Interactions of plant parasitic nematodes and their natural enemies in coastal foredunes

Promotor:

Prof. dr. ir. Wim H. van der Putten

Hoogleraar Functionele biodiversiteit met bijzondere aandacht voor de rol van nematoden in multitrofe interacties, Wageningen Universiteit

Promotiecommissie:

Prof. dr. ir. Ariena H. C. van Bruggen
Wageningen Universiteit, Nederland

Prof. dr. Brian Kerry
Rothamsted Research, United Kingdom

Prof. dr. ir. Maurice Moens
Gent University and Agricultural Research Centre in Merelbeke, Belgium

Prof. dr. Peter C. de Ruiter
Universiteit Utrecht, Nederland

Dit onderzoek is uitgevoerd binnen de onderzoeksschool Production Ecology and Resource Conservation.

Anna Marta Piśkiewicz

Interactions of plant parasitic nematodes and their natural enemies in coastal foredunes

Proefschrift

Ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. M.J. Kropff
in het openbaar te verdedigen
op maandag 26 november 2007
des middags te half twee in de Aula

Piśkiewicz, A.M. (2007)
Interactions of plant parasitic nematodes and their natural enemies in coastal foredunes.

PhD thesis Wageningen University – with a summary in Dutch

ISBN 978-90-8504-798-8

Mamie i Tacie

Abstract

This thesis aimed at revealing the interactions between plant ectoparasitic nematodes, the host plant *Ammophila arenaria* and the natural enemies of the nematodes in a natural dune ecosystem. The ectoparasitic nematode *Tylenchorhynchus ventralis* seems to be a key root-feeding species because it is able to reduce the growth of its host plant. However, usually the numbers of *T. ventralis* in the field soil are too low to influence the performance of the host plant. This suggests that the abundance of *T. ventralis* is controlled to low, non-damaging densities. Nematode control may be due to plant effects ('bottom-up' mechanisms), competition with other nematodes ('horizontal' mechanisms) and the suppression by natural enemies of nematodes ('top-down' mechanisms). Previous studies showed that bottom-up and horizontal effects are not affecting the abundance of *T. ventralis* strong enough to explain the severe control in dune soil.

In the present thesis, I show that the abundance of ectoparasitic nematodes *T. ventralis* can be controlled by microbial enemies. Other soil organisms like nematodes and microarthropods do not play any important role in this control. Suppressive effects of microbial enemies of *T. ventralis* are due to local interactions. These may be caused by microbial parasitism, predation or antagonism or by local induction of defense responses. However, the precise mechanism of these interactions remains unrevealed. I also show that in natural dune soil *T. ventralis* is able to avoid plant roots with detrimental microorganisms. This may occur due to repellence caused by odors produced by roots with microorganisms or stronger attraction to the 'clean' roots.

Finally, I examine top-down control of eight dominant plant parasitic species (six of them are ectoparasites and two endoparasites) by soil microorganisms, nematodes and microarthropods in natural coastal dune soil. I conclude that soil microorganisms are able to exert controlling effects on the majority of root-feeding nematode species from *A. arenaria*. However, some of the nematode species are additionally controlled by other nematodes and / or microarthropods. I combine the results of my experiments with the available published data on nematode control in dune soil. The outcome of this comparison shows that the control of root-feeding nematodes from the *A. arenaria* root zone does not depend only on nematode feeding type, but also on the species of the nematode. I conclude that in the successful control of root-feeding nematodes more than one control factor may be involved.

Contents:

Chapter 1

General Introduction

Nematodes

Nematodes (also known as roundworms) are the most abundant and successful group of metazoans (Bird & Kaloshian 2003). They are common in terrestrial and aquatic ecosystems and include a wide range of feeding types (Yeates et al. 1993). Nematodes can feed on bacteria, fungi, algae, plants, and they can also parasitize insects, animals and humans. The range of nematode body size can vary from 0.5 mm (plant parasites) till several meters (parasites of animals). Nematodes have a huge impact on human life - human parasites cause severe diseases (for example *Ascaris*) and plant parasites lead to huge losses in agriculture, which are estimated worldwide as much as 100 billion US dollars annually (Bird & Kaloshian 2003). All parts of plants can be invaded by plant feeding nematodes, but the root feeders are economically the most important since they are mainly responsible for yield losses. In natural grasslands, plant parasitic nematodes have been estimated to take up around a quarter of the net primary production (Stanton 1988).

The feeding modes of plant parasitic nematodes differ according to the plant parts they feed upon. They can feed from the outside of the roots on outer cortical cell layers, while never entering the roots with more than the feeding stylet. These are ectoparasitic nematodes and they are considered to be feeding generalists (van der Putten et al. 2005; Yeates et al. 1993). Plant parasitic nematodes, which enter and feed inside roots are called endoparasites, some of which are feeding specialists (Yeates et al. 1993).

The traditional methods of reducing plant parasitic numbers in agriculture include cultural practices, crop rotation and the use of chemical pesticides. Pesticides may cause heavy environmental pollution, for example water contamination and toxicity to animals and humans. These negative effects on the environment led to restrictions in nematicide use and are nowadays less widely applied than in the past. Newer methods of nematode suppression include organic matter addition (Akhtar & Alam 1993; Akhtar & Malik 2000; Vawdrey & Stirling 1997; Widmer et al. 2002) and biocontrol practices (Alabouvette et al. 2006; Kerry & Gowen 1995; Kerry & Hominick 2002; Meyer & Roberts 2002; Oka et al. 2000; van Bruggen et al. 2006). Moreover, the studies on plants resistant against nematode infections give promising results (Strauss & Agrawal 1999; Williamson & Kumar 2006).

What affects the abundance of plant parasitic nematodes?

Nematodes may be limited by abiotic and controlled by biotic factors. The term 'regulation' is connected to the factors, which are density-dependent (Hassell et al. 1998). Therefore, I mainly use the term 'control' which is more open to other factors and processes that may limit abundance. The mechanisms that control the abundance of species are one of the main issues in current ecology.

Abiotic factors

Nematodes are present in soils as multi-species communities. The individual species occupy different niches depending on their feeding habits. Nematodes can be limited by chemical and physical soil properties. They are extremely sensitive to the soil water content and soil moisture can be a limiting factor for nematode survival because they inhabit water-filled pore spaces. Soil structure, particle size and pH determine, for example, species composition and nematode numbers (Griffin 1996; Mulder et al. 2005; Zasada et al. 2007). Life cycle and reproduction of the nematodes are connected to the temperature in soil (Papatheodorou et al. 2004; Todd et al. 1999). It is relatively easy to assess how abiotic factors would influence one population of plant parasitic nematodes, but there are some difficulties to assess these effects for the entire nematode community (Norton 1989).

Biotic factors

Nematodes occur in food webs where their abundance can be controlled by different mechanisms (de Ruiter et al. 1993). Populations of plant parasitic nematodes can be suppressed by host plants via so-called 'bottom-up' effects (de Deyn et al. 2004). Furthermore, 'horizontal' effects - competition between different nematode species can lead to suppression of the nematode numbers. Nematodes compete for food and space and the nature of this competition can be physiological, as well as physical. These 'horizontal' effects are often determined by host suitability, pathogenicity, mode of parasitism, time and nematode population density (Khan 1993). In general, endoparasites are more competitive than ectoparasites and in addition, sedentary ectoparasites are more competitive than migratory ones (Eisenback 1993). Finally, the abundance of plant parasitic nematodes can

be suppressed 'top-down' by their natural enemies. The natural enemies of plant parasitic nematodes include fungi, bacteria, predacious invertebrates and viruses. These natural enemies of nematodes are often used in agricultural practices to suppress the populations of plant parasites (Kerry & Hominick 2002).

Fungi, which are detrimental for nematodes, belong to different ecological groups, including endoparasitic, predacious, opportunistic, plant pathogenic and mycorrhizal fungi. They can have suppressive effects on nematode multiplication by parasitism, predation or antagonism (Siddiqui & Mahmood 1996). The suppressive effects of fungi are much better studied for endoparasitic nematodes than ectoparasites. The best described fungal antagonists of root-feeding nematodes are *Verticillium chlamydosporium* (now named *Pochonia chlamydosporia*) (Kerry 1995) and *Paecilomyces lilacinus* (Walters & Barker 1994).

Another important group of soil fauna having detrimental effects on plant parasitic nematodes are bacteria. The suppression of nematodes might be caused by bacterial metabolites which reduce egg hatching or attraction to the roots. These metabolites can also degrade specific root exudates controlling nematode behavior (Siddiqui & Mahmood 1999). Soil bacteria causing the decrease in plant feeding nematodes can be divided into two groups: parasitic and non-parasitic to nematodes. The best studied is *Pasteuria penetrans*, which is an obligate parasite of nematodes (Chen 1998, Davies 1988).

Predacious mites and predacious nematodes (Yeates & Wardle 1996) can be essential control agents for certain nematodes (Imbriani & Mankau 1983). Nevertheless, the practice to use predators as the control agents in agriculture is very rare. Other organisms may be important for nematode abundance – for example in the presence of earthworms the dispersal of the nematodes can be enhanced (Shapiro et al. 1993)

Interactions in coastal dunes

To study the interactions between the soil organisms and host plants, I used the natural coastal dune system with *Ammophila arenaria* as a model. *A. arenaria* (marram grass) is an important species of coastal foredune plants, because it naturally fixes sand, stabilizes foredunes and protects them to be blown away by wind (Huiskes 1979). The wild grass is

artificially established frequently in order to protect coastal dunes from erosion. In mobile foredunes, the grass grows vigorously; however, inland where no sand deposition occurs, it degenerates. Therefore, the performance of *A. arenaria* depends on regular burial by wind-deposited beach sand that occurs regularly in yearly cycles. In autumn and winter, a new sand layer is accreted by wind, the new nodes are produced, the internodes elongate, and then in spring the roots are formed. Afterwards, in summer, the shoots elongate and the roots develop from the buried stems into the new sand layer. In autumn, the sand deposition again occurs and the whole process is repeated.

Different possible explanations were proposed in order to explain how *A. arenaria* benefits from regular sand burial. Some authors concluded that sand accretion enables adventitious root growth (Marshall 1965), the plants are supplied with nutrients in a new layer (Willis 1965) or the roots are associated with mycorrhizal fungi (Ernst et al. 1984; Kowalchuk et al. 2002; Little & Maun 1996) and nitrogen-fixing bacteria (Abdel Wahab 1975; Hassouna & Wareing 1964). However, these explanations did not fully answer the question why *A. arenaria* profits from sand deposition. Another explanation of this phenomenon is that regular burial with sand enables the plants to escape upwards from soil-borne pathogens and root-feeding nematodes (van der Putten & Troelstra 1990; van der Putten et al. 1988). It was demonstrated that the *A. arenaria* decline is not caused by a single pathogen, but rather a combination of pathogenic fungi and nematodes (de Rooij van der Goes 1995). Usually, the presence of nematodes enhances the pathogenicity mechanism of the fungus (Khan 1993). A mixture of all common fungi occurring in coastal dune soil could reduce *A. arenaria* biomass production to about 80% when compared to the plants growing in sterilized soil (de Rooij van der Goes 1995). Thus far, it is unknown if '*Ammophila* decline' is caused by one or some major pathogens, or by a whole community of minor pathogens.

In natural coastal dunes *A. arenaria* is parasitized by a range of plant endo- and ectoparasitic nematodes. However, usually in field these nematodes are suppressed to non-damaging densities (Figure 1). Ectoparasites in natural coastal dunes are represented by *Criconema* sp., *Helicotylenchus pseudorobustus* (Steiner 1914) Golden, 1956, *Hemicycliophora* sp., *Rotylenchus* sp., *Tylenchorhynchus ventralis* (Loof, 1963) Fortuner

and Luc, 1987 and *Tylenchorhynchus microphasmis* (Loof 1960) Jairajpuri and Hunt, 1983. Migratory endoparasites are represented by *Pratylenchus* spp. and sedentary ectoparasites by *Meloidogyne maritima* and *Heterodera arenaria* (van der Stoel et al. 2002).

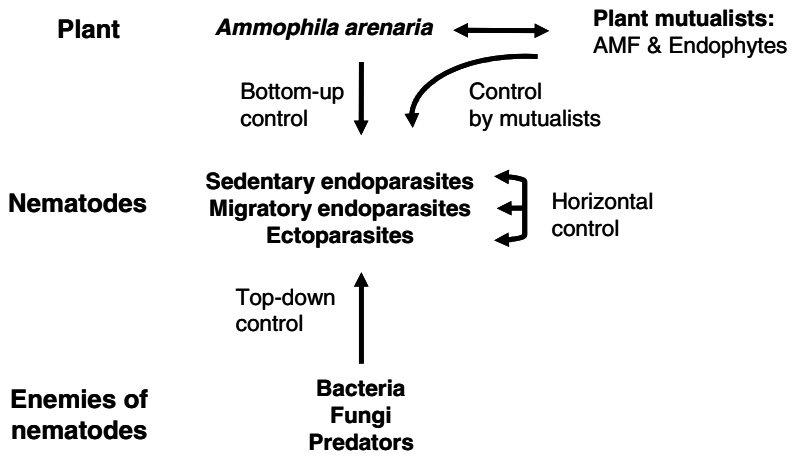

Figure 1. Interaction between host plants, plant parasitic nematodes and their natural enemies in natural coastal dunes (van der Putten 2003).

A. arenaria may suppress nematodes by bottom-up mechanisms. This happens in case of the endoparasitic nematode *Heterodera arenaria* (van der Stoel et al. 2006). Moreover, the plant is protected from nematodes by symbiotic mutualists, notably arbuscular mycorrhizal fungi and plant endophytes (Hol & Cook 2005; Hol et al. 2007; Rodriguez-Echeverria 2006). In coastal foredune soil, this mechanism of control is responsible for suppression of *Pratylenchus penetrans* (de la Peña et al. 2006). Plant parasitic nematodes may compete between each other and this may cause decrease in nematode numbers. Recent studies have shown that it happens in case of *Meloidogyne maritima* (Brinkman et al. 2005). Moreover, nematodes in the field may be also controlled by microorganisms and predators. This way of control was proposed to be the most important for ectoparasitic nematodes (Brinkman et al. 2004), although, not tested yet.

The interactions between *A. arenaria*, plant parasitic nematodes and their natural enemies in natural coastal dunes became a basis for the EcoTrain RTN project and this thesis is a contribution to the project.

Thesis outline

In **Chapter 2**, I examine what controls the populations of the plant ectoparasitic nematode *T. ventralis*. I tested the hypothesis that microorganisms, nematodes and microarthropods, or their interactions, can suppress *T. ventralis* abundance. I show that microbial enemies control the population densities of the ectoparasite *T. ventralis.* I also propose that in natural ecosystems soil microorganisms play a more important role in the top-down control of herbivorous ectoparasitic nematodes than carnivorous soil invertebrates do.

In **Chapter 3,** I question how the microbial enemies of the ectoparasitic nematode *T. ventralis* control these nematodes. I test whether the suppressive effect of the microorganisms on *T. ventralis* is due to a local, or to a non-local (systemic) interaction. I show that microorganisms have direct suppressive effects on these nematodes and that there is no systemic effect detectable. Moreover, I discuss a possible trade-off between nematode control and plant control by the microbial community.

In **Chapter 4,** I study if the ectoparasitic nematodes *T. ventralis* are able to sense and avoid their microbial enemies. I hypothesize that if having a choice between clean roots and the ones inoculated with microorganisms, the nematodes would choose the roots without microorganisms. I show that in semi-natural conditions *T. ventralis* choose 'clean' roots rather that the roots inoculated with microorganisms.

In **Chapter 5,** in order to draw a bigger picture of nematode suppression in nature, I study the top-down control of not only one nematode species as earlier, but eight major root-feeding nematode species, all occurring with the same host plant (*A. arenaria*) in coastal foredunes. I examine if the suppressive effects of microorganisms, nematodes and microarthropods are of the same importance for all these species. I reveal that most root-feeding nematodes potentially are controlled by two or more mechanisms. Nevertheless, soil microorganisms make the most important contribution to the control of the majority of the root-feeding nematode species.

Chapter 6 is devoted to general discussion of nematode control in natural ecosystems, the role of various soil organisms in this control and possible applications in agricultural systems.

References:

Abdel Wahab, A. M. (1975) Nitrogen fixation by *Bacillus* strains isolated from the rizosphere of *Ammophila arenaria*. *Plant and Soil, 42*, 703-708.

Akhtar, M. & Alam, M. M. (1993) Utilization of waste materials in nematode control - a review. *Bioresource Technology, 45*, 1-7.

Akhtar, M. & Malik, A. (2000) Roles of organic soil amendments and soil organisms in the biological control of plant-parasitic nematodes: a review. *Bioresource Technology, 74*, 35-47.

Alabouvette, C., Olivain, C. & Steinberg, C. (2006) Biological control of plant diseases: the European situation. *European Journal of Plant Pathology, 114*, 329-341.

Bird, D. M. & Kaloshian, I. (2003) Are roots special? Nematodes have their say. *Physiological and Molecular Plant Pathology, 62*, 115-123.

Brinkman, E. P., Duyts, H. & van der Putten, W. H. (2005) Consequences of variation in species diversity in a community of root-feeding herbivores for nematode dynamics and host plant biomass. *Oikos, 110*, 417-427.

Brinkman, E. P., van Veen, J. A. & van der Putten, W. H. (2004) Endoparasitic nematodes reduce multiplication of ectoparasitic nematodes, but do not prevent growth reduction of *Ammophila arenaria* (L.) Link (marram grass). *Applied Soil Ecology, 27*, 65-75.

de Deyn, G. B., Raaijmakers, C. E. & van der Putten, W. H. (2004) Plant community development is affected by nutrients and soil biota. *Journal of Ecology, 92*, 824-834.

de la Peña, E., Rodriguez-Echeverria, S., van der Putten, W. H., Freitas, H. & Moens, M. (2006) Mechanism of control of root-feeding nematodes by mycorrhizal fungi in the dune grass *Ammophila arenaria*. *New Phytologist, 169*, 829-840.

de Rooij van der Goes, P. C. E. M. (1995) The role of plant parasitic nematodes and soil borne fungi in the decline of *Ammophila arenaria* (L) Link. *New Phytologist, 129*, 661-669.

de Ruiter, P. C., van Veen, J. A., Moore, J. C., Brussaard, L. & Hunt, H. W. (1993) Calculation of nitrogen mineralization in soil food webs. *Plant and Soil, 157*, 263-273.

Eisenback, J. D. (1993) Interactions between nematodes in cohabitance. *In Nematode interactions* (ed. M. W. Khan), pp. 134-174. London: Chapman and Hall.

Ernst, W. H. O., van Duin, W. E. & Oolbekking, G. T. (1984) Vesicular arbuscular mycorrhiza in dune vegetation. *Acta Botanica Neerlandica, 33*, 151-160.

Griffin, G. D. (1996) Importance of soil texture to the pathogenicity of plant-parasitic nematodes on rangeland grasses. *Nematropica, 26*, 27-37.

Hassell, M. P., Crawley, M. J., Godfray, H. J. C. & Lawton, J. H. (1998) Top-down versus bottom-up and the Ruritanian bean bug. *Proceedings of the National Academy of Sciences of the United States of America, 95*, 10661-10664.

Hassouna, M. G. & Wareing, P. F. (1964) Possible role of rizosphere bacteria in the nitrogen nutrition of *Ammophila arenaria*. *Nature, 202*, 467-469.

Hol, W. H. G. & Cook, R. (2005) An overview of arbuscular mycorrhizal fungi-nematode interactions. *Basic and Applied Ecology, 6*, 489-503.

Hol, W. H. G., de la Peña, E., Moens, M. & Cook, R. (2007) Interaction between a fungal endophyte and root herbivores of *Ammophila arenaria*. *Basic and Applied Ecology, (in press; available on line).*

Huiskes, A. H. L. (1979) Biological flora of the British Isles - *Ammophila arenaria* (L.) Link (*Psamma arenaria* (L.) Roem. et Schult.: *Calmagrostis arenaria* (L.) Roth). *Journal of Ecology, 67*, 363-382.

Imbriani, J. L. & Mankau, R. (1983) Studies on *Lasioseius scapulatus*, a mesostigmatid mite predaceous on nematodes. *Journal of Nematology, 15*, 523-528.

Kerry, B. R. (1995) Ecological considerations for the use of the nematophagous fungus, *Verticillium chlamydosporium*, to control plant parasitic nematodes. *Canadian Journal of Botany, 73*, S65-S70.

Kerry, B. R. & Gowen, S. R. (1995) Biological control of plant parasitic nematodes. *Nematologica, 41*, 362-363.

Kerry, B. R. & Hominick, W. M. (2002) Biological control. London: Taylor & Francis.

Khan, M. W. (1993) Nematode interactions. New York: Chapman & Hall.

Kowalchuk, G. A., De Souza, F. A. & Van Veen, J. A. (2002) Community analysis of arbuscular mycorrhizal fungi associated with *Ammophila arenaria* in Dutch coastal sand dunes. *Molecular Ecology, 11*, 571-581.

Little, L. R. & Maun, M. A. (1996) The '*Ammophila* problem' revisited: A role for mycorrhizal fungi. *Journal of Ecology, 84*, 1-7.

Marshall, J. K. (1965) *Corynephorus canescens* (L.) P. Beauv. as a model for the *Ammophila* problem. *Journal of Ecology, 53*.

Meyer, S. L. F. & Roberts, D. P. (2002) Combinations of biocontrol agents for management of plant-parasitic nematodes and soilborne plant-pathogenic fungi. *Journal of Nematology, 34*, 1-8.

Mulder, C., van Wijnen, H. J. & van Wezel, A. P. (2005) Numerical abundance and biodiversity of below-ground taxocenes along a pH gradient across the Netherlands. *Journal of Biogeography, 32*, 1775-1790.

Norton, D. C. (1989) Abiotic soil factors and plant parasitic nematode communities. *Journal of Nematology, 21*, 299-307.

Oka, Y., Koltai, H., Bar-Eyal, M., Mor, M., Sharon, E., Chet, I. & Spiegel, Y. (2000) New strategies for the control of plant-parasitic nematodes. *Pest Management Science, 56*, 983-988.

Papatheodorou, E. M., Argyropoulou, M. D. & Stamou, G. P. (2004) The effects of large- and small-scale differences in soil temperature and moisture on bacterial functional diversity and the community of bacterivorous nematodes. *Applied Soil Ecology, 25*, 37-49.

Rodriguez-Echeverria, S. (2006) Diversity of AMF associated with *Ammophila arenaria* ssp *arundinacea* in Portuguese san dunes. *Mycorrhiza, 16*, 543-552.

Siddiqui, Z. A. & Mahmood, I. (1996) Biological control of plant parasitic nematodes by fungi: A review. *Bioresource Technology, 58*, 229-239.

Siddiqui, Z. A. & Mahmood, I. (1999) Role of bacteria in the management of plant parasitic nematodes: A review. *Bioresource Technology, 69*, 167-179.

Stanton, N. L. (1988) The underground in grasslands. *Annual Review of Ecology and Systematics, 19*, 573-589.

Strauss, S. Y. & Agrawal, A. A. (1999) The ecology and evolution of plant tolerance to herbivory. *Trends in Ecology & Evolution, 14*, 179-185.

Todd, T. C., Blair, J. M. & Milliken, G. A. (1999) Effects of alerted soil-water availability on a tallgrass prairie nematode community. *Applied Soil Ecology, 13*, 44-45.

van Bruggen, A. H. C., Semenov, A. M., van Diepeningen, A. D., de Vos, O. J. & Blok, W. J. (2006) Relation between soil health, wave-like fluctuations in microbial populations and soil-born disease management. *European Journal of Plant Pathology, 115*, 105-122.

van der Putten, W. H. (2003) Plant defense belowground and spatiotemporal processes in natural vegetation. *Ecology, 84*, 2269-2280.

van der Putten, W. H. & Troelstra, S. R. (1990) Harmful soil organisms in coastal foredunes involved in degeneration of *Ammophila arenaria* and *Calammophila baltica*. *Canadian Journal of Botany, 68*, 1560-1568.

van der Putten, W. H., van Dijk, C. & Troelstra, S. R. (1988) Biotic soil factors affecting the growth and development of *Ammophila arenaria*. *Oecologia, 76*, 313-320.

van der Putten, W. H., Yeates, G. W., Duyts, H., Reis, C. S. & Karssen, G. (2005) Invasive plants and their escape from root herbivory: a worldwide comparison of the root-feeding nematode communities of the dune grass *Ammophila arenaria* in natural and introduced ranges. *Biological Invasions, 7*, 733-746.

van der Stoel, C. D., Duyts, H. & van der Putten, W. H. (2006) Population dynamics of a host-specific root-feeding cyst nematode and resource quantity in the root zone of a clonal grass. *Oikos, 112*, 651-659.

van der Stoel, C. D., van der Putten, W. H. & Duyts, H. (2002) Development of a negative plant-soil feedback in the expansion zone of the clonal grass *Ammophila arenaria* following root formation and nematode colonization. *Journal of Ecology, 90*, 978-988.

Vawdrey, L. L. & Stirling, G. R. (1997) Control of root-knot nematode (*Meloidogyne javanica*) on tomato with molasses and other organic amendments. *Australasian Plant Pathology, 26*, 179-187.

Walters, S. A. & Barker, K. R. (1994) Efficacy of *Paecilomyces lilacinus* in suppressing *Rotylenchulus reniformis* on tomato. *Journal of Nematology, 26*, 600-605.

Widmer, T. L., Mitkowski, N. A. & Abawi, G. S. (2002) Soil organic matter and management of plant-parasitic nematodes. *Journal of Nematology, 34*, 289-295.

Williamson, V. M. & Kumar, A. (2006) Nematode resistance in plants: the battle underground. *Trends in Genetics, 22*, 396-403.

Willis, A. J. (1965) The influence of mineral nutrients on the growth of *Ammophila arenaria*. *Journal of Ecology, 53*, 735-745.

Yeates, G. W., Bongers, T., de Goede, R. G. M., Freckman, D. W. & Georgieva, S. S. (1993) Feeding habits in soil nematode families and genera - an outline for soil ecologists. *Journal of Nematology, 25*, 315-331.

Yeates, G. W. & Wardle, D. A. (1996) Nematodes as predators and prey: Relationships to biological control and soil processes. *Pedobiologia, 40*, 43-50.

Zasada, I., Rogers, S. & Sardanelli, S. (2007) Application of alkaline-stabilised biosolids for *Meloidogyne incognita* suppression in microplots. *Nematology, 9*, 123-129.

Anna M. Piśkiewicz · Henk Duyts · Matty P. Berg · Sofia R. Costa ·
Wim H. van der Putten

Oecologia (2007) 152:505–514

Chapter 2

Soil microorganisms control plant ectoparasitic nematodes in natural coastal foredunes

Summary

Belowground herbivores can exert important controls on the composition of natural plant communities. Until now, relatively few studies have investigated which factors may control the abundance of belowground herbivores. In Dutch coastal foredunes, the root-feeding nematode *Tylenchorhynchus ventralis* is capable of reducing the performance of the dominant grass *Ammophila arenaria* (marram grass). However, field surveys show that populations of this nematode usually are controlled to non-damaging densities, but the control mechanism is unknown. In the present study, we first established that *T. ventralis* populations are top-down controlled by soil biota. Then, selective removal of soil fauna suggested that soil microorganisms play an important role in controlling *T. ventralis*. This result was confirmed by an experiment where selective inoculation of microarthropods, nematodes and microbes together with *T. ventralis* into sterilized dune soil resulted in nematode control when microbes were present. Adding nematodes had some effect, whereas microarthropods did not have a significant effect on *T. ventralis*.

