Specificity, pathogenicity and population dynamics of the endoparasitic nematode *Heterodera arenaria* in coastal foredunes

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STELLINGEN

1. Dynamiek in de buitenduinen, vooral door zandoverstuiving, bevordert de instandhouding van populaties van de cystennematode *Heterodera arenaria* (dit proefschrift).

2. De populatiegrootte van *Heterodera arenaria* wordt in belangrijke mate gereguleerd door de hoeveelheid helmwortels (dit proefschrift).

3. Voor het begrijpen van de rol van nematoden in successie in de vegetatie van de buitenduinen dienen meer soorten, evenals hun samenhang met andere bodemorganismen, bestudeerd te worden dan zijn onderzocht in het onderzoek beschreven in dit proefschrift.

4. De seizoensmobiliteit van plantenparasitaire nematoden is in duinen groter dan die in landbouwgronden.

5. Onderzoek naar interacties tussen planten, bodempathogenen en hun antagonisten in natuurlijke ecosystemen kan bijdragen aan het vaststellen van de mogelijkheden en beperkingen van biologische bestrijding in landbouwsystemen.

6. De doorvoering van een andere puntentelling in sporten zoals volleybal en badminton met het doel de sport aantrekkelijker te maken, maakt het alleen aantrekkelijker voor de zappende sportkijker die geen verstand heeft van de betreffende sport.

7. Onnodig links blijven rijden op de snelweg tijdens spitsuren is een belangrijke veroorzaker van files.

8. De bestrijding van bacteriën en ongedierte in en om het huis neemt ongezonde vormen aan.

Stellingen behorende bij het proefschrift 'Specificity, pathogenicity and population dynamics of the endoparasitic nematode *Heterodera arenaria* in coastal foredunes'
Ineke van der Stoel
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ABSTRACT

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In natural ecosystems hardly any attention has been given to the population dynamics of plant-parasitic nematodes. In coastal foredunes, plant-parasitic nematodes are supposed to be involved in the degeneration and succession of the dominant sand-fixing grass *Ammophila arenaria* (Marram grass). The specificity, pathogenicity and population dynamics of the sedentary endoparasitic nematode *Heterodera arenaria* have been studied to determine if this species might be a key component of the soil pathogen complex of *A. arenaria.*

H. arenaria was found to be specific to *Elymus farctus* and *A. arenaria* in the mobile area of the coastal foredunes. Colonisation of the newly deposited sand layer by *H. arenaria* corresponded well with the development of pathogenicity in a series of bioassays. However, direct addition of the nematode to *A. arenaria* did not result in growth reduction of the plant. So, *H. arenaria* behaves like a biotrophic parasite, which has a high specificity but is not aggressive. Therefore, *H. arenaria* did not seem to be directly involved in the degeneration of *A. arenaria.*

Each year, the majority of the population of new *H. arenaria* cysts develops in the newly deposited sand layers. These layers are colonised by *A. arenaria* roots throughout the growing season. Migration to the new root layer may offer an individual nematode the benefit of early development and a larger potential offspring. The continuous release of juveniles in the field and their development in experiments indicate that release of juveniles from cysts is an ultimately determined process. Juveniles were found to emerge in November and many eggs or juveniles did not survive the winter period. The strategy of release, however, seems effective; the distance of migration could be too large to detect specific cues from the plant and the start of root formation in the field is highly variable. The emergence of juveniles late in the growing season could result in a second generation within the same year. The constant number of cysts per gram of roots suggests that the population density of *H. arenaria* is most likely a bottom-up directed process.

Key words: *Heterodera*, plant-parasitic nematodes, soil pathogens, *Ammophila arenaria*, occurrence, abundance, specificity, population dynamics, life history, pathogenicity, PCR-SSCP, molecular method, escape, sand burial, dispersal, migration, fitness, development time, survival, reproductive success, bottom-up, top-down.

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CHAPTER 1

GENERAL INTRODUCTION

Soil pathogens in natural ecosystems

Soil-borne pathogens in natural vegetation have already briefly been mentioned in a book by Clements (1928), but one of the first reports presenting experimental evidence on the role of soil pathogens in natural vegetation originates from Oremus and Otten (1981). They suggested that plant-parasitic nematodes are involved in the natural decline of the dune shrub *Hippophaë rhamnoides* (Sea Buckthorn). Since then this topic is getting more and more attention, although the actual mechanisms are still poorly understood, and there is uncertainty about the actual species involved (Troelstra *et al.*, 2001).

Most studies in recent years have focused on the influence of pathogenic soil fungi on plant communities. *Pythium* spp. and *Phytophthora* spp. are fungal species known to negatively affect plant growth. In temperate and tropical trees and also in annual plants soil-borne fungi cause seedling mortality, which affects the spatial and temporal distribution of plant species in the vegetation (Augspurger, 1983, 1990; Mihail *et al.*, 1998; Packer and Clay, 2000; Alexander and Mihail, 2000). In later stages of plant growth examples are known from the whole range of vegetation types from grasslands up to forests in which the soil community may negatively affect the growth of specific plant individuals or species, thereby contributing to the spatio-temporal processes in natural vegetation (Bever, 1994; Bever *et al.*, 1997; Holah *et al.*, 1997; Mills and Bever, 1998).

As regards plant-parasitic nematodes in natural vegetation, most is known from grasslands. The role of nematodes in spatio-temporal processes has been studied in coastal dune grasses, meadows, and prairie grasses (Stanton, 1988; Van der Putten *et al.*, 1990; De Rooij-Van der Goes, 1995; Van der Veen, 2000; Blomqvist *et al.*, 2000; Olff *et al.*, 2000; Verschoor, thesis in prep.). For the rest, studies on nematodes in natural vegetation mainly concentrate on two aspects. Some studies examine the taxonomy of species and focus on the morphological description of plant-parasitic nematodes (*e.g.* Sturhan, 1996; Robinson *et al.*, 1996; Karssen *et al.*, 1998a,b, 2000). Others investigate the species composition of whole nematode assemblages and their distribution in space or time, mostly dividing the nematodes in different feeding groups, such as fungal feeders, bacterial feeders or

plant feeders (Wasilewska, 1970, 1971; Magnusson, 1983; Freckman and Virginia, 1989; Bussau, 1991; De Goede *et al.*, 1993; Hodda and Wanless, 1994; Yeates, 1996; De Goede and Bongers, 1998).

To our knowledge very little is known of the population dynamics of plant-parasitic nematodes in natural vegetation, which is the subject of the present thesis.

Plant-parasitic nematodes

Organisms that belong to the phylum of the Nematoda are in general transparent worm-shape invertebrates. Within the Nematoda, the ubiquitous plant-parasitic nematodes are generally too small to be seen with the naked eye. They have a specialised mouth-part (stylet or spear) to penetrate plant roots and are mostly known from diseases in crop plants (*e.g.* Bongers, 1988). The plant-parasitic nematodes exhibit different feeding habits (Yeates *et al.*, 1993), which is generally indicative of their host range and the complexity of their relationship with the plant. Ectoparasites feed on the plant by only penetrating their stylet into the root tissue, whereas endoparasites actually invade the root with their entire body.

Some endoparasitic nematode species are migratory throughout their entire life and can invade and leave the root at any developmental stage. Others invade the root and induce specific feeding structures inside the root as a permanent source of nutrients for further development and reproduction. The endoparasites that induce feeding structures are sedentary for part of their life cycle and have evolved the most complex relationship with their hosts among all other plant-parasitic nematodes (Sijmons *et al.*, 1994; Koenning and Sipes, 1998). Among sedentary endoparasites, quite a number of species are specific, which implies a rather to very narrow host range (Ferris and Ferris, 1998).

The genus *Heterodera* belongs to the sedentary endoparasitic nematodes and is one of the genera that are also mentioned as cyst nematodes. Cyst nematodes are major agronomic pests (Lamberti and Taylor, 1986) and may reduce yields of a wide variety of arable crops including cereals, root crops and many legumes (Stone, 1977; Baldwin and Mundo-Ocampo, 1991). Many of the cyst nematode species have a worldwide distribution. As the cyst is a protective structure in the life cycle of the nematode, it can survive for a number of years in the absence of a suitable host, and only a wide crop rotation may be effective in controlling population densities (Baldwin and Mundo-Ocampo, 1991).

Basic life history of Heterodera species

The general developmental life cycle of *Heterodera* involves various stages, including the egg-stage, four juvenile stages, and the adult nematode (Fig. 1). The second-stage juvenile hatches from the egg in the soil-bound mother cyst. The juvenile, free in the soil, has to move to the roots of a suitable host (Baldwin and Mundo-Ocampo, 1991). After probing with the stylet, the juvenile penetrates into the host root near the growing tip (Seinhorst, 1986a; Von Mende *et al.*, 1998). Within the root the juvenile moves intracellularly in the cortex towards the vascular cylinder where it establishes a feeding site (Baldwin and Mundo-Ocampo, 1991). The so-called syncytium is composed of cells that are fused after cell-wall dissolution. When the juveniles no longer need to move they lose their body musculature and become immobile. For further development they are completely dependent on their host. Soon after feeding begins, the body volume starts to increase (Sijmons *et al.*, 1994). From the second-stage juvenile, the nematode goes through three moults before reaching the adult stage (Seinhorst, 1986a).

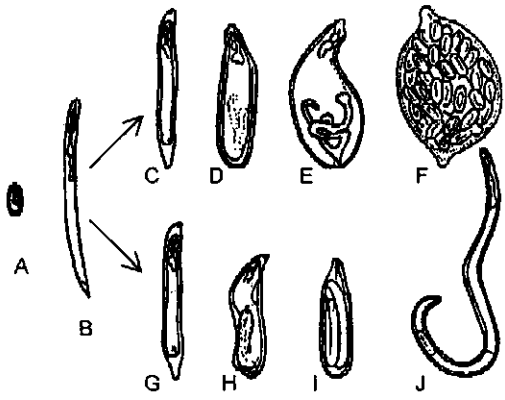

Figure 1. Stages of development of *Heterodera*. A) egg with first-stage juvenile; B) second-stage juvenile (which will penetrate the root); C) third-stage juvenile (female); D) fourth-stage female juvenile; E) immature female; F) cyst filled with eggs; G) third-stage juvenile (male); H) fourth-stage male juvenile; I) immature male inside fourth-stage juvenile; J) mature male. After: Decker (1989).

In a sexually reproducing species, the sexes differentiate mostly after the third juvenile stage. The adult males become vermiform, and leave the root (Seinhorst, 1986a; Sijmons *et al.*, 1994). The female juvenile swells over time and is

flask-shaped in the fourth juvenile stage and splits the cortex of the roots. From that moment onwards the nematode is visible on the roots as a small white or yellow sphere of about 0.5 mm diameter (Seinhorst, 1986a), and may be fertilised by a male that is present in the soil.

In sexually reproducing species, the adult female becomes filled with eggs only after mating. After all eggs have been formed the female dies and becomes a cyst, detached from the root (Seinhorst, 1986a). At the moment the female dies, the cuticle turns brown and is transformed into a thick protective cyst wall (Ferris and Ferris, 1998). In most species the eggs are retained inside the cyst. However, in some species, part of the eggs is placed in a gelatinous egg sac at the posterior end of the cyst, whereas another part remains inside the cyst. The embryos in the eggs develop into first stage juveniles, which moult into second stage juveniles, thus completing the life cycle (Seinhorst, 1986a).

The outer coastal dunes

Ammophila arenaria (L.) Link (Marram grass), a clonal perennial grass species, is the most important natural sand-fixing plant species in the outer coastal dunes of north-western Europe. During autumn and winter, heavy storms deposit freshly windblown sand between the shoots of *Ammophila* (Fig. 2). The plants may be buried by more than a meter of sand, but as long as the leaf tips remain visible new shoots will emerge. Emergence after being buried by sand is an important factor in the vigour of *Ammophila* species. Buried plants show enhanced physiological activity (Yuan *et al.*, 1993), form new nodes and increase the length of the internodes (Disraeli, 1984; Maun and Lapierre, 1984; Baye, 1990; Voesenek *et al.*, 1998). In spring, new roots are produced in the newly deposited sand layer, followed by an increased shoot production during summer. As roots mainly grow horizontally and node-staples mark growth seasons, year-layers of roots can be distinguished, each layer representing one growing season.

As soon as sand accretion ceases, *A. arenaria* starts to degenerate, and other plant species establish. Previous studies have related this degeneration either to ageing of the plant (Marshall, 1965), competition for space with other plant species (Huiskes and Harper, 1979; Huiskes, 1979) and the changing nutritional status of the soil (Willis, 1963, 1965; Maun, 1998). The growth and development of *A. arenaria*, however, was also found to be negatively affected by biotic soil components when the plants had not been buried (Van der Putten *et al.*, 1989). A complex of soil pathogens is thought to contribute to the degeneration of *A. arenaria* (Van der Putten and Troelstra, 1990; De Rooij-Van der Goes, 1995). As

the addition of nematicides reduced the numbers of plant-parasitic nematodes in the soil and subsequently increased the biomass production of *A. arenaria* in greenhouse trials, it has been suggested that plant-parasitic nematodes may contribute to the degeneration process (Van der Putten *et al.*, 1990).

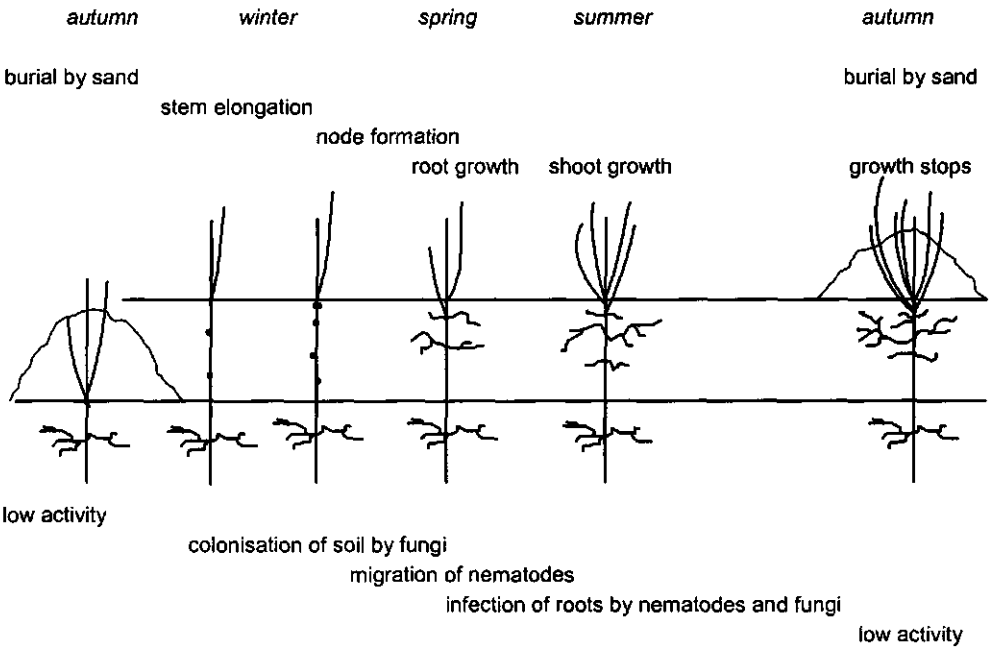

Figure 2. A time axis including the yearly sand deposition, growth of *A. arenaria*, and the migration and development of soil organisms. After: De Rooij - Van der Goes (1996b).

Plant degeneration and vegetation succession in coastal foredunes

In freshly wind-blown beach sand, hardly any harmful soil organisms were found to occur (Van der Putten and Troelstra, 1990). It was, therefore, suggested that enhanced root formation may offer *Ammophila* the possibility to retain its vigour by temporary escape from the soil-borne pathogens that are present in the root layers of previous years (Van der Putten *et al.*, 1988). Experimental burial of *A. arenaria* with sterilised sand resulted in more shoots and biomass than burial with sand from an existing root zone (De Rooij-Van der Goes *et al.*, 1995a). Such burial experiments suggest that *A. arenaria* may benefit from colonisation of freshly deposited windblown sand because of the temporary absence of root pathogens.

