Interactions among endoparasitic root-feeding nematodes; consequences for nematodes and host plant

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Dit onderzoek is uitgevoerd binnen de onderzoeksschool Functionele Ecologie

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
Prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
op maandag 6 december 2004
des namiddags te vier uur in de Aula.

Brinkman, Elsa Pernilla, 2004

Interactions among endoparasitic root-feeding nematodes; consequences for nematodes and host plant.

PhD thesis Wageningen University – with references – with summary in Dutch.

ISBN 90-8504-109-0

Abstract

Brinkman, E.P. 2004. Interactions among endoparasitic root-feeding nematodes; consequences for nematodes and host plant. PhD thesis Wageningen University, Wageningen, The Netherlands.

Plants are influenced by above- and belowground herbivores and their interactions. Root feeders, among which are nematodes, generally occur in multi-species communities. The aim of this study was, to determine whether interspecific interactions among endoparasitic root-feeding nematodes (*Heterodera arenaria*, *Meloidogyne maritima* and *Pratylenchus penetrans*) would influence nematode abundance and dynamics and subsequently biomass of the shared host plant *Ammophila arenaria*.

In a field experiment, different combinations of the endoparasitic nematodes and other soil organisms were added to *A. arenaria*. Subsequently, the feedback of the established soil communities was tested in bioassays. Soil from non-buried plants inoculated with a mixture of organisms from the root zone of *A. arenaria* reduced the biomass of newly planted seedlings. In contrast, a combination of the three endoparasitic nematodes did not affect plant biomass differently from the control.

When in the field experiment *M. maritima* had been added to plants alone, juveniles and males of this species occurred in the new root layer earlier in the year and in higher densities than when *H. arenaria* and *P. penetrans* had been added as well. Addition of the other two species apparently forced *M. maritima* to develop under suboptimal conditions. Addition of the other endoparasite species did not affect *H. arenaria* and *P. penetrans*. Greenhouse experiments aimed at studying interactions between different pairs of the endoparasite species did not explain observations from the field experiment.

Interestingly, in the field experiment *M. maritima* reduced plant biomass more when added alone than when added together with the other two endoparasites, whereas addition of root zone soil reduced plant biomass most. *Heterodera arenaria* and *P. penetrans* did not affect plant biomass. These results support the view that effects of species identity and diversity may be intermingled and that species traits rather than diversity determines the effect.

The ectoparasitic nematode *Tylenchorhynchus ventralis* spontaneously colonised the field experiment in high densities, but the numbers declined when the experiment proceeded. In a greenhouse experiment, numbers of *T. ventralis* were reduced by adding an unnaturally high density of endoparasites. Thus, the observed decline in spontaneously established ectoparasite numbers was unlikely to be caused by the endoparasites, but by other (micro-) organisms that spontaneously colonised the soil. Despite their limiting effect, the endoparasites could not prevent *T. ventralis* from reducing plant biomass.

In conclusion, it seems likely that in the field, abundance and dynamics of *M. maritima* are determined by interspecific competition with *H. arenaria* and *P. penetrans*. The latter two endoparasitic nematodes are less influenced by the presence of the other

endoparasites than is *M. maritima*. Interestingly, *M. maritima*, when added alone, reduced plant biomass, whereas a combination of the three endoparasites did not. Addition of root zone soil of *A. arenaria* reduced plant biomass most, so that the negative soil feedback is likely to be caused by the whole soil community rather than by endoparasitic nematodes alone.

Contents

Chapter 1

General introduction

Plants are influenced by above- and belowground herbivores and their interactions. Root herbivores, mainly soil-inhabiting insect larvae and root-feeding nematodes, directly consume part of the belowground primary production, add to other stress factors that influence plant biomass and predispose plants to attack by other pathogens (Stanton 1988, Masters and Brown 1997). Root-feeding nematodes have mainly been studied in agricultural crops, whereas they have received far less attention in natural systems (Brown and Gange 1990, Mortimer *et al.* 1999). Natural systems provide an opportunity to study interactions between host plants and co-occurring nematode species that have co-evolved for a longer time period than in agricultural systems. Root-feeding nematodes generally occur in multi-species communities, so that interspecific interactions are likely to influence both nematode abundance and dynamics and consequently plant growth. We studied interactions among three endoparasitic root-feeding nematodes that are commonly found together on *Ammophila arenaria* (L.) Link (marram grass). The aim was to determine whether interspecific competition would influence nematode abundance and temporal dynamics and consequently plant biomass.

Influence of soil organisms on natural plant communities

The community of soil organisms has a strong influence on the performance of plants and plant communities (Wardle 2002). Feedback effects of the soil community are a result of specific interactions between the plant community and decomposers (Bardgett and Wardle 2003) and root-associated soil organisms, such as symbiotic mutualists, herbivores and pathogens (Hart *et al.* 2003, Reynolds *et al.* 2003, Van der Putten 2003). Root feeders and pathogens contribute to the structuring of plant communities through their effect on individual plant species. They influence the spatial distribution of plants by affecting seedling establishment (Augspurger and Kelly 1984, Packer and Clay 2000, 2003) and plant vigour (Van der Putten *et al.* 1988, Bever 1994). Plant species specific effects of root feeders and pathogens alter the outcome of competition between plants (Van der Putten and Peters 1997). This may facilitate coexistence of plant species (Holah and Alexander 1999), enhance cyclic patterns in vegetation composition (Holah *et al.* 1997, Olff *et al.* 2000) and succession (Van der Putten *et al.* 1993, De Deyn *et al.* 2003) and contribute to changes in plant species diversity (De Deyn *et al.* 2003) and abundance (Klironomos 2002).

Root herbivores (mainly root-feeding nematodes and soil-inhabiting insect larvae) are able to consume an important part of belowground primary production, although estimates of their effect are inconsistent (Stanton 1988, Brown and Gange 1990). While some studies report an increase in plant biomass of 25 – 59% as a result of nematicide

application (Stanton *et al.* 1981, Ingham and Detling 1990), others only find an increase of 5 – 13% (Seastedt *et al.* 1987, Verschoor 2002, Verschoor *et al.* 2002b). The effect of nematode infection is a general reduction in plant growth, excessive root branching, cessation of root elongation and retardation of root growth (Khan 1993). Besides direct growth inhibition, root-feeding nematodes add to the effect of abiotic stress factors. In addition, they may predispose the plants to attack by pathogens, such as fungi and bacteria, or act as vectors of viruses (Khan 1993).

Multi-species communities of root-feeding nematodes

Generally, plants are infected by several species of root-feeding nematodes at the same time (Oostenbrink 1966 in Eisenback 1993). However, an increase in the diversity of root-feeding nematodes does not necessarily lead to a decrease of plant biomass, as, for example, pathogenicity may be inversely related to competitiveness. In that case, infection of plants by a mixture of several root-feeding nematode species may even be beneficial to the host compared to infection by one root-feeding species (Duncan and Ferris 1983, Umesh *et al.* 1994).

The structure of the nematode community is determined by differences in feeding- and life history strategies and differences in response to abiotic factors like soil texture, moisture and temperature (Ritz and Trudgill 1999, Bardgett 2002). Coexistence of different species of root-feeding nematodes may be facilitated by feeding on different parts of the root or exhibiting different feeding strategies. Root-feeding nematodes generally are categorised into ecto-, migratory endo- and sedentary endoparasites (Yeates *et al.* 1993). Usually, the feeding strategy is related to the degree of host specificity and it expresses the complexity of the relationship with the host plant (Siddiqi 2000). Ectoparasitic nematodes feed by penetrating root cells with their stylet (Yeates *et al.* 1993). The divergence in stylet lengths between species may facilitate coexistence of different ectoparasite species, with nematodes with a longer stylet feeding on deeper cell layers than nematodes with a shorter stylet length (Yeates 1986). Migratory endoparasites enter the root and feed on deeper cell layers. Females of sedentary endoparasites induce specific feeding structures, exhibiting the most complex relationship with the host plant of all root-feeding nematode species.

Population regulation

The population dynamics of herbivores is influenced by abiotic factors (Andrewartha and Birch 1954) and by density-dependent regulating factors (Nicholson 1933). Regulating factors are resource availability (bottom-up) (Lindeman 1942, White 1978) and the occurrence of natural enemies (top-down) (Hairston *et al.* 1960). Studies on population regulation usually focus on either bottom-up or top-down factors, ignoring the fact that the relative importance of these factors might change depending on spatial and temporal factors (Walker and Jones 2001). The role of competition with species on the same trophic level is usually not mentioned in the discussion on population regulation, being part of a

different subset in literature. Competition in the traditional sense occurs when species directly interfere with each other or when they exploit the same resource (Connell 1990). In addition, apparent competition can occur when two species share the same natural enemy (Holt 1977) or via another species on the same trophic level (Connell 1990).

Belowground, most studies on regulation of root-feeding nematodes have been performed in economically important agricultural crop systems. Natural systems though, provide a nice opportunity for studying interactions between different trophic groups that are constantly co-evolving. Bottom-up control of root-feeding nematodes can be attained when plant nutrient composition is unsuitable (Yeates 1987) or by a range of plant secondary compounds (Rohde and Jenkins 1958, Gommers *et al.* 1982, Potter *et al.* 1999). In top-down control of nematodes, several groups of organisms are involved, such as bacteria, fungi, predatory nematodes, insects and mites (Stirling 1991). Predators are supposed to have a relatively small limiting effect on the abundance of root-feeding nematodes (Yeates and Wardle 1996). In contrast, fungi and bacteria in several cases naturally suppress populations of plant parasitic nematodes (Kerry 1987). Most of the studies mentioned report on control of sedentary endoparasites, but migratory endoparasites and ectoparasites can also be attacked (Kerry 1987, Siddiqui and Mahmood 1996, 1999). For practical biological control of root-feeding nematodes the focus has been on top-down control (Kerry 1987), although many studies have assessed interspecific competition between root-feeding nematodes (Eisenback 1985, 1993).

Despite the diversity in feeding strategies, root-feeding nematodes may still affect each other through alteration of plant growth and competition for a limited amount of resources (Siddiqi 2000). Interactions between root-feeding nematodes usually are negative to one or both of the species, although they may be neutral or positive as well (Eisenback 1985, 1993). Competition is supposed to be due to occupation of feeding sites, physical change of the host plant decreasing its suitability or destruction of feeding sites. Competition is supposed to be severest between species with similar feeding habits. Nematodes with a more complex host-parasite relation have a competitive advantage over nematodes with a more simple relation to their host plant. This implies that sedentary endoparasitic nematodes usually are stronger competitors than ectoparasites, and migratory endoparasites are intermediate competitors (Eisenback 1985). A reason might be that sedentary endoparasites only have to penetrate and create a feeding site once, while ectoparasites constantly have to search for new feeding sites. The outcome of competition between nematodes can be affected by the timing of infection of the plant (Eisenback 1985, 1993).

Influence of soil organisms on *Ammophila arenaria*

Ammophila arenaria is the most important natural sand fixing plant species in the outer coastal dunes in Western Europe. In mobile dunes, in autumn and winter *A. arenaria* plants are buried by a layer of sand that is wind-blown from the beach. As long as the tops of the leaves remain above the sand surface, the plants may emerge in the subsequent

growth season. In response to sand burial, new nodes are formed, the internodes elongate and roots are formed in the newly deposited sand layer. For vigorous growth, the grass requires regular sand burial and the plants degenerate when sand burial stops (Huiskes 1979, Wallén 1980, Eldred and Maun 1982). The issue of degeneration of *A. arenaria* in stable dunes has been subject to many studies. Degeneration has been attributed to nutrient deficiency (Willis 1965), competition with other plant species (Huiskes 1979) and morphological constraints inhibiting replacement of old by new roots (Marshall 1965). However, sand deposition caused an increase in growth rate greater than expected from the amount of nutrients present in the soil (Willis 1965) and degeneration also takes place in the absence of other plant species (Hope-Simpson and Jefferies 1966, Disraeli 1984). It has also been hypothesised that soil organisms are responsible for the decline in growth of *A. arenaria* when sand burial ceases (Van der Putten *et al.* 1988).

The root zone of *A. arenaria* contains a complex of plant pathogenic fungi and root-feeding nematodes (De Rooij-van der Goes *et al.* 1995b, Van der Stoel *et al.* 2002). In contrast, beach sand is free from soil pathogens and root feeders (Van der Putten and Troelstra 1990), so that burial with a layer of beach sand may provide the plant with an opportunity to form new roots in sand that is free from its natural belowground enemies. Therefore, the escape from root-feeding nematodes and soil pathogens through sand burial may contribute to vigorous plant growth. However, the escape is only temporary, as the new roots are soon colonised by soil organisms from the deeper soil layers (Van der Putten *et al.* 1989, Van der Stoel *et al.* 2002).

Several greenhouse experiments showed a negative effect of soil organisms, and of root-feeding nematodes in particular, on the growth of *A. arenaria*. Seedlings of the plant produced less biomass when grown in root zone soil from a field stand, suggesting a negative feedback between *A. arenaria* and its soil community (Van der Putten *et al.* 1988, Van der Stoel *et al.* 2002). The addition of nematicides partly counteracted the growth reduction, indicating that root-feeding nematodes may have a negative effect on the growth of *A. arenaria* (Van der Putten *et al.* 1990, Van der Stoel *et al.* 2002). In greenhouse inoculation studies, the ectoparasite *Tylenchorhynchus ventralis* (Loof) Fortuner and Luc (=*Telotylenchus ventralis*) had a considerable effect on plant growth (De Rooij-van der Goes 1995). However, this nematode species only reduced plant biomass when added in unnaturally high densities or in combination with pathogenic soil fungi.

Endoparasitic root-feeding nematodes associated with *Ammophila arenaria*

Endoparasitic nematodes are known to cause severe losses in agricultural crops (Evans *et al.* 1993), so that they may also be responsible for the growth reduction of *A. arenaria*. Three endoparasitic root-feeding nematode species that are frequently found in the root zone of *A. arenaria* are *Heterodera arenaria* (Cooper) Robinson, Stone, Hooper and Rowe, *Meloidogyne maritima* (Jepson) Karssen, van Aelst and Cook and *Pratylenchus penetrans* Cobb (De Rooij-van der Goes *et al.* 1995b).

The cyst nematode *H. arenaria* is a sedentary endoparasite that is specific to the pioneer dune grasses *Elymus farctus* (Viv.) Melderis and *A. arenaria* (Van der Stoel 2001). In greenhouse inoculation studies, *H. arenaria* only had a slightly negative effect on the growth of *A. arenaria* (Van der Stoel 2001). Second stage juveniles of *Heterodera* sp. enter the plant near the growing tip. They pierce cell walls with their stylets and disrupt plant cells while moving to the vascular cylinder (Wyss and Zunke 1986). There, they induce a feeding site by injecting salivary gland secretions (Williamson and Gleason 2003). The feeding site consists of a multinucleate syncytium. This cell with several nuclei is formed by breakdown of cell walls between the initial feeding cell and its neighbouring cells. The syncytium acts as a metabolic sink that funnels plant resources to the nematode. The second stage juveniles go through three further moults before becoming adult. The adult males are vermiform (worm-shaped) and leave the root. Females swell, becoming saccate, and rupture the root while growing. After fertilisation, eggs are formed and retained within the female body. When the female dies, the cuticle becomes a protective cyst wall. The embryos develop into first stage juveniles and within the egg moult into second stage juveniles, which is the infective stage. While some of the juveniles immediately hatch, others remain viable within the cyst for one or several years (Ferris and Ferris 1998, Van der Stoel 2001).

Meloidogyne maritima is a sedentary endoparasite as well. In contract to many other *Meloidogyne* species (root knot nematodes), *M. maritima* is very host specific, as it has only been detected on *A. arenaria* (W.H. van der Putten and H. Duyts, unpublished results). Second stage juveniles of *Meloidogyne* sp. penetrate the root just above the root cap and move intercellularly down the outer root tissue (cortex) towards the root tip (Christie 1936, Hussey 1985). The juveniles then enter the base of the vascular cylinder and migrate up the root. They establish a permanent feeding site in the differentiation zone of the root by inducing nuclear division without cell division (Williamson and Gleason 2003). This results in large, multinucleate cells, which are called giant cells. The giant cells act as a metabolic sink, where the nematode ingests plant cytoplasm. Normally, the plant cells around the feeding site divide and swell, causing the formation of galls or root knots. However, root knots have not been observed on *A. arenaria* after infection by *M. maritima* (pers. obs.). The juveniles go through three moults, after which adult, non-feeding males leave the root (De Guiran and Ritter 1979). Adult females continue feeding, become pear-shaped and form eggs, which are deposited in a gelatinous matrix outside the body. Many *Meloidogyne* species are parthenogenetic and do not require fertilisation, but it is unknown whether this also is the case for *M. maritima*. The embryos develop into first stage juveniles and moult into second stage juveniles, which is the infective stage.

The root lesion nematode *P. penetrans* has a wide host range of agricultural crops. In sand dunes it feeds on foredune grasses, such as *A. arenaria* and *E. farctus*. Although this species may feed ectoparasitically on root hairs (Zunke 1990a), it is primarily a migratory endoparasite (Zunke 1990b). All life stages are able to enter roots mainly in the region of root hair development and to a lesser extent in the zone of elongation. The nematodes

penetrate the root and migrate intracellularly through the cortex, killing the cells that are passed. The nematodes moult four times before becoming adult and all life stages feed and are vermiform. Adult females deposit eggs either inside the cortical tissue or outside the roots (Zunke 1990b).

The three endoparasitic nematode species colonise the new soil layer in sequence, each showing a peak in abundance at different times of the year (Van der Stoel *et al.* 2002). This distribution of nematode activity in time may be determined endogenously, or it may result from interspecific competition for space or feeding sites (Hutchinson 1957, Eisenback 1993, Gouteux and Jarry 1998). The temporal distribution of root-feeding nematodes results in a continuous infection pressure of the plant. However, when the numbers of a more pathogenic species are reduced or nematode activity is shifted towards a period of lower plant activity, then multi-species infection may even be beneficial to the plant (Duncan and Ferris 1983, Umesh *et al.* 1994). For aboveground herbivores, it has been hypothesised that plants may attract specialists, which it can control. Numbers of generalists, which cannot be controlled by the plant, would then be reduced by competition with the specialists (Vinson 1999). When applying this to *A. arenaria*, the plant could use the endoparasites to restrict the numbers of ectoparasitic *Tylenchorhynchus* spp., resulting in less growth reduction.

Aim of this study

In agricultural systems, endoparasitic root-feeding nematodes are an important cause of crop losses. In foredunes, application of nematicides strongly improved the growth of the foredune grass *A. arenaria*. As endoparasitic nematodes are the major component of the root-feeding nematode community, we assumed that they would inhibit the growth of *A. arenaria*. Nematodes generally occur in multi-species communities, so that interspecific interactions are likely to influence both nematode abundance and dynamics and consequently plant growth. In order to analyse the role of endoparasitic nematodes, their interactions, and interactions with ectoparasites on the performance of *A. arenaria*, I focused on three endoparasitic root-feeding nematodes (*H. arenaria*, *M. maritima* and *P. penetrans*). The aim was to determine whether interspecific competition would influence nematode abundance and temporal dynamics and consequently host plant biomass.

Outline of the thesis

The influence of a combination of three endoparasitic nematodes (*H. arenaria*, *M. maritima* and *P. penetrans*) versus the whole soil community on the biomass of *A. arenaria* was studied in a field inoculation experiment (Chapter 2). The hypothesis was tested that sand burial enables the plant to withstand the negative effects of the added soil organisms. Subsequently, the negative feedback of the developed soil communities to *A. arenaria* was tested in controlled conditions.

The consequences of endoparasitic nematode diversity (each species alone compared to a combination of the three species) were studied in a field experiment, again

in relation to sand burial (Chapter 3). The population dynamics of the endoparasites was examined in detail during the growth season and the effects on biomass of *A. arenaria* were determined.

The interaction between *H. arenaria* and *P. penetrans*, as well as between *H. arenaria* and *M. maritima*, was studied in greenhouse conditions, using an additive design (Chapter 4). The influence of timing of inoculation on nematode development and plant biomass was studied, simulating the sequential migration of the nematodes to the new root layer. Further, the hypothesis was tested that at the same inoculation density, both nematode competition and plant inhibition decrease with plant age. It was assumed that nematode competition and plant sensitivity to parasitic nematodes would diminish with increasing size of the root system at the time of inoculation.

Interactions between the three endoparasitic and an ectoparasite nematode (*T. ventralis*) were studied in a greenhouse experiment (Chapter 5). The endoparasites only slightly inhibited plant growth, whereas the ectoparasite is capable to restrict growth of *A. arenaria*. The hypothesis was tested that endoparasitic, feeding-specialist nematodes may protect the plant by restricting multiplication of an ectoparasitic, feeding-generalist nematode through competition.

In chapter 6, the main results of the experimental chapters are discussed and synthesised.

Chapter 2

Unravelling soil feedback effects to the foredune grass *Ammophila arenaria*: influence of endoparasitic root-feeding nematodes versus the whole soil community

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Abstract

The dune grass *Ammophila arenaria* experiences a negative feedback from its soil community and root-feeding nematodes are considered to be among the causal agents. *Ammophila arenaria* grows vigorously when buried regularly by wind-blown beach sand, suggesting that the plants benefit from a temporary escape from root-feeding soil organisms.

In a field experiment, we created series of ceased and continued burial and added three endoparasitic root-feeding nematode species (*Meloidogyne maritima*, *Heterodera arenaria* and *Pratylenchus penetrans*) or root zone soil to the plants. We included a control treatment without addition of soil organisms. During three subsequent years, plant biomass was measured, numbers of nematodes were counted and bioassays were performed with the soil from the field experiment to determine the strength of plant-soil feedback.

In the field, addition of root zone soil had a negative effect on biomass of buried plants. In contrast, in the bioassays, addition of root zone soil had a negative effect on non-buried plants. Neither in the field, nor in the bioassays did the root-feeding nematodes alone influence plant biomass. In the field experiment, the occurrence of *M. maritima* correlated with the addition of root-zone soil, while the other nematodes did not correlate with any of the addition treatments.

In the field, the soil community of *A. arenaria* had a negative effect on buried plants. In contrast, in the bioassays, plants experienced a negative feedback when grown on soil from non-buried plants with the soil community of *A. arenaria*. Endoparasitic root-feeding nematodes alone did not affect plant biomass. Sand burial did not change the effect of additions.

INTRODUCTION

The performance of plants and plant communities is strongly influenced by interactions with the community of soil organisms (Wardle 2002). Feedback effects of the soil community are due to specific interactions between the plant community and root-associated soil organisms, mainly herbivores, pathogens and symbiotic mutualists (Hart *et al.* 2003, Reynolds *et al.* 2003, Van der Putten 2003) and soil organisms belonging to the decomposer subsystem (Bardgett and Wardle 2003). Many studies on plant-soil feedback have considered the composition of the soil community as a black box (Bever *et al.* 1997, Bever 2003), although some have identified specific fungi (Packer and Clay 2000) or groups of fungi as causal agents (Holah and Alexander 1999, Klironomos 2002). Here we present results of a field experiment aimed at unravelling the effects of endoparasitic root-feeding nematodes from that of the whole soil community.

Root herbivores and soil-borne pathogens contribute to the structuring of plant communities through their influence on individual plant species. Shifts in the composition of plant communities are caused by alteration of the outcome of competition between plant species by specific effects of root feeders and soil pathogens (Van der Putten and Peters 1997). Root-feeding and pathogenic soil organisms influence spatial patterns of occurrence by affecting seedling establishment (Augspurger and Kelly 1984, Packer and Clay 2000, 2003) and plant vigour (Van der Putten *et al.* 1988, Bever 1994). This results into species co-existence (Holah and Alexander 1999), vegetation succession (Van der Putten *et al.* 1993, De Deyn *et al.* 2003), cyclic patterns in vegetation composition (Holah *et al.* 1997, Olff *et al.* 2000) and changes in plant species diversity (De Deyn *et al.* 2003) and abundance (Klironomos 2002).

Root herbivores (mainly root-feeding nematodes and soil-dwelling insect larvae) consume an important part of below ground primary production (Stanton 1988, Brown and Gange 1990). The role of root-feeding nematodes in natural communities has indirectly been studied by excluding nematodes through the application of nematicides. The results are, however, inconsistent, ranging from a large effect on plant biomass (Stanton *et al.* 1981, Ingham and Detling 1990) to minor effects (Seastedt *et al.* 1987, Verschoor 2002, Verschoor *et al.* 2002b). Few studies have actually attempted to confirm effects of nematicides in natural vegetation by addition of nematodes. The results of these studies cast doubt on the results obtained from nematicide application, since effects range from not significant (Van der Stoel 2001, Verschoor *et al.* 2002a) to strongly dependent on the densities added (De Rooij-van der Goes 1995). At this moment, it is unclear if root-feeding nematodes may be key species or play a minor role in plant communities.

To study the effect on plant growth of root-feeding nematodes compared to the whole soil community, we used the coastal foredune grass *Ammophila arenaria* as a model. This natural sand-binding species originates from Europe and is used for dune stabilisation (Huiskes 1979). In autumn and winter, *A. arenaria* is covered by a layer of sand that is wind-blown from the beach to the dune. The stem elongates and the plant forms new roots

in the newly deposited layer (Huiskes 1979). Soon after root formation, the new sand layer is colonised by soil organisms from the layer below (De Rooij-van der Goes *et al.* 1995b, Van der Stoel 2001). A time lag between root formation and colonisation by soil organisms is thought to be one of the factors that contribute to vigorous plant growth (Van der Putten *et al.* 1989, Van der Stoel *et al.* 2002).

Several experiments have suggested a negative effect of the soil community in general and of root-feeding nematodes in particular on the growth of *A. arenaria* under greenhouse conditions. Seedlings of *A. arenaria* were hampered in growth when grown in soil from the root zone of a field stand, suggesting a negative feedback from the soil community to its host plant (Van der Putten *et al.* 1988, Van der Stoel *et al.* 2002). The addition of nematicides partly counteracted the growth reduction, indicating that root-feeding nematodes may negatively affect the growth of *A. arenaria* (Van der Putten *et al.* 1990, Van der Stoel *et al.* 2002). Endoparasitic nematode species (*Heterodera arenaria*, *Meloidogyne maritima* and *Pratylenchus penetrans*) that are frequently found in the root zone of *A. arenaria* (De Rooij-van der Goes *et al.* 1995b) might be important causes of negative soil feedback, also because such nematodes are known to cause severe losses in agricultural crops (Evans *et al.* 1993).

In the present study, we tested the effects of the endoparasitic nematodes *H. arenaria*, *M. maritima* and *P. penetrans* on growth of *A. arenaria* and compared these effects to the whole soil community. Our hypothesis was that plants would be equally reduced in growth when exposed to either the three nematode species alone or the soil community as a whole. In addition, we tested the hypothesis that the assumed positive effect of burial by beach sand on plant growth would counteract the negative influence of soil organisms. The hypotheses were tested in a field experiment where we added all soil organisms, or only the three endoparasitic nematode species, to previously uncolonised soil planted with *A. arenaria*. The feedback of the established soil communities was examined in bioassays in a growth chamber.

MATERIALS AND METHODS

Soil, plant material and inoculum

Soil for pre-culturing the plants originated from a sandy beach near Haringvlietdam (51º52' N 04º04' E). The soil was sieved (mesh size 0.5 cm), homogenised and sterilised by gamma-radiation (≥ 25 kGray). Seeds of *A. arenaria* (L.) Link were collected from plants at the same site, dried and stored at 4ºC until use. The seeds were germinated on glass beads at 25ºC/ 10ºC (16 h light/ 8 h dark). For the field experiment, the seedlings were grown in sterilised soil in a greenhouse for about twelve weeks and provided with extra light to ensure a minimum photosynthetic photon fluence rate of 200 µmol m^{-2} s^{-1} (over the waveband 400-700 nm) during 16 h day^{-1}. Temperature was 21 ± 2ºC in the daytime and 18 ± 2ºC at night. Then, they were moved to an unheated greenhouse for four weeks and to outdoor conditions for one week in order to promote their adjustment to field conditions.

Table 2.1. Average number (± s.e.) of endoparasitic nematodes present in each of the root zone soil samples added in May (n = 10) and September (n = 9) of year 1 to *Ammophila arenaria* in the field experiment.

		N inoculation sample^{-1}	
Nematode species	Life stage	May	September
Heterodera arenaria	Female	6.8 ± 0.93	11.4 ± 1.03
	Male	3.9 ± 0.49	0.3 ± 0.17
	Juvenile	21.2 ± 2.69	24.8 ± 8.64
Meloidogyne maritima	Female	4.5 ± 0.45	3.2 ± 0.71
	Male	8.4 ± 1.28	4.1 ± 0.88
	Juvenile	206.4 ± 39.06	112.6 ± 31.32
Pratylenchus penetrans	All together	22.5 ± 5.91	5.1 ± 1.50

The nematodes originated from the same site as the soil and plant material. Cysts of *H. arenaria* (Cooper) Robinson, Stone, Hooper & Rowe were collected from the field and stored at 4°C in a 0.5% NaCl solution until use. The cysts were then crushed to release the eggs and the juveniles were hatched in *A. arenaria* root extract (20 g l^{-1}). Egg masses of *M. maritima* (Jepson) Karssen, van Aelst & Cook were collected from the field and the juveniles were hatched in tap water. In a greenhouse, *P. penetrans* Cobb was cultured on *A. arenaria* and an extract, containing all life stages, was collected from the cultures. Root zone soil containing a mixture of all soil organisms, including endoparasitic nematodes (Table 2.1), was collected from the two uppermost layers of an *A. arenaria* stand in the field. The roots were separated from the soil by sieving (mesh size 1.0 cm), cut into 1.5 cm pieces and homogenised with the soil.

