

Evidence of population differentiation in the dune grass *Ammophila arenaria* and its associated root-feeding nematodes

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Abstract The interactions between herbivores and their host plants determine, to a great extent, the formation, structure and sustainability of terrestrial communities. The selection pressures that herbivores exert on plants and vice versa might vary geographically, leading eventually to population differentiation and local adaptation. In order to test whether there was reciprocal population differentiation among plants and belowground herbivores, we performed a cross-inoculation experiment using combinations of species and populations of root-feeders belonging to the genus *Pratylenchus* and the dune grass *Ammophila arenaria* from different geographic origins. Plant and herbivore responses in terms of growth and multiplica-

tion, respectively, were assessed at the end of the experiment. The 16 plant-herbivore combinations tested showed a high variation in the outcome of the interaction and revealed population differentiation in the responses of both, the host plant and the root-herbivores. The outcome in plant and herbivore performance was strongly case-dependent and for the sympatric combinations tested, support for local adaptation was not found. Nonetheless, the variation in plant-herbivore responses to experimental conditions highlights the plasticity of the interaction and may be pointing at spatial structuring in belowground plant-herbivore interactions.

Keywords Plant-parasitic nematodes · Coastal dunes · Rhizosphere · Host-pathogen · Belowground herbivory · Soil biota · Local adaptation

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Introduction

The development and structure of plant populations and communities depend strongly on soil conditions. There are numerous examples from the literature that show that the edaphic composition has a strong impact on plant fitness (Stratton 1994; Hangelbroek et al. 2003; Abdala-Roberts and Marquis 2007) and its effect may vary spatially leading in some cases to local adaptation, which is a superior fitness of local populations to their home conditions in comparison to non-local populations (Antonovics 1971; Brandon and

Antonovics 1996; Linhart and Grant 1996; Joshi et al. 2001). However, whether interactions between plant and soil biota are able to exhibit variation across sites resulting in population differentiation and local adaptation has only been addressed on a few occasions (Van der Putten et al. 2006; Macel et al. 2007; Hoeksema and Thompson 2007; Piculell et al. 2008).

Herbivory acts as an important selective force in plant communities (Jarosz and Davelos 1995). Plant life-history traits like growth and resistance to herbivores are quantitative traits under constant selection (Ruiz-R. et al. 2006). The forces exerted by herbivores on plant populations, and vice versa, vary spatially and this might result in a geographic structuring of plant-herbivore interactions where certain plant or herbivore genotypes are favoured in one location and not in others (Drès and Mallet, 2002; Thompson 2005; Ferrari et al. 2008; Hufford et al. 2008). Gene flow and random genetic drift ensures a continual remixing of traits among populations. Depending on the balance between selection and gene flow, genetic structuring may lead to the formation of genetically distinct populations. In general terms, low gene flow and divergent selection pressures across sites might result in genetic differentiation and eventually, in local adaptation (Brandon and Antonovics 1996; Kingsolver et al. 2002; Hendry et al. 2002; Urban et al. 2008). Although the importance of plant-herbivore interactions is unquestionable, studies on local adaptation to herbivores are relatively rare and the results are less concluding compared with studies on local adaptation of plants to other environmental variables (Greischar and Koskella 2007). Moreover, studies on plant-herbivore interactions have mainly addressed the effect of plant genotype on herbivore performance and not the other way around (Fornoni and Nuñez-Farfan 2000; Crémieux et al. 2008).

Herbivory takes place on both, the above- and belowground, parts of plants. Although the evolutionary consequences of the variation in the outcome of interactions between herbivores (or parasites) and plants has been analyzed several times, it has been always from an aboveground perspective (Sork et al. 1993; Ballabeni et al. 2003; Thrall et al. 2003; Hufford and Mazer 2003; Zovi et al. 2008). By contrast, the question of whether belowground herbivory might lead to geographic differentiation of plant and herbivore populations remains open, with

only a couple of studies addressing this possibility (Van der Putten et al. 2006; Schreck-Reis et al. 2008).