Soil pathogens are also supposed to contribute to vegetation succession in the coastal foredunes (Van der Putten *et al.*, 1993). *A. arenaria* is generally preceded by *Elymus farctus*, and succeeded by *Festuca rubra* spp. *arenaria*, *Carex arenaria*, *Elymus athericus* and *Calamagrostis epigejos*, respectively. Locally, each of these plant species reaches dominance before being replaced. In a study by Van der Putten and Peters (1997) the replacement of successional plant species was demonstrated to be enhanced by soil pathogens, especially when nutrients were limiting, which indicates that specificity of the soil pathogens causes apparent competition. In addition to *A. arenaria*, soil pathogens were also supposed to be involved in the degeneration of its North-American co-generic species, *Ammophila breviligulata* (Seliskar and Huettel, 1993), although effects of nematodes were found to be counteracted by arbuscular mycorrhizal fungi (Little and Maun, 1996, 1997). The natural degeneration of another dominant coastal dune plant, the shrub species *Hippophaë rhamnoides*, also seems to be affected by soil pathogens (Oremus and Otten, 1981; Maas *et al.*, 1983; Zoon *et al.*, 1993).

Nematodes in the coastal foredunes

So far, studies on the degeneration of *A. arenaria* by plant-parasitic nematodes have been focused on the effects of the ectoparasite *Telotylenchus ventralis* (now *Tylenchorhynchus ventralis*) (De Rooij-Van der Goes, 1995). Furthermore, particular combinations of nematodes and soil-borne fungi that may be harmful to *A. arenaria* were elucidated (De Rooij-Van der Goes *et al.*, 1995b). *Telotylenchus ventralis* was found to reduce plant growth of *A. arenaria* to the same level as growth in non-sterile soil, but only at densities that were 80 times higher than the density observed in non-sterile field soil. In the combinations of organisms that were regularly found to occur together in the field, the nematodes of *Heterodera* spp. were generally present. As a specific complex of soil pathogens and parasites was thought to be present in the rhizosphere of *A. arenaria*, it was hypothesised that species with a high specificity towards the plant would contribute most to the degeneration of *A. arenaria* (Van der Putten and Van der Stoel, 1998). In agricultural crops, many species of the genus *Heterodera* are known to have a narrow host range (Ferris and Ferris, 1998), and cause losses due to deformed roots, necrosis and plant death in major crop species, such as sugar beet and cereals such as oat and wheat (Baldwin and Mundo-Ocampo, 1991). However, natural biotrophic parasites are often characterised by relatively mild aggressiveness towards their host (Lenski and May, 1994), which would predict that specific nematodes do not, or only moderately, negatively affect their host. Therefore, as a

follow-up of the study of De Rooij-Van der Goes (1996a), it was decided to focus the present study on the population dynamics, specificity and pathogenicity of the *Heterodera* species that was most likely involved in the ecology of the grass *A. arenaria* in the outer coastal dunes.

Aim of this study

The main aim of the study presented in this thesis was to study the population dynamics of specialist plant-parasitic nematodes in a natural ecosystem. For that purpose, *Heterodera arenaria* in the coastal foredunes was used as a model. The study considered three detailed objectives. First, molecular techniques were used to identify the different species of *Heterodera* present in the system. Secondly, as specific plant-parasites were thought to be involved in vegetation processes, the abundance, host-specificity and pathogenicity of *H. arenaria*, were determined. The third objective was to study the distribution of *H. arenaria* over the various root layers and the migration to new plant roots that were formed after sand burial.

Outline of the thesis

In Chapter 2 the occurrence of nematodes in different root layers was studied in monthly samplings during the growing season. The presence of nematodes was linked to the development of pathogenicity towards *A. arenaria* in a series of bioassays carried out on root zone samples collected from the field. The period during which *A. arenaria* may grow vigorously and escape from the pathogenic effect of soil organisms is discussed in relation to sand burial.

In Chapter 3 the suitability of the molecular method PCR-SSCP (PCR-Single-Strand Conformational Polymorphism) was established in order to distinguish known species of endoparasitic nematodes on the basis of the ITS2 ribosomal RNA. *Heterodera* species that occur in the coastal foredunes on different plant species were compared with known *Heterodera* species in order to enable identification of the dune cysts to the species level.

In Chapter 4 the host specificity of *Heterodera* and its potential to reduce the growth of *A. arenaria* were addressed. Along the Dutch coast, root zone samples from different plant species were collected to establish the occurrence of *Heterodera*. In addition, an inoculation experiment was carried out to test the host status of the various plant species. In a dose-response experiment, effects of a range of inoculation densities of *H. arenaria* juveniles to *A. areneria* was tested in order to determine pathogenicity.

In Chapter 5 the population dynamics of *H. arenaria* in vigorous stands of *A. arenaria* are described. During two growing seasons, soil and root samples have been collected at monthly intervals from root layers of *A. arenaria* at various depth layers. The consequences of migration to the newly deposited sand layer for the individual juvenile are discussed.

In Chapter 6 two greenhouse experiments are described. The performance of *H. arenaria* cysts formed in the newly deposited sand layer and of cysts from a one-year-old layer was compared. The hypothesis was tested that individuals benefit from migration, *e.g.* resulting in a larger success of reproduction and an earlier start of development of the next generation.

In Chapter 7 the results of the study are discussed. Special attention is paid to the question whether, based on the results on the specificity and pathogenicity, *H. arenaria* may be a key species in the degeneration of *A. arenaria*. Furthermore, the life history strategy of *H. arenaria* is discussed in relation to the dispersal and the possible mechanisms that may control the population density of *H. arenaria* in a natural ecosystem.

CHAPTER 2

COLONISATION OF THE ROOT ZONE OF THE CLONAL GRASS *AMMOPHILA ARENARIA* BY NEMATODES AND THE DEVELOPMENT OF PATHOGENICITY

with W.H. van der Putten and H. Duyts
submitted to *Journal of Ecology*

ABSTRACT

In studies on the role of soil pathogens on spatio-temporal dynamics in natural vegetation, the colonisation process of the soil organisms has received little attention. In mobile outer coastal dunes, the ability to escape from soil pathogens has been suggested to be one of the factors explaining the vigour of *A. arenaria*. Each spring, roots of *A. arenaria* are growing into the newly deposited sand layer whereas the soil pathogens are lagging behind. However, based on the appearance rates, enemy-free space is short as some soil organisms colonise the layer within one month after the first root formation. This would indicate a very narrow window of escape for the plant. In previous studies, however, the colonisation by soil organisms has not been linked to the development of any pathogenic effect to the plant, so that the actual window for escape has not yet been determined.

In the present study, we tested the hypothesis that pathogenicity in the newly colonised sand layer occurs later than the colonisation by the first plant-parasitic nematodes, offering *A. arenaria* a wider window for escape to remain vigorous than the short period during which no nematodes are present in the newly formed root layer. To relate the nematode development in the field to the development of pathogenicity, we collected soil samples from the root zone of *A. arenaria* at monthly intervals and quantified the amount of roots and the number of plant-parasitic nematodes. The same soil samples were also used in a series of bioassays to examine the biomass production of *A. arenaria* seedlings in the natural soil and in soil where the original soil community completely has been eliminated by soil sterilisation or partially by nematicide addition.

Within a month after the first root formation of *A. arenaria*, colonisation of the newly deposited sand layer by soil organisms already resulted in the development of pathogenicity according to the bioassays. Initially, nematicide

addition counteracted growth reduction significantly, suggesting that plant-parasitic nematodes were involved in the observed growth reduction of *A. arenaria* in the bioassays. The nematicide effect coincided with the presence of the plant-parasitic nematodes *Heterodera arenaria* and *Pratylenchus* spp. in the field samples. Later, however, the effectiveness of the nematicide decreased, suggesting that an additional biotic soil component became involved in the growth reduction of *A. arenaria* in the bioassays. In older root layers, where nematodes were present from the start of the sampling period, growth reduction was observed in all bioassays, but nematicide addition did not effectively counteract growth reduction in non-sterilised soil.

Although pathogenicity in the bioassay developed within a month after the first roots were formed in the new sand layer, root biomass of *A. arenaria* in the field increased throughout the growing season, so that the effects of the bioassay do not seem to accurately estimate the window for escape for *A. arenaria*. Our results suggest that plant-parasitic nematodes are involved in the growth reduction of *A. arenaria* in the bioassay, but in the field, escape from pathogens indicates that more complex interactions between various groups of soil organisms are involved, which need further studies.

INTRODUCTION

In recent years, the interest in the role of plant pathogens in natural vegetation has increased considerably. Most of the research has focused on aboveground pathogens (*e.g.*, Burdon, 1987, 1993; Alexander, 1991; Clay *et al.*, 1993; Clay, 1997), but also soil pathogens have been found to influence plant communities (Van der Putten *et al.*, 1993; Bever, 1994; Blomqvist *et al.*, 2000). In the seedling stage of plants, most studies on soil-borne pathogens concentrated on the influence of soil-borne fungi on plant growth. In temperate forests, *Pythium* spp. caused patterns of seedling mortality of *Prunus serotina* (Packer and Clay, 2000). Both *Rhizoctonia solani* and *Pythium irregulare* were found to influence the population dynamics of the host plant *Kummerowia stipulacea*, an annual legume, by reducing seedling survival (Mihail *et al.*, 1998). Seedling mortality of tropical trees was found to be caused by damping-off fungi that may influence spatial distribution patterns and species diversity (Augspurger, 1983, 1990; Augspurger and Kelly, 1984). Also, in later stages of plant development the soil community may influence plant species diversity (Bever *et al.*, 1997; Mills and Bever, 1998; Olff *et al.*, 2000) and

interspecific competition (Turkington and Klein, 1991; Van der Putten and Peters, 1997; Holah and Alexander, 1999). Negative feedback between plant species and their soil communities may lead to either unidirectional or cyclic succession (Van der Putten *et al.*, 1993; Bever, 1994; Holah *et al.*, 1997; D'Hertefeldt and Van der Putten, 1998).

In coastal foredunes, the natural system that has been used in the present study, soil pathogens are supposed to contribute to vegetation succession (Van der Putten *et al.*, 1993). As soon as sand accretion ceases, *Ammophila arenaria* (L.) Link, one of the most important sand-fixing plant species in the outer coastal dunes of north-western Europe, degenerates, to which a specific complex of plant-parasitic nematodes and soil-borne plant-pathogenic fungi is thought to contribute (Van der Putten and Troelstra, 1990; De Rooij-Van der Goes, 1995). In pot trials, the addition of nematicides as well as fungicides caused a reduction in the numbers of plant-parasitic nematodes, and subsequently the biomass production of *A. arenaria* seedlings increased (Van der Putten *et al.*, 1990). As it is expected that nematodes with a narrow host range contribute most to the specificity of the complex of pathogens, it has been suggested, that specific endoparasitic nematodes may be the key species in the soil pathogen complex of *A. arenaria* (Van der Putten and Van der Stoel, 1998).

Burial by fresh windblown sand is an important factor in the vigour of *Ammophila* species. Buried plants show enhanced physiological activity (Yuan *et al.*, 1993), and show an increase in the length of the internodes (Disraeli, 1984; Maun and Lapierre, 1984; Baye, 1990; Voesenek *et al.*, 1998). In spring new roots are produced in the accreted sand layer, followed by increased shoot production during summer. As roots mainly grow horizontally and node-staples mark years of development, distinct layers of roots are produced each new growing season. In fresh wind-blown beach sand, containing few harmful soil organisms, enhanced root formation is supposed to offer *Ammophila* the possibility to retain its vigour by temporary escape from the soil-borne pathogens that are present in the root layers of previous years (Van der Putten *et al.*, 1988). Indeed, *A. arenaria* was more vigorous when buried with sterilised sand than when buried with sand from an existing root zone in which the natural root pathogens are present (De Rooij-Van der Goes *et al.*, 1995a). Such burial experiments support the suggestion that *A. arenaria* may benefit from the absence of root pathogens.

Clonal growth as a strategy to escape from pathogens has already been observed in plants attacked by systemic pathogens. In *e.g. Lactuca sibirica, Trientalis europaea* and *Cirsium arvense*, vigorous growth or the production of rhizomes allowed

at least part of the clone to escape from systemic fungal pathogens (Wennström and Ericson, 1992; Wennström, 1994, 1999; Frantzen, 1994). However, in the case of *A. arenaria*, the window for escape (*i.e.* the time during which the plant roots could grow without negatively being affected by the soil pathogens) appeared to be rather short as the first soil pathogens migrated upwards soon after root formation took place (De Rooij-Van der Goes *et al.*, 1998). In this latter study, however, the migration data had not been linked to the actual development of pathogenicity (*i.e.* the capacity of pathogens to negatively affect plant growth) towards *A. arenaria*.

Once the pathogens have migrated upwards, pathogenicity may develop in the root zone in various ways. One view is that there is an instantaneous relationship between the migration of nematodes and the development of pathogenicity. If the window for escape is indeed narrow, *A. arenaria* will be forced to benefit maximally from a short period of root growth to remain vigorous. Alternatively, pathogenicity may develop later in time when pathogenic species/individuals migrate gradually or occur later than non-pathogenic species, leading to expression of disease incidence when a certain threshold level is exceeded, *e.g.* after reproduction of the nematodes. In that case the window for escape is wider allowing *A. arenaria* to develop freely for almost the full growing season.

In the present study, the hypothesis is tested that pathogenicity in the newly colonised sand layer occurs later than the first migration of plant-parasitic nematodes, so that the window for escape for *A. arenaria* to remain vigorous is wider than the short period during which no nematodes are present in the newly formed root layer. We determined the colonisation of a new sand layer by roots of *A. arenaria* and by nematodes at monthly intervals in the field. In combination with this field study, the soil collected at monthly intervals was used in bioassays to relate the colonisation by nematodes to the development of pathogenicity. A one-year-old root layer of vigorous *A. arenaria* as well as the upper root layer of degenerating *A. arenaria* was included for comparison. Soil sterilisation and nematicide addition were included as treatments in order to study effects of complete and partial elimination of the natural soil organisms. The results of the field data and the bioassays are used to discuss the role of plant-parasitic nematodes and the development of pathogenicity in relation to the ability of *A. arenaria* to retain its vigour and the role of plant-parasitic nematodes therein.

MATERIALS AND METHODS

Collecting soil samples from the field

Samples were collected from the coastal foredunes of Voorne, the Netherlands, at a site north of Haringvlietdam (51°52' N 4°04' E). This location has been used in preceding studies by De Rooij-Van der Goes *et al.* (1995a) and Van der Putten *et al.* (1989). At monthly intervals from April 1997 up to November 1997, samples were collected from the root zone of both vigorous and degenerating *Ammophila arenaria* (Fig. 1). In April 1998, one additional measurement was carried out.

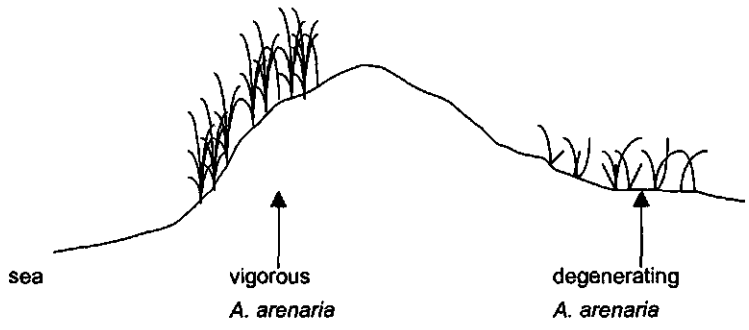

Figure 1. A schematic cross-section of the seaward and landward slope of the outer coastal foredune ridge with vigorous and degenerating *A. arenaria*, respectively.

Vigorous *A. arenaria* occurred on the seaward slope of the first dune ridge. In our study, at the vigorous site, roots and sand from the root zone were collected from a layer colonised in summer 1996 (indicated as 1996 layer), and from a layer with sand deposited in autumn/winter 1996/1997(indicated as 1997 layer). In the 1997 layer, at the start of the sampling period, roots still had to be formed (Fig. 2). Degenerated *A. arenaria* occurred on the landward slope and in the slack behind the first dune ridge (indicated as degenerated). At this site, roots and sand were collected from the top 20 cm layer.

At both the vigorous and the degenerated site, the sampling areas were 100 m long and 10 m wide, parallel to the coastline. At each sampling date in 1997, five random samples of 20x20x20 cm^3 were collected from each soil origin (1997 layer, 1996 layer, and degenerated). In April 1998, additionally, five random samples were collected from the 1998 layer, *i.e.* the layer with sand deposited during

autumn/winter 1997/1998. Replicate samples of the subsequent layers were collected independently by collecting them from different plants.

