Can root-feeders alter the composition of AMF communities?
Experimental evidence from the dune grass *Ammophila arenaria*

Susana Rodríguez-Echeverría, Eduardo de la Peña, Maurice Moens, Helena Freitas, Wim H. van der Putten

**Abstract**

Root herbivores and plant mutualists, such as arbuscular mycorrhizal fungi (AMF), have a significant effect on the structure and dynamic of plant communities. Nevertheless, the interactions between the two groups of organisms in natural ecosystems are far from understood. We carried out an inoculation experiment to examine the effect of two root herbivores, *Pratylenchus penetrans* and *P. dunensis* (Nematoda), on the composition of the AMF communities associated with two populations of the dune grass *Ammophila arenaria*. The outcome of the interaction in terms of plant and nematode performance was also analyzed. The total percentage of AMF colonization was not affected by the presence of root-feeders, but they did alter the composition of the AMF communities inside the roots. These changes were dependent on the root-feeder species and the original AMF community: the most severe alterations were observed in the mycorrhizal plants from Wales attacked by *P. penetrans*. Plant growth was impaired in plants from Wales inoculated with AMF and *P. dunensis*, which suggests a highly species-specific synergistic interaction with negative consequences for the plant. Root infection by the nematodes was reduced in all mycorrhizal plants when compared to non-mycorrhizal plants. However, a significant reduction of the final number of nematodes was observed only in the mycorrhizal plants from one population.

**Zusammenfassung**

Wurzelfresser und Pflanzenmutualisten, wie arbuskuläre Mykorrhizapilze (AMF), haben einen signifikanten Effekt auf die Struktur und Dynamik von Pflanzengesellschaften. Dennoch sind die Interaktionen zwischen den beiden Gruppen von Organismen in natürlichen Ökosystemen weit von einem Verständnis entfernt. Wir führten ein Inokulationsexperiment durch, um die Effekte von zwei Wurzelherbivoren, *Pratylenchus penetrans* und *P. dunensis*...
Introduction

In the last 10 years, there has been an increased research effort to unravel the belowground complexity and to link it with aboveground ecology (Wardle et al., 2004). The interactions between plant roots and soil organisms have a significant effect on the structure and dynamics of both belowground and aboveground communities (Bever, Westover, & Antonovics, 1997; De Deyn et al., 2003; Van der Putten, Vet, Harvey, & Wackers, 2001; Wardle et al., 2004). Root-feeders, pathogens and plant mutualists coexist in the rhizosphere of plants and their interactions can lead to changes in the belowground community, which in turn affect plant performance and community structure (Bezemer et al., 2005; Van der Putten, Van Dijk, & Peters, 1993). Nevertheless, these belowground interactions and their ecological relevance in natural systems are far from being fully understood (Borowicz, 2006).

The role of root-feeders in the decline of *Ammophila arenaria* in coastal sand dunes is one of the best documented cases illustrating how soil biota affect aboveground processes in natural systems (Van der Putten, Maas, Van Gulik, & Brinkman, 1990). This is a good system for ecological studies on belowground interactions because of the large amount of data available on plant–soil interactions and soil diversity (Costa, Davies, Bardgett, & Kerry, 2003; de Rooij-van der Goes, 1995; Kowalchuk, Gerards, & Woldendorp, 1997; Rodriguez-Echeverria & Freitas, 2006; Van der Putten et al., 1990), and because the sandy soil is a relatively easy substrate for soil ecological studies. Initially, it was thought that root-feeding nematodes could play a significant role as belowground grazers in coastal sand dunes (Van der Putten et al., 1990). However, nematode abundance in the foredunes is usually low and subsequent work showed that root-feeding nematodes are strongly controlled in this soil (De Rooij-Van der Goes, 1995). Recent studies have demonstrated that a wide array of nematode control mechanisms can be found in the foredunes. Different root-feeding nematode species are ruled out by bottom-up processes (Van der Stoel & van der Putten, 2006), horizontal competition (Brinkman, Duyts, & van der Putten, 2005), plant mutualists (de la Peña, Rodriguez-Echeverria, van der Putten, Freitas, & Moens, 2006; Greipsson & El-Mayas, 2002; Hol, de la Peña, Moens, & Cook, 2007; Little & Maun, 1996) and/or natural enemies (Piskiewicz, Duyts, Berg, Costa, & van der Putten, 2007). However, little is known about the effect of root-feeding nematodes on other rhizosphere organisms in natural systems. Among these, arbuscular mycorrhizal fungi (AMF) are the most likely to be affected by root herbivores because of the close relationship between them and plant roots.