Root-feeding nematodes are a key component of soil biota. In agricultural systems, they are well known plant pathogens that produce important economic losses in many crops worldwide. Root-feeders contribute indirectly to soil nutrient dynamics, which in turn affect nutrient availability and plant productivity. More importantly, in some instances root-feeders can directly affect the performance of competing plants and, therefore, their role in plant primary and secondary succession, competition and facilitation cannot be neglected (Van der Putten 2003; Maron, 2001; Verschoor et al. 2002). Although several studies have addressed the evolutionary aspects that shape resistance and tolerance of crops towards root-feeders, there are no data available for non-crop plants (Van der Putten et al. 2006).

Ammophila arenaria (L.) Link or marram grass is a clonal species occurring naturally on foredunes along the European and North African coastline. It has been introduced in many other dune areas of the world for dune stabilization purposes (Tutin et al. 1980). Although it is able to produce seeds, the main mechanism of dispersal is clonal growth, which results in low intra-population genetic variation within a site, with only a few related clones per site, and relatively high genetic distances between geographically separated populations (Hol et al. 2008; Rodríguez-Echeverría et al. 2008).

The biotic interactions occurring in the rhizosphere of dune grasses, and of *A. arenaria* in particular, are among the best documented cases illustrating the multitrophic dimension of belowground interactions (de Rooij-van der Goes et al. 1995; Van der Stoel et al. 2002; Brinkman et al. 2005, Pisciewicz et al. 2007). The rhizosphere of plants in coastal dunes contains an ample variety of organisms, root-feeding nematodes among them, involved in the plant-soil feedbacks that modify the long-term fitness of the species of the plant community (Van der Putten 2003). *Pratylenchus brzeskii* and *P. dunensis* are two nematode species restricted to foredunes and typical members of the belowground fauna associated with *A. arenaria* in coastal dunes (de la Peña et al. 2006a, b, 2007). Based on the results of an extensive survey, these two species of *Pratylenchus* were the most common nematode species associated with *A. arenaria* along its whole distribution range in Europe (de la Peña et

al. 2007). These migratory endoparasitic nematodes invade, multiply on, feed on and move through the root cortex of the host plant resulting in necrotic lesions that often promote secondary fungal infections (Back et al. 2002). Experimentally, it has been shown that both species of *Pratylenchus* are able to cause damage at densities resembling those occurring in the field in dunes (Brinkman et al. 2005; de la Peña et al. 2008). The level of tolerance of *A. arenaria* is also different for each of the *Pratylenchus* species (de la Peña et al. 2008). Both facts are in agreement with high reciprocal selection pressures between the plant and root-feeders. Although there are no molecular studies available on the genetic structure of *Pratylenchus* spp. in coastal dunes, the low mobility of soil-borne nematodes probably results in geographic isolation of populations, even though random genetic drift cannot be excluded.

To what degree population differences within plant and nematode species affect the outcome of the interaction is not completely known. Recently, Schreck-Reis et al. (2008) investigated the response of two species of the ectoparasitic genus *Helicotylenchus* towards two plant genotypes of *A. arenaria* and found that these root feeders do not necessarily perform best on their sympatric plant population. However, whether there were differences at intra-specific level for root-feeders was not assessed in that study.

We performed a cross-inoculation experiment using combinations of belowground herbivores (different species and populations of nematodes belonging to the genus *Pratylenchus*) and populations of the dune grass *Ammophila arenaria*. Plant and herbivore responses were assessed to test whether there was population differentiation in the outcome of the interaction. If that was the case, we would expect differences in the multiplication of the root-feeders as a function of the plant population. On the other hand, plants would be differently affected, in terms of biomass production, by the root-feeders.