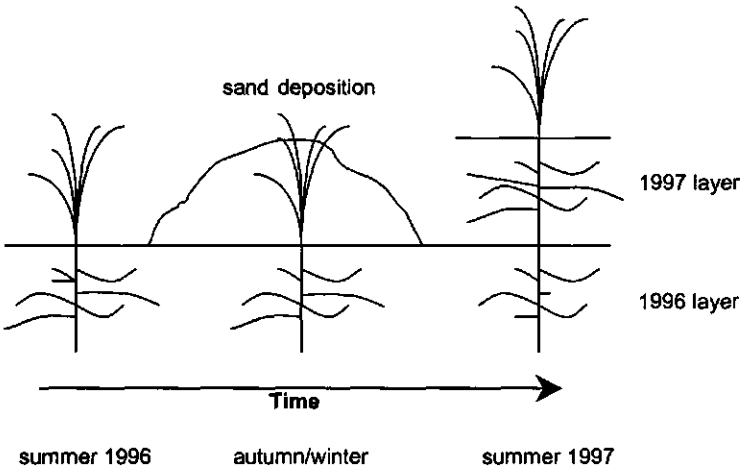

Figure 2. The development of vigorous *A. arenaria* in time with distinct root layers that each have been formed in a different year.

Sample processing

Each sample was sieved (mesh size 0.5 cm) to remove coarse material, and to separate the roots from the soil. The total fresh weight of each rootsample was measured before the roots were divided into three subsamples, each subsample used for a different purpose. From one subsample of roots the free-living nematodes were extracted by the funnel-spray method (Oostenbrink, 1960). From a second subsample, *Heterodera* cysts and *Meloidogyne* root-knots were collected and counted, using a binocular microscope (10-15x magnification). The third subsample was again mixed through the soil that was used for the bioassay. After the nematodes had been extracted from the roots, the subsamples that had been used for nematode extraction and visual counting were dried at 70°C for 48 hours, and weighed.

After sieving, the soil was homogenised gently, and the soil of each sample was used for four purposes. Free-living nematodes were extracted from the first subsample of 250 ml using the Oostenbrink elutriator (Oostenbrink, 1960). Second, *Heterodera* spp. cysts were extracted from another subsample of 1 l of soil, after weighing, by adding 4 l water, stirring the suspension and decanting the water with the floating cysts on a 180 μm mesh sieve. This procedure of adding and decanting was repeated five times. To determine the soil moisture content of each

sample, a third subsample of about 50 g of soil was weighed, dried at 70°C for 48 hours, and weighed again. Finally, about 6 kg of the homogenised soil was used for the bioassay after the remaining root material had been added (as described earlier).

Monthly bioassay with three soil origins

Experimental design

Every month, a bioassay was carried out according to a 3x2x2 factorial design with the factors 'soil origin' (SO) (1997 layer, 1996 layer, and degenerated), 'soil sterilisation' (s, ns), and 'nematicide addition' (+, -). There were five replicates of each treatment corresponding to the samples collected from the field.

Each month, five extra pots were included with a control soil in order to enable an additional analysis of variance with time (month) as factor to check monthly variation in growth conditions. The control soil, about 100 kg, was collected prior to the start of the first bioassay from vigorous and degenerated *A. arenaria* and from *C. epigejos* in the outer coastal foredunes. The soil was sieved (mesh size 0.5 cm) to remove coarse material and roots. Subsequently the soil was thoroughly mixed, placed in 10 plastic bags and sterilised by means of gamma-irradiation (25 kgray). After sterilisation these bags were stored in the dark at 4°C. Each month the five additional pots were filled with this sterilised soil and set at 10% soil moisture content ($w \cdot w^{-1}$).

Soil treatment

Each month, 6 kg of the homogenised soil remaining from the total amount collected was used for the bioassay. Half of each replicate sample was sterilised by autoclaving the soil at 120°C twice for one hour with a 48-hours interval. After sterilisation the soil was stored at 4°C for four days until use. The unsterilised soil was stored at 4°C until use. As a nematicide, oxamyl (100mg/kg Vydate 10% based on dry soil according to Van der Putten *et al.*, 1990) was mixed through the soil, before the pots were filled.

Per replicate four 1.5 l pots were filled with soil (10% soil moisture ($w \cdot w^{-1}$). They received the following treatments: sterilised soil with nematicide (s+), sterilised soil without nematicide (s-), unsterilised soil with nematicide (ns+), and unsterilised soil without nematicide (ns-).

Growing of plants

During the summer of 1995, *A. arenaria* seeds were collected at the earlier described studysite. Prior to the start of each monthly trial, seeds were germinated

for 3 weeks on glass beads at a 16/8 hour light/dark regime with corresponding temperatures of 25/15°C. After germination, four seedlings were planted in each 1.5 l pot. The soil surface was covered with aluminium foil to prevent desiccation of the soil and the pots were randomly placed in a climatised room at a 16/8 hour light/dark regime of 21/18°C. Twice a week, the soil moisture content was reset at 10% ($w \cdot w^{-1}$) with demineralised water. Once every week, a full strength Hoagland nutrient solution was added to all pots. During the first six weeks, 12.5 ml, and in the final two weeks, 25.0 ml were added per week, respectively.

Harvest

Eight weeks after the start of each trial, all pots were harvested. The soil was carefully washed from the roots, and organic matter was removed. Both shoot and root biomass were dried at 70°C for 48 hours and weighed.

April 1998

In April 1998, in addition to the other samples, five samples were collected from the sand layer that had been deposited in autumn/winter 1997/1998. In this particular month, the bioassay was carried out with four soil origins. After collection, the samples were treated similarly as described before.

Data analysis

Nematode development

Numbers of extracted nematodes were expressed as N per 100 g dry soil. For each sample the total number of plant-parasitic nematodes was calculated. Nematode genera that occurred in less than 10% of the soil samples collected per sampling layer in 1997 were discarded from the analysis. Among the genera that had been found rarely were ectoparasites such as *Boleodorus*, *Ecphyadophora*, *Telotylenchus*, *Helicotylenchus*, *Rotylenchus*, *Hemicycliophora*, and *Criconema*. According to the classification based on feeding types by Yeates *et al.* (1993) *Tylenchus*, *Filenchus* and *Ditylenchus* were considered as potential plant-parasites. Data of non-plant feeders are not presented. The nematode data were analysed in a one-way analysis of variance with factors 'month'. To achieve homogeneity in the data, the number of nematodes per 100 g dry soil were log(x+1)-transformed. For each soil origin, it was tested whether the total number of plant-parasitic nematodes showed any differences between months. Treatment means were compared using Tukey's HSD test ($P<0.05$). To test for differences between the layers, Wilcoxon's matched pairs

tests have been carried out, comparing the monthly average values of two soil origins.

Furthermore, for each soil origin, one-way ANOVA's were carried out with 'month' as independent variable, and a particular nematode or nematode life stage as the dependent variable. Also for these analyses, the log(x+1)-transformed numbers per 100 g dry soil have been used. Monthly means were compared using the LSD-test ($P<0.05$).

Bioassay

As variation between months in the control soil was significant, it was decided not to perform any ANOVA's including the sampling date, as possible differences may have been due to other, external conditions such as *e.g.* the initial size of the seedlings. In a 3-way analysis of variance the main effects of the factors 'soil origin', 'soil sterilisation' and 'nematicide addition' were determined. In 2-way analyses of variance with the factors 'soil sterilisation' and 'nematicide addition' data were analysed separately for each layer and month. The data have been tested for normality and homogeneity of variances. If necessary the data were log-transformed to obtain homogeneity. When no homogeneity was obtained, data were analysed in a non-parametric Kruskal-Wallis test. Treatment means were compared using Tukey's HSD test ($P<0.05$).

Root biomass in the field and in the bioassay

In a one-way analysis of variance for each soil origin, the effect of 'month' on the amount of root (g) per kg dry soil was determined. Treatment means were compared using the LSD-test ($P<0.05$).

To check whether plant biomass in the bioassays in the unsterilised soil without nematicide was related to the amount of roots in the field, a regression analysis was carried out for each soil origin. For each sample, the collected root biomass expressed per kilogram of dry soil was used as the first variable. The second variable was the ratio between the biomass of *A. arenaria* in unsterilised soil without nematicide and the biomass in sterilised soil without nematicide.

RESULTS

Nematode development: comparison of genera within sampling layers

In the 1997 layer, significant differences were found between months (P=0.0011), which is due to higher nematode numbers in October and November than in May (Fig. 3). The numbers observed in April and from June until September differed significantly from the numbers in November only. Until November, 100 gram dry soil contained on average about 13 plant-parasitic nematodes. In the 1997 layer, individuals of *Heterodera arenaria* were first observed in July (Fig. 4). The females increased to a maximum of 2.5 per 100 gram dry soil in November. Significantly highest numbers of *Heterodera* juveniles were found in November (P<0.001). Numbers of *Meloidogyne* females were low throughout the year. Juveniles of *Meloidogyne* were present in April and from August onwards, but were not detected from May until July. *Pratylenchus* only occurred in very low numbers.

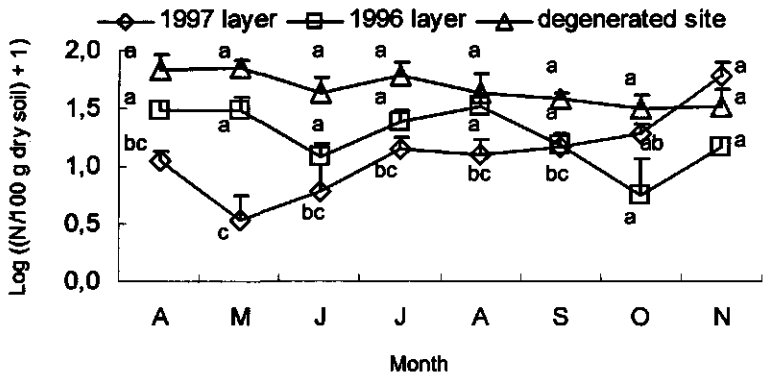

Figure 3. The mean log(x+1)-transformed (+SE) number of obligate plant-parasitic nematodes per month for three soil origins with x being the number per 100 g dry soil. From the site with vigorous *A. arenaria*, soil was collected from a newly colonised layer (1997 layer) and from a one-year-old layer (1996 layer). For each layer, different letters indicate statistically significant differences at P<0.05.

In the 1996 layer, plant-parasitic nematodes were present throughout the whole year, and their total number did not significantly differ between months (Fig. 3). *Heterodera* females were present throughout the whole year. However, only in August newly produced cysts were found (data not shown). Significantly higher numbers of *Heterodera* males were collected in August than in other months. Similar to the 1997 layer also in the 1996 layer *Meloidogyne* juveniles were present already in

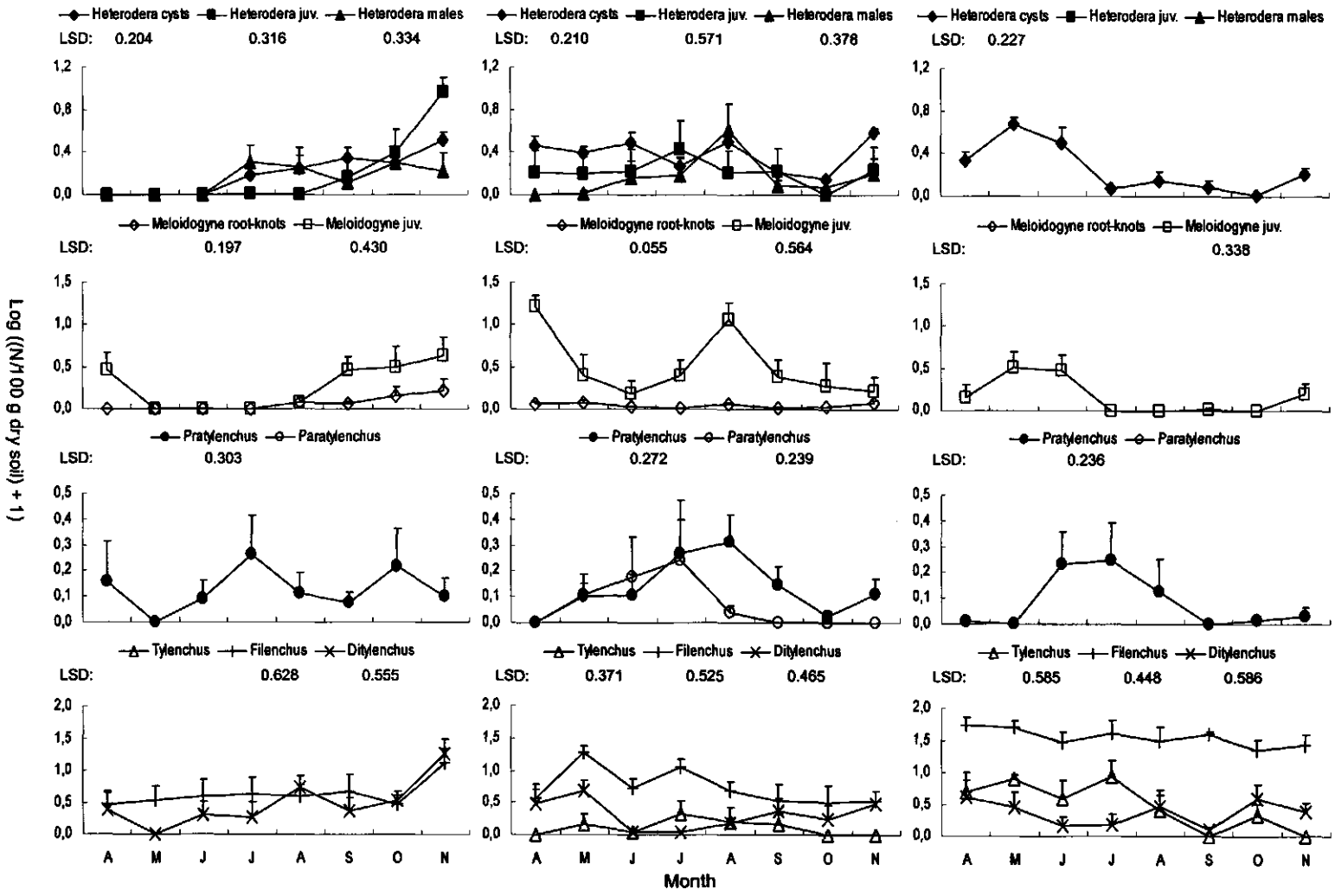

Figure 4. The mean log(x+1)-transformed (+ SE) number of nematodes during the year from April until November for three soil origins. x = the number of a particular genus per 100 g dry soil. Per month the average is calculated over 5 replicates. For the various genera different scales were used on the y-axis.

April. In August, in the 1996 layer, *Meloidogyne* juveniles had a second peak. Both *Pratylenchus* and *Paratylenchus* were present early in the growing season. For both genera a trend was found with the highest numbers occurring during the summer although the population density of *Pratylenchus* lagged behind that of *Paratylenchus*.

From the root zone of *A. arenaria* growing at a degenerated site significantly more plant-parasitic nematodes were collected than from the upper two root layers originating from vigorous *A. arenaria* (Fig. 3) (P=0.017 in the comparison with the 1997 layer; P=0.012 in the comparison with the 1996 layer). The difference was mainly due to the relatively high numbers of individuals that belong to the genera *Tylenchus* and *Filenchus* (Fig. 4). Of the genus *Heterodera* only cysts were found, but these hardly contained any viable eggs. From *Meloidogyne* only juveniles were collected but these did not develop into adult females, as no root-knots were found. *Pratylenchus* numbers tended to be similar to those in the other soils.

Nematode development: comparison of development of genera between layers

In the 1996 layer, *Heterodera* cysts were present throughout the whole year, whereas in the 1997 layer cysts only occurred from July onwards (Fig. 4). As in earlier months no juveniles were extracted from the 1997 layer, the newly formed cysts most likely had developed from juveniles that had migrated vertically from the 1996 to the 1997 layer. Contrary to the site with vigorous *A. arenaria*, there were no *Heterodera* juveniles and males present in the degenerated stand.

