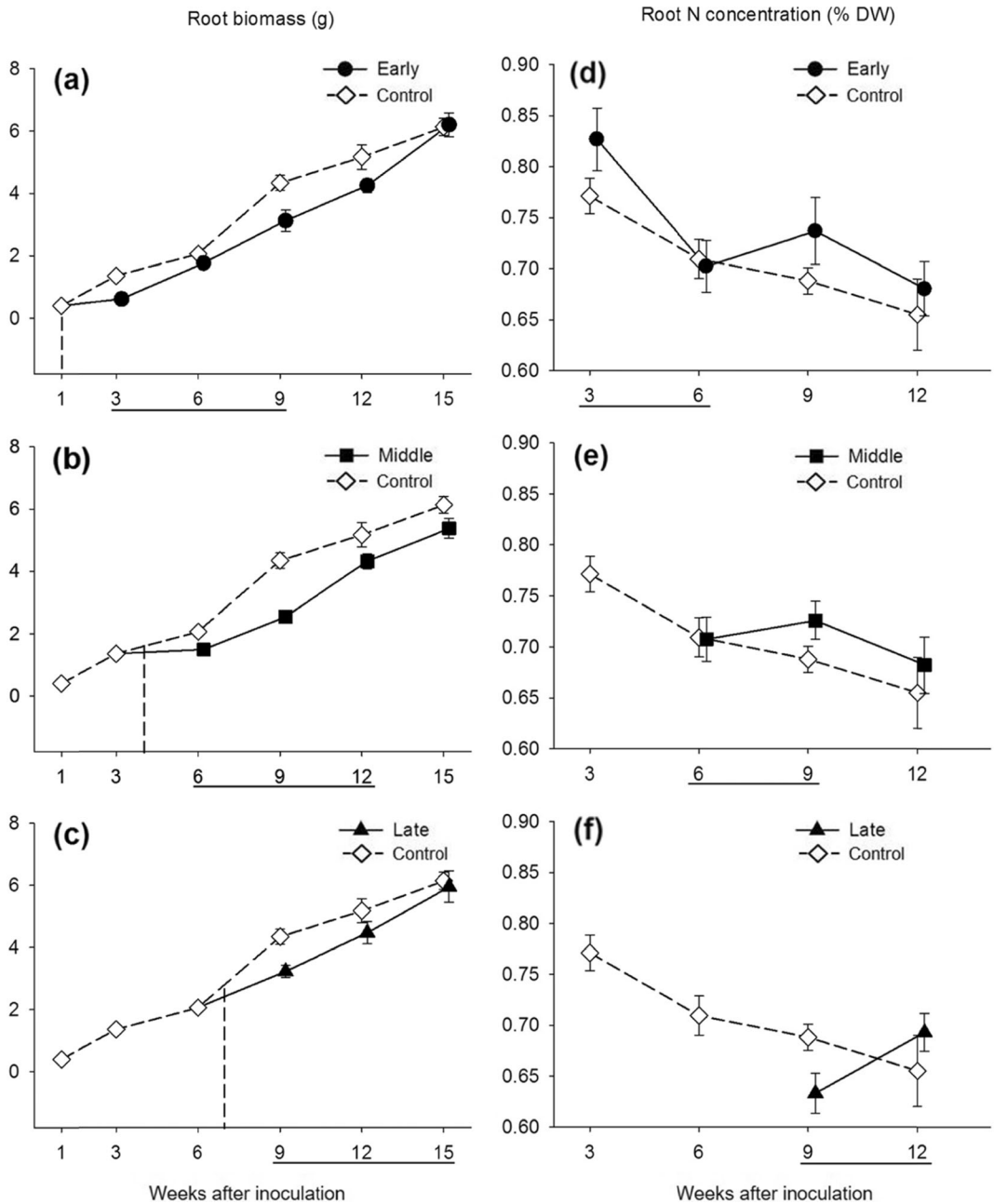

Fig. 5 Mean (±SE) root biomass (g) and N concentration (% DW) in roots of *Holcus lanatus* that were exposed to defoliation (*filled symbols*) or no defoliation (*open diamond*). Plants defoliated at week 1 (*filled circle*), 4 (*filled square*) and 7 (*filled triangle*) after inoculation were regarded as early **a**, **d**, middle **b**, **e** and late **c**, **f** defoliation, respectively. The vertical dashed line indicates the time of defoliation. Data of harvest at underlined weeks were used for statistical analysis. See Table 3 for statistics

which may be caused by a temporal accumulation of N in plant roots due to reduced transport to defoliated aerial tissues. Other studies reported a decrease of root quality following defoliation, because N was transported from roots to shoots for compensatory regrowth (McNaughton 1983; Augustine and McNaughton 1998). These mixed results may be due to differences in responses to defoliation among plant species for example due to different tolerance or defense strategies to tissue losses (Damhoureyeh and Hartnett

2002; Hokka et al. 2004; Del-Val and Crawley 2005). Opposite to the higher N concentration in roots when *Plantago* species were defoliated at an early stage during the growing season (Ilmarinen et al. 2005), our study did not witness a timing effect of defoliation (Defoliation × Timing) on root N concentration, which suggests *H. lanatus* prioritizes N flow to roots regardless of the timing of defoliation.

Conclusion

We conclude that defoliation increased the abundance and density of *P. penetrans* but only increased the density of *T. dubius*. This may be due to a species-specific response of root-feeding nematodes to the same events of defoliation (Wondafrash et al. 2013). However, the observed effects may also be explained by interactions between the two species as they were feeding from the same plant (Brinkman et al. 2008). Further, our study indicates that only when defoliation occurs at a young plant age, soon after nematode inoculation, it causes an increase in the density of *P. penetrans*, suggesting that combined exposure to aboveground and belowground herbivory may increase when plants are grazed upon in a young stage of development. The analysis of plant carbon/nitrogen ratios and biomass indicates that *P. penetrans* may be more sensitive to plant quality alteration, while the species *T. dubius* may be more responsive to changes in root quantity. Our study highlights that the timing of defoliation, in combination with the specific responses of root-feeding nematodes to defoliation, can influence the outcome of combined aboveground and belowground herbivory.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Statement The authors declare that the experiments comply with the current laws of the Netherlands where the experiments were performed.

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