Materials and methods

Nematode cultures and inocula

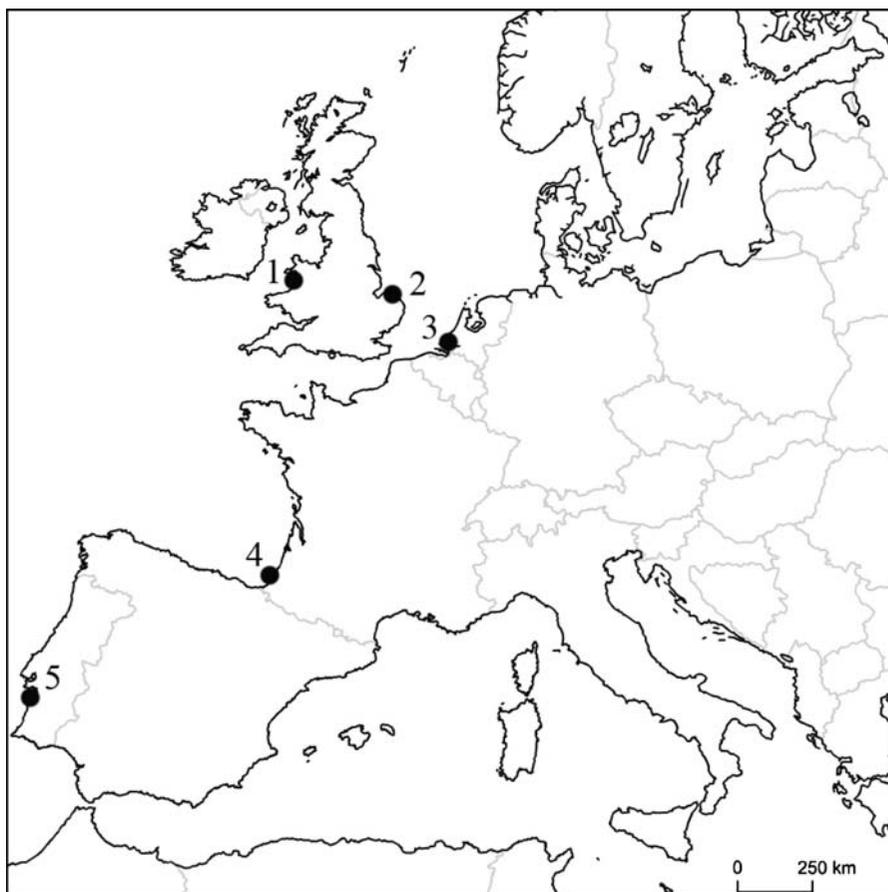
A total of four nematode cultures were used in the experiments; two populations of *P. brzeskii* from Biarritz (France) and Ynyslas (Wales, UK), and two populations of *P. dunensis* from Oostvoorne (The Netherlands) and

Comporta (Portugal) (Fig. 1). Although culturing nematodes could be creating an artificial genetic bottleneck, each population was multiplied for nine months, starting from an initial inoculum of 1200 specimens/population (generation time of approx. 40 days), on *A. arenaria* plants sown in sterilized soil. *Ammophila arenaria* from the same origin as the nematodes were used to rear the population of *P. brzeskii* from Wales and the population of *P. dunensis* from The Netherlands. In order to avoid bias in the set-up and interpretation of the inoculation experiment by culturing nematodes for a year in plants from their own origin, we also included one population of each nematode species that was reared on plants different to their geographic origin; therefore, we multiplied the population of *P. brzeskii* from Biarritz (France) and the population of *P. dunensis* from Comporta (Portugal) on plants from Oostvoorne (The Netherlands). Nematode culture 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 inoculation experiment, soil and plant roots from cultures were sieved through a 0.5-cm mesh and the obtained roots 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 and tapped off every day for one week to collect nematodes in water. Prior to inoculation, nematode identity was checked morphologically and by sequencing the ITS region, and the different nematode stages (adult females, males and juveniles) of all inocula were counted; no significant differences (data not shown) were observed between the juvenile/adult ratio for the cultures.