Design of the field experiment

Field preparation. Spring 1998 (year 0), a field experiment was set up on Brielse Gatdam (51º56' N 04º03' E), a sand dam exposed to the sea wind, providing the natural environment of *A. arenaria*. The soil of an area of 32 m long and 7 m wide was excavated to 0.5 m depth to remove the roots (and root herbivores) of the existing shallowly rooted vegetation. The pit was filled with beach sand that is free from root-feeding nematodes (Van der Putten and Troelstra 1990, De Rooij-van der Goes *et al.* 1995a). The area was divided into ten plots. In each plot, thirty tubes (25 cm diameter, 30 cm high) were placed in a grid of 3 × 10 on top of the beach sand. The tubes, as well as the space in between them, were filled with beach sand.

Planting. June of year 0, one plant was planted in each tube, after which each plant was provided with 0.7 l water (Fig. 2.1). Identical tubes were then placed on top of the initial tubes and the connections were sealed. The upper tube and the surrounding space were filled with beach sand, so that the plants remained vertical with the top of the longest

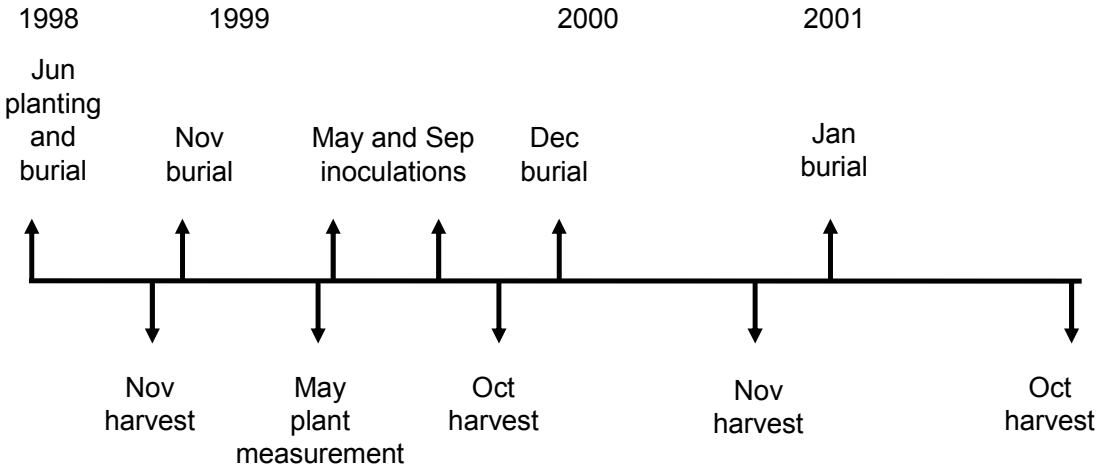

Fig. 2.1. Time scale of the field experiment indicating the events of sand burial, nematode inoculation and harvest of *Ammophila arenaria*.

leaves above the sand surface. This burial procedure mimicked normal sand deposition by wind (Huiskes 1979, Van der Putten *et al.* 1989, Little and Maun 1996). In November, at the end of the growing season, a third layer of tubes was placed on top of the second layer and both tubes and the surrounding space were filled with beach sand. Thus, plants were established that resembled vigorous *A. arenaria* in the field (Van der Putten *et al.* 1989).

Additions. May of year 1, in each plot five plants were inoculated with a mixture of *H. arenaria*, *M. maritima* and *P. penetrans* (200 individuals of each species), five plants with 0.18 l of root zone soil (Table 2.1), and five control plants were inoculated with tap water. The inocula were added at 30 cm depth, between the one-year-old and the newly developing root zone. In September, the inoculations were repeated for the plants that were not harvested in year 1. This time, 274 individuals of each nematode species or 0.35 l root zone soil were added per tube, while the control plants were treated as before. The remaining 15 plants per plot were used for another study.

Burial treatments. In order to mimic the physical growth circumstances of vigorous and degenerating *A. arenaria*, we created series of continued and ceased burial, respectively. Continued burial was achieved by repeating yearly sand burial in half of the plots in December/January of each subsequent winter. To stimulate degeneration of the plants, the other half of the plots was left non-buried after year 1 (Fig. 2.1).

Plant measurements. In May of year 0, before planting, the number of shoots per plant and the length of the longest leaf of each shoot were determined to classify the plants according to size and divide them evenly over the experiment. Immediately after adding the nematodes (May of year 1), the aboveground plant parts were measured again in order to be used as a covariate in further analyses. The number of stems and the length of the longest shoot in each tussock were determined, with tussocks defined as groups of culms at minimally 8 cm distance.

Harvest. In October of year 1, thirty plants from two buried plots were harvested. In November of year 2 and in October of year 3, thirty plants from two buried and thirty plants from two non-buried plots were harvested. The shoots were cut off at ground level and the upper tubes were separated with a sharp blade. The belowground stems and roots were sifted out from the upper compartment, separated, and all plant parts were dried for at least 48 h at 70ºC before weighing.

Nematode sampling and extraction. In year 1, the sieved soil from the tubes was collected, homogenised and a sample of 2-3 kg was taken for nematode isolation, determination and counting. In years 2 and 3, before harvesting the plants, one soil sample of 100-150 g was taken from the upper 0 - 30 cm (at 5 cm from the edge of each replicate tube). Both *Heterodera* cysts and free-living nematodes were extracted from the same sample by decantation (Oostenbrink 1960, Van der Stoel 2001). The water and the suspended nematodes were decanted four times over 1 mm, 180 µm, 75 µm and three 45 µm sieves. Waste material was collected on the 1 mm sieve. The material from the 180 µm sieve was transferred to a coffee filter, air dried at room temperature and stored at 4ºC until counting. Cysts and egg masses were counted using a stereomicroscope (6-50 × magnification). In order to collect free-living nematodes, the material from the 75 µm and 45 µm sieves was transferred to a double cotton milk filter (Hygia rapid, Hartmann AG, Heidenheim, Germany) on a sieve in a dish with a layer of tap water (Oostenbrink 1960, Van der Stoel 2001). The nematodes were allowed to migrate through the filter for 24 h (16-25ºC) and then stored at 4ºC until counting.

The migratory stages of the endoparasites were extracted from the roots by placing them in a mist chamber for 96 ± 2 h (Oostenbrink 1960). In year 1, soil and root extracts were counted separately, while in years 2 and 3, they were combined. The samples were stored at 4ºC until counting, using a reverse light microscope (50-200 × magnification). Sedentary stages of the endoparasitic nematodes (cysts and egg masses) on the roots were counted using a stereomicroscope with 6-50 × magnification.

Bioassays

Each year, a bioassay was performed in order to asses the potential feedback from the soil communities of the experimental field plots in standardised conditions in a growth chamber. At harvest, the soil from the top layer of each of five replicates per treatment was homogenised and samples of 4-5 kg were collected and stored at 4ºC until further processing. The roots were separated from the soil by sieving (0.5 cm mesh size), cut into 1.5 cm pieces and homogenised with the soil. In the second and third year, beach sand was collected to compare plant responses to soil from the experimental units with soil that had been used for the burial treatments. Half of each soil sample was sterilised by autoclaving twice at 120ºC with 48 h time interval. Pots of 1.5 l were filled with either sterilised or non-sterilised soil and the moisture content was set at 10% (w w^{-1}), so that total soil weight was 1400 g in year 1 and 1200 g in years 2 and 3. The soil was covered with aluminium foil to prevent desiccation. Three *A. arenaria* seedlings of three weeks old

were planted in each pot. Three times per week the soil moisture content was reset with demineralised water. Once a week, nutrients were added to the plants as Hoagland solution (Hewitt 1966). Half strength Hoagland solution was added in weeks one and two (12.5 ml pot^{-1}), while full strength Hoagland was added in weeks three and four (12.5 ml pot^{-1}) and in weeks five and six (25 ml pot^{-1}). Double strength Hoagland was added in weeks seven and eight (25 ml pot^{-1}) in order to keep up with the increasing nutrient demand of the developing plants (Van der Putten *et al.* 1988). The plants were grown in a climate cabinet with a photosynthetic photon fluence rate of 260 µmol m^{-2} s^{-1} over the waveband 400-700 nm during 16 h day^{-1}. Temperature was 21ºC in the daytime and 16ºC at night and relative humidity was 70%. Eight weeks after the start of the experiment the plants were harvested. The soil was washed from the roots. Shoot and roots were separated and dried at 70ºC for 48 h before weighing.

Soil analysis

In the third year, soil characteristics were determined of beach sand, non-sterilised soil from non-buried plants (control, addition of nematodes and addition of root zone soil) and sterilised soil from the field experiment. Fifteen replicate soil samples of each treatment were amended with 3.18 ml full strength Hoagland nutrient solution and soil moisture was set to 8% (w w^{-1}), so that total soil weight was 100 g. The amount of nutrients supplied was 20% of the total nutrient supply of the plants in the bioassay, but it was related to the amount of soil used. The samples were incubated in a climate cabinet together with the plants in the bioassay. After one day, four weeks and eight weeks, soil pH and concentration of K^+, NO_3-N and NH_4-N were determined in 0.01 M $CaCl_2$ extracts (w/v 1:10) and Olsen-P (Olsen and Sommers 1982) was measured in $NaHCO_3$ extracts.

Data analysis

Field experiment. Plant biomass was natural logarithm (ln)-transformed to obtain a normal distribution and homogeneity of variances. Two-way analyses of covariance (ANCOVA) of plant biomass (shoot, belowground stem and root) were performed in buried and non-buried series, with addition of soil organisms and year of harvest as main factors. The effect of burial on plant biomass was tested in years 2 and 3 only, using three-way ANCOVA with addition of soil organisms, burial and year of harvest as main factors. Plot was treated as a random factor and it was used as the error term for the effect of burial. Total shoot length in year 1 was used as a covariate to account for differences in size before application of the treatments. Tukey HSD-tests ($P < 0.05$) were performed to compare treatment means within each significant main factor. Nematode numbers were ln(x+1)-transformed, analysed by two- and three-way ANOVA and treatment means were compared by Tukey HSD-tests ($P < 0.05$).

Bioassays. We calculated the ratios between dry biomass of plants grown in non-sterilised soil and in sterilised soil (NS:S ratio) (see Van der Putten *et al.* 1993, Troelstra *et al.* 2001). The NS:S ratios were arcsine square root transformed to obtain a normal

distribution and homogeneity of variances. NS:S ratios of buried and non-buried series were analysed using two-way ANOVAs with addition of soil organisms and harvest year as main factors. Within the main factor addition of soil organisms, the contrasts between control and addition of three endoparasites or a whole soil community, as well as between addition of three endoparasites and addition of the whole soil community were analysed. The effect of burial on NS:S ratios was tested in years 2 and 3, using a three-way ANOVA with addition, burial and harvest year as main factors. Tukey HSD-tests ($P < 0.05$) were performed to compare treatment means within each significant main factor. The correlation of shoot biomass of plants in the field and the corresponding NS:S values in the bioassay was tested with a t-test.

The influence of time, sand burial and addition of soil organisms on the occurrence of individual nematode species was evaluated with ordination techniques (Jongman *et al.* 1995). Redundancy analysis was performed with year, sand burial and addition of soil organisms as independent factors. The burial and addition treatments were digitally coded as dummy variables (0/1). Nematode numbers were log(x+1)-transformed. The effects of the total numbers of *H. arenaria*, *M. maritima*, *P. penetrans* and *Tylenchorhynchus* spp. on the NS:S ratio were determined with multiple regression analysis. As the other ectoparasitic nematode species that were present in the soil correlated with *Tylenchorhynchus*, they were not included in the analysis.

Soil analysis. Soil characteristics were analysed with repeated measures ANOVA with incubation time as dependent factor and soil origin as independent factor. At the end of the bioassay, the effect of soil pH, Olsen-P and NO_3^- on plant biomass was determined with multiple regression analysis. The effects of K^+ and NH_4^+ were excluded from the analysis, as they correlated with the other soil characteristics.

Table 2.2. Ceased sand burial: Dry weight (g) of above- and belowground shoots and roots of *Ammophila arenaria* (back-transformed values). Belowground plant parts were determined at 0 – 30 cm depth. The data of the addition treatments were averaged, because the ANOVA only gave significant year effects. Different letters designate significant differences ($P < 0.05$) within a column (n = 30). Note that the data of year 1 are the same as in Table 2.3.

	Dry weight (g)		
	Aboveground	Belowground	
Year	Shoot	Shoot	Root
1	33 [a]	20 [a]	4.8 [a]
2	71 [b]	41 [b]	7.4 [b]
3	83 [b]	40 [b]	7.8 [b]

RESULTS

Field data

Variation in shoot length at the beginning of the experiment accounted for most of the variation in plant biomass during the course of the experiment ($P < 0.001$) and was, therefore, used as a covariate in further analyses. The addition of soil organisms, sand burial and the year of harvest explained a smaller part of the variation in plant biomass than initial shoot length.

When sand burial was ceased, the biomass of all plant parts significantly increased from the first year to the second, but there was no further increase in the third year, irrespective of the addition treatment (Table 2.2; $P < 0.001$ for all plant parts). There was no significant main effect of addition of nematodes or root zone soil on the biomass of any of the different plant parts. When plants were buried every year, in the second year the addition of root zone soil had a significant negative effect on aboveground shoot biomass (Table 2.3). Belowground shoot and root biomass were not significantly affected by the addition of nematodes or root zone soil, although the effect of addition on belowground shoot biomass came close to significance (Table 2.3; $P = 0.06$). Year of harvest had a significant effect on above- and belowground shoot and on root biomass (Table 2.3), but the direction of the effect differed between the plant parts. Aboveground shoot biomass had increased significantly the third year, belowground shoot biomass had increased significantly in the second and the third year, whereas root biomass was lower in the second and third than in the first year.

The difference between ceased and continued burial could only be determined after year one, when the burial treatments started. Contrary to our hypothesis, cessation of burial did not lead to an immediate decrease in plant growth, as it had a positive effect on above- and belowground shoot biomass in the second year, and no effect in year three (interaction year × burial $F_{1,\ 105} = 5.215$, $P = 0.02$ and $F_{1,\ 105} = 6.143$, $P = 0.01$, respectively). It thus took more than two years after cessation of sand burial before *A. arenaria* produced less shoot biomass. The effect of cessation of burial on root biomass was positive in both years ($F_{1,\ 2} = 1820$, $P = 0.001$), so that a longer period after cessation of sand burial was needed to reduce root biomass than shoot biomass.

The redundancy analysis linked the nematode data from the field experiment to the treatments that had been applied (Fig. 2.2). In the redundancy analysis, the first axis was explained by sand burial and the second axis by the addition of root zone soil. The first axis significantly explained a relatively small percentage (9.7%) of the total variability and the second axis explained an additional 6.4%. The occurrence of *M. maritima* correlated with the addition of root zone soil (Fig. 2.2). Further, *M. maritima* was only detected in the treatments where it had been added, so that this species did not establish spontaneously (Table 2.4). The occurrence of *H. arenaria* and *P. penetrans* was not associated with any of the addition treatments (Fig. 2.2). The reason for this was, that *H. arenaria* established in the treatments where it had been added, but also colonised the control treatment to

which it had not been added, while *P. penetrans* established poorly in all the treatments (Table 2.4). There was no correlation between the occurrence of endoparasites and sand burial (Fig. 2.2). All the treatments had spontaneously been colonised by the ectoparasitic nematodes *Tylenchorhynchus* spp., *Paratylenchus* spp., *Hemicycliophora* spp. and Criconematidae (data not shown). *Tylenchorhynchus* spp., *Paratylenchus* spp. and Criconematidae were correlated with non-buried plants, but not with any addition treatment (Fig. 2.2).

Table 2.3. Continued sand burial: Dry weight (g) of above- and belowground shoots and roots of *Ammophila arenaria* (back-transformed values). Belowground plant parts were determined at 0 – 30 cm depth. Addition treatments were tap water as a control (C), three endoparasitic nematodes *Heterodera arenaria*, *Meloidogyne maritima* and *Pratylenchus penetrans* (HMP) and root zone soil originating from an existing *A. arenaria* stand (R). Different letters designate significant differences ($P < 0.05$) within a column ($n = 10$). The lower part of the table shows the results of two-way ANOVA testing the effects of year and addition on the different plant parts, using shoot length at the start of the experiment as covariable. Note that the data of year 1 are the same as in Table 2.2.

		Dry weight (g)		
		Aboveground	Belowground	
Year	Addition	Shoot	Shoot	Root
1	C	38 ^{ab}	23 ^{ab}	5.4 ^{c}
	HMP	27 ^{ab}	18 ^{a}	4.3 ^{bc}
	R	36 ^{ab}	21 ^{a}	4.9 ^{bc}
2	C	40 ^{bc}	35 ^{bcd}	2.4 ^{ab}
	HMP	42 ^{bc}	36 ^{cd}	2.9 ^{abc}
	R	24 ^{a}	24 ^{abc}	1.5 ^{a}
3	C	74 ^{d}	54 ^{d}	3.1 ^{abc}
	HMP	75 ^{d}	53 ^{d}	3.2 ^{abc}
	R	66 ^{cd}	40 ^{d}	2.8 ^{abc}

	Factor	df	F	P	MSE
Aboveground shoot	Year	2	16.769	***	0.133
	Addition	2	1.024	ns	
	Year × addition	4	2.862	*	
Belowground shoot	Year	2	32.140	***	0.109
	Addition	2	2.846	ns	
	Year × addition	4	0.780	ns	
Root	Year	2	15.340	***	0.322
	Addition	2	0.998	ns	
	Year × addition	4	1.027	ns	

* $P < 0.05$, *** $P < 0.001$, ns = not significant

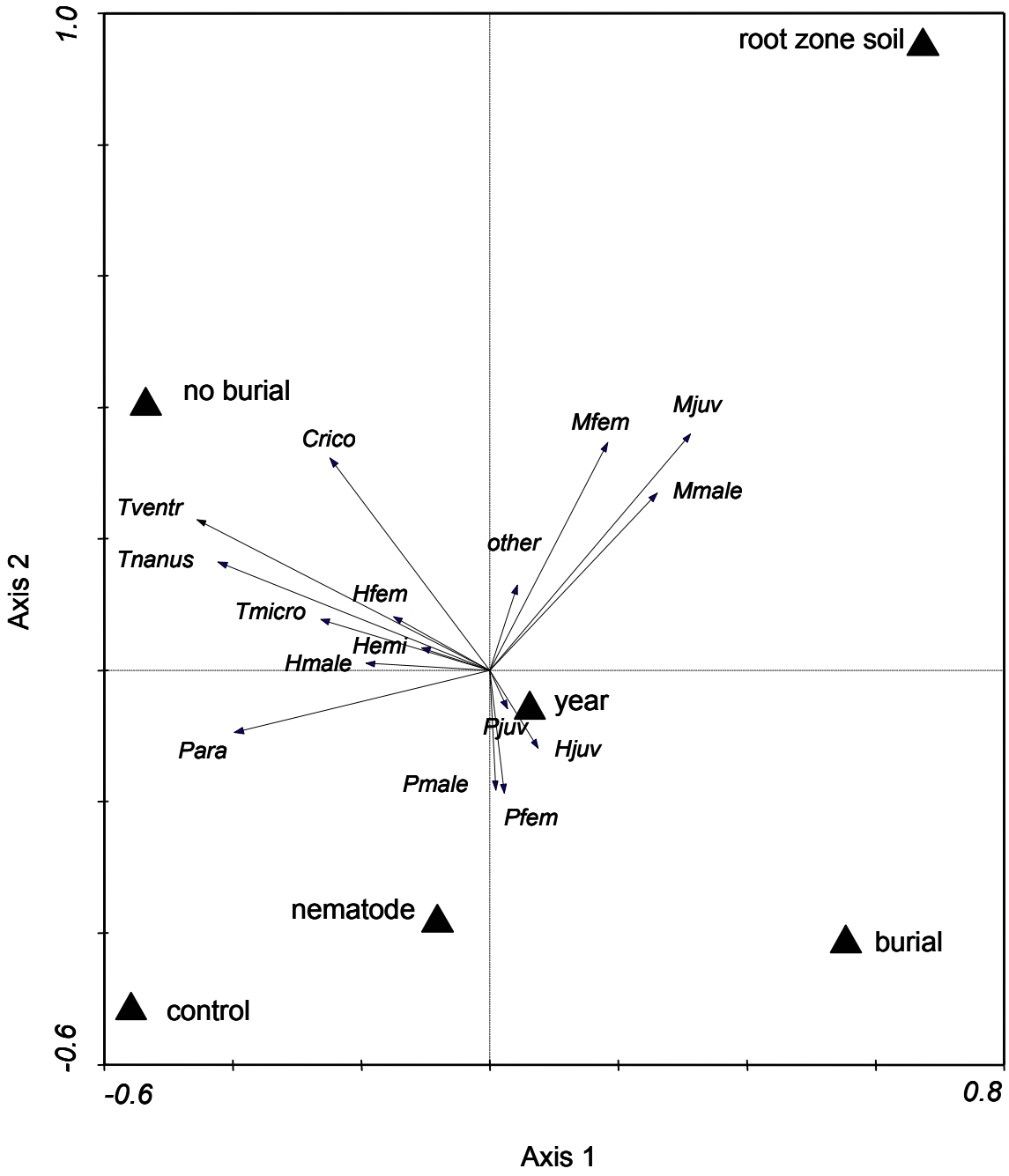

Fig. 2.2. Ordination diagram: Correlation biplot (RDA) of nematode species and environmental variables (axis 1: burial and axis 2: root zone soil) (n = 5). Treatments were year (2, 3), (no) burial with sand and addition (control, nematode = *Heterodera*, *Meloidogyne* and *Pratylenchus*, and root zone soil from an existing *Ammophila arenaria* stand). Endoparasite life stages are abbreviated with first letter of genus name (H = *Heterodera*, M = *Meloidogyne* and P = *Pratylenchus*) and life stage (male, fem = female and juv = juvenile). Tventr = *Tylenchorhynchus ventralis*, Tnanus = *Tylenchorhynchus nanus*, Tmicro = *Tylenchorhynchus microphasmis*, Crico = Criconematidae, Para = *Paratylenchus* spp., Hemi = *Hemicycliophora* spp., other = bacterivorous, fungivorous, predatory and omnivorous nematode species.

Table 2.4. Average number of *Heterodera arenaria*, *Meloidogyne maritima* and *Pratylenchus penetrans* per 100 g dry soil associated with *Ammophila arenaria* in the top 30 cm at the time of harvest (October/November) during three subsequent years. The plants were buried with a layer of beach sand every year (+) or they were left unburied (–). Different letters designate significant differences ($P < 0.05$) in nematode numbers within a column (n = 10). ND = not determined. For further explanation see Table 2.3.

			Average nematode density (N 100 g dry soil^{-1})						
			Heterodera			*Meloidogyne*			*Pratylenchus*
Year	Burial	Addition	Female	Male	Juvenile	Female	Male	Juvenile	All together
1	+	C	ND	0.12^{ab}	0.77^{ab}	ND	0^{a}	0^{a}	0^{a}
		HMP	ND	0.04^{ab}	0.39^{ab}	ND	0.04^{a}	0^{a}	0.04^{a}
		R	ND	0.07^{ab}	0.31^{ab}	ND	0.11^{a}	0.11^{abc}	0^{a}
2	–	C	0.96^{ab}	0.05^{ab}	0.92^{ab}	0.05^{a}	0^{a}	0.14^{a}	0^{a}
		HMP	0.68^{ab}	0.31^{ab}	2.40^{ab}	0.11^{a}	0.21^{a}	0.53^{ab}	0.36^{a}
		R	0.81^{ab}	0.09^{ab}	0.62^{ab}	2.58^{b}	1.09^{a}	14.63^{d}	0.08^{a}
	+	C	0.49^{ab}	1.14^{b}	0.72^{ab}	0^{a}	0^{a}	0^{a}	0.11^{a}
		HMP	1.88^{b}	0.43^{ab}	0.87^{ab}	1.09^{ab}	1.92^{a}	9.09^{abcd}	0.58^{a}
		R	0.27^{ab}	0^{a}	0.90^{ab}	0.24^{a}	0.92^{a}	8.23^{cd}	0.20^{a}
3	–	C	0^{a}	0^{a}	0.11^{a}	0^{a}	0.16^{a}	0.21^{a}	0.10^{a}
		HMP	0.44^{ab}	0^{a}	1.61^{ab}	0.61^{ab}	0.35^{a}	2.25^{abc}	0.05^{a}
		R	0.50^{ab}	0^{a}	0.69^{ab}	1.43^{ab}	0.64^{a}	7.74^{bcd}	0.06^{a}
	+	C	0.17^{ab}	0^{a}	0.77^{ab}	0^{a}	0^{a}	0^{a}	0.31^{a}
		HMP	0.66^{ab}	0^{a}	2.53^{b}	0.49^{a}	0.10^{a}	4.62^{abcd}	0.19^{a}
		R	0.77^{ab}	0^{a}	2.18^{ab}	0.63^{ab}	0.56^{a}	4.56^{abcd}	0^{a}

Bioassays

The NS:S ratio (the biomass of plants grown in non-sterilised soil compared to sterilised soil) in beach sand was 0.65 ± 0.102 (s.e.) in year two and 0.75 ± 0.071 in year three, while the NS:S ratios in the soils from the field experiment did not exceed 0.35 ± 0.032 (Fig. 2.3). Within one year, therefore, a growth inhibiting factor had developed in all the soils from the field experiment. With ceased sand burial, growth inhibition of bioassay plants increased with time (Fig. 2.3a; Table 2.5). In the ANOVA, there was a significant difference between root zone soil and nematode addition (Table 2.5). The addition of root zone soil inhibited the growth of bioassay plants more than the addition of the three endoparasitic nematodes alone, but this effect did not appear in the Tukey's test, which is more conservative than ANOVA (Fig. 2.3a). The difference between adding root zone soil and nematodes was independent of year (Table 2.5). With continued sand burial, the inhibition of plant growth also increased with time, but there was no difference between the

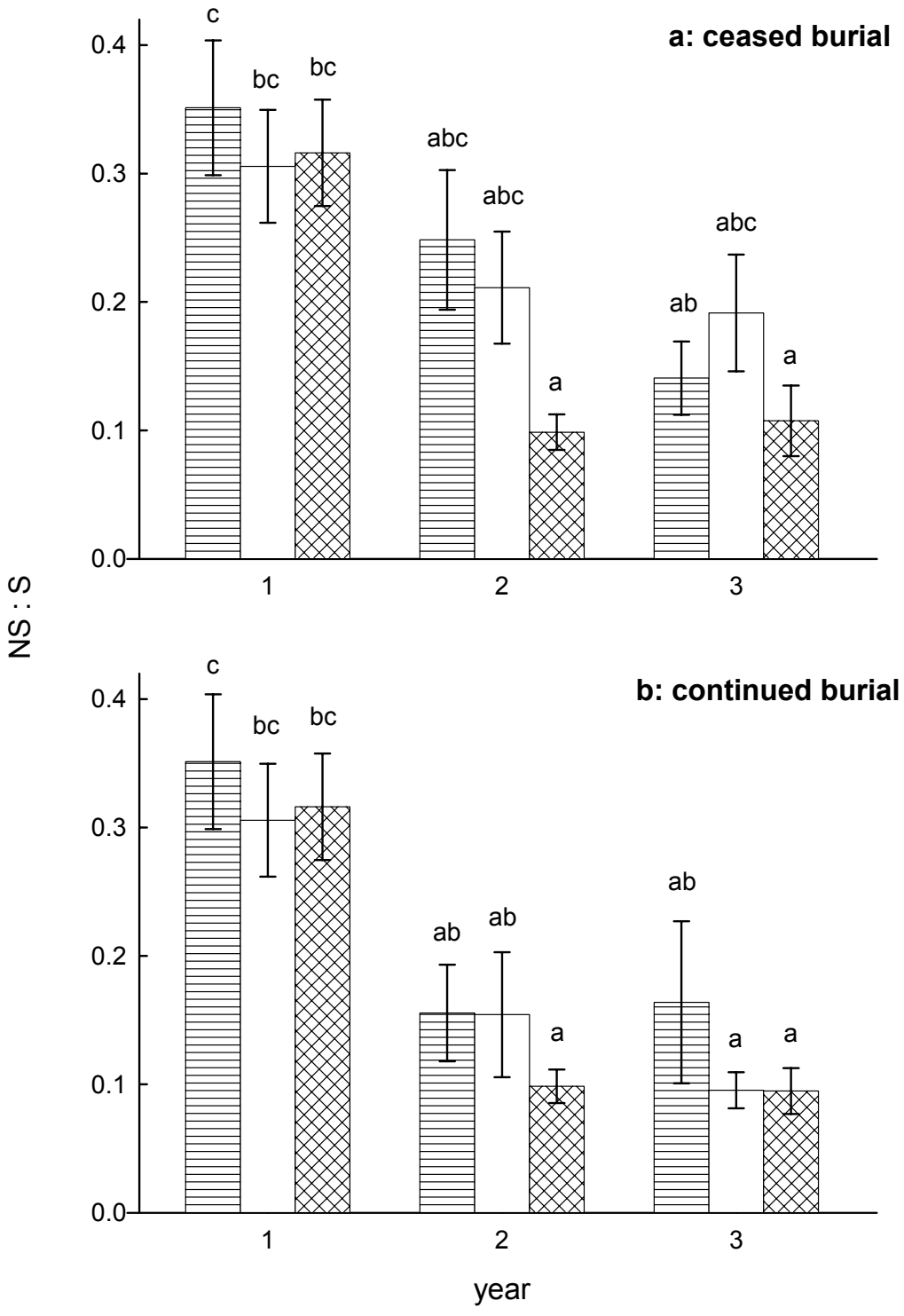

Fig. 2.3. Ratio of biomass of *Ammophila arenaria* (± s.e.; n = 5) grown for eight weeks in non-sterilised soil compared to sterilised soil (NS : S). The soil originated from *A. arenaria* plants in a field experiment that were a) buried with a layer of beach sand in the first year, then left unburied in the second and third year and b) buried with a layer of beach sand in three subsequent years. For explanation of addition treatments (C: hatched, HMP: white and R: cross-hatched) see Table 2.2. Note that in year 1 all plants were buried (same data for non-buried and buried plants).

