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Associational resistance to nematodes and its effects on interspecific interactions among grassland plants

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Abstract

Aims Plants can influence the level of herbivory experienced by neighboring plants. The importance of such belowground associational effects are poorly understood. In this study we examine whether *Jacobaea vulgaris* provides associational resistance against nematodes to neighboring plants.

Methods Thirteen species (6 forbs, 3 grasses and 4 legumes) were each grown in mixtures with *J.*

vulgaris and in monocultures. A nematode community was introduced to half of the pots. After 12 weeks, plant dry mass was assessed for each individual plant in each pot, and the number of nematodes in the soil and roots were identified. We then examined for each plant species its performance in mixtures and in monocultures, in presence and absence of nematodes and analyzed the abundance and composition of nematodes.

Results Forbs produced more, grasses similar, and legumes less biomass in mixtures with *J. vulgaris* than in monocultures. Nematode addition did not influence biomass. There were fewer root-feeding nematodes in the soil in mixtures than in monocultures, but this was only true for plants that were good hosts for nematodes. The community composition of soil nematodes was different in monocultures and mixtures. Densities of migratory endoparasitic nematodes in the roots of neighboring plants were lower in mixtures than in monocultures. Moreover, the presence of nematodes changed the outcome of plant-plant interactions, often in favor of *J. vulgaris*.

Conclusions *Jacobaea vulgaris* provides belowground associational resistance to other plants against migratory endoparasitic nematodes, and the presence of nematodes can change the outcome of plant-plant interactions.

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Keywords *Jacobaea vulgaris* · Associational resistance · Belowground herbivores · Nematodes · Plant-plant interactions

Introduction

Understanding how plants interact with neighboring plants and with their attackers below and aboveground has been a focal topic of research for ecologists for many years (Rees and Brown 1992; Rasmann and Agrawal 2008; Johnson et al. 2016b). Plants compete for nutrients, light and space, and plant species differ greatly in how they use these resources, and hence also in their impact on the availability of resources for competing plants. Plants can also impact the colonization by antagonists such as herbivores or the amount of damage caused by these antagonists on other plants. These effects are called associational resistance or susceptibility depending on whether the focal plant experiences less or more herbivory in the presence of the neighbor (Tahvanainen and Root 1972; White and Andow 2006; Barbosa et al. 2009; Underwood et al. 2014). There are many examples of associational resistance against aboveground herbivores (Underwood et al. 2014). However, if and how neighboring plants influence the susceptibility or resistance of a focal plant to belowground herbivory is not well known (Kos et al. 2015; Jing et al. 2015b).

In the presence of belowground herbivory, associational resistance or susceptibility can arise from a variety of mechanisms. Root herbivory on a neighboring plant can increase the emission of volatiles of this plant and exposure to these volatiles can increase the susceptibility of a focal plant to root herbivores (Huang et al. 2019; Voglar et al. 2019). Alternatively, neighboring plants may also repel root herbivores or reduce their growth rates and this can result in a release from herbivory on the roots of the focal plant. This is particularly relevant for plants with high contents of allelochemicals in the roots such as the plant *Jacobaea vulgaris* that exhibits high root pyrrolizidine alkaloid concentrations (Kostenko et al. 2013). Several studies have shown that these pyrrolizidine alkaloids negatively affect root-feeding nematodes (Thoden et al. 2009a, b; van de Voorde et al. 2012; Harkes et al. 2017). However, it is not known if this has further consequences for nearby focal plants via associational resistance.

Neighboring plants can also greatly influence the performance of a focal plant via competition (Hodge et al. 1999; Craine et al. 2002; Hautier et al. 2009). Besides competition for resources or space, plant-plant interactions can also arise from interactions

between plants and aboveground or soil-dwelling herbivores (Scheublin et al. 2007; Jing et al. 2015a; Cardinaux et al. 2018). While many studies have examined how foliar herbivores alter the outcome of plant-plant interactions (Rees and Brown 1992; Ramsell et al. 1993; Haag et al. 2004), the impact of belowground herbivory is less well studied. Previous studies have shown that root herbivores can relax the negative competitive effect experienced by competitively inferior species simply because root herbivores encounter and feed on the dominant species more often (Verschoor et al. 2002; Niemelä et al. 2008; Oduor et al. 2015, 2017; Wang et al. 2019). We, therefore predict that the competitive advantage of a focal species when growing with a heterospecific neighbor will be reduced in presence of root herbivores.

Nematodes inhabit virtually all soils and root-feeding nematodes are among the most damaging root herbivores with an associated cost of more than \$200 billion annually in agriculture (Brinkman et al. 2005; Elhady et al. 2018). Root-feeding nematodes can be highly specialized and show considerably variance in preference on different plant species (De Deyn et al. 2004; Brinkman et al. 2015). *Jacobaea vulgaris* is considered a poor host for nematodes. Many sedentary endoparasitic nematodes (e.g. *Meloidogyne hapla* and *Subanguina picridis*) cannot reproduce on *J. vulgaris* (Thoden et al. 2009a, b; Thoden and Boppré 2010; Harkes et al. 2017). In a previous study, only *Pratylenchus crenatus* (a migratory endoparasitic nematode species) and root-associated nematodes from the genus *Filenchus* were found to be associated with the roots of *J. vulgaris* growing in the field. This also indicates that this plant species is a poor host for other root-feeding nematodes as there are other root-feeding nematode species found underneath other plant species in the same soil (Van de Voorde et al. 2012). If the presence of *J. vulgaris* increases nematode mortality or deters root feeding nematodes, growing together with *J. vulgaris* may lead to reduced nematode pressure on other plants. However, this will also depend on how susceptible the focal plant itself is to nematodes as all nematodes may then prefer to feed on the focal plant resulting in more damage than when the focal plant competes with a conspecific neighbor.

In this study, we examine associational resistance against root-feeding nematodes implied by *J. vulgaris* to a range of plant species. We performed a greenhouse experiment with 13 grassland species

planted each in monocultures and in mixtures with *J. vulgaris*. All plant species co-occur in the grasslands where the nematodes were collected from. The plants were grown in sterilized soil and half of the pots were inoculated with a nematode community that contained a high density of root-feeding nematodes. *Jacobaea vulgaris* is often considered a poor competitor (Jing et al. 2015b; Kostenko et al. 2016). The plant contains pyrrolizidine alkaloids (PAs), a well-studied group of secondary plant compounds that play an important role in plant–insect and plant–soil interactions (Hol et al. 2004; Macel 2011; Kostenko et al. 2013). Other work with some of the studied plant species and the same soil, showed that root-feeding nematodes are found in high densities on the legume, *Lotus corniculatus*, and in lower densities on the two grasses (*Anthoxanthum odoratum* and *Festuca rubra*) and the three forbs (*Plantago lanceolata*, *Tanacetum vulgare* and *Leucanthemum vulgare*) (Bezemer et al. 2010). We hypothesized that (i) The other plants produce more biomass when they are grown with *J. vulgaris* than with conspecific individuals but that this effect will be strongest in absence of nematodes; (ii) Densities of root-feeding nematodes in the soil will be lower in mixtures with *J. vulgaris* than in monocultures of the other species. Further, densities of root-feeding nematodes in soils of mixtures can be predicted by those in the soils of monocultures; (iii) The density of root-feeding nematodes inside the roots of the other species will be lower in mixtures with *J. vulgaris* than in monocultures, but only for plants that are susceptible to nematodes i.e. that are good hosts; (iv) The presence of nematodes will reduce the competitive advantage of other species when growing with *J. vulgaris*, since *J. vulgaris* is a competitively inferior species and nematodes will feed preferentially on the roots of other species.