Our results have important implications for the appreciation of herbivore controls in natural soils. Soil food web models assume that herbivorous nematodes are controlled by predaceous invertebrates, while many biological control studies focus on managing nematode abundance by soil microorganisms. We propose that soil microorganisms play a more important role than carnivorous soil invertebrates in the top-down control of herbivorous ectoparasitic nematodes in natural ecosystems. This is opposite to many studies on factors controlling root-feeding insects, which are supposed to be controlled by carnivorous invertebrates, parasitoids or entomopathogenic nematodes.

Our conclusion is that the ectoparasitic nematode *T. ventralis* is potentially able to limit productivity of the dune grass *A. arenaria*, but that soil organisms, mostly microorganisms, usually prevent the development of growth-reducing population densities.

Keywords: *Ammophila arenaria*, multitrophic interactions, root herbivory, top-down control, *Tylenchorhynchus ventralis*

Introduction

Root herbivores play an important role in shaping the composition of natural plant communities (Brown & Gange 1990). Nematodes and insects represent the vast majority of the belowground herbivores (Brown & Gange 1990; Stanton 1988). Nematodes are more abundant than soil insects and in some grassland ecosystems nematodes are the dominant herbivores (Ingham & Detling 1986). Root-feeding nematodes have been estimated to take up as much as one quarter of the net primary production of grassland vegetation (Stanton 1988) and they affect plant quality (Davis et al. 1994; Troelstra et al. 2001), plant diversity and vegetation succession (de Deyn et al. 2003). Root-feeding nematodes can also indirectly affect plant performance by their influence on bottom-up and top-down control of aboveground invertebrate herbivores (Bezemer et al. 2005). However, in spite of the increasing knowledge on the significant role of belowground herbivores in the control of plant abundance and plant community composition, relatively few studies have investigated which factors control the abundance of the belowground herbivores in natural ecosystems (Strong et al. 1996; Strong et al. 1999).

Herbivore abundance can be influenced by natural enemies (top-down), by the host plant (bottom-up), and by competition with other herbivores (horizontal control). In (semi-)natural ecosystems, most studies on the control of root-feeding nematodes have focused on plant quality (Yeates 1987), interspecific competition (Brinkman et al. 2005; Brinkman et al. 2004), plant community composition (de Deyn et al. 2004), plant succession and soil conditions (Verschoor et al. 2002) and mycorrhizal fungi (de la Peña et al. 2006). Soil food web models assume root-feeding nematodes to be controlled by carnivorous nematodes and microarthropods (Hunt et al. 1987; Neutel et al. 2002). However, most biological control studies in agricultural systems focus on managing nematode abundance by parasitic soil microorganisms (Kerry 2000; Sikora 1992) or mycorrhizal fungi (Hol & Cook 2005), suggesting that root-feeding nematodes are mainly controlled by microorganisms. Therefore, previous studies show little agreement and do not clearly predict how root-feeding nematodes will be controlled in natural ecosystems.

Empirical data for top-down mechanisms are rare for terrestrial ecosystems relative to the many studies in aquatic systems (Walker & Jones 2001). In general, trophic

cascades have been argued to be less common on land than in water (Polis & Strong 1996). Nevertheless, there is empirical evidence supporting the existence of trophic cascades in terrestrial plant-predator-prey systems (Schmitz et al. 2004). Tritrophic systems of plants, aboveground insect herbivores and their natural aboveground enemies are the best studied terrestrial examples of top-down and bottom-up herbivore controls (Carson & Root 1999; Rosenheim 1998). Below ground, tritrophic interactions may not essentially differ from what is known above ground (Bezemer & van Dam 2005), although rates of dispersal of organisms and chemical compounds will be lower than is mostly the case above ground (Rasmann et al. 2005; van der Putten 2003). Therefore, the challenge is, similar to that above ground (Schmitz et al. 2004), to assess what controls the abundance of root herbivores. This knowledge will enhance our understanding of belowground multitrophic interactions and their influences on plant performance and plant community composition.

In the present study, the role of microarthropods, nematodes and microorganisms in controlling the abundance of the root-feeding nematode *Tylenchorhynchus ventralis* (Loof, 1963) Fortuner and Luc (synonym *Telotylenchus ventralis*) was experimentally compared. This nematode is a polyphagous ectoparasite, which means that it is a quite generalistic root feeder that penetrates outer cortical cells with its stylet to collect and ingest cell contents (Yeates et al. 1993). *Tylenchorhynchus ventralis* is a root parasite of the dominant coastal foredune grass *Ammophila arenaria* (marram grass). In field soil, *T. ventralis* reaches densities that are 80 times lower than achieved when inoculated into sterilized dune soil (de Rooij van der Goes 1995). While *T. ventralis* can strongly reduce growth of *A. arenaria* in sterilized soil, field densities in non-sterilized soil are too low to directly influence plant performance (de Rooij van der Goes 1995). The roots of *A. arenaria* are parasitized by an array of herbivorous nematodes ranging from ectoparasites to sedentary endoparasites (de Rooij van der Goes et al. 1995). The control mechanisms of root herbivorous nematodes in dunes appear to highly depend on the feeding type of the nematode, and even on the species of nematode. While the sedentary root knot nematode *Meloidogyne maritima* (Jepson, 1987) Karssen, van Aelst and Cook is controlled by competition (Brinkman et al. 2005), the sedentary cyst nematode *Heterodera arenaria* (Cooper, 1955) Robinson, Stone, Hooper and Rowe appears to be controlled by bottom-up

processes (van der Stoel et al. 2006). The migratory endoparasitic root lesion nematode *Pratylenchus penetrans* (Cobb, 1917) is controlled by arbuscular mycorrhizal fungi (de la Peña et al. 2006). Thus far, the factors that control the ectoparasitic nematode *T. ventralis* associated with *A. arenaria* are unknown.

Previous studies showed bottom-up control of *A. arenaria* to occur only when the plants were severely growth reduced (de Rooij van der Goes et al. 1995). Alternatively, competition with cyst and root lesion nematodes is a potential factor controlling ectoparasitic nematodes (Eisenback 1993). However, endoparasitic nematodes did not control abundance of *T. ventralis* (Brinkman et al. 2004). In the present study, the top-down factors that may be involved in the control of *T. ventralis* populations were investigated in order to determine how belowground trophic interactions may influence plant performance and vegetation composition.

To assess the top-down control of *T. ventralis*, three experiments were performed. The aim of experiment 1 was to elucidate the potential top-down control of *T. ventralis* by the dune soil community. In experiment 2, the particular role of microorganisms was investigated by selective elimination of soil fauna (nematodes and microarthropods). In experiment 3, the hypothesis emerged from experiment 2, that soil microorganisms are the main cause of top-down control of *T. ventralis* was tested. Here, we applied Koch's postulates by collecting microorganisms, nematodes and microarthropods from dune soil and adding them to sterilized soil inoculated with *T. ventralis*. New evidence that top-down control by soil microorganisms is the most important factor controlling the abundance of ectoparasitic nematodes in dune soil is presented and discussed.

Materials and methods

Soil

In summer 2003, soil samples were collected from mobile and stable foredunes at Voorne, The Netherlands (Latitude 51°55'N – Longitude 04°05'E). The samples were collected along six transects parallel to the beach and 50 m apart. At each sampling station in the mobile and stable dune, 60 kg of soil was collected from the youngest root zone of *A.*

arenaria. The soil was sieved (0.5 cm mesh size) to remove plant parts and debris, and stored in plastic bags at 4°C until used (van der Stoel et al. 2002).

Plants

Seeds of *A. arenaria* were collected from the same foredune area and stored dry until used. In order to obtain seedlings, the seeds were germinated for 2 weeks on moist glass beads in a climate room at a 16/8 hours light/dark regime at a temperature of 25/15°C, respectively. When the first leaf was 2-3 cm long the seedlings were transplanted to 1.5 l plastic pots filled with 1500 g of dune soil. In each pot 4 seedlings of *A. arenaria* were planted and the soil surface was covered with aluminum foil to protect the soil from desiccation. The soil moisture was adjusted to 10 % $w \cdot w^{-1}$ and maintained at this level throughout the experiment by weighing the pots twice a week and resetting their initial weight using demineralized water. Once a week full-strength Hoagland nutrient solution was added at a weekly rate of 12.5 $ml \cdot pot^{-1}$ for the first three weeks and then 25 $ml \cdot pot^{-1}$, subsequently (Brinkman et al. 2004). This nutrient supply rate was effective to compensate for effects of nutrient release as a result of soil sterilization in dune soil (Troelstra et al. 2001; van der Putten et al. 1988). The experiments were carried out in a greenhouse at a day temperature of 21°C $\pm$ 2°C (day length 16 hours) with additional light (to maintain a minimum of 225 $\mu mol\ m^{-2} \cdot s^{-1}$ PAR with SON-T Agro lamps) and a night temperature of 16°C. These temperatures are comparable to summer conditions in the field and they are optimal for both plant and nematode development (S.R. Troelstra and R. Wagenaar, unpublished results).

Experiments

Experiment 1: Multiplication of *T. ventralis* in sterilized and non-sterilized dune soil.

In this experiment the effect of soil origin (mobile and stable dunes) and soil organisms on multiplication of the ectoparasitic nematode *T. ventralis* was tested. Half of the soil was sterilized by gamma irradiation at an average dose of 25 kGray, which eliminates microorganisms and nematodes effectively from dune soil (de Rooij van der Goes et al. 1998). One week after the seedlings of *A. arenaria* had been transplanted, half the pots were inoculated with 50 *T. ventralis* · pot^{-1}. The non-inoculated pots served as controls for

effects of *T. ventralis* on plant biomass production. There were 6 replicates of each treatment.

Experiment 2: Reproduction of *T. ventralis* in partially sterilized soil.
Multiplication of *T. ventralis* was studied in soils from which microarthropods and nematodes had been selectively removed by stirring the soil for 15 minutes at 1500 rpm. This method has proven to effectively kill the soil fauna (de Rooij van der Goes et al. 1998). We confirmed this by inspecting the soil following stirring and did not find any live nematodes or microarthropods. The experiment was carried out as described above, but now the soil was completely sterilized by gamma irradiation (average 25 kGray), partially sterilized by stirring to remove the soil fauna, or non-stirred in order to have a non-sterilized control soil. Each soil was inoculated with 0, 25 or 250 *T. ventralis* · pot^{-1} in order to examine any interaction between the effect of type of soil sterilization and nematode inoculation density. There were 6 replicates of each treatment.

Experiment 3: Re-inoculation of microorganisms, nematodes and microarthropods into sterilized soil with *T. ventralis*.
In order to completely apply Koch's postulates, microarthropods, nematodes and microorganisms were extracted from the soil of mobile and stable coastal foredunes and inoculated alone and in all factorial combinations, into sterilized dune soil. Then, seedling plants of *A. arenaria* were grown as in the previous experiment and every pot was inoculated with 50 *T. ventralis*. All treatments were carried out in 6 replicates.
The microorganisms were obtained by shaking soil samples of 100 g with demineralized water (1:1 $w \cdot w^{-1}$) for 10 minutes and filtering the supernatant through a 20 µm mesh (Klironomos 2002). Prepared microbial filtrate contained no nematodes, but bacteria and fungi had readily passed through the filter. The pots with microorganisms were inoculated with 10 ml of the filtrate, which was 1/15 of the original soil density. For each pot nematodes had been extracted from 1500 g of non-sterile soil by Cobb's method (Oostenbrink 1960), and added in a suspension of 10 ml · pot^{-1}, so that nematode inoculation density corresponded with the density of nematodes in field soil. The nematode

community added to the pots was analyzed microscopically (magnification 200 x) and consisted of plant parasites (*T. ventralis*, *Tylenchorhynchus microphasmis, Pratylenchus spp, Paratylenchus* spp., *Meloidogyne* sp., *Rotylenchus* spp., Criconematidae), bacterivores (*Acrobeles* spp., *Acrobeloides* spp., *Chiloplacus* spp., Cephalobidae, *Plectus* spp.), omnivores (*Aporcelaimellus* spp., *Microdorylaimus* spp.) and carnivores (*Choanolaimus* spp.).

Microarthropods were collected from non-sterile dune soil by wet sieving through 180 µm mesh and added as 10 ml of suspension · pot^{-1}, which corresponded with the field density of microarthropods. Demineralized water was added to all pots in equal amounts.

Assessing the presence of microbial enemies on nematodes in field soil

In order to confirm whether microbial enemies may occur on *T. ventralis* in the field, we extracted mobile nematodes from 100 cm^3 of the field soil from each of the sampling sites using an adaptation of the Tray Method (Whitehead & Hemming 1965). Half of the resulting nematode suspension was inspected using an inverted microscope (magnification 200 x) and the nematodes were checked for symptoms of infection by bacteria or fungi. Nematodes infected by fungi, were picked from the suspension and transferred to a Corn Meal Agar plate with antibiotics to encourage sporulation (Smith & Onions 1994), making possible the identification of the fungi that were previously found in a vegetative state. Identification of fungal natural enemies was done by observing mycelia and spore structure morphology and comparing this to the descriptions of Barron (1977). Endospores of the parasitic bacterium *Pasteuria* spp. were recorded when observed attached to the nematode cuticle. Symptoms of infection by a non-lethal bacterial parasite, *Microbacterium nematophilum* were assessed according to Sulston and Hodgkin (1988).

To detect whether nematode natural enemies may occur as dormant forms in the soil, nematode-baited sprinkle-plates were used. Soil (1 g) from each of the samples was sprinkled on Water Agar (1%) in a 9 cm diameter Petri dish. A concentrated suspension of an estimated 500 *Caenorhabditis elegans* synchronized in young adult stage (Sulston & Hodgkin 1988) was added to the plates. A negative control containing nematodes only in Water Agar (1%) was used. The plates were sealed, kept at room temperature and observed

after two weeks and subsequently at weekly intervals up to 5 weeks (Barron 1977). Identification of fungal natural enemies was done as described above.

Harvest

All three experiments were harvested 12 weeks after inoculation of *T. ventralis* allowing this nematode to complete two reproductive cycles (de Rooij van der Goes 1995). The nematodes were extracted from soil by Cobb's decantation method and from the roots using a mistifier (Oostenbrink 1960). The numbers of *T. ventralis* were counted using a microscope (magnification 200 x) and expressed as numbers · 100 g^{-1} of dry soil. The roots and shoots of *A. arenaria* were dried for 48 hours at 75°C and weighed.

Data analysis

Normal distribution of data and homogeneity of variance were checked by inspection of the residuals after model fit (using the package Statistica 7). To obtain the normal distribution of data and homogeneity of variances, numbers of *T. ventralis* were log transformed in experiment 1 and square root transformed in experiment 2. In all three experiments the soil origin (stable or mobile dune) did not significantly ($P > 0.05$) affect the measured variables. Therefore, all data from treatments with those two soil origins was pooled, resulting in 12 replicates per treatment. Numbers of *T. ventralis* of experiment 1 were analyzed using one-way ANOVA with main factor 'soil treatment'. Two-way ANOVAs with the main factors 'soil sterilization' and 'nematode inoculation' were performed for root and shoot biomass. Three-way ANOVAs with the main factors 'stirring', 'sterilization' and 'inoculation density' were performed for analyzing the numbers of *T. ventralis*, shoot and root biomass in experiment 2. Experiment 3 was analyzed by three-way ANOVA with the main factors 'invertebrates', 'nematodes' and 'microorganisms'. The treatments were compared by posthoc analysis using Tukey HSD tests ($P < 0.05$).

Results

Experiment 1

The numbers of *T. ventralis* at harvest differed significantly between sterilized and non-sterilized soils ($F_{2,33}$ = 77.9 and P < 0.001). In the non-sterilized soil, addition of *T. ventralis* resulted in a significant increase of numbers at the end of the experiment as compared to non-sterilized non-inoculated soil (Figure 2). However, there were five times more *T. ventralis* in the inoculated sterilized soil than in the inoculated non-sterilized soil (Figure 2; P < 0.05; sterilized soil without *T. ventralis* was not included because the nematodes were absent).

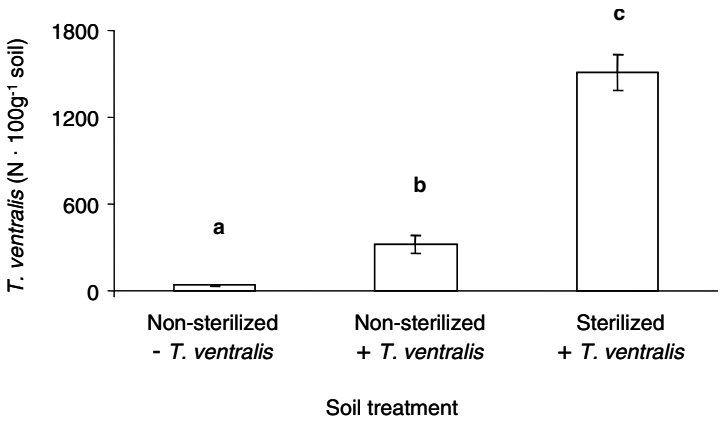

Figure 2. Numbers of *Tylenchorhynchus ventralis* in 100 g of non-sterilized and sterilized dune soil 12 weeks after inoculation with *T. ventralis*. Error bars indicate standard error and different letters above the bars indicate significant difference at P < 0.05 (Experiment 1)

These results show that multiplication of *T. ventralis* in non-sterilized soil was significantly enhanced by inoculation, but that *T. ventralis* multiplication was significantly reduced by some factor in the non-sterilized soil that could be excluded by soil sterilization.

Soil sterilization influenced shoot biomass more than *T. ventralis* inoculation ($F_{1,44}$ = 117, P < 0.001 for soil sterilization and $F_{1,44}$ = 4.17, P < 0.05 for inoculation, Figure 3), and the effect of *T. ventralis* inoculation depended on soil sterilization ($F_{1,44}$ = 7.06, P < 0.05). Most shoot biomass was produced in sterilized soil, while *T. ventralis* inoculation

significantly reduced shoot biomass (Figure 3). As expected, least shoot biomass was produced in non-sterile soil; however, addition of *T. ventralis* caused no further reduction in growth (Figure 3). As expected, root biomass was also strongly influenced by soil sterilization ($F_{1,44}$ = 56.1 and P < 0.001), whereas the effect of *T. ventralis* addition was greater than for shoot biomass ($F_{1,44}$ = 16.8 and P < 0.001). As for shoot biomass, the effect of *T. ventralis* inoculation on root biomass depended on soil sterilization ($F_{1,44}$ = 9.87 and P < 0.005), which reflects that shoot biomass significantly reduced by *T. ventralis* inoculation in the sterilized soil only (Figure 3).

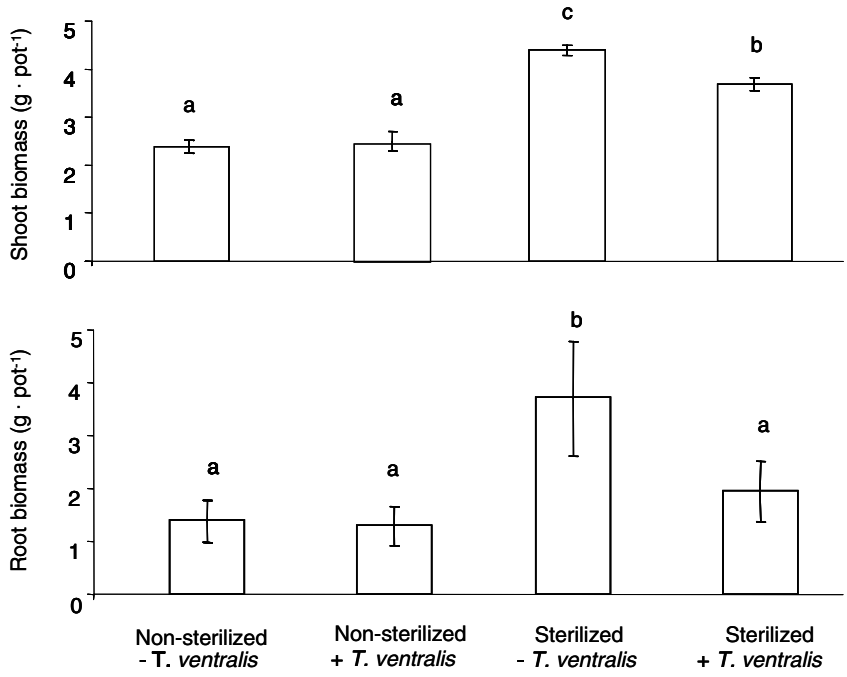

Figure 3. Shoot and root biomass of *Ammophila arenaria* in sterilized and non-sterilized soil after 12 weeks from inoculation with *Tylenchorhynchus ventralis*. Error bars show standard error and letters above indicate significant differences at P < 0.05 (Experiment 1)

Experiment 2

Significantly greater populations of *T. ventralis* developed in sterilized than in non-sterile soil, at both inoculation densities (Table 1). At the low inoculation density, the number of the nematodes in non-sterile soil was 30 times less than in sterilized soil and 15 times less at the high inoculation density. There was no significant effect of soil stirring on the numbers of *T. ventrali*s, although there was a trend (P = 0.06) that stirring reduced *T. ventralis* multiplication.

Table 1. Top: Three-way ANOVA of the numbers of *Tylenchorhynchus ventralis* in non-sterilized and sterilized, stirred and non-stirred dune soil at three inoculation rates (0, 25, 250 · pot^{-1}) after 12 weeks from inoculation to *Ammophila arenaria*. The data has been square root transformed to achieve normal error distribution. **Bottom:** The effects of soil sterilization on numbers of *T. ventralis* in 100 g of soil (± 1SE) 12 weeks from inoculation. Letters indicate significant differences at P < 0.05 (Experiment 2)

	DF	F	P
Stirring (1)	1	3.481	0.06
Sterilization (2)	1	137.41	< 0.001
Inoculation density (3)	2	95.55	< 0.001
1 * 2	1	0.858	0.36
1 * 3	2	1.222	0.30
2 * 3	2	43.94	< 0.001
1 * 2 * 3	2	1.499	0.23
Error	125		

	Non-sterilized	Sterilized
0 *T. ventralis* added	0.77 ± 0.22 *a*	0.08 ± 0.03 *a*
25 *T. ventralis* added	12.3 ± 2.62 *b*	390 ± 73.03 *c*
250 *T. ventralis* added	69.2± 16.76 *bc*	1162 ±203.7 *d*

As expected, both soil stirring and sterilization influenced shoot biomass (Figure 4, Table 2). Shoot biomass was greater in sterilized than in non-sterile soil and in stirred than in non-stirred soil, however, inoculation density of *T. ventralis* did not influence the shoot biomass (Figure 4). Root biomass was affected by stirring, soil sterilization and inoculation density of *T. ventralis*, whereas effects of inoculation density depended on soil stirring, as well as on soil sterilization (Table 2). If no nematodes were inoculated to the pots, soil sterilization almost doubled the root biomass. However, if inoculated with 25 or 250 *T. ventralis* per pot, the roots in sterilized soil with *T. ventralis* did not produce more biomass than in non-sterilized soil (Figure 4). Root biomass was significantly increased by soil stirring when no or few ($25 \cdot pot^{-1}$) *T. ventralis* were added to the pots, but there was no increase in root weight at the high inoculation rate.

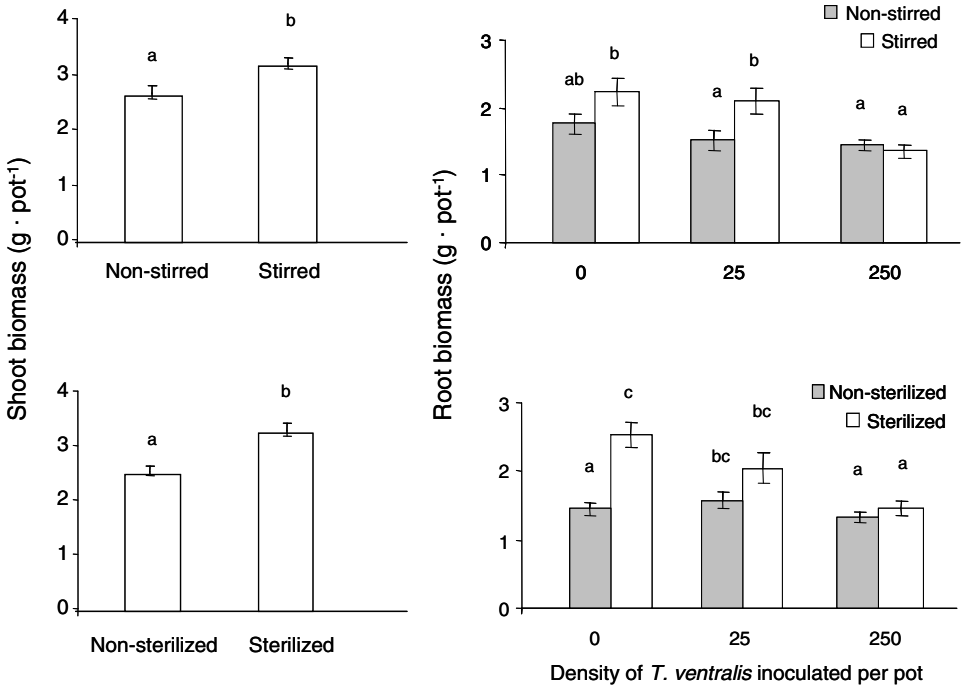

Figure 4. The effects of soil stirring, soil sterilization and addition of *Tylenchorhynchus ventralis* on shoot and root biomass of *Ammophila arenaria*. Error bars show standard error and letters above indicate significant differences at $P < 0.05$ (Experiment 2)

Table 2. Shoot and root biomass of *Ammophila arenaria* 12 weeks after inoculation with nematodes. The results of a factorial ANOVA with factors 'stirring', 'sterilization' and 'inoculation density' (Experiment 2)

		Shoot biomass		Root biomass	
	DF	F	P	F	P
Stirring (1)	1	14.13	< 0.001	8.69	< 0.01
Sterilization (2)	1	30.49	< 0.0001	26.8	< 0.001
Inoculation density (3)	2	1.998	0.139	10.53	< 0.001
1 * 2	1	3.288	0.072	1.91	0.169
1 * 3	2	1.916	0.151	3.622	< 0.05
2 * 3	2	2.145	0.121	6.452	< 0.01
1 * 2 * 3	2	1.921	0.151	1.911	0.152
Error	132				

Experiment 3

The multiplication of *T. ventralis* numbers was significantly reduced by adding a mixture of soil nematodes, however, the effect of adding microorganisms was far greater (Figure 5; Table 3). If microorganisms were present alone or in combination with other soil organisms the number of *T. ventralis* was always less than when microorganisms were absent. On average, adding a suspension of nematodes reduced final numbers of *T. ventralis* by 15 %, while adding microorganisms reduced numbers of *T. ventralis* by 55 % (Figure 5). Therefore, the effect of adding nematodes on *T. ventralis* multiplication was substantially weaker than the effect of microorganisms. Microarthropods did not have a significant effect on the numbers *T. ventralis*. Adding microarthropods, nematodes and microorganisms did not influence shoot or root biomass ($P > 0.05$; data not shown).

