

Analysis of the specificity of three root-feeders towards grasses in coastal dunes

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Abstract Among the root-feeding nematodes that accumulate in the rhizosphere of grasses in European dunes, the genus *Pratylenchus* is of special relevance given its diversity and distribution. Although different species of *Pratylenchus* have been reported in dune grasses, the specificity towards dune plants, a fundamental aspect of the biology of the species, has hitherto not been studied. Two inoculation experiments using different combinations of grasses and nematodes were performed. The multiplication and the effect on plant growth of *P. dunensis* and *P. brzeskii*, two species which only occur in dune areas was compared with

that of *P. penetrans*, a broad host-range species. The three *Pratylenchus* spp. could multiply under all hosts; however, there was a clear host-dependent response. The species-specific response observed might account for the shift of *Pratylenchus* spp. detected in the field. Although, a negative effect on the growth of *A. arenaria* was demonstrated for the three nematode species, different densities were needed to observe the same effects in plant biomass which point at nematode-specific tolerance. While the typical dune species needed very high densities to produce damage, *P. penetrans* needed very few specimens. The results obtained indicate that species with similar feeding adaptations show very different multiplication abilities on co-occurring hosts, an aspect that is usually overlooked for belowground herbivores in natural systems. The obtained results might suggest a coevolutionary relationship between specific nematode species and *Ammophila arenaria*.

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Introduction

The interactions between plant roots and soil organisms have a significant effect on the structure and dynamic of both, belowground biota and plant

communities (Reinhart et al. 2003; De Deyn et al. 2003; Wardle et al. 1999). Although belowground interactions play a key role in regulating the structure and functioning of plant communities, they are far from being fully understood. The interactions occurring in the rhizosphere of dune grasses, and of *Ammophila arenaria* L. (Link) in particular, are one of the best documented cases illustrating the multi-trophic dimension of soil biota and the influence of root-feeders in plant community development (Van der Putten 2001; Zoon et al. 1993; Maas 1983). The sand and the rhizosphere of plants in coastal dunes contain a wide variety of organisms, root-feeding nematodes among them, that contribute to the plant–soil feedbacks that modify the fitness and long-time establishment of competing plant species (Van der Stoel et al. 2002; de Rooij-van der Goes et al. 1998). Therefore, primary succession in coastal dunes is determined in a great extent by the net effect of different soil-borne organisms.

Root-lesion nematodes (*Pratylenchus* spp.) are a frequent component of nematode communities in crops (Duncan and Moens 2006), in temperate grassland ecosystems (de Goede and Bongers 1998) and also in coastal dunes (de la Peña et al. 2007). These migratory endoparasitic nematodes invade, multiply, feed and move through the root cortex of the host plant (Zunke 1990) resulting in necrotic lesions which often promote secondary fungal infections (Back et al. 2002). *Pratylenchus brzeskii* and *P. dunensis* are commonly found associated with two fore-dune grasses, *A. arenaria* and *Elymus farctus* (Viv.) Runemark ex Melderis, along the European Atlantic coast (de la Peña et al. 2007; de la Peña et al. 2006a) whereas *P. penetrans* is a cosmopolite species reported feeding in the roots of more than 400 plant species (Duncan and Moens 2006) and in dunes is usually associated with degenerating *A. arenaria* stands and *Hyppophäe rhamnoides* L. (Zoon et al. 1993). Natural densities in foredune soils of *Pratylenchus* spp. are relatively low and range from 0.003–0.15 nematodes-per gram in soils with vigorous *A. arenaria* (André Van der Wurff, unpublished results; Schreck-Reis et al. 2005; de Rooij-van der Goes et al. 1995); in inner dunes densities up to 0.15–1 nematodes-per gram are found in the rhizosphere of *A. arenaria* and *H. rhamnoides* (Zoon et al. 1993). Complexes of multiple *Pratylenchus* species are common; however, results from sampling surveys suggest a

shift in nematode composition from foredunes to inner dunes (Wall et al. 2002; Zoon et al. 1993).