Materials and methods

Plants

Jacobaea vulgaris Geartn. subs. *vulgaris* (syn. *Senecio Jacobaea* L.; Asteraceae) is a monocarpic perennial that is native in Europe (Harper and Wood 1957). It is a weak competitor (Jing et al. 2015b) but can be highly abundant locally when the soil is disturbed or bare (van de Voorde

et al. 2012). Seeds of *J. vulgaris* were collected from a population in a natural grassland area, “De Mossel”, at Planken Wambuis, Ede, The Netherlands (52.06 N, 5.75 E).

Thirteen plant species that all co-occur with *J. vulgaris* in natural grasslands in the Netherlands were used in this study: three grasses (*Anthoxanthum odoratum* L., *Agrostis capillaris* L., *Festuca rubra* L.), six forbs (*Achillea millefolium* L., *Hypochaeris radicata* L., *Leucanthemum vulgare* Lamb., *Plantago lanceolata* L., *Tanacetum vulgare* L., *Tripleurospermum maritimum* (L.) Koch) and four legumes (*Lotus corniculatus* L., *Trifolium arvense* L., *Trifolium pratense* L., *Trifolium repens* L.). Seeds of these 13 species were purchased from Cruydt-Hoeck (Nijeberkoop, The Netherlands), a supplier of seeds obtained from wild plants. Seeds from all species were sterilized (1 min in 2.5% sodium hypochlorite solution and rinsed with water afterwards), and germinated in containers (10×10×4 cm) filled with a layer of sterilized glass beads submerged in water and in a climate chamber at 16/8 h light–dark regime and a 20/15 °C temperature regime. After germination (c. one to two weeks later depending on the species), seedlings were stored at 4 °C until further use.

Nematode collection

Nematodes were extracted from 20 kg of soil collected at a grassland adjacent to the campus of Wageningen University (Bornsesteeg, Wageningen) which is known to have a high density of root-feeding nematodes in late April (the start of the growth season of plants), using the Cobbs’ decantation and sieving method (1×180 mm, followed by 1×75 mm, and 3×45 mm). We collected the nematodes from the 75-mm and 45-mm sieves. The total density of root-feeding nematodes was counted in two 2 ml samples and was on average 456.

Greenhouse experiment

Top soil was collected from the natural grassland area “De Mossel”. The soil is a holt podzol, sandy loam (94% sand, 4% silt, 2% clay, and 4% organic matter). The soil was sieved (0.5 cm mesh) to

remove pebbles and large root fragments, homogenized and sterilized using gamma irradiation (> 25 Kgray, Isotron, Ede, The Netherlands). Pots ($13 \times 13 \times 13$ cm) were filled with 2 kg sterilized soil. Soil moisture was set at 17%. For each of the 13 co-occurring species, pots were then planted with 4 seedlings of a single species (monoculture) or with two individuals of a species and two individuals of *J. vulgaris* (mixture). *Jacobaea vulgaris* was also planted in monocultures. There were 10 replicate pots for each combination and in total 270 pots (14 monocultures \times 10 replicates + 13 mixtures \times 10 replicates). All pots were then placed in a greenhouse compartment (60% relative humidity; 16 h light (20 °C) and 8 h dark (16 °C) photo regime). Natural day-light was supplemented by 400 W metal halide lamps ($225 \text{ mmol m}^{-2} \text{ s}^{-1}$ PAR, 1 lamp per 1.5 m^2). To minimize the effects of local differences in microclimate in the greenhouse, pots were randomly placed on trolleys in the greenhouse and the trolleys were randomly redistributed within the greenhouse once a week. Seedlings that died during the first week of the experiment were replaced. Twenty-eight days after planting, nematodes were added to five of the ten replicate pots of each treatment. Into each pot, at two positions 1 ml nematode suspension was injected into the soil with a pipette. The other five replicate pots received 2×1 ml tap water.

Plants were watered regularly, and soil moisture was reset to 17% once a week. Fifty-four days after nematode inoculation (12 weeks after planting) three soil cores (0.5 cm diameter) were collected from each pot. The soil was homogenized so that there was one soil sample for each pot. In pots with plant mixtures the two other plants were first clipped individually at soil level and stored in separate paper bags. Soil was then removed from each pot and the roots were washed. Subsamples from roots of each species were collected from all pots to be used for nematode extraction (see below). In mixtures, roots of the two species were separated (the shoots of *J. vulgaris* plants were still attached to the roots). Hereafter, the two *J. vulgaris* shoots were clipped and stored individually in paper bags. We were not able to separate the roots of both species entirely, and hence for each pot all roots were collected at once, and only data on total root biomass is available. All plant material was then dried at 60 °C and dry weight was determined.

Nematode extraction

For pots with soil where nematodes had been inoculated, the nematodes were extracted from the soil and from the root sample. Extractions of three pots with soil without nematode addition revealed that no nematodes were found in these pots. From each homogenized soil sample from each pot, nematodes were extracted from 70 g of soil by Oostenbrink elutriators (Oostenbrink 1960). All nematodes present in 3 ml (of a total of 10 ml) were then identified to sub-order or family level (Bongers 1988; see Table S1 for details). We distinguished nematodes as plant feeders, bacterivores, fungivores, carnivores and omnivores based on Yeates et al. (1993). For each pot where nematodes had been inoculated, the number of nematodes belonging to each feeding guild per 100 g soil was then calculated.

Nematodes from root samples were extracted from approximately 0.6 g dry weight of roots using a mistifier and an extraction time of 48 h. All nematodes were then identified, and root dry weight of each sample was determined. For pots with monocultures there was one root sample per pot and for pots with mixtures, nematodes were extracted from the roots of both species separately. The number of plant feeders and bacterivores per gram root were determined. Fungivores were not and omnivores rarely observed in the root samples, therefore they were excluded from analyses (see Table S2). Meanwhile, root-feeding nematodes in the soil and in the roots were categorized into feeding guilds based on Yeates et al. (1993) (see Table S3). After extraction, root samples were dried at 60 °C and the biomass was added to the total root biomass of the pot.

Data analysis and statistics

Plant dry mass: Individual shoot dry mass per plant species and total root dry mass per pot was calculated. During the experiment, in 17 pots one or more plants died and these pots were excluded from the analyses (see table S4 for details). The effect of nematode addition (yes/no) and whether the species was grown in monoculture or in mixture with *J. vulgaris* (mixture, hereafter) on shoot dry mass of the other species was tested with three-way ANOVA with competing species (13 levels), mixture (monoculture/mixture) and nematode addition (yes/no) as main factors.