AMF are ubiquitous plant mutualists which represent a large fraction of the fungal biomass of the rhizosphere. AMF are particularly beneficial for plants in sand dunes because they facilitate nutrient uptake in these low-fertility soils and play a key role in sand stabilization (Koske & Gemma, 1997). AMF can also act as mediators between plants and above and belowground herbivores (Brown & Gange, 2002). The outcome of this interaction depends mainly on the life history traits of the herbivore, the physiological status of the host plant and the composition of the AMF community (Hol & Cook, 2005). Different AMF species can have different effects on plant growth and chemistry and, therefore, on herbivore performance (Gange, Brown, & Aplin, 2005). In addition, the effect of mixtures of AMF species on herbivore performance can differ from the effects of single AMF (Gange, 2001). In terms of the consequences of the interaction for the AMF community, aboveground herbivory can reduce the extent of root colonization by the fungal mutualists (Gange, Bower, & Brown, 2002), although this effect seems to be
species specific (Klironomos, McCune, & Moutoglis, 2004). For belowground herbivores, de la Peña et al. (2006) showed that the root-feeding nematode Pratylenchus penetrans did not affect root colonization by AMF. Nevertheless, whether belowground herbivores also have differential effects on different fungal species is not clear yet (Borowicz, 2006). The composition of the AMF communities in natural systems is relevant for ecosystem dynamics because of the functional and physiological differences among AMF (Maherali & Klironomos, 2007). In the case of A. arenaria, distinctive AMF communities have been found in association with the plant roots in either different locations or dune succession stages (Blaszkowski, 1994; Kowalchuk, De Souza, & Van Veen, 2002; Rodriguez-Echeverria, Hol, Freitas, Eason, & Cook, 2007). Kowalchuk et al. (2002) also showed that the AMF communities associated with A. arenaria in healthy stands were different from those of degenerating plants, suggesting a link between plant performance, changes in the AMF community and changes in the populations of other rhizosphere organisms. When A. arenaria degenerates, it is replaced by later successional plant species (Van der Putten et al., 1993). Therefore, the causes that lead to changes in the AMF communities can have important consequences for the ecosystem.

In the present study, we tested the hypothesis that root-feeders can change the AMF community structure in the roots of the attacked plant. In order to test this hypothesis, we designed an experiment using two genetically different populations of A. arenaria (Rodriguez-Echeverria, Freitas, & van der Putten in press) inoculated with their native AMF communities and two species of root-feeding nematodes, P. penetrans and P. dunensis, which occur frequently in the rhizosphere of A. arenaria (de la Peña, Karssen, & Moens, 2007). We discuss our results in relation to the dynamics of root herbivores and AMF communities in natural ecosystems and the aboveground consequences of this interaction.

Materials and methods

Collection and preparation of plants, AMF and nematodes

Seeds of A. arenaria were collected in Ynyslas (Wales, UK) and Het Zwin Nature Reserve (Knokke, Belgium). Seeds were germinated in a glasshouse with a 16 h/8 h light/dark regime and 25 °C/16 °C day/night temperature on 2-mm-diameter glass beads with demineralized water. Two-week-old seedlings with 2-cm-tall shoots were used in the bioassays.