Table 2.5. Results of a two-way ANOVA testing the effect of year (1-3) and addition on the biomass NS:S ratio of *Ammophila arenaria* plants grown in non-sterilised soil versus sterilised soil with additions C, HMP and R (for explanation see Table 2.3). Within the main factor addition, the contrasts C versus HMP and R, and HMP versus R were analysed (n = 5).

	Ceased sand burial				Continued sand burial			
Factor	df	F	P	MSE	df	F	P	MSE
Year	2	15.887	***	0.0119	2	24.117	***	0.0121
Addition	2	3.256	0.050		2	1.456	ns	
C vs. HMP - R	1	2.282	ns		1	2.732	ns	
HMP vs. R	1	4.375	*		1	0.249	ns	
Year × addition	4	1.298	ns		4	0.310	ns	

P < 0.05, *** P < 0.001, ns = not significant

addition of nematodes or root zone soil (Fig. 2.3b; Table 2.5). In the second and third year, growth inhibition of bioassay plants did not differ between continued and ceased burial treatments, although the effect was close to significance (comparison of data of Fig. 2.3a, b; $F_{1, 47} = 3.593$, P = 0.06).

The established soil communities had different effects on plants in the field and in the bioassay, as we did not find a significant correlation between the shoot or root biomass of *A. arenaria* in the field experiment and the growth inhibition of plants in the bioassay (results of multiple regression not shown). There was an effect of addition of root zone soil on growth inhibition in the bioassay (Fig. 2.3a, Table 2.5) and a significant correlation between addition of root zone soil and nematode numbers in the soil (Fig. 2.2). However, there was no correlation between the abundance of *H. arenaria*, *M. maritima*, *P. penetrans* or *Tylenchorhynchus* spp. and plant growth inhibition (results of multiple regression not shown).

Soil analysis

At the start of the final bioassay, the soils did not differ significantly in the concentration of NO_3^- (Table 2.6). During the course of the experiment, the concentration of NO_3^- became significantly greater in sterilised soil and in non-sterilised beach sand than in non-sterilised soil from the field experiment. The soils did not differ significantly in concentration of NH_4^+ or Olsen-P, but in sterilised soil, Olsen-P decreased with time. The concentration of K^+ was significantly greater in beach sand than in the other soils. Soil pH increased during the course of the experiment and was significantly higher in beach sand than in non-sterilised control soil and soil inoculated with root zone soil. Multiple regression analysis showed a positive correlation between soil pH, Olsen-P and NO_3^- and plant biomass in the bioassay (data of analysis not shown). The concentration of NO_3^- did not differ between non-sterilised soils from the field experiment, while it was greater in sterilised soil and beach sand. Therefore, the concentration of NO_3^- may only explain the greater plant biomass in

Table 2.6. Soil characteristics of beach sand, sterilised soil from the field experiment, and of soil with different addition treatments C, HMP and R (for explanation see Table 2.3) after one day, four and eight weeks of incubation. Soil samples were collected in year 3 at final harvest and were amended with full strength Hoagland solution. Different letters designate significant differences ($P < 0.05$) within a column (n = 5). The lower part of the table shows the results of a repeated measures ANOVA testing the effects of incubation time and soil origin on the different soil characteristics.

Time	Soil	pH (CaCl2)	K (µg g^{-1})	NH_4-N (µg g^{-1})	NO_3-N (µg g^{-1})	Olsen P (µg g^{-1})
t=0	Beach	8.0 abcdef	38 bc	0.2 a	7.0 g	0.8 ab
	Sterilised soil	8.0 abcde	16 a	0.6 a	6.6 g	0.9 b
	C	7.9 abd	17 a	0.3 a	6.8 g	0.7 ab
	HMP	7.9 abc	14 a	0.1 a	6.5 fg	0.7 ab
	R	7.8 a	17 a	0.1 a	6.9 g	0.7 ab
t=4	Beach	8.1 h	35 b	0.3 a	6.1 efg	0.7 a
	Sterilised soil	8.0 defgh	15 a	0.2 a	6.2 efg	0.7 a
	C	8.0 cefgh	16 a	0.2 a	4.1 cd	0.6 a
	HMP	8.1 efgh	14 a	0.4 a	3.5 bc	0.7 a
	R	8.0 bcdefgh	16 a	0.1 a	4.3 cde	0.7 a
t=8	Beach	8.1 gh	45 c	0.5 a	5.9 efg	0.7 a
	Sterilised soil	8.1 fgh	14 a	0.2 a	4.9 cdef	0.7 a
	C	8.0 bcdefgh	15 a	0.2 a	2.0 ab	0.7 a
	HMP	8.0 defgh	15 a	0.0 a	1.7 a	0.7 a
	R	8.0 bcdefg	15 a	0.1 a	2.1 ab	0.7 a

	Factor	F		P	MSE
pH	Soil	$F_{4,20}$=	6.900	**	0.008
	Time	$F_{2,40}$=	68.900	***	0.002
	Time × soil	$F_{8,40}$=	1.900	ns	
K	Soil	$F_{4,20}$=	63.762	***	27.61
	Time	$F_{2,40}$=	1.013	ns	13.72
	Time × soil	$F_{8,40}$=	2.084	ns	
NH_4-N	Soil	$F_{4,20}$=	2.107	ns	0.095
	Time	$F_{2,40}$=	0.720	ns	0.051
	Time × soil	$F_{8,40}$=	2.675	*	
NO_3-N	Soil	$F_{4,20}$=	25.038	***	0.686
	Time	$F_{2,40}$=	146.228	***	0.505
	Time × soil	$F_{8,40}$=	9.190	***	
Olsen-P	Soil	$F_{4,20}$=	1.638	ns	0.015
	Time	$F_{2,40}$=	14.529	***	0.005
	Time × soil	$F_{8,40}$=	2.880	*	

$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = not significant

the bioassay in sterilised soil and beach sand, but not the differences in plant biomass between the non-sterilised soils.

DISCUSSION

The addition of root zone soil to beach sand in some cases had a negative effect on the growth of *A. arenaria*, both in the field and in the bioassays, whereas the combination of the three endoparasitic nematodes did not differ from the control. As we added only a small amount of root zone soil, some biological rather than a chemical or physical factor in the soil must have been responsible for the growth reduction. Besides endoparasitic root-feeding nematode species, root zone soil contains other (ectoparasitic) root-feeding nematodes, plant-pathogenic micro-organisms (De Rooij-van der Goes *et al.* 1995b, Van der Stoel *et al.* 2002) and arbuscular mycorrhizal (AM) fungi (Kowalchuk *et al.* 2002). Consequently, the addition of root zone soil with a whole soil community introduces more potential growth reducing biota than the combined addition of three endoparasitic nematodes. The spontaneous colonisation of nematodes in the controls might have reduced the difference between nematode addition and control soils.

Our study supports the conclusion by Bever (2003) that there is increasing evidence for net negative feedback effects from soil communities to their host plants. The novelty of our experiment is the introduction of different combinations of soil organisms to plants under field conditions and subsequently testing the feedback of the established soil communities in bioassays. Klironomos (2002) found that rare plants showed a stronger negative feedback when grown in soils with a history of the same plant species than dominant or invasive plants, but the culturing of the soil communities took place in the greenhouse. Studies that added biocides to field soil to suppress the influence of fungi (Van der Putten *et al.* 1990, Frank *et al.* 2003) or nematodes (Van der Putten *et al.* 1990, Van der Stoel *et al.* 2002) only indicate, but do not prove, the role of the suppressed organisms. In some cases, specific organisms have been shown to be the cause of the negative feedback (Holah and Alexander 1999, Packer and Clay 2000). Our results show that a combination of three endoparasitic root-feeding nematodes, although parasitizing the plant, not necessarily cause a negative feedback.

Addition of root zone soil to *A. arenaria* in the field only had a negative effect on shoot biomass in the second year. In the first year, the time between soil addition and harvest may have been too short for the soil organisms to become established and affect plant growth. In the third year, the effects of our experimental treatments may have become masked by spontaneous colonisation of all treatments (including the controls) by soil organisms from the surrounding area, most likely by wind (De Rooij-van der Goes *et al.* 1997).

Cessation of burial did not immediately lead to decreased plant growth. On the contrary, it led to an increase in plant growth in the second year, but not in the third year. Burial did not lead to an increase in shoot biomass in the second year, but it did so in the

third year. Other authors showed that *Ammophila* spp. responded positively to burial with sand, both in field experiments with degenerated plants and in greenhouse experiments with young plants (Yuan *et al.* 1993, De Rooij-van der Goes *et al.* 1995a, Little and Maun 1996), and described a long-term decrease in vigour when burial ceased (Huiskes 1979, Wallén 1980, Eldred and Maun 1982, Disraeli 1984). Our study showed that in the field, plants respond with a time lag of two or more years to burial or cessation of burial treatments. The plants in our experiment initially showed a negative response to burial, perhaps because we used an instantaneous and relatively thick layer (30 cm) of sand to bury young plants. Other field studies did not, as we did, start with plant material with a standardised growth history. We show that the initial plant size explains a considerable amount of the treatment effects observed.

Besides root-feeding nematodes and pathogens, the addition of root zone soil also will have introduced organisms with a positive effect on plant growth. AM fungi have been shown to have a positive effect on growth of the similar North-American dune grass *A. breviligulata* and may protect the plant from infection by root-feeding nematodes (Little and Maun 1996). Both the diversity and the incidence of infection with AM fungi is lower in stable dunes than in mobile dunes (Kowalchuk *et al.* 2002), so that reduced diversity and abundance of AM fungi may contribute to degeneration of plants in stable dunes. Although the homogenisation of the root zone soil used in our field experiment and bioassays may have disrupted the mycorrhizal hyphal network, infection with AM fungi may have had a positive effect on the growth of the plants that had been inoculated with root zone soil in the longer-term field experiment. In addition, dune soils contain microbial antagonists that may suppress growth of and infection with plant pathogenic fungi in the root zone of *A. arenaria* (De Boer *et al.* 1998a,b).

The NS:S ratio of *A. arenaria* in the bioassay with soil from the field experiment decreased when the field experiment proceeded, but in all cases it was lower than the NS:S ratio of plants grown in beach sand. Apparently, already in the first year after planting a plant growth inhibiting factor had established in the soil, even in the non-inoculated control. This growth inhibiting factor may be of biological origin (Van der Putten 1993), but Troelstra *et al.* (2001) pointed at the importance of changes in nutrient availability when comparing plants grown in sterilised and in non-sterilised dune soil, even at apparently adequate nutrient supply. Indeed, in our experiment the concentration of inorganic N was higher in beach sand and in sterilised soil than in non-sterilised soil, which may have contributed to a higher plant biomass in these soils in the bioassay. However, the concentration of the nutrients that we measured did not differ significantly among the non-sterilised soils in the bioassay, so that it is likely that some biotic factor caused the observed negative effect of addition of soil organisms.

We found differences in composition of the community of plant parasitic nematodes between the addition of root zone soil on the one hand and the addition of endoparasitic nematodes or the control on the other hand, but endoparasitic nematode abundance did not correlate with plant inhibition in the bioassay. Thus, neither changes in nutrient

concentration nor endoparasitic nematodes are likely to be the main cause of the difference in growth inhibition between the addition of root zone soil and the addition of nematodes or the control. Probably, pathogenic fungi that may reduce biomass of *A. arenaria* (De Rooij-van der Goes 1995) have contributed to this extra growth reduction.

The negative effect of addition of root zone soil in the field was observed when plants had been buried, but in the bioassay it was observed in the field soil from the non-buried plants. This may explain the lack of correlation between shoot or root biomass in the field and biomass production in the bioassay, as also has been observed when correlating field data with results from bioassays using soil from natural stands of *A. arenaria* (Van der Stoel *et al.* 2002). Apparently, the effect of the soil organisms on the growth of plants in the field was not the same as the effect on seedlings in the bioassay, which emphasises the need for combined field and bioassay studies as we have presented.

Van der Stoel *et al.* (2002) showed that during the growing season, the growth inhibiting factor migrates to the newly formed upper layer. This may explain why in our bioassays, which were performed at the end of the growing season, also soil from buried plants caused considerable growth inhibition. Van der Stoel *et al.* (2002) also found that, during the course of the growing season, soil from vigorous (buried) *A. arenaria* inhibited plant growth more than soil from degenerated (non-buried) plants. In our experiment, however, the time after cessation of burial may have been too short to develop a difference in inhibition response between bioassay plants in soil from buried and non-buried plants.

We conclude that the whole soil community of *A. arenaria* in some cases caused a negative feedback to the plant, both in the field and under controlled conditions. Addition of a combination of the endoparasitic nematodes *H. arenaria*, *M. maritima* and *P. penetrans*, however, did not have a negative effect on plant growth, so that the combined effect of these endoparasites cannot substitute that of the whole soil community. Plant growth in the bioassays differed among the control and the addition of endoparasitic nematodes or root zone soil, while the concentration in their available form of three major nutrients in the soil did not differ among treatments. This indicates that a biotic factor other than endoparasitic nematodes caused the increased growth reduction of *A. arenaria* when the whole soil community was added.

Acknowledgements

We gratefully thank the Water and Civil Board De Brielse Dijkring, which provided the field locality and assistance of R. Dekker, A. Voskamp and J. van Eersel. We thank W. Smant for analysing soil characteristics and assistance with processing the plant material, H. Duyts for nematode identification and A. Kamp for performing the bioassay in year 1. This work involved a great effort in the field and many colleagues and friends helped us. Herewith we thank A. de Bever, M. Bezemer, G. Doodeman, H. Duyts, N. van der Feest, A. Kamp, M. Keus, G. Kowalchuk, F. Menting, C. van Santen, N. Scheerder, C. Schreck Reis, D. Tap, J. van Veen, J.W. van der Vegte, T. van Veldhuisen, R. Wagenaar and L. Witjes. We also thank T.M. Bezemer for performing the redundancy analysis and J.A. van Veen for discussion and comments on earlier versions of the manuscript. This project was supported by the Research Council for Earth and Life Sciences

(ALW) of the Netherlands Organisation for Scientific Research (NWO) contract 805-35.352 and by the European Commission INCO-DC contract IC18CT97014.

Chapter 3

Consequences of species diversity in a community of root-feeding herbivores for nematode dynamics and host plant biomass

E. Pernilla Brinkman, Henk Duyts and Wim H. van der Putten

Abstract

To date, no study has explicitly addressed effects of species diversity of root-feeding herbivores on host plant biomass. Root-feeding nematodes typically occur in multi-species communities. In a three-year field experiment, we investigated effects of species diversity of root-feeding nematodes on nematode dynamics and biomass production of the dune grass *Ammophila arenaria*. The plant needs regular burial by fresh beach sand, suggesting that *A. arenaria* benefits from a temporary escape from root-feeding soil organisms. We created series of ceased and continued sand burial and added the endoparasitic nematodes *Meloidogyne maritima*, *Heterodera arenaria* and *Pratylenchus penetrans* alone or in combination. We included treatments with and without the whole soil community, measured plant biomass and quantified numbers of nematodes.

Addition of *H. arenaria* and *P. penetrans* decreased numbers of *M. maritima* juveniles and delayed the first appearance in time of both juveniles and females. Numbers of males were only affected when plants had been buried. Burial with sand and addition of the other two endoparasites affected numbers of *H. arenaria* juveniles, while numbers of *P. penetrans* were low and not affected. Shoot biomass was lower when *M. maritima* had been added alone than when the three species had been added together. Addition of root zone soil decreased biomass of all plant parts. Burial with sand decreased aboveground shoot biomass, whereas it increased belowground shoot and root biomass.

Our results indicate idiosyncratic effects of nematode diversity on *A. arenaria* biomass. Suppression of *M. maritima* by *H. arenaria* and *P. penetrans* has important consequences, as two root-feeding nematodes seem to protect their host against a third species. We conclude that understanding consequences of root-feeding nematode diversity requires analysing temporal dynamics of the species involved.

INTRODUCTION

Most studies on consequences of diversity in soil communities have focused on organisms belonging to the decomposer subsystem (Wardle 2002). In experimental conditions, effects of species diversity of decomposer organisms on plant biomass level off at relatively low species diversity (Laakso and Setälä 1999, Liiri *et al.* 2002). An increase in the diversity of soil organism body sizes affects several ecosystem processes, although effects on plant communities were quite small (Bradford *et al.* 2002). As far as root-associated soil organisms are concerned, arbuscular mycorrhizal diversity was found to enhance plant productivity and plant community diversity indices (Van der Heijden *et al.* 1998), although these results were criticised for potential sampling effects (Wardle 1999). For ectomycorrhizal fungi, effects of diversity were context-dependent, as soil fertility influenced the outcome of diversity treatments (Jonsson *et al.* 2001). Some previous studies on soil pathogen and root feeder diversity show that different subsets of the soil community may have similar plant growth reducing effects (De Rooij-van der Goes 1995).

We studied consequences of species diversity of root-feeding nematodes, as root-feeders, alone or together with plant pathogens, have substantial effects on plant community processes (Brown and Gange 1990, Van der Putten 2003). Root herbivores and pathogens contribute to the structuring of natural vegetation through their influence on individual plants. They affect the place of seedling establishment (Augspurger and Kelly 1984, Packer and Clay 2003) and plant vigour (Van der Putten *et al.* 1988, Bever 1994) and alter the outcome of competition between plants (Van der Putten and Peters 1997). This results in species coexistence (Holah and Alexander 1999), maintenance of plant species diversity (De Deyn *et al.* 2003) and enhancement of vegetation succession (Van der Putten *et al.* 1993, De Deyn *et al.* 2003).

Root herbivores (mainly root-feeding nematodes and soil-inhabiting insect larvae) are able to consume an important part of belowground primary production, although estimates of their effect are inconsistent (Stanton 1988, Brown and Gange 1990). While some studies report a large effect on plant biomass (Stanton *et al.* 1981, Ingham and Detling 1990), others only find minor effects (Seastedt *et al.* 1987, Verschoor 2002, Verschoor *et al.* 2002b). Generally, plants are infected with several species of root-feeding nematodes (Oostenbrink 1966 in Eisenback 1993). However, an increase in the diversity of root herbivores does not necessarily lead to a decrease of plant biomass. When pathogenicity is inversely related to competitiveness, then multi-species infection by root-feeding nematodes may even be beneficial to the plant (Duncan and Ferris 1983, Umesh *et al.* 1994).

Multi-species communities of root-feeding nematodes often show temporal variation in abundance of the individual species (Yeates *et al.* 1985) and spatial variation in colonisation patterns of the root system (Van der Stoel *et al.* 2002). The partitioning in time and space may be optimal for each of the species or caused by interspecific competition for space or feeding sites (Hutchinson 1957, Eisenback 1993). An increase in the species

diversity of root-feeding nematodes may therefore force (some of) the nematodes involved to inhabit suboptimal temporal and spatial conditions, which in turn may affect growth of the host plant.

To study the effect of species diversity of root-feeding nematodes on nematode and plant performance, we used the dune grass *Ammophila arenaria* (L.) Link and its concomitant endoparasitic nematodes as a model. For vigorous growth, in autumn and winter *A. arenaria* requires to be covered by a layer of sand that is wind-blown from the beach. In response to burial, the stem internodes elongate and the plant forms new roots in this upper layer (Huiskes 1979). Soon after root formation, the new sand layer is colonised by nematodes and micro-organisms (De Rooij-van der Goes *et al.* 1995b, Van der Stoel *et al.* 2002). This time lag between root formation and colonisation with soil organisms is thought to be one of the positive effects of sand burial that contributes to vigorous plant growth (Van der Putten *et al.* 1989).

Three endoparasitic root-feeding nematodes that are frequently found with *A. arenaria* are *Meloidogyne maritima* (Jepson) Karssen, van Aelst & Cook, *Heterodera arenaria* (Cooper) Robinson, Stone, Hooper & Rowe and *Pratylenchus penetrans* Cobb (De Rooij-van der Goes *et al.* 1995b). These nematodes colonise the new root layer in sequence, each showing a peak in abundance at different times of the year (Van der Stoel *et al.* 2002). Absence of the other endoparasite species may lead to a change in temporal dynamics and a different effect on plant growth. In greenhouse experiments, the three endoparasitic nematodes had very little or no effect on plant growth when added alone or in combination (Van der Stoel 2001, Brinkman *et al.* 2004, Chapters 4 and 5). However, the results of these pot trials needed to be verified in the field in natural environmental conditions.

In the present study, we examined under field conditions how the temporal dynamics of each of the endoparasitic nematodes *M. maritima*, *H. arenaria* and *P. penetrans* is affected by the presence of the other two species. We also investigated the consequences of infection with one or three endoparasitic nematode species as compared to the whole soil community for the growth of their host plant *A. arenaria*. Finally, we assessed whether burial with sand enables the plant to overcome possible negative effects of the endoparasitic nematodes.

MATERIALS AND METHODS

Soil, plant material and inoculum

Soil for preculturing the plants was collected from a sand beach near Haringvlietdam (the Netherlands; 51º52' N 04º04' E). The soil was sieved (mesh size 0.5 cm), homogenised and sterilised by gamma-radiation (≥ 25 kGray). Seeds of *A. arenaria* were collected from plants at the same site, dried and stored at 4ºC until use. The seeds were germinated on glass beads at 25ºC/ 10ºC (16 h light/ 8 h dark) for three weeks. The seedlings were grown in sterilised soil in a greenhouse for about twelve weeks and provided with extra

light to ensure a minimum photosynthetic photon fluence rate of 200 µmol m^{-2} sec^{-1} over the waveband 400-700 nm during 16 h day^{-1}. Temperature was 21 ± 2ºC in the daytime and 18 ± 2ºC at night. Then, the plants were transferred to an unheated greenhouse for four weeks and outdoors for one week to promote their chance of survival when transferred to the field.

The nematodes originated from the same site as the soil and plant material. Cysts of *H. arenaria* were collected from the field and stored at 4°C in a 0.5% NaCl solution until use. The cysts were then crushed and the juveniles were hatched in *A. arenaria* root extract (20 g l^{-1}). Egg masses of *M. maritima* were collected from the field and the juveniles were hatched in tap water. In the greenhouse, *P. penetrans* was cultured on *A. arenaria* and an extract, containing all life stages, was collected from the cultures. Root zone soil, containing all naturally occurring soil organisms, amongst which were endoparasitic nematodes, was collected from the two uppermost layers of an existing *A. arenaria* stand. The soil was sieved (mesh size 1.0 cm) to collect the roots, which were cut into 1.5 cm pieces and homogenised with the soil.

Design of the field experiment

Field preparation and planting. Spring 1998 (year 0), a field experiment was set up on Brielse Gatdam (the Netherlands; 51º56' N 04º03' E), a sand dam exposed to the sea wind providing the natural abiotic environment of *A. arenaria*. The soil of an area of 32 m long and 7 m wide was excavated to 0.5 m depth to remove the roots (and root herbivores) of the existing shallowly rooting vegetation. The pit was filled with beach sand that is normally free from root-feeding nematodes (Van der Putten and Troelstra 1990, De Rooij-van der Goes *et al.* 1995a). The area was divided into ten plots. In each plot, thirty tubes (25 cm diameter, 30 cm high) were placed in a grid of 3 × 10 on top of the beach sand. The tubes, as well as the space in between them, were filled with beach sand.

Planting. June of year 0, one plant was planted in each tube, after which each plant was provided with 0.7 l water. New tubes were then placed on top of the standing tubes and the connections were sealed. The upper tube and the surrounding space were filled with beach sand, so that the plants remained vertical with the top of the longest leaves above the sand surface. This burial was to mimic normal sand deposition by wind (Huiskes 1979, Van der Putten *et al.* 1989, Little and Maun 1996). In November, at the end of the growing season, a third layer of tubes was placed on top of the second and both tubes and the surrounding space were filled with beach sand. Thus, plants were established that were similar to vigorous *A. arenaria* in the field (Van der Putten *et al.* 1989).

Addition treatments. May of year 1, in each plot five plants were inoculated with 200 *H. arenaria*, 200 *M. maritima*, 200 *P. penetrans*, a mixture of the three nematodes (200 individuals of each species), 0.18 l root zone soil (Table 3.1) or tap water (control). The inocula were added at 30 cm depth, between the one-year-old and the newly developing root zone. In September, the inoculations were repeated for the plants that were not

Table 3.1. Number (average ± s.e.) of endoparasitic nematodes present in each of the root zone soil samples added to individual tubes in May (n = 10) and September (n = 9) of year 1 to *Ammophila arenaria* in the field experiment.

		N tube^{-1} (added as root zone soil)			
Nematode species	Life stage	May		September	
Heterodera arenaria	Female	6.8 ±	0.93	11.4 ±	1.03
	Male	3.9 ±	0.49	0.3 ±	0.17
	Juvenile	21.2 ±	2.69	24.8 ±	8.64
Meloidogyne maritima	Female	4.5 ±	0.45	3.2 ±	0.71
	Male	8.4 ±	1.28	4.1 ±	0.88
	Juvenile	206.4 ±	39.06	112.6 ±	31.32
Pratylenchus penetrans	All together	22.5 ±	5.91	5.1 ±	1.50

harvested in year 1. This time, 274 individuals of each nematode species or 0.35 l root zone soil were added per tube, while the control plants were treated as before.

Burial treatments. In order to mimic the physical growth circumstances of vigorous and degenerating *A. arenaria*, we created one series of continued and one of ceased burial. Continued burial was achieved by repeating yearly sand burial in half of the plots in December/January of each subsequent winter. In order to stimulate degeneration of the plants, the other half of the plots were left unburied after year 1.

Plant measurements. In May of year 0, before planting, the number of shoots per plant and the longest leaf of each shoot were determined to classify the plants according to size and divide them evenly over the experiment. Immediately after adding the nematodes (May of year 1), the aboveground plant parts were measured again to be used as a covariate in further analyses. The number of tussocks (tussocks defined as groups of culms at minimally 8 cm distance) and the number of stems in each tussock were counted and the longest shoot in each tussock was measured. These measurements were regarded as the size of the plants before application of the different treatments and they were used as covariate in further analyses.

Harvest. In October of year 1, sixty plants from two buried plots were harvested. In November of year 2 and in October of year 3, sixty plants from two buried and sixty plants from two non-buried plots were harvested. The shoots were cut off at ground level, the tubes were separated with a sharp blade and the belowground stems and roots were sifted out from each compartment (the upper tube for non-buried plants and the two uppermost tubes for buried plants) and separated. All plant parts were dried for at least 48 h at 70ºC before weighing.

Nematode sampling and extraction. In year 1, the sieved soil from the tubes was collected, homogenised and a sample of 2-3 kg was taken for nematode isolation, determination and counting. In each month (May-November) of year 2 and in October of year 3, one soil sample of 100-150 g was taken from the upper 0-30 cm at 5 cm from the

edge of each replicate tube. Both *H. arenaria* cysts, *M. maritima* egg masses and free-living nematodes were extracted from the same sample by decantation (Oostenbrink 1960, Van der Stoel 2001). The water and suspended nematodes were decanted four times over 1 mm, 180 µm, 75 µm and three 45 µm sieves. Waste material was collected on the 1 mm sieve. The material from the 180 µm sieve was transferred to a coffee filter, air dried at room temperature and stored at 4ºC until counting. Cysts and egg masses were isolated and counted using a stereomicroscope (6-50 × magnification). To collect free-living nematodes, the material from the 75 µm and 45 µm sieves was transferred to a double cotton milk filter (Hygia rapid, Hartmann AG, Heidenheim, Germany) on a sieve in a dish with a layer of tap water (Oostenbrink 1960, Van der Stoel 2001). The nematodes were allowed to migrate through the filter for 24 h (16-25ºC) and then stored at 4ºC until counting.

The migratory stages of the endoparasites were extracted from the roots by placing them in a mist chamber for 96 h (Oostenbrink 1960). In year 1, soil and root extracts were counted separately, while in years 2 and 3, they were combined. The samples were stored at 4ºC until counting, using a reverse light microscope (50-200 × magnification). Sedentary stages of the endoparasitic nematodes (cysts and egg masses) on the roots were counted using a stereomicroscope with 6-50 × magnification.

Data analysis

Plant biomass was natural logarithm (ln)-transformed to obtain a normal distribution and homogeneity of variances. Plant biomass (shoot, belowground stem and root) in year 1 was analysed using a two-way analysis of covariance (ANCOVA) with addition of soil organisms as main factor. Total shoot length in year 1 was used as a covariate for this, as well as the subsequent analyses, to account for differences in size before application of the treatments. Plant biomass in years 2 and 3 was analysed using a two-way ANCOVA with sand burial and addition of soil organisms as main factors. Plots were nested within the burial treatment and used as error term for the effect of burial. Tukey HSD-tests ($P < 0.05$) were performed to compare treatment means within each significant main factor.