Meloidogyne root-knots were found in low numbers in both root layers of vigorous *A. arenaria*, and they were not present in the degenerated stand (Fig. 4). Although in April *Meloidogyne* juveniles were present in both root layers of the vigorous stand, they did not contribute to an increase in adult root-knots. Also a second peak of juveniles in August in the 1996 layer did not lead to a significant increase in the number of root-knots. In the 1996 layer, the number of *Meloidogyne* juveniles was significantly lower from September onwards than the number of juveniles in August, whereas in the 1997 layer, juvenile numbers tended to increase from September onwards (Fig. 4).

In both root layers the numbers of *Pratylenchus* varied largely between months, occasionally leading to significant differences between months, but not between root layers (Fig. 4). *Paratylenchus* and *Tylenchus* were present in the vigorous stand, but only in the one-year-old root layer. In the root layer of the degenerated stand, the numbers of *Filenchus* and *Tylenchus* tended to be higher than in the root layers of the vigorous stand.

Monthly bioassay

Although abiotic conditions were not supposed to vary over time, the biomass of the *A. arenaria* plants grown in the control soil varied significantly between months (data not shown). Apparently not all growth factors could be controlled between the monthly trials. For that reason, the factor time has not been included in the analyses. In a 3-way analysis with the factors soil origin, soil sterilisation and nematicide addition, each factor had a significant effect on the biomass. Due to the lack of homogeneity of variance in the data, it was not possible to test interaction effects. Therefore, a more detailed two-way ANOVA was carried out for every individual month and soil origin, using soil sterilisation and nematicide addition as factors (Table 1).

In the 1997 layer no significant differences in the total biomass between the treatments were found when soil was used collected in April, May, and June (Table 1; Fig. 5A). In the remaining months the biomass of *A. arenaria* was significantly lower in unsterilised soil than in sterilised soil. Until October, addition of nematicide to unsterilised soil counteracted growth reduction such that biomasses were not different from those in sterilised soil with nematicide. From October onwards, addition of nematicide to unsterilised soil did no longer yield biomasses of *A. arenaria* as high as in nematicide-treated sterilised soil.

In the 1996 layer, for each month biomass of *A. arenaria* was higher in sterilised than in unsterilised soils, irrespective of nematicide addition (Table 1; Fig. 5B). Although not significant for each month, addition of nematicide to unsterilised soil resulted in a higher biomass as compared to the biomass in unsterilised soil without nematicide. However, there was no month in which nematicide addition to unsterilised soil resulted in biomasses as high as in the sterilised soils. The results of nematicide addition to soil from the 1996 layer resemble those of the last few months of the 1997 layer.

In the soil collected from the root zone of degenerating *A. arenaria*, biomass in the unsterilised soil was also reduced as compared to that in the sterilised soil (Table 1; Fig. 5C). Addition of nematicide to unsterilised soil enhanced productivity in three months (June, August and September) as compared to productivity in unsterilised soil without nematicide. Only in April, July and August nematicide addition to unsterilised soil did enhance productivity up to the level of sterilised soil with nematicide.

Table 1. The results of a two-way analysis of variance with the factors soil sterilisation (S) and nematicide addition (N) and the mean values of the total biomass (g/ pot) per treatment for the 1997 and 1996 layers of vigorous *A. arenaria* and for the degenerated (deg.) stage of *A. arenaria*. For each soil origin and each month the F-, and P-value, and the results of Tukey's HSD test are presented. * P<0.05; ** P<0.01; *** P<0.001; -- no significant difference. Different letters indicate significant differences.

			Month							
Soil origin	Factor		A	M	J	J	A	S	O	N
	S	F	1.77	3.79	2.13	20.9	15.6	21.1	85.8	50.5
		P	--	--	--	***	**	***	***	***
1997	N	F	0.09	0.90	30.5	10.6	9.10	20.0	6.14	20.0
layer		P	--	--	--	**	**	***	*	***
	SxN	F	2.25	0.26	1.49	4.88	5.12	11.2	3.53	8.23
		P	--	--	--	*	*	**	--	*
Sterile + nematicide			1.63^{a}	2.00^{a}	1.88^{a}	1.33^{a}	1.03^{a}	0.58^{a}	1.11^{a}	2.69^{a}
Sterile – nematicide			1.75^{a}	1.87^{a}	1.81^{a}	1.20^{a}	0.95^{a}	0.53^{a}	1.14^{a}	2.44^{ab}
Non-sterile + nematicide			1.65^{a}	1.79^{a}	1.85^{a}	1.04^{a}	0.85^{a}	0.53^{a}	0.66^{b}	2.02^{b}
Non-sterile – nematicide			1.46^{a}	1.75^{a}	1.47^{a}	0.35^{b}	0.29^{b}	0.17^{b}	0.39^{c}	0.85^{c}
	S	F	35.7	21.0	103.5	18.9	98.9	11.0	86.5	97.9
		P	***	***	***	***	***	**	***	***
1996	N	F	3.34	3.71	5.97	9.32	14.9	1.48	2.20	6.80
layer		P	--	--	*	**	**	--	--	*
	SxN	F	7.00	0.18	2.25	2.05	12.5	0.70	3.55	0.05
		P	*	--	--	--	**	--	--	--
Sterile + nematicide			1.83^{ab}	2.38^{a}	2.38^{a}	1.68^{a}	1.08^{a}	0.83^{a}	1.91^{a}	3.03^{a}
Sterile – nematicide			1.97^{a}	2.09^{ab}	2.26^{a}	1.47^{a}	1.06^{a}	0.78^{a}	1.96^{a}	2.59^{a}
Non-sterile + nematicide			1.26^{b}	1.57^{bc}	1.26^{b}	1.30^{a}	0.69^{b}	0.53^{ab}	0.98^{b}	1.25^{b}
Non-sterile – nematicide			0.49^{c}	1.11^{c}	0.75^{b}	0.72^{b}	0.24^{c}	0.29^{b}	0.56^{b}	0.72^{b}
	S	F	7.36	47.2	52.1	17.0	19.0	56.4	33.7	41.0
		P	*	***	***	***	***	***	***	***
deg.	N	F	2.86	7.02	7.32	5.84	7.69	9.11	4.59	1.33
stage		P	--	*	*	*	*	**	*	--
	SxN	F	4.33	1.13	6.05	2.25	2.12	1.34	1.78	2.89
		P	--	--	*	--	--	--	--	--
Sterile + nematicide			1.79^{a}	3.51^{a}	3.10^{a}	2.49^{a}	1.19^{a}	1.49^{a}	2.88^{a}	4.05^{a}
Sterile – nematicide			1.87^{a}	3.19^{a}	3.06^{a}	2.18^{a}	0.95^{a}	1.30^{a}	2.72^{ab}	4.25^{a}
Non-sterile + nematicide			1.67^{ab}	2.32^{b}	2.21^{b}	1.61^{ab}	0.88^{a}	0.84^{b}	1.99^{bc}	2.39^{b}
Non-sterile – nematicide			0.96^{b}	1.55^{b}	1.24^{c}	0.29^{b}	0.32^{b}	0.42^{c}	1.30^{c}	1.38^{b}

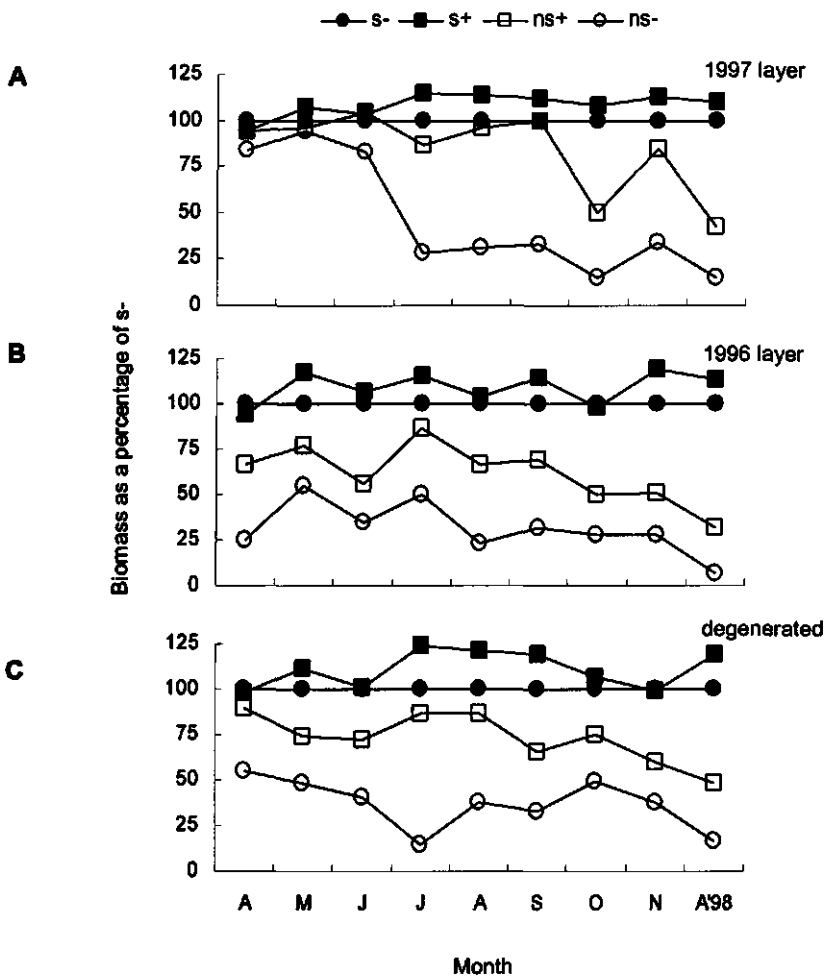

Figure 5. The total biomass of *A. arenaria* as a percentage of the total biomass in sterilised soil without nematicide (s-). As each replicate sample from the field was divided over the four treatments in the bioassay, for each replicate the total biomass of each of the treatments s+ (sterilised soil with nematicide), ns+ (unsterilised soil with nematicide) and ns- (unsterilised soil without nematicide), was divided by the total biomass of that particular replicate in the treatment s-, and multiplied by 100. The mean was calculated based on 5 replicates. A'98 indicates April 1998.

In April 1998, for all soil origins, the productivity of *A. arenaria* was significantly lower in unsterilised soil than in sterilised soil (Table 2). However, in the 1998 layer, the sterilisation effect was relatively small and there were no further significant differences between the biomass in the unsterilised soil with nematicide

and the sterilised treatments. Addition of nematicide to the unsterilised soil did only result in a significantly higher biomass in soil collected from the degenerated stand (Table 2).

Table 2. The results of a two-way analysis of variance with the factors soil sterilisation (S) and nematicide addition (N) and the mean values of the total biomass (g/pot) per treatment for the April 1998 sampling. The data of the 1997 layer and the 1996 layer were analysed by a Kruskal-Wallis non-parametric test, which does not allow to test more than one factor at a time. For each soil origin the F-, and P-value, and the results of Tukey's HSD test are presented. * P<0.05; ** P<0.01; *** P<0.001; -- no significant difference. Different letters indicate significant differences.

		April 1998			
		1998	1997	1996	degenerated
Factor		layer	layer	layer	layer
S	*F*	12.6	14.3	14.3	133.1
	P	*	***	***	***
N	*F*	3.96	1.37	0.97	13.7
	P	--	--	--	**
SxN	*F*	0.52			1.12
	P	--			--
Sterile + nematicide		2.10^{a}	2.18^{a}	2.34^{a}	2.92^{a}
Sterile – nematicide		2.02^{a}	1.99^{a}	2.16^{a}	2.47^{a}
Non-sterile + nematicide		1.86^{ab}	0.84^{b}	0.63^{b}	1.17^{b}
Non-sterile – nematicide		1.55^{b}	0.30^{b}	0.13^{b}	0.37^{c}

In the field samples collected in April 1998, a small amount of roots had been found in the 1998 layer (Fig. 6), whereas in 1997, the first roots in the 1997 layer had not been found yet at the sampling date in early May. Therefore, considerable variability may exist between years in the moment *A. arenaria* starts to produce new roots in the newly deposited sand layer. Furthermore, in the 1997 layer the quantity of roots per kg of soil gradually increased during the growing season with significantly most roots in November (Fig. 6). In a regression analysis it was determined whether the reduction in growth in unsterilised soil as compared to sterilised soil in the bioassay correlated with the presence of roots in the field. For none of the soil origins, however, a significant correlation was found.

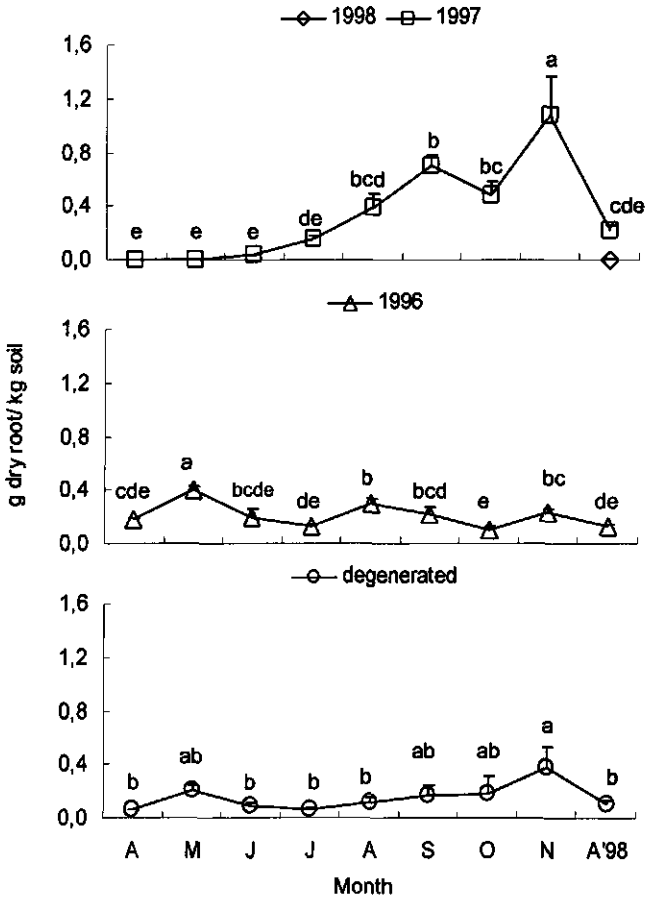

Figure 6. The amount of roots of *A. arenaria* per kg of soil in the layers 1998, 1997, 1996, and degenerated in various months. The mean was calculated based on 5 replicates. Different letters indicate statistically significant differences at $P<0.05$. A'98 indicates April 1998.

DISCUSSION

In 1997, within a month after the formation of the first roots of *A. arenaria* in the newly deposited sand layer, colonisation by soil organisms had already resulted in the development of pathogenicity in bioassay conditions. Initially, nematicide addition enhanced biomass production in unsterilised soil, suggesting that plant-parasitic nematodes were responsible for the observed growth reduction in the bioassays. Numbers of plant-parasitic nematodes in the field in this study were of the same order of magnitude as those in other studies on nematodes in dunes, although in previous studies numbers have been expressed per square metre

(Yeates, 1968; De Rooij-Van der Goes *et al.*, 1995b). The abundance of the cyst nematode *Heterodera arenaria*, and less strongly of the root-lesion nematode *Pratylenchus* spp., increased between June and July when the growth reduction was observed for the first time. Later in the growing season, however, the effectiveness of the nematicide diminished, suggesting that another biotic factor became involved in growth reduction of *A. arenaria* in the bioassay. Such reduced effects of nematicides in the late season were also observed in the one-year-old root layer, as well as in the soil from degenerating *A. arenaria*.

As nematicide effects correlated well with the root colonisation by *H. arenaria* and *Pratylenchus*, the results suggest that these nematode species contribute to the growth reduction of *A. arenaria*. Based on the numbers of nematodes in the field soil, we calculated about 180 plant parasites to be present in each pot in the bioassays (about 13 plant-parasitic nematodes were extracted per 100g dry soil; each pot in the bioassay contained about 1350 g dry soil). However, inoculation experiments with *H. arenaria* and *Pratylenchus* sp., including densities up to 23 and 8 times higher, respectively, than in the field, did not result in any growth reduction of *A. arenaria* (Van der Stoel and Van der Putten, see Chapter 4; E.P. Brinkman, unpubl. results). In contrast, inoculation experiments with ectoparasites, although with densities as high as eighty times the field density, did result in growth reduction of *A. arenaria* (De Rooij-Van der Goes, 1995). Therefore, it is not likely that the observed growth reduction in the bioassay is due to direct effects of *H. arenaria* and *Pratylenchus*, in spite of the correlation between their colonisation of the new root layer and the growth compensating effect of nematicide addition. Alternatively, Little and Maun (1996) observed direct growth reducing effects of species of the same genera on the North-American *Ammophila breviligulata*, so that findings for these related species concerning plant-endoparasitic nematode interactions may not be consistent.