Coastal dunes are a typical example of primary succession, where a replacement in species abundance and composition is observed in relation to the development of soil horizons, the decrease in sand accretion and the effect of the soil community (Van der Putten 2003; Imbert and Huele 2001). *Elymus farctus* occupies embryonic dunes in the North Sea and it is replaced in foredunes by *A. arenaria*. The latter species gradually degenerates when sand accretion diminishes, as in stabilized dunes, and it is subsequently replaced either by other grass species or by shrubs such as *H. rhamnoides*. The ecology of root-feeding nematodes associated with dune plants has been thoroughly studied during the last decade. These studies illustrate how different root-feeding species are ruled out either by bottom–up processes (Van der Stoel et al. 2002; Brinkman et al. 2005a), horizontal competition (Brinkman et al. 2005b) or top–down control by natural enemies (Pisciewicz et al. 2007) and plant mutualists (Hol et al. 2008; de la Peña et al. 2006b) and explain the population dynamics and the low densities observed in the field (Brinkman et al. 2005a). However, the multiplication and specificity of root feeders on different dune hosts has only been studied up to now for the sedentary endoparasite *Heterodera arenaria* (Van der Stoel et al. 2006). To understand the biology and the effect of root-feeders in coastal dunes, and of *Pratylenchus* spp. in particular, the multiplication abilities of different species and the consequences for their dune hosts should be elucidated.

In the present study we address two aspects of the interaction between root-feeding nematodes and dune grasses. First, we test the hypothesis of a species-specific response of *Pratylenchus* spp. towards different foredune grasses. In order to test this hypothesis we compared the multiplication of three species (*P. dunensis*, *P. brzeskii* and *P. penetrans*) which are found in coastal dunes on three species of grasses: *Elymus farctus* and *A. arenaria* which were selected as typical species from embryonic and foredunes respectively; and *Lolium perenne* which represented a species of inner grasslands. Second, we investigated whether *A. arenaria* show a different level of tolerance to each *Pratylenchus* species and therefore, we compared for the same three nematode species their multiplication capacities and the effect on this host plant.

Materials and methods

Seeds

Seeds of *A. arenaria* and *E. farctus* were collected at mid August 2004 in foredunes in the nature reserve of Het Zwin (Belgium). They were air dried at room temperature and then stored in a dry place at 4°C until use. Seeds of *L. perenne* were obtained from the ILVO (Merelbeke, Belgium) seed collection. All seeds were germinated in a growth chamber in plastic pots containing a 2-cm layer of glass beads (2 mm diameter) covered with demineralised water.

Nematode cultures and inocula

Both *P. dunensis* and *P. brzeskii* were isolated originally from *A. arenaria* stands in Oostvoorne (The Netherlands) and Ynyslas (Wales, UK), respectively. *Pratylenchus penetrans* was recovered from *Zea mays* in Zandhoven (Belgium). The three nematode populations were multiplied for 9 months on *A. arenaria* sown in sterilized dune sand. Pots were watered twice a week and fertilized once a month with 120 ml half-strength Hoagland's nutrient solution. To obtain nematodes for the experiments, both soil and plant roots from cultures were sieved through a 0.5-cm sieve; the roots retained by the sieve were chopped in 1-cm fragments and placed in a funnel over a cotton filter. The funnels were placed in a mist chamber at 20°C (Seinhorst 1950) and tapped off every day during 1 week to collect nematodes migrating out of the roots. Nematode identity was checked prior to inoculation by means of morphology and sequencing of the ITS region following the procedure described by de la Peña et al. (2007). Prior to their use, the proportion of the different mobile nematode stages (adult females, males and juveniles) was estimated in all inocula. No significant differences (data not shown) were observed between the juvenile/adult and female/male ratios for each nematode culture.

