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# Interspecific differences in nematode control between range-expanding plant species and their congeneric natives

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ABSTRACT

Climate change enables range expansions of plants, animals and microbes to higher altitudes and latitudes. Plants may benefit from range expansion when they escape from natural enemies. However, range expansion becomes a disadvantage when plants become disconnected from organisms that control enemies in the new range. Here, we examined nematode control in the root zone of range-expanding plant species and congeneric natives. In a greenhouse, we determined bottom-up (by the plants) and top-down (by natural enemies of the nematodes) control of two root-feeding nematode species (*Helicotylenchus pseudorobustus* and *Meloidogyne hapla*) in the rhizospheres of two range-expanding plant species, *Centaurea stoebe* and *Geranium pyrenaicum*, and two congeneric natives, *Centaurea jacea* and *Geranium molle*. Pots with plants growing in sterilized soil were inoculated with either a microbial soil community from the newly colonized natural habitat, a mixture of native microbial nematode antagonists, or a combination of these two communities. We tested the hypotheses that bottom-up control of root-feeding nematodes would be strongest in the root zone of range expanders and that top-down control would be strongest in the root zone of native plant species. We observed profound intra- and interspecific differences in bottom-up and top-down control among all four plant species. Bottom-up control by the range-expanding plant species was either strong or weak. Top-down control by microbes was strongest in native *Centaurea*. The addition of a mixture of both microbial communities reduced control of *M. hapla* in the root zones of the native plant species, and enhanced its control in the root zones of range-expanding plant species. We conclude that there was species-specific bottom-up and top-down control of root-feeding nematodes among the four plant species tested. Range-expanding plant species influenced their microbial rhizosphere community differently compared to native plant species, but top-down control in the root zone of natives was not systematically superior to that of range-shifting plant species.



## 1. Introduction

Recent climate warming has enabled altitudinal and latitudinal range expansions of many animal and plant species (Parmesan, 2006; Chen et al., 2011). Such range expansions can lead to disruptions of co-evolved biotic interactions, as individual species shift range at contrasting rates (Berg et al., 2010). While some plant species, aboveground vertebrate and invertebrate species may be able to shift range relatively quickly, belowground organisms are likely to lag behind (Berg et al., 2010). Eventually, such complex interactions might become re-established in the new range, when slower range-expanding species colonize the new areas. However, it is currently unknown what happens in the initial phases of range-expansion, when plant species are colonizing new areas and encounter novel enemies and their antagonists, which are both non-adapted to the introduced plant species.

Some recent studies have shown that climate warming-induced range-expanding plant species or populations can be less strongly affected by belowground enemies in their new range than in their old range (van Grunsven et al., 2010; De Frenne et al., 2014). Moreover, these range expanders may experience less negative effects of soil organisms in their new range than congeneric natives (van Grunsven et al., 2007; Engelkes et al., 2008). This suggests that range shifts result in a release from natural enemies, which has

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been proposed as an important cause of invasiveness of introduced exotic species (Keane and Crawley, 2002; Mitchell and Power, 2003). However, compared to exotic species introduced from geographically isolated areas, plant species expanding their range within a continent are less likely to be completely released from natural enemies as some of these enemies might be widespread in a larger geographical area.

Despite the presence of natural enemies, successful range-expanding plant species might have a benefit over native plants, as range expanders have been shown to be more strongly defended against naïve aboveground herbivores than congeneric natives (Engelkes et al., 2008). This stronger defense against generalists by the range-expanding plant species could be due to increased resource allocation to general defense mechanisms in response to reduced specialist herbivore and pathogen pressure (Müller-Schärer et al., 2004; Joshi and Vrieling, 2005; Oduor et al., 2011; Lin et al., 2015). Additionally, range expanders might possess certain allelochemicals in roots or shoots, to which the native soil community is not well adapted (Cappuccino and Arnason, 2006; Schaffner et al., 2011). Indeed, range expanders produce more unique metabolites than related natives (Macel et al., 2014). Together, these defense mechanisms may provide the range-expanding plant species with a competitive benefit over native plant species, as they suffer less from specialist herbivores and their generalist enemies are not well adapted to their novel defense mechanisms (Bossdorf, 2013; Uesugi and Kessler, 2013).

Also belowground range-expanding plants may be better defended against generalist herbivores from the new range than their native congeners. In soil from the new range, range expanders indeed were shown to accumulate fewer root-feeding nematodes per unit root mass than congeneric species that are native in the new range (Morrien et al., 2012). Such reduced densities of root-feeding nematodes might be due to either enhanced control by the plant roots (also named bottom-up, or resource control) or control by natural enemies (also named top-down or predator control), or a combination of both mechanisms. Previous studies in other systems have shown that bottom-up control by direct plant defense mechanisms (van der Stoel et al., 2006) and top-down control by fungi, bacteria, micro-arthropods and protists are all possible (Kerry, 2000; Piskiewicz et al., 2008; Costa et al., 2012; Geisen et al., 2015). These control mechanisms can operate on nematodes in species-specific ways (Piskiewicz et al., 2008). Range-expanding plant species have been shown to accumulate different microbial communities in their rhizospheres compared to closely related natives (Morrien and van der Putten, 2013). However, it is unknown whether these community differences have consequences for root-feeding nematode control, for example due to longer shared co-evolutionary histories of microbial nematode antagonists with native than with range-expanding plant species.