Nematode numbers were ln(x+1)-transformed to obtain homogeneity of variances. For the monthly sampling in year 2, nematode numbers were analysed using a two-way repeated measures ANOVA with burial and additions as main factors and with months as the different levels of the repeated measures factor. The month when the first representative of a developmental stage of a nematode appeared was chosen as the first month of the repeated measures factor. In the case of *H. arenaria* females, May and November of year 2 were used as repeated measures factors, since the soil and root samples were checked for the occurrence of females in these two months only. In May of year 2, no *M. maritima* females occurred. A t-test was used to test whether the observed numbers of *M. maritima* females in November of year 2 differed from zero. The effect of burial and addition of other endoparasites on *M. maritima* females in November of year 2 was tested with a two-way ANOVA. The effect of the presence of other endoparasites on

the occurrence of each nematode species over the different years (1-3) in buried and non-buried series was tested with a two-way ANOVA with year and addition as main factors. Tukey HSD-tests ($P < 0.05$) were performed to compare treatment means within each significant main factor.

RESULTS

Nematodes

Spontaneous colonisation. All the tubes had been colonised spontaneously by some species of root-feeding nematodes (Table 3.2). In years 0 and 1, the numbers of *Tylenchorhynchus* Cobb spp. were relatively highest, but their numbers declined in the second year. At the same time, the numbers of *Paratylenchus* Micoletzky spp. and Criconematidae (Taylor) Thorne increased. The densities of these three ectoparasitic nematode species were higher when plants remained non-buried than when plants had been buried (data not shown). Of the three endoparasitic nematodes that had been added to the plants, *H. arenaria* also spontaneously colonised experimental units where it had not been added, while *M. maritima* and *P. penetrans* did not, or only in very low numbers, spread to treatments where they had not been added (Table 3.2).

Table 3.2. Average density (N 100 g dry soil^{-1}) of spontaneously established root-feeding nematodes associated with *Ammophila arenaria* plants without nematode addition, in November of year 0 and in October of years 1-3. Samples were taken from the top 30 cm soil of buried plants (n = 10, except in year 2 n = 9). Different letters designate significant differences ($P < 0.05$) within a row.

		Year			
Nematode species	Life stage	0	1	2	3
Tylenchorhynchus spp.	All together	18.0 ^{b}	32.7 ^{b}	1.3 ^{a}	1.9 ^{a}
Paratylenchus spp.	All together	0.0 ^{a}	0.2 ^{a}	4.4 ^{a}	25.5 ^{b}
Criconematidae	All together	0.0 ^{a}	0.0 ^{a}	9.3 ^{a}	5.4 ^{a}
Heterodera arenaria	Male	0.0 ^{a}	0.1 ^{a}	0.6 ^{a}	0.0 ^{a}
	Juvenile	0.0 ^{a}	0.8 ^{a}	2.0 ^{a}	0.8 ^{a}
Meloidogyne maritima	Male, juvenile	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
Pratylenchus penetrans	All together	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}	0.3 ^{a}

Development of added endoparasites over the years. When plants had been buried yearly, the numbers of *M. maritima* juveniles increased in the second year, whereas the numbers of *H. arenaria* males decreased in the third year (Fig. 3.1, Table 3.3). The addition of other endoparasites had a significant negative effect on the numbers of *M. maritima* juveniles and males, and on *H. arenaria* juveniles, whereas *H. arenaria* males

were not affected. In the root zone of non-buried plants, time affected the numbers of *M. maritima* juveniles (Fig. 3.1, Table 3.3), but Tukey's HSD did not reveal which years differed. The only effect of addition of other endoparasites was a decrease in the numbers of *H. arenaria* males in the first year. In all the treatments, *P. penetrans* established poorly and the numbers were neither affected by time, nor by addition of other endoparasites (Fig. 3.1, Table 3.3).

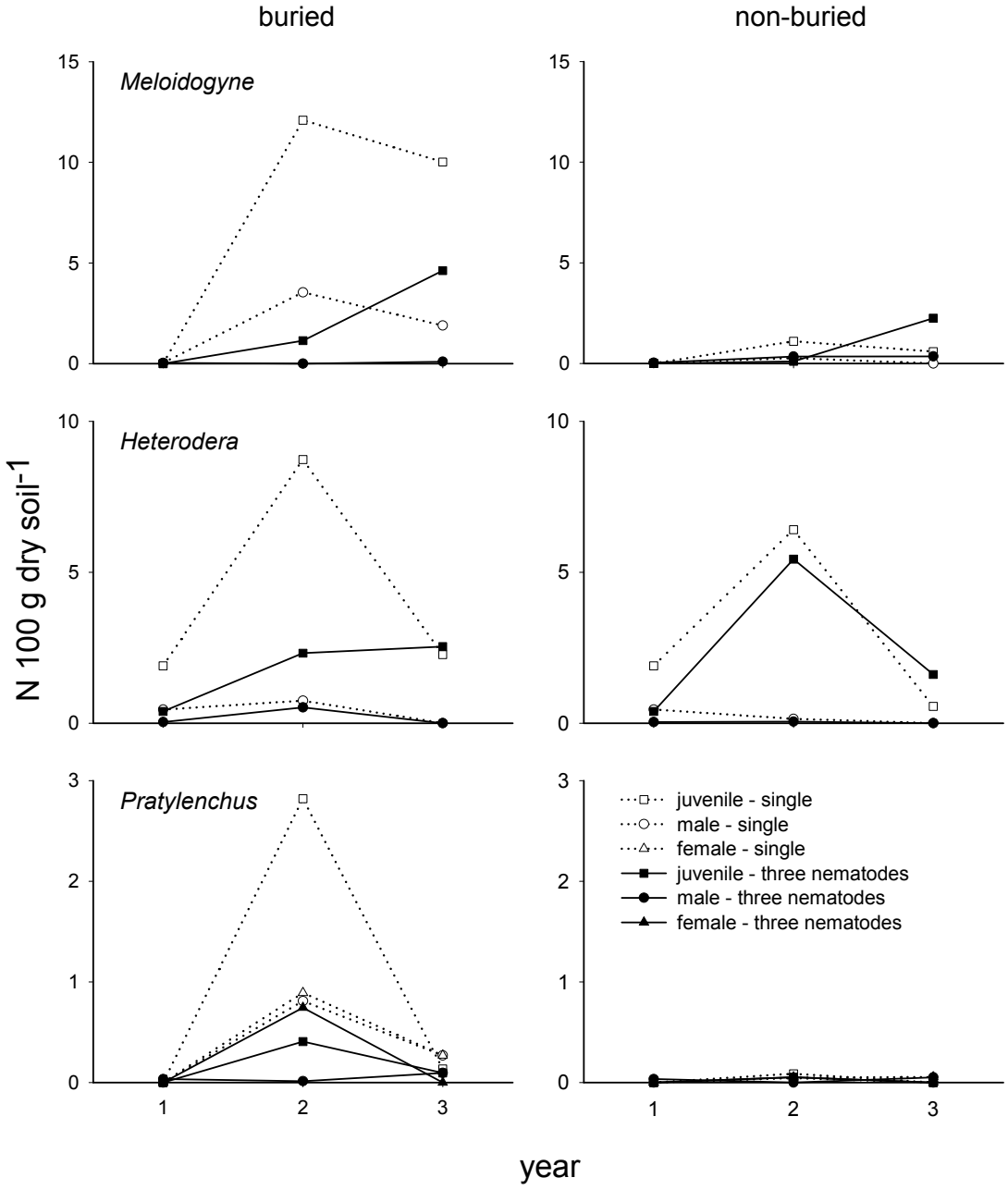

Fig. 3.1. Density (N 100 g dry soil^{-1}) of the endoparasitic nematodes *Meloidogyne maritima* (upper), *Heterodera arenaria* (middle) and *Pratylenchus penetrans* (lower) on *Ammophila arenaria* in October from the years 1 – 3 (n = 10). The left column shows the numbers when plants had been buried, the right column when plants had been left unburied. The development of juveniles (□,■), males (○,●) and females (Δ,▲) of each endoparasite species is presented when inoculated as single species (□,○,Δ; ···) or together with the other two endoparasites (■,●,▲; —). Note the different scales on the y-axes.

Table 3.3. Results of a two-way ANOVA testing the effect of year (1-3) and nematode addition (single species or three species) on the numbers of juveniles and males of *Meloidogyne maritima* and *Heterodera arenaria* associated with *Ammophila arenaria* plants that had been buried yearly or left unburied after year 1 (n = 10). None of the effects on *Pratylenchus penetrans* were significant (data not shown).

				Buried			Non-buried		
Nematode	Life stage	Factor	df	F	P	MSE	F	P	MSE
M. maritima	Juvenile	Year	2	8.65	***	0.184	3.29	*	0.049
		Addition	1	7.61	**		0.89	ns	
		Year×addition	2	1.94	ns		2.20	ns	
	Male	Year	2	2.60	ns	0.064	1.62	ns	0.015
		Addition	1	8.57	**		1.05	ns	
		Year×addition	2	2.93	ns		0.77	ns	
H. arenaria	Juvenile	Year	2	2.81	ns	0.115	1.57	ns	0.144
		Addition	1	8.34	**		0.70	ns	
		Year×addition	2	2.04	ns		1.18	ns	
	Male	Year	2	5.97	**	0.019	4.83	*	0.006
		Addition	1	2.77	ns		6.40	*	
		Year×addition	2	1.01	ns		3.63	*	

P < 0.05, ** P < 0.01, *** P < 0.001, ns = not significant

In year 2, the population development of the nematodes was assessed monthly. The numbers of all developmental stages of *M. maritima* increased over the second year, however, the numbers of males only increased when no other endoparasites had been added and when plants had been buried (Fig. 3.2, Table 3.4; females P < 0.01). Numbers of juveniles of *M. maritima* were lower when *H. arenaria* and *P. penetrans* had been added, while the numbers of females were not affected significantly. The addition of *H. arenaria* and *P. penetrans* delayed the first appearance of both *M. maritima* juveniles and females, but not of males (Fig. 3.2).

The numbers of both juveniles and females of *H. arenaria* increased over the growth season of year 2, whereas the numbers of males were highest in May in the case of non-buried plants and in October in the case of buried plants (Fig. 3.2, Table 3.4; females P < 0.05). The magnitude of the increase of *H. arenaria* juveniles depended on both burial with sand and addition of *M. maritima* and *P. penetrans* (Fig. 3.2, Table 3.4), but Tukey's HSD-test did not reveal which treatments differed, so that the effects will have been marginally significant.

In year 2, the numbers of *P. penetrans* were not influenced by the presence of the other two endoparasite species, neither did burial with sand change the population dynamics of this species significantly (Fig. 3.2). The repeated measures ANOVA did not detect a change in numbers over the second year due to high variability of the data.

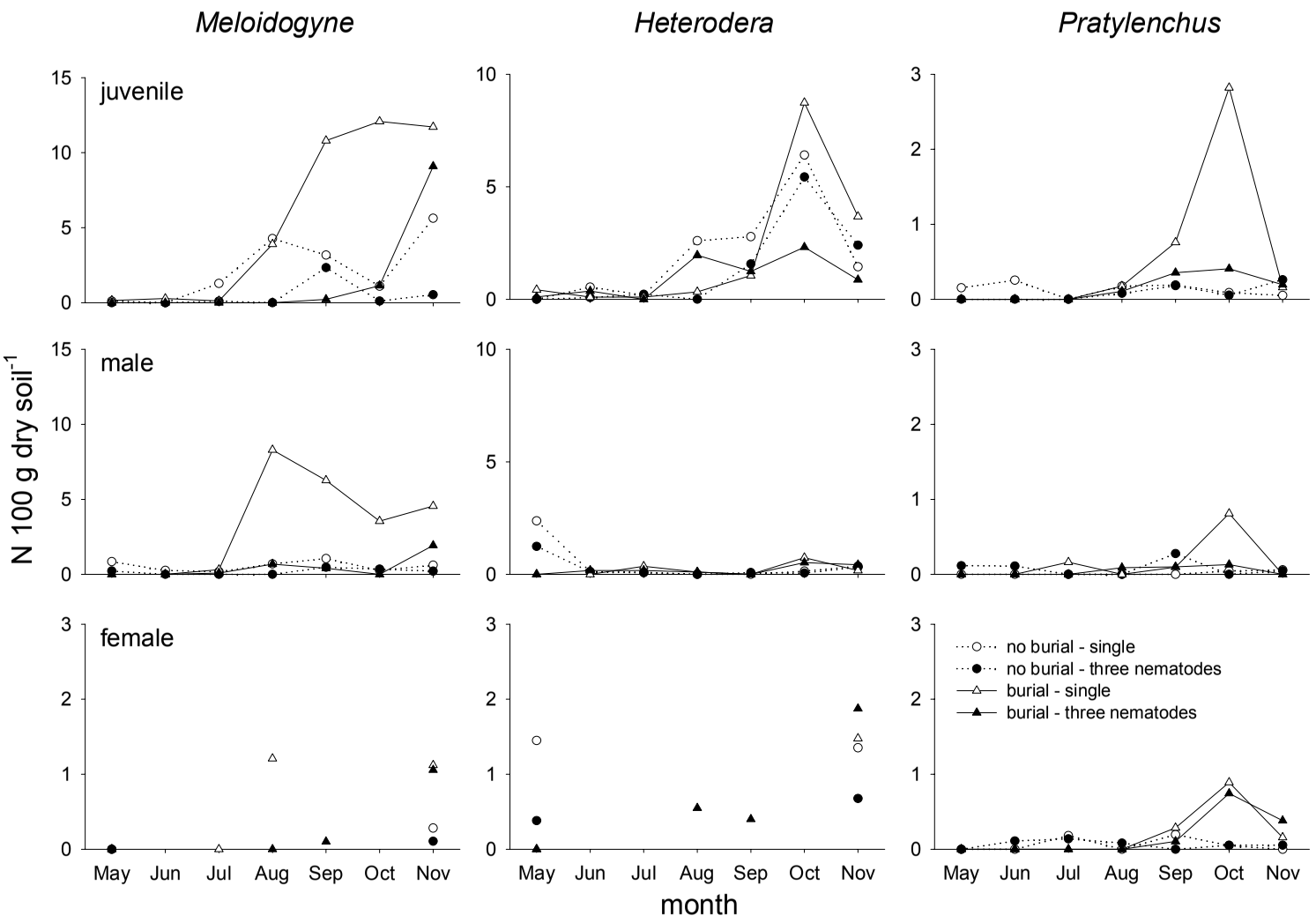

Fig. 3.2. The development (N 100 g dry soil^{-1}) of the endoparasitic nematodes *Meloidogyne maritima* (left), *Heterodera arenaria* (middle) and *Pratylenchus penetrans* (right) on *Ammophila arenaria* in a growing season (May – November of year 2) (n = 10). The development of each endoparasite species is presented when inoculated as single species (○,Δ) or together with the other two endoparasites (●,▲), and when plants were buried with sand (Δ▲; —) or left unburied (○,●; ···). The upper row shows the numbers of juveniles, the middle row the numbers of males and the lower row the numbers of females. Note the different scales on the y-axes.

Table 3.4. Results of a repeated measures ANOVA testing the effect of burial with sand (+/–), nematode addition (single species or three species) and time (months) on the numbers of juveniles and males of *Meloidogyne maritima* and *Heterodera arenaria* associated with *Ammophila arenaria* in year 2 (n = 10). Plot (nested within burial) was used as the error term for burial. None of the effects on *Pratylenchus penetrans* were significant (data not shown).

		Juvenile			Male		
Nematode	Factor	F	P	MSE	F	P	MSE
M. maritima							
	Burial	$F_{1,2}$ = 1.91	ns	1.828	$F_{1,2}$ = 1.68	ns	1.853
	Plot (burial)	$F_{2,34}$ = 0.84	ns	2.189	$F_{2,33}$ = 2.21	ns	0.837
	Addition	$F_{1,34}$ = 12.18	**		$F_{1,33}$ = 7.94	**	
	Burial×addition	$F_{1,34}$ = 0.38	ns		$F_{1,33}$ = 0.73	ns	
	Time	$F_{4,136}$ = 7.42	***	0.532	$F_{6,198}$ = 7.37	***	0.250
	Time×burial	$F_{4,136}$ = 2.22	ns		$F_{6,198}$ = 4.27	***	
	Time×plot (burial)	$F_{8,136}$ = 1.34	ns		$F_{12,198}$= 0.95	ns	
	Time×addition	$F_{4,136}$ = 1.59	ns		$F_{6,198}$ = 2.49	*	
	Time×burial×addition	$F_{4,136}$ = 1.73	ns		$F_{6,198}$ = 2.03	ns	
H. arenaria							
	Burial	$F_{1,2}$ = 0.00	ns	2.058	$F_{1,2}$ = 0.51	ns	0.375
	Plot (burial)	$F_{2,32}$ = 1.99	ns	1.034	$F_{2,32}$ = 2.28	ns	0.165
	Addition	$F_{1,32}$ = 2.78	ns		$F_{1,32}$ = 0.32	ns	
	Burial×addition	$F_{1,32}$ = 0.00	ns		$F_{1,32}$ = 0.28	ns	
	Time	$F_{6,192}$ = 12.27	***	0.407	$F_{6,192}$ = 5.94	***	0.100
	Time×burial	$F_{6,192}$ = 0.48	ns		$F_{6,192}$ = 10.11	***	
	Time×plot (burial)	$F_{12,192}$= 0.60	ns		$F_{12,192}$= 1.22	ns	
	Time×addition	$F_{6,192}$ = 0.90	ns		$F_{6,192}$ = 0.72	ns	
	Time×burial×addition	$F_{6,192}$ = 2.21	*		$F_{6,192}$ = 0.54	ns	

P < 0.05, ** P < 0.01, *** P < 0.001, ns = not significant

Plants

The effect of the addition treatments did not differ between buried and non-buried plants, so that the data were combined. Only in the second year, the addition treatments significantly affected above- and belowground shoot and root biomass (Fig. 3.3, Table 3.5). Interestingly, plants with *M. maritima* alone had less aboveground shoot biomass than plants with the three endoparasites together (P < 0.05), but the effect on belowground shoot and roots was not significant. Still, *M. maritima* alone had a close to significant negative effect on total shoot (= above- and belowground) biomass (P = 0.059). In contrast, *H. arenaria* and *P. penetrans* alone did not affect biomass production. Root zone soil had a negative effect on all plant parts as compared to plants with all three

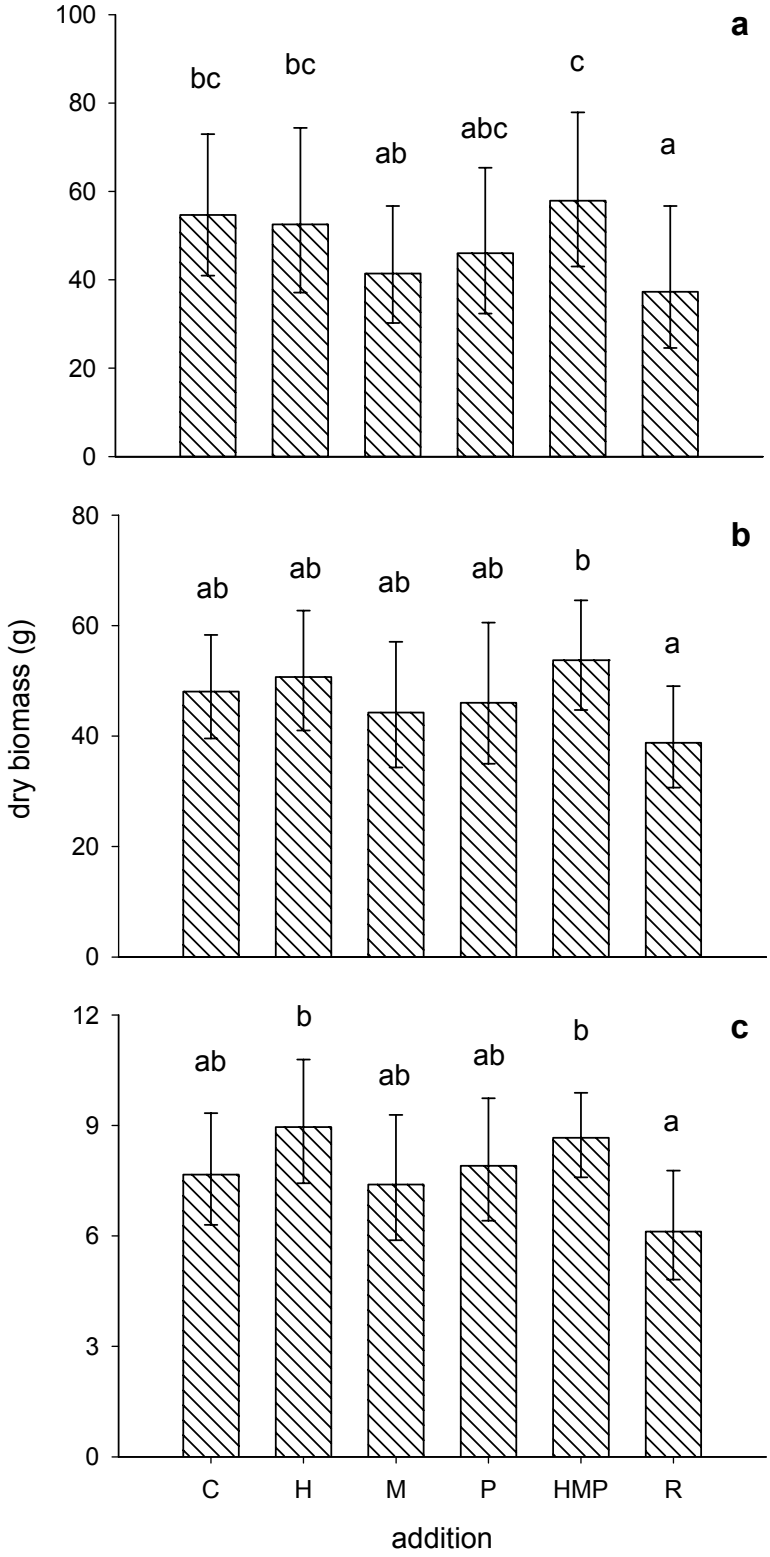

Fig. 3.3. The influence of sand burial and additions on the dry biomass (g) ± 95% confidence interval of a) shoot, b) belowground shoot and c) root of *Ammophila arenaria* (back-transformed values; n = 20). Additions: C = no nematodes, H = *Heterodera arenaria*, M = *Meloidogyne maritima*, P = *Pratylenchus penetrans*, HMP = all three nematodes, R = root zone sand from an existing *A. arenaria* stand containing amongst others the three nematodes. Different letters indicate significant differences ($P < 0.05$). Note the different scales on the y-axes.

endoparasites. However, neither in the first, nor in the third year, did the addition treatments affect above- and belowground shoot or root biomass (data not shown).

In the second year, burial with sand did not significantly affect total shoot biomass, but it altered the distribution into above- and belowground stem parts (Table 3.5). Contrary to our expectation, sand burial did not have a positive, but a negative effect on aboveground shoot biomass, whereas it had a positive effect on belowground stem parts and on root biomass (Table 3.6).

Table 3.5. Results of a two-way ANCOVA testing the effect of burial with sand and addition of soil organisms on the dry biomass of different plant parts of *Ammophila arenaria* in the second year of the field experiment (n = 10). Plant lengths before additions in year 1 were used as a covariate and plot (nested within burial) was used as error term for burial.

Plant part	Factor	df	F	P	MSE
Aboveground shoot	Burial	1	22.79	*	0.131
	Plot (burial)	2	1.50	ns	0.196
	Addition	5	2.55	*	0.131
	Burial × addition	5	1.51	ns	
Belowground shoot	Burial	1	559.78	***	0.073
	Plot (burial)	2	0.15	ns	0.011
	Addition	5	2.59	*	0.073
	Burial × addition	5	0.35	ns	
Root	Burial	1	25.59	*	0.098
	Plot (burial)	2	0.57	ns	0.056
	Addition	5	0.01	*	0.098
	Burial × addition	5	0.48	ns	

* P < 0.05, *** P < 0.001, ns = not significant

Table 3.6. The influence of sand burial (+/–) on *Ammophila arenaria* above- and belowground shoot and root biomass (g). Belowground shoot and root biomass was determined from the upper 60 cm (buried plants) or 30 cm (non-buried plants) in the second year of the field experiment (n = 60). Different letters indicate significant differences within columns (P < 0.05).

	Shoot (g)		Root (g)
Burial	Aboveground	Belowground	
+	46 [a]	63 [b]	9.1 [b]
–	76 [b]	42 [a]	7.7 [a]

DISCUSSION

The present study is one of the few that examines the effect of nematode species diversity on their temporal distributions in the field (Quénéhervé 1990, Eisenback 1993, Lasserre *et al.* 1994). When *M. maritima* had been added to the plants as single species, juveniles and males of this species were present in the newly deposited sand layer earlier in the year and reached higher densities than when *P. penetrans* and *H. arenaria* had been added as well. Apparently, the addition of the other two nematode species forced *M. maritima* to use suboptimal temporal conditions for reproduction. A forced partitioning of space, but not of time, has been suggested in a field experiment with banana plants, where concomitant plant parasitic nematodes reduced densities and restricted the area of infection of *Radopholus similis* (Cobb) Thorne (Quénéhervé 1990). Unlike in our experiment though, the other plant parasitic nematodes had not been inoculated, but had spontaneously colonised the soil. In our experiment, *H. arenaria* and *P. penetrans* were not significantly affected by the presence of the other endoparasites, so that their effects on *M. maritima* seem one-sided.

We showed that an increase in the number of root herbivore species did not necessarily lead to a stronger effect on plant biomass. Interestingly, *M. maritima* alone affected plant biomass more than a combination of three endoparasitic nematode species, while the whole soil community had the strongest negative effect. On the contrary, when added alone, *H. arenaria* and *P. penetrans* did not affect plant biomass. This would be a typical example of an idiosyncratic relationship between the diversity of the root-feeding soil community and plant biomass production as the responding ecosystem function (Lawton 1994). It has been acknowledged that the effects of species identity and diversity may be intermingled (Huston 1997) and that species traits rather than species diversity seem to affect ecosystem function (Bardgett 2002). Still, it is not possible to conclude that *M. maritima* is the key species that is responsible for most of the effect of root zone soil on plant biomass. Root zone soil of *A. arenaria* contains a wealth of pathogenic organisms that together may reduce plant biomass (De Rooij-van der Goes *et al.* 1995b). It also contains beneficial organisms (Little and Maun 1996, De Boer *et al.* 1998a,b) and the response of the plants is to be considered a net effect of pathogenic and beneficial soil organisms. As we did not determine nematode numbers in the root zone soil treatment throughout the year, we cannot directly compare the population dynamics of *M. maritima* in absence and in presence of the whole soil community. In the natural field situation, *M. maritima* colonises new root layers later in the growth season and their densities are relatively low (Van der Stoel *et al.* 2002).

Our finding of a positive effect of multi-species infection on plant growth is in line with some experiments testing combinations of a *Meloidogyne* species and other endoparasitic nematode species (Estores and Chen 1970, Eisenback 1993, Umesh *et al.* 1994). However, most cases conclude that in such conditions the suppression of plant growth increases (Eisenback 1993). In contrast to these agricultural studies, we used a naturally

occurring and co-evolved combination of a host plant and root-feeding nematodes. Also in humans and animals, multi-parasite infection may reduce the pathogenic effect of individual species, although mostly the opposite has been reported (Petney and Andrews 1998). Co-occurrence of an aboveground herbivore and an aerial pathogen may not be negative for the abundance of plants in populations, as the herbivore may suppress pathogen outbreaks (Ericson and Wennström 1997). Such non-additive effects of herbivores or pathogens may be a more general phenomenon in the root zones of natural plant communities than has been supposed thus far.

The effect of *M. maritima* on plant biomass was detected in the second year only, despite the positive effect of absence of other endoparasites on *M. maritima* numbers in all years. In the first year, the time between inoculation of the endoparasitic nematodes and harvest may have been too short to find an effect on plant biomass. Moreover, in the first year all the treatments were spontaneously colonised by unnaturally high numbers of *Tylenchorhynchus* spp. (De Rooij-van der Goes *et al.* 1995b, Van der Stoel *et al.* 2002), which may have a negative effect on biomass of *A. arenaria* in the greenhouse (De Rooij-van der Goes 1995, Brinkman *et al.* 2004: Chapter 5). In the second year, the numbers of ectoparasitic nematodes were relatively low, while in the third year numbers of *Paratylenchus* spp. increased. Thus, effects of the nematode additions will have been clearest in the second year.

In greenhouse studies, *H. arenaria*, *M. maritima* and *P. penetrans* together had a negative effect on the growth of *A. arenaria*, although the effect was small and not continuously present during the course of the experiment (Chapter 5). When added alone, *P. penetrans* had a negative effect on shoot biomass, while *H. arenaria* decreased root biomass only in the case of young seedlings (Van der Stoel 2001, Chapter 4). Attempts to determine the effect of *M. maritima* alone on plant growth failed, since this nematode established very poorly in greenhouse experiments (Chapter 4). Thus, the present study contradicted greenhouse experiments when finding *M. maritima* a stronger pathogen than *H. arenaria* and *P. penetrans*. As the occurrence of a nematode species is determined both by abiotic conditions and competition with other nematode species (Griffiths *et al.* 2002), this emphasises that observations in the greenhouse may not necessarily substitute for field experiments.

The presence of *H. arenaria* in vigorous and early declining *A. arenaria*, but absence in degenerating stands (Clapp *et al.* 2000, Van der Stoel 2001), suggests that a situation without sand accretion is not optimal for this nematode species (De Rooij-van der Goes *et al.* 1995b, Van der Stoel *et al.* 2002). Both *M. maritima* and *Pratylenchus* sp. seem to be less influenced by sand burial, as they are found in vigorous as well as in degenerating *A. arenaria* (De Rooij-van der Goes *et al.* 1995b, Van der Stoel *et al.* 2002). We therefore assumed that cessation of sand burial would lead to a decrease in density of *H. arenaria*, but not of *M. maritima* and *P. penetrans*. On the contrary, when sand burial ceased, *H. arenaria* numbers only decreased temporarily, while the already low numbers of *P. penetrans* were not affected. When sand burial ceased, *M. maritima* did not benefit from

the absence of *H. arenaria* and *P. penetrans* and the numbers remained lower than when plants had been buried. This suggests that in stabilised dunes, *M. maritima* does not contribute to the degeneration of *A. arenaria*.