Cross-inoculation experiment

Population differentiation in species interactions is revealed by a genotype×environment experimental design; in our case different environments would be represented by different populations of herbivores or plants. Therefore, in a cross-inoculation experiment two populations of *P. dunensis* and two populations of *P. brzeskii* were inoculated on four *A. arenaria* populations from different geographical origins (Oostvoorne, Blakeney Point, Ynyslas, Comporta; Fig. 1) that previously have shown distinctive genotypic profiles in molecular studies (Rodríguez-Echeverría et al. 2008). There was a total of 16 nematode-*A. arenaria* combinations (four *A. arenaria* populations×four *Pratylenchus* populations) and in three cases sympatric

Fig. 1 Geographic origin of the populations of *Pratylenchus* spp. and *Ammophila arenaria* used in the cross inoculation experiment where 1: Ynyslas, Wales (UK); 2: Blakeney Point, UK; 3: Oostvoorne, The Netherlands; 4: Biarritz, France; 5: Comporta, Portugal



plant-herbivore combinations could be tested. Both nematode species had been reported previously on the selected plant populations (de la Peña et al. 2007). Pots and seedlings were prepared as follows: each *Pratylenchus* population was inoculated in each of the *A. arenaria* populations; 150 nematodes were inoculated per pot and pots with un-inoculated seedlings were used as controls. This density was selected since it resembles natural densities of *Pratylenchus* spp. on foredunes. Six replicates were used for each plant population-nematode combination and for the control treatment. We used a completely randomized design. The experiment was run in a glasshouse for 16 weeks between June and September of 2005 with a day/night supplementary illumination ($250 \mu\text{mol m}^{-2}\text{h}^{-1}$) regime of 16/18 h and a temperature of 22/18°C. Plants were fertilized with 50 ml of half strength Hoagland solution every 20 days and water content was reset at 5–10% every 2 days using 100–150 ml of demineralized water. At harvest plant growth parameters and nematode numbers were assessed. The fresh weight of shoots and roots was

measured for each plant. After taking the root fraction for assessing nematode numbers, the remaining plant material was dried at 72°C for 48 h to estimate plant above- and belowground biomass (taking in account the excised root fragment). Nematode numbers were calculated at harvest as follows: a portion of each root was weighed and blended in 100 ml of distilled water for 30s in a commercial blender. The volume of the suspension was made up to 1000 ml and nematodes were extracted from the root suspension by automated zonal centrifugation following Hendrickx's (1995) protocol. Nematodes were counted in 120 ml of the elutriated suspension. Nematodes of any mobile developmental stage were taken as a positive count.

Statistical analysis

We used aboveground dry biomass and belowground dry biomass as plant response variables. Herbivore multiplication was estimated by counting the total

number of nematodes in roots at the end of the experiment ($\text{nem} \cdot \text{g}^{-1} \text{ root}$) and was used as the response variable for the root-feeders. All data were $\log(X+1)$ transformed and checked for normality and homogeneity of variance with the Kolmogorov-Smirnov test and Levene's test, respectively. Statistical analyses were performed with the statistical package SAS 9.01. Firstly, a one-way ANOVA with the type of inoculation as factor (control and inoculation with the two species of nematodes) was used to find out whether there were differences in plants due to the inoculation with root feeders. Secondly, and in order to disentangle the main and interaction effects, a General Linear Mixed Model (GLIMMIX) was implemented with *Ammophila arenaria* population (4 populations e.g: Oostvoorne, Ynyslas, Het Zwin, Comporta) and nematode species (*P. dunensis* and *P. brzeskii*) as main factors, and nematode population (two populations per species) was nested within nematode species. Post-hoc comparisons were performed using Bonferroni's method ($p \leq 0.0125$)

Results

Inoculation of *A.arenaria* plants with nematodes did not have an effect on aboveground dry biomass (Table 1, Fig. 2a, b). However, for root dry biomass the ANOVA analysis indicated that nematodes had a detrimental effect overall (Table 1, Fig. 2c, d); control plants had a mean root dry biomass of 0.12 g, whereas plants inoculated with nematodes had only 0.08 g. The effect of nematodes on root dry biomass depended on the nematode species (Table 1) and the interaction between nematode species and plant population (Table 1). Post-hoc comparisons revealed that the interaction effect between nematode species and plant populations was due to the remarkable difference in the quantity of root dry biomass produced by *A. arenaria* from Ynyslas when inoculated with *P. dunensis* from Comporta compared with *P. dunensis* from Oostvoorne ($t=3.47$, $p=0.0013$, Fig. 3b). In the case of *P. brzeskii*, all pairwise comparisons between nematode populations for each plant population were not significant (all $t < 1.33$; $p \geq 0.19$, Fig. 3a).