Soil was collected from the rhizosphere of four different A. arenaria plants in Het Zwin and Ynyslas to set up separate trap cultures of the AMF community with corn (Zea mays L.) as host plant. Spores were extracted from the trap cultures by wet sieving and inspected under a stereoscopic microscope (Leica MZ 8). Most spores from both sites belonged to Glomus spp. Healthy spores of both cultures were collected, washed and re-suspended in autoclaved distilled water to obtain suspensions with a final concentration of 100 spores ml⁻¹ for each origin.

Pratylenchus dunensis was isolated originally from A. arenaria stands in Oostvoorne, the Netherlands, and P. penetrans from Zea mays in Zandhoven, Belgium. Both populations were multiplied on A. arenaria plants in sterilized soil for 9 months before the experiment was set up. To obtain nematodes for the inoculation experiments, soil and plant roots from cultures were sieved through a 0.5-mm 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 ºC and tapped off every day during 1 week to collect nematodes in water. Nematode identity was checked prior to inoculation by morphology and sequencing of the ITS region (de la Peña et al., 2007).

Experimental set-up

For each of the two A. arenaria plant populations, we set up six treatments with five replicated pots per treatment: non-inoculated control (C), inoculation with AMF (F), inoculation with P. penetrans (Pp), inoculation with P. dunensis (Pd), inoculation with AMF and P. penetrans (F-Pp) and inoculation with AMF and P. dunensis (F-Pd). Four seedlings of A. arenaria were planted in 1.5-l pots filled with 1800 g of autoclaved dune sand. In the inoculation treatments, each pot received 700 nematodes (mobile stages) and/or 550 AMF spores. Nematodes and AMF were inoculated at the same time to mimic natural conditions and give nematodes and AMF the same chance to colonize plant roots. Pots were covered with tin foil to prevent desiccation and watered every second day to keep the moisture content at 5–10% based on pot weight. Every 2 weeks all pots were watered with 120 ml half-strength modified (P-free) Hoagland’s nutrient solution. The pots were placed in the greenhouse in a full randomized design and repositioned every 2 weeks, after each fertilizer application. The experiment was conducted for 14 weeks, from June 2004 until September 2004.

Harvest, data collection and statistical analyses

After harvest, fresh root weights were obtained and a portion of each root was subsequently cut, weighed and stained using acid fuchsin for nematode and AMF assessment (Baker & Gowen, 1996). After taking the
root fraction for assessing colonization, the remaining plant material was dried at 72°C for 48 h to determine plant biomass. Total root biomass was estimated including the root fraction taken for assessing root colonization. Using a compound microscope, root infection by AMF and nematodes was estimated as the percentage of 1-cm root fragments containing structures of these organisms. Nematodes were counted in each root fragment to estimate the total number of nematodes per gram of root. Nematodes were also extracted from soil by zonal centrifugation following Hendrickx (1995). Nematodes in any developmental stage, eggs included, were counted. The total number of nematodes per pot was calculated by adding up the number of nematodes in both roots and soil.

A three-way ANOVA was used to test if the effect of plant origin, AMF and nematode species on herbivore performance and plant growth were independent of local conditions and nematode identity. Differences in AMF infection were examined using a two-way ANOVA with plant origin and nematode as fixed factors. Tukey’s HSD post hoc tests were used to analyze the statistical differences between groups. Prior to analysis, data were checked for normality and homogeneity of variances. The biomass data were square root transformed to meet ANOVA assumptions. All statistical analyses were performed using SPSS 14.0.