Experiment 1: Host suitability

In a cross inoculation experiment three plant species, viz. *A. arenaria*, *E. farctus* and *L. perenne* were inoculated with *P. dunensis*, *P. brzeskii* or *P. penetrans*. A 2-week old seedling of each host was sown in a

500-ml pot filled with 500 ml of sterilized dune soil. Each pot was inoculated with 150 mobile stages of one of the *Pratylenchus* species. This density was selected since it resembles natural densities of *Pratylenchus* spp. on foredunes. Pots with un-inoculated seedlings of each of the plant species were used as control. Each of the different plant–nematode combinations and controls were replicated seven times. Pots were placed randomly on a bench in a glasshouse. The experiment ran for 12 weeks between July and September 2005 under ambient light conditions and 25/18°C mean day/night temperatures. Plants were fertilized three times during the experiment, at 4 weeks interval, with 50 ml of half-strength Hoagland's solution. Water content was reset to 5–10% every 2 days adding by 100–120 ml of demineralised water. At harvest (after 12 weeks), total fresh plant biomass was recorded. At the same time nematode numbers were estimated as follows: a portion of each root was weighed and macerated in 100 ml of distilled water for 30 s in a commercial blender. The volume of the suspension was taken to 1,000 ml and nematodes were extracted from the root suspension by automated zonal centrifugation following Hendrickx (1995) protocol. Similarly, 100 ml of sand were sampled from each pot and nematodes were extracted. The soil was stirred in 900 ml of water and the suspension was extracted following the above mentioned procedure. Nematodes in all mobile stages (juveniles + adults) present in 120 ml of the eluted suspension were counted. The plant parts remaining after sampling were dried at 72°C for 48 h. Eventually the dry weight of the above and belowground plant parts was measured taking into account the sampled roots. For each species the multiplication rate was determined as the ratio of the final number of nematodes in roots and soil (Pf) and the initial inoculation density on that pot (Pi).

Experiment 2: Nematode density—*Ammophila arenaria* growth

Pots were prepared according to the procedure described above, but this time only *A. arenaria* was used. For each nematode species 3,000, 1,500, 750, 375, 190, 90 or 45 nematodes were inoculated per pot resulting in seven nematode densities (viz. 5, 2.5, 1.2, 0.6, 0.3, 0.15 and 0.075 nematodes-per gram sand). Inoculation densities and control (no inoculation) were replicated six times per nematode species. Pots

were distributed randomly on the bench of a growth chamber. The experiment was run for 16 weeks between June and September 2005 with a day/night illumination ($250 \mu\text{mol m}^{-2} \text{h}^{-1}$) regime of 16/18 h and a temperature of 25/18°C. Plants were fertilized with 50 ml of half strength Hoagland solution every 20 days and water content was reset at 10% every 2 days using 100–150 ml of demineralised water. At harvest plant growth parameters and nematode numbers were assessed (see above).

Statistical analysis

Experiment 1: Host suitability

All data were checked for normality with the Kolmogorov–Smirnov test and homogeneity of variance with Levene's test. Variables that did not meet ANOVA model assumptions were $\text{Log}(x+1)$ transformed. Nematode multiplication (Pf/Pi) and total dry biomass, were compared with two-way ANOVA using inoculation (I; i.e. control, *P. dunensis*, *P. brzeskii* and *P. penetrans*) and plant species (P; i.e. *A. arenaria*, *E. farctus* and *L. perenne*) as factors. Tukey's multiple range test for overall comparison were conducted to estimate differences in nematode multiplication and total number of nematodes for each host plant separately.

Experiment 2: Nematode density—*Ammophila arenaria* growth

The correlation between plant-growth related parameters and initial and final nematode numbers for each species were estimated using Spearman Rank correlations. Differences in plant biomass and final number of nematodes (Pf) with respect to control treatments were done using a non-parametric Mann–Whitney test in which pair-wise comparison between control pots and the different nematode densities for each species were performed.