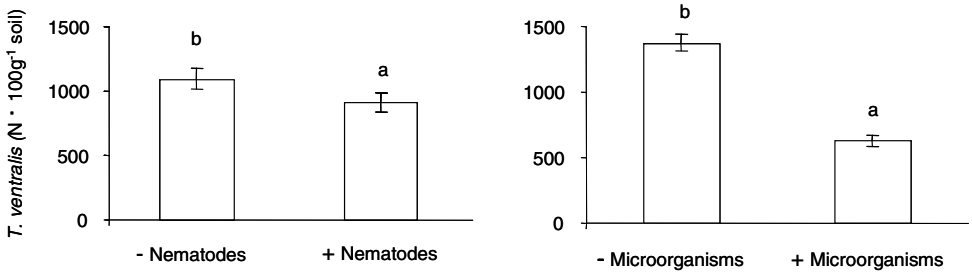

Figure 5. The effects of mixed nematode inoculum and microorganisms on *Tylenchorhynchus ventralis* multiplication. Error bars show standard error and letters above indicate significant differences at $P < 0.05$ (Experiment 3)

Table 3. The numbers of *Tylenchorhynchus ventralis* after 12 weeks from inoculation. The results of a three-way ANOVA with factors 'microarthropods', 'nematodes' and 'microorganisms' (Experiment 3)

	DF	F	P
Microarthropods (1)	1	0.0006	0.981
Nematodes (2)	1	5.167	< 0.05
Microorganisms (3)	1	87.84	< 0.0001
1 * 2	1	1.736	0.191
1 * 3	1	0.618	0.434
2 * 3	1	0.672	0.415
1 * 2 * 3	1	0.047	0.828
Error	88		

In the suspension of nematodes obtained from the pots to which microorganisms had been added, 47.3 % showed signs of infection by culturable microbial enemies (Table 4). The fungal parasite *Catenaria* sp. was found infecting 16 out of 110 *T. ventralis* inspected, and another, unidentified fungus was detected inside 30 destroyed of 110 *T.*

ventralis checked. Bacterial attachment to the cuticle was also observed for 6 nematodes, a *Paenibacillus*-like organism was found on four nematodes, and *Pasteuria* sp. on two out of 110 nematodes.

Table 4. Microbial enemies in or attached to *Tylenchorhynchus ventralis* in a suspension obtained from microorganism treatment pots in Experiment 3

Microbial enemy detected	Fraction of affected nematodes (%)
Unidentified assimilative hyphae	27.3
Paenibacillus-like	3.6
Catenaria sp.	14.5
Pasteuria sp.	1.8
TOTAL	47.3
(Healthy)	52.7
Total nematodes examined	100 (n=110)

Assessing the presence of microbial enemies on nematodes in field soil

The fungal natural enemies *Catenaria* sp., *Harposporium* sp., and *Myzocytium* sp. were found infecting nematodes extracted by the Tray Method. The bacterium *P. penetrans* was attached to root knot nematodes (*Meloidogyne maritima*). The fungal genera were also detected using nematode-baited sprinkle plates. Some of the nematodes on the plates had a swollen region behind the anus not observed in the original culture or in the negative control. This is a symptom of infection by a non-lethal bacterial parasite, *Microbacterium nematophilum*. An unidentified trapping fungus with non-constricting rings was also detected in the sprinkle plates, but could not be identified as it did not sporulate. These identifications may not have been exhaustive, but they confirmed the presence of antagonistic microorganism species on *T. ventralis*, as well as in the soil from the field.

Discussion

In coastal foredunes the root-feeding ectoparasitic nematode *T. ventralis* would significantly influence the pioneer grass *A. arenaria* if the density of this nematode was not naturally controlled. Our study strongly suggests that the natural control of *T. ventralis* in coastal foredune soil is mostly due to soil microorganisms. When inoculated into sterilized soil, numbers of *T. ventralis* were more than five times greater than when inoculated into non-sterilized soil, while selective elimination of soil fauna by stirring did not affect nematode numbers. These results from selective elimination studies were confirmed by isolating microarthropods, nematodes and microbes and adding these together with *T. ventralis* to sterilized soil. Inoculation with soil microorganisms reduced *T. ventralis* more strongly than inoculation with a nematode community consisting of other plant parasites, bacterivores, omnivores and carnivores. The negative effect of the nematode community on *T. ventralis* density might have been due to competition with other root-feeders. However, competition between *T. ventralis* and endoparasitic nematodes (i.e. *Heterodera arenaria*, *Meloidogyne maritima* and *Pratylenchus penetrans*) occurs only if numbers of the competitors strongly exceed present field densities (Brinkman et al. 2004). Therefore, that the observed effect of adding nematodes on reducing *T. ventralis* has been due to soil microorganisms co-introduced with the nematode suspension, carnivorous nematodes (Jairajpuri & Bilgrami 1990), or by effects of other non-plant feeding nematodes. Our results highlight an important discrepancy in thinking about control mechanisms of plant-feeding (also called plant-parasitic) nematodes between biocontrol studies on the one hand and soil food web studies on the other. The majority of studies on biocontrol of nematodes in agricultural ecosystems mostly focus on parasitic bacteria, such as *Pasteuria penetrans* and fungi (Kerry 2000), for example *Arthrobotrys* spp. and *Pochonia* spp. (Stirling & Smith 1998). Biological control practice usually does not consider microarthropods to be relevant for parasitic nematode control (Kerry & Gowen 1995), while the role of carnivorous nematodes has been considered (Mankau 1980; Yeates & Wardle 1996), but not successfully used. According to the food web model used by Neutel et al. (2002), root-feeding nematodes in coastal ecosystems are affected by predaceous mites and carnivorous nematodes. The role of microorganisms in nematode control is generally ignored in

prominent soil food web models (de Ruiter et al. 1993; Hunt et al. 1987). In our study system, however, soil microorganisms appear to play a more important role than soil fauna in the control of plant ectoparasitic nematodes. In dune grasslands the densities of soil fauna are usually rather low, perhaps too low to control significantly the abundance of nematodes (Petersen & Luxton 1982). Our results, therefore, support the view of biological control studies more than of soil food web models for the control of the ectoparasitic nematode *T. ventralis*.

Our results suggest that top-down control by natural enemies is more important for ectoparasitic feeding-generalist such as *T. ventralis* than are competition (Brinkman et al. 2004) or mycorrhizal fungi (A.M. Piśkiewicz and W.H.G. Hol, unpublished results), although these control mechanisms have been suggested for the other endoparasitic root-feeding nematode species in the same study system (Brinkman et al. 2005; de la Peña et al. 2006; van der Stoel et al. 2006).

The control of *T. ventralis* in non-sterilized soil to which *T. ventralis* was added was not as good as in non-sterile soil with only the background population of *T. ventralis* present. Earlier studies have shown that multiplication of *T. ventralis* is density- and time-dependent (de Rooij van der Goes 1995). Over time, a low inoculation density of nematodes may result in population increase, while a high inoculation density may result in population decline (de Rooij van der Goes 1995). However, we have no information on how the microbial control shown in the present study may depend on nematode density and subsequent long-term studies are required to further explore density-dependence of nematode top-down control by microbes.

The two screening methods used to detect microbial enemies of nematodes in the soil yielded a diversity of fungal and bacterial antagonists. Tray method permitted the extraction of mobile stages of nematodes only, and therefore dead or dying nematodes could not be screened. The nematode-baited sprinkle plate method, although useful for detecting microbial enemies in dormant forms in the soil, produced biased results that reflected the choice of nematode added, the bacterial-feeding nematode *C. elegans*. The fungal endoparasite *Harposporium* sp. infects nematodes that ingest its spores and therefore would not be able to infect plant-parasites, due to their narrow lumen of the stylet (Barron

1977). The bacterium *Microbacterium nematophilum* is thought to be a specialist parasite of *C. elegans* (Hodgkin et al. 2000). *Tylenchorhynchus ventralis* was attacked by a subset of the microbial enemy genera found in the soil, which reflects some specificity in the action of these microbes. Some microorganisms, namely *Pasteuria* sp. and the *Paenibacillus*-like organism, have not been detected in the dune soil, although they are widespread in the dune sites. This may be due to their absence or to presence in small numbers that are below the level of detection. The unidentified assimilative hyphae could be the result of attack by generalist trapping fungi. This was a first assessment to confirm the presence of nematode antagonistic microorganisms on *T. ventralis* and further studies are needed to isolate culture and inoculate those antagonists in order to evaluate their contribution in root-feeding nematodes control.

Soil sterilization always led to increased root and shoot biomass. Previous studies have already shown that soil biota may reduce performance of *A. arenaria* (van der Putten et al. 1988; van der Stoel et al. 2002). The effect of soil sterilization was greater for root biomass than for shoot biomass. In sterilized soil, enhanced plant growth can be caused by nutrient release as a result of the soil sterilization process (Troelstra et al. 2001). We avoided different nutrient status of the sterilized and non-sterilized treatments by adding nutrient solution (van der Putten et al. 1988). When added to sterilized soil, the root-feeding nematode *T. ventralis* reduced root and shoot biomass of *A. arenaria* and the effect of nematodes on root biomass increased with increasing inoculation density. Addition of *T. ventralis* to the non-sterile soil did not change root biomass, showing that the contribution of this nematode species to growth reduction of *A. arenaria* is limited (de Rooij van der Goes 1995). Our study shows that the dune soil not only contains biotic factors that reduce growth of *A. arenaria*, but that there are also (micro) organisms that control population abundance of *T. ventralis*. In prairie grassland ecosystems root-feeding nematodes have been assessed to account for reducing 58% in aboveground biomass (Stanton 1988). These estimates are based on elimination trials using soil biocides. However, these studies did not verify the biocide effects by inoculation trials and they also did not account for natural top-down control of the root-feeding nematodes. Our results suggest that when assessing the

effects of root feeders on plant production interactions of the root feeders with their natural predators need to be taken into account as well.

We conclude that soil microorganisms contribute to controlling the plant ectoparasitic nematode *T. ventralis* at a low population density in natural coastal foredunes. When not controlled, *T. ventralis* would be a key control factor for *A. arenaria* performance. Addition of other soil fauna, i.e. nematodes and microarthropods, did not influence the abundance of *T. ventralis* as much as microorganism addition did, which confirms the marginal effects of soil fauna removal on reproduction of *T. ventralis*. Our results suggest that belowground multitrophic interactions can be crucial for plant performance. Revealing the precise identity of the microorganisms which have negative effects on *T. ventralis* population as well as the mechanisms and involvement of the host plant need further studies.

Acknowledgements

This study is part of the EcoTrain project (HPRN-CT 2002 00210), which is funded by the European Union.

References:

Barron, G. L. (1977) The nematode destroying fungi. Ontario: Canadian Publications Ltd.

Bezemer, T. M., de Deyn, G. B., Bossinga, T. M., van Dam, N. M., Harvey, J. A. & van der Putten, W. H. (2005) Soil community composition drives aboveground plant-herbivore-parasitoid interactions. *Ecology Letters, 8*, 652-661.

Bezemer, T. M. & van Dam, N. M. (2005) Linking aboveground and belowground interactions via induced plant defenses. *Trends in Ecology & Evolution, 20*, 617-624.

Brinkman, E. P., Duyts, H. & van der Putten, W. H. (2005) Consequences of variation in species diversity in a community of root-feeding herbivores for nematode dynamics and host plant biomass. *Oikos, 110*, 417-427.

Brinkman, E. P., van Veen, J. A. & van der Putten, W. H. (2004) Endoparasitic nematodes reduce multiplication of ectoparasitic nematodes, but do not prevent growth

reduction of *Ammophila arenaria* (L.) Link (marram grass). *Applied Soil Ecology, 27*, 65-75.

Brown, V. K. & Gange, A. C. (1990) Insect herbivory below ground. *Advances in Ecological Research, 20*, 1-58.

Carson, W. P. & Root, R. B. (1999) Top-down effects of insect herbivores during early succession: influence on biomass and plant dominance. *Oecologia, 121*, 260-272.

Davis, R. F., Noel, G. R. & Wilkinson, H. T. (1994) Pathogenicity of *Tylenchorhynchus nudus* to creeping bentgrass and annual bluegrass. *Plant Disease, 78*, 169-173.

de Deyn, G. B., Raaijmakers, C. E., van Ruijven, J., Berendse, F. & van der Putten, W. H. (2004) Plant species identity and diversity effects on different trophic levels of nematodes in the soil food web. *Oikos, 106*, 576-586.

de Deyn, G. B., Raaijmakers, C. E., Zoomer, H. R., Berg, M. P., de Ruiter, P. C., Verhoef, H. A., Bezemer, T. M. & van der Putten, W. H. (2003) Soil invertebrate fauna enhances grassland succession and diversity. *Nature, 422*, 711-713.

de la Peña, E., Rodriguez-Echeverria, S., van der Putten, W. H., Freitas, H. & Moens, M. (2006) Mechanism of control of root-feeding nematodes by mycorrhizal fungi in the dune grass *Ammophila arenaria*. *New Phytologist, 169*, 829-840.

de Rooij van der Goes, P. C. E. M. (1995) The role of plant parasitic nematodes and soil borne fungi in the decline of *Ammophila arenaria* (L) Link. *New Phytologist, 129*, 661-669.

de Rooij van der Goes, P. C. E. M., Peters, B. A. M. & van der Putten, W. H. (1998) Vertical migration of nematodes and soil-borne fungi to developing roots of *Ammophila arenaria* (L.) Link after sand accretion. *Applied Soil Ecology, 10*, 1-10.

de Rooij van der Goes, P. C. E. M., van der Putten, W. H. & van Dijk, C. (1995) Analysis of nematodes and soil borne fungi from *Ammophila arenaria* (Marram grass) in Dutch coastal foredunes by multivariate techniques. *European Journal of Plant Pathology, 101*, 149-162.

de Ruiter, P. C., van Veen, J. A., Moore, J. C., Brussaard, L. & Hunt, H. W. (1993) Calculation of nitrogen mineralization in soil food webs. *Plant and Soil, 157*, 263-273.

Eisenback, J. D. (1993) Interactions between nematodes in cohabitance. *In Nematode interactions* (ed. M. W. Khan), pp. 134-174. London: Chapman and Hall.

Hodgkin, J., Kuwabara, P. E. & Corneliussen, B. (2000) A novel bacterial pathogen, *Microbacterium nematophilum*, induces morphological change in the nematode *C. elegans*. *Current Biology, 10*, 1615-1618.

Hol, W. H. G. & Cook, R. (2005) An overview of arbuscular mycorrhizal fungi-nematode interactions. *Basic and Applied Ecology, 6*, 489-503.

Hunt, H. W., Coleman, D. C., Ingham, E. R., Ingham, R. E., Elliott, E. T., Moore, J. C., Rose, S. L., Reid, C. P. P. & Morley, C. R. (1987) The detrital food web in a shortgrass prairie. *Biology and Fertility of Soils, 3*, 57-68.

Ingham, R. E. & Detling, J. K. (1986) Nematode population dynamics from different aged prairie dog colonies. *Journal of Nematology, 18*, 638-638.

Jairajpuri, M. S. & Bilgrami, A. L. (1990) Predatory nematodes. *In Nematode bio-control: Aspects and prospects* (ed. M. S. Jairajpuri, M. M. Alam & I. Ahmad). New Delhi: CBS Publishers & Distributions Pvt. Ltd.

Kerry, B. R. (2000) Rhizosphere interactions and the exploitation of microbial agents for the biological control of plant parasitic nematodes. *Annual Review of Phytopathology, 38*, 423-441.

Kerry, B. R. & Gowen, S. R. (1995) Biological control of plant parasitic nematodes. *Nematologica, 41*, 362-363.

Klironomos, J. N. (2002) Feedback with soil biota contributes to plant rarity and invasiveness in communities. *Nature, 417*, 67-70.

Mankau, R. (1980) Biological control of nematode pests by natural enemies. *Annual Review of Phytopathology, 18*, 415-440.

Neutel, A. M., Heesterbeek, J. A. P. & de Ruiter, P. C. (2002) Stability in real food webs: Weak links in long loops. *Science, 296*, 1120-1123.

Oostenbrink, M. (1960) Estimating nematode populations by some selected methods. *In Nematology* (ed. J. N. Sasser & W. R. Jenkins), pp. 85-102. Chapel Hill: The University of North Carolina Press.

Petersen, H. & Luxton, M. (1982) A comparative analysis of soil fauna populations and their role in decomposition processes. *Oikos, 39*, 287-388.

Polis, G. A. & Strong, D. R. (1996) Food web complexity and community dynamics. *American Naturalist, 147*, 813-846.

Rasmann, S., Kollner, T. G., Degenhardt, J., Hiltpold, I., Toepfer, S., Kuhlmann, U., Gershenzon, J. & Turlings, T. C. J. (2005) Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature, 434*, 732-737.

Rosenheim, J. A. (1998) Higher order predators and the regulation of insect herbivore populations. *Annual Review of Entomology, 43*, 421-447.

Schmitz, O. J., Krivan, V. & Ovadia, O. (2004) Trophic cascades: the primacy of trait-mediated indirect interactions. *Ecology Letters, 7*, 153-163.

Sikora, R. A. (1992) Management of the antagonistic potential in agricultural ecosystems for the biological control of plant parasitic nematodes. *Annual Review of Phytopathology, 30*, 245-270.

Smith, D. & Onions, A. H. S. (1994) The preservation and maintenance of living fungi. Wallingford, UK: CAB International.

Stanton, N. L. (1988) The underground in grasslands. *Annual Review of Ecology and Systematics, 19*, 573-589.

Stirling, G. R. & Smith, L. J. (1998) Field tests of formulated products containing either *Verticillium chlamydosporium* or *Arthrobotrys dactyloides* for biological control of root-knot nematodes. *Biological Control, 11*, 231-239.

Strong, D. R., Kaya, H. K., Whipple, A. V., Child, A. L., Kraig, S., Bondonno, M., Dyer, K. & Maron, J. L. (1996) Entomopathogenic nematodes: Natural enemies of root-feeding caterpillars on bush lupine. *Oecologia, 108*, 167-173.

Strong, D. R., Whipple, A. V., Child, A. L. & Dennis, B. (1999) Model selection for a subterranean trophic cascade: Root-feeding caterpillars and entomopathogenic nematodes. *Ecology, 80*, 2750-2761.

Sulston, J. & Hodgkin, J. (1988) Methods. *In The nematode Caenorhabditis elegans* (ed. W. B. Wood), pp. 587-606. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Troelstra, S. R., Wagenaar, R., Smant, W. & Peters, B. A. M. (2001) Interpretation of bioassays in the study of interactions between soil organisms and plants: involvement of nutrient factors. *New Phytologist, 150*, 697-706.

van der Putten, W. H. (2003) Plant defense belowground and spatiotemporal processes in natural vegetation. *Ecology, 84*, 2269-2280.

van der Putten, W. H., van Dijk, C. & Troelstra, S. R. (1988) Biotic soil factors affecting the growth and development of *Ammophila arenaria*. *Oecologia, 76*, 313-320.

van der Stoel, C. D., Duyts, H. & van der Putten, W. H. (2006) Population dynamics of a host-specific root-feeding cyst nematode and resource quantity in the root zone of a clonal grass. *Oikos, 112*, 651-659.

van der Stoel, C. D., van der Putten, W. H. & Duyts, H. (2002) Development of a negative plant-soil feedback in the expansion zone of the clonal grass *Ammophila arenaria* following root formation and nematode colonization. *Journal of Ecology, 90*, 978-988.

Verschoor, B. C., de Goede, R. G. M. & Brussaard, L. (2002) Do plant parasitic nematodes have differential effects on the productivity of a fast- and a slow-growing grass species? *Plant and Soil, 243*, 81-90.

Walker, M. & Jones, T. H. (2001) Relative roles of top-down and bottom-up forces in terrestrial tritrophic plant-insect herbivore-natural enemy systems. *Oikos, 93*, 177-187.

Whitehead, A. G. & Hemming, J. R. (1965) A comparison of some quantitative methods of extracting small vermiform nematodes from soil. *Annals of Applied Biology, 55*, 25-38.

Yeates, G. W. (1987) How plants affect nematodes. *Advances in Ecological Research, 17*, 61-113.

Yeates, G. W., Bongers, T., de Goede, R. G. M., Freckman, D. W. & Georgieva, S. S. (1993) Feeding habits in soil nematode families and genera - an outline for soil ecologists. *Journal of Nematology, 25*, 315-331.

Yeates, G. W. & Wardle, D. A. (1996) Nematodes as predators and prey: Relationships to biological control and soil processes. *Pedobiologia, 40*, 43-50.

Anna M. Piśkiewicz · Henk Duyts · Wim H. van der Putten

Functional Ecology, under revision

Chapter 3

Soil microorganisms in coastal foredunes control ectoparasitic root-feeding nematodes by local interactions

Summary

Root herbivores control the diversity and succession of natural vegetation, however, little is known about what controls root herbivores and how this operates. In coastal foredunes, the ectoparasitic nematode *Tylenchorhynchus ventralis* would be a major root herbivore if not strongly controlled by soil microorganisms.

Here, we show how soil microorganisms suppress the abundance of *T. ventralis* in the root zone of the grass *Ammophila arenaria*. In a split-root experiment, we compared local *versus* non-local (systemic) control of the nematodes by a natural community of soil microorganisms. Local interactions can be due to predation, parasitism or antagonism, or to local induction of defense responses in the plant root. Non-local interactions will be due to a defense response induced by the microorganisms. The split-root experiment revealed that microorganisms affected *T. ventralis* numbers only when present in the same root compartment. Therefore, the effects of microorganisms on *T. ventralis* are due to local interactions and not due to induction of a systemic defense signal. As the nematodes which were inoculated together with microorganisms were heavily infected with unknown bacteria and with fungi that resembled the genus *Catenaria*, we conclude that microorganisms control nematodes mainly through parasitism. However, local defense induction cannot be completely excluded. We also analyzed if the soil microorganisms might influence shoot and root biomass, because the root zone *A. arenaria* also contains soil pathogens. As root biomass was reduced by nematode infection, but also by the combination of nematodes and microorganisms, we conclude that there may be a trade-off between nematode control and pathogenic effects caused by the soil microbial community.

Keywords: biological control, nematodes, root herbivory, soil microorganisms, split-root, trade-off

Introduction

In natural grassland ecosystems, root-feeding nematodes and insects are the dominant belowground herbivores (Brown & Gange 1990; Stanton 1988). Nematodes and insects influence plant productivity and plant community composition (de Deyn et al. 2004; de Deyn et al. 2007; Verschoor et al. 2002). In grasslands, herbivorous nematodes take up as much as one quarter of the net primary production (Stanton 1988) and in agricultural crops, these nematodes are major soil-borne pests which cause significant yield reduction. There are different feeding types of herbivorous nematodes: ectoparasites that feed on root hairs and outer cortex tissue, semi-endoparasites that feed on deeper cortex layers, and endoparasites that enter and feed inside roots (Yeates et al. 1993). Whereas many studies have shown how plants are controlled by root-feeding nematodes, we examined how root-feeding nematodes are controlled by their natural enemies. Such biological control is an important prerequisite for the persistence of wild plant populations, as well as for sustainable agriculture. Most of the existing studies concentrate on nematode control mechanisms in agricultural fields, but nematode controls in natural systems hardly have been explored.

In general, in a natural system the control mechanisms of root-feeding nematodes appear to be dependent on the feeding type of the nematode, as well as on the species of nematode (Brinkman et al. 2005a; de la Peña et al. 2006; van der Stoel et al. 2006). Plants may defend themselves against nematodes directly, which is called bottom-up control (Walker & Jones 2001), nematodes may control each other by competition (horizontal control) (Eisenback 1993), or nematodes can be controlled by their natural enemies (top-down effects) like carnivorous nematodes, microarthropods (Imbriani & Mankau 1983; Yeates & Wardle 1996) and microorganisms (Kerry 2000). Finally, complex controls are possible that can be a mix of bottom-up, horizontal and top-down control. This applies to interactions between nematodes and mycorrhizal fungi (Hol & Cook 2005), or above- and belowground insects (de Deyn et al. 2007).

In agriculture, the usual mode of nematode control, besides resistance breeding, is nematode suppression by microorganisms (Sikora 1992). The suppressive effects of microbial enemies of root-feeding nematodes may be local or non-local (systemic). The

local response is due to the direct interactions between root-feeding nematodes and microorganisms through predation, parasitism or antagonism, or by local induction of plant defense. In case of non-local response, the microorganisms in one section of the plant roots can affect nematode control in another section of the root system. This involves a systemic induction of the plant defense system (Bakker et al. 2003; Pieterse et al. 1998). In that case, the effect of microorganisms would be transferred through the host plant to other parts or organs than originally affected.

Previous studies have shown that ectoparasitic nematodes *Tylenchorhynchus ventralis* (former name *Telotylenchus ventralis* (Loof, 1963) Fortuner and Luc, 1987) a keystone species in natural coastal foredune, can be controlled by soil microorganisms (Piśkiewicz et al. 2007). If these nematodes are not controlled they cause severe growth reduction to the wild foredune grass *Ammophila arenaria* (de Rooij van der Goes 1995). In mobile coastal foredunes, performance of *A. arenaria* depends on burial by wind-deposited beach sand, which enables the plants to escape from soil-borne pathogens and root-feeding nematodes (van der Putten & Troelstra 1990). Therefore, the role of the microbial community in the rhizosphere in plant defense against the nematode *T. ventralis* could have a trade-off. Whereas some of the microorganisms can improve plant growth by suppressing nematodes, others can be antagonistic against the plant. Our study aimed at detecting if the suppressive effect of microorganisms on *T. ventralis* was due to a local, or a non-local (systemic) interaction. To reveal whether the effects of plant-nematode-microorganism interactions were local or systemic, we performed a split-root experiment with different combinations of inoculated nematodes and microorganisms. As a side question, we examined if the benefit of nematode control by the microbial community would outweigh the negative effect of reducing shoot or root biomass. We present new evidence that microorganisms have direct suppressive effects on the ectoparasitic nematode *T. ventralis* and discuss a possible trade-off between nematode control and plant control by the microbial community.

Materials and methods

Experimental setup

We tested the local *versus* non-local effects of microorganisms on *T. ventralis* abundance in a split-root experiment. We prepared sixty 1.5 l pots filled with dune soil that was sterilized by gamma irradiation. Sets of two pots were attached to each other and the roots of single plants were split and one half of each plant root system was planted into each of both compartments (named A and B). The treatment combinations were: 1). Control ↔ Control; 2). Control ↔ *T. ventralis*; 3). Microorganisms ↔ *T. ventralis*; 4). *T. ventralis* ↔ *T. ventralis*; 5). *T. ventralis* ↔ *T. ventralis* + microorganisms; 6). *T. ventralis* + microorganisms ↔ *T. ventralis* + microorganisms. Every treatment combination was replicated ten-fold.