Since the nematicide effects varied in time, and proper controls were included, consistent side effects may be excluded. There are two possible explanations of the nematicide effects as observed in the series of bioassays. Nematodes may enhance the sensitivity of the plant to other soil organisms, or they may serve as a vector of pathogenic organisms such as viruses or bacteria (Khan, 1993; Barker and McGawley, 1998). Pathogenic fungi were already known to occur in the rhizosphere soil of *A. arenaria* (Van der Putten *et al.*, 1990; Kowalchuk *et al.*, 1997), although synergism with nematodes has not been proven. When plant-parasitic nematodes are being kept away from the roots due to nematicide addition, their transmission of other soil pathogens is prevented, which

could explain the positive effects of the nematicide addition even though plant-parasitic nematodes have no direct negative effect on plant growth. Later in the growing season, addition of nematicide no longer compensated for the entire growth reduction in unsterilised soil. As in unsterilised soil growth was reduced even stronger in the presence of nematodes, there is circumstantial evidence for some synergistic interaction between nematodes and other pathogenic soil organisms in this experiment. Soil pathogens may have accumulated and reduce growth of *A. arenaria*, irrespective of the reduction due to possible transmitted soil pathogens by nematodes. In such a synergistic interaction, fairly low numbers of nematodes interacting with other micro-organisms, could still be important for the activity of the complex of pathogens (Van der Putten and Van der Stoel, 1998). Further study is required, however, to unravel the direct and indirect pathogenic effects of soil-borne fungi in combination with plant-parasitic nematodes.

In the field, in the newly colonised layer, root biomass continued to increase throughout the growth season in spite of pathogen activity measured in the bioassays. Results from the bioassays may not be directly extrapolated to the field situation (Troelstra *et al.*, 2001). The poor correlation between the start of pathogenicity development in the newly colonised sand layer according to bioassays and the pattern of root development has important consequences for our view on the length of the period during which plants may escape from their soil pathogens. According to the bioassay, pathogenicity had developed within a month after the first roots had been formed, so that in the field other factors may be involved that can explain vigorous growth of *Ammophila*.

Arbuscular mycorrhizal fungi (AMF) are known to protect plants from pathogenic fungi (Carey *et al.*, 1992; Newsham *et al.*, 1994, 1995a,b), as well as to protect the roots from nematode attack (Francl, 1993). These fungi may be involved in the vigorous growth of *A. arenaria* in the field. In a study on *Ammophila breviligulata*, the presence of AMF in combination with sand burial was related to enhanced vigour in plants after exposure to the endoparasitic nematode genera *Heterodera* and *Pratylenchus* (Little and Maun, 1996; Maun, 1998). Our bioassays may have discouraged plant-mycorrhizal associations as we supplied nutrient solution, although soil sterilisation effects in dune sand are consistent along a gradient of nutrient supply rates (Van der Putten and Peters, 1997). Furthermore, the homogenisation of the soil that disrupts the hyphal networks, and the relatively short duration of the bioassay (8 weeks) may have limited plant-mycorrhizal associations. Also the natural suppressiveness of dune soils to soil pathogens (De Boer *et al.*, 1998) may be involved in the more complex situation in the field, which

may explain the vigorous growth of *A. arenaria* in the field in spite of the observed pathogenicity in the bioassays.

Another possibility why *A. arenaria* may grow vigorously following sand deposition, is that the plants may use the short period of low pathogenic activity or the incompleteness of the soil pathogen complex to acquire sufficient additional resources to remain vigorous throughout the growing season. In spite of the many studies on *A. arenaria* in relation to sand burial, its physiology in relation to resource acquisition is still poorly understood.

In conclusion, after sand burial and colonisation of the new root layer, according to a bioassay pathogenicity may develop within a month after the first roots are formed. However, in the field the window for escape for *A. arenaria* seems to be wide enough to remain producing new roots, thereby developing vigorously. Our results suggest that nematodes are involved in the development of pathogenicity, but the mechanism remains unclarified. *Heterodera arenaria* and *Pratylenchus* spp. are not able to reduce growth of *A. arenaria* directly (Van der Stoel and Van der Putten, see Chapter 4). However, the nematodes may act as a vector for another soil pathogen (fungal or bacterial). Possibly, in the field AMF may protect the plants from pathogenic activity (Little and Maun, 1996). The escape of clonal plants from soil pathogens, therefore, seems the result of a more complex set of interactions than in case of the escape from systemic fungal pathogens (Wennström and Ericson, 1992; Wennström, 1994, 1999). Future studies need a wider approach focusing on various trophic groups of organisms that may influence the growth of *A. arenaria.*

CHAPTER 3

RAPID IDENTIFICATION OF CYST (*HETERODERA* SPP., *GLOBODERA* SPP.) AND ROOT-KNOT (*MELOIDOGYNE* SPP.) NEMATODES ON THE BASIS OF ITS2 SEQUENCE VARIATION DETECTED BY PCR-SSCP (PCR-SINGLE-STRAND CONFORMATIONAL POLYMORPHISM) IN CULTURES AND FIELD SAMPLES

Based on J.P. Clapp, C.D. van der Stoel and W.H. van der Putten, 2000.
Molecular Ecology **9**, 1223-1232.

ABSTRACT

Cyst and root-knot nematodes show high levels of gross morphological similarity. This presents difficulties for the study of their ecology in natural ecosystems. In the present study, cyst and root-knot nematode species as well as some ectoparasitic nematode species were identified using ITS2 sequence variation detected by PCR-SSCP. The ITS2 region was sufficiently variable within the taxa investigated to allow species to be separated on the basis of minor sequence variation. The PCR primers used in this study were effective for 12 species with three genera within the Heteroderinae (*Globodera pallida*, *G. rostochiensis*, *Heterodera arenaria/avenae*, *H. ciceri*, *H. daverti*, *H. hordecalis*, *H. mani*, *H. schachtii*, *H. trifolii*, *Meloidogyne ardenensis*, *M. duytsi*, and *M. maritima*). However, pathotypes of *Globodera pallida* and *G. rostochiensis* could not be distinguished. The method was tested at two coastal dune locations in the Netherlands (one in the lime-poor dunes of the north and one in calcareous dunes of the south) to determine the population structure of cyst nematodes. At each site, cyst nematodes were associated with three plant species: two plant species on the foredune (*Elymus farctus* and *Ammophila arenaria*) and one plant species occurring further inland (*Calamagrostis epigejos*). The PCR-SSCP results showed that two species of cyst nematodes were found: *H. arenaria* and *H. hordecalis*. *H. arenaria* associated with vigorous *A. arenaria*, and *H. hordecalis* in association with degenerating *A. arenaria* and *C. epigejos*. The field survey demonstrated that in coastal dunes abiotic factors may be important in affecting the distribution of cyst nematodes.

INTRODUCTION

Nematodes are an important biotic component of the rhizosphere (Nickle, 1991). Plant-parasitic nematodes are well known pests in agroecosystems and are also thought to exert an important influence on the structure and stability of natural plant communities (Stanton, 1988; Van der Putten and Van der Stoel, 1998). Precise identification of the components of natural plant-parasitic nematode communities is a prerequisite for these studies.

Several classical techniques have been used for nematode identification including host range tests and the use of morphological characters. Despite having specialised ecological functions, the overall morphology of many nematode taxa is conservative, especially as variation in juveniles frequently ranges across species divisions. This is particularly true for closely related species with high morphological similarity such as *Globodera rostochiensis* and *G. pallida* (Morgan Golden, 1986). The use of morphology has therefore been augmented by techniques based on molecular characters, which generally result in simple band patterns that are easy to interpret by non-specialists.

PCR-SSCP (PCR-Single-Strand Conformational Polymorphism) (Orita *et al.*, 1989) has considerable advantages over many other molecular techniques used for taxonomic characterisation. A major advantage is that taxonomic differentiation utilises the sum of all nucleotide sequence variation *between* PCR primers sites rather than the taxon-specific annealing of primers or differences in restriction sites (often revealing no differences unless a suite of several enzymes are tested). The design of PCR primers can therefore be based on conserved regions and allows a single primer pair to differentiate species across more than one genus combining the advantages of PCR with a sensitivity (over defined regions) on a par with DNA sequencing (Hayashi and Yandell, 1993). The technique relies on differences in the mobility of single stranded PCR amplicons in non-denaturing polyacrylamide gels. The length, position, and extent of self-complimentary base pairing affect the conformation taken up by the molecules and thus their electrophoretic mobility. Single base differences between amplicons can affect the tertiary conformation of the molecules and allow differentiation. This effect is enhanced by minor length polymorphisms and increasing amounts of sequence variation. SSCP patterns are highly reproducible between gels and generate two markers from each DNA sequence present, enabling identification to take place on the basis of minor nucleotide differences across several hundred bases of sequence,

but without recourse to sequencing (Lessa and Applebaum, 1993). The target region in this study has been the second internal transcribed spacer (ITS2) of the ribosomal RNA (rRNA) gene clusters. The ribosomal genes are most frequently used for taxonomic work as they are present in all organisms and sequence data are available which enable phylogenetic affiliations to be rooted in a background of related taxa. The ITS2 was chosen over the small sub-unit rRNA genes because it was likely to show less sequence conservation and thus enable the discrimination of closely related species on this basis.

PCR-SSCP has been exploited for the identification of fungi (Simon *et al.*, 1993; Clapp, 1999), bacteria (Lee *et al.*, 1996; Schwieger and Tebbe, 1998) and carabid ground beetles (Boge *et al.*, 1994). Gasser (1997), Gasser and Monti (1997), and Gasser *et al.* (1997) have applied PCR-SSCP to distinguish veterinary parasites but its potential for application to free-living and plant-parasitic nematode communities has not been investigated. Cyst and root-knot nematodes are major agronomic pests (Lamberti and Taylor, 1986) and may also be involved in natural soil pathogen complexes such as those occurring in coastal foredunes (De Rooij-Van der Goes *et al.*, 1995b). The aims of the present study were to determine the suitability of PCR-SSCP for identification of cyst and root-knot nematodes and to establish the identity of *Heterodera* spp. that occurred on plant species in a field investigation in the coastal foredunes, carried out by Van der Stoel and Van der Putten (see Chapter 4).

MATERIALS AND METHODS

Nematodes: identified species

Three *Globodera* and eight *Heterodera* species from cultures together with three species of *Meloidogyne* from the Haringvliet (51°52' N 4°04' E) field locality were investigated in this study (Table 1). Twenty individual cysts were analysed from each pathotype of *G. pallida* and *G. rostochiensis* in addition to extractions from multiple cysts and individual juveniles. Five cysts of *G. tabacum* were also analysed. Similarly, five to fifteen individual cysts were analysed for each cultured *Heterodera* species (total 58). DNA was extracted from egg masses (total n=6) and juveniles (total n=106) of the *Meloidogyne* species from maritime dune locations. *M. maritima*, *M. duytsi* and *M. ardenensis* were associated with *Ammophila arenaria, Elymus farctus* and a *Salix* sp. respectively and identified microscopically by Henk Duyts.

Table 1. List of nematode isolates and samples used in this study. – indicates no culture available or no known host. PD refers to the Plantenziektenkundige Dienst, Wageningen, The Netherlands. *H. avenae* and *H. mani* were isolated from sites at Grafenreuth and Hamminkeln, Germany respectively and provided by Dr. D. Sturhan, Biologische Bundesanstalt, Institut für Nematologie und Wierbeltierkunde, Toppheideweg 80, D-48161, Münster, Germany. *H. arenaria* was provided by J.A. Rowe, Department of Entomology and Nematology, Institute of Arable Crops Research, Rothamsted Experimental Station, Harpenden, Hertfordshire, UK. *H. hordecalis* was provided by Dr. S. Andersson, National Swedish Institute for Plant Protection, S-230 47, Åkarp, Sweden. *G. rostochiensis* A-50 was collected from Mierenbos and provided by J. van Bezooijen, Department of Nematology, Wageningen Agricultural University, Wageningen, The Netherlands.

Nematode species	Pathotype	Culture code	Host plant	Origin
Globodera pallida	Pa2	D-381	*Solanum tuberosum*	PD
Globodera pallida	Pa2	D-475	*Solanum tuberosum*	PD
Globodera pallida	Pa3	E-412	*Solanum tuberosum*	PD
Globodera rostochiensis	Ro1	A-50	*Solanum tuberosum*	J. v. Bezooijen
Globodera rostochiensis	Ro1	A-56	*Solanum tuberosum*	PD
Globodera rostochiensis	Ro3	B-140	*Solanum tuberosum*	PD
Globodera rostochiensis	Ro4	F-539	*Solanum tuberosum*	PD
Globodera rostochiensis	Ro5	G-1526	*Solanum tuberosum*	PD
Globodera tabacum		C-6876	*Nicotiana* sp.	PD
Heterodera trifolii		A1-1	*Trifolium repens*	PD
Heterodera ciceri		Pot 30	*Phaseolus vulgaris*	PD
Heterodera avenae		Field	-	D. Sturhan
Heterodera arenaria		Field	*Ammophila arenaria*	J.A. Rowe
Heterodera mani		Field	-	D. Sturhan
Heterodera daverti		LU68	*Trifolium repens*	PD
Heterodera schachtii		Pot 7	*Brassica* sp.	PD
Heterodera hordecalis		-	*Hordeum vulgare*	S. Andersson
Meloidogyne ardenensis		Field	*Salix* sp.	Haringvliet
Meloidogyne maritima		Field	*Ammophila arenaria*	Haringvliet
Meloidogyne duytsi		Field	*Elymus farctus*	Haringvliet
Heterodera sp.		Field	*Calamagrostis epigejos*	Haringvliet
Heterodera sp.		Field	vigorous *A. arenaria*	Haringvliet
Heterodera sp.		Field	degenerated *A. arenaria*	Haringvliet
Heterodera sp.		Field	*Calamagrostis epigejos*	Texel
Heterodera sp.		Field	degenerated *A. arenaria*	Texel
Heterodera sp.		Field	vigorous *A. arenaria*	Texel
Heterodera sp.		Field	degenerated *A. arenaria*	Texel

Nematodes: field samples

Comparisons were made between nematode populations occurring at three locations along the coast of the Netherlands in this pilot study using one way analysis of variance. Based on an earlier field study at six locations along the Dutch coast, two field sites were chosen, one at Texel (53°07' N 4°45' E; lime-poor), and one at Haringvliet (51°52' N 4°04' E; calcareous). A third site, Walcheren (51°35' N 3°32' E; calcareous) was included to obtain additional information on the population density of cysts and juveniles. Eight soil samples (1 kg each), subsequently combined, were collected from the rhizosphere of a series of dominant plant species: *Elymus farctus*, *Ammophila arenaria*, *Festuca rubra* ssp. *arenaria*, *Carex arenaria*, *Elymus athericus* and *Calamagrostis epigejos*. Soil was sampled from both vigorous and degenerating stands of *A. arenaria*. Cysts were extracted by flotation and decantation over a 180 µm mesh size sieve, quantified to establish the specificity of the host-plant association (Van der Stoel and Van der Putten, see Chapter 4), and subsequently used for molecular analysis.

DNA extraction

The DNA-extraction protocol for individual cysts was based on that of Caswell-Chen *et al.*, (1992). However, the volumes were doubled since more diluted DNA was acceptable for the PCR. Nematode juveniles were picked individually and placed into 10µl sterile water on glass slides and disrupted manually with a needle. The juvenile fragments were then diluted in 45µl sterilised water of which 5µl was used in the subsequent PCR. Extractions of both cysts and juveniles were flash frozen in liquid nitrogen and stored at -20°C until required.