Results

Experiment 1: Host suitability

The two-way ANOVA and Tukey's test showed significant differences in total dry biomass ($P < 0.01$,

$F_{2,67}=54.183$) for the three plant species that were compared (Fig. 1). *Lolium perenne* produced more biomass than the other two species; however, no significant differences for total dry biomass were observed between *A. arenaria* and *E. farctus* (Fig. 1, Table 1). No significant differences in total dry biomass were found between inoculations (I), or in the interactions between plant identity and inoculation ($P \times I$; Table 1).

With respect to the nematode multiplication (Pf/Pi), the two-way ANOVA indicated significant differences between plant species (P; $P \leq 0.01$, $F_{2,52}=34.794$, Table 1), inoculations (I) and in the interaction between plant and nematode species ($P \times I$; $P \leq 0.01$, $F_{2,52}=5.206$ and $F_{2,52}=12.145$ respectively, Table 1). Significant differences in Pf/Pi and total number of nematodes were found between nematode species for each of the host plant species (Fig. 2). On *L. perenne* the highest multiplication was found for *P. penetrans*; *P. dunensis* and *P. brzeskii* showed a similar but lower multiplication ($P \leq 0.05$, Fig. 2). On *E. farctus* the inverse situation was observed; the lowest multiplication was obtained for *P. penetrans* whereas *P. dunensis* and *P. brzeskii* had a higher and

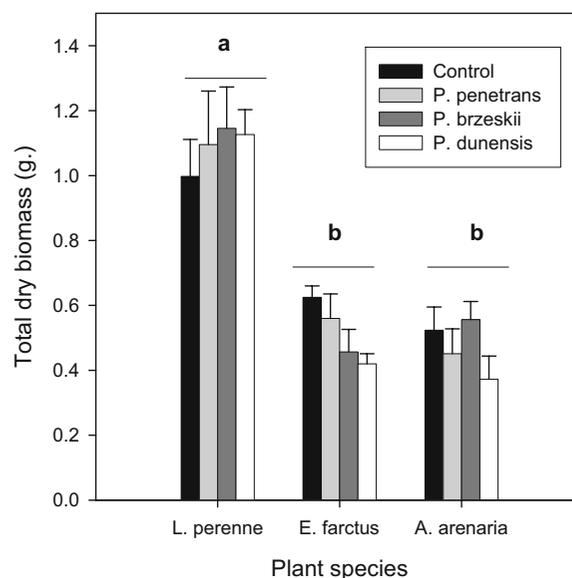


Fig. 1 Total dry biomass (grams per-plant) produced by *Lolium perenne*, *Elymus farctus* and *Ammophila arenaria* in controls (no inoculation) or after inoculation with *Pratylenchus penetrans*, *P. brzeskii* and *P. dunensis*. Data are mean \pm SE. Different letters indicate significant differences according to two-way ANOVA Tukey's test

Table 1 Statistics of the host suitability experiment: mean squares (MS), degrees of freedom (d.f.), *F*-ratios of different measured variables for two-way ANOVA

Factor	Dependent variable								
	Multiplication (Pf/Pi)			Total number of nematodes (roots + soil)			Total dry biomass		
	MS	F	d.f.	MS	F	d.f.	MS	F	d.f.
P	2.000	34.794**	2	2.966	33.210**	2	0.184	54.183**	2
I	0.299	5.206**	2	0.324	3.625*	2	2.301 E-03	0.705	3
P × I	0.691	12.145**	4	1.154	12.924**	4	3.88 E-03	1.144	6

The analysis compared the effect of plant identity (P; *Lolium perenne*, *Elymus farctus*, *Ammophila arenaria*), and inoculation (I; Control, *Pratylenchus penetrans*, *P. brzeskii* and *P. dunensis*) and the interaction of the two factors (P × N).