Soil

In summer 2004, soil was collected from the mobile foredunes of Voorne, The Netherlands (Latitude 51° 55'N – Longitude 04° 05'E). The samples were collected from ten points parallel to the beach and 50 m apart from each other. At all sampling points 30 kg of soil was collected from the youngest root zone of *A. arenaria* (van der Stoel et al. 2002). The soil was sieved (0.5 cm mesh size) in order to remove plant parts and debris and after that homogenized. The collected soil was sterilized by gamma irradiation apart from 10 kg that was kept for microorganism extraction. Gamma irradiation was performed with a minimal dosage of 25 kGray, which is effective to eliminate all soil organisms from dune soil (de Rooij van der Goes et al. 1998). The soil was stored in plastic bags at 4°C until used (van der Stoel et al. 2002).

Plants

Seeds of *A. arenaria* were collected from random plants at the same field site and stored dry until used. The seeds were germinated for 2 weeks on moist glass beads at 16/8 hour light/dark regime at 25/15°C. To allow sufficient root growth seedlings with the first leaf of 2-3 cm long were transplanted to plastic tubes filled with 250 g of sterilized dune soil. The soil moisture was set at 10 % $w \cdot w^{-1}$. After 4 weeks when the roots were about 10 cm long, the plants were transplanted to pots, consisting of two 1.5 l components, which were attached to each other. The roots of every plant were split into two halves, which were

planted one in each of the two compartments. Subsequently, the soil surface was covered with aluminum foil to protect the surface from desiccation. The soil moisture was maintained at 10 % during the whole experiment by weighing the two-component pots twice a week and re-setting the initial weight by adding demineralized water. We could not maintain soil moisture of each compartment separately. Once a week full-strength Hoagland nutrient solution was added; for the first 3 weeks 12.5 ml · pot^{-1} and later 25 ml · pot^{-1} (Brinkman et al. 2004). The experiment was carried out in a greenhouse at a day temperature of 21°C ± 2°C and the day length was minimally 16 hours by providing additional light to ensure minimally 225 µmol · m^{-2} · s^{-1} PAR with SON-T Agro lamps. The night temperature was 16 ± 2°C.

Soil organisms

The root-feeding nematode *T. ventralis* was collected from the same coastal foredune area as where soil and plants originated from. The nematodes were cultured in a greenhouse with *A. arenaria* as host plant. The nematodes were extracted from the cultures by Cobb's decantation method (Oostenbrink 1960) and inoculated to the pots in tap water at a density of 25 *T. ventralis* · pot^{-1}, similar to the density in foredune soil (de Rooij van der Goes et al. 1995).

Microorganisms were extracted from field soil by shaking 100 g soil samples with demineralized water (1:1 w · w^{-1}) for 10 minutes and filtering the supernatant through a 20 µm mesh (Bezemer et al. 2005; Klironomos 2002). Prepared microbial filtrate contained bacteria and fungi, but no nematodes and no arbuscular mycorrhizal fungi. The microorganisms were inoculated at a rate of 10 ml · pot^{-1} of the filtrate, which was 1/15 of the original density of the microorganisms in the field soil.

Harvest

The pots were harvested 12 weeks after inoculation of the soil organisms to allow *T. ventralis* passing minimally two reproductive cycles (de Rooij van der Goes et al. 1995). The nematodes from soil of both compartments were extracted separately by Cobb's decantation method and from the roots using a mistifier (Oostenbrink 1960). The numbers

of nematodes were counted by microscope (magnification 200 x) and expressed as numbers · 100 g^{-1} of soil. The nematodes were also inspected by microscope for the signs of direct infections with detrimental microorganisms. The roots and shoots of *A. arenaria* were dried for 48 hours at 70°C and weighed.

Statistical analysis

Normal distribution of data and homogeneity of variance were checked by inspection of the residuals after model fit (using the package Statistica 7). The numbers of nematodes extracted from the pots were analyzed first by T-test to compare multiplication between compartments within experimental treatments. In this test, we established if the numbers of nematodes between compartments did not differ when the treatments of both compartments were the same and whether numbers of nematodes differed between opposite compartments when having different treatments.

Then, we examined if the presence of microorganisms in the compartment had direct (local) effect on final numbers of *T. ventralis*. We used two-way ANOVA with main factors 'nematode inoculation' (with or without *T. ventralis*) and 'microorganism inoculation' (with or without microorganisms). The treatments were compared by posthoc analysis using Tukey HSD tests ($P < 0.05$).

Finally, we established if the effect of the microorganisms was transmitted through the plant as a systemic response. To test whether the treatment in compartment A had an effect on nematode numbers in compartment B, we performed one-way ANOVA with a factor 'compartment A treatment' (4 levels: control, microorganisms, nematodes, nematodes + microorganisms), whereas in all cases compartment B was inoculated with the nematode *T. ventralis*.

In order to test how nematode reduction could affect plant biomass, we used one-way ANOVA and compared dry biomass of shoots and roots in treatments with double control, double *T. ventralis* and double *T. ventralis* + microorganisms. By using these identical treatments in both compartment, we were sure that the shoots were exposed to the same treatment and that the total root biomass of the two compartments could be summed

up. The treatments were then compared by posthoc analysis using Tukey HSD tests ($P < 0.05$).

Results:

Nematode numbers

The T-tests results showed highly significant differences in numbers of *T. ventralis* when the nematodes were added to one compartment and not to the attached compartment ($P < 0.001$; Figure 6). Moreover, there were twice as many nematodes in compartments without than with microorganisms ($P < 0.01$). There were no significant differences in numbers of nematodes ($P > 0.05$) if both compartments were inoculated with nematodes alone, or when both compartments were inoculated with nematodes and microbes. Therefore, our experimental treatments were well established.

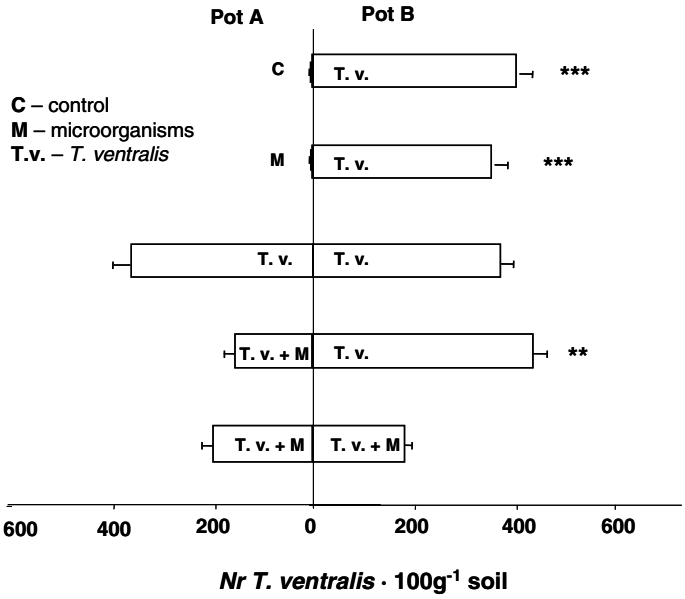

Figure 6: Numbers of *Tylenchorhynchus ventralis* which were extracted from the roots and soil of *Ammophila arenaria* 12 weeks after inoculation. The nematodes were extracted from sets of two compartment pots of the split root experiment. Then the nematode numbers in both compartments were compared by T-test results. The numbers of nematodes are expressed as $N \cdot 100g^{-1}$ of soil. Error bars show standard error. Two asterisks show significance at $P < 0.01$, three at $P < 0.001$.

Two-way ANOVA of *T. ventralis* and microorganisms revealed a significant difference in *T. ventralis* abundance as affected by microorganism addition ($F_{115,1}$ = 15 and P < 0.001). There were half as many nematodes in compartments with as compared to without microorganisms present in the same compartment (Figure 7). Therefore, microorganisms had local effects on *T. ventralis* and microscopic analyses provided further support for the local effects hypothesis. Nematodes which were inoculated together with microorganisms showed signs of infections with unknown bacteria (Figure 8A) and *Catenaria* fungi (Figure 8B).

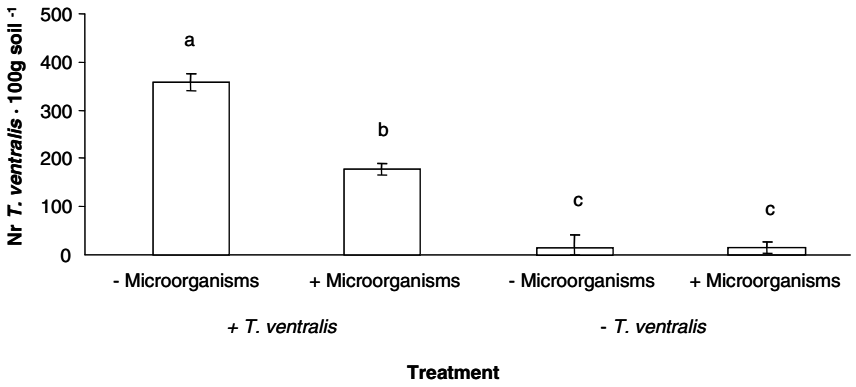

Figure 7: Numbers of *Tylenchorhynchus ventralis* extracted from the root zone of *Ammophila arenaria* 12 weeks after inoculation, with or without microorganisms added. The nematode numbers are expressed as N · $100g^{-1}$ of soil. Error bars show standard error and letters above indicate significant differences at P < 0.05.

One-way ANOVA showed that the number of *T. ventralis* in one half of the root system was not influenced by the treatment of the other half of the roots ($F_{56,3}$ = 2.5; P > 0.07). Therefore, microorganisms did not have systemic effects on *T. ventralis*.

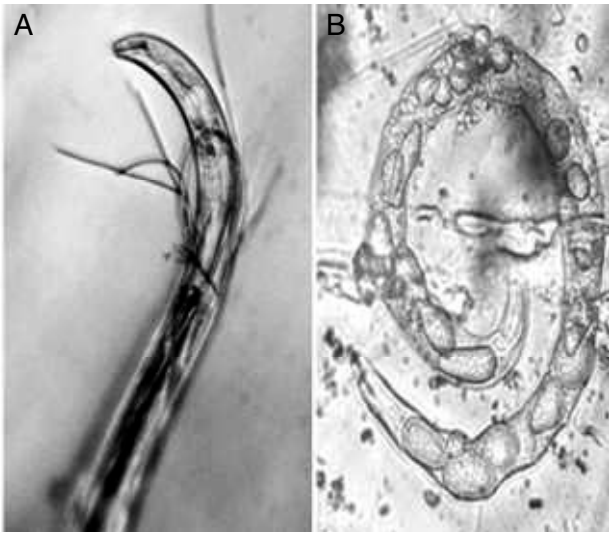

Figure 8: Nematode *Tylenchorhynchus ventralis* infected with unknown bacterium (A), or with *Catenaria*-like fungi (B).

Plant biomass

One-way ANOVA showed that root biomass was significantly lower when both *T. ventralis* and microorganisms were present than of plants without the nematodes and microorganisms ($F_{27,2} = 4.3$ and $P < 0.05$; Figure 9).

The root biomass with microorganisms and nematodes was almost half as low as in the control, whereas adding *T. ventralis* without microorganisms had intermediate effects on root biomass. Therefore, the beneficial effect of the microorganisms by reducing nematode abundance had a trade-off in that root biomass was more severely reduced than with nematodes alone. Shoot biomass did not change when the plant was inoculated with nematodes and microorganisms ($F_{27,2} = 1.3$ and $P > 0.05$).

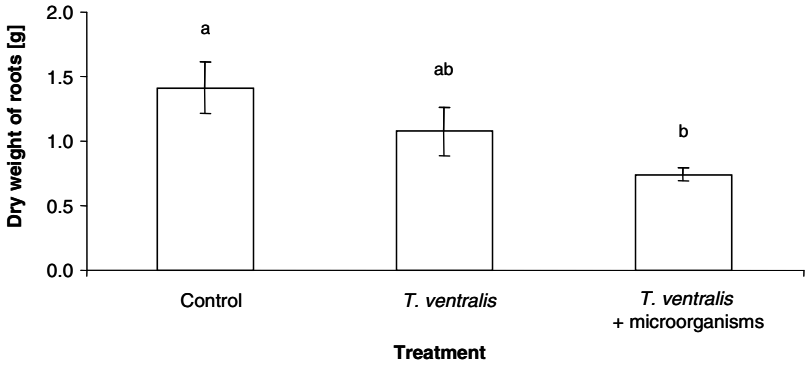

Figure 9: Dry biomass of *Ammophila arenaria* roots growing in sterilized dune soil and with *Tylenchorhynchus ventralis* alone or a combination of *T. ventralis* and microorganisms 12 weeks after inoculation. Error bars show standard error and letters above indicate significant differences at $P < 0.05$.

Discussion

In this study, we show that the suppressive effects of microorganisms on the ectoparasitic nematode *T. ventralis* were due to local interactions. These local interactions could be due to both parasitic relations and local induction of defense responses in the plant root as induced by the microorganisms. We did not find any non-local (systemic) response, so the effects of microorganisms were not transferred through the plant to other distant organs. This did not exclude the possibility of local defense induction. However, microscopic observations of nematodes which were inoculated together with microorganisms showed heavy infections with unknown bacteria and fungi form the genus *Catenaria* (S. Costa pers. comm.). Therefore, we conclude that direct parasitism of the microorganisms on the ectoparasitic nematode *T. ventralis* is an important mechanism of nematode control. Whether or not plants may play an active role in this process, such as has been observed for the attraction of entomopathogenic nematodes by plant roots infested by soil-dwelling insect larvae (Rasmann et al. 2005; van Tol et al. 2001) is open to further studies.

We show that the suppressive effects of microorganisms on root-feeding nematodes, which are known to occur in agricultural and horticultural soils, also take place

in a natural ecosystem. In our model system, the root zone of the wild coastal foredune grass *A. arenaria*, microorganisms suppress the population of ectoparasitic nematodes by direct parasitism, predation or antagonism. In dune soil, microbial enemies are the main source of antagonism against the ectoparasitic nematode *T. ventralis*. Microarthropods or other nematodes did not have significant effects (Piśkiewicz et al. 2007), whereas competition by other, for example sedentary endoparasitic nematodes only had weak effects on *T. ventralis* abundance (Brinkman 2005b). *Catenaria* which parasitized *T. ventralis* in our assay is a well-known genus of fungi which parasitize nematodes in crop systems (Rodriguez Kabana 1991; Singh et al. 1996). Interestingly, the natural control of ectoparasitic root herbivorous nematodes in dune soil is so severe (de Rooij van der Goes 1995; Piśkiewicz et al. 2007) that this would meet all targets of biological control when setting over these principles to crop systems.

It is widely known that soil microorganisms are able to efficiently suppress plant parasitic nematode populations (Kerry 2000). Nevertheless, most studies that have shown microbial nematode control thus far come from the agricultural systems. The role of soil microorganisms against plant infections in natural ecosystems is still poorly explored. Natural coastal dune soil is suppressive to fungal pathogens (de Boer et al. 1998; de Boer et al. 2003). In this case the suppression of fungi pathogenic to *A. arenaria* was due to anti-fungal compounds produced by microorganisms. Now, we show that some of the soil microorganisms are able to exert local control to nematodes, most likely due to direct parasitism. Future studies should point out what other nematode control mechanisms are present in the soil microbial community of natural coastal foredunes.

Inoculation of the plants with nematodes did not influence shoot biomass but tended to decrease root biomass. However, the root biomass was significantly lower than the control when both nematodes and microorganisms were present, although the density of the nematodes was half that in compartments without microorganisms added. The root zone of *A. arenaria* is known to contain plant pathogenic fungi (de Rooij van der Goes 1995). In our study, the presence of microbial enemies of nematodes and suppression of nematode numbers did not enhance plant biomass production. Plants with lower numbers of *T. ventralis* should produce more biomass (de Rooij van der Goes 1995). Probably the

microbial community in this case also contained pathogens, which contributed to reduced plant biomass production. Such additive effects have also been shown by experiments where nematodes and specific soil pathogens were added to *A. arenaria* planted in previously sterilized dune soil (de Rooij van der Goes 1995). Therefore, we conclude that the rhizosphere of *A. arenaria* contains microorganisms that contribute to control of herbivorous nematodes, as well as microorganisms that are pathogenic to the plants. Whether the plants are able to modify the rhizosphere communities to their own benefit is open to further studies, however, our results suggest a trade-off between positive and negative factors in the microbial community.

The outcome of positive and negative interactions in the rhizosphere is crucial for plant performance (Wardle et al. 2004). A negative feedback on plant growth can be caused by accumulation of parasites, pathogens and root-herbivores (van der Stoel et al. 2002). On the other hand, positive influence on plant performance is directly due to symbionts, for example mycorrhizal fungi (Smith & Read 1997) and indirectly to decomposers (Barot et al. 2007; Partsch et al. 2006). The net effects of all these positive and negative interactions will determine how the soil microbial community influences plant performance and plant community development.

We conclude that microorganisms suppress the abundance of the ectoparasitic nematodes *T. ventralis* due to local interactions. Predation, parasitism or antagonism- these are the mechanisms that most likely lead to the very low numbers of root-feeders that have been observed in natural coastal foredunes. A possible local defense induction can not be completely excluded because this effect and that of direct parasitism cannot be teased apart, however, microscopic analysis showed the nematodes to be parasitized by microorganisms. Belowground microorganisms may play a complex role in plant performance - some of the microorganisms can help protecting the plants from root-herbivore damage, some other microorganisms act as plant pathogens. In our study, suppression of nematodes did not result into improved plant performance, which suggests that negative effects of the microbial community were stronger than positive effects.

Acknowledgements

We thank Sofia Costa for useful comments on this study. This work is part of the EcoTrain project (HPRN-CT 2002 00210), which was funded by the European Union rtn program.

References:

Bakker, P., Ran, L. X., Pieterse, C. M. J. & van Loon, L. C. (2003) Understanding the involvement of rhizobacteria-mediated induction of systemic resistance in biocontrol of plant diseases. *Canadian Journal of Plant Pathology, 25*, 5-9.

Barot, S., Ugolini, A. & Brikci, F. B. (2007) Nutrient cycling efficiency explains the long-term effect of ecosystem engineers on primary production. *Functional Ecology, 21*, 1-10.

Bezemer, T. M., de Deyn, G. B., Bossinga, T. M., van Dam, N. M., Harvey, J. A. & van der Putten, W. H. (2005) Soil community composition drives aboveground plant-herbivore-parasitoid interactions. *Ecology Letters, 8*, 652-661.

Brinkman, E. P., Duyts, H. & van der Putten, W. H. (2005a) Consequences of variation in species diversity in a community of root-feeding herbivores for nematode dynamics and host plant biomass. *Oikos, 110*, 417-427.

Brinkman, E. P., Duyts, H., van der Putten W.H. (2005b) Competition between endoparasitic nematodes and effect on biomass of *Ammophila arenaria* (marram grass) as affected by timing of inoculation and plant age. *Nematology, 7*, 169-178.

Brinkman, E. P., van Veen, J. A. & van der Putten, W. H. (2004) Endoparasitic nematodes reduce multiplication of ectoparasitic nematodes, but do not prevent growth reduction of *Ammophila arenaria* (L.) Link (marram grass). *Applied Soil Ecology, 27*, 65-75.

Brown, V. K. & Gange, A. C. (1990) Insect herbivory below ground. *Advances in Ecological Research, 20*, 1-58.

de Boer, W., Gunnewiek, P. & Woldendorp, J. W. (1998) Suppression of hyphal growth of soil-borne fungi by dune soils from vigorous and declining stands of *Ammophila arenaria*. *New Phytologist, 138*, 107-116.

de Boer, W., Verheggen, P., Gunnewiek, P., Kowalchuk, G. A. & van Veen, J. A. (2003) Microbial community composition affects soil fungistasis. *Applied and Environmental Microbiology, 69*, 835-844.

de Deyn, G. B., Raaijmakers, C. E. & van der Putten, W. H. (2004) Plant community development is affected by nutrients and soil biota. *Journal of Ecology, 92*, 824-834.

de Deyn, G. B., van Ruijven, J., Raaijmakers, C. E., de Ruiter, P. C. & van der Putten, W. H. (2007) Above- and belowground insect herbivores differentially affect soil nematode communities in species-rich plant communities. *Oikos, 116*, 923-930.

de la Peña, E., Rodriguez-Echeverria, S., van der Putten, W. H., Freitas, H. & Moens, M. (2006) Mechanism of control of root-feeding nematodes by mycorrhizal fungi in the dune grass *Ammophila arenaria*. *New Phytologist, 169*, 829-840.

de Rooij van der Goes, P. C. E. M. (1995) The role of plant parasitic nematodes and soil borne fungi in the decline of *Ammophila arenaria* (L) Link. *New Phytologist, 129*, 661-669.

de Rooij van der Goes, P. C. E. M., Peters, B. A. M. & van der Putten, W. H. (1998) Vertical migration of nematodes and soil-borne fungi to developing roots of *Ammophila arenaria* (L.) Link after sand accretion. *Applied Soil Ecology, 10*, 1-10.

de Rooij van der Goes, P. C. E. M., van der Putten, W. H. & van Dijk, C. (1995) Analysis of nematodes and soil borne fungi from *Ammophila arenaria* (Marram grass) in Dutch coastal foredunes by multivariate techniques. *European Journal of Plant Pathology, 101*, 149-162.

Eisenback, J. D. (1993) Interactions between nematodes in cohabitance. *In Nematode interactions* (ed. M. W. Khan), pp. 134-174. London: Chapman and Hall.

Hol, W. H. G. & Cook, R. (2005) An overview of arbuscular mycorrhizal fungi-nematode interactions. *Basic and Applied Ecology, 6*, 489-503.

Imbriani, J. L. & Mankau, R. (1983) Studies on *Lasioseius scapulatus*, a mesostigmatid mite predaceous on nematodes. *Journal of Nematology, 15*, 523-528.

Kerry, B. R. (2000) Rhizosphere interactions and the exploitation of microbial agents for the biological control of plant parasitic nematodes. *Annual Review of Phytopathology, 38*, 423-441.

Klironomos, J. N. (2002) Feedback with soil biota contributes to plant rarity and invasiveness in communities. *Nature, 417*, 67-70.

Oostenbrink, M. (1960) Estimating nematode populations by some selected methods. *In Nematology* (ed. J. N. Sasser & W. R. Jenkins), pp. 85-102. Chapel Hill: The University of North Carolina Press.

Partsch, S., Milcu, A. & Scheu, S. (2006) Decomposers (*Lumbricidae, Collembola*) affect plant performance in model grasslands of different diversity. *Ecology, 87*, 2548-2558.

Pieterse, C. M. J., van Wees, S. C. M., van Pelt, J. A., Knoester, M., Laan, R., Gerrits, N., Weisbeek, P. J. & van Loon, L. C. (1998) A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *Plant Cell, 10*, 1571-1580.

Piśkiewicz, A. M., Duyts, H., Costa, S. R., Berg, M. P. & van der Putten, W. H. (2007) Soil microorganisms control plant ectoparasitic nematodes in natural coastal foredunes. *Oecologia, 152*, 505-514.

Rasmann, S., Kollner, T. G., Degenhardt, J., Hiltpold, I., Toepfer, S., Kuhlmann, U., Gershenzon, J. & Turlings, T. C. J. (2005) Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature, 434*, 732-737.

Rodriguez Kabana, R. (1991) Biological control of plant parasitic nematodes. *Nematropica, 21*, 111-122.

Sikora, R. A. (1992) Management of the antagonistic potential in agricultural ecosystems for the biological control of plant parasitic nematodes. *Annual Review of Phytopathology, 30*, 245-270.

Singh, K. P., Stephen, R. A. & Vaish, S. S. (1996) Pathogenicity and development of *Catenaria anguillulae* on some nematodes. *Mycological Research, 100*, 1204-1206.

Smith, S. E. & Read, D. J. (1997) Mycorrhizal symbiosis. London: Academic Press.

Stanton, N. L. (1988) The underground in grasslands. *Annual Review of Ecology and Systematics, 19*, 573-589.

van der Putten, W. H. & Troelstra, S. R. (1990) Harmful soil organisms in coastal foredunes involved in degeneration of *Ammophila arenaria* and *Calammophila baltica*. *Canadian Journal of Botany-Revue Canadienne De Botanique, 68*, 1560-1568.

van der Stoel, C. D., Duyts, H. & van der Putten, W. H. (2006) Population dynamics of a host-specific root-feeding cyst nematode and resource quantity in the root zone of a clonal grass. *Oikos, 112*, 651-659.

van der Stoel, C. D., van der Putten, W. H. & Duyts, H. (2002) Development of a negative plant-soil feedback in the expansion zone of the clonal grass *Ammophila arenaria* following root formation and nematode colonization. *Journal of Ecology, 90*, 978-988.

van Tol, R. W. H. M., van der Sommen, A. T. C., Boff, M. I. C., van Bezooijen, J., Sabelis, M. W. & Smits, P. H. (2001) Plants protect their roots by alerting the enemies of grubs. *Ecology Letters, 4*, 292-294.

Verschoor, B. C., de Goede, R. G. M. & Brussaard, L. (2002) Do plant parasitic nematodes have differential effects on the productivity of a fast- and a slow-growing grass species? *Plant and Soil, 243*, 81-90.

Walker, M. & Jones, T. H. (2001) Relative roles of top-down and bottom-up forces in terrestrial tritrophic plant-insect herbivore-natural enemy systems. *Oikos, 93*, 177-187.

Wardle, D. A., Bardgett, R. D., Klironomos, J. N., Setala, H., van der Putten, W. H. & Wall, D. H. (2004) Ecological linkages between aboveground and belowground biota. *Science, 304*, 1629-1633.

Yeates, G. W., Bongers, T., de Goede, R. G. M., Freckman, D. W. & Georgieva, S. S. (1993) Feeding habits in soil nematode families and genera - an outline for soil ecologists. *Journal of Nematology, 25*, 315-331.

Yeates, G. W. & Wardle, D. A. (1996) Nematodes as predators and prey: Relationships to biological control and soil processes. *Pedobiologia, 40*, 43-50.

Anna M. Piśkiewicz · Maarten J.K. de Milliano · Henk Duyts · Wim H. van der Putten

Submitted

Chapter 4

Plant ectoparasitic nematodes prefer roots without their microbial enemies

Summary

The abundance of the ectoparasitic nematodes *Tylenchorhynchus ventralis* in coastal foredune soil is controlled by microbial enemies. If not controlled, *T. ventralis* becomes so abundant, that it reduces growth and performance of the host plant *Ammophila arenaria*. In the present study, we determined if the nematodes may defend themselves against soil microorganisms by avoiding microbial hotspots. We examined if the presence or absence of soil microorganisms influences the choice of *T. ventralis* when selecting roots to feed upon. First, we performed an experiment using artificial agar medium to examine if *T. ventralis* is able to choose between *A. arenaria* seedlings inoculated with or without microorganisms. We also checked if the nematodes were attracted to the microbial inoculum alone when no plants were present. Then, we optimized nematode choice conditions using Y-tubes filled with sterilized dune soil. Again, we examined if *T. ventralis* could choose between *A. arenaria* with or without microorganisms. In addition, we studied how nematodes responded to a microbial-free solution obtained from a microbial suspension.