When looking at nematode multiplication ($\text{nem} \cdot \text{g}^{-1} \text{ root}$) the GLMM revealed significant differ-

ences according to plant population, nematode population (within a nematode species), and also the interaction between plant population and nematode population (Table 1). Moreover, the interaction between plant population and nematode species was nearly significant (Table 1). As can be deduced from Fig. 4a and the post-hoc comparisons, the multiplication of the two *P. brzeskii* populations differed in all of the *A. arenaria* populations ($t > 2.42$, $p \leq 0.0125$) except for Blakeney Point where, after the Bonferroni correction, the difference was not significant ($t = 2.42$, $p = 0.021$, Fig. 4a). In the case of *P. dunensis* only in *A. arenaria* from Comporta there was a difference in multiplication between the two nematode populations ($t = 4.42$, $p = 0.001$, Fig. 4b)

Discussion

The results obtained from the cross-inoculation experiment indicate that population differentiation occurs within both species tested of root-feeders and *A. arenaria*. In this sense, the results obtained matched our expectations and root-feeder multiplication was different depending of the *A. arenaria* population used as host, and on the other hand, *A. arenaria* root-biomass differed as a function of the nematode population inoculated. Interestingly, the outcome of the interactions was strongly idiosyncratic (case-dependent). For the three sympatric nematode-plant combinations studied we did not find evidence supporting local adaptation between plant and belowground herbivores. Nevertheless, population differentiation within the plant and the herbivores demonstrates genetic-based differences mediating the belowground biotic interactions in the rhizosphere of *A. arenaria* and, it suggests that there may be spatial structuring in plant-nematode interactions within the studied system.

Overall, nematodes had a negative effect on root growth (biomass) of *A. arenaria*, but the outcome depended on the interaction between nematode and plant population. This observation might explain the inconsistent responses in different experiments of *A. arenaria* plants to inoculation with the same species (Brinkman et al. 2005; Hol et al. 2007; Rodríguez-Echevarría et al. 2009). Although those differences can be accounted for by the use of different densities of nematodes in the experimental set-ups, the fact that

Table 1 Statistics for the One-Way ANOVA and the General Linear Mixed Model (GLMM) for the variables above-, below-ground biomass and nematode multiplication

	Statistics			
	Num df	den df	F	P
<i>Aboveground biomass</i>				
ANOVA				
Inoculation	2	117	0.54	0.585
GLMM				
Nematode species	1	80	0.47	0.453
Plant population	3	80	1.79	0.154
Nematode population (within nematode species)	2	80	0.13	0.882
Plant population*nematode population	6	80	1.23	0.299
Plant population*nematode species	3	80	0.47	0.702
<i>Belowground biomass</i>				
ANOVA				
Inoculation	2	117	7.86	<0.001
GLMM				
Nematode species	1	80	4.17	0.045
Plant population	3	80	2.57	0.06
Nematode population (within nematode species)	2	80	1.74	0.182
Plant population*nematode population	6	80	0.29	0.835
Plant population*nematode species	3	80	2.35	0.039
<i>Nematode multiplication</i>				
GLMM				
Nematode species	1	80	3.11	0.082
Plant population	3	80	12.41	<0.0001
Nematode population (within nematode species)	2	80	28.71	<0.0001
Plant population*nematode population	6	80	3.25	0.026
Plant population*nematode species	3	80	2.21	0.051

different *A. arenaria* and herbivore populations were used in each of the studies may also have influenced the results obtained.