Burial with sand had a negative effect on aboveground shoot biomass, whereas it had a positive effect on belowground shoot and on root biomass. Burial with sand did not affect total (above- and belowground) shoot biomass. In contrast, degenerate *A. arenaria* in the field and young *Ammophila* plants in the greenhouse responded positively to burial with sand (Huiskes 1979, Wallén 1980, Eldred and Maun 1982, Disraeli 1984, Yuan *et al.* 1993, De Rooij-van der Goes *et al.* 1995a, Little and Maun 1996). Thus, plants seem to respond faster to sudden burial with sand than to cessation of burial. We expected the burial treatment to undo any negative effect of nematode and root zone soil additions on plant biomass, but did not find an interaction between burial and addition treatments. Therefore, we did not find evidence for the escape hypothesis as an explanation for the remained vigour of *A. arenaria* in mobile dunes (Van der Putten and Troelstra 1990).

In conclusion, *M. maritima* was suppressed, both in abundance and in time of first appearance in the new root layer, by the addition of the endoparasitic nematodes *H. arenaria* and *P. penetrans*, while the other two were not affected. When present alone, *M. maritima* colonised the newly deposited sand layer earlier in the year and reached higher densities. The host plant benefited from this suppression, since it was less reduced in biomass when infected by all three endoparasites together than when exposed to *M. maritima* alone. Addition of the whole soil community caused biomass reduction of the plants. In this experiment we did not find evidence for the hypothesis that sand burial enables vigorous growth of *A. arenaria* through a temporary escape from its parasites and pathogens.

Acknowledgements

We gratefully thank the Water and Civil Board De Brielse Dijkring, which provided the field locality and assistance of R. Dekker, A. Voskamp and J. van Eersel. We thank W. Smant for assistance with processing the plant material. This work involved a great effort in the field and many colleagues and friends helped us. Herewith we thank A. de Bever, M. Bezemer, G. Doodeman, N. van der Feest, A. Kamp, M. Keus, G. Kowalchuk, F. Menting, C. van Santen, N. Scheerder, C. Schreck Reis, D. Tap, J. van Veen, J.W. van der Vegte, T. van Veldhuisen, R. Wagenaar and L. Witjes. We also thank J.A. van Veen for discussion and comments on earlier versions of the manuscript. This project was supported by the Research Council for Earth and Life Sciences (ALW) of the Netherlands Organisation for Scientific Research (NWO) contract 805-35.352 and by the European Commission INCO-DC contract IC18CT97014.

Chapter 4

Competition between endoparasitic nematodes and effect on biomass of *Ammophila arenaria* (marram grass) as affected by timing of inoculation and plant age

E. Pernilla Brinkman, Henk Duyts and Wim H. van der Putten

Abstract

We studied the effects of intra- and interspecific competition on the abundance of endoparasitic nematodes and assessed consequences for biomass production of the natural dune grass *Ammophila arenaria*. *Pratylenchus penetrans* was limited by intraspecific competition and it suppressed the abundance of *Heterodera arenaria*, while the interaction between *H. arenaria* and *Meloidogyne maritima* was neutral. *Pratylenchus penetrans* and *H. arenaria* reduced plant biomass, whereas *M. maritima* did not. Plant biomass was not differently affected by adding one or two nematode species. When added to older plants, numbers of *H. arenaria* and *M. maritima* were higher, but numbers of *P. penetrans* were lower, resulting in less reduction of plant biomass. We discuss our results on this natural system with respect to patterns of interspecific nematode competition observed in agricultural systems.

INTRODUCTION

Interactions between plant parasitic nematodes have mainly received attention in agricultural systems (Eisenback 1993). These studies provide valuable information for cropping systems, however, the combinations of host plants and their parasites usually have not naturally co-evolved for a long time. This may be why generalizations from interactions between nematodes with different feeding habits are difficult to be made and why results of nematode competition studies are quite contradictory (Eisenback 1993). In natural systems, host plants and their parasites have co-evolved for a longer period of time, however, these systems have been rarely considered when studying nematode competition. We used the dune grass *Ammophila arenaria* (L.) Link (marram grass) and its cohabiting endoparasitic nematodes to study these interactions.

Ammophila arenaria needs regular burial by wind-blown beach sand to grow vigorously (Huiskes 1979), suggesting that the grass benefits from a temporary escape from root parasites and pathogens (Van der Putten *et al.* 1989). Three endoparasitic nematodes that frequently co-occur with *A. arenaria* are *Heterodera arenaria* (Cooper) Robinson, Stone, Hooper and Rowe, *Meloidogyne maritima* (Jepson) Karssen, van Aelst and Cook and *Pratylenchus penetrans* Cobb (De Rooij-van der Goes *et al.* 1995b, Van der Stoel *et al.* 2002). In greenhouse experiments, *H. arenaria* and *P. penetrans* slightly decreased plant biomass (Van der Stoel 2001, E.P. Brinkman, unpublished results), whereas in a field experiment, only *M. maritima* restricted plant biomass (Chapter 3). As *H. arenaria* is the first endoparasitic nematode to migrate to the new root layer (Van der Stoel *et al.* 2002), we studied it in competition with an endoparasitic nematode species with a similar, sedentary, (*M. maritima*) and one with a different, migratory, feeding behaviour (*P. penetrans*).

Competition for feeding sites is one of the factors that limit nematode establishment and reproduction (Duncan and Ferris 1983). Generally, both migratory and sedentary endoparasites have the potential to limit each other. Migratory endoparasites disturb feeding by sedentary endoparasites by disrupting plant tissue (Zunke 1990b), whereas the latter alter host suitability through a change in plant physiology (Eisenback 1993, Williamson and Gleason 2003). Both the sedentary endoparasites *Heterodera* spp. and *Meloidogyne* spp. penetrate the plant at the root tip (Christie 1936, Wyss and Zunke 1986), whereas the migratory endoparasite *P. penetrans* enters the root mainly in the region of root hair development and, to a lesser extent, in the root elongation zone (Zunke 1990b). In a field experiment, neither *H. arenaria*, nor *P. penetrans* were affected by interspecific competition with each other or with *M. maritima*, whereas *M. maritima* was limited by the presence of the other two species (Chapter 3).

Timing is known to have an important effect on the outcome of competition between nematodes (Eisenback 1993). In field conditions, the observed variation in the time of migration among the three endoparasitic nematode species (Van der Stoel *et al.* 2002)

may be caused by competition. This effect on timing in turn may influence plant production, as suggested by results of an inoculation experiment in the field (Chapter 3).

We performed two indoor competition experiments in controlled conditions to test the ecological consequences of our observations in previous studies (Van der Stoel *et al.* 2002) and outdoor inoculation experiments (Chapter 3). First, we examined whether the three nematode species are limited by intraspecific competition. Then, we examined the role of interspecific competition in combinations of *H. arenaria* with *P. penetrans* and *M. maritima*. We investigated the consequences of interactions for plant biomass. Then, we examined if serial inoculation of nematode species affects competition between nematodes and plant biomass. Finally, we tested the hypothesis that at the same inoculation density, both competition between nematodes and inhibition of biomass production decrease with the age of the plants at the time of inoculation. We assumed that nematode competition and plant sensitivity to parasitic nematodes would diminish with increasing size of the root system at the time of inoculation.

MATERIALS AND METHODS

Soil, plant material and nematode inoculum

Soil was collected from a sandy beach near Haringvlietdam, the Netherlands (51º51' N 04º04' E). The soil was sieved (mesh size 0.5 cm), homogenised and sterilised by gamma-radiation (≥ 25 kGray). Seeds of *A. arenaria* were collected from an existing stand at the same site. The seeds were germinated on glass beads in a growth cabinet at 25ºC/ 10ºC (16 h light/ 8 h dark). The seedlings were then precultured in cones filled with ca. 35-40 g sterilised soil in a greenhouse at 21 ± 2ºC/ 18 ± 2ºC (Experiment 1) and 21 ± 2ºC/ 16 ± 2ºC (Experiment 2) (16 h light/ 8 h dark) for 3 weeks. The soil was moistened with demineralised water and 1 week after planting, the seedlings were provided with 1.4 ml full-strength Hoagland nutrient solution (Hewitt 1966).

Cysts of *H. arenaria* were either collected from the same site as the plant material (Experiment 1), or after collection cultured in the greenhouse on *A. arenaria* (Experiment 2). Cysts of *H. arenaria* were collected and then stored at 4ºC in a 0.5% NaCl solution until use. To collect eggs for hatching, cysts were crushed and the juveniles were hatched in *A. arenaria* root extract (20 g l^{-1}). A mixture of eggs and juveniles of *H. arenaria* was added (1:1 and 1:2 for the first and second inoculation, respectively (Experiment 1) and 3:1 and 99:1 for the first and second inoculation, respectively (Experiment 2)). Egg masses of *M. maritima* were collected from the same site as the plant material and the juveniles were hatched in tap water. A mixture of females, males and juveniles of *P. penetrans* was extracted directly from the *A. arenaria* cultures.

Experimental design

Two experiments were performed to study interactions between *H. arenaria* and *P. penetrans* (Experiment 1) and between *H. arenaria* and *M. maritima* (Experiment 2). In

both experiments, three aspects were addressed: simultaneous inoculation, serial inoculation and plant age.

Simultaneous inoculation. Intra- and interspecific competition between nematodes and the effect of nematodes on plant growth was studied in two experiments using an additive design with the nematode species as factors. Pots of 1.5 l were filled with sterilised soil and planted with three precultured *A. arenaria* seedlings, resulting in 1280 g soil per pot (moisture content of 10% (w w^{-1})). The soil surface was covered with aluminium foil to prevent desiccation. Two days after transferring the plants to the pots, the nematodes were added in three different densities: 0, 150 and 1500 per pot. The nematodes were added in 5.0 ml tap water through straws, which were placed around each plant, so that the nematodes were introduced near the roots. In order to ensure that all nematodes had entered the soil, the straws were flushed with 5.0 ml tap water and removed.

Three times per week, the soil moisture content was adjusted to 10% (w w^{-1}) with demineralised water. Once a week, nutrients were added as Hoagland solution. The nutrient dosage to all plants was gradually increased in time to meet enhanced plant demand (Van der Putten *et al.* 1988). In weeks one and two, half-strength Hoagland solution was added (12.5 ml pot^{-1}). Full-strength Hoagland solution was added in weeks three and four (12.5 ml pot^{-1}) and in weeks five and six (25 ml pot^{-1}). Double-strength Hoagland solution was added in weeks seven and eight (25 ml pot^{-1}), in weeks nine to twelve (38 ml pot^{-1}) and in week thirteen (50 ml pot^{-1}). The experiment was carried out in a greenhouse and provided with extra light to ensure a minimum photosynthetic photon fluence rate of 200 µmol m^{-2} s^{-1} over the waveband 400-700 nm during 16 hours day^{-1}. Temperature was 21 ± 2ºC at daytime and 18 ± 2ºC (Experiment 1) or 16 ± 2ºC (Experiment 2) at night. Thirteen-fourteen weeks after inoculation, the plants were harvested and the numbers of nematodes were determined.

Serial inoculation. Experiments were carried out to examine if interspecific competition and the effect on plant growth changed when the nematodes were added in a sequence with a time interval of 9-10 days. These experiments were carried out in parallel with the simultaneous inoculation in a simple pairwise design (Gibson *et al.* 1999) with nematode density as one factor and time of nematode inoculation as the second. The nematodes were added in densities of 0, 150 and 1500 individuals of each species per pot. The experimental conditions and other technical details of the serial inoculation experiment were the same as for the simultaneous inoculation treatment.

Plant age. In addition to the simultaneous inoculation, experiments were carried out to examine if interspecific competition and the effect on plant growth differed when the nematodes were added to plants of different ages. The experiments were carried out in a simple pairwise design (Gibson *et al.* 1999) with nematode density as one factor and plant age as the other. The plants were precultured in the cones for 3, 5 and 7 weeks (referred to as 3-, 5- and 7-weeks-precultured plants, respectively) and 1, 3 and 5 weeks after planting, the seedlings were provided with 1.4 ml full-strength Hoagland nutrient solution. Root biomass of individual pre-cultured plants at the beginning of experiment 1 was 11.7 ±

s.e. 0.6 mg DM, 24.9 ± s.e. 2.7 mg DM and 57.0 ± s.e. 5.0 mg DM, respectively, whereas it was not determined in experiment 2. The nematodes were added in densities of 0, 150 and 1500 individuals of each species per pot. The experimental conditions and other technical details of the serial inoculation experiment were the same as for the simultaneous inoculation treatment.

Harvest

Plants. When harvested, the sand was washed from the roots and shoot and roots were separated. One third (experiment 1) or all roots (experiment 2) were used for nematode extraction. Shoot and roots were dried at 70ºC for 48 hours and weighed.

Nematode extraction. Nematodes were extracted from the sand by decantation (Van der Stoel *et al.* 2002). The sand with nematodes was washed into a bucket and tap water was added to achieve 4-5 l suspension. The suspension was stirred and after waiting 5-10 seconds, the water and the suspended nematodes were decanted through 1 mm, 180 µm, 75 µm and three 45 µm sieves. This procedure was carried out four times. The 1 mm sieve removed waste material that did not contain cysts or free-living nematodes. The material from the 180 µm sieve was transferred to a coffee filter in order to quantify cysts. The filters were dried at room temperature, and stored at 4ºC until numbers of cysts were counted at 6-50 × magnification. To collect free-living nematodes, the material from the 75 and 45 µm sieves was transferred to a double cotton milk filter (Hygia rapid, Hartmann AG, Heidenheim, Germany) on a sieve in a dish with a layer of tap water (Oostenbrink 1960). The nematodes were allowed to pass through the filter to the water for 24 h (16-25 ºC), then stored at 4ºC until counting.

The migratory stages of endoparasites in the roots were extracted by the funnel-spray method (Oostenbrink 1960) for 96 ± 2 h. Free-living nematodes extracted from the sand and the roots were counted separately (experiment 1) or the extracts were combined (experiment 2). The suspensions were stored at 4ºC until the nematodes were determined and counted at 40-200 × magnification.

The numbers of *M. maritima* egg masses were not determined, as they could have been washed off the roots when harvesting and egg masses are difficult to be determined in the sand. For example, in samples where no egg masses had been recorded, we observed newly formed juveniles, suggesting that egg masses actually had been formed.

Data analysis

Homogeneity of variances and fit to normal distribution were checked for with Hartley's F_{max}-test and the Kolmogorov-Smirnov test, respectively. Plant biomass was natural logarithm (ln)-transformed and analysed using two-way ANOVA. The main factors in the different experiments were '*H. arenaria*' and '*P. penetrans*' (experiment 1) and '*H. arenaria*' and '*M. maritima*' (experiment 2) (simultaneous inoculation), 'nematode density' and 'time' (serial inoculation) and 'nematode density' and 'plant age' (plant age). When there were no

significant interactions, treatment means within each main factor were compared using Tukey's HSD-test ($P < 0.05$).

The relative increase of the different life stages of the nematodes was determined as the ratio of final numbers to inoculated numbers (Pf/Pi) and it was used as standardisation of different inoculation densities. When Pf/Pi's decreased significantly with increasing inoculation density, it was concluded that competition took place. If possible, and when needed after ln-transformation, the data were analysed using two-way ANOVA with the same main factors as for plant biomass. When the data did not meet the conditions for ANOVA, they were analysed by the non-parametric Scheirer-Ray-Hare extension to the Kruskal-Wallis test (Sokal and Rohlf 1995). Approximate P-values for the H-statistic were obtained from Rohlf and Sokal (1981).

RESULTS

Experiment 1

Nematodes

Simultaneous inoculation. Intraspecific competition reduced the Pf/Pi's of females and males of *P. penetrans* (Table 4.1; H_1 (female) = 7.89, H_1 (male) = 12.70, $P < 0.001$). The Pf/Pi's of *H. arenaria* and of juveniles of *P. penetrans* were not influenced by intraspecific competition. Interspecific competition with *H. arenaria* did not affect *P. penetrans*, whereas interspecific competition with *P. penetrans* limited the Pf/Pi of *H. arenaria* males (H_1 = 6.49, $P < 0.05$), but not of females and juveniles (Table 4.1).

Serial inoculation. Contrary to our expectation, serial inoculation neither influenced the outcome of competition of *H. arenaria*, nor of *P. penetrans* (data not shown).

Plant age. The effect of intra- and interspecific competition on *H. arenaria* females was highest on 3-weeks-precultured plants (Table 4.2; H_2 = 11.62, $P < 0.01$), whereas plant age did not influence the effect of competition on *H. arenaria* males and juveniles and *P. penetrans* males. In contrast to our expectation, the effect of competition on *P. penetrans* females was lowest on 3-weeks-precultured plants and the effect of competition on *P. penetrans* juveniles was lower on 3-weeks-precultured than on 5-weeks-precultured plants (Table 4.2; $F_{2, 54}$ (female) = 4.99, $F_{2, 54}$ (juvenile) = 3.54, $P < 0.05$). The male to female ratio of *P. penetrans* was lower on 3-weeks-precultured than on 7-weeks-precultured plants (Table 4.2; $F_{2, 54}$ = 6.83, $P < 0.01$), also suggesting that older plants were less favourable for the development of *P. penetrans* than younger plants.

Plant

Simultaneous inoculation. Addition of *H. arenaria* tended to decrease shoot biomass (Fig. 4.1a; $F_{2, 81}$ = 2.88, $P = 0.06$) and reduced root biomass significantly ($F_{2, 81}$ = 3.14, $P < 0.05$), however, Tukey's HSD did not reveal which treatments differed. Addition of *P. penetrans* reduced shoot biomass significantly (Fig. 4.1b; $F_{2, 81}$ = 4.92, $P < 0.01$) and tended to decrease root biomass ($F_{2, 81}$ = 2.56, $P = 0.08$). There was no interaction

Table 4.1. Relative increase (Pf/Pi; ratio of final to inoculated numbers) of *Heterodera arenaria* and *Pratylenchus penetrans* at two densities (150 and 1500 per pot) when added alone or together with the other species (competitor density 0, 150 or 1500 per pot) to *Ammophila arenaria*. Different letters indicate significant differences within a column ($P < 0.05$; n = 10, except *H. arenaria* female n = 5).

Density (N per pot)		*Heterodera*			*Pratylenchus*		
Target species	Competitor	Female	Male	Juvenile	Female	Male	Juvenile
150	0	0.067 ^{a}	0.007 ^{a}	0.051 ^{a}	23.2 ^{a}	35.1 ^{ab}	45.5 ^{a}
	150	0.164 ^{a}	0.113 ^{a}	0.213 ^{a}	35.2 ^{a}	43.4 ^{ab}	67.4 ^{a}
	1500	0.079 ^{a}	0 ^{a}	0.100 ^{a}	36.0 ^{a}	47.6 ^{b}	85.0 ^{a}
1500	0	0.049 ^{a}	0.003 ^{a}	0.039 ^{a}	17.8 ^{a}	25.8 ^{a}	53.6 ^{a}
	150	0.056 ^{a}	0.003 ^{a}	0.059 ^{a}	17.3 ^{a}	23.7 ^{a}	46.9 ^{a}
	1500	0.051 ^{a}	0.003 ^{a}	0.067 ^{a}	19.7 ^{a}	26.1 ^{a}	53.1 ^{a}

Table 4.2. Relative increase (Pf/Pi; ratio of final to inoculated numbers) of different life stages and ratio male/female (m/f) of *Heterodera arenaria* and *Pratylenchus penetrans* on *Ammophila arenaria* that had been precultured for 3, 5 and 7 weeks before nematode inoculation. Different letters indicate significant differences within a column ($P < 0.05$; n = 20, except *H. arenaria* female and m/f n = 10).

Plant age	*H. arenaria*				*P. penetrans*			
(weeks)	Female	Male	Juvenile	m/f	Female	Male	Juvenile	m/f
3	0.107 ^{a}	0.058 ^{a}	0.140 ^{a}	0.32 ^{a}	27.5 ^{b}	34.8 ^{a}	60.3 ^{b}	1.37 ^{a}
5	0.161 ^{b}	0.003 ^{a}	0.077 ^{a}	0.02 ^{a}	15.7 ^{a}	23.7 ^{a}	34.7 ^{a}	1.62 ^{ab}
7	0.176 ^{b}	0.038 ^{a}	0.061 ^{a}	0.27 ^{a}	13.5 ^{a}	23.7 ^{a}	38.4 ^{ab}	1.80 ^{b}

between the two species, implying that plants inoculated with both nematode species were not differently affected than plants inoculated with only one of the species.

Serial inoculation. Serial inoculation of *H. arenaria* and *P. penetrans* did not influence plant biomass (data not shown).

Plant age. Roots of older plants were relatively less affected by nematode addition than younger plants. This was shown by a significant interaction between nematode density and plant age on root biomass, as the high density reduced root biomass of 3-weeks-precultured plants, whereas it increased the root biomass of 5-weeks-precultured plants (Table 4.3; $F_{2, 81} = 3.09$, $P < 0.05$). Addition of the high nematode density decreased shoot biomass independent of plant age (Table 4.3; $F_{2, 81} = 8.60$, $P < 0.001$).

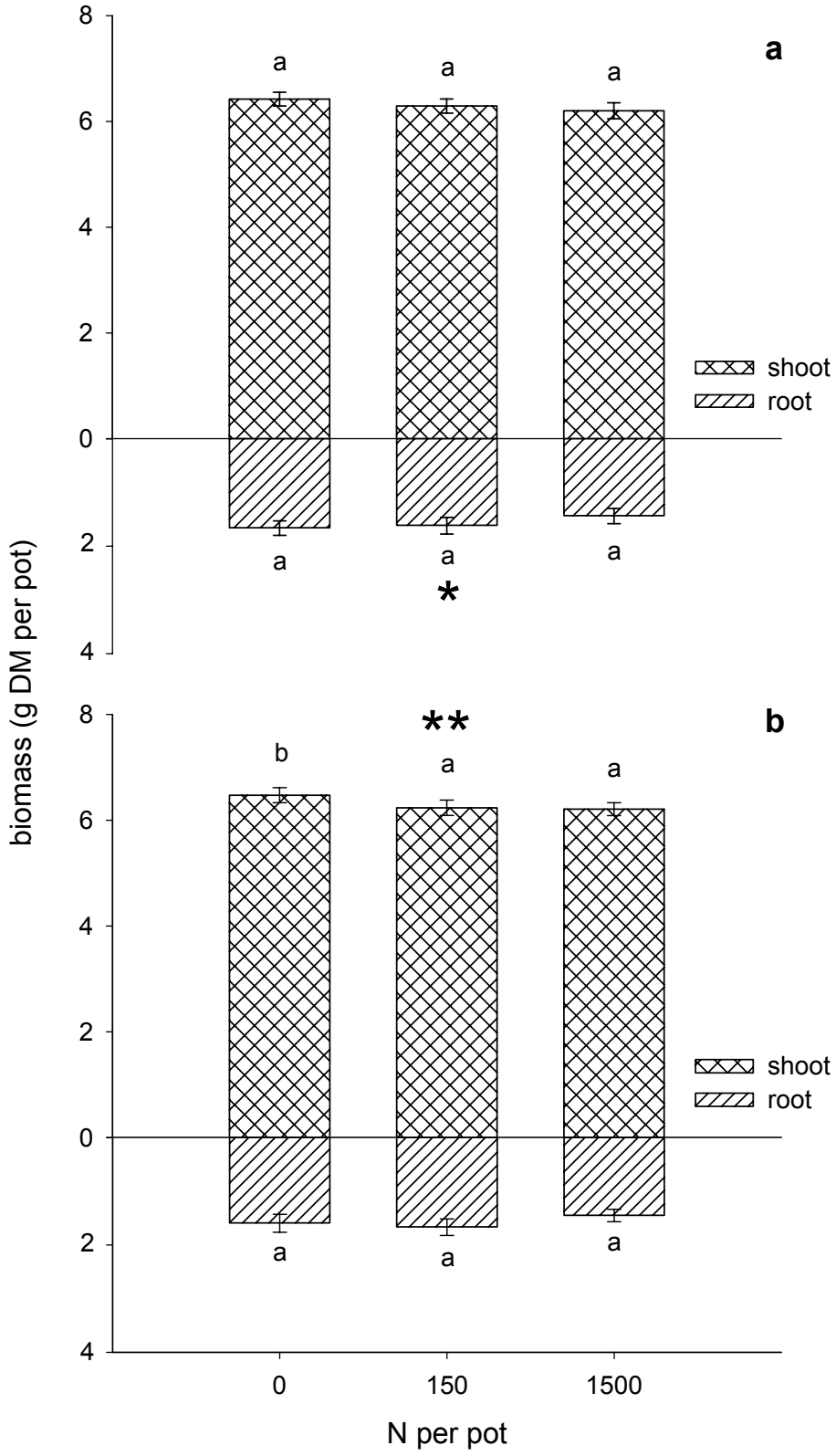

Fig. 4.1. Shoot and root dry biomass (g DM per pot ± s.e.) of *Ammophila arenaria* at different densities (N per pot) of a) *Heterodera arenaria* and b) *Pratylenchus penetrans*. Different letters indicate significant differences within each plant part (P < 0.05). Asterisks indicate significant effects of nematode additions (* P < 0.05, ** P < 0.01; n = 30).

Table 4.3. Effect of addition of different densities (N per pot; equal numbers of each species) of *Heterodera arenaria* and *Pratylenchus penetrans* or *H. arenaria* and *Meloidogyne maritima* on shoot and root dry biomass (expressed as a fraction of the biomass of control plants without nematode addition) of *Ammophila arenaria* that had been precultured for 3, 5 or 7 weeks. Different letters indicate significant differences within a column ($P < 0.05$; $n = 10$).

Plant age (weeks)	Density (N per pot)	Biomass as fraction of control without nematodes			
		H. arenaria + *P. penetrans*		*H. arenaria* + *M. maritima*	
		Shoot	Root	Shoot	Root
3	0 - 0	1.00 ^{b}	1.00 ^{ab}	1.00 ^{a}	1.00 ^{b}
	150 - 150	0.96 ^{ab}	1.06 ^{ab}	1.03 ^{a}	0.85 ^{ab}
	1500 - 1500	0.93 ^{a}	0.85 ^{a}	0.97 ^{a}	0.72 ^{a}
5	0 - 0	1.00 ^{b}	1.00 ^{ab}	1.00 ^{a}	1.00 ^{b}
	150 - 150	0.98 ^{ab}	0.98 ^{ab}	1.00 ^{a}	0.75 ^{a}
	1500 - 1500	0.97 ^{ab}	1.23 ^{b}	0.99 ^{a}	0.83 ^{ab}
7	0 - 0	1.00 ^{b}	1.00 ^{ab}	1.00 ^{a}	1.00 ^{b}
	150 - 150	0.99 ^{b}	1.10 ^{ab}	0.98 ^{a}	0.75 ^{a}
	1500 - 1500	0.97 ^{ab}	0.95 ^{ab}	0.95 ^{a}	0.66 ^{a}

Experiment 2

Nematodes

Simultaneous inoculation. Neither *H. arenaria*, nor *M. maritima* was affected by intra- or interspecific competition, as the relative increase did not change significantly with increasing density of either species (data not shown).

Serial inoculation. To account for differences in nematode numbers as a result of time of development, the ratio was determined of final nematode numbers in mixture to numbers in monoculture (with the same time of inoculation and development). A lower ratio thus represented a stronger effect of competition. The effect of serial inoculation on *H. arenaria* males depended on the inoculation density, however, Tukey's HSD did not reveal how (Table 4.4; $F_{2, 53} = 3.64$, $P < 0.05$). Still, when inoculated after *M. maritima*, *H. arenaria* males tended to increase at low densities, but decrease at high densities. At the low inoculation density, addition of *H. arenaria* enhanced the number of *M. maritima* males, whereas at the high inoculation density, the number of *M. maritima* males was decreased ($H_1 = 6.37$, $P < 0.05$). Serial inoculation did not significantly change the effect of competition on *H. arenaria* females or juveniles or *M. maritima* males or juveniles (Table 4.4).

Plant age. Neither plant age, nor nematode density influenced the effect of competition on *H. arenaria* males and juveniles (Table 4.5). In contrast, the effect of competition on *M. maritima* juveniles was lowest on 7-weeks-precultured plants (Table 4.5; $F_{2, 54} = 12.04$, $P < 0.001$) and the effect of competition on both *M. maritima* males and

juveniles decreased with nematode density (H_1 (male) = 11.63, $P < 0.001$ and $F_{1, 54}$ (juvenile) = 5.16, $P < 0.05$).

Table 4.4. Effect of the timing of inoculation on females of *Heterodera arenaria* and on males and juveniles (ratio of mixture to monoculture) of *H. arenaria* and *Meloidogyne maritima* on *Ammophila arenaria*. Equal densities (N per pot) of both species were added to the plants simultaneously or one of the species was added 9 days later. Different letters indicate significant differences within a column ($P < 0.05$; N = 10, except *H. arenaria* female N = 5). ND = not determined.

Density		*H. arenaria*			*M. maritima*	
(N per pot)	Inoculation time	Female	Male	Juvenile	Male	Juvenile
150-150	Simultaneous	0.92 [a]	2.06 [a]	1.09 [a]	8.00 [ab]	0.73 [a]
	Heterodera later	0.85 [a]	2.53 [a]	1.67 [a]	3.00 [ab]	1.00 [a]
	Meloidogyne later	1.31 [a]	1.03 [a]	1.35 [a]	1.00 [a (x)]	ND [(y)]
1500-1500	Simultaneous	1.00 [a]	1.41 [a]	1.29 [a]	0.85 [b]	0.87 [a]
	Heterodera later	1.17 [a]	0.83 [a]	1.37 [a]	0.52 [ab]	0.50 [a]
	Meloidogyne later	1.35 [a]	1.58 [a]	1.13 [a]	0.83 [ab]	4.80 [(ND)]

[x] *M. maritima* males only in one sample.
[y] in monoculture, no *M. maritima* juveniles were observed.