The effect of plant functional groups (grasses, forbs, legumes), mixture with *J. vulgaris*, nematodes addition and their interactions on the shoot dry mass was tested with a linear mixed effect model with species as random effect. A Tukey's post hoc test was used for pair-wise comparisons for each species and functional group.

The shoot dry mass of *J. vulgaris* in presence and absence of nematodes was analyzed separately for plants in monoculture and mixtures using two-way ANOVA with nematode addition (yes/no) and competing species (14 levels) as main factors. A Dunnett post-hoc test was then used to compare individual shoot biomass of *J. vulgaris* in mixtures and monocultures. All ANOVA was carried out with the "aov" function and the post-hoc test was performed using the "glht" function with the "multcomp" package (Hothorn et al. 2008).

Linear mixed models were used to test the effect of the functional group the competing species belonged to, nematodes addition and their interaction on *J. vulgaris* biomass. In this analysis, species identity was added as random effect. The same analysis was carried out for total root dry mass per pot. In all analyses, residuals were checked for homogeneity of variance using a Levene's test and normality by a Shapiro Wilk test. Root dry weight was square root transformed to fulfil requirements of normality. Linear mixed models were performed using the "lme" function with the "nlme" package (Pinheiro et al. 2019). The Levene's test and Shapiro Wilk test were performed using the "levene_test" and "shapiro_test" function with the "rstatix" package (Kassambara 2021).

Nematode densities: The effects of competing species (13 levels), mixture with *J. vulgaris* (monoculture / mixture) and their interaction, on the number of soil nematodes and root nematodes (different groups of plant feeders and bacterivores) were tested using generalized linear models with a negative binomial distribution. Generalized linear mixed models, with a negative binomial distribution and species as random effect, were used to test the effect of the functional group the other species belonged to, mixture with *J. vulgaris* (monoculture / mixture), and their interaction on the number of soil nematodes and root nematodes (plant feeders and bacterivores). Significance of factors was assessed by comparing models with and without the factor using a Chi-squared Likelihood Ratio (LR) test on the residual deviance. A Tukey's

post hoc test was used for pair-wise comparisons between monocultures and mixtures for each species and functional group. To examine the effects of species identity on the number of plant feeders and bacterivores of *J. vulgaris*, a generalized linear model with a negative binomial distribution with species as the fixed effect (14 levels, including *J. vulgaris*) was used. Significance of the factor was assessed as described above. A Dunnett post-hoc test was then used to compare nematodes in root tissues of *J. vulgaris* growing in mixtures and monoculture. Generalized linear mixed models with a negative binomial distribution were performed using the "glm.nb" function with the "MASS" package (Venables and Ripley 2002).

Nematodes composition: Unconstrained principal component analysis (PCA) and constrained redundancy analysis (RDA) were used to analyze soil nematode composition with Hellinger transformed count data (Legendre and Gallagher 2001). The PCA analysis was performed using the "prcomp" function. In order to test whether changes in the community composition of nematodes could be explained by species, mixture or their interaction, we conducted constrained redundancy analysis (RDA), a permutational multivariate analysis of variance (PERMANOVA, 999 permutations) and variance partitioning. The RDA, permutational multivariate analysis and variance partitioning were carried out with the "rda", "adonis" and "varpart" function with the "vegan" package (Oksanen et al. 2019).

To examine whether the number of nematodes in the soil in mixtures could be predicted from the densities in monocultures, we used the following formula for each species combination:

$$N = N_o \times SO_{mix}/SO_{mono} + N_j \times SJ_{mix}/SJ_{mono} \quad (1)$$

where N represents the predicted number of root feeders or bacterivores in soil; N_o and N_j the mean number of plant feeders or bacterivores in the soil in the monocultures of the other species and *J. vulgaris* respectively; SO_{mono} and SO_{mix} the mean shoot biomass of other species in monocultures and mixtures respectively; and SJ_{mono} and SJ_{mix} the mean shoot biomass of *J. vulgaris* in monocultures and mixtures. We also predicted the number of nematodes in the soil using the following formula:

$$N = N_o \times 0.5 + N_j \times 0.5 \quad (2)$$

In this formula, the predicted number of soil nematodes (N) in mixtures was determined based on an equal contribution of both species in the mixtures. Linear regression (based on mean values per species) was then used to analyze the relationship between the observed and predicted number of nematodes.

Plant-plant interactions: To test whether the presence of nematodes altered plant-plant interactions, the relative interaction intensity (RII, hereafter) was calculated for each species separately for pots with and without nematode addition (Armas et al. 2004). We used the following formula to calculate RII:

$$RII = (B_W - B_0)/(B_W + B_0) \quad (3)$$

where B_W and B_0 represent the individual shoot biomass in mixture and in monoculture of plant species. B_W can be seen as a function of the interaction factor which is $\alpha \times B_0$. Therefore the formula of RII can be converted to:

$$\begin{aligned} RII &= (B_W - B_0)/(B_W + B_0) \\ &= (\alpha \times B_0 - B_0)/(\alpha \times B_0 + B_0) \\ &= (\alpha - 1)/(\alpha + 1) \end{aligned} \quad (4)$$

Thus, RII only depends on the interaction factor α . If $\alpha < 0$ ($RII < 0$), competition occurs, while $\alpha > 0$ ($RII > 0$) facilitation occurs. When $\alpha = 1$ ($RII = 0$), the interaction outcome is neutral (Armas et al. 2004). Linear regression (based on mean values per species) was then used to examine the relationship between *J. vulgaris* and the other species. Differences in slopes

and intercepts of regressions for the two relationships (with and without nematodes addition) were examined using a t-test. A paired t-test was performed to examine the effects of nematode addition on RII of the other plant species and on *J. vulgaris*. The linear regression analysis and the paired t-test were performed using the “lm” and “t.test” function.

All analyses were performed using the R statistical language, version 4.0.2 (R Core Team 2020).

Results

Individual shoot biomass

There were no significant main or interaction effects of nematode addition on shoot biomass (Table 1 and S5, Fig. 1a, S1a and S2a). Individual shoot biomass was significantly influenced by whether the plants were grown in monocultures or in mixtures with *J. vulgaris* (Table 1 and S5, Fig. 1a, S1a and S2a). However, this effect was not the same for all species resulting in a significant interaction between species and mixture (Table 1 and S5). In particular forb species produced more shoot biomass in pots with mixtures than in monocultures, legumes produced less shoot biomass, while biomass of grasses did not differ in monocultures and mixtures (Fig. S1a).

Individual shoot biomass of *J. vulgaris* varied significantly depending on which species it was growing with in the pot, and the functional group the other plants belonged to, and this was not affected by nematode addition (Table 2 and S6, Fig. 1b, S1b and S2b).