PCR

Forward and reverse primers were designed for the second ITS ribosomal RNA spacer region (ITS2) based upon all available Heteroderinae sequences: *CysNFwd*1 (5'GATCGATGAAGAACGCAGC), *CysNRvs*1 (5'TCCTCCGCTAAATG ATATG) respectively. The ITS2 was chosen as it was expected to show interspecific variation, and as sequence data were available for several species in the literature and sequence databases. The expected amplicon sizes based on available sequence information were in the following ranges and varied according to genus: *Globodera* - 394bp, *Meloidogyne* - 292-298bp, *Heterodera* - 392-401bp. All amplifications were carried out in a volume of 20µl using 5µl DNA extraction, 20µM dNTP's, 0.4U DNA polymerase (DynaZyme™, Finnzymes) and 20pmol of

each primer. The amplifications were carried out in a PTC-200 thermocycler (MJ-Research) with heated lid and did not require an oil overlay. Product quality was checked by agarose gel electrophoresis in 2% gels, stained with ethidium bromide. The PCR parameters used were as follows: 96°C for 55 seconds, 53°C for 55 seconds, 72°C for 45 seconds-10 cycles; next 20 cycles - anneal temperature reduced to 51°C and extension time increased to 2 minutes; final 15 cycles - anneal temperature reduced to 50°C and extension time increased to 3 minutes.

SSCP

Optimisation (maximising band separation) of PCR-SSCP is empirical (Orita *et al.*, 1989) since the conformations adopted by PCR amplicons cannot be predicted in advance, even if sequence data are available. Therefore although different conditions were tried (such as altered gel concentration and running temperature), no significant benefit could be gained across all species tested. It should be stressed that in this application the technique is designed to profile unknown samples where the optimal conditions for a particular sequence are unknown and cannot be met in a single gel. Therefore, we advocate that a compromise set of conditions is used. This may not be optimal for an individual sequence but worked well generally. The conditions described for this study enabled the differentiation of all species investigated with different ITS2 sequence. MDE (0.5x, FMC BioProducts, Biozym) non-denaturing polyacrylamide gels were poured on a wide H03 system (Pharmacia) as recommended by the manufacturer. The TBE running buffer was cooled to 4°C before sample application. Two microlitres of PCR product was combined with 8µl of denaturing loading buffer (95% formamide, 10mM NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol) followed by a 3 minute denaturation at 94°C. The samples were snap cooled on ice before loading and running at 6W for 20,000 Volt.hours (Vhrs). The electrophoresis was carried out at 4°C. Bands were detected by silver staining or by incorporation of ^{32}P labelled dATP following established procedures. PCR-SSCP gels were highly reproducible under the condition described.

Sequencing

To address the possibility that additional bands of equal intensity seen in some species arose from the presence of multiple ITS2 sequences, silver stained bands were excised from a dried polyacrylamide gel, resuspended in water and reamplified by PCR. Four bands were excised from the profile of a *H. arenaria* cyst

and 3 from a cyst of *H. hordecalis* (Fig. 4). The double-stranded products were cloned into pGem-T and two recombinants sequenced in both directions using an ALF sequencer (Pharmacia). Sequences, excluding the primer sites, were aligned with published sequences using ClustalW (http://www2.ebi.ac.uk/clustalw/) (Thompson *et al.*, 1994, 1997) with some manual adjustment using JalView. A distance matrix with Kimura's two-parameter correction for multiple substitutions (Kimura, 1980) was used to construct a Neighbour-Joining tree (Saitou and Nei, 1987). The ClustalW.dnd file was displayed using TreeView (Page, 1996). The tree was rooted using *Meloidogyne* spp. sequences as an outgroup.

RESULTS AND DISCUSSION

Globodera

Figure 1A shows representative SSCP profiles obtained from individual cysts of the three *Globodera* species in this study. SSCP profiles were identical between different populations within each species. The lack of ITS2 variation (Thiéry and Mugniéry, 1996) did not allow pathotypes to be distinguished, presumably reflecting sequence identity in this region. Both *G. tabacum* (lanes 1-5) and *G. pallida* (lanes 11-15) profiles were composed of two bands, however the *G. rostochiensis* (lanes 6-10) profile was consistently comprised of multiple bands. These could be due to the formation of metastable (secondary) conformations by this strand (see below) but could also be due to the presence of multiple sequences in the ITS2 region. Identical profiles were obtained from single juveniles for all three species (results not shown). The profiles obtained from *Globodera* were distinct and easily distinguished from those of the *Heterodera* species (Fig. 1C). This allowed the relative mobilities of *Globodera* and *Heterodera* to be directly compared, and demonstrates pattern reproducibility between gels and samples.

Meloidogyne

PCR-SSCP profiles of the ITS2 allowed the three *Meloidogyne* spp. in this study to be distinguished (Fig. 1B). The sympatrically occurring dune species (*M. maritima* and *M. duytsi*) were easily separated (Fig.1B, lanes 1, 2 and 7; lanes 5 and 6). The PCR products from these two species stained to a similar intensity with ethidium bromide after agarose gel electrophoresis, but *M. ardenensis* showed a much stronger SSCP signal after radiolabelling (Fig. 1B, lanes 3 and 4). This may

be due to the higher AT-richness of the *M. ardenensis* ITS2 as compared to the ITS2 of the other species.

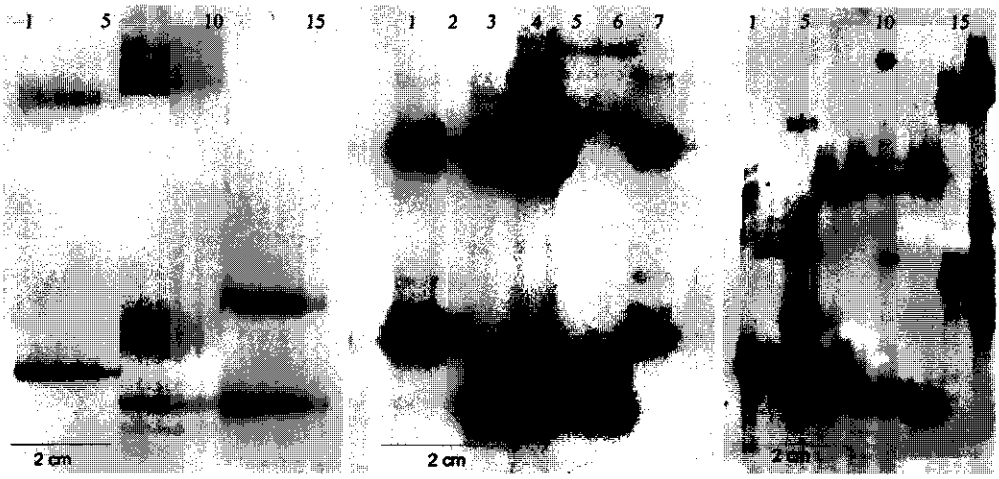

Figure 1. PCR-SSCP profiles of ITS2 regions from a selection of nematode species included in this study. A). *Globodera* spp.. Lanes 1-5: *G. tabacum* (C-6876); Lanes 6-10: *G. rostochiensis*, (A-50, A-56, B-140, F-539 and G-1526); Lanes 11-15: *G. pallida*, (D-381, D-475, E-412, J2 juveniles D-381, single J2 juvenile D-381). B) *Meloidogyne* spp.. Lanes 1, 2 and 7: *M. maritima*; Lanes 3 and 4: *M. ardenensis*; Lanes 5 and 6: *M. duytsi*. C) *Heterodera* spp.. Lanes 1 and 7: *H. ciceri* (Pot 30); Lanes 2 and 3: *H. trifolii* (A1-1); Lanes 4 and 5: *H. daverti* (LU68); Lanes 6, 8 and 9: *H. avenae* (D.Sturhan); Lanes 10 and 11: *H. arenaria* (J. Rowe); Lanes 12 and 13: *H. mani* (D. Sturhan); Lanes 14 and 15: *Globodera tabacum* (C-6876); Lane 16: *G. rostochiensis* (A-50).

Heterodera

The identity of PCR-SSCP profiles within species reflected sequence conservation of the ITS2 region which has been noted in sequencing studies (Ferris *et al.*, 1993, 1994, 1995; Thiéry and Mugniéry, 1996; Bekal *et al.*, 1997). The sequence of the ITS2 is more conserved than the ITS1 (Ferris *et al.*, 1993, 1994, 1995) but there is sufficient inter-specific variation to make it a prime target for species differentiation. All the *Heterodera* species investigated (Figures 1C, 2 and 4) could be differentiated on the basis of ITS2 PCR-SSCP with the exception of *H. avenae* (Fig. 1C, lanes 6, 8-9) and *H. arenaria* (Fig. 1C, lanes 10-11). The inability to separate these species reflects their close taxonomic relationship and almost certain sequence identity in the ITS2 spacer region. This supports restriction enzyme data showing that there are no enzymes capable of separating European

populations of *H. arenaria* and *H. avenae* in this region (Subbotin *et al.*, 1999). *H. arenaria* was originally described as *H. major* var. *arenaria* (Cooper, 1955), although '*avenae*' was later preferred to '*major*' (Cooper, 1968) before being raised to a full species by Kirjanova and Krall (1971). Due to its larger size, *H. arenaria* may be a polyploid of *H. avenae*, however no difference in ploidy level has yet been detected (Karssen and Van der Beek, pers. comm.). A single cyst of *H. arenaria* (Fig. 1C, lane 10) showed two strong additional bands in its SSCP profile. The origin of these additional bands is unknown but they possibly come from a parasitising nematode within the cyst. The SSCP profile of *H. avenae* is consistently different from that of *H. mani* (Fig. 1C, lanes 12 and 13) and does not support the synonymy of *H. mani* with *H. avenae* proposed by Ebsary (1991).

The SSCP profiles of *H. ciceri* (Fig. 1C, lanes 1 and 7) and *H. trifolii* (Fig. 1C, lanes 2 and 3, Fig. 2, lanes 6, 7 and 9) tended to be less intense than those of other species, although a diagnostic pattern could be identified for each. The single stranded bands from *H. ciceri* may be superimposed under the conditions used. This could be determined by end labelling of single primers and comparing the relative mobilities of the individual bands. The SSCP profiles of *H. trifolii* cysts were less intense particularly when visualised by radiolabelling as opposed to silver-staining (not shown). In the latter case two bands of equal intensity were obtained. It is probable that the method of labelling is responsible and the high signal generated by the lower band due to it being proportionally richer in labelled bases. *H. daverti* cysts (Fig. 2, lanes 1-5) consistently showed four bands, two being shared with *H. trifolii* (Fig. 2, lanes 6, 7 and 9). There were however exceptions where individual *H. daverti* cysts did not have the two bands diagnostic of *H. trifolii* (Fig. 2, lane 2). It would appear that many cysts of this *H. daverti* isolate contained eggs/juveniles with two distinct sequences or a mixture of juveniles. Hybridisation between nematode species is not an uncommon event and frequently results in the production of viable inter-specific hybrids (Mulvey, 1958; Mugniéry, 1979; Ferris and Ferris, 1992; Thiéry *et al.*, 1996). *H. daverti* and *H. trifolii* are closely related with most members of the *H. trifolii* complex being described as non-sexual species or members of a parthenogenic species complex (Mulvey, 1958; Triantaphyllou and Hirschmann, 1978; Sikora and Maas, 1986). *H. daverti* has also been described as a sexual form of *H. trifolii* (Wouts and Sturhan, 1978) and could be expected to produce viable offspring with *H. trifolii.* Cysts containing hybrid progeny containing sequences from both species may have been observed in this study.

SSCP, as well as allowing identification to species level, may also allow the parentage and frequency of inter-specific hybridisation to be studied in nematodes.

Cysts obtained from a culture of *H. schachtii* showed two distinct SSCP profiles, which represented both *H. schachtii* and *H. avenae* (result not shown). On further investigation it emerged that the culture had been isolated from an area where *H. schachtii* was abundant, rather than initiated with pure identified material.

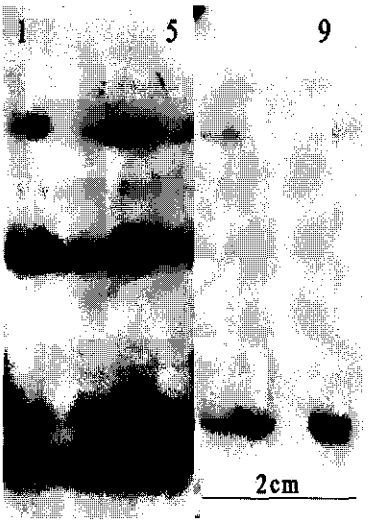

Figure 2. PCR-SSCP profiles of ITS2 regions from cysts of *H. daverti* and *H. trifolii*. Lanes 1-5: *H. daverti* (LU68); Lanes 6, 7 and 9: *H. trifolii* (A1-1). Lane 2 shows a *H. daverti* cyst without the "*trifolii*" bands.

Field samples

There was no overall effect of collection site on the distribution of cysts and juveniles. Variations in the host plant associations of *Heterodera* species were seen between the sites but no consistent pattern was observed. At all locations, *Heterodera* cysts and juveniles were found mainly associated with *E. farctus*, *A. arenaria* and *C. epigejos* (Fig. 3).

Although not statistically significant (P=0.173), there tended to be more cysts/gram root associated with *E. farctus* and *A. arenaria* than any other plant. Similarly, cysts/litre soil showed a tendency to be greatest in rhizosphere soil of *E. farctus*, *A. arenaria* and *C. epigejos* (P=0.113). The number of juveniles/litre of soil was however significantly (P=0.039) greater from the rhizosphere of *E. farctus* and *A. arenaria* than in that of other plants. Based on this survey, it was decided to

omit the second calcareous dune site (Walcheren) from the SSCP-analysis because it was similar to that of Haringvliet.

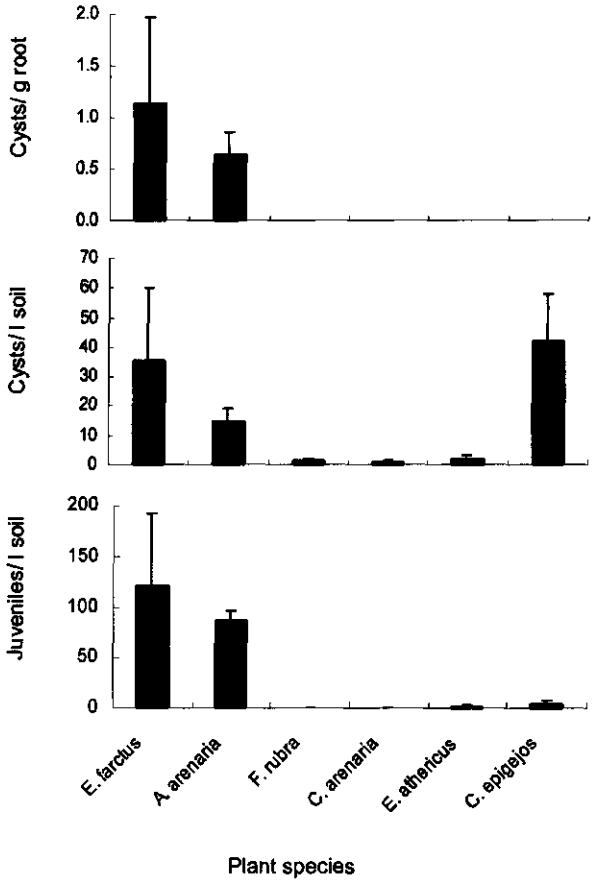

Figure 3. Numbers and distribution of cysts and juveniles of *Heterodera* spp. associated with coastal sand dune plants. Plant species occurring earliest in the dune succession, left to right. Bars represent the standard error of each sample mean. Data were obtained from Van der Stoel and Van der Putten (see Chapter 4).

H. arenaria/avenae and *H. hordecalis* cysts, identified by PCR-SSCP, occurred at both sites investigated in this study. The identification of field cysts by PCR-SSCP demonstrated differences in cyst nematode population structure between field sites and between host plant species (Fig. 4). At Haringvliet, distinct nematode populations were associated with vigorous and degenerating stands of *Ammophila arenaria.* Degenerating stands of *A. arenaria* were favoured by *H. hordecalis* whereas vigorous stands were populated by *H. arenaria/H. avenae,* the former considered the most likely (Cook, 1982; Robinson *et al.*, 1996). This

differentiation was not marked at Texel where *A. arenaria* was parasitised by *H. arenaria* alone, although *H. hordecalis* was still present associated with *Calamagrostis epigejos* further back in the dune succession. This is most likely to reflect the different abiotic environments at these sites. At Texel, the trajectory of degeneration of *A. arenaria* occurs over a much larger distance and the transition of vigour is therefore not as abrupt as at Haringvliet (Van der Putten *et al.*, 1989). The extended vegetation succession at Texel also allows the spatial separation of *C. epigejos* from different stages of the *A. arenaria* degeneration sequence. The distribution of *H. arenaria/avenae* and *H. hordecalis* seems therefore to be correlated with abiotic environmental dynamics. This initial study shows that *H. arenaria* may be better adapted to mobile dunes with regular influxes of wind-transported beach sand but is succeeded by *H. hordecalis* in more stable areas, where sand deposition is lower. A single cyst originating from degenerating *A. arenaria* at Haringvliet (Fig. 4, lane 17) had a PCR-SSCP profile identical to *H. mani*.