Here, we quantify and compare effects of top-down and bottom-up control of root-feeding nematodes in the rhizosphere of range-expanding plant species and congeneric natives. We tested the hypotheses that 1) if top-down control of nematodes by soil microbes is plant-species specific, we expect this control within congeneric pairs to be stronger in the native than in the range expander and 2) range-expanding plant species exert stronger bottom-up control on root-feeding nematodes than congeneric natives. In order to test the hypotheses, we conducted a greenhouse experiment to examine the microbial control of two native generalist root-feeding nematode species, *Meloidogyne hapla* and *Helicotylenchus pseudorobustus*, in the rhizospheres of two range-expanding plant species and their native congeners. This experiment will provide insights in how complex multi-trophic interactions may function in the rhizospheres of climate-driven range-expanding plant species in their new range, and how these interactions differ from those of related native plant species. The experimental results will contribute to enhanced insights in how multi-trophic interactions of non-native plant species may become assembled in their new range.

## 2. Methods

### 2.1. Plant species and seed collection

We tested our hypotheses using two range-expanding plant species that originate from southern Europe, *Centaurea stoebe* L. and *Geranium pyrenaicum* Burm. f., and two congeneric species that are native in the newly colonized range in north-western Europe, *Centaurea jacea* L. and *Geranium molle* L.. *Centaurea stoebe* originates from the Danube area and since the late 1990's invaded the Rhine valley and some suitable habitats in The Netherlands (FLORON, 2014). *Geranium pyrenaicum* originally has a more widespread south-European distribution and although it colonized Northwestern Europe already in the 19th century, it only showed a strong expansion in the Netherlands since the 1980's, where it now is common (FLORON, 2014). Both congeneric native species *C. jacea* and *G. molle* are common throughout northern and southern Europe.

All seeds used for the present study originated from plant populations from the Netherlands. Seeds of *C. stoebe* and *G. molle* were collected directly from the field. Seeds of *C. jacea* originated from an experimental garden in Wageningen. They were collected from first generation plants grown from seeds of plants growing in Dutch field sites. Seeds of *G. pyrenaicum* were delivered by the seed production company Cruydthoeck (Nijeberkoop, The Netherlands), where plant species are cultured from seeds collected in Dutch field sites. Seeds of all plant species were surface-sterilized by washing them for 3 min in 10% bleach solution, after which they were rinsed with demineralized water, and germinated on glass beads in a growth cabinet (20/10 °C; 16 h light/8 h dark).

### 2.2. Nematode cultures

Two generalist root-feeding nematodes that commonly occur throughout Europe were extracted from cultures originating from Dutch field sites. An inoculum of the sedentary endoparasite *Meloidogyne hapla* Chitwood (hereafter referred to as *Meloidogyne*) was collected from a field near Bovensmilde (Drenthe, The Netherlands), subsequently cultured on tomato (*Solanum lycopersicum* L.) at PPO-AGV (Lelystad, The Netherlands) and extracted using a mistifier (Funnel-spray method; Oostenbrink, 1960). A population of the ectoparasite *Helicotylenchus pseudorobustus* Steiner (hereafter referred to as *Helicotylenchus*), originating from coastal sand dunes, was cultured on Marram grass (*Ammophila arenaria* L.) at NIOO-KNAW (Wageningen, the Netherlands) and extracted using an Oostenbrink elutriator (Oostenbrink, 1960).

### 2.3. Microbial inocula

We prepared three different microbial inocula and tested their effects on root-feeding nematode abundance on range expanders and congeneric natives: a general microbial inoculum obtained from field soil, a specific nematode antagonist inoculum and a combination of the two. The used field soil was collected from riverine grasslands where most of the plant species used in the present study are present in the immediate surroundings. To obtain the general microbial inoculum, we used a serial wet-sieving approach to establish a community of predominantly microbes <20 μm (see: van de Voorde et al., 2012). We used nine batches of 2 kg top soil collected from 3 sites (6 kg per site) in a riverine

grassland (Wageningen, The Netherlands; 51°57′N, 5°39′E) that were mixed with 1.5 l demineralized water, stirred and left for 15 min. This stirring procedure was then repeated for each batch, after which the supernatant went through sieves with mesh sizes of 1 mm, 180 μm, 75 μm, 45 μm (twice) and 20 μm. Hence, we obtained 12.5 l inoculum with a general microbial wash from 18 kg of field soil.

The inoculum of nematode antagonists included three nematophagous fungi and the nematophagous amoeba *Cryptodifflugia operculata*, which was cultured on a mixed prokaryotic community in a liquid wheat grass medium (Geisen et al., 2015). The nematophagous fungi were obtained from field soil from a riverine grassland (Millingerwaard, Netherlands; 51°52′N, 6°0′E), by adding 0.1 g of soil to three Petri dishes filled with water that contained a free-living nematode community from different trophic groups, which was collected from the same grassland. After one week, an inverted microscope (Olympus CK40) at 100 and 200× magnification was used to detect killed or parasitized nematodes. Dead nematodes with hyphae or spores of potentially nematophagous fungal or oomycete origin were transferred individually to 1% water agar for subsequent cultivation. Three well-growing monoclonal fungal cultures were selected and used for the experiment. We collected spores using a sterile metal cell-scraper after adding 1 ml double-distilled water. Spore numbers were determined using an inverted microscope (Olympus CK40) at 400× magnification. The amoebae were acquired by detaching one week old, well active cultures from the surface of five 10 cm Petri dishes by vigorous shaking. The amoebae-suspension then immediately was transferred to 50 ml centrifuge tubes and carefully centrifuged at 800 rpm for 5 min. The supernatant was then decanted, after which the suspensions were pooled and enumerated. The three fungal and the amoebae cultures were combined and named nematode antagonist inoculum. Each pot inoculated with the nematode antagonist mixture received 1.4 ml suspension containing $1.6 \times 10^6$ *C. operculata* amoebae, as well as $3.4 \times 10^6$, $1.3 \times 10^6$ and $1.5 \times 10^6$ spores of fungal isolates Mil3, Mil4, and Mil5b, respectively.