We show that in foredune soil, the plant parasitic nematode *T. ventralis* chooses roots without soil microorganisms when having roots with a soil microbial community as alternative. No choice was made when microorganisms were present or absent without plants. Therefore, nematode choices require both plants and microorganisms to be present.

We conclude that the plant parasitic nematode *T. ventralis* is able to avoid microbial hotspots in the rhizosphere, thereby avoiding its natural microbial antagonists. Thus far, studies on the distribution of root feeders have paid very little attention to the capacity of root feeders to avoid their natural enemies. Our results suggest that avoiding top-down control can structure belowground communities, as it does with aboveground herbivores.

Keywords: coastal dunes, plant parasitic nematodes, predator avoidance, repellence, root herbivore community structure, soil microorganisms, top-down control, tri-trophic interaction, Y-tubes

Introduction

Root-feeding nematodes are the main belowground herbivores in natural grassland ecosystems and take up as much as a quarter of the net primary production (Stanton 1988). Besides their influence on plant productivity, root-feeding nematodes also influence plant community composition (de Deyn et al. 2004; de Deyn et al. 2007; Verschoor et al. 2002). In agricultural systems, root-feeding nematodes are major pests and cause severe crop losses. Nematodes are difficult to be suppressed and the use of nematicides leads to severe environmental contamination (Oka et al. 2000), which causes opposition against chemical nematode control (Doran et al. 1996). On the other hand, biological control is still unreliable and, therefore, not widely practiced (Alabouvette et al. 2006; Kerry 2000; Kerry & Hominick 2002). One of the benefits of studying how the abundance of root-feeding nematodes is controlled in natural ecosystems is that it may help to understand why biological control of nematodes in agro-ecosystems is so unpredictable (van der Putten et al. 2006).

In natural ecosystems, root-feeding nematodes are suppressed in a species-specific way. For example, in coastal foredunes competition between nematodes may lead to low numbers of the root knot nematode *Meloidogyne maritima* (Brinkman et al. 2005), arbuscular mycorrhizal fungi can suppress the root lesion nematode *Pratylenchus* spp. (de la Peña et al. 2006), the plant can control the cyst nematode *Heterodera arenaria* by bottom-up mechanisms (van der Stoel et al. 2006) and microbial enemies are involved in suppression of the ectoparasitic nematode *Tylenchorhynchus ventralis* (Piśkiewicz et al. 2007). Thus far, in these and other studies on root feeders, very little attention has been given to the capacity of the root feeders to avoid their natural enemies. Here, we study if ectoparasitic nematodes are able to avoid feeding on roots where their microbial enemies are present.

Below ground, when searching for suitable feeding sites, plant parasitic nematodes are attracted to the roots by diverse chemical cues, for example CO_2, inorganic ions and salts (Abou-Setta & Duncan 1998; Le Saux & Queneherve 2002; Robinson 1995). Without the presence of cues parasitic nematodes would show random movement. Contact with the chemosensory stimulants or deterrents produced by host roots, would determine if a

nematode will commence feeding or whether it keeps searching for a better feeding site (Johnson & Gregory 2006). Nematodes sense environmental cues by a specialized nervous system that includes chemo, thermo-, and mechanosensory neurons (Bird & Bird 1991). There is some evidence that after exposure to harmful organisms, nematodes are able to change their olfactory preferences. For example, *Caenorhabditis elegans* modifies its preferences after exposure to pathogenic bacteria, avoiding odors from the pathogenic and increasing its attraction to odors from familiar non-pathogenic bacteria. This allows *C. elegans* to avoid infections with detrimental microorganisms (Zhang et al. 2005).

In a previous study, we have shown that microorganisms from the rhizosphere of the coastal foredune grass *Ammophila arenaria* (marram grass) are able to control ectoparasitic root-feeding nematodes *T. ventralis* (former name *Telotylenchus ventralis* (Loof, 1963) Fortuner and Luc, 1987) (Piśkiewicz et al. 2007). These nematodes are key root herbivores in natural coastal foredunes, as they are able to decrease the biomass of *A. arenaria* (de Rooij van der Goes 1995) if not controlled by their microbial enemies (Piśkiewicz et al. 2007). In the field *T. ventralis* usually does not reach population levels that can affect plant growth (van der Stoel et al. 2002), but numbers vary locally (de Rooij van der Goes et al. 1995). In the present study, we examine if plant ectoparasitic nematodes *T. ventralis* are able to sense and avoid detrimental microorganisms.

To examine if the ectoparasitic nematodes avoid their microbial enemies, we first studied the choice of *T. ventralis* on agar in Petri dishes with or without *A. arenaria* seedlings inoculated with or without dune soil microorganisms. Then, we tested the hypothesis that the nematodes would be attracted more to roots without than to roots with soil microorganisms. In order to test this hypothesis, we performed two experiments in soil using a Y-tube olfactometer filled with sterilized dune soil (Boff et al. 2001) with or without *A. arenaria* seedlings that had been inoculated without or with soil microorganisms. We also studied whether microorganisms alone or microbial filtrate would give the same result as that of a combination of plant roots and microorganisms. We present and discuss new evidence that in dune soil the ectoparasitic nematode *T. ventralis* is able to avoid its microbial enemies.

Materials and Methods

We designed three experiments to study the choice behavior of the nematode *T. ventralis*. Experiment 1 was performed in Petri dishes on agar medium, allowing us to make observations of the nematode behavior in a non-destructive way. In this experiment we examined the choice of *T. ventralis* between *A. arenaria* seedlings inoculated with microorganisms or clean (no microorganisms added) seedlings. We also checked if the nematodes were attracted to microbial inoculum alone (without plants in Petri dishes). Experiments 2a and 2b were performed in Y-tubes filled with sterilized dune soil, which simulated semi-natural conditions more effectively than the agar Petri dishes. In experiment 2a, vertical nematode migration was optimized and in experiment 2b, we examined the choice behavior of *T. ventralis* between *A. arenaria* without or inoculated with microorganisms originating from the natural foredune soil.

Experiment 1: choice of *T. ventralis* in Petri dishes

Plants

In summer 2005, seeds of *A. arenaria* were collected from a natural coastal foredune at Voorne, The Netherlands (Latitude 51°55'N – Longitude 04°05'E) and stored dry until used. The seeds were separated from their seed coat and subsequently sterilized by soaking in 95% ethanol and 8% bleach (85 ml bleach water, 92 ml H_2O, 150 µl tween-20) for 2 min and 15 min, respectively. Sterilized seeds were washed 5 times for at least 2 minutes with sterilized tap water and then dried. In order to obtain *A. arenaria* seedlings, the seeds were placed on thirty Petri dishes of 8.5 cm diameter, each containing 10 ml of 0.5% microbial agar (Merck kGaA, De.). The seeds were placed on both sides of the dish, 2.5 cm away from the center. Seedlings on the agar matrix were grown for 8 days and after this time the treatments were applied. The assays were performed in a climate chamber with a 16/8 hours light/dark regime at a temperature of 25/15°C, respectively.

Nematodes and soil microorganisms

Specimen of *T. ventralis* were collected from the same coastal foredune area as plants and soil and cultured on the roots of *A. arenaria* plants in a greenhouse. One day before usage,

the nematodes were extracted from the cultures by Cobb's decantation method (Oostenbrink 1960). Microorganisms were extracted from the collected dune soil by shaking 100 g soil samples with demineralized water (1:1 $w \cdot w^{-1}$) for 10 minutes and filtering the supernatant through a 20 µm mesh (Bezemer et al. 2005; Klironomos 2002). Prepared microbial solutions were checked by microscope to establish that they did not contain nematodes or spores of arbuscular mycorrhizal fungi.

Experimental setup

In case of the Petri dishes with and without seedlings, the treatments consisted of the following combinations: Control ↔ Control; Control ↔ Microorganisms; Microorganisms ↔ Microorganisms. On each half of the Petri dish, depending on the inoculation treatment, 80 µl of microbial suspension or sterile tap water was applied. Each treatment combination was carried out in 5 replicate Petri dishes. Three days after adding the microbial suspension or sterilized water, 40 *T. ventralis* were inoculated in a 100 µl water suspension at the center of the Petri dishes. Migration of the nematodes from the centre of the agar plate towards the edge was recorded 3 and 16 hours, 1 day, 2, 4 and 6 days after inoculation.

Experiments 2a and b: choice of *T. ventralis* in Y-tubes with dune soil

Soil

In summer 2005, soil samples were collected from the natural coastal foredune at Voorne. The soil was collected from ten transects parallel to the beach and 50 m apart. At each sampling point, 10 kg of soil was collected from the youngest root zone of *A. arenaria* (van der Putten et al. 1988). The soil was sieved (0.5 cm mesh size) to remove plant parts and debris, and stored in plastic bags at 4°C until used (van der Stoel et al. 2002).

Plants

Seeds of *A. arenaria* were collected from the same foredune area and stored dry until used. In order to obtain seedlings, the seeds were germinated for 2 weeks on moist glass beads in a climate chamber at a 16/8 hours light/dark regime and 25/15°C to provide day/night conditions. When the first leaf was 2-3 cm long, the seedlings were transplanted to the arms

of vertically positioned Y-tubes filled with sterilized dune soil. The soil moisture was adjusted to 10 % $w \cdot w^{-1}$ and maintained at this level until the nematodes and microorganisms were inoculated. After inoculation, the tubes were moistened with 2 ml of demineralized water once a day. This amount of water prevented that the migrating nematodes were flushed back to the bottom of the Y-tube. The experiments were carried out in a greenhouse at a day temperature of 21°C $\pm$ 2°C (day length 16 hours) with additional light (to maintain a minimum of 225 µmol $m^{-2} \cdot s^{-1}$ PAR with SON-T Agro lamps) and a night temperature of 16°C. These temperatures are comparable to summer conditions in the field and they are optimal for both plant and nematode development (S.R. Troelstra and R. Wagenaar, unpublished results).

Soil organisms

The nematodes and microbial suspensions were collected and prepared as described as in experiment 1. The nematodes were inoculated at the bottom of the lower parts of Y-tubes in 2 ml of tap water at a density of 300 *T. ventralis* · $Y\text{-tube}^{-1}$ in case of experiment 2a and 500 *T. ventralis* · $Y\text{-tube}^{-1}$ in case of experiment 2b. The microorganisms were inoculated at a rate of 5 ml suspension · $Y\text{-tube arm}^{-1}$. The filtrate was prepared by filtering a microbial solution by 0.2 µm sieve. This filtrate did not contain any nematodes or microorganisms, but only the compounds that could be extracted from the soil by water, including those produced by the soil organisms.

Harvest

Five days after inoculation with nematodes and microorganisms, the arms and the lower part of Y-tubes were dissembled and the soil from each of those parts was collected separately. *T. ventralis* from each soil portion were collected by Cobbs' decantation method (Oostenbrink 1960) and counted by microscope (200 x magnification).

Experimental setup

Experiment 2a:

Thirty Y-shape vertical plastic tubes were constructed. The lower part was 15 cm long and both arms 10 cm. The arms were closed at the bottom with a 0.5-mm mesh to protect the sand to leak and to prevent root growth into the lower part of the Y-tube, while still enabling the nematodes to migrate through the mesh towards the plant roots. The bottom of Y-tube was closed by a plastic cap. Prior to the choice experiment, the Y-tubes were filled with the sterilized dune soil and *A. arenaria* seedlings were planted, one into each arm. After 3 weeks, when the shoots of the seedlings were about 10 cm long, 300 nematodes were inoculated at the bottom of the lower part of each Y-tube and closed with the plastic cap. Then the Y-tubes were randomly divided into three groups and the arms of each group were inoculated with different treatment combinations. The first treatment combination consisted of both arms receiving no microorganisms, but sterilized tap water. In the second treatment combination, one arm was inoculated with microorganisms and the other arm with sterilized tap water. Finally, both arms of the Y-tubes from the third treatment combination were inoculated with microorganisms.

Experiment 2b:

Based on the results of experiment 2a, ninety Y-tubes were constructed having 10 cm long lower parts to facilitate nematode migration towards the arms. The arms, similarly as in Experiment 2a, were 10 cm long. All Y-tubes were filled with sterilized dune soil and *A. arenaria* seedlings were planted in sixty of them. Thirty tubes contained no plants. Three weeks after the seedlings were planted, 500 nematodes were inoculated. They were suspended into 2-ml tap-water, and added at the bottom of the lower part of each Y-tube. Then, the Y-tubes were closed with plastic caps. Sixty Y-tubes with *A. arenaria* were divided randomly into 6 groups and the arms were inoculated with treatment combinations: Control ↔ Control; Control ↔ Microorganisms; Control ↔ Filtrate; Microorganisms ↔ Filtrate; Microorganisms ↔ Microorganisms; Filtrate ↔ Filtrate. The Y-tubes without plants were randomly divided into three groups and the arms were inoculated with the treatment combinations: Control ↔ Control; Control ↔ Microorganisms; Microorganisms ↔ Microorganisms.

Statistical analyses

Normal distribution of data and homogeneity of variance were checked by inspection of the residuals after model fit (using the package Statistica 7). In case of experiment 1, the numbers of nematodes counted on both halves of Petri dishes were compared by T-test. First, we tested if the numbers of migrated nematodes did not differ when the treatments of both halves of Petri dishes were the same. Then, we tested if the numbers of nematodes differed between both halves of Petri dishes when having received different treatments. Finally, using one-way ANOVA (three levels: Control ↔ Control; Control ↔ Microorganisms; Microorganisms ↔ Microorganisms) we tested if total migration of the nematodes away from the inoculation point was dependent on the microorganism addition.
In case of the experiments 2a and 2b, T-tests were used to test if the migrated nematodes differed between both arms of the Y-tube. Then, by one-way ANOVA we checked if the total migration of the nematodes was influenced by microorganism addition into the Y-tubes.

Results

Experiment 1: choice of T. ventralis in Petri dishes

We did not observe any nematode migration in each of three treatment combinations if there were no *A. arenaria* seedlings growing in the Petri dishes (data not shown). The nematodes clustered in the centre of the Petri dish close to the inoculation point and did not move more than 0.5 cm. After 7 days, the nematodes started to degenerate and microorganisms covered the plates, which prevented the nematodes from moving further.

When the plates contained *A. arenaria* seedlings, the nematode started to migrate towards the seedlings soon after inoculation. Although there was a trend in nematode migration towards the non-inoculated plants, the effects were not statistically significant ($P > 0.05$). The migration tended to be the highest 1 day after nematode inoculation and reached 61% on the plates where the seedlings did not contain any microorganisms, 53% on plates with single-sided microbe inoculation and 41% on the plates with microorganisms on both sides. After 4 days, the number of live nematodes counted on both sides of the Petri dishes was lower than on the 2nd day because of nematode mortality.

At any time point, we did not observe any significant differences in the percentage of nematodes that had migrated from the place of inoculation towards the plants inoculated with or without microorganisms (Figure 10). The only difference that came close to significance (P = 0.06) in the numbers of migrated nematodes was observed after 1 day in case of seedlings inoculated with microorganisms and other seedlings without microorganisms (Figure 10C). More nematodes moved towards seedlings without microbial inoculation.

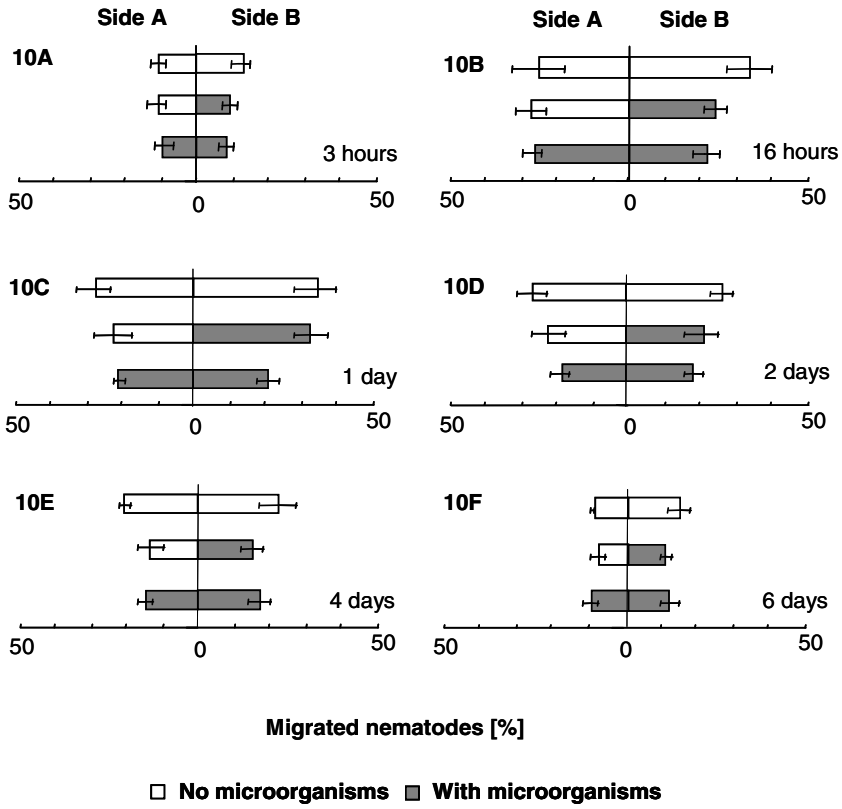

Figure 10. Experiment 1. Percentages of nematodes migrated towards *Ammophila arenaria* seedlings on two halves of agar Petri dishes inoculated with or without microorganisms. The nematodes were scored 3 hours (10A), 16 hours (10B), 1 day (10C), 2 days (10D), 4 days (10E) and 6 days (10F) after inoculation with nematodes. Error bars indicate standard errors.

Experiment 2a (pilot): choice of T. ventralis in Y-tubes with dune soil

T-tests showed no differences in numbers of migrated nematodes between two control arms ($P = 0.23$), or between two arms inoculated with microorganisms ($P = 0.8$) (Figure 11). However, there were significantly less nematodes in arms inoculated with microorganisms when the other arms had not received microbial suspension (Figure 11). In this case, twice as many nematodes moved towards the plant without than to the plant with microorganisms ($P < 0.01$). There was a trend, although not significant, that if one or both arms were inoculated with microorganisms, less nematodes migrated upwards.

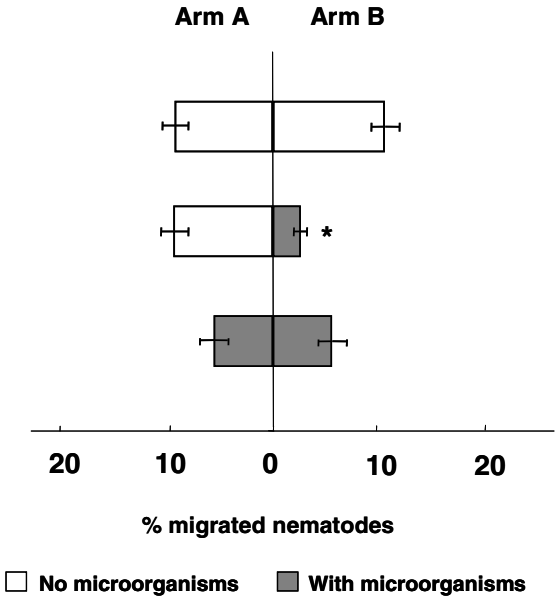

Figure 11. Experiment 2a. Percentages of nematodes migrated towards *Ammophila arenaria* seedlings growing in two arms of Y-tubes filled with sterilized dune soil and inoculated with or without microorganisms. Error bars indicate standard errors. The asterisks show significance at $P < 0.05$.

Experiment 2b: choice of *T. ventralis* in Y-tubes with dune soil

If no *A. arenaria* seedlings were present in the arms of the Y-tubes, the total nematode migration was lower than 5% and the majority of the nematodes stayed in the lower part of Y-tubes (Figure 12). Besides, there were no differences in total numbers of migrated nematodes ($P > 0.05$) between different treatment combinations.

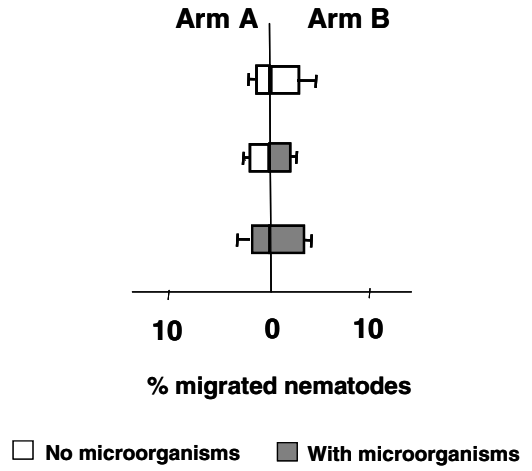

Figure 12. Experiment 2b. Percentages of nematodes migrated upwards in two arms of Y-tubes filled with sterilized dune soil and inoculated with microorganisms or without microorganism addition (no plant seedlings present in the arms). Error bars indicate standard errors. The asterisks show significance at $P < 0.05$.

If *A. arenaria* seedlings were present in the Y-tubes, T-tests comparing the number of nematodes that had migrated upwards into both arms revealed that the only significant difference was if one arm was inoculated with microorganisms and other arm not ($P = 0.01$) (Figure 13). There was no difference in nematode numbers in case of any other treatment combination ($P > 0.05$). Therefore, the microorganism effect on the nematode choice was observed only when both plant roots and microorganisms were present at the same time. Microbial filtrates did not influence the nematode choice.

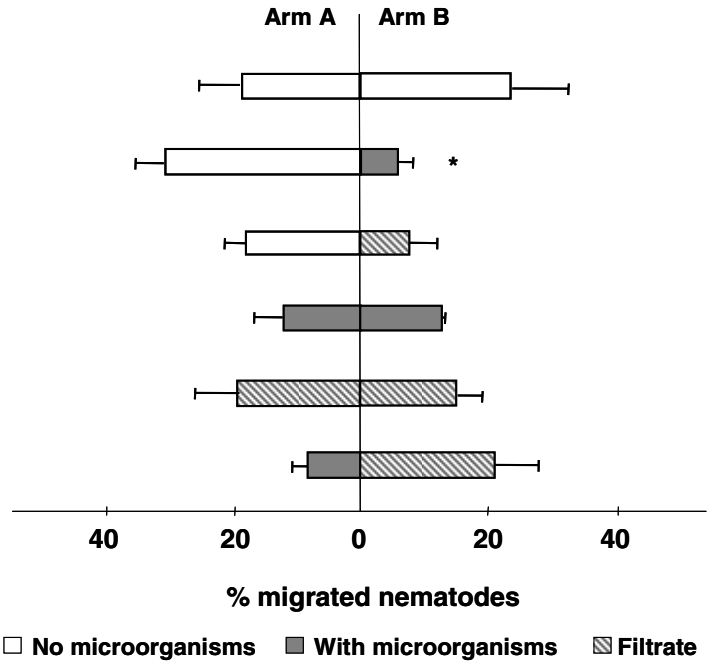

Figure 13. Experiment 2b. Percentages of nematodes migrated towards *Ammophila arenaria* seedlings growing in Y-tubes filled with sterilized dune soil and inoculated with microorganisms, inoculated with microbial filtrate or clean (no microorganisms, no filtrate). Error bars indicate standard errors. The asterisks show significance at $P < 0.05$.

One-way ANOVA showed that there were no differences between treatments in the total number of migrated nematodes. However, the total migration tended to be the highest (42%) when both arms were not inoculated with microorganisms and lowest (24%) in case both arms had been inoculated with microorganisms. This suggests that the microorganisms make roots less attractive, or repellant, for the nematodes.

Discussion

In this study, for the first time we show that the choice of the plant ectoparasitic nematode *T. ventralis* for plant roots may be influenced by the presence of a microbial

community, which contains all, including nematode-antagonistic microorganisms. Our results do not reveal whether this choice effect is due to either reduced attractiveness or to enhanced repellence of roots with soil microorganisms. However, it is clear that the microbial effects on nematode choice depend on the combined presence of both plant roots and microorganisms. Microorganisms alone or roots with microbial filtrate did not influence nematode choice significantly. There are several possible explanations for our results. The first is that the microorganisms, when feeding on root exudates, mask the attractive effects of CO_2-related cues produced by the plant roots. The second is that the microorganisms themselves, or easily decomposable products, produce compounds that actively repel the nematodes. We can exclude the possibility that in the sites inoculated with microorganisms, the nematodes were suppressed by parasitism, predation or antagonism. The duration of the experiments was simply too short to allow predatory activities to reduce nematode numbers.

The trials using the artificial agar medium did not show any differences in choice behavior of the nematodes, whereas the Y-tubes did. This may stress the importance of performing such choice experiments in a more natural environment, such as Y-tubes with dune soil. Moreover, another possible difficulty of the experiments using soil microorganisms on artificial agar medium is that the microorganisms may not develop as in a natural rhizosphere. Previous studies showed that the ectoparasitic nematodes *T. ventralis* are parasitized mostly by *Pasteuria* sp., *Catenaria* sp. and *Paenibacillus*-like organisms and other unidentified bacteria (Piśkiewicz et al. 2007). Until now, there are no proper methods to successfully culture *Pasteuria* sp. on agar medium (Davies 2005). Our results suggest being careful with nematode choice experiments carried out in highly artificial environments, such as Petri dishes with agar medium.

When the sensory interaction between plant roots and ectoparasitic nematodes is influenced by the microbial communities on or near the plant roots, such altered signaling can have influences on the distribution of nematodes in the soil. In our experiments, we have used a whole microbial community, however, in the rhizosphere the microbial community is known to vary in composition along plant roots (Duineveld & Van Veen 1999). It will, therefore, be of interest to further unravel whether the altered signals are due

to the microbial community as a whole, or to specific components of the microbial community. In case of the latter option, it is of interest to study where these components occur in the rhizosphere. For example, when hot spots of microbial repellents would occur near root tips, where active growth takes place, or a bit further up where active uptake of nutrients takes place, but not towards the basal part of the root, plants could be actively defending the most valuable parts of their root system against ectoparasitic nematodes in a tri-trophic way. Tri-trophic indirect defense is commonly known for aboveground plant defense; however, such phenomena in soil have received limited attention, usually for entomopathogenic nematodes (Rasmann et al. 2005; van Tol et al. 2001).

We conclude that plant ectoparasitic nematodes of the species *T. ventralis* have the capacity to sense and avoid their microbial enemies. This may be due to the attraction to the cues produced by the roots without dune soil microorganisms, or by repellence of roots which contain these microorganisms. The precise mechanisms of these interactions is yet unknown. The results of the microbial filtrate addition suggested that the repelling effects are not caused by microbial products, or their interactions with plant roots. Therefore, our results suggest that the possible repellence is due to direct interactions between plant roots and rhizosphere microorganisms. The questions whether in the field plants may direct their generalist root feeding nematodes to less sensitive parts of their root system, and which components of the microbial community are involved in these tri-trophic interactions require further studies.

Acknowledgements

This work is part of the EcoTrain project (HPRN-CT 2002 00210), which was funded by the European Union rtn program.

References

Abou-Setta, M. M. & Duncan, L. W. (1998) Attraction of *Tylenchulus semipenetrans* and *Meloidogyne javanica* to salts in vitro. *Nematropica, 28*, 49-59.