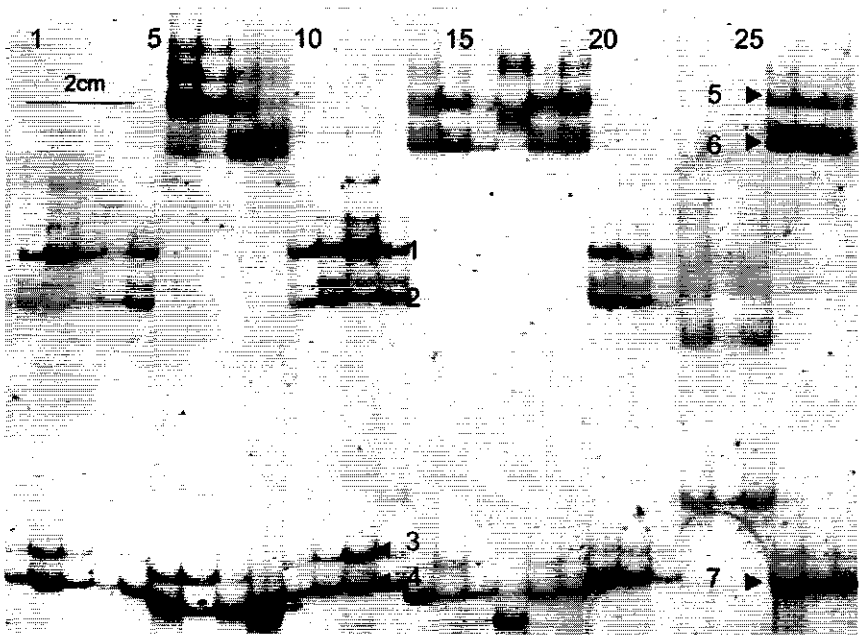

Figure 4. An example of the PCR-SSCP profiles obtained from the ITS2 of field cysts collected from Texel and Haringvliet. Lanes 1-9, Texel field sites. Lane 1: cyst from vigorous *A. arenaria*; Lanes 2-5 cysts from degenerating stands of *A. arenaria*; Lanes 6-9: cysts obtained from the root zone of *Calamagrostis epigejos*; Lanes 10-19, Haringvliet field sites. Lanes 10-13: cysts obtained from the root zone of vigorous *A. arenaria*; Lanes 14-19: cysts obtained from the root zone of degenerating *A. arenaria*. Lanes 20-28, Control profiles. Lanes 20-22: *H. avenae* (D. Sturhan); Lanes 23-25: *H. trifolii* (A1-1); Lanes 26-28: *H. hordecalis* (S. Andersson). Arrowheads indicate bands excised and sequenced.

Presence of extra bands

SSCP analysis of a PCR product, originating from a single cyst, was expected to give rise to two single bands, however this seldom occurred. The majority of SSCP bands showed the presence of additional less intense bands. There are a number of possible reasons for their presence: samples may have been incompletely denatured or partially renatured prior to loading, there may be multiple sequences present (the result of hybridisation, polymorphic PCR target sequences or heterozygous loci) or the single strands may have formed metastable conformers. Incomplete denaturation/partial renaturation were considered to be unlikely for several reasons. The foremost being that the presence of extra bands was reproducible between PCRs, different samples of the same species and between gels.

Since the denaturing conditions used in the PCR to obtain the samples for SSCP were 55 seconds at 96°C, the time used to denature the samples (in a high percentage of formamide) for SSCP was considered adequate for complete denaturation and undenatured samples were found to migrate much faster through the gels in control experiments and were usually electrophoresed off the bottom. In addition, the denaturing and loading conditions were rigorously reproduced from gel to gel, with samples being immediately cooled after heat denaturation in wet ice and loaded rapidly through cold (4°C) buffer. Faint additional bands were attributed to the presence of metastable conformers (Zehbe *et al.*, Pharmacia Application Note 384). These are identical in sequence to those of the primary bands but have an alternative conformation, which affects their mobility relative to the primary conformer. Metastable conformers were therefore considered to be the most likely explanation for fainter bands but stronger bands merited further investigation.

The presence of multiple sequences was a distinct possibility, particularly where bands of equal intensity were observed. An analysis of ITS2 regions of *H. avenae* (Subbotin *et al.*, 1999) revealed the presence of two ITS2 types (A and B). Type A being European and B from an Indian population. However, both types were detected in three French populations. To address the possibility of multiple ITS2 sequences indicated by additional PCR-SSCP bands, seven were excised from *H. arenaria* and *H. hordecalis* SSCP patterns (indicated by arrows, Fig. 4), re-amplified and sequenced. A phylogenetic tree showing the relationship of these sequences in relation to ITS2 sequences of related nematodes, are shown in Fig. 5. The sequencing data indicated that two ITS2 sequences were present in both *H. arenaria* and *H. hordecalis* cysts. The difference was minor, a single base but considering the overall conservation of ITS sequences in these nematodes reported previously, is

nevertheless significant. The presence of multiple sequences has been inferred from the RFLP patterns of other nematodes (Zijlstra *et al.*, 1995). The presence of additional bands did not, however, affect the ability of SSCP to effect an identification.

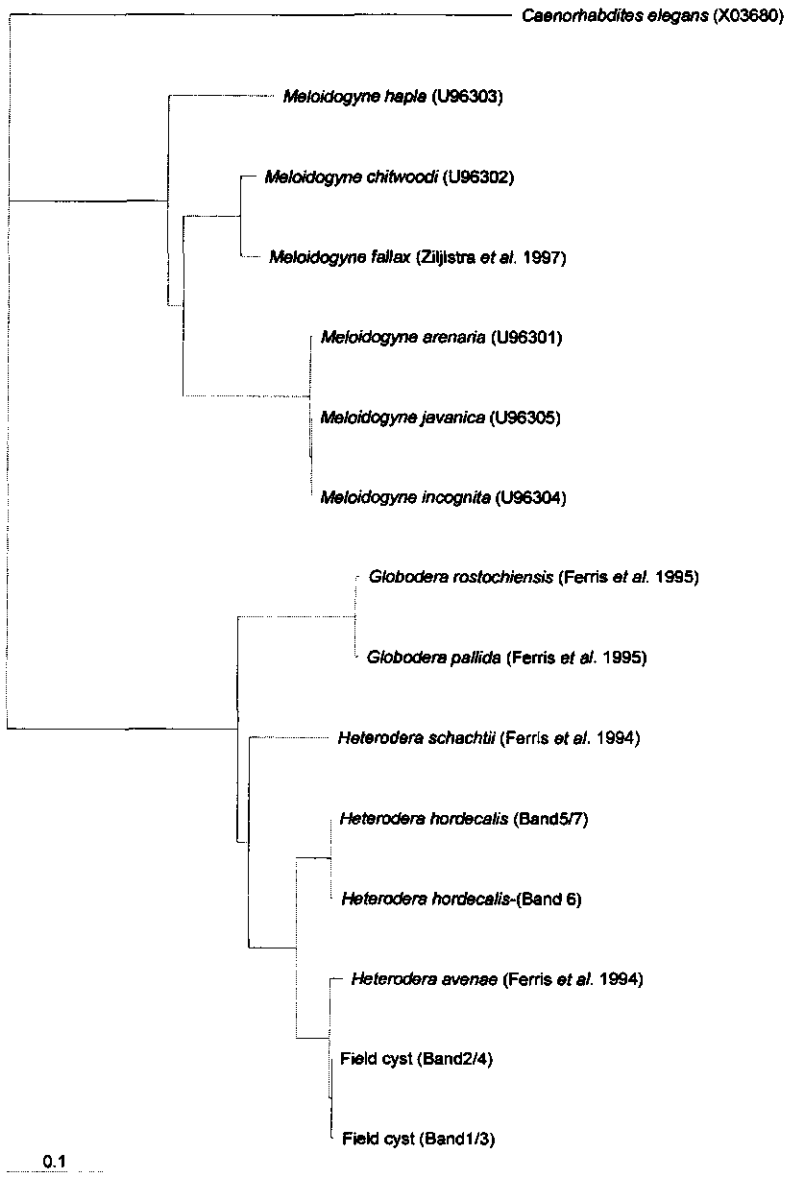

Figure 5. Phylogenetic tree showing the relationships of the excised bands with sequences of related species. Accession numbers for the bands are: field collected *H. arenaria*, bands 1/3 (AF239233), bands 2/4 (AF239234). *H. hordecalis*, bands (5/7 AF239235) and band 6 (AF239236).

CONCLUSIONS

This work was initiated to develop a method that would enable the identification of adult cysts and root-knot nematodes for ecological studies in natural ecosystems. It is clear from the success of the field investigation, that a single PCR primer pair used in conjunction with SSCP across a variable region has major diagnostic potential for nematodes. SSCP profiles were reproduced from different cysts and juveniles of the same species, different PCR amplifications and on different gels. The primers were found to amplify well from all species tested within the three genera in this investigation in addition to *Rotylenchus* and *Filenchus* spp. (data not shown). This suggests that the primers may be suitable for several genera allowing the application to be widened. Since the technique can utilise broad specificity primers it is likely that cryptic species could be detected if encountered. Band position alone allowed identification when nematodes of known identity were available for comparison. However, where profile could not be matched to controls, bands could be excised and sequenced. PCR-SSCP is simpler to perform, broader in application and more economic in terms of time and resources than many other techniques. From the viewpoint of ecological investigation and plant protection, the use of this sensitive and highly discriminatory PCR based technique allows rapid and routine identifications of a broad range of species with minimal resources and development time.

CHAPTER 4

HOST STATUS OF SUCCESSIONAL COASTAL FOREDUNE PLANT SPECIES AND PATHOGENICITY OF THE ENDOPARASITIC NEMATODE *HETERODERA ARENARIA*

with W. H. Van der Putten

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ABSTRACT

Several studies have demonstrated effects of soil-borne pathogens on the species composition of natural vegetation. Most of these studies have focused on pathogenic soil fungi and some on complexes of plant-parasitic nematodes and pathogenic soil fungi. In coastal foredunes, plant-parasitic nematodes are supposed to play a role in plant competition and plant succession. As specificity of soil pathogens is assumed to be a prerequisite in vegetation processes, endoparasitic nematodes may be the key species in soil pathogen complexes.

In previous studies on coastal foredunes, the endoparasitic nematode *Heterodera arenaria* was found to be ubiquitous on *Ammophila arenaria* (L.) Link, one of the pioneer grass species dominating coastal foredune vegetation. In the present study, *H. arenaria* was used as a model organism of the obligate plant-parasites, to test host specificity of endoparasitic nematodes on a natural plant species, and effects of the nematode on the growth of dominant plant species that occur in the various stages of succession in the coastal foredune vegetation.

In a field survey at three locations along a 150-km stretch of coastline in the Netherlands, the root zones of various plant species have been sampled to establish the occurrence of *Heterodera* spp. *H. arenaria* was most abundant on the early successional pioneers *Elymus farctus* and *A. arenaria*. In later stages of succession, dominated by *Festuca rubra* ssp. *arenaria*, *Carex arenaria*, *Elymus athericus* and *Calamagrostis epigejos*, another endoparasite, *H. hordecalis* was found. Both *Heterodera* species occurred on *A. arenaria*, with *H. arenaria* confined mainly to vigorous plants in mobile dunes, and *H. hordecalis* assuming dominant in degenerated *A. arenaria* in stable dunes. *H. hordecalis* however, was only found in the root zone soil and not on the roots themselves.

In pot trials, reproduction of *H. arenaria* was highest on *E. farctus* and *A. arenaria*, suggesting that the occurrence of *H. arenaria* in the field is due mostly to host specificity. The growth of the susceptible hosts was not affected negatively by nematode inoculation, but root biomass of the poor, non-susceptible, hosts *F. rubra* and *E. athericus* grown in sterilised soil was reduced when *H. arenaria* was added. Furthermore, the inoculation of *A. arenaria* with greater densities of *H. arenaria* did not result in more abundant cysts or further growth reduction of the host, in comparison to smaller densities. Thus, *H. arenaria* has low pathogenicity towards the host plants they occur on naturally. The tolerance of the natural hosts is suggested to be due to co-adaptation of host plant and parasite. The reduced growth of non-hosts is due possibly to a higher degree of pathogenicity or virulence of *H. arenaria* or to the fact that those plants in the field could not have developed tolerance in the absence of *H. arenaria*.

In the coastal foredunes, *H. arenaria* is part of a complex of parasites and pathogens. The results demonstrate that biotrophic parasites may be moderately pathogenic even in a community containing a complex of parasitic and pathogenic organisms. The moderate effects on its natural hosts suggest that *H. arenaria*, alone, is not the main component in the complex of soil pathogens influencing the foredune vegetation. The specificity of successive pathogen complexes, as observed in previous studies, may, therefore, not be due to single species, but rather be due to specific species interactions or to the specific composition of the complex of parasites and pathogens as a whole.

INTRODUCTION

The number of studies on the role of soil pathogens in processes of natural plant communities in which space and time are involved, the so-called spatio-temporal processes, is steadily increasing. Activity of soil pathogens has been related to spatio-temporal processes in old fields (Bever, 1994; Mills and Bever, 1998), prairies and natural pastures (Holah and Alexander, 1999; Blomqvist *et al.*, 2000; Olff *et al.*, 2000), tropical rain forest (Augspurger, 1990), temperate forest (Packer and Clay, 2000), annual plants (Carey *et al.*, 1992; Newsham *et al.*, 1995a; Mihail *et al.*, 1998), and coastal sand dunes (*e.g.* Oremus and Otten, 1981; Seliskar and Huettel, 1993; Van der Putten *et al.*, 1993; Zoon *et al.*, 1993). The role of soil pathogens in cyclic and directional succession has been visualised by conceptual models on plant-soil feedback (Bever *et al.*, 1997; Van der Putten and Van der

Stoel, 1998). If we want to further understand plant-soil feedback, however, we need to obtain information on how plants may selectively interact with the soil community, such as demonstrated for pathogenic soil fungi by Mills and Bever (1998), Holah and Alexander (1999), and Packer and Clay (2000), and for arbuscular mycorrhizal fungi by Bever *et al.* (1996).

In most of the known cases on soil pathogens and parasites in natural ecosystems, the prime attention has been on pathogenic soil fungi. Many soil fungi are facultative saprotrophs, which can be highly aggressive (Jarosz and Davelos, 1995), thereby causing strongly negative plant-soil feedback. Little is known on the aggressiveness or pathogenicity (in this study both used as the capacity to cause damage or disease leading to growth reduction) of natural plant-parasitic nematodes, although nematodes are notorious because of yield reduction in a wide variety of arable crops (*e.g.* Stone, 1977; Baldwin and Mundo-Ocampo, 1991). Biocide treatments of prairie soils resulted in considerable increase of net primary production of prairie grasses (Stanton, 1988) and in coastal foredunes, endoparasitic nematodes are supposed to play a key role in the specificity of successional soil pathogen complexes (Van der Putten and Van der Stoel, 1998). Specific endoparasitic nematodes may be regarded as biotrophic parasites for which theory predicts mild aggressiveness towards their natural hosts (Lenski and May, 1994). However, plant-parasitic nematodes are known to occur in multi-species complexes, which could lead to enhanced levels of aggressiveness because this requires competitive ability at the expense of the shared host (Lenski and May, 1994).