Table 4.5. Relative increase (Pf/Pi; ratio of final to inoculated numbers) of males and juveniles of *Heterodera arenaria* and *Meloidogyne maritima* on *Ammophila arenaria* that had been precultured for 3, 5 and 7 weeks before nematode inoculation. Different letters indicate significant differences within a column ($P < 0.05$; n = 10).

Plant age	Density	*H. arenaria*		*M. maritima*	
(weeks)	(N per pot)	Male	Juvenile	Male	Juvenile
3	150 - 150	0.021 [a]	0.019 [a]	0.005 [a]	0.11 [a]
	1500 - 1500	0.012 [a]	0.025 [a]	0.005 [ab]	0.19 [abc]
5	150 - 150	0.008 [a]	0.019 [a]	0.003 [a]	0.16 [ab]
	1500 - 1500	0.010 [a]	0.032 [a]	0.009 [ab]	0.31 [bc]
7	150 - 150	0.011 [a]	0.040 [a]	0.005 [a]	0.38 [c]
	1500 - 1500	0.013 [a]	0.025 [a]	0.012 [b]	0.39 [c]

Plant

Simultaneous inoculation. Neither *H. arenaria*, nor *M. maritima* affected shoot or root biomass (7.81 ± s.e. 0.054 g DM and 1.082 ± s.e. 0.028 g DM, respectively) (effect of *H. arenaria* on shoot $F_{2, 81} = 2.60$, $P=0.08$). There was no interaction between the two species, implying that plants inoculated with both nematode species were not affected differently than plants inoculated with only one of the species.

Serial inoculation. Serial inoculation of the nematode species affected plant biomass differently at low and high nematode densities ($F_{2, 54}$ (shoot) = 3.66, $F_{2, 54}$ (root) = 3.67, $P < 0.05$), although Tukey's HSD did not show significant differences. At low nematode densities, serial inoculation tended to decrease shoot and root biomass, whereas at high densities it tended to increase shoot and root biomass (data not shown).

Plant age. Nematode addition did not affect older plants less than younger plants (Table 4.3). Shoot biomass was not affected by nematode addition, whereas both the low and the high nematode densities reduced root biomass of all plant age groups ($F_{2, 81}$ = 21.4, $P < 0.001$). Combined addition of *H. arenaria* and *M. maritima* reduced root biomass more than combined addition of *H. arenaria* and *P. penetrans*, whereas the effects of both nematode combinations on shoot biomass were similar.

DISCUSSION

In our experiments with naturally co-evolved nematode species, the migratory endoparasite *P. penetrans* was a stronger competitor than the sedentary endoparasites *H. arenaria* and *M. maritima*. Several studies in agricultural systems also describe that *Pratylenchus* spp. inhibit both *Heterodera* spp. and *Meloidogyne* spp. (Eisenback 1993, Lasserre *et al.* 1994, Umesh *et al.* 1994), whereas the reverse is reported depending on host suitability (Eisenback 1993). We showed that *P. penetrans* was limited by intraspecific competition, but not by interspecific competition with *H. arenaria*. There was some response of *H. arenaria* to interspecific competition with *P. penetrans*, but this was expressed only as a reduction in the numbers of males. In our study, the *P. penetrans* culture contained an unknown proportion of *P. brzeskii* Karssen, Waeyenberge and Moens, which is specific to sand dunes. This may have had some effect on the results. There seems to be no general pattern in interactions between *Heterodera* spp. and *Meloidogyne* spp., which range from inhibition to stimulation (Eisenback 1993). We showed that neither *M. maritima*, nor *H. arenaria* were affected by intra- or interspecific competition. In this experiment, as well as in other greenhouse experiments (Brinkman *et al.* 2004: Chapter 5), *M. maritima* established poorly compared to the other endoparasites, so that poor establishment probably is the first limitation in exerting an effect on *H. arenaria*.

Serial inoculation did not affect competition between *H. arenaria* and *P. penetrans*, whereas it had a minor effect on competition between *H. arenaria* and *M. maritima*. When inoculated later than *M. maritima*, males of *H. arenaria* tended to increase at low densities, but decrease at high densities. Similarly, but independent of the timing of inoculation, interaction with *H. arenaria* enhanced the number of *M. maritima* males at low densities, whereas it reduced the number of *M. maritima* males at high densities. It should be noted that the data were highly variable and in most samples, no *M. maritima* males were encountered. Generally, a large number of males is regarded to be an indication of suboptimal feeding circumstances (Yeates 1987). However, in our case more males were

observed particularly at the low inoculation density, when competition was supposed to be minimal.

Older plants supported a larger population of *H. arenaria* and *M. maritima* than plants that had been pre-cultured for a shorter time period. However, older plants were less favourable for *P. penetrans* when competing with *H. arenaria*. It has been shown that root exudates of older plants stimulated more hatching of some *Globodera* spp. (Lamondia 1995, Byrne *et al.* 2001), which could also have influenced *H. arenaria*. As we added a mixture of eggs and juveniles of *H. arenaria*, root exudates therefore may have affected hatching of the eggs. In addition, older plants also may provide a larger number of feeding sites for *H. arenaria* and *M. maritima*. On the other hand, *M. javanica* invaded older *Brassica napus* plants less and produced fewer eggs on older then on young plants (McLeod *et al.* 2002), whereas *M. graminicola* equally infected onion plants of different ages (Gergon *et al.* 2002). In the field, a higher proportion of *Pratylenchus* spp. was found in younger than in older roots of *A. arenaria* (C.D. van der Stoel, pers. comm.) and penetration by *P. penetrans* decreased with increasing age of alfalfa roots (Olthof 1982). Our method of pre-culturing all plants in relatively small cones may have limited the formation of new roots of the older plants, explaining why *P. penetrans* multiplication may have been limited on these plants.

In the simultaneous inoculation experiments, *H. arenaria* and *P. penetrans* had a small and sometimes close to significant negative effect on the biomass of *A. arenaria*, whereas *M. maritima* did not at all affect plant biomass. In neither of the two experiments did concomitant infection with two nematode species alter the effect on plant biomass. The small, but not always significant effect of *H. arenaria* supports earlier findings (Van der Stoel 2001), whereas the lack of effect of *M. maritima* on plant biomass in greenhouse conditions may be attributed to the low infection of the plants. However, it contradicts results of a field experiment, where *M. maritima* reduced plant biomass, whereas *H. arenaria* and *P. penetrans* or a combination of the three nematodes did not reduce plant biomass (Chapter 3). This may be explained by the fact that both *H. arenaria* and *P. penetrans* in the field reached lower densities than in greenhouse conditions, whereas *M. maritima* in the field reached higher densities than in the greenhouse (Van der Stoel *et al.* 2002, Chapter 3). Also, we studied interactions between combinations of two endoparasitic nematode species, while in the field, interactions are not limited to two nematode species. In addition, the timing of infection in relation to the developmental stage of the plant and the dry soil conditions in summer in the field may be important determinants of the effect of the nematodes on plant biomass.

Serial inoculation of *H. arenaria* and *P. penetrans* did not affect plant biomass. This is in line with the observation that serial inoculation did not influence nematode numbers. In contrast, serial inoculation of either *H. arenaria* or *M. maritima* tended to decrease shoot and root biomass at low nematode densities, but to increase shoot and root biomass at high densities. The only correlation between plant and nematode data was an increase in *H. arenaria* males at low densities and a decrease at high densities when inoculated later

than *M. maritima*, although *Heterodera* males do not feed (Siddiqi 2000) and thus are not expected to have a strong effect on plant biomass.

We showed that adding a combination of *H. arenaria* and *P. penetrans* stronger reduced root biomass of 3- than of 5-weeks-precultured plants, whereas shoot biomass of the different age classes was not affected differently. In contrast, adding a combination of *H. arenaria* and *M. maritima* equally reduced the biomass of plants that had been precultured for different periods of time. The results only partly supported both our expectation and other studies, which showed a reduced effect of *Meloidogyne* spp. with increasing plant age (Canals *et al.* 1992, Fernandez *et al.* 1995, Ploeg and Phillips 2001). The simultaneous increase in *H. arenaria* and decrease in *P. penetrans* numbers was correlated with the reduced inhibition of root biomass of older plants. The simultaneous increase in *H. arenaria* and *M. maritima* numbers on older plants resulted in an equal effect of nematodes on younger and older plants. It is impossible to contribute the effect to one of the species, since we changed the numbers of the added nematode species simultaneously.

In conclusion, in our greenhouse trials the migratory endoparasite *P. penetrans* was a stronger competitor than the sedentary endoparasites *H. arenaria* and *M. maritima*. *Pratylenchus penetrans* and *H. arenaria* influenced biomass production of *A. arenaria*, but effects fluctuated around the significance level of P = 0.05. The poorly established *M. maritima* did not affect plant biomass. Thus, *A. arenaria* may be considered to be relatively tolerant to its naturally co-evolved endoparasitic nematodes. Serial inoculation of *H. arenaria* and *P. penetrans* did not affect nematode numbers or plant biomass, whereas serial inoculation of *H. arenaria* and *M. maritima* gave variable results, depending on inoculation densities. When added to older plants, numbers of *H. arenaria* and *M. maritima* were higher, whereas numbers of *P. penetrans* were lower. Combined addition of *H. arenaria* and *P. penetrans* resulted in less biomass reduction of older than of younger plants, whereas addition of *H. arenaria* and *M. maritima* equally reduced biomass of plants of different initial age.

Acknowledgements

We thank W. Smant for assistance with processing the plant material. This project was supported by the Research Council for Earth and Life Sciences (ALW) of the Netherlands Organisation for Scientific Research (NWO) contract 805-35.352.

Chapter 5

Endoparasitic nematodes reduce multiplication of ectoparasitic nematodes, but do not prevent growth reduction of *Ammophila arenaria* (L.) Link (marram grass)

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Published in *Applied Soil Ecology* 27: 65-75, 2004

Abstract

Several studies have suggested that plants are able to control the development of specialist herbivorous invertebrates, but not that of generalists. Plants are alleged to have evolved tolerance against specialists in order to suppress the development of more damaging generalists through competition. Here, we tested whether specialist plant parasitic nematodes in the root zone of the natural dune grass *Ammophila arenaria* are able to suppress the development of a generalist plant parasitic nematode and therewith protect the plant. We added a generalist ectoparasite (*Tylenchorhynchus ventralis*) and specialist endoparasites (*Heterodera arenaria*, *Pratylenchus penetrans*, *Meloidogyne maritima*) in different densities to *A. arenaria*. We also tested whether sequential inoculation of the specialists had an additional competitive effect on *T. ventralis*.

Our results show that the specialist endoparasitic nematodes indeed suppressed the development of the generalist *T. ventralis*, but only when the specialists were added to the plant in relatively high densities that exceeded the field density of the specialist endoparasitic nematodes. Therefore, we conclude that competition by specialist nematodes is not a likely mechanism for the regulation of the generalist plant parasite *T. ventralis*. Sequential inoculation of endoparasites did not influence the development of *T. ventralis* more than inoculation at the same time. Despite their inhibiting effect on the development of *T. ventralis*, the endoparasites did not counteract the negative effect of *T. ventralis* on plant biomass. On the contrary, they themselves had a negative effect on shoot biomass of *A. arenaria*, although no effect was found on root biomass. We discuss our results in relation to other mechanisms that may regulate the population density of *T. ventralis*.

INTRODUCTION

Studies on population regulation usually focus on either bottom-up or top-down factors (Walker and Jones 2001). In soil, most studies on factors that control nematode population abundance have been performed in agricultural systems (Akhtar and Malik 2000, Kerry 2000), whereas natural systems have received little, if any, attention. In natural systems, plant parasitic nematodes influence primary production (Stanton 1988, Ingham and Detling 1990). In the first instance, there was no support for the idea that nematodes may influence plant succession (Van der Putten and Van der Stoel 1998, Verschoor *et al.* 2002a). Lately, there has been more evidence that plant parasitic nematodes can enhance succession, on a local scale (Verschoor 2002, Verschoor *et al.* 2002b) or in combination with other soil invertebrates (De Deyn *et al.* 2003). Since in natural systems the host plants, their nematodes, and their potential antagonists may have co-evolved for a longer time span than in agricultural systems, these natural systems provide an interesting opportunity for studying interactions between and within trophic groups in order to unravel how nematode abundance is controlled naturally.

Competition between nematodes is one of the factors that influences the structure of nematode communities (Griffiths *et al.* 2002). An extensive review shows that interactions between plant parasitic nematodes usually are negative to one or both of the species, but in a few cases they were found to be neutral or positive (Eisenback 1993). Competition between plant parasitic nematodes is due to occupation of space, physical change of the host plant that decreases its suitability, or destruction of feeding sites, and it is most severe between species with similar feeding habits. Sedentary endoparasitic nematodes, which have a relatively complex host-parasite relationship, usually are stronger competitors than are ectoparasites, whereas migratory endoparasites are intermediate competitors (Eisenback 1985). An explanation for the competitive advantage of sedentary endoparasites might be that they only have to penetrate and create a feeding site once, while ectoparasites constantly have to search for new feeding sites.

For insect herbivores, it has been hypothesised that plants might tolerate specialists, because these can be controlled. The development of generalists, which cannot be controlled by the plant, may then be limited through competition with the specialists (Vinson 1999). This hypothesis is in line with overviews for plant parasitic nematodes, such as Eisenback (1993). It has been hypothesised (Duncan and Ferris 1982) and observed that infection with only one nematode species may result in greater suppression of plant growth than infection with two or more strongly competitive nematode species (Estores and Chen 1970, Duncan and Ferris 1983, Umesh *et al.* 1994). This is possible when the less pathogenic nematodes occupy feeding sites that can no longer be used by the more pathogenic nematodes.

We used the coastal dune grass *Ammophila arenaria* (L.) Link and some of the major endo- and ectoparasitic nematodes inhabiting its root zone (Yeates *et al.* 1993, Van der Putten and Van der Stoel 1998). The nematode feeding types were assigned according to

Yeates *et al.* (1993). The sedentary endoparasites *Heterodera arenaria* (Cooper) Robinson, Stone, Hooper & Rowe and *Meloidogyne maritima* (Jepson) Karssen, van Aelst & Cook and the migratory endoparasite *Pratylenchus penetrans* Cobb are specialist feeders on *A. arenaria*, but they are not harmful to the plant (Van der Stoel 2001, E.P. Brinkman, unpublished results). In contrast, the ectoparasite *Tylenchorhynchus ventralis* (Loof) Fortuner & Luc (= *Telotylenchus ventralis*) may be considered a generalist plant parasite, while feeding on a broader range of dune grasses (W.H. van der Putten, unpublished results), and it can damage *A. arenaria* (De Rooij-van der Goes 1995). The multiplication of the endoparasitic nematodes is limited by *A. arenaria* (Van der Stoel 2001), whereas the plant cannot directly control the ectoparasite (De Rooij-van der Goes 1995).

We tested the hypothesis (1) that specialist endoparasitic nematodes reduce the multiplication of ectoparasitic nematodes through interspecific competition. We suppose that as a result of the occurrence of both endo- and ectoparasitic nematodes, *A. arenaria* is less damaged than when exposed to the generalist ectoparasite alone. Since in the field the three endoparasitic nematode species tend to succeed each other throughout the growing season (Van der Stoel *et al.* 2002), we also tested the hypothesis (2) that the resulting continuous infection of the plant with different specialist endoparasites may act as an additional protection to the plants. We have tested these two hypotheses by performing a competition experiment in the greenhouse where we added different densities of endo- and ectoparasites to the plants. In addition, we added the endoparasites in the natural temporal sequence.

MATERIALS AND METHODS

Soil, plant material and nematode inoculum

Soil was collected from a sandy beach at Haringvlietdam, the Netherlands (51º51' N 04º04' E). The soil was sieved (mesh size 0.5 cm) and homogenised in a concrete mixer. Before use, the soil was sterilised by gamma-radiation (≥ 25 kGray).

Seeds of *A. arenaria* were collected from an existing stand at the dunes north of Haringvlietdam, landward of the soil-sampling site. The seeds were germinated on moist glass beads in a growth cabinet at 25ºC/ 10ºC (16 h light/ 8 h dark) for 18-19 days. The seedlings were then precultured in cones filled with ca. 35-40 g sterilised soil in a greenhouse at 21 ± 2ºC/ 16 ± 2ºC (16 h light/ 8 h dark) for 19-20 days. The soil in the cones was supplied with demineralised water and 14 days after planting the seedlings were provided with 2 ml full-strength Hoagland nutrient solution (Hewitt 1966).

All nematode species were collected from the same site as the plant material and then cultured in the greenhouse on *A. arenaria*. *Heterodera arenaria* cysts were collected from the cultures and stored at 4ºC in a 0.5% NaCl solution until use. To collect eggs for hatching, cysts were crushed and the juveniles were hatched in *A. arenaria* root extract (20 g l^{-1}). *Heterodera arenaria* was added as a mixture of eggs and juveniles (74:26).

Meloidogyne maritima egg masses were collected from the cultures and the juveniles were hatched in tap water. *Pratylenchus penetrans* and *T. ventralis* were extracted directly from the *A. arenaria* cultures. A mixture of females, males and juveniles (16:25:59 and 7:8:85 respectively) was added.

Experimental design

Simultaneous inoculation experiment

Interspecific competition between nematodes and the effect of nematodes on plant growth (hypothesis 1) was tested in an experiment using a factorial design with the three endoparasites as one factor and the ectoparasite *T. ventralis* as another. Pots of 1.5 l were filled with sterilised soil and planted with three pre-cultured *A. arenaria* seedlings, resulting in 1280 g soil per pot (moisture content of 10 % (w w^{-1})). The soil surface was covered with aluminium foil to prevent desiccation and the nematodes were added three days after transferring the plants to the pots. *Tylenchorhynchus ventralis* was added to pots in three different densities: 0, 25 and 255 per pot. The endoparasites were added in individual densities of 0, 25, and 255 per pot, adding up to total endoparasite densities of 0, 75 and 765 per pot. The nematodes were added in 5.0 ml tap water through straws, which were placed around each plant, so that the nematodes were introduced near the plant roots. In order to ensure that all nematodes had entered the soil, the straws were flushed with 5.0 ml tap water before pulling them out.

Three times per week, the soil moisture content was adjusted with demineralised water to 10 % (w w^{-1}). Once a week, nutrients were added as Hoagland solution. The nutrient dosage to all plants was gradually increased in time to meet enhanced plant demand (Van der Putten *et al.* 1988). Full-strength Hoagland solution was added in weeks one to four (12.5 ml pot^{-1}) and in weeks five and six (25 ml pot^{-1}), whereas double-strength Hoagland solution was added in weeks seven and eight (25 ml pot^{-1}) and in weeks nine to twelve (37.5 ml pot^{-1}). The experiment was carried out in a greenhouse and provided with extra light to ensure a minimum photosynthetic photon fluence rate of 200 µmol m^{-2} s^{-1} over the waveband 400-700 nm during 16 hours day^{-1}. Temperature was 21 ± 2ºC at daytime and 16 ± 2ºC at night.

Four, eight and twelve weeks after inoculation, one third of all plants were harvested, so that six replicates of every treatment were obtained at each harvest. The number of *T. ventralis* was determined at all harvests, whereas the number of established endoparasites was determined at the final harvest.

Serial inoculation experiment

A parallel experiment was carried out to examine if competition between the endoparasite and ectoparasite species and the effect on plant growth differed when the endoparasites were added in a sequence with time intervals of four weeks (hypothesis 2). The

experiment was carried out in a factorial design with nematode density as one factor and time of endoparasite inoculation as the second. The order of endoparasite inoculation was according to their order of appearance when colonising new root layers in the field (Van der Stoel *et al.* 2002): *P. penetrans* was added simultaneously with *T. ventralis*, *H. arenaria* four weeks later and *M. maritima* a further four weeks later. The nematodes were added in densities of 25 and 255 individuals per species per pot. Therefore, the total endoparasite densities inoculated were 75 and 765 individuals per pot. The experimental conditions and other technical details of the serial inoculation experiment were the same as for the simultaneous inoculation treatment.

Harvest

Plants

When harvested, the sand was washed from the roots. Shoot and roots were separated, dried at 70ºC for 48 hours and weighed.

Nematode extraction

Nematodes were extracted from the sand by decantation (Van der Stoel *et al.* 2002). The sand with nematodes was washed into a bucket and tap water was added to achieve 4-5 l suspension. The suspension was stirred and after waiting 5-10 seconds, the water and the suspended nematodes were decanted through 1 mm, 180 µm, 75 µm and three 45 µm sieves. This procedure was carried out four times. The 1 mm sieve removed waste material that did not contain cysts, egg masses or free-living nematodes. The material from the 180 µm sieve was transferred to a coffee filter in order to quantify cysts and egg masses. The filters were dried at room temperature, and stored at 4ºC until numbers of cysts and egg masses were counted at 6-50 × magnification. The material from the 75 and 45 µm sieves was transferred to a double cotton milk filter (Hygia rapid, Hartmann AG, Heidenheim, Germany) on a sieve in a dish with a layer of tap water (Oostenbrink 1960). The nematodes were allowed to pass through the filter to the water for 24 hours at 20 ºC, which delivered clean suspensions for nematode counting.

The migratory stages of endoparasites in the roots were extracted by the funnel-spray method (Oostenbrink 1960) for 96 hours and added to the free-living nematodes that were extracted from the sand. The suspensions were stored at 4ºC until the nematodes were determined and counted at 40-200 × magnification. Sedentary endoparasites (cysts and egg masses) on the roots were counted at 6-50 × magnification.

Data analysis

Homogeneity of variances and fit to normal distribution were checked for with Hartley's F_{max}-test and the Kolmogorov-Smirnov test, respectively. Plant biomass and numbers of *T. ventralis* were log-transformed to meet the conditions for analysis of variance (ANOVA). For the simultaneous inoculation experiment, the effect of the main factors 'time', '*T. ventralis*' and 'endoparasites' was determined in a three-way ANOVA. For the serial

inoculation experiment, the effect of the main factors 'time', 'nematode density' and 'endoparasite timing' was determined in a three-way ANOVA. When there were no significant interactions, treatment means within each main factor were compared using Tukey's HSD-test ($P < 0.05$). When one of the interactions with time was significant, the effect of the other two main factors was determined in two-way ANOVAs for each harvest date. The relative increase rate (RIR) of *T. ventralis* was calculated as the logarithmic increase in nematode numbers within each time interval ($\ln (N_t) - \ln (N_t - 4)$) divided by the number of weeks between harvests (4). Thus, RIR = $(\ln (N_t) - \ln (N_t - 4)) / 4$. The effect of the main factors 'time', '*T. ventralis*' and 'endoparasites' on relative increase rate was determined in a three-way ANOVA.

At the last harvest date, the endoparasite numbers did not meet the conditions for an ANOVA. Therefore, the effect of the main factors '*T. ventralis*' and 'endoparasites' on the ranked endoparasite multiplication numbers (= final nematode numbers divided by inoculated numbers) was determined in a Scheirer-Ray-Hare extension to the Kruskal-Wallis test (Sokal and Rohlf 1995). Approximate P-values for the H-statistic were obtained from Rohlf and Sokal (1981).

RESULTS

Simultaneous inoculation experiment

Plants

In the first eight weeks of the experiment, *T. ventralis* did not have an effect on shoot biomass (Table 5.1). Twelve weeks after inoculation, a high number of *T. ventralis* increased shoot biomass significantly compared with a low number or no *T. ventralis*. After four weeks, the shoot biomass was marginally decreased by the addition of a low number of endoparasites compared to a high number or no endoparasites. This effect disappeared and after eight weeks the endoparasites did not affect shoot biomass. After twelve weeks, a high number of endoparasites decreased shoot biomass, however, the addition of endoparasites did not change the effect of *T. ventralis* (Table 5.1; Tyl × endo not significant).

After the first four weeks of the growth experiment, *T. ventralis* had not influenced root biomass (Table 5.1). From eight weeks onwards, addition of the highest number of *T. ventralis* decreased the root biomass significantly compared to the low number or no *T. ventralis* added. This effect of *T. ventralis* on root biomass was stronger and opposite to the effect on shoot biomass. After four weeks, the root biomass of plants inoculated with a low number of endoparasites was smaller than of plants without endoparasites. However, this effect disappeared at later stages of the experiment. The addition of endoparasites did not significantly counteract the effect of *T. ventralis* on root biomass. On the contrary, the trend was a decrease in root biomass when both ecto- and endoparasites were added (Table 5.1).

Table 5.1. Simultaneous inoculation experiment. The effect of the addition of *Tylenchorhynchus ventralis* ('Tyl') and endoparasites (*Pratylenchus penetrans*, *Heterodera arenaria* and *Meloidogyne maritima*; abbreviated as 'endo') on shoot and root dry biomass (g DM pot^{-1}) (± s.e.) of *Ammophila arenaria* at different harvest dates (t = 4, 8 and 12 weeks). Each nematode species was added in three densities (0, 25 and 255 nematodes pot^{-1}), resulting in total endoparasite densities of 0, 75 and 765 nematodes pot^{-1}. The lower part of the table shows the results of a two-way ANOVA at each harvest date with values for shoot and root dry biomass (df, F, P and MSE) (n = 6). * P < 0.05, *** P < 0.001, ns = not significant.

		shoot (g DM pot^{-1})			root (g DM pot^{-1})		
		T. ventralis					
date	endo	0	25	255	0	25	255
t=4	0	0.568 (± 0.0269)	0.608 (± 0.0235)	0.563 (± 0.0165)	0.202 (± 0.0164)	0.198 (± 0.0153)	0.170 (± 0.0134)
	75	0.541 (± 0.0236)	0.530 (± 0.0151)	0.516 (± 0.0416)	0.160 (± 0.0142)	0.148 (± 0.0156)	0.147 (± 0.0219)
	765	0.536 (± 0.0179)	0.583 (± 0.0194)	0.556 (± 0.0283)	0.172 (± 0.0150)	0.177 (± 0.0142)	0.156 (± 0.0152)
t=8	0	2.415 (± 0.0810)	2.409 (± 0.0990)	2.359 (± 0.0978)	0.732 (± 0.0483)	0.696 (± 0.0532)	0.580 (± 0.0230)
	75	2.446 (± 0.0910)	2.316 (± 0.0761)	2.301 (± 0.1034)	0.861 (± 0.0653)	0.754 (± 0.0625)	0.560 (± 0.0526)
	765	2.576 (± 0.0455)	2.361 (± 0.0174)	2.299 (± 0.0794)	0.793 (± 0.0174)	0.781 (± 0.0364)	0.611 (± 0.0462)
t=12	0	5.893 (± 0.0549)	6.281 (± 0.0925)	6.366 (± 0.1424)	1.384 (± 0.1035)	1.379 (± 0.0508)	0.926 (± 0.0660)
	75	5.748 (± 0.0718)	5.959 (± 0.1999)	6.271 (± 0.0729)	1.340 (± 0.1299)	1.194 (± 0.0884)	0.893 (± 0.0559)
	765	5.665 (± 0.1221)	5.723 (± 0.1388)	6.262 (± 0.1491)	1.260 (± 0.0520)	1.213 (± 0.0808)	0.818 (± 0.0963)

	shoot				root			
	df	F	P	MSE	df	F	P	MSE
Tyl	2	1.260	ns	0.012	2	1.585	ns	0.060
endo	2	3.297	*		2	4.569	*	
Tyl x endo	4	0.443	ns		4	0.144	ns	
Tyl	2	2.942	ns	0.007	2	16.964	***	0.028
endo	2	0.424	ns		2	1.207	ns	
Tyl x endo	4	0.522	ns		4	0.716	ns	
Tyl	2	13.535	***	0.003	2	23.130	***	0.042
endo	2	4.490	*		2	1.759	ns	
Tyl x endo	4	0.915	ns		4	0.282	ns	

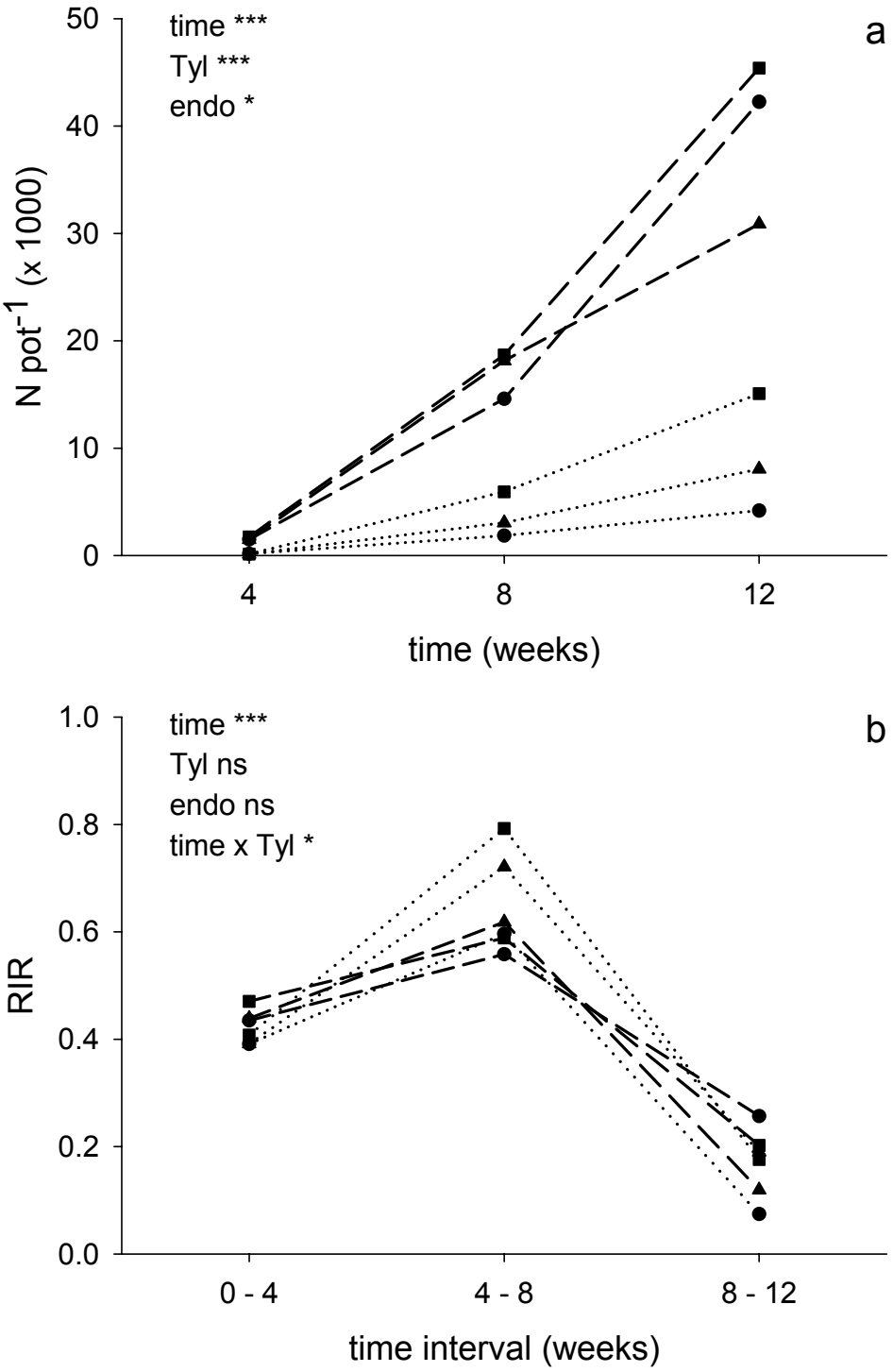

Fig. 5.1. Simultaneous inoculation experiment. The average number (a) and relative increase rate (b; RIR = $(\ln(N_t) - \ln(N_t - 4)) / 4$) of *Tylenchorhynchus ventralis* developed on *Ammophila arenaria* inoculated with different combinations and densities of *T. ventralis* (Tyl; 25 (···) and 255 (---) nematodes pot^{-1}) and *Pratylenchus penetrans*, *Heterodera arenaria* and *Meloidogyne maritima* (endo; 0 (■), 25 (▲) and 255 (●) of each endoparasite species pot^{-1}) at four, eight and twelve weeks after inoculation (n = 6). * P < 0.05, *** P < 001, ns = not significant. Second- and third-order interactions that are not mentioned were not significant.