### 2.4. Experimental set-up

A three-factor pot experiment was set up using 4 plant species (*C. jacea*, *C. stoebe*, *G. molle and G. pyrenaicum*), 3 nematode treatments (*Helicotylenchus*, *Meloidogyne* and a control without root-feeding nematodes), and 4 soil treatments (microbial inoculum, nematode antagonist inoculum, combined microbial and nematode antagonist inoculum and a control without live inoculum), with each treatment replicated 5 times, resulting in 240 pots. Sandy clay soil was collected from a former agricultural field in the riparian area of the same river system as Millingerwaard (Beneden-Leeuwen, The Netherlands; N51° 53.952, E05° 33.670). This soil was homogenized with sand (2:1 soil:sand) and sterilized using gamma-sterilization (McNamara et al., 2003; 25 KGray, Syngenta bv, Ede, The Netherlands). Pots of 1 l were filled with 830 g of the sterilized soil. Of each plant species 60 seedlings were planted in individual pots. After 10 days, two thirds of all pots were inoculated with 2 ml water suspension containing 200 juveniles of either *Meloidogyne* or *Helicotylenchus*. One third of all pots did not receive any nematodes. Next, microbial treatments were established: pots received either 50 ml of the general microbial inoculum, 1.4 ml of the nematode antagonist inoculum, or a combined inoculum of both the general microbial (50 ml) and the nematode antagonist inoculum (1.4 ml). Control pots did not receive any live inoculum. To compensate for potential nutrient and moisture effects control pots received 50 ml sterilized general microbial inoculum and 1.4 ml sterilized nematode antagonist inoculum, pots containing the general microbial community received sterilized 1.4 ml nematode antagonist inoculum and pots with the nematode antagonist community received 50 ml sterilized general microbial inoculum. The pots were placed in a greenhouse compartment at 16 h light (20 °C), 8 h dark (15 °C) and 60% relative humidity according to a randomized block design on carts, which were rotated weekly. Throughout the experiment the pots were watered twice per week. Once a week, pots were reset to a weight of 860 g by adding demineralized water, representing a moisture content of approximately 15%.

### 2.5. Harvest

Fifteen weeks after inoculation, the aboveground plant parts were harvested and dried at 70 °C until constant weight. Subsequently, all soil from every pot was collected for nematode extraction, and 2-ml centrifuge tubes with well-homogenized soil were stored at −20 °C for DNA extraction. To reduce the loss of nematodes from the rhizosphere, roots were first washed in 200 ml water, after which the washout was stored at 4 °C until nematode extraction. Root systems from *Helicotylenchus* pots were placed in a mistifier (Funnel-spray method; Oostenbrink, 1960) for 24 h to extract remaining root-attached nematodes of this ectoparasitic species. The roots were dried at 70 °C until they reached constant weight. Root systems from pots containing the endoparasitic *Meloidogyne* were split: one half was placed in a mistifier for 4 weeks in order to extract nematodes from developing eggs inside the roots, and the other half was weighed fresh, dried at 70 °C until constant weight, and weighed again. Once per week nematodes were collected from the mistifier and stored at 4 °C. After 4 weeks, all nematode subsamples harvested from the same root sample were combined into one single pot and concentrated to 10 ml. For both the pots with *Helicotylenchus* or *Meloidogyne*, as well as 3 replicates of the non-nematode treatments, free-living nematodes were extracted from the bulk soil and the rhizosphere soil suspension using an Oostenbrink elutriator (Oostenbrink, 1960), and concentrated to 10 ml prior to counting.

### 2.6. Nematode counting

Nematodes were counted alive using an inverted microscope (Olympus CK40, 40× and 100× magnification). Either the full sample was counted or, in case of high densities, 2 subsamples of 1 ml, each diluted 10 times. During nematode counting, all samples were carefully checked for contamination with other root-feeding nematodes. Because of contamination with *Meloidogyne*, 2 samples from pots inoculated with *Helicotylenchus* were excluded from further analysis. In all samples bacterivorous nematodes were found, which could originate from both co-inoculations of bacterivorous nematode eggs with the microbial inocula and natural colonization of the pots via air.

### 2.7. Bacterial and fungal quantification

We quantified bacteria and fungi using quantitative (q)PCR in the pots containing *Meloidogyne*, as we found stronger inoculum effects on this nematode species than on *Helicotylenchus*. Soil DNA was extracted using the PowerSoil DNA isolation kit (Mo Bio Laboratories Inc, Carlsbad, USA) and stored at −20 °C. Bacterial 16s rDNA copy numbers were quantified using the primer combination 515F and 806R (Caporaso et al., 2011). The qPCR mastermix contained 0.25 μl BSA (Roche Diagnostics, Basel, Switzerland), 10 μl SensiFAST SYBR® No-ROX (Bioline, Taunton, USA), 0.25 μl 515F (10 μM; Apha DNA, Montréal, Canada), 0.25 μl 806R (10 μM; Apha DNA) and 5 μl DNA template in a total volume of 20 μl. Cycling conditions were the following: initiation for 3 min at 95 °C,