Table 1 Results of a three-way ANOVA testing the effects of plant species (13 species), nematode addition (yes/no), and whether the species was grown in monoculture or in mixture with *J. vulgaris* (mixture) and their interactions on individual

shoot dry mass and total root dry mass per pot. Presented are degrees of freedom (df; treatment, error) and F values. *** indicates significant difference at $P < 0.001$, ** indicates significant difference at $P < 0.01$

	Individual shoot dry mass		Total root dry mass	
	df	F	df	F
Species	12, 193	6.41***	12, 192	21.57***
Nematodes	1, 193	3.18	1, 192	0.50
Mixture	1, 193	2.07	1, 192	0.19
Species × Nematodes	12, 193	0.94	12, 192	1.52
Species × Mixture	12, 193	2.37**	12, 192	5.36***
Nematodes × Mixture	1, 193	1.09	1, 192	0.09
Species × Nematodes × Mixture	12, 193	0.52	12, 192	1.10

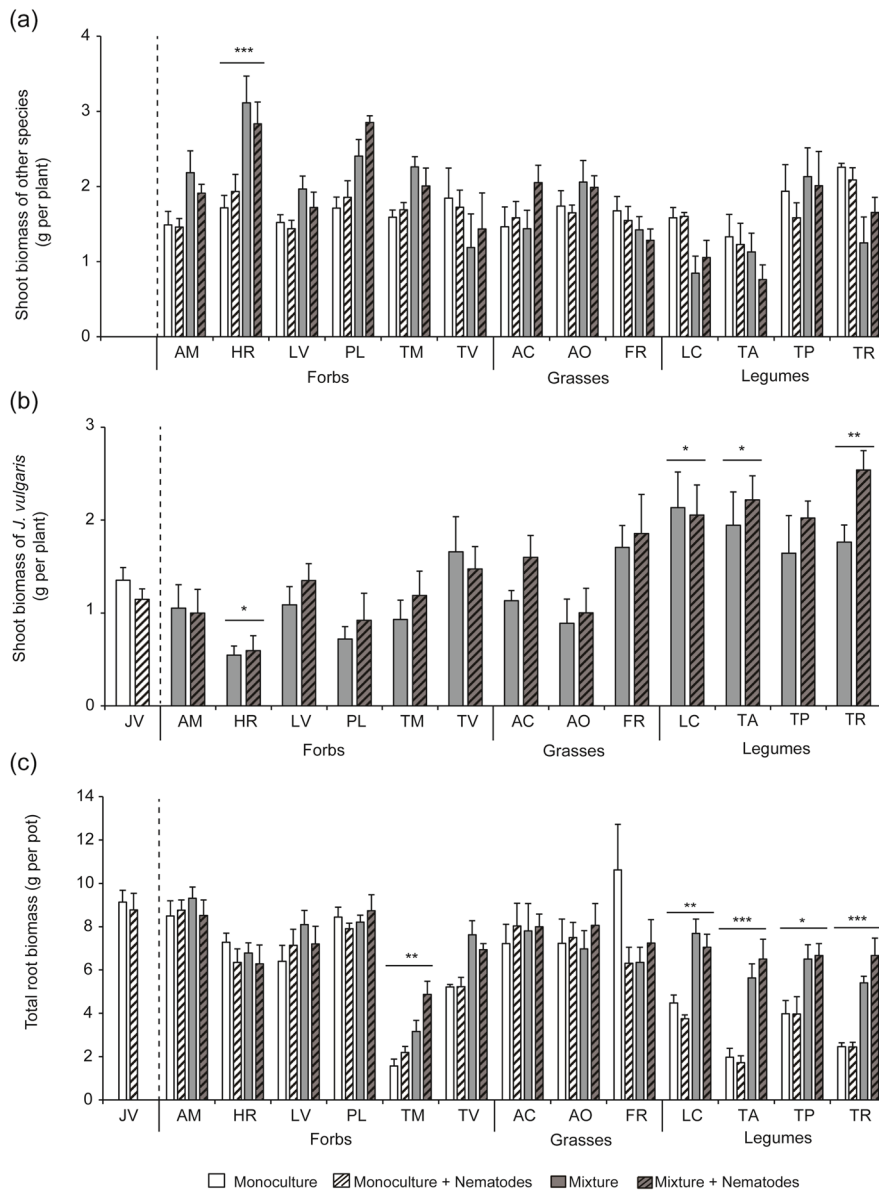


Fig. 1 Mean (+SE) individual shoot biomass of plants grown in monocultures and in mixtures of (a) the other species (b) *J. vulgaris*, and (c) total root biomass per pot. Plants were grown with or without nematodes. In (a) and (c) asterisks above each set of bars indicate significant difference between monoculture and mixture for each species based on one-way ANOVA across species and mixture (nematodes had no effect on biomass and the raw data of four treatments in species level were shown). In (b) asterisks indicate significant differences between shoot biomass of *J. vulgaris* in mixtures and mono-

cultures based on a Dunnett post hoc test. *, **, *** indicates significant differences at $P < 0.05$, 0.01 or 0.001, respectively. Abbreviations of species: JV = *Jacobaea vulgaris*, AM = *Achillea millefolium*, HR = *Hypochoeris radicata*, LV = *Leucanthemum vulgare*, PL = *Plantago lanceolata*, TM = *Tripleurospermum maritimum*, TV = *Tanacetum vulgare*, AC = *Agrostis capillaris*, AO = *Anthoxanthum odoratum*, FR = *Festuca rubra*, LC = *Lotus corniculatus*, TA = *Trifolium arvense*, TP = *Trifolium pratense*, TR = *Trifolium repens*

Overall, shoot dry mass was highest in mixtures with legumes (Table S6, Fig. S1b).

Total root dry mass per pot

Total root biomass per pot differed strongly between monocultures and mixtures and this was true at species and functional group level (Table 1 and S5, Fig. 1c, S1c and S2c). Overall, total root biomass was higher in mixtures than in monocultures, but this was particularly so for legumes, to a lesser extent for forbs, and not true for grasses (Fig. 1c, S1c and S2c). Root biomass was not affected by nematode addition (Table 1 and S5).

Soil nematodes

The number of root-feeding nematodes in the soil varied significantly between plant species and was highest in grass species (Table 3 and S7, Fig. 2a and S3a). The number of plant feeders in the soil in *J. vulgaris* monocultures was low (Fig. 2a). In mixtures with *J. vulgaris* there were fewer root-feeding nematodes than in monocultures of the other species but this was only true for species where the number of root-feeding nematodes was high in monocultures, resulting in a significant interaction between plant species and the planting treatment (Table 3, Fig. 2a). Specifically, the numbers of ectoparasitic and migratory endoparasitic nematodes in the soil were lower in mixtures than in monocultures for species where the number of these nematodes was high in monocultures (Table 4, Fig. 3a, c). The number of bacterivores in the soil varied significantly between species but did not differ significantly between monocultures and mixtures

Table 2 Results of a two-way ANOVA testing the effects of the other plant species present in the pot (13 other species or *J. vulgaris*) and nematode addition (yes/no), on individual shoot dry mass of *J. vulgaris*. Presented are degrees of freedom (df; treatment, error) and F values. *** indicates significant difference at $P < 0.001$

	Shoot biomass of <i>J. vulgaris</i>	
	df	F
Species	13, 99	8.77***
Nematodes	1, 99	3.90
Species × Nematodes	13, 99	0.57

(Table 3, Fig. S4a). The number of plant feeders in the soil in mixtures was highly positively correlated with the predicted number of plant feeders in both monocultures, but this was not the case for bacterivores (Fig. 4 and S5).