Table 5.2. Simultaneous inoculation experiment. The effects of the addition of *Tylenchorhynchus ventralis* ('Tyl'; three densities 0, 25 and 255 pot^{-1}) and the endoparasites *Pratylenchus penetrans*, *Heterodera arenaria* and *Meloidogyne maritima* ('endo'; 25 and 255 of each species pot^{-1}, resulting in total endoparasite densities of 75 and 765 nematodes pot^{-1}) on the numbers (± s.e.) of *P. penetrans* and *H. arenaria* after twelve weeks. Results of the Scheirer-Ray-Hare extension of the Kruskal-Wallis test (degrees of freedom, H-, P- and MS_{total}-values) comparing the multiplication values (final nematode number divided by inoculated nematode number) are presented in the lower part of the table (n = 6). * P < 0.05, ** P < 0.01, ns = not significant.

	P. penetrans		*H. arenaria*	
Tyl (N pot^{-1})	75 endo pot^{-1}	765 endo pot^{-1}	75 endo pot^{-1}	765 endo pot^{-1}
0	300 (± 81)	5019 (± 798)	3.8 (± 0.91)	34.8 (± 9.36)
25	267 (± 74)	5370 (± 401)	5.0 (± 0.45)	36.7 (± 4.01)
255	540 (± 190)	7950 (± 992)	4.7 (± 1.12)	23.7 (± 2.64)

	effect	df	H	P	MS_{total}
P. penetrans	Tyl	2	4.983	ns	110.93
	endo	1	8.113	**	
	Tyl x endo	2	0.103	ns	
H. arenaria	Tyl	2	3.907	ns	110.46
	endo	1	5.071	*	
	Tyl x endo	2	0.888	ns	

Nematodes

The numbers of *T. ventralis* increased significantly over time at both low and high inoculation densities (Fig. 5.1a). At all harvest dates, the high inoculation density produced more *T. ventralis* than the low inoculation density. However, the final numbers were not proportional to the initial inoculation densities, suggesting that intraspecific competition for resources increased with initial inoculation density (Fig. 5.1a). This was confirmed by the relative increase rate of *T. ventralis*, which changed during the course of the experiment and depended on the inoculation density (Fig. 5.1b). The addition of high numbers of endoparasites limited the development of *T. ventralis*, while low numbers of endoparasites did not (Fig. 5.1a). Considering the levels of significance, the influence of intraspecific competition on the development of *T. ventralis* was stronger than the influence of interspecific competition with the endoparasites (Fig. 5.1a).

Twelve weeks after inoculation, the multiplication of neither *P. penetrans* nor *H. arenaria* were affected significantly by the presence of *T. ventralis* (Table 5.2). After twelve weeks, the total numbers of *P. penetrans* and *H. arenaria* were positively affected by endoparasite inoculation density (Table 5.2). *Meloidogyne maritima* had established relatively poorly and no significant differences between low and high inoculation densities could be observed (results not shown).

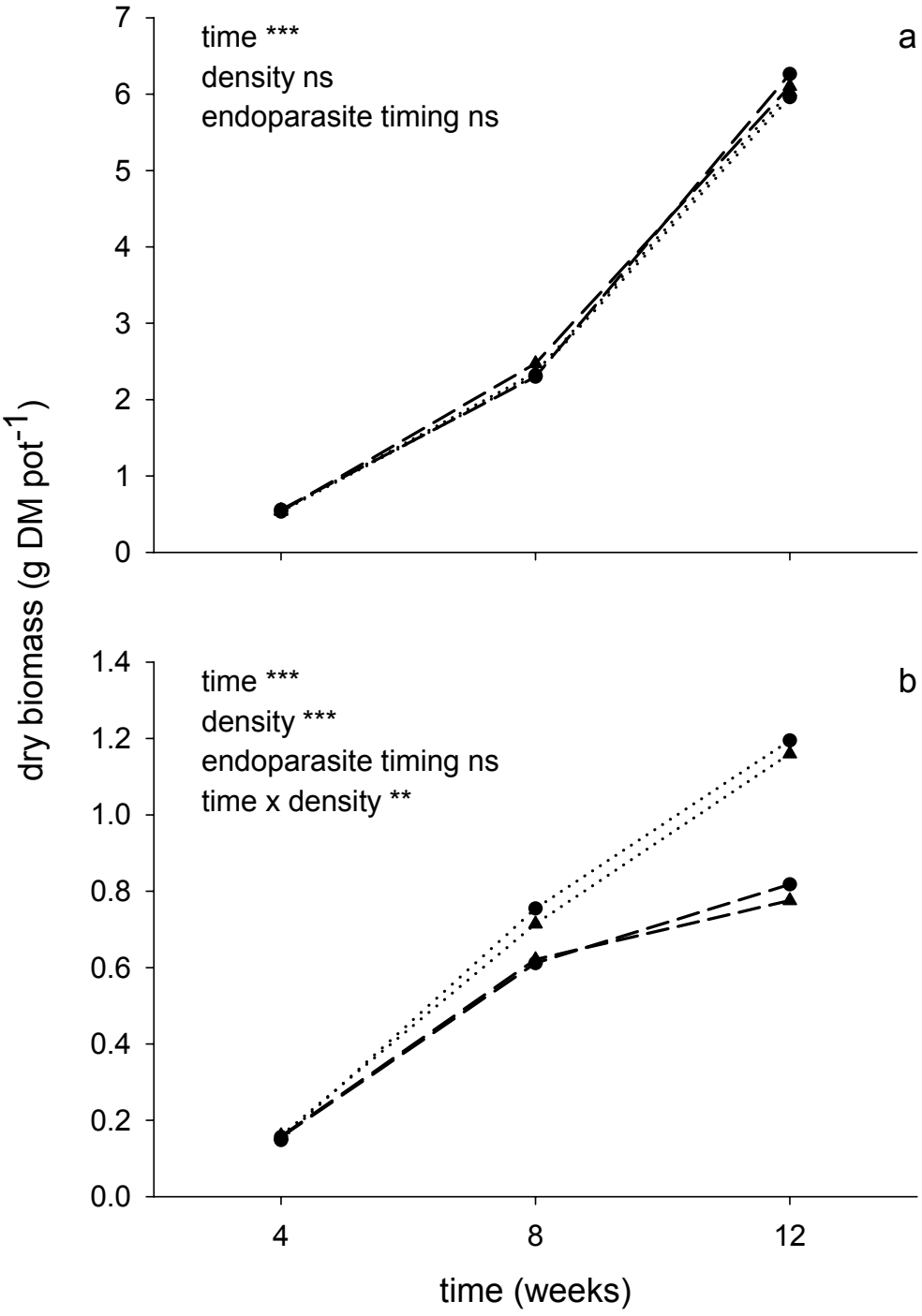

Fig. 5.2. Serial inoculation experiment. The average amount of shoot (a) and root (b) dry biomass (g pot^{-1}) of *Ammophila arenaria* inoculated with different densities (25 (···) and 255 (---) of each nematode species pot^{-1}) of *Tylenchorhynchus ventralis* and the endoparasites *Pratylenchus penetrans*, *Heterodera arenaria* and *Meloidogyne maritima* at four, eight and twelve weeks after inoculation. The endoparasites were added at the same time as was *T. ventralis* (simultaneous (●)) or with time intervals (serial (▲); *P. penetrans* at the same time, *H. arenaria* four weeks later and *M. maritima* a further four weeks later) (n = 6). ** P < 0.01, *** P < 001, ns = not significant. Second- and third-order interactions that are not mentioned were not significant.

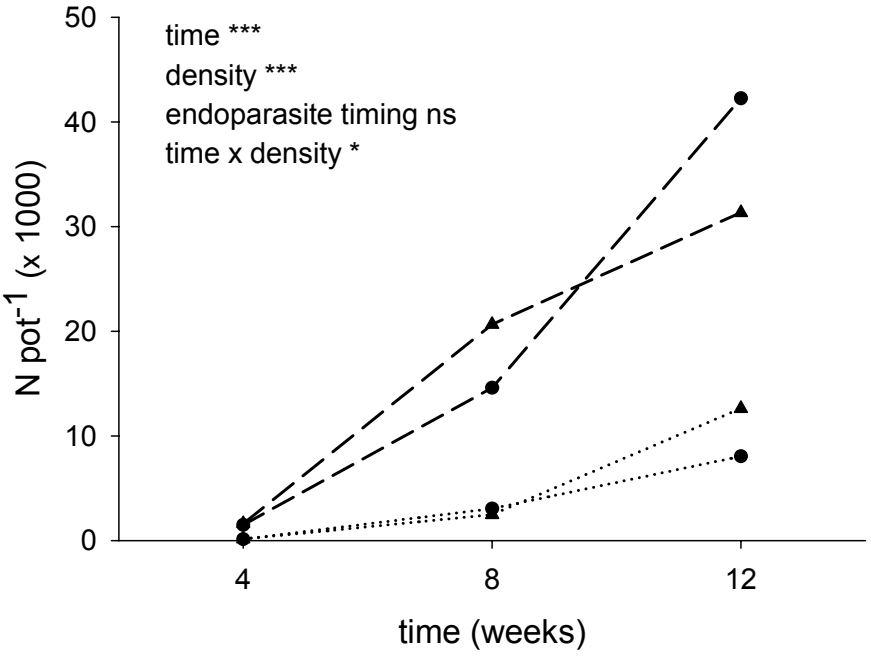

Fig. 5.3. Serial inoculation experiment. The average number of *Tylenchorhynchus ventralis* developed on *Ammophila arenaria* inoculated with different densities (25 (···) and 255 (---) of each nematode species pot^{-1}) of *T. ventralis* and the endoparasites *Pratylenchus penetrans*, *Heterodera arenaria* and *Meloidogyne maritima* at four, eight and twelve weeks after inoculation. The endoparasites were added at the same time as was *T. ventralis* (simultaneous (●)) or with time intervals (serial (▲); *P. penetrans* at the same time, *H. arenaria* four weeks later and *M. maritima* a further four weeks later) (n = 6). * P < 0.05, *** P < 001, ns = not significant. Second- and third-order interactions that are not mentioned were not significant.

Serial inoculation experiment

Plants

Neither nematode density nor timing of endoparasite inoculation influenced shoot biomass (Fig. 5.2a, ANOVA not shown). There were no significant interactions between the main factors.

After four weeks, the treatments had not resulted in significant differences in root biomass (Fig. 5.2b). After eight and twelve weeks, the high nematode inoculation density had reduced root biomass significantly compared to the low inoculation density ($P < 0.01$ and $P < 0.05$, respectively). However, timing of endoparasite inoculation had no influence on root biomass.

Nematodes

At all harvest dates, compared to the lowest inoculation density, the highest number of *T. ventralis* inoculated resulted in the highest number recovered (Fig. 5.3; $P < 0.001$). Apparently, intraspecific competition was not strong enough to eliminate the difference between the inoculation densities under the experimental conditions. The timing of endoparasite inoculation did not influence the multiplication of *T. ventralis* significantly (Fig.

5.3). The relative increase rate of *T. ventralis* changed during the course of the experiment, but was also dependent on the inoculation density of *T. ventralis* and, interestingly, the timing of endoparasite inoculation (significant three-way interaction; data not shown).

Serial inoculation of the endoparasites had a positive effect on the numbers of *P. penetrans* at low densities, but no effect at high densities (Table 5.3). Possibly, competition between *T. ventralis* and *P. penetrans* was already so high, that it did not matter whether *H. arenaria* and *M. maritima* were inoculated at the same time or not. Serial inoculation of the endoparasites limited the multiplication of *H. arenaria* at both densities, probably due to the shorter development time of *H. arenaria* when inoculated four weeks later. When corrected for inoculation density, the multiplication of *H. arenaria* at the lower inoculation density was higher than at the higher inoculation density (Table 5.3). It was not possible to find any significant differences in the numbers of *M. maritima*, because a development time of four weeks did not allow the young females to develop to a size which could be observed (results not shown).

Table 5.3. Serial inoculation experiment. The effects of the addition of *Tylenchorhynchus ventralis* ('Tyl') and *Pratylenchus penetrans*, *Heterodera arenaria* and *Meloidogyne maritima* ('endo')(25 and 255 of each species pot^{-1}) and the timing of endoparasite addition (simultaneous = all at the same time, serial = *P. penetrans* at the same time, *H. arenaria* four weeks later and *M. maritima* a further four weeks later) on the numbers (± s.e.) of *P. penetrans* and *H. arenaria* after twelve weeks. Results of the Scheirer-Ray-Hare extension of the Kruskal-Wallis test (degrees of freedom, H-, P- and MS_{total}-values) comparing the multiplication values (final nematode number divided by inoculated nematode number) are shown in the lower part of the table (n = 6). * P < 0.05, ** P < 0.01, ns = not significant.

	P. penetrans		*H. arenaria*	
nematodes (N pot^{-1})	simultaneous	serial	simultaneous	serial
25 Tyl, 75 endo	267 (± 74)	1103 (± 275)	5.0 (± 0.45)	2.5 (± 0.62)
255 Tyl, 765 endo	7950 (± 992)	7253 (± 1509)	23.7 (± 2.64)	13.7 (± 1.26)

	effect	df	H	P	MS_{total}
P. penetrans	density	1	1.470	ns	50.00
	timing	1	3.630	ns	
	density x timing	1	5.333	*	
H. arenaria	density	1	6.220	*	49.54
	timing	1	8.579	**	
	density x timing	1	0.189	ns	

DISCUSSION

Vinson (1999) hypothesised that a plant may recruit specialist herbivorous insects to compete with generalist herbivorous insects that the plant can not manage itself, therewith

protecting the plant against these generalists. We tested whether this hypothesis might apply to plant parasitic nematodes in a natural host-nematode system. In the present case, the endoparasites were considered as specialist based on their specific occurrence in the field (Van der Putten and Van der Stoel 1998), whereas an ectoparasitic nematode was the generalist. We found that the endoparasitic nematodes *P. penetrans*, *H. arenaria* and *M. maritima* inhibited the multiplication of the ectoparasite *T. ventralis*, which partly supports Vinson's (1999) hypothesis. The outcome of interspecific competition between endo- and ectoparasites is also in line with general observations on mostly agricultural host-nematode systems, where nematodes with the most complex relationship with their host usually are superior in competition (Eisenback 1993). Inhibition of ectoparasite multiplication only occurred when the endoparasites were inoculated in high numbers. The actual density of endoparasites therefore seemed to be more important than the ratio between *T. ventralis* and endoparasites. As the density of endoparasites in the field usually is comparable with the low inoculation density used in this experiment (Van der Stoel *et al.* 2002), the low numbers of *T. ventralis* in the field are not likely to be regulated by interspecific competition with endoparasites. Bottom-up control by the plant is highly unlikely, considering the fast multiplication and super-abundance of *T. ventralis* in this and earlier greenhouse experiments (De Rooij-van der Goes 1995), as well as in outdoor experimental conditions (Chapter 3). We therefore conclude that top-down control is the most likely regulation mechanism that limits the density of *T. ventralis* in the root zone of *A. arenaria*. Possible regulatory biota are bacteria and fungi (Kerry 2000), but also predatory nematodes, insects and mites may be involved in this process (Akhtar and Malik 2000). A challenge for subsequent studies is to test the relative contribution of top-down control factors to the vigorous growth of *A. arenaria* in mobile dunes. Also, it needs to be examined whether plants play an active role in their indirect defence by attracting nematode antagonists (Van der Putten *et al.* 2001, Van Tol *et al.* 2001).

Based on field surveys (Van der Stoel *et al.* 2002), successive infection with different endoparasitic nematodes was supposed to result in a stronger suppression of generalist nematodes than when all endoparasites were present at the same time. The presence of at least one of the endoparasitic nematode species at any stage of the growth season was also reported for white clover, but without reference to consequences for other plant parasitic nematodes (Yeates *et al.* 1985). We hypothesised that this may lead to more continuous interspecific competition than when all endoparasites would compete with ectoparasites and with themselves at the start of the experiment, leaving ample opportunities for the ectoparasites to develop and multiply later on in the growth experiment. However, our results showed that serial inoculation of the endoparasites did not cause additional inhibition of *T. ventralis* multiplication compared to pots that had all endoparasites present at the start. Possibly, the later inoculation of *Heterodera* and *Meloidogyne* endoparasites was less effective because of the relatively small number of individuals added compared to the relatively large root system of the plant. From the first experiment, we could conclude that the actual density of endoparasites was more

important for the outcome of competition than the ratio between endoparasites and *T. ventralis*.

Our experiment is not conclusive about the mechanism of competition between the endoparasites and *T. ventralis*. Migratory endoparasites are antagonistic to ectoparasites by altering both root morphology and physiology, whereas sedentary endoparasites suppress ectoparasites merely through a physiological change of the plant (Eisenback 1985). Direct competition for feeding sites seems unlikely, because behavioural observations showed that the endoparasites feed on different parts of the roots than *T. ventralis* (Christie 1936, Wyss and Zunke 1986, Zunke 1990a,b, De Rooij-van der Goes 1995).

In our experiments, *T. ventralis* had a negative effect on the growth of the roots of *A. arenaria*, as was reported by De Rooij-van der Goes (1995). Despite the suppressing effect of endoparasites on the multiplication of *T. ventralis*, the negative effect on plant growth could not be counteracted by the presence of endoparasites. On the contrary, the endoparasites had a negative effect on plant biomass themselves, albeit that this was a minor effect that appeared only occasionally, confirming results by Van der Stoel (2001). *Tylenchorhynchus ventralis* did not influence the multiplication of any of the three endoparasites significantly, although there was a tendency towards stimulation of *P. penetrans* in the presence of *T. ventralis*. The positive effect of serial inoculation on *P. penetrans* at low densities may either be caused by a lack of competition with the other two endoparasites in the first four weeks, or indirectly via a negative effect of *T. ventralis* on the other two endoparasites, especially on *H. arenaria* in the last eight weeks. The latter case would be a special form of apparent competition (Holt 1977), through another species on the same trophic level instead of through a shared enemy (Connell 1990). The negative effect of serial inoculation on *H. arenaria* may be attributed to the shorter development time. *Meloidogyne maritima* established so poorly, that it was not possible to find any differences between the treatments.

Greenhouse experiments differ from the field situation on several points, which should be acknowledged when extrapolating these results to field conditions. Sterilisation of soil leads to enhanced nutrient availability to the plant and enhanced plant growth (Powlsen and Jenkinson 1976, Troelstra *et al.* 2001). Therefore, plants in the greenhouse may be a better food source that can support higher nematode densities while showing limited growth reduction (Den Toom 1988). Also, the elimination of both parasitic as well as beneficial soil organisms may change both the relations between the nematodes themselves, and between the nematodes and the plant. Nevertheless, the great multiplication of *T. ventralis* in sterilised soil in a greenhouse experiment compares well to the abundance of this species in an outdoor experiment in the field with non-sterilised soil from the beach that also lacked possible natural enemies (Chapter 3). Therefore, we conclude that Vinson's (1999) hypothesis does not apply to the suppression of the ectoparasitic nematode *T. ventralis* in the root zone of the natural grass *A. arenaria* and that interspecific competition with endoparasitic nematodes is of minor importance for the

natural control of this generalist nematode species. When present in high numbers, the endoparasitic nematodes suppress the generalist *T. ventralis* to some extent, but in the field endoparasite densities (Van der Stoel *et al.* 2002) are too low to provide a realistic threat to the development of *T. ventralis* and to provide a realistic benefit to the host plant. Therefore, top-down control seems a more likely explanation for the low *T. ventralis* abundance in the root zone of *A. arenaria*.

Acknowledgements

We thank Nanda Scheerder for technical assistance with the experiment and Hans Peter Koelewijn for advice on statistical analysis. This research was supported by the Research Council for Earth and Life Sciences (ALW) with financial aid from the Netherlands Organisation for Scientific Research (NWO).

Chapter 6

General discussion

Root herbivores, among which are root-feeding nematodes, influence plant growth both directly through consumption and indirectly by adding to other stress factors and predisposing plants to attack by other pathogens. Root-feeding nematodes generally occur in multi-species communities. Interspecific interactions are likely to influence both nematode abundance and dynamics and consequently plant growth. In order to analyse the role of endoparasitic root-feeding nematodes and their interactions on the growth of the dune grass *Ammophila arenaria*, I focused on three endoparasitic nematodes (*Heterodera arenaria*, *Meloidogyne maritima* and *Pratylenchus penetrans*). The aim was to determine the influence of interspecific competition on nematode abundance and temporal dynamics and consequently on host plant biomass. In a field experiment, it was determined whether added soil organisms would inhibit *A. arenaria* less when plants were buried yearly by beach sand. In addition, interactions between endoparasitic nematodes and the ectoparasitic nematode *Tylenchorhynchus ventralis* were studied in relation to their effects on plant biomass.

Do endoparasitic nematodes contribute to plant-soil feedback?

Plants change the composition of the community of beneficial and pathogenic soil organisms, which in turn affects plant growth (Bever 1994). This so-called plant-soil feedback may be positive or negative, however, the soil community that causes the feedback effect is generally treated as a black box. I identified the role of three endoparasitic root-feeding nematodes in the development of the soil feedback to *A. arenaria*. In a field experiment, soil organisms were added to *A. arenaria* and subsequently the feedback of the established soil communities was tested in bioassays.

In the bioassays, soil from non-buried plants inoculated with root zone soil reduced the biomass of newly planted *A. arenaria* seedlings (Chapter 2). In contrast, the addition of a combination of the three endoparasitic nematodes did not have an effect on plant biomass different from the control. Thus, it is unlikely that co-occurrence of the three endoparasitic nematodes caused the development of a negative soil feedback to *A. arenaria*. We added only a small amount of root zone soil to previously uncolonised soil, so that some biological rather than a physical or chemical soil factor must have been responsible for the reduction of plant growth. Although root zone soil also contains soil organisms that directly or indirectly are beneficial for plant growth (De Boer *et al.* 1998a,b, Kowalchuk *et al.* 2002), a variety of plant-pathogenic micro-organisms and root-feeding nematodes is present besides the three endoparasitic nematodes (De Rooij-van der Goes 1995, Van der Stoel *et al.* 2002). Consequently, the addition of root zone soil with a whole soil community

introduces more potentially growth reducing soil organisms, including microbial pathogens, than addition of a combination of three endoparasitic nematodes.

The ratio of dry biomass of plants grown in non-sterilised soil to that in sterilised soil (NS:S ratio) of *A. arenaria* in the bioassays with soil from the field experiment was lower than the NS:S ratio of plants grown in beach sand. Apparently, in all the treatments in the field experiment, a growth inhibiting factor had established in the soil already in the first year after planting (Chapter 2). Sterilisation eliminates soil organisms, so that a low NS:S ratio indicates that pathogenic soil organisms reduce plant growth in non-sterilised soil (Van der Putten *et al.* 1993). However, sterilisation also causes changes in nutrient availability, which obscures comparisons between plants grown in sterilised and in non-sterilised soils (Troelstra *et al.* 2001). Indeed, in the bioassay the concentration of inorganic N in beach sand and in sterilised soil was higher than in non-sterilised soil, which may have contributed to a larger plant biomass in these soils. However, the origin of the soil in which *A. arenaria* was planted in the field experiment was the same and the concentration of the measured nutrients did not differ among the inoculation treatments. Therefore, it is likely that a biotic factor rather than differences in soil chemistry caused the observed negative effect of adding soil organisms. Although the composition of the root-feeding nematode community differed between the addition of root-zone soil and the addition of endoparasitic nematodes or the control, the abundance of endoparasitic nematodes did not correlate with plant inhibition in the bioassays. Pathogenic fungi (De Rooij-van der Goes 1995) or bacteria probably have contributed to this growth reduction.

Does co-occurrence of endoparasites influence nematode abundance and dynamics?

The three endoparasitic nematodes show variation in abundance and in timing of colonisation of the new root layer (Van der Stoel *et al.* 2002). The observed partitioning in time and space may be genetically determined and optimal for each of the nematode species. Alternatively, interspecific competition for space or feeding sites may force (some of) the species to inhabit suboptimal temporal and/or spatial conditions (Hutchinson 1957, Eisenback 1993). Consequently, the abundance and dynamics of the endoparasitic nematodes in a multi-species community may be different than in a single-species community.

Indeed, when in the field experiment *M. maritima* had been added to *A. arenaria* as single species, juveniles and males of this species were present in the new root layer earlier in the year and reached higher densities than when *P. penetrans* and *H. arenaria* had been added as well (Chapter 3). Apparently, the addition of the other two species forced *M. maritima* to use suboptimal temporal conditions for reproduction. In contrast, *H. arenaria* and *P. penetrans* were not significantly affected by addition of the other endoparasites. In a greenhouse experiment, *M. maritima* was not affected by adding *H. arenaria*, while the effect of *P. penetrans* has not been tested (Chapter 4). Interspecific competition with *P. penetrans* reduced the numbers of males of *H. arenaria*, but not other

life stages. Both in natural field stands and in the field experiment, the density of *P. penetrans* was much lower than in the greenhouse experiment, whereas the reverse was true for *M. maritima* (Van der Stoel *et al.* 2002, Chapters 3, 4 and 5). Differences in densities, in duration of the experiments and the presence of different root layers in the field may all together explain the divergence of the results of the field and greenhouse experiments.

We did not determine the nematode numbers in the root zone soil treatment throughout the year, so that we cannot directly compare the population dynamics of *M. maritima* in presence and in absence of the whole soil community. In the natural field situation, as well as in our three-species inoculation, *M. maritima* colonised the new root layer later in the growth season and the density was lower than in our single species inoculation treatment (Van der Stoel *et al.* 2002, Chapter 3). Thus, it seems likely that in the field, abundance and dynamics of *M. maritima* are determined by interspecific competition with the other endoparasites, whereas the population dynamics of *H. arenaria* and *P. penetrans* is independent of the presence of the other endoparasite species.

Does co-occurrence of endoparasites influence plant biomass?

As the abundance and dynamics of *M. maritima* was influenced by adding *H. arenaria* and *P. penetrans*, co-occurrence of the endoparasites may in turn influence the growth of the host plant.

Interestingly, in the field experiment *M. maritima* alone reduced plant biomass more than a combination of the three endoparasitic nematodes, while the whole soil community had the strongest effect (Chapter 3). On the other hand, when added alone, *H. arenaria* and *P. penetrans* did not affect plant biomass. In greenhouse experiments, the influence of nematode additions on plant biomass was quite the reverse: *M. maritima* did not reduce biomass of *A. arenaria*, whereas *H. arenaria* and *P. penetrans* did (Chapter 5). Another dissimilarity was that in the greenhouse, adding one or two endoparasite species had the same, albeit small, effect on plant biomass. The low density of *M. maritima* in the greenhouse and high density in the field experiment, and the opposite for *P. penetrans*, may explain part of the difference. In the field and when added alone, *M. maritima* colonised the new root layer earlier in the season, possibly infecting the new roots of *A. arenaria* at a more sensitive growth stage.