The interaction between root-feeders and their host plants depends on their reciprocal genetic background. Recently, Schreck-Reis et al. (2008) reported that nematode performance on *A. arenaria* is affected by host genotype, but the effect of different populations within a root-feeding species was not tested. As in this study, no evidence for local adaptation was found. Instead, both our study and the latter, highlight the possibility of completely opposite effects, with nematodes performing worst on sympatric hosts (as occurred with *P. dunensis* from Comporta, Fig. 4b). This observation is not unusual and a better plant or herbivore performance for local combinations should not always be assumed (Gandon and Michalakis 1996; Lively 1999; Thrall et al. 2003). Different phenomena may account for this fact: (i) Evolutionary rates do not differ because generation times or

recombination rates are similar among the interacting species (Gandon and Michalakis 2002). (ii) Migration, the patterns of adaptation or maladaptation are highly variable over time because of the stochastic nature of the arrival of new favorable alleles. Low dispersal and divergent selection pressures across sites usually result in genetic differentiation and eventually, in local adaptation (Kingsolver et al. 2002; Hendry et al. 2002). The molecular characterization of *A. arenaria* populations indicates that there are marked genetic differences between populations separated by large geographical distances (Rodríguez-Echeverría et al. 2008). However, it is quite likely that gene flow occurs among close populations of *A. arenaria* due to long-distance dispersal of rhizome fragments and in consequence, it limits the occurrence of local adaptation. (iii) Environmental heterogeneity may further complicate the process and pattern of local adaptation. This latter point can be particularly relevant in the case of *A. arenaria*.