The composition of the nematode community in the soil varied between grass, forb and legume species as depicted in the PCA (Fig. 5). For most of the forb and legume species the composition varied between monocultures and mixtures (Fig. 5b, d). The composition of nematodes in soil of monocultures and mixtures of grasses and *J. vulgaris* was relatively similar. Species identity accounted for 18% ($F_{12, 97} = 3.47$, $P < 0.001$; Table S8) of the total variation in soil nematode composition, while the mixture treatment explained 3% ($F_{1, 97} = 5.39$, $P < 0.001$; Table S8).

Root nematodes

The number of root-feeding nematodes associated to the roots of the other species varied significantly between plant species but did not differ depending on whether the plant was growing in monocultures or mixtures (Table 3, Fig. 2b). The number of migratory endoparasitic nematodes was lower in roots of other species in mixtures than in monocultures

Table 3 Results of a generalized linear model testing the effects of plant species (13 species), and whether the species was grown in monoculture or in mixture with *J. vulgaris* (mixture) and their interaction on plant feeders and bacterivores in the soil, and in root tissues of the other species and of *J. vulgaris*. Presented are degrees of freedom (df) and Likelihood Ratio Chi-squares obtained from a generalized linear model. *** indicates significant difference at $P < 0.001$, * indicates significant difference at $P < 0.05$

		Plant feeders	Bacterivores
Nematodes in soil	df	χ^2	χ^2
Species	12	53.04***	43.78***
Mixture	1	0.70	1.05
Species × Mixture	12	23.28*	18.99
Nematodes in roots of other species			
Species	12	230.61***	85.94***
Mixture	1	0.18	0.01
Species × Mixture	12	19.69	23.70*
Nematodes in roots of <i>J. vulgaris</i>			
Species	13	19.62	41.235***

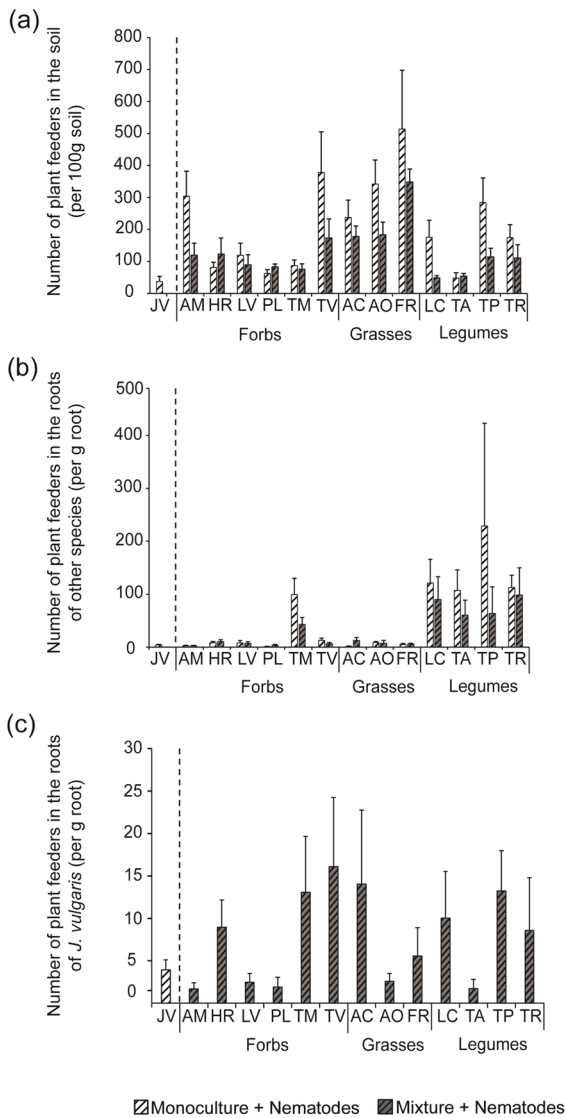


Fig. 2 Mean (+SE) number of root-feeding nematodes in the soil (a) and in the roots (b) of the 13 species in monocultures and mixtures, and in roots of *J. vulgaris* (c). In (a, b) there was no significant differences between monocultures and mixtures ($p < 0.05$) based on a Tukey HSD post hoc test. In (c) there was no significant differences ($p < 0.05$) between nematode densities in the root of *J. vulgaris* in mixtures and monocultures based on a Dunnett post hoc test. Species abbreviations are described in the legend of Fig. 1

(Table 4, Fig. 3d). The number of bacterivores recovered from the root samples also differed between species (Table 3, Fig. S4b). For some species, numbers of bacterivores were higher in monocultures than in mixtures resulting in a significant interaction between

species and planting treatment (Table 3, Fig. S4b). The number of plant feeders in roots of *J. vulgaris* did not significantly differ among co-occurring species but the number of bacterivores recovered from root samples from *J. vulgaris* plants varied significantly depending on the identity of the neighboring plants (Table 3, Fig. 2c and S4c).

Plant-plant interactions

Independent of nematode addition, forbs exhibited a competitive advantage over *J. vulgaris*, while this was reversed for legumes (Fig. 6). The slope of the relationship between the RII of *J. vulgaris* and that of the other species did not differ between pots with or without nematodes (Fig. S6). In general, nematode presence resulted in a significant increase in the RII of *J. vulgaris* in mixtures (arrows pointing to the right in Fig. 6) indicating that the relative competitive ability of *J. vulgaris* in mixtures increased in presence of nematodes (Table S9).

Discussion

The aim of this study was to examine whether neighbor plants provide associational resistance against root-feeding nematodes and how root-feeding nematodes in the soil change plant-plant interactions. We measured the biomass of individual plants growing in monocultures and in mixtures, and recorded the number of nematodes in the roots and in the soil. Three main findings arise from this study. First, the number of migratory endoparasitic nematodes in the roots of other species is lower in mixtures than in monocultures. And in soil of mixtures with plants that are good hosts for nematodes (i.e. where root-feeding nematode densities were relatively high), the number of the root-feeding nematodes in the soil surrounding the roots is lower than in soil from monocultures of those species. Second, soil nematodes can change the outcome of plant-plant interactions, but this is often beneficial to *J. vulgaris* instead of the other species. Third, forb individuals produced more biomass, grass biomass did not change and legume plants produced less biomass when growing in mixtures with *J. vulgaris* than with conspecific individuals showing that responses to *J. vulgaris* differ among functional groups. Overall, our study provides evidence for

Table 4 Results of a generalized linear model testing the effects of plant species (13 species), and whether the species was grown in monoculture or in mixture with *J. vulgaris* (mixture) and their interaction on different groups of plant feeders in the soil and in root tissues of the other species. Presented