The effect of *M. maritima* on plant biomass occurred in the second year (Chapter 3). In the first year, the time between inoculation of the endoparasitic nematodes and harvest may have been too short to find an effect on plant biomass and all the treatments were spontaneously colonised by unnaturally high numbers of ectoparasitic *Tylenchorhynchus* spp. (De Rooij-van der Goes *et al.* 1995b, Van der Stoel *et al.* 2002). In the third year, numbers of spontaneously established *Paratylenchus* spp. increased. In all years, there was a positive effect of absence of other endoparasites on *M. maritima* numbers. The effect of *M. maritima* on plant biomass was most pronounced in the second year, when the soil was least contaminated with ectoparasitic nematodes.

Our results from the field experiment support the view that the effects of species identity and diversity may be intermingled (Huston 1997) and that species traits rather than diversity determines the effect (Bardgett 2002). However, the question remains if *M. maritima* is responsible for most of the effect of root zone soil on plant biomass. In the natural field situation, the three endoparasites usually occur together and abundance and dynamics of *M. maritima* are similar to our three-species inoculation treatment (Van der Stoel *et al.* 2002, Chapter 3). Most likely, the combination of other pathogenic (De Rooij-van der Goes *et al.* 1995b) and beneficial soil organisms (Little and Maun 1996, De Boer *et al.* 1998a,b) reduces plant biomass. In natural soils, such negative soil feedbacks appear to be more common than are positive soil feedbacks (Bever 2003).

What is the effect of sand burial on nematodes and plant biomass?

Burial with beach sand was assumed to have a positive effect on biomass of *A. arenaria* and it was supposed to counteract the negative influence of soil organisms.

The presence of *H. arenaria* in vigorous and early declining *A. arenaria*, but absence in degenerating stands (Clapp *et al.* 2000, Van der Stoel 2001), suggests that a situation without sand accumulation is not optimal for this nematode species (De Rooij-van der Goes *et al.* 1995b, Van der Stoel *et al.* 2002). Both *M. maritima* and *P. penetrans* are found in vigorous as well as in degenerating *A. arenaria*, so that they seem to be less influenced by sand burial. We therefore assumed that cessation of sand burial would result in decreased numbers of *H. arenaria*, but not of *M. maritima* and *P. penetrans*. On the contrary, when sand burial ceased, *H. arenaria* numbers only decreased temporarily, while as expected, the already low numbers of *P. penetrans* were not affected (Chapter 3). When sand burial ceased, *M. maritima* did not benefit from the absence of *H. arenaria* and *P. penetrans* and the numbers remained lower than when plants had been buried. This suggests that *M. maritima* does not contribute to the degeneration of *A. arenaria* in stabilised dunes.

We expected that burial with sand would enable the plants to withstand negative effects of added soil organisms, but did not find an interaction between sand burial and addition treatments. Cessation of burial did not immediately lead to decreased plant growth. On the contrary, it led to an increase in plant growth in the second year, but not in the third year (Chapters 2 and 3). Sand burial did not lead to an increase in aboveground shoot biomass in the second year, but it did so in the third year. Burial with sand had a positive effect on belowground shoot and root biomass, while it did not affect total (above- and belowground) shoot biomass (Chapter 3). On the other hand, degenerate *A. arenaria* in the field and young *Ammophila* plants in the greenhouse responded positively to burial with sand (Yuan *et al.* 1993, De Rooij-van der Goes *et al.* 1995a, Little and Maun 1996). On the long term, *Ammophila* stands are known to decrease in vigour when burial ceased (Huiskes 1979, Wallén 1980, Eldred and Maun 1982, Disraeli 1984). Thus, *Ammophila* spp. seems to respond faster to sudden burial with sand than to cessation of burial.

What is the effect of timing on competition between endoparasites and plant biomass?

In spring, *H. arenaria* is the first endoparasitic nematode to move to a newly developing root layer (Van der Stoel *et al.* 2002), so that we studied competition of this species with an endoparasitic nematode with a similar (*M. maritima*) and one with a different feeding behaviour (*P. penetrans*). The observed partitioning in time in the field may reduce the effect of competition, so that inoculation in the natural order of appearance may have a positive effect on nematode abundance.

Serial inoculation did not affect competition between *H. arenaria* and *P. penetrans*, whereas it had a minor effect on competition between *H. arenaria* and *M. maritima* (Chapter 4). When inoculated later than *M. maritima*, males of *H. arenaria* tended to increase in numbers at low densities, but decrease at high densities. Generally, a large number of males is regarded to be an indication of suboptimal feeding circumstances (Yeates 1987). However, in this case more males were observed particularly at the low density, when competition was supposed to be minimal. These two-species competition experiments showed that the effect of competition is not reduced by partitioning in time, as observed in natural field stands. On the contrary, the field experiment showed that interspecific competition is the cause of partitioning in time (Chapter 3). Therefore, I conclude that care should be taken to unravel biotic interactions observed in the field by greenhouse experiments.

Serial inoculation of *H. arenaria* and *P. penetrans* did not affect plant biomass (Chapter 4). This is in line with the observation that serial inoculation did not influence nematode numbers. In contrast, serial inoculation of either *H. arenaria* or *M. maritima* tended to decrease shoot and root biomass at low densities, but to increase shoot and root biomass at high densities. These observations suggest that, depending on the inoculation densities, serial inoculation may enlarge or decrease the infection pressure on the plant. The effects on plant biomass are not explained by the nematode data, may be due to the fact that we determined nematode numbers only at the end of the experiment.

What is the effect of plant age on competition between endoparasites and plant biomass?

Competition for feeding sites is one of the factors that limit nematode establishment and reproduction (Duncan and Ferris 1983). Therefore, we examined whether nematode competition and plant sensitivity to parasitic nematodes would diminish with increasing size of the root system at the time of inoculation.

Older plants supported a larger population of *H. arenaria* and *M. maritima* than plants that had been pre-cultured for a shorter period of time (Chapter 4). However, older plants were less favourable for *P. penetrans* when in competition with *H. arenaria*. Root exudates of older plants, which stimulated more hatching of some *Globodera* spp. (Lamondia 1995, Byrne *et al.* 2001), may have affected hatching of the added eggs of *H. arenaria* as well. In contrast, the effect of plant age on some *Meloidogyne* spp. was either neutral or negative

(Gergon *et al.* 2002, McLeod *et al.* 2002). In the field, numbers of *Pratylenchus* sp. were higher in younger than in older roots (Olthof 1982, C.D. van der Stoel, pers. comm.). The results show that, besides the size of the root system (Yeates 1987), also the age of the roots determines the size of the nematode population.

The simultaneous increase in *H. arenaria* and decrease in *P. penetrans* numbers was correlated with a reduced inhibition of root biomass of older plants (Chapter 4). The simultaneous increase in *H. arenaria* and *M. maritima* numbers on older plants resulted in an equal effect of nematodes on younger and older plants. Thus, depending on the combination of nematode species, older plants may be less or equally sensitive to infection.

Can endoparasites regulate the abundance of ectoparasites?

At the start of the field experiment, before and just after addition of the endoparasites to the plants, the numbers of *T. ventralis*, *T. microphasmis* and *T. nanus* were higher than ever recorded in natural field stands (De Rooij-van der Goes *et al.* 1995b, Chapter 3, H. Duyts, pers. comm.). When the experiment proceeded and the added endoparasitic nematodes established, the density of the three *Tylenchorhynchus* spp. declined. This decline may have been caused by the endoparasites, but it may also have been due to other (micro-)organisms that had colonised the soil spontaneously.

In order to establish whether the correlation between endoparasite inoculation and the decline of *Tylenchorhynchus* spp. abundance was due to competitive exclusion, a greenhouse experiment was performed. My hypothesis was that endoparasitic nematodes, which may be bottom-up controlled by *A. arenaria*, through competition reduce the numbers of *T. ventralis*, which cannot be controlled by the plant. This hypothesis has also been postulated for aboveground specialist and generalist insects (Vinson 1999). In the greenhouse experiment, endoparasites reduced the numbers of *T. ventralis*, but only when the endoparasites were present in numbers that exceed normal field densities (Van der Stoel *et al.* 2002, Brinkman *et al.* 2004: Chapter 5). It is, therefore, not likely that endoparasites reduce the abundance of *T. ventralis* in the field by interspecific competition. The density of *T. ventralis* is neither bottom-up controlled by the plant, unless the plant suffers from extreme growth reduction (De Rooij-van der Goes 1995, Chapters 3 and 5). Hence, top-down control by bacteria and fungi (Kerry 2000), predatory nematodes, insects or mites (Akhtar and Malik 2000) is more likely limiting the density of *T. ventralis* in the root zone of *A. arenaria*.

The temporal distribution of endoparasitic nematodes in the field (Van der Stoel *et al.* 2002) was assumed to have an additional suppressive effect on *T. ventralis*. The presence of at least one of the endoparasitic nematode species at any time of the growth season would ascertain continuous interspecific competition. However, my results showed that serial inoculation of the endoparasites did not cause additional inhibition of *T. ventralis* multiplication compared to simultaneous inoculation of all endoparasites at the start of the experiment (Chapter 5). Possibly, the later inoculation of *H. arenaria* and *M. maritima* was

less effective because the root system had increased in size, decreasing the likeliness of competition with *T. ventralis*. Since this may also be the case in the field, it seems quite unlikely that interspecific competition, even by a multi-species community of endoparasites, limits the abundance of *T. ventralis* in the field.

Although in the greenhouse experiment the endoparasites reduced the numbers of *T. ventralis*, they did not diminish the effect of *T. ventralis* on plant biomass (Chapter 5). On the contrary, the endoparasitic nematodes themselves had a slightly negative effect on plant biomass. Thus, our results support Vinson's (1999) hypothesis in that specialist endoparasites can reduce numbers of a generalist nematode. However, opposite to Vinson's (1999) assumption for insects, endoparasitic nematodes are not capable to protect the plant from growth reduction by generalist nematodes.

This study showed, that:

- In the field, *M. maritima* was less abundant and delayed in development because of interspecific competition with *H. arenaria* and *P. penetrans*. When *M. maritima* was present alone, high abundance and early development correlated with reduced biomass of *A. arenaria*.
- *Heterodera arenaria* and *P. penetrans* were not significantly influenced by the presence of the other endoparasites. When *H. arenaria* or *P. penetrans* was added alone or when the three endoparasitic nematodes were added together, plant biomass was not reduced.
- The whole soil community of *A. arenaria* reduced plant biomass more than one or three endoparasitic nematodes.
- Greenhouse inoculation experiments did not always represent effects of outdoor interactions well, which was partly due to differences in performances of endoparasitic nematodes in the greenhouse compared to outdoors.
- Inoculation of endoparasitic nematode species with a time interval had a similar effect on nematode numbers and plant biomass as simultaneous inoculation.
- Both size and age of the root system determined the size of the nematode population. Depending on the combination of nematode species, older plants were equally or less sensitive to infection than younger plants.
- The endoparasites, when present in higher numbers than in the field, reduced the numbers of *T. ventralis*. However, they did not protect the plant from growth reduction by *T. ventralis*.

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Summary

Plants are influenced by above- and belowground herbivores and their interactions. Root feeders, among which are nematodes, directly consume part of the belowground primary production and interact with other biotic and abiotic factors that influence plant growth. Natural ecosystems provide an opportunity to study interactions between host plants and co-occurring root-feeding nematode species that have co-evolved for a longer period than in agricultural systems. Root-feeding nematodes generally occur in multi-species communities, so that interspecific interactions are likely to influence nematode abundance and dynamics and, subsequently, plant growth. I studied interactions among three endoparasitic root-feeding nematodes that generally co-occur on the pioneer dune grass *Ammophila arenaria* (marram grass).

In a field experiment, different combinations of endoparasitic nematodes and other soil organisms were added to *A. arenaria*. Subsequently, the feedback of the established soil communities was tested in bioassays. Soil from non-buried plants inoculated with a mixture of organisms from the root zone of *A. arenaria* reduced the biomass of newly planted seedlings. In contrast, addition of a combination of the three endoparasitic nematodes *Heterodera arenaria*, *Meloidogyne maritima* and *Pratylenchus penetrans* did not affect plant biomass differently from the control. Thus, it is unlikely that co-occurrence of the three endoparasitic nematodes caused the development of a negative soil feedback to *A. arenaria*. Most likely, (interactions with) other soil organisms from the root zone soil have been responsible for the reduction in plant biomass.

When in the field experiment *M. maritima* had been added to the plants alone, juveniles and males of this species occurred in the new root layer earlier in the year and in higher densities than when *H. arenaria* and *P. penetrans* had been added as well. Apparently, addition of the other two species forced *M. maritima* to develop under suboptimal conditions. In contrast, *H. arenaria* and *P. penetrans* were not significantly affected by addition of the other endoparasite species.

In greenhouse experiments, *M. maritima* and *H. arenaria* did not affect each other, whereas *P. penetrans* reduced the numbers of males of *H. arenaria*. Thus, the greenhouse experiments, aimed at studying interactions between different pairs of nematode species, did not explain the observations from the field experiment. Nevertheless, in natural field stands, the usually observed abundance and timing of migration of *M. maritima* is quite comparable to the inoculation treatment with three nematode species. Moreover, in the greenhouse *M. maritima* did not establish well. Therefore, I conclude that in the natural situation, the abundance and dynamics of *M. maritima*, but not of *H. arenaria* and *P. penetrans*, are determined by interspecific competition with the other two endoparasites.

Interestingly, in the field experiment *M. maritima* reduced plant biomass more when added alone than when added together with the other two endoparasites, whereas addition of root zone soil reduced plant biomass most. In the field experiment, *H. arenaria* and *P. penetrans* did not affect plant biomass. In greenhouse experiments, the influence of

nematode additions on plant biomass was the reverse: *M. maritima* did not influence plant biomass, whereas *H. arenaria* and *P. penetrans* did. In the greenhouse, addition of two nematode species did not enhance the growth reducing effect on plant biomass. The density of *M. maritima* was low in the greenhouse and high in the field experiment, while the density of *P. penetrans* was high in the greenhouse and low in the field experiment. Therefore, the results of the greenhouse experiments may not necessarily stand for the magnitude of effects to be expected in the field.

My results from the field experiment support the view that the effects of species identity and diversity may be intermingled and that species traits rather than diversity determines the effect. In the natural field situation, the three endoparasites usually occur together and abundance and timing of migration of *M. maritima* are similar to our three-species inoculation treatment. It is therefore more likely that in field stands, the wealth of pathogenic and beneficial soil organisms together reduces plant biomass rather than *M. maritima* alone.

In greenhouse experiments, serial inoculation did not affect competition between *H. arenaria* and *P. penetrans*, whereas it had a minor effect on competition between *H. arenaria* and *M. maritima*. This was expressed only in a change in the numbers of *H. arenaria* males. These results do not support the assumption that the partitioning in time, as observed in previous studies, reduces the effect of competition. The field experiment showed that the partitioning in time, especially of *M. maritima*, is a result of interspecific competition. In addition, the age of the plants influenced the development of the nematodes. When added to older plants, numbers of *H. arenaria* and *M. maritima* were higher, whereas numbers of *P. penetrans* were lower. This shows that, besides the size of the root system, also the age of the roots influences the size of the nematode population. Depending on the combination of nematode species, older plants were less or equally sensitive to infection.

At the start of the field experiment, the densities of ectoparasitic *Tylenchorhynchus* spp. were unnaturally high. When the experiment proceeded and the endoparasites established, the densities of *Tylenchorhynchus* spp. declined. In a greenhouse experiment, the numbers of *T. ventralis* could only be limited by adding an unnaturally high density of endoparasites. Thus, the decline in the field experiment was unlikely to be caused by the endoparasites, but by other (micro-) organisms that spontaneously colonised the soil. Despite their limiting effect, the endoparasites could not prevent *T. ventralis* from reducing plant biomass. In contrast, the endoparasites themselves had a minor limiting effect on plant biomass.

In conclusion, it seems likely that in the field, abundance and dynamics of *M. maritima* are determined by interspecific competition with *H. arenaria* and *P. penetrans*. The latter two endoparasitic nematodes are less influenced by the presence of the other endoparasites than is *M. maritima*. Interestingly, *M. maritima*, when added alone, reduced plant biomass, whereas a combination of the three endoparasites did not. Addition of root

zone soil of *A. arenaria* reduced plant biomass most, so that the negative soil feedback is likely to be caused by the whole soil community.

Samenvatting

Planten worden beïnvloed door boven- en ondergrondse planteneters en hun interacties. Wortelherbivoren, waaronder nematoden, verbruiken direct een deel van de ondergrondse primaire productie en staan in wisselwerking met andere biotische en abiotische factoren die de plantengroei beïnvloeden. Natuurlijke ecosystemen bieden de mogelijkheid om de wisselwerking te bestuderen tussen waardplanten en daarop voorkomende wortelherbivore nematodensoorten met een langere co-evolutie dan in agrarische systemen. Wortelherbivore nematoden komen meestal voor in gemeenschappen die bestaan uit meerdere soorten, zodat interspecifieke interacties waarschijnlijk van invloed zijn op de dichtheid en de dynamica van de nematoden en uiteindelijk op de plantengroei. Ik heb interacties onderzocht tussen drie endoparasitaire wortelherbivore nematoden die meestal gezamenlijk voorkomen op *Ammophila arenaria* (helm), een pioniersgras in de duinen.

In een veldexperiment werden verschillende nematodencombinaties aan *A. arenaria* toegevoegd. Vervolgens werd de terugkoppeling van de gevestigde bodemgemeenschappen in biotoetsen bepaald. Grond van niet-overstoven planten, waaraan wortelzonezand met de hele bodemgemeenschap van *A. arenaria* was toegevoegd, verminderde de biomassa van daarin geplante zaailingen. Daarentegen had toevoegen van een combinatie van de drie endoparasitaire nematoden *Heterodera arenaria*, *Meloidogyne maritima* en *Pratylenchus penetrans* hetzelfde effect op plantenbiomassa als de controle. Het is daarom onwaarschijnlijk dat het gezamenlijk voorkomen van de drie endoparasitaire nematoden een negatieve terugkoppeling naar *A. arenaria* veroorzaakt. Waarschijnlijk zijn (interacties met) andere bodemorganismen in het wortelzonezand verantwoordelijk voor het afnemen van de plantenbiomassa.

Als in het veldexperiment *M. maritima* alleen aan planten werd toegevoegd, waren juvenielen en mannetjes van deze soort vroeger in het jaar aanwezig in de nieuwe wortellaag en behaalden hogere dichtheden dan wanneer *H. arenaria* en *P. penetrans* eveneens aanwezig waren. Blijkbaar dwong het toevoegen van de andere twee soorten *M. maritima* ertoe om zich onder suboptimale omstandigheden te ontwikkelen. De aanwezigheid van de andere endoparasieten had echter geen aantoonbare invloed op *H. arenaria* en *P. penetrans*.

In kasexperimenten hadden *M. maritima* en *H. arenaria* geen invloed op elkaar, terwijl het aantal *H. arenaria* mannetjes lager was in aanwezigheid van *P. penetrans*. De kasexperimenten, waarin paarsgewijze interacties tussen nematodensoorten werden bestudeerd, verklaarden de waarnemingen uit het veldexperiment dus niet. Op grond van voorgaand onderzoek is het bekend dat de dichtheid en het migratietijdstip van *M. maritima* in natuurlijke helmbestanden vergelijkbaar zijn met die in het door mij uitgevoerde veldexperiment met de drie nematodensoorten. Vermoedelijk zijn onder natuurlijke omstandigheden de dichtheid en de dynamica van *M. maritima*, maar niet van

H. arenaria en *P. penetrans*, bepaald door interspecifieke competitie met de andere twee endoparasieten.

Opvallend genoeg remde *M. maritima* in het veldexperiment de plantenbiomassa sterker wanneer deze nematode alleen was toegevoegd dan wanneer de andere twee endoparasieten aanwezig waren, terwijl toevoegen van wortelzonezand de plantenbiomassa het sterkst remde. Toevoegen van alleen *H. arenaria* of *P. penetrans* had echter geen invloed op de plantenbiomassa. In kasexperimenten was het effect van toevoegen van nematoden tegenovergesteld: niet *M. maritima*, maar *H. arenaria* en *P. penetrans* hadden negatieve invloed op de plantenbiomassa. In de kas had het toedienen van één of twee nematodensoorten hetzelfde, hoewel geringe, effect op de plantenbiomassa. De dichtheid van *M. maritima* was laag in de kas en hoog in het veld, terwijl de dichtheid van *P. penetrans* hoog was in de kas en laag in het veld, hetgeen het verschil tussen kas- en veldexperimenten gedeeltelijk zou kunnen verklaren.

De resultaten uit het veldexperiment ondersteunen de opvatting dat het effect van soortidentiteit en –diversiteit verstrengeld kan zijn en dat soortkenmerken sterker bepalend zijn voor het effect dan diversiteit per sé. In de veldsituatie komen de drie endoparasieten gewoonlijk gezamenlijk voor en de dichtheid en het tijdstip van migratie lijken op die van onze experimentele behandeling met drie soorten in het veldexperiment. Het is daarom waarschijnlijker dat het totaal aan ziekteverwekkende en nuttige bodemorganismen samen de plantenbiomassa reduceren dan dat de groeiremming van helm het gevolg is van de activiteit van *M. maritima* alleen.

In kasexperimenten had opeenvolgende inoculatie geen effect op de competitie tussen *H. arenaria* en *P. penetrans*, maar wel een klein effect op de competitie tussen *H. arenaria* en *M. maritima*. Dit tijdseffect kwam echter alléén tot uiting in een verandering van het aantal *H. arenaria* mannetjes. Deze resultaten vormen geen ondersteuning van de aanname dat de waargenomen verdeling in de tijd het effect van competitie vermindert; uit het veldexperiment bleek immers we dat de verdeling in tijd juist een gevolg is van interspecifieke competitie. Daarnaast speelde de leeftijd van de planten nog een rol bij de ontwikkeling van de nematoden. Bij toevoegen aan oudere planten was het aantal *H. arenaria* en *M. maritima* hoger, maar het aantal *P. penetrans* lager. Dit laat zien dat naast de grootte van het wortelsysteem ook de leeftijd van de wortels van invloed is op de grootte van de nematodenpopulatie. Afhankelijk van de nematodencombinatie waren oudere planten minder of even gevoelig voor infectie.

Aan het begin van het veldexperiment was de dichtheid van de ectoparasitaire *Tylenchorhynchus* spp. ongewoon hoog. Terwijl het experiment voortduurde en de endoparasieten zich vestigden, namen de aantallen *Tylenchorhynchus* spp. af. In een kasexperiment konden de aantallen *T. ventralis* slechts worden beperkt door het toedienen van een onnatuurlijk hoge dichtheid endoparasieten. Daarom werd de aantalsafname in het veldexperiment waarschijnlijk niet veroorzaakt door de endoparasieten, maar door andere (micro-) organismen die de bodem spontaan koloniseerden. Ondanks hun beperkende invloed, konden de endoparasieten niet voorkomen dat *T. ventralis* de

plantenbiomassa beperkte. De endoparasieten hadden zelf een gering groeiremmend effect op de plant.

Concluderend is het aannemelijk dat de dichtheid en de dynamica van *M. maritima* worden bepaald door interspecifieke competitie met *H. arenaria* en *P. penetrans*. De laatste twee endoparasitaire nematoden worden minder beïnvloed door de aanwezigheid van de andere endoparasieten dan dat bij *M. maritima* het geval is. Het was opvallend dat *M. maritima* alléén wel de plantenbiomassa reduceerde, maar een combinatie van de drie endoparasieten niet. Toevoegen van wortelzonezand van *A. arenaria* verminderde de plantenbiomassa het meeste, zodat het waarschijnlijk is dat de gehele bodemgemeenschap verantwoordelijk is voor de negatieve terugkoppeling.

Nawoord

Een proefschrift komt niet door één persoon tot stand. Een aantal mensen wil ik met name bedanken voor de hulp en steun die zij hebben verleend.

Het waterschap De Brielse Dijkring stelde de locatie voor de veldproef ter beschikking en zorgde voor ondersteuning tijdens overstuiven en oogsten. Ruud Dekker, Aart Voskamp en Johan van Eersel, bedankt voor het regelen van materieel, hulp met het werk, jullie praktische inzicht en de belangstelling voor de resultaten van dit werk.

Daarnaast wil ik mijn begeleiders bedanken. Wim van der Putten, als directe begeleider en inmiddels ook promotor had ik het meeste met jou te maken. Met jouw praktische aanpak lukte het om een groot veldexperiment uit te voeren. Ik waardeer je grenzeloze optimisme en zonder je snelle correcties had het schrijven zeker langer geduurd. Hans van Veen, jij hield overzicht en wist me op het juiste moment te stimuleren om het schrijven weer serieus op te pakken. Sep Troelstra, ik waardeer je interesse, je oog voor detail en de gedrevenheid waarmee je een probleem aanpakt.

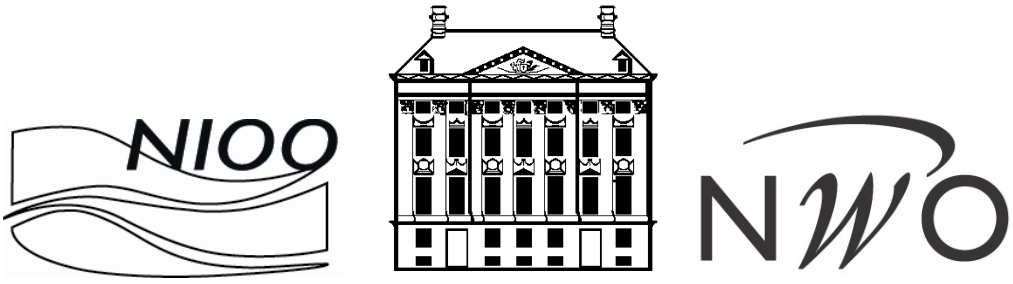
Henk Duyts, dankjewel voor het tellen van vele nematodenmonsters en het bijspringen waar nodig. Leuk dat je me liet meegenieten als je een bijzonder beest had gevonden. De stagiaires Diana Tap, Nico van der Feest en Nanda Scheerder hebben veel werk verzet. Bedankt voor de fijne samenwerking. Het veldexperiment was een grote klus en veel mensen waren bereid om ‘s morgens vroeg te vertrekken en de kou te trotseren. André, Arjan, Catarina, Cindy, Diana, Frank, George, Gerard, Hans, Henk, Jan Willem, Leontien, Martijn B., Martijn K., Nanda, Nico, Roel, Sep, Tycho en Wim, dankjewel. Gregor, bedankt dat je steeds ruimte wist te vinden in het fytotron om experimenten uit te voeren en zandmonsters te bewaren. Hans Peter Koelewijn en Martijn Bezemer hielpen me op weg met statistische analyses, veel dank daarvoor. Ineke, Gerlinde en later Anna en An, mede-AIO’s en OIO’s met nematoden als studie-object, bedankt voor alle goede gesprekken over werk en andere zaken. Jullie enthousiasme zorgde voor een fijne werksfeer.

Alle kamergenoten door de jaren heen, Michel, Ingeborg, Jan Willem, Frank, Gerard, Nicole, Anna, An en Hanneke, bedankt voor de vrolijke en ernstige gesprekken en jullie relativerende woorden. Agata, Christel en Ivonne, bedankt voor de gezelligheid als fietsmaatjes en eetgroepgenoten. Alle andere collega’s, jullie zorgden voor een omgeving waarin het plezierig werken was.

Het laatste jaar bracht werken in het weekeinde met zich mee dat ik weinig tijd had om familie en vrienden te bezoeken. Bedankt voor jullie begrip daarvoor. Lieve Arjan, dankjewel voor alle steun van begin tot eind. Je hebt me steeds gestimuleerd om door te gaan met schrijven, ondanks dat we elkaar daardoor het laatste jaar weinig zagen. Ik geniet er nu extra van om bij elkaar te zijn. Lennart, nu zijn we weer vaker ‘samen’.

Curriculum Vitae

Elsa Pernilla (Pella) Brinkman werd op 18 juli 1972 geboren te Ede. In 1991 begon ze de studie Plantenziektekunde aan de toenmalige Landbouwuniversiteit Wageningen. In een eerste afstudeervak aan het toenmalige Proefstation voor de Akkerbouw en de Groenteteelt in de Vollegrond (PAGV) te Lelystad onderzocht ze de biologische bestrijding van de akkeraardslak (*Deroceras reticulatum*) met de parasitaire nematode *Phasmarhabditis hermaphrodita*. In een tweede afstudeervak bij de vakgroep Fytopathologie van de Landbouwuniversiteit Wageningen deed ze onderzoek naar het bepalen van de dichtheid van *Verticillium dahliae* in de grond. Tijdens haar stageperiode op het Natural Resource Ecology Laboratory aan Colorado State University (VS) bestudeerde ze de invloed van extractietechnieken op de verdeling van nematoden over verschillende trofische groepen. Hier werd ze direct na haar afstuderen in 1996 aangesteld als 'research associate'. In 1998 werd zij aangesteld als onderzoeker in opleiding bij het Centrum voor Terrestrische Ecologie van het Nederlands Instituut voor Ecologie (NIOO-KNAW) te Heteren. De resultaten van dat onderzoek zijn beschreven in dit proefschrift.



The research described in this thesis (NIOO Thesis 32) was conducted at the department of Multitrophic Interactions, Centre for Terrestrial Ecology of the Netherlands Institute of Ecology (NIOO-KNAW) in Heteren. The project was supported by the Research Council for Earth and Life Sciences (ALW) with financial aid from the Netherlands Organisation for Scientific Research (NWO).