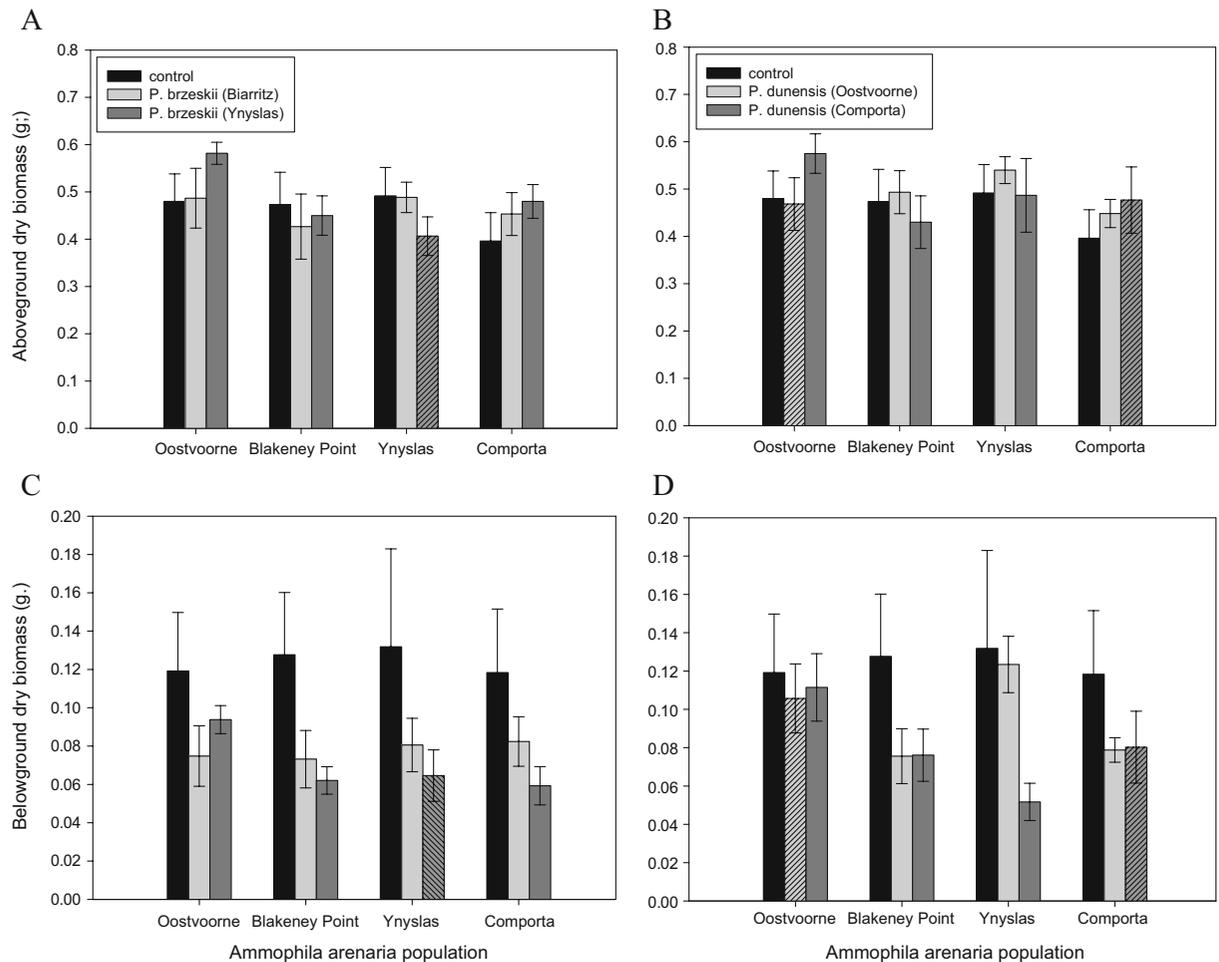


Fig. 2 Mean aboveground dry biomass (shoot) and belowground dry biomass (root) for different *Ammophila arenaria* populations inoculated with different populations of *P. brzeskii*

(a) or *P. dunensis* (b). Error bars indicate \pm SD. Fine striped bars indicate a sympatric plant-nematode combination

The composition of the root-feeder community associated with *A. arenaria* varies significantly across sites and, therefore, plant adaptation to herbivory might be occurring with other root-feeding nematodes (e.g. *Heterodera* or *Meloidogyne*). Moreover, the interaction between *A. arenaria* and root-feeders is mediated to a great extent by plant mutualists and soil microbes. Previous studies have shown that a wide array of organisms (AMF, microbes) not only affects plant performance, but also controls nematode multiplication in coastal dunes (Van der Stoel et al. 2002; de la Peña et al. 2006a, b; Pisciewicz et al. 2007). This set of complex interactions is likely to shape plant-nematode interactions in different ways across sites as seen for other artificial systems (Tzortzakakis 2008; Davies and Williamson 2006). In these sit-

uations, the analysis of population differentiation and local adaptation plant and herbivores should take this in account. Future studies should investigate this aspect in depth and attempt to unravel how population differentiation at both plant and herbivore level is affected by different microbial (AMF, microbes) and abiotic environments. As already seen for other types of complex belowground interactions, the outcome of interactions between plants and soil biota might differ drastically depending on the microbial or abiotic environment in which they occur (Hoeksema and Thompson 2007; Piculell et al. 2008).

Marram grass is a keystone species for dune formation and is used as a natural fixer of mobile dunes (Maun 1998). Transfer of native plant material is common in ecological restoration of dune areas.