Alabouvette, C., Olivain, C. & Steinberg, C. (2006) Biological control of plant diseases: the European situation. *European Journal of Plant Pathology, 114*, 329-341.

Bezemer, T. M., de Deyn, G. B., Bossinga, T. M., van Dam, N. M., Harvey, J. A. & van der Putten, W. H. (2005) Soil community composition drives aboveground plant-herbivore-parasitoid interactions. *Ecology Letters, 8*, 652-661.

Bird, A. F. & Bird, J. (1991) The structure of nematodes. San Diego: Academic Press.

Boff, M. I. C., Zoon, F. C. & Smits, P. H. (2001) Orientation of *Heterorhabditis megidis* to insect hosts and plant roots in a Y-tube sand olfactometer. *Entomologia Experimentalis Et Applicata, 98*, 329-337.

Brinkman, E. P., Duyts, H. & van der Putten, W. H. (2005) Consequences of variation in species diversity in a community of root-feeding herbivores for nematode dynamics and host plant biomass. *Oikos, 110*, 417-427.

Davies, K. G. (2005) Interactions between nematodes and microorganisms: Bridging ecological and molecular approaches. *Advances in Applied Microbiology,* 57, 53-78.

de Deyn, G. B., Raaijmakers, C. E. & van der Putten, W. H. (2004) Plant community development is affected by nutrients and soil biota. *Journal of Ecology, 92*, 824-834.

de Deyn, G. B., van Ruijven, J., Raaijmakers, C. E., de Ruiter, P. C. & van der Putten, W. H. (2007) Above- and belowground insect herbivores differentially affect soil nematode communities in species-rich plant communities. *Oikos, 116*, 923-930.

de la Peña, E., Rodriguez-Echeverria, S., van der Putten, W. H., Freitas, H. & Moens, M. (2006) Mechanism of control of root-feeding nematodes by mycorrhizal fungi in the dune grass *Ammophila arenaria*. *New Phytologist, 169*, 829-840.

de Rooij van der Goes, P. C. E. M. (1995) The role of plant parasitic nematodes and soil borne fungi in the decline of *Ammophila arenaria* (L) Link. *New Phytologist, 129*, 661-669.

de Rooij van der Goes, P. C. E. M., van der Putten, W. H. & van Dijk, C. (1995) Analysis of nematodes and soil borne fungi from *Ammophila arenaria* (Marram grass) in Dutch coastal foredunes by multivariate techniques. *European Journal of Plant Pathology, 101*, 149-162.

Doran, J. W., Sarrantonio, M. & Liebig, M. A. (1996) Soil health and sustainability. *Advances in Agronomy, 56,* 1-54.

Duineveld, B. M. & van Veen, J. A. (1999) The number of bacteria in the rhizosphere during plant development: relating colony-forming units to different reference units. *Biology and Fertility of Soils, 28*, 285-291.

Johnson, S. N. & Gregory, P. J. (2006) Chemically-mediated host-plant location and selection by root-feeding insects. *Physiological Entomology, 31*, 1-13.

Kerry, B. R. (2000) Rhizosphere interactions and the exploitation of microbial agents for the biological control of plant parasitic nematodes. *Annual Review of Phytopathology, 38*, 423-441.

Kerry, B. R. & Hominick, W. M. (2002) Biological control. London: Taylor & Francis.

Klironomos, J. N. (2002) Feedback with soil biota contributes to plant rarity and invasiveness in communities. *Nature, 417*, 67-70.

Le Saux, R. & Queneherve, P. (2002) Differential chemotactic responses of two plant-parasitic nematodes, *Meloidogyne incognita* and *Rotylenchulus reniformis*, to some inorganic ions. *Nematology, 4*, 99-105.

Oka, Y., Koltai, H., Bar-Eyal, M., Mor, M., Sharon, E., Chet, I. & Spiegel, Y. (2000) New strategies for the control of plant-parasitic nematodes. *Pest Management Science, 56*, 983-988.

Oostenbrink, M. (1960) Estimating nematode populations by some selected methods. *In Nematology* (ed. J. N. Sasser & W. R. Jenkins), pp. 85-102. Chapel Hill: The University of North Carolina Press.

Piśkiewicz, A. M., Duyts, H., Costa, S. R., Berg, M. P. & van der Putten, W. H. (2007) Soil microorganisms control plant ectoparasitic nematodes in natural coastal foredunes. *Oecologia, 152*, 505-514.

Rasmann, S., Kollner, T. G., Degenhardt, J., Hiltpold, I., Toepfer, S., Kuhlmann, U., Gershenzon, J. & Turlings, T. C. J. (2005) Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature, 434*, 732-737.

Robinson, A. F. (1995) Optimal release rates for attracting *Meloidogyne incognita*, *Rotylenchulus reniformis*, and other nematodes to carbon dioxide in sand. *Journal of Nematology, 27*, 42-50.

Stanton, N. L. (1988) The underground in grasslands. *Annual Review of Ecology and Systematics, 19*, 573-589.

van der Putten, W. H., Cook, R., Costa, S., Davies, K. G., Fargette, M., Freitas, H., Hol, W. H. G., Kerry, B. R., Maher, N., Mateille, T., Moens, M., de la Peña, E., Piśkiewicz, A. M., Raeymaekers, A. D. W., Rodriguez-Echeverria, S. & van der Wurff, A. W. G. (2006) Nematode interactions in nature: Models for sustainable control of nematode pests of crop plants? *Advances in Agronomy, 89*, 227-260.

van der Putten, W. H., van Dijk, C. & Troelstra, S. R. (1988) Biotic soil factors affecting the growth and development of *Ammophila arenaria*. *Oecologia, 76*, 313-320.

van der Stoel, C. D., Duyts, H. & van der Putten, W. H. (2006) Population dynamics of a host-specific root-feeding cyst nematode and resource quantity in the root zone of a clonal grass. *Oikos, 112*, 651-659.

van der Stoel, C. D., van der Putten, W. H. & Duyts, H. (2002) Development of a negative plant-soil feedback in the expansion zone of the clonal grass *Ammophila arenaria* following root formation and nematode colonization. *Journal of Ecology, 90*, 978-988.

van Tol, R. W. H. M., van der Sommen, A. T. C., Boff, M. I. C., van Bezooijen, J., Sabelis, M. W. & Smits, P. H. (2001) Plants protect their roots by alerting the enemies of grubs. *Ecology Letters, 4*, 292-294.

Verschoor, B. C., de Goede, R. G. M. & Brussaard, L. (2002) Do plant parasitic nematodes have differential effects on the productivity of a fast- and a slow-growing grass species? *Plant and Soil, 243*, 81-90.

Zhang, Y., Lu, H. & Bargmann, C. I. (2005) Pathogenic bacteria induce aversive olfactory learning in *Caenorhabditis elegans*. *Nature, 438*, 179-184.

Anna M. Piśkiewicz · Henk Duyts · Wim H. van der Putten

Submitted

Chapter 5

Multiple species-specific controls of root-feeding nematodes in natural soils

Summary

One of the major limitations of enhancing sustainability of crop production systems is the inability to control root-feeding nematodes without using chemical biocides. In soils under wild vegetation, root-feeding nematodes affect plant performance and plant community composition varying from substantially to insignificantly. In order to learn from natural systems, we examined nematode control in the root zone of a wild coastal foredune grass by microorganisms, other nematodes and microarthropods. We show that almost each of the eight dominant root-feeding nematodes could be controlled by more than one mechanism and that most nematode species were controlled in a species-specific way. Our results strongly suggest that sustainable agriculture will benefit from using a range of biological control mechanisms when controlling root-feeding nematodes, rather than relying on single control agents. We conclude that conserving soil biodiversity is crucial in order to ensure biological crop protection.

Keywords: root-herbivory, biological control, nematodes, multitrophic interactions, sustainable agriculture

Introduction

Belowground herbivores have a profound influence on the productivity and species composition of plant communities (de Deyn et al. 2003; Gange & Brown 1989; Gange & Brown 2002; Wäckers & Bezemer 2003). The majority of belowground herbivores are root-feeding nematodes and insect larvae (Gange & Brown 2002). In some grassland ecosystems, nematodes are the dominant herbivores (Ingham & Detling 1986). Most information on root-feeding nematodes, however, stems from agricultural systems, where root-feeding nematodes are notorious pests (Freckman & Caswell 1985). Because chemical control of nematodes involves the use of persistent broad-activity chemicals, nematode control may disrupt complete soil food webs. Therefore, many efforts have been spent to improve biological control (Kerry & Gowen 1995; Rodriguez Kabana 1991). However, the results of biological control are notoriously unpredictable and most efforts have been dedicated to a limited subset of nematodes, whereas solving one nematode problem often creates another one (Barker & Koenning 1998). Therefore, in order to enhance the sustainability of crop production methods, studying nematode control in nature may serve to ultimately enhance effectiveness of nematode control in agriculture (van der Putten et al. 2006).

Root-feeding nematodes have been estimated to take up as much as one quarter of the net primary production in prairie grasslands (Stanton 1988). When present at low amounts, root-feeding nematodes may increase the allocation of assimilated carbon to roots, leading to increased root exudation and microbial activity in the rhizosphere (Bardgett et al. 1999). In this case, root-feeding nematodes may even enhance plant productivity through their positive feedback effects on plant nutrition. Opposite to prairie grasslands, the abundance of root-feeding nematodes in coastal foredunes appears too low to account for any plant growth reduction (de Rooij van der Goes 1995). Some factors have been suggested as controlling root-feeding nematodes, such as competition (Brinkman et al. 2005), arbuscular mycorrhizal fungi (de la Peña et al. 2006) and bottom-up control by the plant (van der Stoel et al. 2006). Whereas these processes are mainly driven by limitation of resource availability (so-called bottom-up and horizontal interactions), nematodes may also be controlled by predatory or parasitic soil organisms (so-called top-down effects) (Kerry &

Hominick 2002). To date, no study has attempted to analyze all potential factors involved in root herbivore control in a comparative way.

Soil organisms play an important role in soil suppressiveness against plant diseases. However, what exactly makes soils suppressive for soil-borne pathogens has been an objective of intensive studies (Jaffee 1993; Mazzola 2002; Termorshuizen et al. 2006). In most cases suppressiveness is caused by the interactions between soil-borne pathogens and soil microorganisms (Alabouvette 1999; Weller et al. 2002). Many studies have shown soil suppressiveness against fungal diseases (Alabouvette et al. 1993; Amir & Alabouvette 1993) and a few studies have shown similar suppressiveness against root-feeding nematodes (Dicklow et al. 1993; Esnard et al. 1995; Kluepfel et al. 1993). Nematode suppression may be caused, similarly as for fungi, by soil microorganisms. However, those studies are usually based on agricultural systems, whereas only a few studies have examined pathogen or nematode suppression in natural soils. Natural coastal dune soil, which has become a model for studying plant-soil pathogen interactions, is suppressive to fungal pathogens (de Boer et al. 1998; de Boer et al. 2003). In this case the suppression of fungi pathogenic to *Ammophila arenaria* is due to the anti-fungal compounds produced by the microorganisms.

The root zone of the wild coastal foredune grass *Ammophila arenaria* contains an array of different nematode feeding types. They range from ectoparasites that feed on the outer cortical cell layers and root hairs to sedentary endoparasites that establish a feeding site to which females get attached in order to complete their life cycle (van der Putten & van der Stoel 1998). In the present study, we explore the potential contribution of soil microorganisms, the whole nematode community and microarthropods to top-down control of all eight major root-feeding nematodes that co-occur on *A. arenaria* roots in the central part of its native range (van der Putten et al. 2005). The aim of our study is to determine if different nematode species are controlled by the same top-down mechanism. Alternatively, different mechanisms may actively control different nematode species.

Six of the root-feeding nematode species were ectoparasites, which feed on outer cortical cell layers from the outside of the roots. These ectoparasitic root-feeding nematodes are considered to be feeding generalists (van der Putten et al. 2005; Yeates et al. 1993).

Two other species were endoparasitic nematodes, which are feeding specialists. Moreover, one of these endoparasites was a sedentary and the other a migratory endoparasite. We extracted microorganisms, nematodes and microarthropods from non-sterile dune soil. Subsequently we added the microorganisms, nematodes and microarthropods into the root zone of *A. arenaria* plants that were grown in previously sterilized dune soil to which the different root-feeding nematode species had been added, species by species. The effects of adding the various soil organisms on nematode abundance and plant biomass were determined at the end of the experiment.

Our null hypothesis was that there were no differences in top-down control among the plant parasitic nematode species. Alternatively, we hypothesized that root-feeding nematode species are controlled in a species-specific way, so that control mechanisms will differ among the nematode species. We conclude that most root-feeding nematodes potentially are controlled by two or more mechanisms. Nevertheless, soil microorganisms make the most important contribution to the control of the majority of the root-feeding nematode species. We discuss our results in relation to soil biodiversity and its importance for sustainable crop protection in agriculture.

Materials and methods

Soil, plants, nematodes and potential control organisms

Soil was collected from the mobile foredunes of Voorne, The Netherlands (51°55'N – 04°05'E) at peak growing season. The samples were collected from ten points parallel to the beach and 50 m apart from each other. At all sampling points, 50 kg of soil was collected from the youngest root zone of *A. arenaria*, which is situated on top of older root zones from previous growth seasons (van der Stoel et al. 2002). The soil was sieved (0.5 cm mesh size) to remove plant parts and debris and after that homogenized and stored in plastic bags at 4°C until usage (van der Stoel et al. 2002). One half of the collected soil was sterilized by gamma irradiation with an average dose of 25 kGray, which is effective to eliminate all living soil organisms from dune soil (de Rooij-van der Goes et al. 1998).

Seeds of *A. arenaria* were collected from random plants at the same field site and stored dry until usage. The seeds were germinated for 2 weeks on moist glass beads at 16/8

hour light/dark regime at 25/15°C. Seedlings with the first leaf of 2-3 cm long were transplanted to plastic 1.5 l pots filled with 1500 g sterilized dune soil (4 seedlings of *A. arenaria* per pot) with 10 % w · w^{-1} soil moisture. Subsequently, the soil surface was covered with aluminum foil to protect the surface from desiccation. The soil moisture was maintained during the whole experiment by weighing the pots twice a week and re-setting the initial weight by adding demineralized water. Once a week full-strength Hoagland nutrient solution was added; first 3 weeks 12.5 ml · pot^{-1} and later 25 ml · pot^{-1} (Brinkman et al. 2004). The experiment was carried out in a greenhouse at a day temperature of 21°C ± 2°C and the day length was minimally 16 hours by providing additional light to ensure minimally 225 µmol · m^{-2} · s^{-1} PAR with SON-T Agro lamps. The night temperature was 16 ± 2 °C.

Eight different plant parasitic species of nematodes were used. Among those species six were ectoparasitic: *Criconema* sp., *Helicotylenchus pseudorobustus* (Steiner 1914) Golden, 1956, *Hemicycliophora* sp., *Rotylenchus* sp., *Tylenchorhynchus ventralis* (Loof, 1963) Fortuner and Luc, 1987 and *Tylenchorhynchus microphasmis* (Loof 1960) Jairajpuri and Hunt, 1983. Two other species were endoparasitic: sedentary *Heterodera arenaria* (Cooper, 1955) Robinson, Stone, Hooper and Rowe and migratory *Pratylenchus* sp. All these species originated from Dutch coastal foredunes and the cultures were from the NIOO at Heteren, The Netherlands, except *Pratylenchus* sp., which was cultured at CLO, Merelbeke, Belgium.

Microorganisms, a full nematode community and microarthropods (mites and Collembola) were extracted from field soil. The microorganisms were obtained by shaking 100 g soil samples with demineralized water (1:1 w · w^{-1}) for 10 minutes and filtering the supernatant through a 20 µm mesh (Bezemer et al. 2005; Klironomos 2002). Prepared microbial filtrate contained bacteria and fungi, but no nematodes and no AMF. The pots with microorganisms were inoculated with 10 ml of the filtrate, which was 1/15 of the original density of the microorganisms in the field soil. For each pot with 'full nematode community' treatment, the nematodes were extracted from 1500 g of non-sterilized soil by Cobb's method (Oostenbrink 1960) and inoculated as a suspension of 10 ml · pot^{-1}, at a rate 1/1 of original field density. The nematode community added to the pots was analyzed

microscopically (magnification 200 x) and consisted of plant parasites (*T. ventralis*, *Tylenchorhynchus microphasmis, Pratylenchus spp, Paratylenchus* spp., *Meloidogyne* sp., *Rotylenchus* spp., Criconematidae), bacterivores (*Acrobeles* spp., *Acrobeloides* spp., *Chiloplacus* spp., Cephalobidae, *Plectus* spp.), omnivores (*Aporcelaimellus* spp., *Microdorylaimus* spp.) and carnivores (*Choanolaimus* spp.).

Microarthropods were collected by wet sieving of dune soil through 180 µm mesh. The microarthropods were added in a suspension of 10 ml · pot^{-1} at a rate 1/1 of their dune soil density.

The experiment

The experiment was carried out with 8 nematode species (mentioned above) and 4 nematode control treatments: microorganisms, a full nematode community, microarthropods and a control. There were 7 replicates of each treatment. In total, the experiment involved 8+1 (nematode species + control) times 4 (three nematode control organism groups + one control) times 7 (replicates) = 252 pots. The root-feeding nematode species *Criconema* sp., *H. pseudorobustus, Hemicycliophora* sp., *H. arenaria, Pratylenchus* sp., *Rotylenchus* sp., *T. ventralis* and *T. microphasmis* were all added in tap water suspension at a rate of 100 nematodes · pot^{-1}. We added demineralized water to the pots in order to make up the difference in water supply between the treatments.

The pots were harvested 12 weeks after inoculation of the soil organisms to allow minimally one reproductive cycle (*H. arenaria*) or two (*T. ventralis* and *T. microphasmis*). All other root-feeding nematode species passed through minimally one reproductive cycle. The nematodes were extracted from soil by Cobb's decantation method and from the roots using a mistifier (Oostenbrink 1960). *H. arenaria* cysts were extracted from soil by wet sieving through 180 µm mesh and from fresh roots by collecting the cysts using a microscope (Oostenbrink 1960). The numbers of nematodes and cysts were counted by microscope (magnification 200 x) and were expressed as numbers · 100 g^{-1} of soil. The roots and shoots of *A. arenaria* were dried for 48 hours at 70°C and weighed.

Data analysis

Normal distribution of data and homogeneity of variances were checked by inspection of the residuals after model fit (using the package Statistica 7, StatSoft Inc.). To obtain normal distributions, the relative suppression of the nematodes and their multiplication factors were log-transformed. To test if the suppression caused by soil organisms depended on nematodes species, two-way ANOVA was performed. In this case the main factors were 'nematode species added' (with and without) and 'soil fauna added' (microorganisms, nematodes, microarthropods and control). Subsequently, for each of eight nematode species one-way ANOVA was performed with the main factor 'treatment' (three levels: 'microorganisms', 'nematodes' and 'microarthropods') and those latter were compared with the control.

The multiplication factor of the nematodes was analyzed first by two-way ANOVA similarly as relative suppression of the nematodes. After that one-way ANOVA was performed for each of four 'addition of soil organisms' separately (no organisms added, microorganisms, nematodes or arthropods) with the main factor 'nematode species inoculated'. Shoot and root biomass was analyzed by two-way ANOVA with main factors 'nematode species added' (with and without) and 'soil fauna added' (microorganisms, nematode community, microarthropods and control). The treatments were compared by posthoc analysis using Tukey HSD tests ($P < 0.05$).

Results

Nematodes

Two-way ANOVA of relative suppression of nematodes revealed significant interaction between nematode species and soil organisms addition ($F_{18, 166} = 9.3$; $P < 0.001$), showing that different nematode species were differently controlled by soil organisms. Therefore, we rejected our null hypothesis and accepted the alternative hypothesis that root-feeding nematodes in this natural system were controlled in a species-specific way.

Subsequent one-way ANOVAs showed significant suppression of six out of eight nematode species when compared to the control treatments (Figure 14): *Criconema* sp. ($F_{3, 23} = 4.9$; $P < 0.05$), *Helicotylenchus pseudorobustus* ($F_{3, 23} = 5.6$; $P < 0.05$),

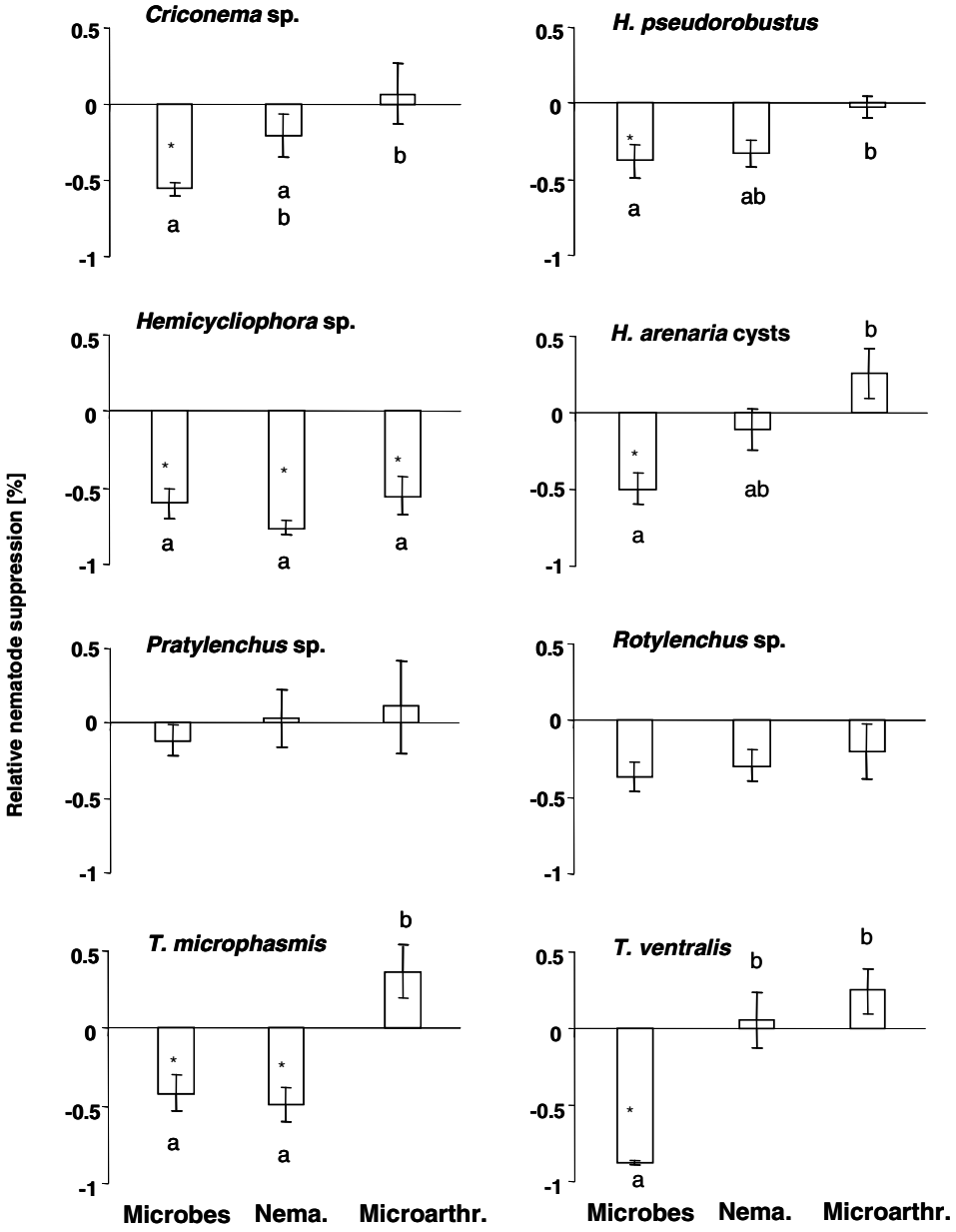

Figure 14. Effects of microorganisms, nematodes or arthropods on nematode multiplication 12 weeks after inoculation. Numbers of nematodes were recalculated for 100 g of dune soil. Error bars indicate standard error and different letters above the bars indicate significant difference among the treatments at $P < 0.05$.

Hemicycliophora sp. ($F_{3, 23}$ = 12.2; P < 0.001), *Heterodera arenaria* cysts ($F_{3, 23}$ = 5.8; P < 0.05), *Tylenchorhynchus ventralis* ($F_{3, 23}$ = 12.2; P < 0.001) and *Tylenchorhynchus microphasmis* ($F_{3, 23}$ = 10.4; P < 0.001). No effects of soil organisms were found for *Pratylenchus* sp ($F_{3, 23}$ = 0.14; P > 0.05) and *Rotylenchus* sp. ($F_{3, 23}$ = 1.9; P > 0.05). Microorganisms significantly suppressed the populations of *T. ventralis, T. microphasmis*, *Criconema* sp., *H. pseudorobustus, Hemicycliophora* sp. and *H. arenaria* (P < 0.05; Figure 14).

The strongest decrease in nematode numbers was observed in the case of *T. ventralis*, where microorganisms reduced multiplication by 87%, whereas other multiplication reductions were more modest. Adding the mixture of all soil nematodes from a dune soil, which included carnivores, plant feeders, bacterial and fungal feeders, significantly reduced the numbers of *Hemicycliophora* sp. and *T. microphasmis* (P < 0.05; Figure 14). *Hemicycliophora* sp. was the only nematode to be significantly reduced by microarthropods, which reduced multiplication around 60 % (P < 0.05; Figure 14).

Multiplication factor depended on nematode species (Table 5).

Table 5. Multiplication factor of the nematodes 12 weeks after inoculation with soil organisms. One-way ANOVAs with a factor 'species inoculated' performed for each of the soil organism additions (no organisms, microbes, nematodes, arthropods) ± 1SE. Different letters indicate the significant differences between treatments within rows at P < 0.05.

Species inoculated	Addition of soil organisms			
	No organisms added	+ Microbes	+ Nematodes	+ Microarthrop.
Criconema sp.	12.8 ± 1.25	5.70 ± 0.53	10.2 ± 1.68	13.8 ± 2.40
H. pseudorobustus	6.54 ± 0.73	3.69 ± 0.58	3.97 ± 0.49	5.74 ± 0.39
Hemicyclio. sp.	7.34 ± 0.79	2.93 ± 0.69	1.77 ± 0.31	3.30 ± 0.85
H. arenaria cysts	0.13 ± 0.02	0.06 ± 0.01	0.11 ± 0.02	0.16 ± 0.02
Pratylenchus sp.	6.20 ± 1.19	3.57 ± 0.59	4.83 ± 1.13	2.81 ± 0.69
Rotylenchus sp.	2.87 ± 0.16	1.82 ± 0.24	2.03 ± 0.26	2.29 ± 0.47
T. microphasmis	131 ± 19.7 *a*	76.8 ± 14.93 *b*	67.1 ± 13.8 *b*	179 ± 21.3 *a*
T. ventralis	57.2 ± 12.2 *a*	3.42 ± 0.65 *b*	41.8 ± 6.59 *a*	48.9 ± 5.26 *a*

In case of all four 'soil organism additions' (no addition, microorganisms, nematodes or arthropods) multiplication was always the highest for *T. microphasmis* and the lowest for *H. arenaria.* However, the cysts of *H. arenaria* may contain even more than 200 eggs, so that our figure for this species represents females completing their life cycle successfully, rather than reproduction. Multiplication factors of *T. microphasmis* and *T. ventralis* were significantly different from the other species. For *T. microphasmis* multiplication factor was two times higher than of *T. ventralis* and ten times higher than of *Criconema* sp. There were no differences between the multiplication factors of *Criconema* sp., *H. pseudorobustus*, *Hemicycliophora* sp., *H. arenaria*, *Pratylenchus* sp. and *Rotylenchus* sp.