followed by 40 cycles of 30 s at 95 °C, 30 s at 50 °C, 1 min at 72 °C with a final elongation for 5 min at 72 °C. Fungal ITS copy numbers were quantified using the primer combination ITS4 and ITS9 targeting the fungal ITS2 region (White et al., 1990; Ihrmark et al., 2012). The qPCR mix contained 1 μl $MgCl_2$ (Roche Diagnostics), 0.25 μl forward ITS4 primer (30 μM; Alpha DNA), 0.25 μl reverse ITS9 primer (30 μM; Alpha DNA), 10 μl SensiFAST SYBR® No-ROX, 5 μl DNA template in a total volume of 20 μl. Cycling conditions were the following: initiation for 3 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 1 min at 72 °C with a final elongation for 5 min at 72 °C. Both qPCR approaches were replicated twice for each sample. Analyses of the qPCRs were done using Biorad CFX manager (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). The average number of PCR-cycles needed to reach a threshold value determined by the software was used to calculate total abundances of bacteria (16S rDNA copy numbers) and fungi (ITS2 copy numbers) in each sample. The ratio between the inverse of these abundance measures was used to calculate the bacterial/fungal-ratio.

### 2.8. Statistical analyses

All statistical analyses were performed in R Studio (Version 0.98.507; R Core Development Team, 2012). Nematode count data were analyzed using negative binomial generalized linear models, as the data were strongly overdispersed (Hilbe, 2014). *Helicotylenchus* and *Meloidogyne* counts were analyzed separately. We modeled total numbers per pot and numbers per gram root as the response of each nematode species to the fixed factors block, plant species and inoculum, as well as to the interaction between plant species and inoculum. Because of the use of only 2 species pairs, we did not include the factor origin (range-expander or native). As negative binomial generalized linear models have to be provided with integer values, and *Helicotylenchus* numbers were low, we expressed *Helicotylenchus* numbers per 10 g root to avoid introduction of zeroes in the model. Model fit was checked using residual plots and AIC-values. Using post-hoc Wald tests performed with the R-package 'phia' (De Rosario-Martinez, 2013) we determined for each plant species the pairwise differences in nematode numbers between the different inocula and overall differences in nematode numbers between plant species. A general linear model and subsequent post-hoc Wald tests were used to test the effects of nematode species, inoculum and plant species on total plant biomass data. Two-way ANOVA models were used to analyze the effect of plant species and inocula on the relative abundance of bacteria, fungi and the bacterial/fungal ratio for the pots inoculated with *Meloidogyne hapla*. Residual plots and Shapiro-Wilk normality tests were used to confirm that model assumptions were not violated.

## 3. Results

### 3.1. Plant biomass

There was a significant main treatment effect of inoculum addition ($F = 2.68$, $p < 0.05$), because plants receiving the combined microbial and nematode antagonist community produced significantly less biomass than plants receiving the nematode antagonist and the microbial communities alone (Fig. S1). However, this effect size was relatively minor.

### 3.2. Root-feeding nematode numbers

#### 3.2.1. Meloidogyne hapla

We found strong differences in total *Meloidogyne* numbers among plant species ($\chi2 = 111.89$, $p < 0.001$). Total numbers of *Meloidogyne* were significantly ($\chi2 = 434.54$, $p < 0.01$) higher in native *C. jacea* than in range-expanding *C. stoebe* (Fig. 1). *Meloidogyne* also performed significantly poorer on *C. stoebe* than on both native and range-expanding *Geranium* species (Fig. 1). However, the range-expander *G. pyrenaicum* was a better host for *Meloidogyne* than the native *G. molle* ($\chi2 = 51.76$, $p < 0.01$; Fig. 1). Effects of plant species on the total numbers of *Meloidogyne* depended on soil inoculum (interaction effect: $\chi2 = 86.53$, $p < 0.01$). The nematode antagonist community significantly reduced *Meloidogyne* numbers in *C. jacea* ($\chi2 = 4.58$, $p < 0.05$; Fig. 2a). This reduction, however, disappeared when the nematode antagonists were added in combination with the general microbial community; in that case *Meloidogyne* numbers were significantly higher than in pots with only the nematode antagonist community ($\chi2 = 5.91$, $p < 0.05$; Fig. 2a). There were no strong inoculum effects in the root zone of *C. stoebe*. However, in this species, the combined microbial and nematode antagonist community significantly reduced *Meloidogyne* numbers compared to the general microbial community ($\chi2 = 8.94$, $p < 0.01$; Fig. 2b). In *G. molle*, pots with nematode antagonists added had significantly lower numbers of *Meloidogyne* than pots with the combined microbial and nematode antagonist community added ($\chi2 = 4.65$ $p < 0.05$; Fig. 2c). In *G. pyrenaicum* the opposite pattern ocurred: pots with the combined microbial and nematode antagonist community had lower numbers of *Meloidogyne* than pots with only nematode antagonists (total: $\chi2 = 4.24$, $p < 0.05$; Fig. 2d). Overall, patterns of *Meloidogyne* numbers per gram root strongly corresponded with total *Meloidogyne* numbers per pot with some minor exceptions: while *C. jacea* was found to accumulate the highest *Meloidogyne* numbers per pot, numbers of *Meloidogyne* per gram root were higher in *G. pyrenaicum* than in *C. jacea* (Fig. S2). Furthermore, in *G. molle*, pots inoculated with the nematode antagonists did not have lower *Meloidogyne* numbers per gram root than pots inoculated with the combined microbial and nematode antagonist community (Fig. S3).