Significant differences according to ANOVA are indicated by * $P \leq 0.05$, ** $P \leq 0.01$.

comparable multiplication ($P \leq 0.01$, Fig. 2). On *A. arenaria* a significant difference in multiplication was observed between *P. penetrans* and *P. dunensis*, ($P \leq 0.05$, Fig. 2).

Experiment 2: Nematode density—*Ammophila arenaria* growth

Pratylenchus dunensis and *P. brzeskii* multiplied at all densities examined; at the highest Pi *P. penetrans* hardly increased in number and reached an equilibri-

um (Pf=Pi; Fig. 3). A clear negative effect on *A. arenaria* growth was observed at high densities of the three species studied (Fig. 4). According to the Spearman rank test negative correlations were found between nematode density and *A. arenaria* total dry biomass, root dry biomass and aboveground dry biomass for the three species analyzed (Table 2). The slope variable (rho coefficient) showed that the correlation was stronger with respect to the above-ground biomass with values ranging from -0.744 to -0.572 while for the root biomass the values ranged between -0.381 and -0.510 (Table 2). With respect to uninoculated control pots, significant differences ($P \leq 0.05$) in plant dry biomass were only observed for initial populations (Pi) higher than 375 (0.6 g^{-1}) nematodes per pot for the three species compared (*P. dunensis* $Z = -2.242$; *P. brzeskii* $Z = -2.562$; *P. penetrans* $Z = -2.242$; Fig. 4). Differences in the final number of nematodes causing reduction in plant

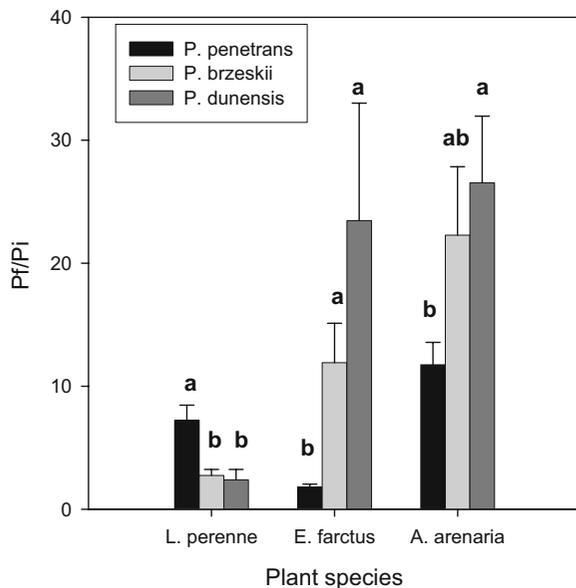


Fig. 2 *Pratylenchus* spp. (*P. penetrans*, *P. dunensis* and *P. brzeskii*) multiplication (Pf/Pi) on three different hosts *Lolium perenne*, *Elymus farctus* and *Ammophila arenaria*. Data are mean \pm SE. Significant differences according to two-way ANOVA and Tukey's test within a given species are indicated by different letters

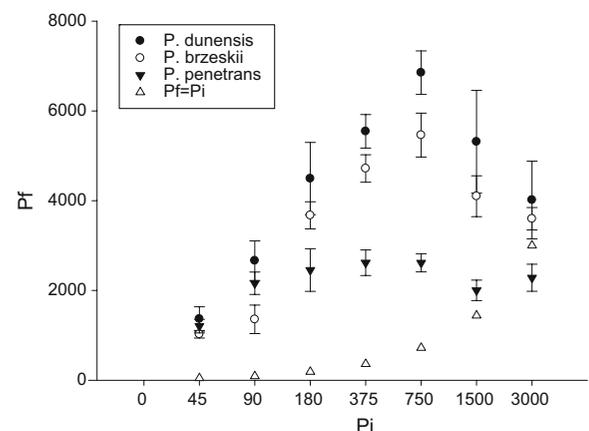


Fig. 3 Relationship between initial *Pratylenchus* spp. density (Pi) and final number of nematodes (Pf) in *Ammophila arenaria*