Plant biomass

Two-way ANOVA of shoot biomass showed a significant interaction between nematode species inoculated and soil organism addition ($F_{24, 208} = 2.66$; $P < 0.001$). Shoot biomass was significantly enhanced if nematodes or arthropods were inoculated with *Pratylenchus* sp. ($P < 0.05$; Table 6). On the other hand, addition of microarthropods together with *H. pseudorobustus* decreased shoot biomass ($P < 0.05$; Table 6). No change in shoot biomass was observed in case of *T. ventralis, T. microphasmis*, *Criconema* sp.*, Hemicycliophora* sp.*, Rotylenchus* sp. and *H. arenaria* with or without soil organisms inoculated.

Similarly to shoot biomass, two-way ANOVA of root biomass showed a significant interaction between nematode species inoculated and soil organisms added. Root biomass was significantly lower if *T. ventralis, T. microphasmis* and *Hemicycliophora* sp. were inoculated together with the whole nematode suspension or microarthropods (Table 7). The root biomass was twice as low when inoculated with *Hemicycliophora* sp. or other nematodes as with *Hemicycliophora* sp. alone. Microorganisms caused significantly lower root biomass if added together with *T. microphasmis* and *Hemicycliophora* sp. than when plants were interacting with those nematodes alone.

Table 6. Dry biomass (g) of *Ammophila arenaria* shoots 12 weeks after inoculation with soil organisms ± 1 SE. Two-way ANOVA with factors 'species inoculated' and 'addition of soil organisms'. Asterix (*) indicates the significant difference between the treatment and the control at $P < 0.05$.

Species inoculated (factor 1)	Addition of soil organisms (factor 2)			
	No organisms added	+ Microbes	+ Nematodes	+ Microarthrop.
Control	1.21 ± 0.16	1.55 ± 0.07	1.40 ± 0.11	1.64 ± 0.08
Criconema sp.	1.59 ± 0.05	1.52 ± 0.06	1.53 ± 0.15	1.58 ± 0.09
H. pseudorobustus	1.85 ± 0.08 (*)	1.57 ± 0.12	1.78 ± 0.12	1.50 ± 0.06
Hemicycliophora sp.	1.82 ± 0.07 (*)	1.56 ± 0.10	1.59 ± 0.10	1.65 ± 0.05
H. arenaria cysts	1.87 ± 0.11 (*)	1.83 ± 0.06	1.76 ± 0.06	2.01 ± 0.04
Pratylenchus sp	1.40 ± 0.07	1.57 ± 0.11	1.85 ± 0.11	1.85 ± 0.12
Rotylenchus sp.	1.61 ± 0.16	1.71 ± 0.05	1.55 ± 0.12	1.30 ± 0.08
T. microphasmis	1.89 ± 0.08 (*)	1.65 ± 0.09	1.57 ± 0.11	1.61 ± 0.09
T. ventralis	1.75 ± 0.08	1.47 ± 0.11	1.69 ± 0.09	1.39 ± 0.14

Table 7. Dry biomass (g) of *Ammophila arenaria* roots 12 weeks after inoculation with soil organisms ± 1 SE. Two-way ANOVA with factors 'species inoculated' and 'addition of soil organisms'. Asterix (*) indicate the significant difference between the treatment and the control at $P < 0.05$.

Species inoculated (factor 1)	Addition of soil organisms (factor 2)			
	No organisms added	+ Microbes	+ Nematodes	+ Microarthrop.
Control	0.90 ± 0.09	1.09 ± 0.09	0.79 ± 0.1	0.81 ± 0.07
Criconema sp.	0.81 ± 0.10	0.75 ± 0.04	0.60 ± 0.07	0.64 ± 0.05
H. pseudorobustus	0.96 ± 0.07	0.66 ± 0.09	0.68 ± 0.09	0.75 ± 0.06
Hemicycliophora sp.	0.99 ± 0.04	0.65 ± 0.07	0.50 ± 0.06	0.74 ± 0.06
H. arenaria cysts	0.82 ± 0.06	0.54 ± 0.05	0.73 ± 0.06	0.49 ± 0.06
Pratylenchus sp.	0.49 ± 0.05	0.69 ± 0.08	0.60 ± 0.9	0.71 ± 0.09
Rotylenchus sp.	0.85 ± 0.10	0.82 ± 0.06	0.60 ± 0.08	0.58 ± 0.08
T. microphasmis	0.70 ± 0.07	0.45 ± 0.07 (*)	0.43 ± 0.06	0.51 ± 0.05
T. ventralis	0.77 ± 0.05	0.65 ± 0.06	0.49 ± 0.04	0.53 ± 0.06

Discussion

Our results show that in the root zone of the wild grass *A. arenaria* most of the eight co-occurring root-feeding nematodes could be controlled by soil microorganisms. In addition to the supreme effects of soil microorganisms, a full nematode community and microarthropods were able to control some of the root-feeding nematode species. Therefore, we rejected our null hypothesis that all the nematodes are controlled by the same top-down control factor and accepted the alternative hypothesis that a multitude of factors can be involved in controlling nematode abundance. Moreover, the effectiveness of different soil organisms like microorganisms, nematodes and microarthropods in controlling nematode abundance varied profoundly. The most effective was a control of *T. ventralis* by microorganisms, which reduced multiplication by 87 %.

Our findings suggest that nematode control is much more complicated than thought before and depends not only on the feeding group to which the nematodes belong (ecto- or endoparasitic), but also on the nematode species. There does not seem to be a relationship between multiplication factor of nematodes and suppression type. In the field, probably, *A. arenaria* is a better host plant for some species, especially for *T. microphasmis* and *T. ventralis*. Populations of these nematodes reach very high numbers if there are no natural enemies present. (de Rooij van der Goes 1995).

Nematode suppression by the natural enemies should theoretically enhance plant biomass production, as the plant does not suffer from damage caused by those root-feeders. Adding antagonistic soil organisms to plants with specific root-feeding nematode species, however, did not cause a significant increase of shoot or root biomass. Probably, the initial densities of the nematodes were too low to demonstrate significant growth reduction of the plants. Our approach of applying of whole mixtures of, for example, soil microorganisms also introduced pathogenic species which are able to reduce performance of *A. arenaria* (van der Putten et al. 1988; van der Stoel et al. 2002). Increased root biomass was achieved by soil sterilization, which removed all soil biota. Interestingly, shoot growth was enhanced if some of the root herbivores were present.

Our results point at an important role of microorganisms in nematode control which is generally not accounted for in soil food web studies (de Ruiter et al. 1993; Hunt et

al. 1987; Neutel et al. 2002). According to those models, root-feeding nematodes in coastal ecosystems should be controlled by predaceous mites and carnivorous nematodes. However, in our study the effects of microarthropods were generally weak, possibly because in dune grasslands the densities of microarthropods are usually too low to control significantly the abundance of nematodes (Petersen & Luxton 1982).

We conclude that different root-feeding nematode species can be controlled by more than one mechanism and that they are controlled in a species-specific way. Nematode control did not relate to multiplication rate or feeding type, however, more studies including different wild systems are needed to draw more general conclusions about if nematode traits relate to their control. For the majority of root-feeding nematode species soil microorganisms may act as a control factor. The multiple control possibilities of root-feeding nematodes should be taken into consideration while managing nematode communities in sustainable agricultural systems.

Acknowledgements

This study is part of the EcoTrain project (HPRN-CT 2002 00210), funded by the European Union rtn-programme.

References

Alabouvette, C. (1999) *Fusarium* wilt suppressive soils: an example of disease-suppressive soils. *Australasian Plant Pathology, 28*, 57-64.

Alabouvette, C., Lemanceau, P. & Steinberg, C. (1993) Recent advances in the biological control of *Fusarium* wilts. *Pesticide Science, 37*, 365-373.

Amir, H. & Alabouvette, C. (1993) Involvement of soil abiotic factors in the mechanisms of soil suppressiveness to *Fusarium* wilts. *Soil Biology & Biochemistry, 25*, 157-164.

Bardgett, R. D., Cook, R., Yeates, G. W. & Denton, C. S. (1999) The influence of nematodes on below-ground processes in grassland ecosystems. *Plant and Soil, 212*, 23-33.

Barker, K. R. & Koenning, S. R. (1998) Developing sustainable systems for nematode management. *Annual Review of Phytopathology, 36*, 165-205.

Bezemer, T. M., de Deyn, G. B., Bossinga, T. M., van Dam, N. M., Harvey, J. A. & van der Putten, W. H. (2005) Soil community composition drives aboveground plant-herbivore-parasitoid interactions. *Ecology Letters, 8*, 652-661.

Brinkman, E. P., Duyts, H. & van der Putten, W. H. (2005) Consequences of variation in species diversity in a community of root-feeding herbivores for nematode dynamics and host plant biomass. *Oikos, 110*, 417-427.

Brinkman, E. P., van Veen, J. A. & van der Putten, W. H. (2004) Endoparasitic nematodes reduce multiplication of ectoparasitic nematodes, but do not prevent growth reduction of *Ammophila arenaria* (L.) Link (marram grass). *Applied Soil Ecology, 27*, 65-75.

de Boer, W., Gunnewiek, P. & Woldendorp, J. W. (1998) Suppression of hyphal growth of soil-borne fungi by dune soils from vigorous and declining stands of *Ammophila arenaria*. *New Phytologist, 138*, 107-116.

de Boer, W., Verheggen, P., Gunnewiek, P., Kowalchuk, G. A. & van Veen, J. A. (2003) Microbial community composition affects soil fungistasis. *Applied and Environmental Microbiology, 69*, 835-844.

de Deyn, G. B., Raaijmakers, C. E., Zoomer, H. R., Berg, M. P., de Ruiter, P. C., Verhoef, H. A., Bezemer, T. M. & van der Putten, W. H. (2003) Soil invertebrate fauna enhances grassland succession and diversity. *Nature, 422*, 711-713.

de la Peña, E., Rodriguez-Echeverria, S., van der Putten, W. H., Freitas, H. & Moens, M. (2006) Mechanism of control of root-feeding nematodes by mycorrhizal fungi in the dune grass *Ammophila arenaria*. *New Phytologist, 169*, 829-840.

de Rooij van der Goes, P. C. E. M., Peters, B. A. M. & van der Putten, W. H. (1998) Vertical migration of nematodes and soil-borne fungi to developing roots of Ammophila arenaria (L.) link after sand accretion. *Applied Soil Ecology, 10*, 1-10.

de Rooij van der Goes, P. C. E. M. (1995) The role of plant parasitic nematodes and soil borne fungi in the decline of *Ammophila arenaria* (L) Link. *New Phytologist, 129*, 661-669.

de Ruiter, P. C., van Veen, J. A., Moore, J. C., Brussaard, L. & Hunt, H. W. (1993) Calculation of nitrogen mineralization in soil food webs. *Plant and Soil, 157*, 263-273.

Dicklow, M. B., Acosta, N. & Zuckerman, B. M. (1993) A novel *Streptomyces* species for controlling plant parasitic nematodes. *Journal of Chemical Ecology, 19*, 159-173.

Esnard, J., Potter, T. L. & Zuckerman, B. M. (1995) *Streptomyces costaricanus* sp nov, isolated from nematode suppressive soil. *International Journal of Systematic Bacteriology, 45*, 775-779.

Freckman, D. W. & Caswell, E. P. (1985) The ecology of nematodes in agroecosystems. *Annual Review of Phytopathology, 23*, 275-296.

Gange, A. C. & Brown, V. K. (1989) Insect herbivory affects size variability in plant populations. *Oikos, 56*, 351-356.

Gange, A. C. & Brown, V. K. (2002) Soil food web components affect plant community structure during early succession. *Ecological Research, 17*, 217-227.

Hunt, H. W., Coleman, D. C., Ingham, E. R., Ingham, R. E., Elliott, E. T., Moore, J. C., Rose, S. L., Reid, C. P. P. & Morley, C. R. (1987) The detrital food web in a shortgrass prairie. *Biology and Fertility of Soils, 3*, 57-68.

Ingham, R. E. & Detling, J. K. (1986) Nematode population dynamics from different aged prairie dog colonies. *Journal of Nematology, 18*, 638-638.

Jaffee, B. A. (1993) Density dependent parasitism in biological control of soil-borne insects, nematodes, fungi and bacteria. *Biocontrol Science and Technology, 3*, 235-246.

Kerry, B. R. & Gowen, S. R. (1995) Biological control of plant parasitic nematodes. *Nematologica, 41*, 362-363.

Kerry, B. R. & Hominick, W. M. (2002) Biological control. London: Taylor & Francis.

Klironomos, J. N. (2002) Feedback with soil biota contributes to plant rarity and invasiveness in communities. *Nature, 417*, 67-70.

Kluepfel, D. A., McInnis, T. M. & Zehr, E. I. (1993) Involvement of root colonizing bacteria in peach orchard soils suppressive of the nematode *Criconemella xenoplax*. *Phytopathology, 83*, 1240-1245.

Mazzola, M. (2002) Mechanisms of natural soil suppressiveness to soilborne diseases. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 81*, 557-564.

Neutel, A. M., Heesterbeek, J. A. P. & de Ruiter, P. C. (2002) Stability in real food webs: Weak links in long loops. *Science, 296*, 1120-1123.

Oostenbrink, M. (1960) Estimating nematode populations by some selected methods. *In Nematology* (ed. J. N. Sasser & W. R. Jenkins), pp. 85-102. Chapel Hill: The University of North Carolina Press.

Petersen, H. & Luxton, M. (1982) A comparative analysis of soil fauna populations and their role in decomposition processes. *Oikos, 39*, 287-388.

Rodriguez Kabana, R. (1991) Biological control of plant parasitic nematodes. *Nematropica, 21*, 111-122.

Stanton, N. L. (1988) The underground in grasslands. *Annual Review of Ecology and Systematics, 19*, 573-589.

Termorshuizen, A. J., van Rijn, E., van der Gaag, D. J., Alabouvette, C., Chen, Y., Lagerlof, J., Malandrakis, A. A., Paplomatas, E. J., Ramert, B., Ryckeboer, J., Steinberg, C. & Zmora-Nahum, S. (2006) Suppressiveness of 18 composts against 7 pathosystems: Variability in pathogen response. *Soil Biology & Biochemistry, 38*, 2461-2477.

van der Putten, W. H., Cook, R., Costa, S., Davies, K. G., Fargette, M., Freitas, H., Hol, W. H. G., Kerry, B. R., Maher, N., Mateille, T., Moens, M., de la Peña, E., Piśkiewicz, A. M., Raeymaekers, A. D. W., Rodriguez-Echeverria, S. & van der Wurff, A. W. G. (2006) Nematode interactions in nature: Models for sustainable control of nematode pests of crop plants? *In Advances in Agronomy, Vol 89*, vol. 89, pp. 227-260.

van der Putten, W. H. & van der Stoel, C. D. (1998) Plant parasitic nematodes and spatio-temporal variation in natural vegetation. *Applied Soil Ecology, 10*, 253-262.

van der Putten, W. H., van Dijk, C. & Troelstra, S. R. (1988) Biotic soil factors affecting the growth and development of *Ammophila arenaria*. *Oecologia, 76*, 313-320.

van der Putten, W. H., Yeates, G. W., Duyts, H., Reis, C. S. & Karssen, G. (2005) Invasive plants and their escape from root herbivory: a worldwide comparison of the root-feeding nematode communities of the dune grass *Ammophila arenaria* in natural and introduced ranges. *Biological Invasions, 7*, 733-746.

van der Stoel, C. D., Duyts, H. & van der Putten, W. H. (2006) Population dynamics of a host-specific root-feeding cyst nematode and resource quantity in the root zone of a clonal grass. *Oikos, 112*, 651-659.

van der Stoel, C. D., van der Putten, W. H. & Duyts, H. (2002) Development of a negative plant-soil feedback in the expansion zone of the clonal grass *Ammophila arenaria* following root formation and nematode colonization. *Journal of Ecology, 90*, 978-988.

Wäckers, F. L. & Bezemer, T. M. (2003) Root herbivory induces an above-ground indirect defence. *Ecology Letters, 6*, 9-12.

Weller, D. M., Raaijmakers, J. M., Gardener, B. B. M. & Thomashow, L. S. (2002) Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annual Review of Phytopathology, 40*, 309-+.

Yeates, G. W., Bongers, T., de Goede, R. G. M., Freckman, D. W. & Georgieva, S. S. (1993) Feeding habits in soil nematode families and genera - an outline for soil ecologists. *Journal of Nematology, 25*, 315-331.

Chapter 6

General Discussion

Why is the ectoparasitic nematode *T. ventralis* so low abundant in dune soil?

The plant ectoparasitic nematode *Tylenchorhynchus ventralis* is a key species in coastal dunes because it is able to reduce growth and performance of its host plant *Ammophila arenaria* (de Rooij van der Goes 1995). However, in natural dune soil, the abundance of *T. ventralis* usually is too low to affect the performance of *A. arenaria* (de Rooij van der Goes 1995). The suppression of the ectoparasitic nematode might be caused by bottom-up (plant) effects (Walker & Jones 2001), by horizontal mechanisms (competition with other nematodes) (Chase et al. 2002) and by top-down control from natural enemies (Kerry & Hominick 2002). The results of my experiments have shown that the ectoparasitic nematode *T. ventralis* can be controlled by the natural microbial enemies, whereas the role of other soil organisms like nematodes and microarthropods is not substantial (Chapter 2, this thesis). Moreover, mycorrhizal fungi and plant endophytes have no effects on *T. ventralis* populations (A. M. Piśkiewicz and W. H. G. Hol, unpublished results). The microorganisms that are involved in nematode control include bacteria and fungi. In our experiments, *T. ventralis* were infected with *Pasteuria* sp, *Paenibacillus*-like organisms and *Catenaria* sp. Besides, other bacteria and fungi, so far unidentified, have been involved in the suppression of *T. ventralis*. The precise identity of microbial enemies of nematodes may need to be established by using molecular methods (Davies 2005). Currently it is unknown what the contribution of bacteria versus fungi in the suppression of *T. ventralis* is. I made a first attempt to separate bacterial and fungal fractions (Moller et al. 1999) and inoculate them alone or together with *T. ventralis*, but this requires further effort in subsequent studies.

In spite of the relatively large amount of studies on control of plant parasitic nematodes, there is no common answer how these pests are suppressed in natural systems. Soil food web models propose plant parasitic nematodes to be suppressed by predators, for example predacious mites and nematodes (Neutel et al. 2002). On the other hand, for biocontrol practices it is assumed that plant parasitic nematodes are mostly suppressed by their microbial enemies (Kerry & Hominick 2002). Surprisingly, these approaches have never met. My results that ectoparasitic nematodes *T. ventralis* in nature are controlled by soil microorganisms support the point of view of biocontrol studies rather than soil food

web models. However, it must be underlined, that food-web models are made for another purpose than determining what controls organisms in ecosystems, for example to assess the flow size of nutrients and energy (de Ruiter et al. 1993).

Microbial enemies of nematodes could affect their hosts by local or systemic (non-local) interactions. Local effects are caused by direct interactions between nematodes and soil microorganisms. Non-local effects occur if the microorganisms present in one section of the root system suppress the nematodes in another section, involving a systemic transfer of control factors or signals. The results of my experiments reveal that *T. ventralis* is suppressed due to local interactions of plant parasitic nematodes and their microbial enemies. Nematode suppression may be caused by parasitic characteristics of these microorganisms or local induction of defense responses. The direct effects of microorganisms were proven by microscopic observations of nematodes - the nematodes which were inoculated together with microorganisms were infected with unknown bacteria and with fungi that resembled the genus *Catenaria*. The studies on local or non-local effects of microorganisms were performed in the split-root experiments in which the roots of *A. arenaria* were divided into two compartments (two pots attached to each other) filled with sterilized dune soil. It can be assumed that the potential contamination with microorganisms was not present because the reducing effects of microorganisms were observed only when inoculated with *T. ventralis*. In all the pots where microorganisms were not added, there was no suppression of the nematodes.

Some soil organisms have been shown to sense and avoid their enemies, for example the bacterial feeder *Caenorhabditis elegans* can avoid odors from pathogenic bacteria (Zhang et al. 2005). My results suggest that in the natural dune soil this phenomenon also occurs in the case of *T. ventralis*. This nematode feeds on outside root tissues and moves along the roots to find suitable feeding sites (de Rooij van der Goes et al. 1998). My experiments revealed that in natural dune soil the ectoparasite *T. ventralis* prefers to feed upon roots without its microbial enemies. This may suggest that in the field, nematodes would sense and avoid the sites which are occupied by microorganisms. If the nematodes are able to sense their enemies, they may avoid the risk of being parasitized or consumed. However, based on our study it can not be concluded which microorganisms

(antagonistic or not to the nematodes) are responsible for the avoidance behavior. The choice behavior of *T. ventralis* could have important ecological relevance in the case of the existence of 'microbial hotspots' (Pietramellara et al. 2002) along the root surface. It would be essential is to examine if in the field soil the 'hotspots' of antagonistic microbial activity exist, at which parts of the root system they occur and if they correlate negatively with the nematode presence. The avoidance phenomenon was never studied for other root-feeding nematode species; therefore it can not be concluded that other nematodes are also able to avoid their microbial enemies. As mentioned before, the effects of microorganisms act only locally and their distraction of nematodes could be functional from both - the plant and the nematode point of view. In this case, if the microbial enemies affect nematodes by local interactions, then indeed, nematodes would be able to avoid microorganisms. On the other hand, if microorganisms would have systemic effects, then nematodes cannot avoid microbes by choosing another part of the root system. This phenomenon might occur in case of defense against other plant parasitic nematode species.

If microorganisms suppress plant parasitic nematodes, do they improve plant growth?
The microbial community in natural soil contains bacteria and fungi which may be detrimental for nematodes, but also microorganisms which are detrimental for the host plant. The plants are parasitized by an array of different organisms, for example soil-borne fungi (de Rooij van der Goes et al. 1995). These organisms are involved in plant degeneration and die out (van der Stoel et al. 2002). Probably, the proportion of these nematode antagonistic and plant-pathogenic microorganisms in the entire microbial community would define if the nematode suppression ultimately may result in the increased plant growth (Wardle et al. 2004). If inoculated into the sterilized soil, the nematode *T. ventralis* lead to lower shoot and root growth. Potentially, if the microorganisms would reduce the number of the parasitic nematodes, the plant growth would be enhanced. However, in spite of suppressed nematode numbers after microorganism addition, improved plant growth was not observed.

In case of my experiments, the microorganism community extracted from natural dune soil was inoculated to the pots with *A. arenaria* at one time point. However, in field,

soil microorganisms may attack the newly developed *A. arenaria* root zone in a sequence (de Rooij van der Goes et al. 1998). The order in which the roots zone is colonized by the microorganisms may be important for the negative or positive effects on plant growth. If the plant pathogens colonize the roots before the nematode enemies are present, then perhaps the negative effects on plants would predominate.

Is there a unifying control mechanism for all nematode species?

The results which come from the studies on *T. ventralis* can not be extrapolated to all nematode species in the root zone of *A. arenaria*. There is no general pattern in the control mechanism for all the species but the way nematodes are controlled seems to be dependent on nematode species, not only on the feeding group. Nevertheless, soil microorganisms appear to be very important for top-down control, because of their capacity to suppress the majority of the root-feeding nematode species associated with *A. arenaria*. Other soil organisms like predatory mites and nematodes may be important for the control of some plant nematode species (Imbriani & Mankau 1983), however, not for those of *A. arenaria*.

In order to obtain an even more complete insight into nematode control mechanisms in coastal foredunes, I produced an overview of all knowledge on nematode control factors in the root zone of the grass *A. arenaria*. Combining my results with published data on the same model system provides further evidence that a multitude of top-down, bottom-up and horizontal control factors may be involved in reducing the actual nematode load on this wild grass (Table 8). The sedentary cyst nematode *H. arenaria* can be controlled by bottom-up processes (van der Stoel et al. 2006) and by microorganisms. The sedentary root knot nematode *Meloidogyne arenaria*, which is not included in the present study, is controlled by microorganisms (Costa, unpublished) and competition with other nematodes, most likely *H. arenaria* (Brinkman et al. 2005). The migratory endoparasitic root lesion nematode *Pratylenchus penetrans* can be suppressed by arbuscular mycorrhizal fungi, when these infect the plants prior to the nematodes (de la Peña et al. 2006) and endophytic fungi may relax competition between *P. penetrans* and *Pratylenchus dunensis*. This complex nature of root-feeding nematode control strongly suggests that any solution to crop protection and, therefore, to sustainable agriculture, cannot be reached by a

one problem-one solution approach. Solving one nematode problem might very well enhance others, so that an integral approach should be preferred over case by case approaches (van der Putten et al. 2006).

Table 8. An overview of potential control mechanisms of different plant parasitic nematodes in natural costal dunes. S- suppression, n.e.- no effect. [(1)] van der Stoel et al. 2006; [(2)] Costa, unpublished; [(3)] Brinkman et al. 2005; [(4)] de la Peña et al. 2006; [(5)] Piśkiewicz, unpublished; [(6), (7)] Piśkiewicz and Hol, unpublished, [(8)] Piśkiewicz et al. 2007; indications without superscript are based on the Chapter 5 of this thesis.

	Control mechanisms					
	Bottom-up (and by plant mutualists)			**Top-down**		**Horizontal / top-down**
Nematode species	**Plant effects**	**AMF**	**Endo-phytes**	**Micro-organisms**	**Micro-arthropods**	**Nematodes**
Criconema				**S**	n.e	n.e.
H. pseudorobustus				**S**	n.e.	n.e.
Hemicycliophora				**S**	**S**	**S**
H. arenaria	**S** [(1)]			**S**	n.e	n.e
Meloidogyne				**S** [(2)]		**S** [(3)]
Pratylenchus		**S** [(4)]		n.e.	n.e.	n.e.
Rotylenchus				n.e.	n.e.	n.e.
T. microphasmis				**S**	n.e.	**S**
T. ventralis	n.e. [(5)]	n.e. [(6)]	n.e. [(7)]	**S** [(8)]	n.e. [(8)]	n.e. [(8)]

Future directions

Biocontrol of plant parasitic nematodes is a complicated research field and still requires intensive studies (Kerry & Hominick 2002). The potential of soil-borne microorganisms in nematode control is well realized, but the practical use of nematode enemies is difficult

(Alabouvette et al. 2006; Meyer & Roberts 2002). Some of the organisms that are pathogenic to the nematodes can not be grown on artificial media. Even if this obstacle is resolved, often the laboratory-cultured enemies of nematodes are difficult be successfully grown in the field. The control of plant parasitic nematodes using their predators (mites and carnivorous nematodes) until now has not been very successful. In natural coastal dune soil most the nematode species are potentially controlled by microorganisms. However, the precise information which microorganisms - bacteria or fungi are more important is not known. Using molecular methods the identity of microbial enemies that reduce nematode populations can be elucidated. This information could help in developing nematode species – specific control for agricultural purposes. In my thesis I showed that the plant ectoparasitic nematode *T. ventralis* might avoid its enemies. Further studies are needed to unravel the precise mechanism underlying this phenomenon, as well as the relevance for other nematodes which are potentially controlled by soil microorganisms.