At the end of the experiment, the AMF communities associated with A. arenaria in each treatment were analyzed by PCR-DGGE. DGGE (Denaturing Gradient Gel Electrophoresis) separates DNA fragments of similar size but with different base sequences by exploiting the melting behavior of DNA. Put simply, DNA with different base sequences (i.e. different G+C content) melt at different positions in a polyacrylamide gel containing a linear gradient of denaturants (DGGE). The DNA fragments are amplified from environmental DNA with different base sequences (i.e. different G+C content) melt at different positions in a polyacrylamide gel containing a linear gradient of denaturants (DGGE). The DNA fragments are amplified from environmental samples using specific primers for the studied fungal or bacterial group. Therefore, as with many molecular techniques, the information obtained by PCR-DGGE is limited by the variability of the selected DNA region and the availability of specific primers. Nevertheless, it is a powerful technique to observe the composition of fungal or bacterial communities, which otherwise are impossible to analyze (Anderson & Cairney, 2004).

Samples containing approximately twenty, 1-cm root pieces from each pot were used for DNA extraction and subsequent molecular analysis. Total DNA was extracted using the Nucleon Phytopure Plant DNA extraction kit (Amersham Biosciences, Freiburg, Germany) following the manufacturer’s protocol. A nested-PCR was used to selectively amplify the fungal LSU rDNA sequences from the extracts using the primers LR1 and FLR2 (Gollotte, van Tuinen, & Atkinson, 2004) in the first reaction and FLR3-GC (FLR3 plus a GC clamp at the 5′ end) and FLR4 for the second reaction. FLR3 and FLR4 were described by Gollotte et al. (2004) as specific for Glomeromycota fungi. All reactions were carried out in a GeneAmp PCR 9700 system (Perkin-Elmer, CA, USA).

The products from the second PCR were examined by standard 1% (w/v) agarose gel electrophoresis with ethidium bromide staining, to confirm product integrity and estimate yield. One band of 380 bp was obtained in all samples. Approximately 0.4 μg of DNA from each sample was used for DGGE analysis. Gels contained 8% (w/v) polyacrylamide (37:1 acrylamide/bis-acrylamide) and 1 x TAE. The linear gradient used was from 27% to 43% denaturant, where 100% denaturing is defined as containing 7 M urea and 40% (v/v) formamide (Muyzer & Smalla, 1998). Electrophoresis was run at 20 V for 15 min, and afterwards at 200 V for 330 min with a constant temperature of 60°C in a DGGE-2401 system from CBS Scientific (CA, USA). Afterwards, gels were stained with ethidium bromide and visualized in a UV transilluminator. Digital images were captured using a Vilber Loumat system and analyzed with GelCompar II version 4.0 (Applied Maths, Kortrijk, Belgium). UPGMA dendrograms were constructed using the Pearson correlation coefficient to calculate the similarities between samples. The result was statistically tested using one-way ANOVA on the values from the similarity matrix.

Results

Effect of AMF on the root-feeders

Root infection by nematodes was reduced from values of 70% in non-mycorrhizal plants to about 50% in mycorrhizal plants (Fig. 1A). The three-way ANOVA showed a significant effect of the presence of AMF on root colonization by nematodes (F1,32 = 41.41, P < 0.001) (Table 1, Fig. 1B). The two other factors considered in the analysis, origin of plant/AMF and identity of the herbivore species, did not have any significant effect on this variable (Table 1).

The final number of nematodes in all treatments from Belgium was about 5600 with the exception of P. penetrans in mycorrhizal plants, which had a final number of 4000 individuals (Fig. 2A). In non-mycorrhizal plants from Wales, there were 6600 and 4900 nematodes of P. penetrans and P. dunensis, respectively. These numbers were reduced to about 2300 in mycorrhizal plants (Fig. 2A). The three-way ANOVA showed a significant effect of the presence of AMF (F1,32 = 15.33, P < 0.001), the origin of plant/AMF (F1,32 = 8.69, P < 0.01) and an interaction between the two factors (F1,32 = 5.07, P < 0.05) on the final number of nematodes (Table 1). The Tukey HSD post hoc test
on the interaction AMF\slash C2 origin found a significant reduction of the final number of nematodes in the mycorrhizal plants from Wales (Fig. 2B).