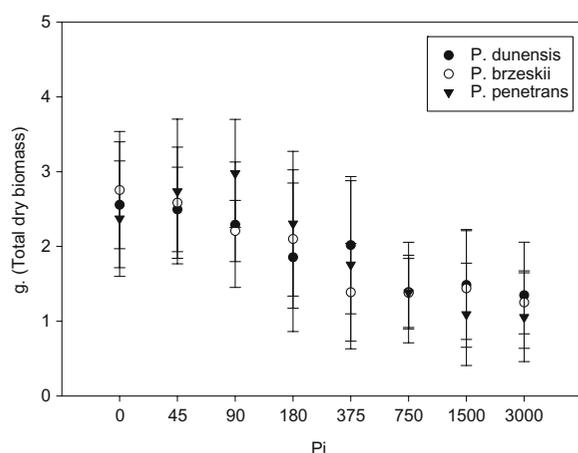


Fig. 4 Relationship between initial *Pratylenchus* spp. density (Pi) and *Ammophila arenaria* dry biomass (g)

biomass were observed for each nematode species. When inoculated with *P. penetrans* only an average of 2,400 nematodes were necessary to produce significant damage on *A. arenaria* ($P \leq 0.05$), whereas inoculations with *P. dunensis* and *P. brzeskii* required an average of 5,250 and 4,900 to cause damage, respectively (*P. dunensis* $Z = -2.242$; *P. brzeskii* $Z = -2.562$; *P. penetrans* $Z = -2.242$, Fig. 3).

Discussion

Multiplication was observed for the three nematode species on the three hosts. This was expected for *P. penetrans* since it is a species reported in many different crops and grasses (Duncan and Moens 2006); far less knowledge was available for *P. brzeskii* and *P. dunensis* (de la Peña et al. 2006a, b). Our results highlight clear differences in nematode multiplication as a function of the host and provide clear evidence for host specialization among the three species of *Pratylenchus* compared. When analyzing in detail the multiplication on different grasses *P.*

penetrans showed the highest multiplication on *L. perenne* whereas *P. brzeskii* and *P. dunensis* hardly multiplied on this host. The reverse situation occurred on *E. farctus* on which *P. dunensis* and *P. brzeskii* developed the highest multiplication. On *A. arenaria*, *P. dunensis* showed the highest multiplication followed by *P. brzeskii* and *P. penetrans*. The first experiment also confirms previous field observations (de la Peña et al. 2007). Sampling surveys along the European coast showed *P. penetrans* to be a very rare species in foredunes (de la Peña et al. 2007); this nematode seems to occupy inner or stabilized areas where other plant species are better hosts. The results here obtained provide also empirical evidence to the observations in which *P. brzeskii* and *P. dunensis* were extracted from *E. farctus* roots (de la Peña et al. 2006a, b; Karssen et al. 2000). Specificity of nematodes towards dune grasses has been previously suggested. Two root-knot nematode species, *Meloidogyne duytsi* and *M. arenaria*, occur in dunes of Western Europe and although both species are present in foredunes, each one exploits a different host plant (*E. farctus* and *A. arenaria*, respectively; Karssen et al. 1998a, b).

Van der Stoel et al. (2006) analyzed the pathogenicity and host-range of the cyst nematode *Heterodera arenaria* in coastal dunes. *Heterodera arenaria* is found only in foredunes on *E. farctus* and *A. arenaria* but disappears in inner dunes where other hosts are present. Our results together with the examples of *Meloidogyne* spp. and *H. arenaria* might indicate that the specialization to exploit a narrow set of hosts might be common pattern of root herbivores in coastal dunes and this could be extensive to other type of natural grassland. Therefore, this fact should be taken into account in order to understand belowground biotic interactions in natural systems, and in coastal dunes in particular.