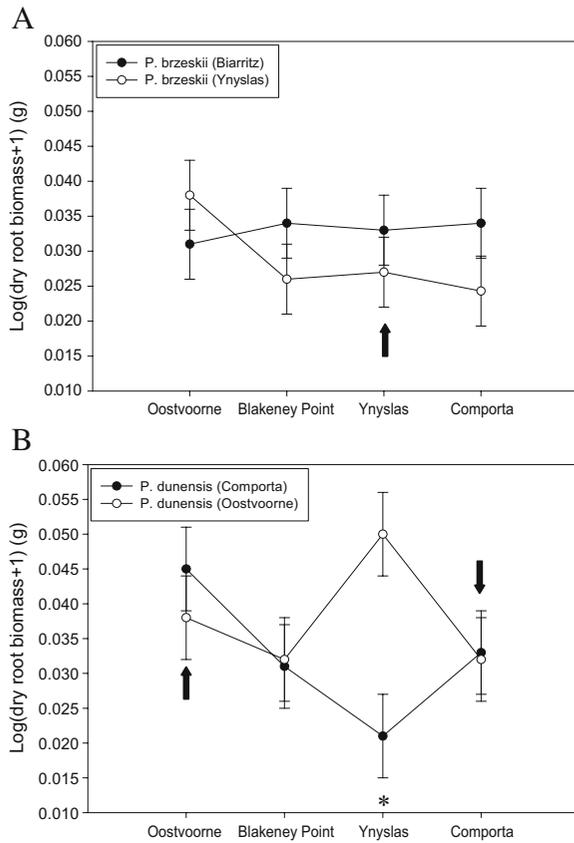


Fig. 3 Mean dry root biomass of *Ammophila arenaria* populations (Blakeney Point, Comporta, Oostvoorne, Ynyslas) after inoculation with *Pratylenchus brzeskii* (a) or *P. dunensis* (b). Asterisk indicates significant differences between nematode populations for a given plant population according to post-hoc test (Bonferroni, $p \leq 0.0125$). Error bars = \pm SD. Black arrows indicate sympatric plant-nematode combinations

The successful germination and establishment of plant populations might be hampered by selecting plant genotypes that are inadequate for the soil conditions of the area to be restored. The results obtained in our study highlight that generalizations cannot be made when studying belowground herbivores. In consequence, plant genotype should be taken into account for restoration practices since the origin of the plant material will affect the responses of soil fauna and can influence the success of establishment of the introduced populations (Crémieux et al. 2008).

The idiosyncrasy of many plant studies dealing with plants and their associated nematode community might be due to the effects of population differentiation at those levels. The responses observed might depend on the site where the study was conducted

(Maun 1998; Van der Stoel et al. 2002; Hedlund et al. 2003). For other groups of soil biota, such as arbuscular mycorrhizal fungi, it has been shown that there are remarkable differences in plant and fungal performance when using local vs non-local inocula and, in consequence, the use of local combinations in experimental approaches is currently encouraged (Klironomos and Hart 2002). In the same way, studies involving root-feeders should take account of this aspect.

Population differentiation and local adaptation in the outcome of the interactions between plants and soil biota is a topic that has until recently been ignored. In order to understand the functioning of terrestrial ecosystems and the dynamics of plant

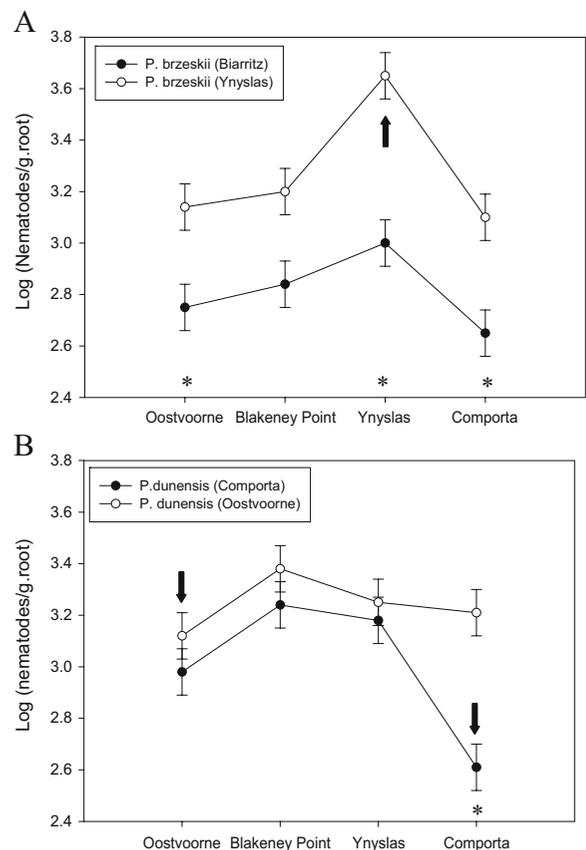


Fig. 4 Multiplication of *Pratylenchus brzeskii* populations (Biarritz and Ynyslas) (a) and *P. dunensis* (Comporta and Oostvoorne) (b) on different *Ammophila arenaria* populations (Blakeney Point, comporta, Oostvoorne, Ynyslas). Error bar indicates \pm SD. Asterisks indicate significant differences between nematode populations for a given plant population after post-hoc comparison (Bonferroni) ($p \leq 0.0125$). Black arrows indicate sympatric plant-nematode combinations

communities, more research is needed to unravel the evolutionary ecology of plants and the different biotic components of the soil.

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