**Effect of the root-feeders on AMF**

Root colonization by AMF was about 50\% in plants from Belgium and 40\% in plants from Wales (Fig. 3). The two-way ANOVA showed a significant effect of plant/AMF origin on mycorrhizal infection ($F_{1,24} = 5.08$, $P < 0.05$) but no effect of the presence of nematodes on the percentage of root colonized by AMF (Fig. 3, Table 2).

In spite of this, the PCR-DGGE showed that root-feeders altered the composition of the AMF communities colonizing the roots of *A. arenaria* (Fig. 4A and B). The variation in the structure of the AMF communities differed in both plant/AMF origins. The overall similarity of samples was higher in plants from Belgium than from Wales. Even so, the analysis of the PCR-DGGE data separated the three treatments: mycorrhizal plants, mycorrhizal plants inoculated with *P. penetrans* and mycorrhizal plants inoculated with *P. dunensis* (Fig. 4A). The three groups were statistically different (one-way ANOVA, $P < 0.001$). In the case of plants from Wales, the AMF community structure of plants inoculated with *P. penetrans* was significantly different ($P < 0.001$) than that of the other treatments (Fig. 4B). Only one PCR product could be obtained from roots inoculated with AMF and *P. dunensis* and this sample grouped with the mycorrhizal plants not inoculated with nematodes (Fig. 4B).

**Plant growth**

Plant biomass ranged between 0.22 and 0.40 g per plant for Belgium and between 0.21 and 0.46 g for Wales (Fig. 5). Because the statistical analysis of the data for root and shoot biomass produced the same results as those for total biomass, only the latter are presented. The three-way ANOVA showed a significant effect of inoculation with nematodes ($F_{2,48} = 3.78$, $P < 0.05$) and of the interaction between the three factors (nematode, plant/AMF origin and AMF inoculation, $F_{2,48} = 4.65$, $P < 0.05$) on plant biomass (Table 3). The *post hoc* comparisons with Tukey HSD test revealed that the simultaneous inoculation with AMF and *P. dunensis* lead to a significant reduction of plant growth in Wales (Fig. 5).

**Discussion**

The presence of AMF always reduced root infection by both nematode species, a result that agrees with
previous data for *P. penetrans* (de la Peña et al., 2006). Nevertheless, the multiplication of the two nematode species was differently affected by AMF. One of the AMF communities only suppressed the generalist *P. penetrans* while the AMF from Wales impaired the reproduction of both root-feeding nematodes. Direct competition for space might be responsible for the suppression of root infection by nematodes (de la Peña et al., 2006; Roncadori, 1997). The suppression of nematode reproduction, however, might also depend on other mechanisms such as local changes in root biochemistry of mycorrhizal plants (Smith, 1987), which could change with the specific composition of the AMF community. The fact that both root-feeders declined significantly only with AMF from Wales suggests that nematode reproduction suppression depends on the AMF community and also on nematode sensitivity. This result agrees with the species-specific effect observed in studies done with single AMF species and herbivorous insects (Gange, 2001; Gange et al., 2005).

Experimental evidence for the role of AMF as a mediator of root-herbivore interactions in natural and

---

**Fig. 2.** (A) Total number (mean± S.E.) of *P. penetrans* and *P. dunensis* in each experimental treatment (*N* = 5). Treatments as in Fig. 1A. (B) Pooled data for total number of both nematode species for non-mycorrhizal plants (*−F*) and mycorrhizal plants (*+F*) from Belgium and Wales (*N* = 10). Different letters mean significant differences (Tukey HSD post hoc tests after three-way ANOVA).

**Fig. 3.** (A) Root colonization (mean± S.E.) by AMF in each experimental treatment (*N* = 5). F: inoculation with a natural mixed culture of AMF; F-Pp and F-Pd as in Fig. 1A. (B) Pooled data for AMF colonization for each origin (*N* = 20). Different letters mean significant differences (Tukey HSD post hoc tests after two-way ANOVA).