Lessons from nature for agriculture?

My thesis aimed at studying interactions between plant parasitic nematodes and their enemies like bacteria, fungi and predatory mesofauna in a natural ecosystem. Studying nematode control in natural ecosystems may help explaining mechanisms which control the abundance of root herbivores in natural field. It is sometimes assumed that in natural systems the outbreaks of nematodes are pretty rare. Whether or not this is generally true, in coastal foredunes the suppressive mechanisms are quite strong and result in suppressing root-feeding nematode abundance. As a result, direct effects of root-feeding nematodes in the decline of *A. arenaria* in stabilized dunes seem less likely, unless the nematodes may escape, spatially or temporally, from being controlled. Ultimately this knowledge about interactions between pests and their natural enemies may help understanding and solving 'nematode problems' in agriculture (van der Putten et al. 2006). However, one should remember that natural and agricultural ecosystems differ in one fundamental aspect: in natural ecosystems the plants and soil organisms co-evolved for a long time and this did not happened in case of agricultural systems.

Main findings of this thesis:

- The plant ectoparasitic nematode *Tylenchorhynchus ventralis* is controlled by microbial enemies.
- Microbial enemies of *T. ventralis* suppress nematode abundance due to local interactions.
- The ectoparasite *T. ventralis* is able to sense and avoid microbial communities, also including nematode enemies.
- In general, the nematode suppression depends on the species of nematodes.
- Most of the important nematode species in coastal dune soil may be controlled by more than one mechanism.
- Among possible top-down control mechanisms, microorganisms seem to play a more important role in nematode control than other soil organisms (notably predacious soil fauna).
- Biological control of plant parasitic nematodes requires soil biodiversity that minimally needs to include all control options.

References

Alabouvette, C., Olivain, C. & Steinberg, C. (2006) Biological control of plant diseases: the European situation. *European Journal of Plant Pathology, 114*, 329-341.

Brinkman, E. P., Duyts, H. & van der Putten, W. H. (2005) Consequences of variation in species diversity in a community of root-feeding herbivores for nematode dynamics and host plant biomass. *Oikos, 110*, 417-427.

Chase, J. M., Abrams, P. A., Grover, J. P., Diehl, S., Chesson, P., Holt, R. D., Richards, S. A., Nisbet, R. M. & Case, T. J. (2002) The interaction between predation and competition: a review and synthesis. *Ecology Letters, 5*, 302-315.

Davies, K. G. (2005) Interactions between nematodes and microorganisms: Bridging ecological and molecular approaches. *Advances in Applied Microbiology, 57*, 53-78.

de la Peña, E., Rodriguez-Echeverria, S., van der Putten, W. H., Freitas, H. & Moens, M. (2006) Mechanism of control of root-feeding nematodes by mycorrhizal fungi in the dune grass *Ammophila arenaria*. *New Phytologist, 169*, 829-840.

de Rooij van der Goes, P. C. E. M. (1995) The role of plant parasitic nematodes and soil borne fungi in the decline of *Ammophila arenaria* (L) Link. *New Phytologist, 129*, 661-669.

de Rooij van der Goes, P. C. E. M., Peters, B. A. M. & van der Putten, W. H. (1998) Vertical migration of nematodes and soil-borne fungi to developing roots of *Ammophila arenaria* (L.) Link after sand accretion. *Applied Soil Ecology, 10*, 1-10.

de Rooij van der Goes, P. C. E. M., van der Putten, W. H. & van Dijk, C. (1995) Analysis of nematodes and soil borne fungi from *Ammophila arenaria* (Marram grass) in Dutch coastal foredunes by multivariate techniques. *European Journal of Plant Pathology, 101*, 149-162.

de Ruiter, P. C., van Veen, J. A., Moore, J. C., Brussaard, L. & Hunt, H. W. (1993) Calculation of nitrogen mineralization in soil food webs. *Plant and Soil, 157*, 263-273.

Imbriani, J. L. & Mankau, R. (1983) Studies on *Lasioseius scapulatus*, a mesostigmatid mite predaceous on nematodes. *Journal of Nematology, 15*, 523-528.

Kerry, B. R. & Hominick, W. M. (2002) Biological control. London: Taylor & Francis.

Meyer, S. L. F. & Roberts, D. P. (2002) Combinations of biocontrol agents for management of plant-parasitic nematodes and soilborne plant-pathogenic fungi. *Journal of Nematology, 34*, 1-8.

Moller, J., Miller, M. & Kjoller, A. (1999) Fungal-bacterial interaction on beech leaves: influence on decomposition and dissolved organic carbon quality. *Soil Biology & Biochemistry, 31*, 367-374.

Neutel, A. M., Heesterbeek, J. A. P. & de Ruiter, P. C. (2002) Stability in real food webs: Weak links in long loops. *Science, 296*, 1120-1123.

Pietramellara, G., Ascher, J., Ceccherini, M. T. & Renella, G. (2002) Soil as a biological system. *Annals of Microbiology, 52*, 119.

van der Putten, W. H., Cook, R., Costa, S., Davies, K. G., Fargette, M., Freitas, H., Hol, W. H. G., Kerry, B. R., Maher, N., Mateille, T., Moens, M., de la Peña, E., Piśkiewicz, A. M., Raeymaekers, A. D. W., Rodriguez-Echeverria, S. & van der Wurff, A. W. G. (2006) Nematode interactions in nature: Models for sustainable control of nematode pests of crop plants? *Advances in Agronomy, 89*, 227-260.

van der Stoel, C. D., Duyts, H. & van der Putten, W. H. (2006) Population dynamics of a host-specific root-feeding cyst nematode and resource quantity in the root zone of a clonal grass. *Oikos, 112*, 651-659.

van der Stoel, C. D., van der Putten, W. H. & Duyts, H. (2002) Development of a negative plant-soil feedback in the expansion zone of the clonal grass *Ammophila arenaria* following root formation and nematode colonization. *Journal of Ecology, 90*, 978-988.

Walker, M. & Jones, T. H. (2001) Relative roles of top-down and bottom-up forces in terrestrial tritrophic plant-insect herbivore-natural enemy systems. *Oikos, 93*, 177-187.

Wardle, D. A., Bardgett, R. D., Klironomos, J. N., Setala, H., van der Putten, W. H. & Wall, D. H. (2004) Ecological linkages between aboveground and belowground biota. *Science, 304*, 1629-1633.

Zhang, Y., Lu, H. & Bargmann, C. I. (2005) Pathogenic bacteria induce aversive olfactory learning in *Caenorhabditis elegans*. *Nature, 438*, 179-184.

Summary

Plant ectoparasitic nematodes are important herbivores below ground. This thesis aimed at revealing the interactions between the plant ectoparasitic nematode *Tylenchorhynchus ventralis*, the host plant *Ammophila arenaria* (marram grass) and the natural enemies of the nematode. The work has been carried out in the frame of the EU-EcoTrain project, in which the main nematode control processes in coastal foredunes have been studied.

I examined how the populations of plant ectoparasitic nematode *T. ventralis* are controlled in the rhizosphere of the wild grass *Ammophila arenaria*. These nematodes are able to strongly reduce the performance of their host plant if nematode abundance is not controlled. However, in the field the number of *T. ventralis* is usually too low to affect negatively the growth of the host plant. This suggests that the populations of *T. ventralis* are suppressed to non-damaging densities but the mechanisms of control were not known. My study was initiated to elucidate this control mechanism(s).

The nematode control may be due to resource limitation, caused by the host plant ('bottom-up' mechanisms), competition with other nematodes ('horizontal' mechanisms), or the suppression by the natural enemies of nematodes ('top-down' mechanisms). I showed that the populations of the ectoparasitic nematode *T. ventralis* are controlled by their microbial enemies. Other soil organisms like nematodes and microarthropods do not play any important role in these interactions.

Further, I examined how the populations of the ectoparasitic nematode *T. ventralis* are controlled by soil microorganisms. The suppressive effects of microbial enemies of nematodes may be due to local or systemic interactions. Local interactions would be caused by microbial parasitism, predation or antagonism to nematodes or by local induction of defense responses. Systemic interactions can be due to a defense response induced by the microorganisms in other plant parts than where the microorganisms were present. I showed that the suppressive effects of microorganisms on *T. ventralis* are caused by local interactions.

Potentially, *T. ventralis* is able to sense the cues produced by their microbial enemies and avoid them. I studied whether this phenomenon may occur in the case of *T. ventralis* in the experiments on artificial agar medium and in natural dune soil in a Y-tube

olfactometer. I showed that in natural dune soil *T. ventralis* is able to avoid plant roots with microorganisms. This may occur due to repellence caused by odors produced by microorganisms or stronger attraction to the 'clean' roots, or to repellence by roots with microorganisms. Microorganisms alone did not influence nematode choice. I used the whole microbial community extracted from the dune soil which was also lethal to the nematodes. The question, which components in the microbial community are responsible for the nematode repellence, remains to be answered.

Finally, I examined top-down control by soil microorganisms, nematodes and microarthropods of eight dominant plant parasitic species (six of them were ectoparasites and two endoparasites) in natural coastal dune soil. This study was carried in order to obtain a more complete view on nematode control mechanisms in coastal foredunes. Each of the nematode species was inoculated with microorganisms, nematodes or microarthropods extracted from natural dune soil, or sterilized water as a control. I concluded that the most important top-down mechanisms controlling a majority of nematode populations are soil microorganisms. Nevertheless, two out of eight examined nematode species were not affected by microorganisms at all, which suggests that for some species microorganisms are not important as a control factor. Moreover, some of the nematode species are additionally controlled by other nematodes and/or microarthropods or, as pointed out in the literature, by competition, arbuscular mycorrhizal fungi or endophytic fungi.

When I combined the results of my experiments with the available published data on nematode control in dune soil, nematode control by their natural enemies turns out to be much more complicated than thought before. It does not depend only on feeding group, but also on the nematode species. I conclude that more than one factor is often involved in the successful nematode control. Although the nematode populations are effectively controlled in natural ecosystem, this control in agriculture is not always that successful. My results strongly suggest that a variety of control mechanisms may be required for nematode suppression in agriculture and other production systems.

Samenvatting

Plant-ectoparasitaire nematoden zijn belangrijke bodembewonende planteneters. Het doel van dit proefschrift was de interacties tussen de ectoparasiet *Tylenchorhynchus ventralis*, de gastheerplant *Ammophila arenaria* (helmgras) en de natuurlijke vijanden van de nematode op te helderen. Het werk is uitgevoerd in het kader van het EU-EcoTrain project, waarin de belangrijke nematode-controlerende processen in buitenduinen zijn onderzocht.

Ik onderzocht hoe de populaties van de ectoparasitaire nematode *T. ventralis* worden gecontroleerd in de rhizosfeer van het natuurlijke gras *A. arenaria*. Deze nematode is in staat de groei van het gras sterk te onderdrukken, indien de aantallen nematoden niet worden beperkt. Echter, in het veld is het aantal *T. ventralis* gewoonlijk te gering om de groei van de gastheerplant negatief te beïnvloeden. Dit suggereert dat de populatiedichtheid van *T. ventralis* wordt onderdrukt tot niet-schadelijke aantallen. Tot op heden, waren de mechanismen van deze onderdrukking niet bekend. Mijn studie was opgezet om de controlemechanismen op te helderen.

De onderdrukking van nematoden kan het gevolg zijn van voedingsstofbeperking, veroorzaakt door de gastheerplant (zogenaamde 'bottom-up' mechanismen), competitie met andere nematoden ('horizontale' mechanismen), of onderdrukking door de natuurlijke vijanden van de nematoden ('top-down' meachanismen). Ik toonde aan, dat de populaties van de ectoparasiet *T. ventralis* worden beperkt door hun microbiële vijanden. Andere organismen, zoals nematoden en micro-arthropoden spelen geen belangrijke rol in deze interacties.

Daarna onderzocht ik hoe de populaties van de ectoparasiet *T. ventralis* worden gecontroleerd door bodemmicroörganismen. De onderdrukkende effecten van de microbiële vijanden van nematoden kunnen het gevolg zijn van locale, dan wel van systemische interacties. Locale interacties zouden worden veroorzaakt door microbiële parasitisme, predatie of antagonisme, of door locale inductie van verdedigingresponsen tegen nematoden. Systemische interacties kunnen worden veroorzaakt door verdedigingsresponsen, die worden geïnduceerd door de microörganismen in andere delen van de plant dan waar de microörganismen aanwezig zijn. Ik toonde aan dat de

onderdrukkende effecten van de microörganismen op *T. ventralis* worden veroorzaakt door locale interacties.

Het is mogelijk dat *T. ventralis* in staat is om signalen, die uitgestuurd worden door de microbiële vijanden, waar te nemen en daardoor de vijanden te ontwijken. Ik onderzocht of dit fenomeen kan plaatsvinden bij *T. ventralis* en gebruikte daarvoor experimenten op kunstmatig agar medium en in duinzand gebruik makend van een Y-buis olfactometer. Ik toonde aan dat in het duinzand *T. ventralis* in staat is plantenwortels met microörganismen te ontwijken. Dit kan worden veroorzaakt door afweer ten gevolge van geurstoffen die door de microörganismen worden geproduceerd, door sterkere aantrekking naar 'schone' wortels, of door afweer door wortels met microörganismen. Microörganismen alléén hadden geen invloed op de keuze van de nematoden. Ik gebruikte de gehele microbiële gemeenschap, die uit een duinbodem was gehaald door extractie en deze gemeenschap was ook dodelijk voor de nematoden. De vraag welk deel van de microbiële gemeenschap verantwoordelijk is voor de nematodenafweer, dient nog te worden beantwoord.

Tot slot heb ik de top-down onderdrukking van acht dominante plantenparasitaire nematoden uit buitenduinen (zes ecto- en twee endoparasitaire nematodensoorten) door bodemmicroörganismen, nematoden en microarthropoden onderzocht. Deze studie was bedoeld om een meer compleet overzicht te krijgen van nematode-onderdrukkingmechanismen in buitenduinen. Aan elk van de nematodensoorten werden microörganismen, nematoden of microarthropoden, afkomstig van buitenduinbodem, of gesteriliseerd water als controle toegediend. Ik concludeerde dat microörganismen de belangrijkste top-down controlefactor vormden voor de meerderheid van de plantenparasitaire nematoden. Niettemin werden twee van de acht nematodensoorten in het geheel niet door microörganismen beïnvloed, hetgeen suggereert dat voor sommige nematodensoorten microörganismen geen belangrijke controlefactor vormen. Daarnaast werden sommige nematodensoorten eveneens gecontroleerd door andere nematoden en/of microarthropoden of, zoals in de literatuur is aangegeven, door competitie, arbusculaire mycorrhizaschimmels of endofytische schimmels.

Als ik alle resultaten van mijn experimenten met de beschikbare gegevens uit de literatuur over nematodencontrole in buitenduinen combineer, blijkt de controle van

nematoden veel complexer zijn dan tevoren werd verondersteld. Het controlemechanisme hangt niet alleen af van het voedseltype, maar ook van de soort nematode. Ik concludeer dat vaak meer dan één factor betrokken is in succesvolle onderdrukking van nematoden. Hoewel nematodenpopulaties succesvol onderdrukt kunnen worden in natuurlijke ecosystemen, is nematodeonderdrukking in de landbouw niet altijd succesvol. Mijn resultaten suggereren sterk dat een variëteit aan controlemechanismen nodig zou kunnen zijn voor nematode-onderdrukking in de landbouw en in andere productiesystemen.

Acknowledgements

Time flies too fast - this is what you learn (among other useful things) during the PhD studies…

I would like to express my gratitude to Prof. dr. ir. Wim H. van der Putten, who supervised my work. Wim, you are the best supervisor one can wish for. You are an extremely busy person but you've always had time for me. Your optimism and fantastic sense of humor made practical work and writing process much easier. Henk Duyts introduced me to the world of nematology. Henk, I'm grateful for all those hours you spent on telling me about nematodes, working together in the greenhouse and counting tiny worms under the microscope. Without your help this thesis would never exist.

I had a pleasure to carry out my PhD research in the frames of the EcoTrain EU – project. Our meetings were always scientifically superb and the social life in the EcoTrain was fantastic!

Many thanks to my colleagues from the Laboratory of Nematology at Wageningen University! I would like to thank Prof. dr. ir. Jaap Bakker for introducing me to the NIOO and for further collaboration.

Great contribution to my thesis was provided by the students I supervised: Arne Elskamp, Francesca Rinaldi and Maarten de Milliano.

All at CTE (and especially MTI ☺) people, thank you for a great atmosphere in the Institute!
My office-mates: Pella, Hanneke and Susanne- it was a pleasure to share the room with you! Pella, your experience and comments on my work were always profitable. Hanneke, thanks for all our chats and good luck with your PhD thesis! Susanne, I will never forget our mysterious and exciting "R" experience ☺! Good luck in Berlin!
Ania K., Annemieke, Bibi, Ciska, Elly, Etienne, Eva, Fernando, Francesca, Gera, Hanneke, Jeroen, Joanneke, Katrin, Paul, Radim, Roxi, Sachie, Stein, Susanne, Tess, Tim, Tom - I enjoyed so much our "after work" life! I hope we will still be visiting cinemas and pubs together.

People, who made my private life in the Netherlands so wonderfully colorful, thank you a lot. Aga, Aneta, Ania F.-T., Ania K., Ania M., Kamila, Marysia T-K, Ula, Andre, Erik, Hassan, Patrick, Piotrek, Richard, Tomek – our social activities, especially 'dress-up' parties are unforgettable! Those who were in Wageningen but left for other places around the world (or other parts of the Netherlands) - Asia, Marysia S., Monika Z., Marcin, Sylwia, Dominika and Sewer - you still belong to Wageningen!
I am proud and happy to have friends like you!

Ziemek, you appeared in my life in a very interesting way ☺. Thanks for all the 'projects' that made me laugh until tears. Thanks for your love, care and support.

Wielkie podziękowania dla moich Przyjaciół z Krakowa: Agi & Rafała, Ani & Bogusia, Oli & Piotrka. Dzięki Wam za każdym razem kiedy przyjeżdżam do Krakowa, czuję, jakbym zawsze tam z Wami była i nigdzie nie wyjeżdżała.

Mamo, Tato, Andrzejku i reszto mojej najkochańszej Rodziny, dziękuje Wam za Wasze wsparcie i wiarę we mnie. Gdziekolwiek będę, mój dom zawsze będzie z Wami w Krakowie.

Ania

Wageningen, 15th October 2007

Curriculum Vitae

Anna Marta Piśkiewicz was born on 27th November 1978 in Cracow, Poland. In 1993 - 1997 she was a student of XI LO in Cracow, Poland. In 1997 she began Environmental Protection studies at the Jagiellonian University of Cracow. She obtained a BSc diploma with a major in environmental chemistry and a thesis entitled 'Analysis of chromium compounds in tannery waste waters' in 2000. From 2000 until 2003 she was a master student of Environmental Protection at the Jagiellonian University. She carried out a part of her studies at the Santiago de Compostela University in Spain. In 2002 she received a scholarship at the Laboratory of Nematology at Wageningen University in The Netherlands where she worked on the effects of toxic mixtures on life history traits of *Caenorhabditis elegans*. She obtained a MSc diploma at the Jagiellonian University with a major in environmental biology and a thesis entitled 'Effects of Cadmium and zinc on life history parameters of ground beetles' in 2003. From 2003 until 2007 she was a PhD student at the Netherlands Institute of Ecology, Department of Multitrophic Interactions in Heteren. Her research was conducted within the EcoTrain EU-RTN project which aimed at studying the interactions between plants, their parasitic nematodes and the enemies of these nematodes in natural coastal dune system.

List of publications

- Mozdzer T., Kramarz P., **Piśkiewicz A.M.**, Niklińska M., 2003. Effects of cadmium and zinc on larval growth and survival in the ground beetle, Pterostichus oblongopunctatus. Environment International, vol. 28-3, pp. 737-742
- Jonker M.J., **Piśkiewicz A.M.**, Ivorra N., Kammenga J.E., 2004. Toxicity of binary mixtures of cadmium- copper and carbendazim- copper to the nematode Caenorhabditis elegans. Environmental Toxicology and Chemistry: vol. 23-6, pp. 1529–1537
- van der Putten W.H., Cook R., Costa S., Davies K.G., Fargette M., Freitas H., Hol W.H.G., Kerry B.R., Maher N., Mateille T., Moens M., de la Peña E., **Piśkiewicz A.M.**, Raeymaekers A., Rodriguez-Echeverria S., van der Wurff A.W.G., 2006. Nematode interactions in nature: models for sustainable control of nematode pests of crop plants? Advances in Agronomy, vol. 89, pp. 227-260
- **Piśkiewicz A.M.**, Duyts H., Berg M.P., Costa S.R., van der Putten W.H., 2007. Soil microorganisms control plant ectoparasitic nematodes in natural coastal foredunes. Oecologia, vol. 152, pp 505-514

Submitted manuscripts

- **Piśkiewicz A.M.**, Duyts H., van der Putten W.H., Soil microorganisms in coastal foredunes control ectoparasitic root-feeding nematodes by local interactions. Functional Ecology, under review
- **Piśkiewicz A.M.**, Duyts H., van der Putten W.H., Multiple species-specific controls of root-feeding nematodes in natural soils, submitted
- **Piśkiewicz A.M.**, van der Putten W.H., Plant parasitic nematodes sense and avoid detrimental microorganisms, submitted

Posters with published abstracts

- Mozdzer T., Kramarz P., **Piśkiewicz A.M.**, Niklińska M., 20-24 August 2001. Accumulation of cadmium and zinc in the larval stage of the ground beetle Pterostichus oblongopunctatus F. SECOTOX World Congress, Cracow, Poland

- Kramarz P., **Piśkiewicz A.M.**, Mozdzer T. and Niklińska M., 12-16 May 2002. Two-generation response of ground beetle (Pterostichus oblongopunctatus) from metal-polluted site to cadmium and zinc intoxication. XXII Annual Meeting of SETAC: Challenges in Environmental Risk Assessment and Modelling: Linking Basic and Applied Research, Vienna, Austria
- **Piśkiewicz A.M.**, van der Putten W.H. & EcoTrain Team, 25-27 March 2003. Ecology of plant parasitic nematodes, their hosts and antagonists in European coastal dunes (EcoTrain Project). Annual Meeting of British Ecological Society, Lancaster, United Kingdom
- **Piśkiewicz A.M.**, van der Putten W.H. 12-16 June 2004. How the plant ectoparasitic nematodes are regulated in natural foredune systems? XXVII Symposium of European Society of Nematologists, Rome, Italy
- **Piśkiewicz A.M.**, van der Putten W.H., 07-09 September 2004. Top-down control of plant ectoparasitic nematodes in natural foredunes. Annual Meeting of British Ecological Society (BES), Lancaster, United Kingdom
- Hol W.G.H., **Piśkiewicz A.M.**, Cook R., 07-09 September 2004. Arbuscular mycorrhizal fungi - nematode interactions in Ammophila arenaria, Annual Meeting of British Ecological Society (BES), Lancaster, United Kingdom
- **Piśkiewicz A.M.**, van der Putten W.H., 17-19 December 2004. Top-down control of plant ectoparasitic nematodes by soil microbes in natural coastal dunes. Advances in Applied Biology, Oxford, United Kingdom
- **Piśkiewicz A.M.**, van der Putten W. H., 06-08 June 2005. Top- down control of plant ectoparasitic nematodes in natural foredune systems. International Organization of Biological Control, Wageningen, The Netherlands
- **Piśkiewicz A.M.**, van der Putten W.H., 17-23 July 2005. Top-down control of plant ectoparasitic nematodes by soil microbes in natural coastal dunes. XVII International Botanical Congress, Vienna, Austria
- **Piśkiewicz A.M.**, Costa S.R, van der Putten W.H., 20-25 August 2006. Microorganisms control plant parasitic nematodes in natural coastal foredunes. XI Symposium of International Society of Microbial Ecology, Vienna, Austria

Oral presentations at the international conferences

- **Piśkiewicz A.M.**, 5-9 June 2006. Top-down control of plant parasitic nematodes in natural coastal dunes. XXVIII Symposium of European Society of Nematologist, Blagoevgrad, Bulgaria
- **Piśkiewicz A.M.**, 11-14 September 2006. Top-down control of plant parasitic nematodes in natural coastal dunes. XXXVI Annual Conference of the Ecological Society of Germany, Austria and Switzerland, Bremen, Germany

PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of Literature (5.6 credits)
Control of the plant parasitic nematodes

Laboratory Training and Working Visits (4.4 credits)
AMF-nematodes interactions; IGER, Aberystwyth, UK (2004)
Methods in nematology; Nematology WUR, NL (2003-2006)

Post-Graduate Courses (9.2 credits)
Soil ecology: linking theory to practise; FE, SENSE, PE&RC (2003)
Basic and advanced statistics; PE&RC (2003/2004)
General linear modelling; PE&RC (2006)
Nematode identification; Nematology, WUR (2005)

Deficiency, Refresh, Brush-up and General Courses (2.8 credits)
Basic course in nematotology; Nematology, WUR (2004)

Competence Strengthening / Skills Courses (1.4 credits)
Goal oriented working and planning; FOM/KNAW (2004)
Internet platform training; TopShare (2004)

Discussion Groups / Local Seminars and other Scientific Meetings (7.1 credits)
NIOO CTE discussion group (2003-2007)
NIOO PhD discussion group (2003-2007)
Current themes in ecology, biological invasions (2004)
IOBC meeting multitrophic interactions, Wageningen (2005)
EcoTrain project workshops (2003-2007)

PE&RC Annual Meetings, Seminars and the PE&RC Weekend (1 credits)
Annual meeting graduate school functional ecology (2005)

International Symposia, Workshops and Conferences (9.3 credits)
BES symposium “Soil biodiversity and function, Lancaster, UK (poster:2003)
XXVII international symposium European society of nematology; Rome (2004)
XVII international botanical congress; Vienna, Austria (poster: 2005)
XXVII symposium of European society of nematologists; Blagoevgrad, Bulgaria (2006)
XXXVI annual conference of the ecological society of Germany, Austria and Switzerland, Bremen, Germany (2005)

Courses in which the PhD Candidate has worked as a Teacher
Nematology - practicals; Nematology, WUR, 15 days

Supervision of MSc student(s)
Research topic: influence of natural dune soil microbes on host choice and survival of *Tylenchorhynchus ventralis*; 1 student supervised, 10 days

This work is a part of the EcoTrain project (HPRN-CT 2002 00210), which was funded by the European Union rtn program.

The research presented in this thesis was performed at the Netherlands Institute of Ecology, Centre for Terrestrial Ecology, Department of Multitrophic Interactions in Heteren.

NIOO Thesis 60

Cover: View of a sandy beach and sea from the top of a grass covered dune
Photo by David Chadwick
Design: Ziemowit Czerep