#### 3.2.2. Helicotylenchus pseudorobustus

Numbers of the ectoparasite *Helicotylenchus* in all 4 plant species were substantially lower than numbers of *Meloidogyne* (Fig. 1). Nevertheless, we found a significant plant species effect on total *Helicotylenchus* numbers ($\chi2 = 104.85$, $p < 0.01$); there were significantly higher numbers of *Helicotylenchus* on *G. pyrenaicum* than on all other plant species (all p-values <0.01). Effects of plant species on total numbers of *Helicotylenchus* depended on soil inoculum (significant species × inoculum interaction; $\chi2 = 85.11$, $p < 0.05$). Inoculum type did not have a significant effect on numbers of *Helicotylenchus* in both native species *C. jacea* and *G. molle* (Fig. 2e and g). In *C. stoebe*, nematode antagonists significantly reduced total numbers of *Helicotylenchus* compared to the combined microbial and nematode antagonist community ($\chi2 = 5.22$, $p < 0.05$; Fig. 2f). In *G. pyrenaicum*, the total number of *Helicotylenchus* was significantly lower in pots with the combined microbial and nematode antagonist community than in pots with the general microbial inoculum ($\chi2 = 6.66$, $p < 0.01$), or in pots with the nematode antagonists ($\chi2 = 6.01$, $p < 0.05$; Fig. 2h). *Helicotylenchus* densities per gram root were significantly different among plant species (all p-values < 0.05; Fig. S2). Both range-expanding plant species contained more *Helicotylenchus* per gram root than their native congenerics (Fig. S2), and plant species effects did not depend on inoculum, which differs from total numbers per pot. There was also no main effect of inoculum when *Helicotylenchus* density was expressed as numbers per g root.

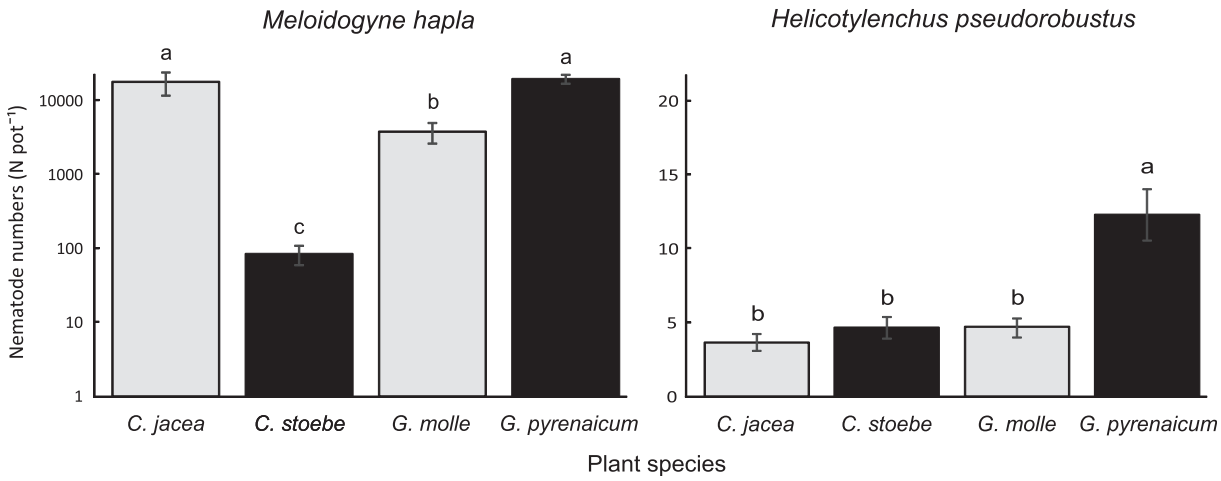


**Fig. 1.** Mean total numbers (N pot$^{-1}$) of root-feeding nematodes *Meloidogyne hapla* (left; logarithmic scale) and *Helicotylenchus pseudorobustus* (right; linear scale) on range-expanding (black) plants *Centaurea stoebe* and *Geranium pyrenaicum* species and related natives *Centaurea jacea* and *Geranium molle* (grey). Vertical bars show means ± standard errors. Different letters indicate significant ($p < 0.05$) pairwise post-hoc Wald tests between plant species.

### 3.3. Bacterial and fungal abundances

Abundances of soil bacteria, expressed as 16S rDNA copy numbers, were significantly different between plant species ($F = 3.18$, $p < 0.05$; Fig. 3). *Geranium* species harboured more bacteria than *Centaurea* species, whereas differences within species pairs were not significant. Fungal abundances, based on ITS copy numbers, depended on a combination of plant species and soil inoculum (species × inoculum interaction $F = 2.19$,$p < 0.05$). *Centaurea stoebe* had fewer fungi in the control than in the three soil inoculation treatments (all p-values < 0.05), and fungal abundance was lower in the combined microbial and nematode antagonist community than in the nematode antagonist community ($F = 4.91$, $p < 0.05$; Fig. 4). The *C. stoebe* control treatment had a lower fungal abundance than the control treatments of *C. jacea* ($F = 8.71$, $p = 0.052$) and *G. molle* ($F = 11.82$, $p < 0.01$; Fig. S4). Overall, the bacterial/fungal ratio was significantly ($F = 3.45$, $p < 0.05$) influenced by soil inoculation, and the bacterial/fungal ratio in the nematode antagonist treatment was significantly lower than in the control and other inoculum treatments (Fig. 5). This change in bacterial/fungal ratio occurred due to both a relatively low bacterial abundance and a relatively high fungal abundance.