		Ectoparasitic nematodes	Migratory endoparasitic nematodes	Root-associated nematodes	Semi-endoparasitic nematodes
Nematodes in soil	df	χ^2	χ^2	χ^2	χ^2
Species	12	71.34***	34.09***	20.48	6.34
Mixture	1	0.34	3.45	2.10	0.08
Species × Mixture	12	21.90*	22.50*	12.86	11.23
Nematodes in roots of other species					
Species	12	36.57***	91.16***	47.71***	11.19
Mixture	1	2.77	18.64***	0	0.34
Species × Mixture	12	9.89	29.59**	17.61	6.51

are degrees of freedom (df) and Likelihood Ratio Chi-squares obtained from a generalized linear model. *** indicates significant difference at $P < 0.001$, ** indicates significant difference at $P < 0.01$, * indicates significant difference at $P < 0.05$

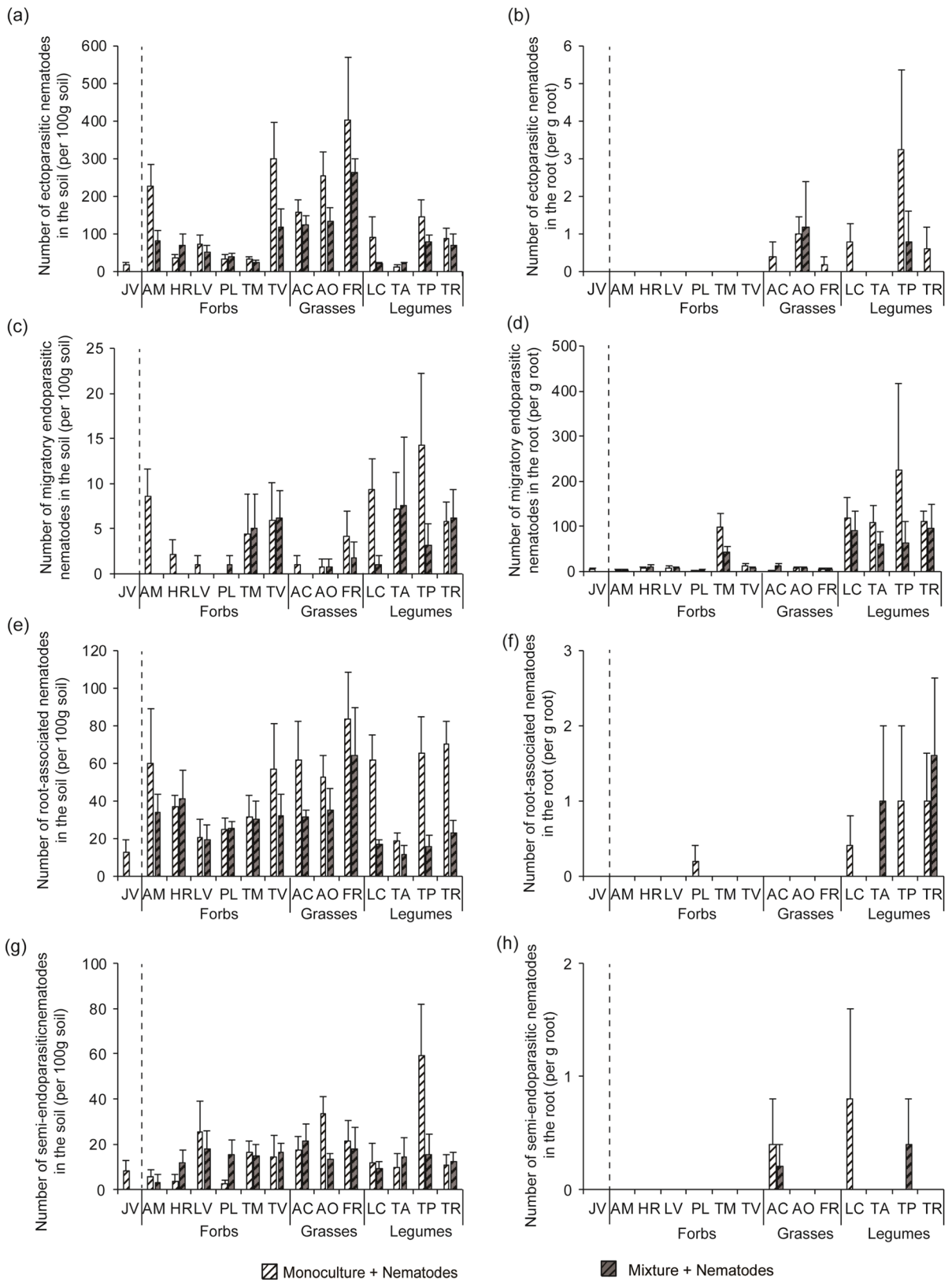
belowground associational resistance, and it shows that the presence of belowground herbivores can change the outcome of plant-plant interactions.

Plant competition, not the presence of nematodes, influenced plant biomass with forbs benefiting from interspecific competition with *J. vulgaris*, while grasses were not affected and legumes exhibited the opposite pattern. In our study most forb species benefited from interspecific competition while legumes suffered in interspecific competition independent of nematode addition. *Jacobaea vulgaris* produces a rosette aboveground and has a fibrous root system (Harper and Wood 1957). Recent evidence has shown that competition can promote root aggregation of individual plants and this may especially true for species with fibrous roots which have a higher morphological plasticity in rooting (Fry et al. 2018; Lepik et al. 2020). Other forb species in our study, most of them having tap roots, benefitted from growing with *J. vulgaris* instead of intraspecific neighbors. This is because these plants occupied more belowground space over *J. vulgaris* than with intraspecific neighbors. Grasses often have fibrous roots and this is relatively similar to the roots of *J. vulgaris*, and this may have resulted in similar competitive abilities in mixtures (Ravenek et al. 2016). This may explain why the shoot biomass of grass species did not differ in monocultures and mixtures. Although for grasses overall there was no significant difference in biomass between mixtures with *J. vulgaris* and monocultures, *A. odoratum* benefitted, while *F. rubra* suffered in

interspecific competition with *J. vulgaris*. Previous studies have found that *A. odoratum* has a higher root physiological plasticity for acquiring soil nitrogen, while this is not the case for *F. rubra* (Fransen et al. 1998, 1999). This may explain why *A. odoratum* and *F. rubra* performed opposite in interspecific competition with *J. vulgaris*. Legumes, in our study, produced relatively little root biomass and *J. vulgaris* benefited from this in the mixtures. Interestingly, *T. maritimum* is a forb that also produced relatively little root biomass. However, in mixtures with *T. maritimum*, *J. vulgaris* was not able to benefit from the extra belowground space and/or resources. We hypothesize that this may be due to allelopathic compounds released in the soil by *T. maritimum* or stimulation of specific soil microbes by *T. maritimum* that hamper the growth of *J. vulgaris*, but further studies are needed that examine the mechanisms behind the competition between these two species.