**Table 2.** Results of the two-way ANOVA for treatment effects on root infection by AMF. Significant *P* values are in bold.

<table>
<thead>
<tr>
<th></th>
<th>d.f.</th>
<th><em>F</em></th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>1</td>
<td>5.08</td>
<td>0.034</td>
</tr>
<tr>
<td>Nematode</td>
<td>2</td>
<td>2.00</td>
<td>0.158</td>
</tr>
<tr>
<td>Origin × nematode</td>
<td>2</td>
<td>2.80</td>
<td>0.081</td>
</tr>
<tr>
<td>Error</td>
<td>24</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>
Agricultural systems has been shown in previous studies (Borowicz, 2001; Hol & Cook, 2005). In contrast, the effect of root-feeders in the composition of AMF colonizing the roots has hardly been addressed before. Our results show that root-herbivores can alter the AMF community associated with a plant, even if the total percentage of root colonization by AMF is not affected. Some AMF species are known to be more...
sensitive to herbivory than others (Klironomos et al., 2004). Therefore, the differential detrimental effect of herbivores in some AMF species could benefit others in terms of root colonization. Interestingly, the results were different for the two AMF communities tested. In the case of plants and AMF from Belgium, the three treatments – mycorrhizal plants without nematodes, mycorrhizal plants with *P. penetrans* and mycorrhizal plants with *P. dunensis* – resulted in three separate groups of AMF communities (Fig. 4A). *P. dunensis* had a bigger impact on the AMF community obtained from Belgium than *P. penetrans*. In contrast, the AMF community from Wales was more sensitive to the presence of *P. penetrans* (Fig. 4B). This root-feeder was responsible for major alterations in the structure of the fungal community associated with *A. arenaria*. The differential effect of the root-feeders in both plant/AMF origins highlights the complexity of the belowground interactions in natural systems.

The herbivore densities used in our experiments did not result in a reduction of plant growth and the presence of AMF did not increase plant biomass. However, the combination of AMF and *P. dunensis* in plants from Wales caused a significant reduction of plant biomass. AMF can become parasitic to plants (Johnson, Graham, & Smith, 1997) and exacerbate the detrimental effect of foliar herbivory (Gange et al., 2002). To our knowledge, it has not previously been shown that AMF can have negative synergistic interactions with root-feeding nematodes. This effect depends on the particular combination of plant, AMF and nematode species. Environmental conditions might also affect this result because previous studies did not detect the detrimental effect of inoculating AMF and nematodes together (de la Peña et al., 2006). It is noteworthy that plant biomass was reduced in the cases where the structure of the AMF communities was less affected by the root-feeder. This opens the question whether the changes in the AMF community happened because of a direct effect of the root-feeder on the fungal species or through physiological changes in the plant caused by the herbivores.

In the field, different AMF communities and nematode species have been found in early and late successional stages of *A. arenaria* (Kowalchuk et al., 2002; Wall, Skene, & Neilson, 2002). The differential effect of AMF on both *Pratylenchus* species is likely to affect competition between root-feeding nematodes, which is one of the mechanisms controlling the relative abundance of nematodes in the rhizosphere of *A. arenaria* (Brinkman et al., 2005). However, the presence of different nematode species might also modify the structure of the AMF community leading to further changes for the plant, and subsequently influencing other herbivores. If our results are to be extrapolated to the field, it seems clear that these interactions between root herbivores and AMF could drive the belowground succession of both organisms and, ultimately, affect the composition of plant communities.

**Acknowledgments**

This study is part of the EcoTrain project (HPRN-CT 2002 00210) funded by the European Union. The authors thank Kris Struyf, curator of Het Zwin Nature Reserve, for allowing collection of samples. Eduardo de la Peña is a post doctoral fellow of the Flemish Science Foundation (FWO).

**References**