## 4. Discussion

Our results show species-specific patterns of bottom-up and top-down control of generalist root-feeding nematodes, both between and within two pairs of range-expanding and related native plant species. Our hypothesis that bottom-up control of root-feeding nematodes is stronger in the root zone of range-expanding plant species than of their congenerically related natives was supported in the case of the range-expander *C. stoebe*. This plant species had considerably stronger bottom-up defense against the endoparasite *Meloidogyne* than the congeneric native *C. jacea* (Fig. 1). However, *Meloidogyne* showed stronger multiplication on roots of the range-expanding *G. pyrenaicum* than on the native *G. molle* (Fig. 1). *Geranium pyrenaicum* was also a better host for the ectoparasitic *Helicotylenchus* than *G. molle*. *Helicotylenchus* numbers did not differ between the two *Centaurea* species. When expressed per unit of root weight, *Helicotylenchus* densities tended to be higher on both range expanders than on related natives (Fig. S2), which is not in support of our hypothesis. On all plant species, numbers of *Helicotylenchus* were relatively low.

Although range-expanding plant species are thought to benefit when released from their specialized soil-borne enemies after latitudinal range expansion (van Grunsven et al., 2010; De Frenne et al., 2014), plants will still be exposed to natural enemies in the new range, including widespread generalist enemies. Both *Meloidogyne hapla* and *Helicotylenchus pseudorobustus* are widespread throughout Europe (Bongers, 1988), which does not exclude a co-evolutionary history with all four plant species. However, the limited dispersal capacity of nematodes and low gene flow between nematode populations (Blouin et al., 1999) could have led to local adaptation of the nematodes to native plant species of Northwest European populations. A similar event of local adaptation of a natural enemy was also found for range-expanding butterflies and their parasitoids in Great Britain (Menendez et al., 2008). Therefore, plant-nematode interactions that are established when range-expanding plant species encounter individuals of these root-feeding nematodes populations in newly colonized areas, may at least to some extent result in novel interactions when the plants encounter non-adapted populations of the same herbivores in the new range.

Both strong suppression by, or release from aboveground and belowground herbivores has been argued a possible outcome of novel plant-herbivore interactions, as both plant and herbivore might be maladapted to their new host or enemy (Verhoeven et al., 2009). The strong bottom-up control of *Meloidogyne* by *C. stoebe* corresponds with low levels of herbivory on this plant species as found in several studies in North America (Cappuccino and Carpenter, 2005; Schaffner et al., 2011), where *C. stoebe* is an invasive exotic. Native generalist moths grow poorer on *C. stoebe* than European generalists (Schaffner et al., 2011). Moreover, *C. stoebe* is less prone to aboveground herbivory than the non-invasive exotic *C. jacea* (Cappuccino and Carpenter, 2005), indicating that *C. stoebe* may produce secondary compounds to which the native community is not adapted. In our study, the strong bottom-up control of *Meloidogyne* by *C. stoebe* suggests a similar maladaptation of the nematode to the root compounds of this plant species. Interestingly, we also found evidence for lower fungal abundances in the control soils of *C. stoebe* than in control soils grown with the other plant species, suggesting an inhibiting effect of root compounds or exudates of *C. stoebe* on fungal growth. In contrast to the strong direct defense of *C. stoebe*, the high *Meloidogyne* numbers found in *G. pyrenaicum* point to a non-existent or weak bottom-up defense of the plant, allowing herbivores