The non-significant impact of root-feeding nematodes on plant biomass in our study is somewhat unexpected and in contrast with other studies (e.g. Kaplan et al. 2008, 2009; de la Peña et al. 2009). In

Fig. 3 Mean (+SE) number of root-feeding nematodes in different groups in the soil and in the roots of the 13 other species in monocultures and mixtures. Sedentary endoparasitic nematodes were not plotted due to it rarely presented in the soil (only found in one pot) and completely absent in roots of other species. In (a–h) there was no significant differences between monocultures and mixtures ($p < 0.05$) based on a Tukey HSD post hoc test. Species abbreviations are described in the legend of Fig. 1



a study by Brinkman et al. (2005), addition of nematodes significantly affected shoot and root biomass of the dune grass *Ammophila arenaria*, but only in the second year probably when populations had built up to high levels. In our (short-term) study the populations of nematodes were likely too low to have an impact on plant growth (Piśkiewicz et al. 2008). Unfortunately, due to space limitation we were limited to use pots that contained only 2 kg of soil, and after 3 months pot size limited plant growth and this necessitated harvesting of the experiment. Further studies should examine the longer-term effects of nematodes on plant growth and plant-plant interactions.

Our study contained a series of plant species that ranged from relatively unsuitable to suitable for nematodes, as we can conclude from the density of root-feeding nematodes in the plant roots in monocultures. The numbers of root-feeding nematodes in the soil and in the roots of *J. vulgaris* in monoculture were relatively low and other studies have also shown that *J. vulgaris* has a negative effect on nematodes (Thoden et al. 2009a, b; Sikder and Vestergård 2020). Although *J. vulgaris* is a poor host for nematodes, the presence of *J. vulgaris* in mixtures did not significantly reduce nematodes in the roots of the other species when we simply look at the overall abundance. However, migratory endoparasitic nematodes which contributed the most (97%, on average of nematodes in the roots), were clearly reduced by the presence of *J. vulgaris*. This finding reveals that *J. vulgaris*

provides belowground associational resistance to other plants against migratory endoparasitic nematodes. This effect was detected for migratory endoparasitic nematodes, and this may depend on the way that this group of nematodes interacts with plants. During their life, migratory endoparasitic nematodes retain their mobility both outside and inside the plant roots, moving from one feeding location to another within the same root or among roots of different host plants (Moens and Perry 2009). The neighboring plant *J. vulgaris* may mask or delay the migration pattern and host location, and this may have resulted in a lower abundance of migratory endoparasitic nematodes in the roots of other species. This was also confirmed by our results on migratory endoparasitic nematodes in the soil. The numbers of ectoparasitic and migratory endoparasitic nematodes in the soil were lower in mixtures than in monocultures for some species. The vast majority of ectoparasitic nematodes was found in the soil, as they stay in soil and feed from outside the roots. Our study shows that non-host character of *J. vulgaris* does lead to suppression of migratory endoparasitic nematodes in roots of neighboring plants and of both ectoparasitic and migratory endoparasitic nematodes in the soil. Further studies should examine the mechanism behind this belowground associational resistance in more detail. Interestingly, the presence of *J. vulgaris* changed the structure of the nematode community in the soil for forb and legume species, but not for grasses. In line with previous studies, we

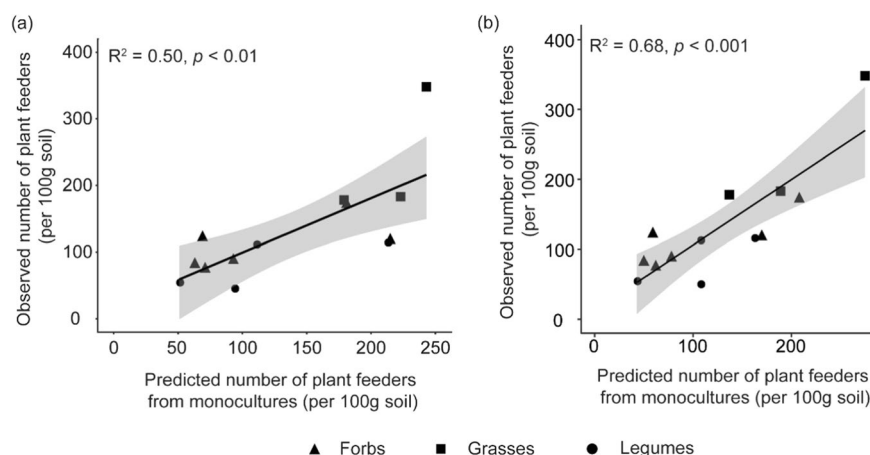


Fig. 4 Relationship between the predicted number of root-feeding nematodes in the soil (a) based on the shoot biomass and densities in monocultures ($N = N_o \times SO_{mix} / SO_{mono} + N_j \times SJ_{mix} / SJ_{mono}$) and (b) only based on densities in

monocultures ($N = 0.5 \times N_o + 0.5 \times N_j$) and the observed number of nematodes in mixtures. R^2 , P-values and the confidence interval from a linear regression analysis are also presented

found that root-feeding nematodes had relatively high densities on grasses even when mixed with a bad neighboring host (Viketoft et al. 2005).

Interestingly, in the presence of nematodes plant-plant interactions changed such that overall it became beneficial to *J. vulgaris*. Both above- and below-ground herbivory can greatly influence the outcome of plant competition (Schädler et al. 2007; Kim et al. 2013; Jing et al. 2015b). An earlier study suggests that generalist root herbivores can relax the competitive asymmetry between two plant species if the root herbivore suppresses the dominant species more (Borgström et al. 2016). In our study, most of the tested forbs were the dominant species in mixtures with *J. vulgaris*.

We assume that these forbs also occupied most of the space belowground, and hence we hypothesize that, although the impact of nematodes overall was low, the competing species experienced overall more root herbivores than *J. vulgaris*, and that this may have benefited *J. vulgaris* in the mixtures. However, we should notice that *J. vulgaris* was the dominant species in mixtures with legumes and that the competitive advance of *J. vulgaris* also increased in presence of nematodes in competition with legumes. Several studies have shown that nematodes can cause substantial damage on roots of legumes (Barker 1998; Davis and Mitchum 2005; Ye et al. 2020) and this was also the case in our study. This could probably explain

Fig. 5 Principal component analysis (PCA) of the soil nematode community showing the species plot (a) and sample scores (b–d). For clarity, the sample scores are shown separately for forbs (b), grasses (c) and legumes (d) only soil nematodes with a contribution larger than 2.5% for PC1 or PC2 axis are shown to avoid the overlap of arrows (a). In b–d, Squares represent mean sample scores for each other species in monocultures (white) and mixtures (grey; n = 5) and for *J. vulgaris* monocultures (light grey). Error bars represent the SE of the mean PCA scores for the first or second axis. Species abbreviations are described in the legend of Fig. 1