The first experiment using a nematode density resembling those present in the foredunes did not show any effect on growth of any of the grass species

Table 2 Spearman's rho coefficients for correlations between initial nematode density and total biomass, root biomass, aboveground biomass for *Pratylenchus* spp

	Total dry biomass	Root dry biomass	Aboveground dry biomass
<i>P. dunensis</i>	-0.528**	-0.381**	-0.616**
<i>P. brzeskii</i>	-0.628**	-0.395**	-0.667**
<i>P. penetrans</i>	-0.616**	-0.510*	-0.510**

** $P \leq 0.01$, * $P \leq 0.05$

compared. However, the second experiment showed a clear detrimental effect of the three *Pratylenchus* spp. on the growth of *A. arenaria*. Earlier inoculation studies have shown a variable impact of plant-parasitic nematodes on the growth of *A. arenaria*. Either inoculation of *A. arenaria* seedlings with *H. arenaria* did not affect plant growth (Van der Stoel 2001) or negative effects in the growth of *A. arenaria* were observed after inoculation with the ectoparasitic nematode *Tylenchorhynchus ventralis* (de Rooij-van der Goes et al. 1995). However, the nematode densities used in the latter experiment were considerably higher than those reported from coastal dunes (Pisciewicz et al. 2007). More recent experiments using *P. penetrans* were not conclusive about the effect of this species on the growth of *A. arenaria*. Initial inoculation densities of 0.2 and 1 nematodes-per gram reduced aboveground biomass while root-biomass remained unaffected (Brinkman et al. 2005b).

Sampling surveys focused on *Pratylenchus* spp. and other plant-parasitic nematodes in coastal dunes of Western Europe have shown that the density of this group range between 0.003–0.15 nematodes-per gram soil in foredunes with vigorous *A. arenaria* stands (Schreck-Reis et al. 2005; de Rooij-van der Goes et al. 1995) and 0.15–1 nematodes-per gram soil in the rhizosphere of *A. arenaria* and *H. rhamnoides* in inner dunes (Zoon et al. 1993). At the range of natural densities detected in foredunes, no detrimental effect on plant biomass is observed for any of the three species used in our experiment. Only when densities resembling those found in inner dunes were used (0.2 nematodes-per gram soil) a negative effect was found. Interestingly, to observe the same level of damage in *A. arenaria* much higher nematode numbers were needed for the species only found in coastal dunes than for *P. penetrans*. This fact might suggest *A. arenaria* to be more tolerant to specific dune nematodes than to *P. penetrans*.

Pratylenchus species in foredunes can occur in species complexes in which two to three species of the genus share the same host (de la Peña et al. 2007). It has been hypothesized that biotrophic parasites would show higher pathogenicity in multispecies complexes to be able to compete with other parasitic species (Jarosz and Davelos 1995). The results presented here showing the negative effect of *Pratylenchus* spp. and especially of *P. penetrans* are in agreement with this hypothesis.

The effect of interspecific competition between species of different root-feeding nematode has been analyzed for *A. arenaria* (Hol et al. 2008; Brinkman et al. 2005a). These studies demonstrated that *Pratylenchus* spp. were strong competitors when compared to other endoparasitic nematodes but suffered from intraspecific competition. In our density-growth experiment, a negative effect on plant growth was shown at high initial nematode densities, however in field conditions it is unlikely to find patches where only one species dominant, so probably interspecific competition between *Pratylenchus* spp. is an important mechanism controlling the multiplication of these root feeders. In absence of competitors or control agents (such as AMF, or horizontal competition) *Pratylenchus* spp. might reach high densities that potentially damage the host. However, the results obtained in this study should be taken with caution and although a negative effect was observed at high densities, a causal-effect association between *Pratylenchus* spp. and the decline of *A. arenaria* should not be directly assumed and the interaction with other soil components should also be taken into account.

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