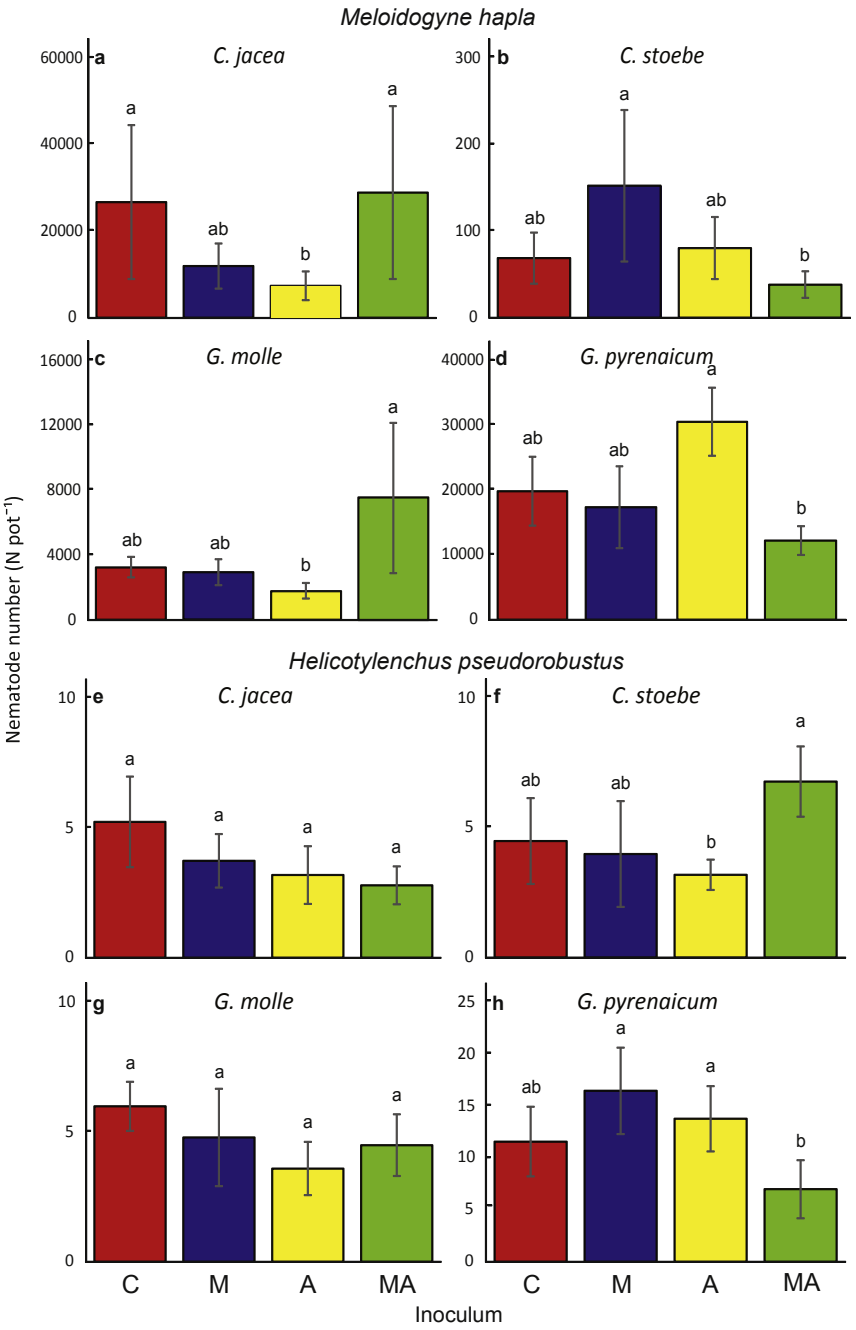


**Fig. 2.** Microbial inoculum effects on mean total numbers of root-feeding nematodes *Meloidogyne hapla* (A,B,C,D) and *Helicotylenchus pseudorobustus* (E,F,G,H) on native plant species *Centaurea jacea* and *Geranium molle* (left) and range-expanding plant species *Centaurea stoebe* and *Geranium pyrenaicum* (right). Vertical bars show means ± standard errors. Different letters indicate significant ($p < 0.05$) pairwise post-hoc Wald tests between plant species. Per panel, the four bars represent following inoculum treatments: control (C; red), general microbial community (M; blue), nematode antagonists (A; yellow) and the mixed community (MA; green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

associated with related native plants to easily exploit the new host (Louda et al., 1997).

We found strong plant species-specific effects on top-down control of both root-feeding nematode species (Fig. 2). We expected the microbial communities to have strong nematode control potential in the rhizospheres of the native plant species. However, the nematode antagonist community effectively controlled *Meloidogyne* numbers only in the root zone of the native *C. jacea*. Therefore, we found mixed evidence to support the hypothesis that top-down control of root-feeding nematodes is strongest in native plant species. Remarkably, unlike in other experiments on nematode control by microbial communities (Piskiewicz et al., 2007; Viketoft and van der Putten, 2014) there was no effective top-down control of the two root-feeding nematode species by the general microbial inoculum. Interestingly, the controlling effect of the nematode antagonists in the root zone of *C. jacea* was lost when they were added in combination with the general microbial community (Fig. 2). In both native plant species, numbers of *Meloidogyne* were higher in the presence of the combined microbial and nematode antagonist community than in pots with the nematode

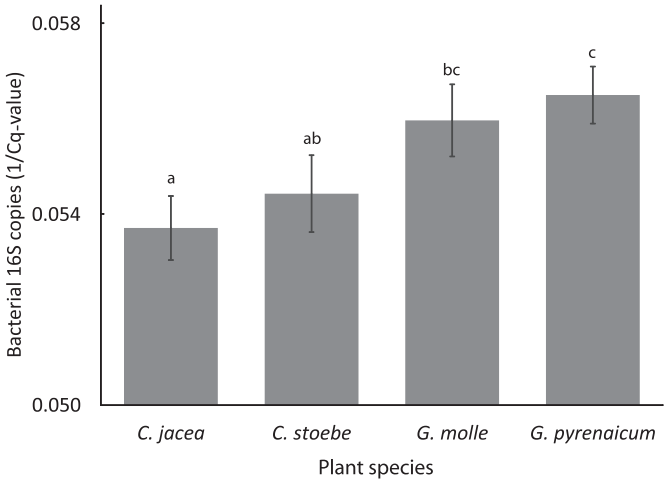


**Fig. 3.** Plant species effects on bacterial abundances (1/qPCR threshold value) of native plant species *Centaurea jacea* and *Geranium molle* and range-expanding plant species *Centaurea stoebe* and *Geranium pyrenaicum*. Vertical bars show means ± standard errors. Different letters indicate significant ($p < 0.05$) pairwise post-hoc Wald tests between plant species per inoculum treatment.

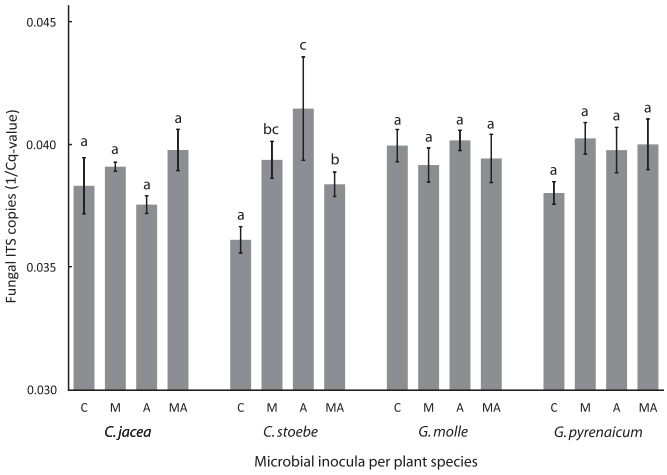


**Fig. 4.** Microbial community effects on fungal abundances (1/qPCR threshold value) per plant species. Vertical bars show means ± standard errors. Different letters indicate significant ($p < 0.05$) pairwise post-hoc Wald tests between inoculum treatments per plant species. Microbial treatments are abbreviated: control (C), general microbial community (M), nematode antagonists (A) and the combined microbial and nematode antagonist community (MA).