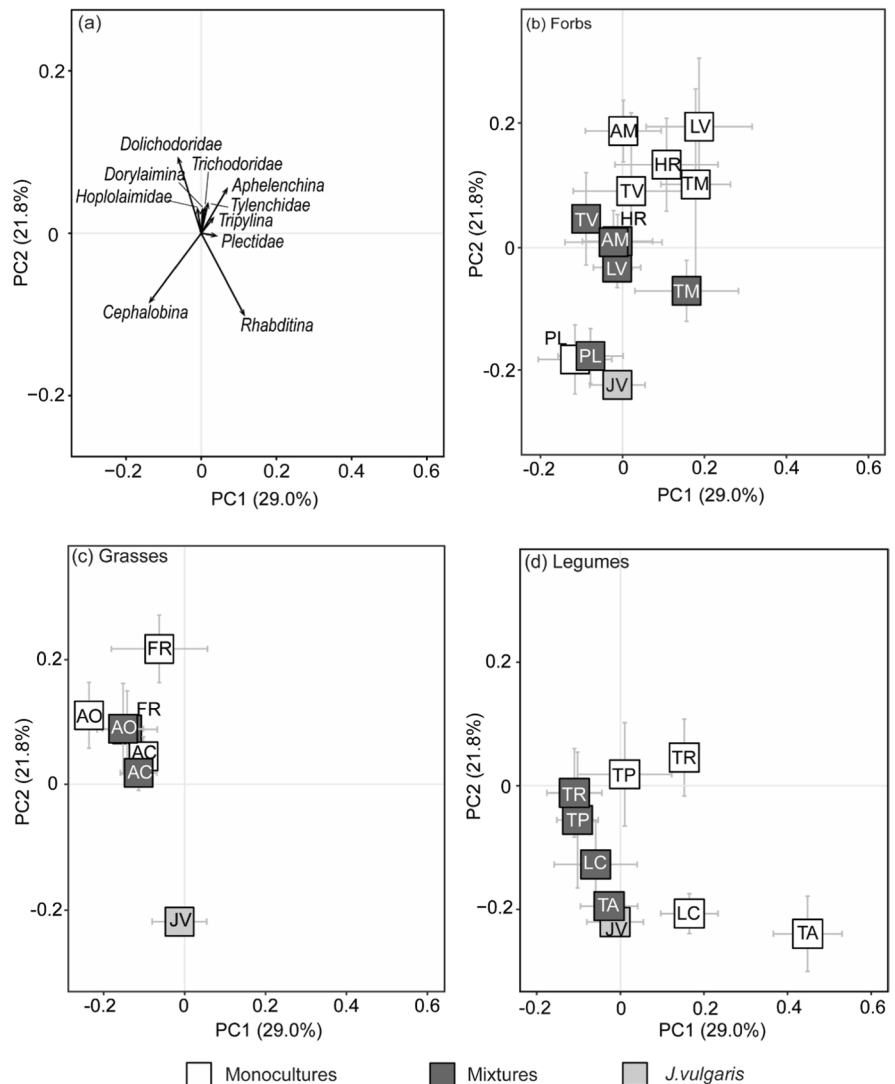
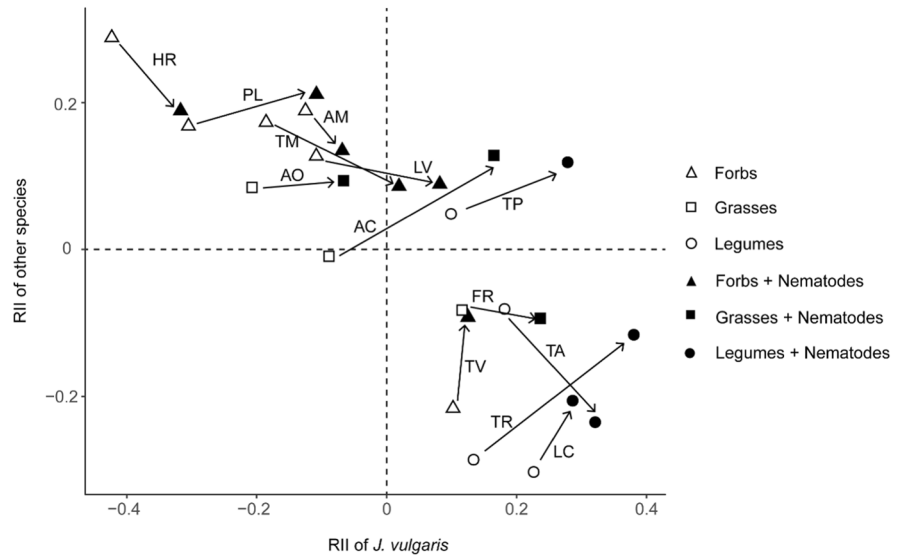


Fig. 6 The change of the outcome of plant-plant interactions between *J. vulgaris* and the other species with or without nematodes. The arrows direct the change in plant-plant interactions in mixture in presence of nematodes for each species. Species abbreviations are described in the legend of Fig. 1



the competitive advantage of *J. vulgaris* in the presence of nematodes when growing with legumes. Alternatively, it is also possible that *J. vulgaris* responded to exposure to root-feeding nematodes with increased root growth independent of the response of the neighboring plants to root herbivores. Increased root growth of *J. vulgaris* in response to root herbivores has been noted in several studies (Kostenko et al. 2013; Jing et al. 2015b; Lin et al. 2018). Our study also exemplifies that the presence of root herbivores, nematodes in this case, can increase the coexistence between two species. Previous studies have found negative (Fraser and Grime 1999; Wang et al. 2019), positive (De Deyn et al. 2003; Stein et al. 2010; Borgström et al. 2017) and neutral (Wurst and Rillig 2011) effects of belowground herbivory on the diversity of plant communities and we still poorly understand how the presence of root herbivores impact coexistence (Carson and Root 2000; Körner et al. 2014).

In mixtures consisting of *J. vulgaris* and the forb *L. vulgare*, the grass *A. capillaris* or the legume *T. pratense* the presence of root-feeding nematodes resulted in a facilitative interaction between the two plant species or increased this interaction. Similar results have been shown for other belowground herbivores and other plant species, and this shows that root herbivores can not only alleviate competitive interactions between plants but can even result in facilitative

effects (Graff and Aguiar 2011; Louthan et al. 2014; de Matos et al. 2019; He et al. 2019). Further studies should examine how root herbivores cause or increase these facilitative interactions. Overall, our study exemplifies that soil dwelling nematodes can exert a diverse range of outcomes of plant-plant interactions. Further, it is important to notice that soil microbes such as AMF and endophytes can play a vital role in regulating root-herbivore interactions (Hol et al. 2010; Schouteden et al. 2015; Johnson et al. 2016a, b; Wang et al. 2019). In our study we used sterilized soil. Even though microbes inevitably were introduced when we added the nematode extractions, many potential interactions with soil microbes were omitted and this may have influenced the effects we observed. Further studies should examine plant-plant interactions in the presence of both nematodes and microbes.

In conclusion, our study demonstrates that *J. vulgaris* provides belowground associational resistance to neighboring plants against migratory endoparasitic nematodes. While nematodes did not influence plant biomass in our study, they changed the outcome of plant-plant interactions and interestingly often so that it favored *J. vulgaris* instead of the neighboring plant.

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Author's contribution T.M.B designed the experiment. C.R. maintained the experiment and extracted and identified nematodes and C.R. and T.M.B. collected biomass data. X.Y.L., K.V. and S.T.E.L. and T.M.B. developed and discussed analysis and presentation of the data, and X.Y.L. analyzed all data. X.Y.L. and T.M.B. wrote the first version of the manuscript; and all authors revised and improved subsequent versions of the manuscript.

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