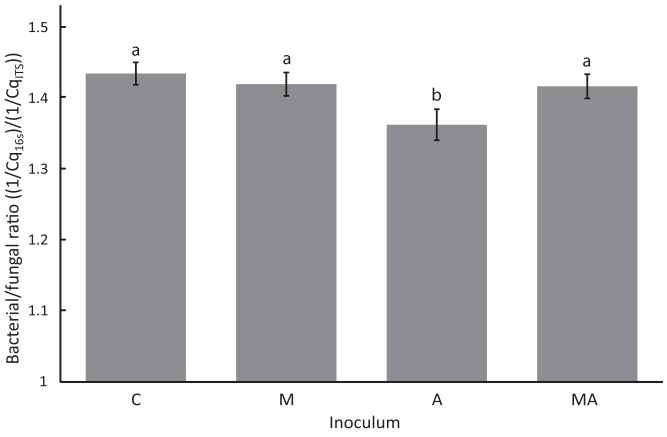


**Fig. 5.** Ratios of bacterial and fungal abundances per inoculum treatment, quantified by the ratio between the inverse qPCR Cq-values of bacterial 16s and fungal ITS copy numbers. Vertical bars show means ± standard errors. Different letters indicate significant ($p < 0.05$) pairwise post-hoc Wald tests between inoculum treatments. X-axis labels represent inoculum treatments: control (C), general microbial community (M), nematode antagonists (A) and the mixed community (MA).

antagonist community alone. Possibly the nematode antagonists could have been outcompeted by micro-organisms from the general microbial community resulting in a reduced top-down control of the nematodes. Alternatively, as none of the inoculated nematode antagonists are obligatory nematophagous (the three fungi can grow purely saprophytically, the amoeba merely on bacteria and fungi (Geisen et al., 2016)), they could predominantly feed on other food sources in the presence of a diverse microbial community, thereby releasing the nematodes from their control. Interestingly, in both range expanders *Meloidogyne* numbers were found to be reduced by the combined microbial and nematode antagonist community compared to the other microbial communities, suggesting a synergistic effect of potential nematode antagonists from both communities. Overall, as in some plant species the nematodes performed better in the presence of the combined microbial and nematode antagonist community than in the presence of either one of the microbial communities alone, our results suggest that it is crucial to perform nematode control studies under soil microbial conditions as natural as possible.

Top-down control of *Helicotylenchus* differed from *Meloidogyne*. While there were no top-down control effects in both native plants, *Helicotylenchus* was effectively controlled in the root zone of *G. pyrenaicum*, both by the combined microbial and nematode antagonist community and by the nematode antagonist community in *C. stoebe* (Fig. 2). The overall differences in top-down control patterns of two root-feeding nematode species in four plant species indicate that interactions between soil microbes, nematode antagonists and root-feeding nematodes are strongly plant and also nematode species-specific. Such plant species-specific interactions in the rhizosphere can probably be best explained by plant species-specific root chemistry, influencing rhizosphere communities differently (Shi et al., 2011), by which top-down control of nematodes is altered. As bacterial or fungal abundances do not seem to explain differences in root-feeding nematode abundances, it is likely that interspecific differences in top-down control effects on root-feeding nematodes are caused by differences in the microbial rhizosphere community composition rather than sheer microbial abundances (Figs. 3–5).

In a recent study (Viketoft and van der Putten, 2014) native microbes showed effective top-down control of root-feeding nematodes in the root zones of both native and range-expanding plant species, although top-down control effects were highly plant species-specific. We show such plant species-specific top-down control effects as well, but we also show that range-expanding plant species interact with their microbial community differently than their related natives. As a result, patterns of top-down (and bottom-up) control turned out to be highly species-specific. As in the experiment of Viketoft and van der Putten (2014) root-feeding nematodes did not decrease plant biomass. Only plants treated with the combined microbial and nematode antagonist community tended to produce less plant biomass, potentially caused by an increased competition for nutrients between the plants and the microbial community (Clarholm, 1985) or by mild pathogenic effects only affecting plant biomass when the combined microbial and nematode antagonist community was added. The absence of a negative effect of *Meloidogyne* on plant biomass might be explained by the low nematode densities in the early phases of the experiment. The strong differences in nematode

densities between the root zones of native *C. jacea* and range-expanding *C. stoebe* that build up over the course of time might have strong effects on next generations of conspecifics, but we did not test such feedback effects.

In conclusion, we show that range-expanding plant species influence top-down control of root-feeding nematodes in their root zones differently than related native plant species. Our results add to the findings that range-expanding plant species accumulate different soil microbial communities compared to related native species (Morrien and van der Putten, 2013), as we provide novel evidence that these different soil communities affect root-feeding nematodes differently. Furthermore, we show that bottom-up control of root-feeding nematodes can both be strong and weak in the root zones of range-expanding plant species. The root-feeding nematode abundance patterns indicate that range-expanding plant species influence root-feeding nematode populations in a plant species-specific manner, which likely will result in strongly different plant-soil feedback outcomes. Range-expanding plant species that escaped their specialized enemies and have strong defense mechanisms, even against generalist nematodes, could eventually become increasingly abundant. Thereby they might negatively influence the native vegetation, while other range-expanding plant species are more likely to develop similar negative plant-soil feedbacks as related natives.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2016.06.025.

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