In the present study, we address the question if endoparasitic nematodes may be key species in the soil pathogen complex of *Ammophila arenaria* (Marram grass) because of its specificity and pathogenicity towards its natural host. This grass species is one of the early successional dominant pioneers of coastal foredunes that degenerates and becomes replaced when dunes get stabilised (Huiskes, 1979). In mobile foredunes, plants are vigorous when constantly colonising new layers of freshly deposited sand, which is supposed, among others, to enable escape from natural soil pathogens (Van der Putten *et al.*, 1988). Little and Maun (1996) have demonstrated for the North-American cogener *Ammophila breviligulata* that sand burial may enhance protection of plants by arbuscular mycorrhizal fungi against the endoparasitic nematodes *Heterodera* sp. (cyst nematode) and *Pratylenchus* sp. (root-lesion nematode). Reduced sand deposition would then make plants more susceptible to endoparasitic nematodes because plants are less protected by arbuscular mycorrhizal fungi (Little and Maun, 1996).

In the case of *A. arenaria*, the endoparasitic nematode *Heterodera arenaria* has been found abundantly in the root zone (Cook, 1982; De Rooij-Van der Goes *et al.*, 1995b) and appeared not to produce cysts on the later successional grass *Festuca rubra* ssp. *arenaria* (Sand Fescue) (Van der Putten and Peters, 1997). This makes *H. arenaria* an interesting candidate for further studying the host status of dominant successional foredune plants and the pathogenicity of the nematode to hosts and non-hosts.

We tested the hypothesis that the cyst nematode *H. arenaria* is host specific for the early pioneer *A. arenaria* and that it has the potential to reduce the growth of its natural host. We used a sequence of natural coastal foredune plant species that are locally dominant: *Elymus farctus*, the first coloniser of the beach, *A. arenaria* (vigorous in mobile foredunes and degenerating in stabilised foredunes), and their successors *Festuca rubra* ssp. *arenaria*, *Carex arenaria*, *Elymus athericus* and *Calamagrostis epigejos*. Each of these species reaches dominance locally. We started with a pilot field survey to determine the occurrence of *Heterodera* species on the above-mentioned six dominant plant species. Based on the results of this pilot survey, a second, more detailed survey was conducted at several locations along the Dutch coast, which included vigorous and degenerating stages of *A. arenaria* in the foredunes. Molecular identification of the cysts to species level was carried out by Clapp *et al.* (2000) and described in Chapter 3. An inoculation experiment was carried out to test the host status of each plant species for the cyst nematode *H. arenaria* collected at *A. arenaria*. The completion of a life cycle (*i.e.* the production of new cysts) was used to qualify the host status of the plants, and biomass production was measured to determine potential effects of the nematodes on plant growth. As *A. arenaria* allowed the cyst nematodes to complete their life cycle, but did not show any growth reduction, a dose-response experiment was carried out to determine if cyst nematodes may have any direct effects on their natural host plant. The results are used to discuss pathogenicity of biotrophic parasites, occurring in a multi-parasite and –pathogen environment, to their host, as well as the possible key role of *H. arenaria* in foredune soil pathogen complexes.

MATERIALS AND METHODS

Heterodera arenaria

Cooper (1955) included the species *Heterodera major* var. *'arenaria'* from Marram grass in his key to British *Heterodera* species. In 1996, Robinson *et al.*

redescribed the species as *H. arenaria*. In general, nematodes of the genus *Heterodera* are known to be specialised plant-parasites with sophisticated interactions with their hosts (Cook, 1991; Sijmons *et al.*, 1994; Trudgill, 1997). After penetration of the root as a juvenile, the nematode injects secretions in the root cells that induce the plant to form a syncytium. This is a multinucleate cell of high metabolic activity, which is formed after cell walls breakdown and the protoplasm of the cells fuse due to nematode stylet penetration (Endo, 1971; Wyss, 1987; Burrows, 1992). The cyst nematode depends upon this syncytium as its sole source of nutrients for further development and reproduction (Baldwin and Mundo-Ocampo, 1991; Sijmons *et al.*, 1994). Cyst nematodes include several major agricultural pests (Stone, 1977), such as the sugar beet cyst nematode *H. schachtii*, the soybean cyst nematode *H. glycines*, and the oat cyst nematode *H. avenae* (Baldwin and Mundo-Ocampo, 1991).

Field surveys

Pilot survey

To survey the occurrence of *Heterodera* spp. on the various foredune plant species, rhizosphere samples were collected in December at three locations along a stretch of about 150 km of Dutch coastal dunes: Haringvlietdam (51°52' N 4°04' E), Walcheren (51°35' N 3°32' E), and the island of Texel (53°07' N 4°45'E). Samples were collected from the rhizosphere of the successional series of dominant monocotyledonous plant species from the beach towards inner dunes. Sampled species were *Elymus farctus*, *Ammophila arenaria*, *Festuca rubra* ssp. *arenaria*, *Carex arenaria*, *Elymus athericus* and *Calamagrostis* epigejos. For each plant species eight random samples of about 1 kg of soil and roots were collected from an area parallel to the coastline of 50 m long and 10 m wide. These samples were pooled to form composite samples representative of the sampling area.

The samples were sieved (mesh size 1 cm) to remove coarse material and separate roots from soil, and the soil was homogenised. *Heterodera* spp. cysts were extracted from soil by adding about 4 l of water to a subsample of 1 l of soil. The soil-water mixture was stirred, and the water with the cysts was decanted onto a 180 µm-mesh sieve. This procedure was repeated four times. In addition, males and juveniles of *Heterodera* spp. were isolated from a subsample of 250 ml of soil by elutriation (Oostenbrink, 1960). *Heterodera* spp. cysts on roots were counted using a binocular microscope (10-15x magnification).

Detailed survey

Based on the results of the pilot survey, a limited number of plant species was chosen for a more detailed survey in December of a subsequent year at the same three locations. Samples were collected from the rhizosphere of *E. farctus*, vigorous *A. arenaria*, degenerated *A. arenaria* and *C. epigejos*. At each location, along a transect perpendicular to the beach, four sites of 50 m long and 10 m wide parallel to the coastline were chosen such that each site was dominated by one of the plant species. For each plant species, 6 random samples of 15 x 15 x 15 cm^3 were collected with a spade. Each sample represented an individual tussock and contained about 5 kg of soil and roots. In the cases of *E. farctus* and vigorous *A. arenaria*, the top 10 cm of soil was removed prior to sampling to reach the upper root zone underneath the wind-deposited sand layer.

Each sample was sieved with a mesh size of 1 cm to remove coarse material and separate the roots from the soil. Each soil sample was mixed gently. Cysts and free-living nematodes were extracted from the soil in the same way as described in the pilot survey. Part of the roots was used to extract nematodes by the funnel-spray method according to Oostenbrink (1960). The remaining fraction was used for the visual counting of *Heterodera* cysts on the roots using a binocular microscope (10-15x magnification).

Identification of cysts

After DNA extraction of the single adult cysts, the nematodes were identified to species level on the basis of ITS2 sequence variation detected by PCR-SSCP (PCR Single-Strand Conformational Polymorphism). The identification of the field cysts has been described by Clapp *et al.* (2000) (see Chapter 3).

Host specificity of H. arenaria

Soil was collected from the foredunes of Voorne (51°52' N 4°04' E) in the rhizosphere of six dominant successional plant species (*E. farctus*, vigorous *A. arenaria*, *F. rubra* ssp. *arenaria*, *C. arenaria*, *E. athericus*, and *C. epigejos*). After sieving (mesh size 1 cm), the soil was mixed gently and part of it was sterilised by means of gamma-irradiation (25 kgray). In earlier studies, soil that had received this dose appeared to be sterile (Oremus and Otten, 1981). The unsterilised soil of each origin was checked for the occurrence of *Heterodera* spp. juveniles. The seeds of all plant species were collected from the same sampling site at Voorne. The seeds were allowed to germinate for 10 days on glass beads with a 16/8 hour light/dark regime at 25/15°C. After germination, seedlings were pre-cultured for 7 days in

cones of 30 ml in a greenhouse with a 16/8 hour light/dark regime at 23/19 (±2)°C. After pre-culture, each plant species was planted in soil that was collected from the successional zone dominated by that particular plant species. Four seedlings of one plant species were planted per 1.5 l pot filled with 1500 g of soil, containing 10% soil moisture ($w \cdot w^{-1}$). The soil surface was protected against desiccation by an aluminium foil cover. Half of the pots were filled with sterilised soil whereas the other half was filled with a mixture of 60% sterilised soil and 40% of unsterilised soil. Sterilised soil was mixed through the unsterilised soil, to prevent excessive suffering of the seedlings from growth reduction by soil pathogens. This ratio of 60% of sterilised and 40% of unsterilised soil was based on earlier studies by D' Hertefeldt and Van der Putten (1998). The pots were placed in a greenhouse. Twice a week, the soil moisture content was adjusted to 10% ($w \cdot w^{-1}$) with demineralised water. Once every week, a full strength Hoagland nutrient solution was added to all pots in the following amounts: weeks 1 to 6, 12.5 ml; weeks 7 to 11, 25 ml; and weeks 12 and 13, 50 ml per pot.

The juveniles used in this experiment had hatched from cysts collected from vigorous *A. arenaria* at Voorne. Crushed roots (10 mg of fresh roots of *A. arenaria* seedlings were crushed in a mortar and suspended in 500 ml tap water) stimulated the hatching of *Heterodera* juveniles from these cysts. Cysts were placed in Petri dishes containing 5 ml of this suspension. One week after planting the seedlings, half the pots were inoculated with 1700 juveniles, whereas no juveniles were added to the other half. All treatments were carried out in six replicates.

Thirteen weeks after inoculation, all pots were harvested. Total shoot biomass was dried for 48 hours at 70°C. Half of the root biomass was dried immediately after harvesting at 70°C for 48 hours, whereas the other half was first examined for the occurrence of adult cysts of *Heterodera* before being dried. To extract *Heterodera* cysts from the soil, the soil was washed into a 10 l bucket, and the water was decanted onto a 180 µm-mesh sieve. All replicates of the non-sterilised treatments as well as the pots with the sterilised soil inoculated with *Heterodera* were decanted to collect cysts. Non-inoculated pots with *A. arenaria* in sterilised soil were decanted to look for cysts to test the completeness of sterilisation.

Response of A. arenaria to various inoculation densities of H. arenaria

Soil was collected from the foredunes of Voorne, the Netherlands, at a site north of Haringvlietdam (51°52' N 4°04' E) in the successional zone dominated by vigorous *Ammophila arenaria*. After sieving (mesh size 1 cm), the soil was mixed

thoroughly and sterilised by means of gamma-irradiation (25 kgray). *A. arenaria* seeds were collected from the same area and were treated as described for the previous experiment. After pre-culturing, four seedlings were planted per 1.5 l pot filled with 1500 g of soil containing 10% soil moisture ($w \cdot w^{-1}$). The soil surface was covered with aluminium foil to prevent desiccation of the soil, and the pots were placed in the greenhouse. Watering and nutrient addition were the same as in the previous experiment.

The juveniles used in this experiment had hatched from *Heterodera arenaria* cysts collected from vigorous *A. arenaria* and from *H. arenaria* cysts harvested from an earlier greenhouse-experiment. All inoculation material originated from the same area as the seeds and soil. The hatching of the *Heterodera arenaria* juveniles from these cysts was stimulated in the same way as in the experiment described earlier. One week after the seedlings had been planted, different concentrations of *Heterodera* juveniles were inoculated: 0, 1380, 2760 and 4140 individuals per pot.

After both 6 and 13 weeks, five pots of each inoculum concentration were harvested. Total shoot biomass was dried for 48 hours at 70°C. The roots were first examined for the occurrence of adult cysts of *Heterodera* before they were dried for 48 hours at 70°C. The soil was washed into a 10 l bucket, the water decanted onto a 180 μm-mesh sieve, and examined for cysts of *Heterodera* using a binocular microscope with a 10-15x magnification.

Data analysis

Nematode abundance in the field survey and the numbers of cysts in the experiment testing the response of *A. arenaria* to various inoculation densities were analysed by a Kruskal-Wallis non-parametric test. Cyst data of the inoculated pots in the host specificity experiment were analysed by a two-way analysis of variance with plant species and sterilisation as factors after natural-log-transformation in order to achieve homogeneity of variances.

The plant biomass data of each plant species in the host specificity experiment were analysed by a two-way analysis of variance using soil sterilisation and nematode inoculation as independent factors. Plant biomass data of *A. arenaria* in the dose-response experiment were analysed statistically by a one-way analysis of variance using inoculation density as the factor. Data were tested for normality of distribution by means of the Kolmogorov-Smirnov test and for homogeneity of variances by means of Bartlett's test. When necessary, data were natural-log-transformed to obtain homogeneity. Treatment means were compared using Tukey's HSD test ($P < 0.05$).

RESULTS

Field survey

Pilot survey

In the root zone soil, *Heterodera* cysts were confined mainly to sites dominated by *Elymus farctus*, *Ammophila arenaria* and *Calamagrostis epigejos* (Table 1). Of these plant species, cysts on roots were only found in stands dominated by *E. farctus* and *A. arenaria* and not in those with *C. epigejos*. Numbers of juveniles were greatest in the soil of the early successional plant species *E. farctus* and *A. arenaria*. The root zone soil of *Festuca rubra* ssp. *arenaria*, *Carex arenaria* and *Elymus athericus* rarely contained cysts and juveniles of *Heterodera*. The general pattern of occurrence of *Heterodera* was similar at the three locations.

Table 1. Numbers of *Heterodera* cysts and juveniles found in the pilot survey in the rhizosphere of six plant species at three locations along the Dutch coast. Numbers are expressed per gram root dry weight or per volume of soil.

		Locations along the coast		
Plant species		Haringvliet	Walcheren	Texel
Elymus farctus	Cysts/g root	0.2	0.4	2.8
	Cysts/ l soil	9	13	85
	Juveniles/ l soil	60	40	264
Ammophila arenaria	Cysts/g root	0.2	0.9	0.8
	Cysts/ l soil	24	10	10
	Juveniles/ l soil	84	72	104
Festuca rubra ssp. *arenaria*	Cysts/g root	0.0	0.0	0.0
	Cysts/ l soil	2	2	1
	Juveniles/ l soil	0	0	0
Carex arenaria	Cysts/g root	0.0	0.0	0.0
	Cysts/ l soil	2	1	0
	Juveniles/ l soil	0	0	0
Elymus athericus	Cysts/g root	0.0	0.0	0.0
	Cysts/ l soil	4	0	2
	Juveniles/ l soil	4	0	0
Calamagrostis epigejos	Cysts/g root	0.0	0.0	0.0
	Cysts/ l soil	60	56	10
	Juveniles/ l soil	0	12	0

Detailed survey

Heterodera cysts and Heteroderidae males (identified to family level) isolated from the roots occurred significantly more often on *E. farctus* and vigorous *A. arenaria* (mobile foredunes), than on degenerating *A. arenaria* and *C. epigejos* (stabilised foredunes) (Table 2). Numbers of cysts and males extracted from the soil did not differ among plant species. Few *Heterodera* juveniles were extracted from the roots, and the numbers extracted from the soil were not different among plant species. No significant differences in the numbers of the various *Heterodera* life-stages were found between the three locations.

At all locations, the cysts isolated from *E. farctus* and vigorous *A. arenaria* were, based on their PCR-SSCP profile identified as *H. arenaria*, and cysts collected from *C. epigejos* as *H. hordecalis* (see Chapter 3). Cysts from the root zone soil of the degenerating stage of *A. arenaria* at Haringvliet and Walcheren were identified as *H. hordecalis*, and at Texel both *Heterodera* species were isolated from the degenerating *A. arenaria* stand. However, from the roots, only *H. arenaria* cysts were isolated.

Table 2. Mean numbers of *Heterodera* cysts, *Heterodera* juveniles and Heteroderidae males extracted from roots (expressed as the mean number per gram root dry weight) and soil (expressed as the mean number per liter soil) of the root zone of four plant species (vig. = vigorous; deg.= degenerated) at the locations Haringvliet (Hv), Walcheren (Wch) and Texel (Tx). The results of the Kruskal-Wallis analyses on the effects of location and plant species are presented directly underneath the data. ** $P < 0.01$.

		Cysts			Juveniles			Males		
	Location:	Hv	Wch	Tx	Hv	Wch	Tx	Hv	Wch	Tx
	Plant species									
Root	*E. farctus*	0.6	2.0	0.0	0.0	0.0	0.0	0.0	9.7	130.7
	vig. *A. arenaria*	8.5	2.0	1.1	0.0	7.9	0.0	176.7	27.9	16.3
	deg. *A. arenaria*	0.0	0.0	0.3	0.0	0.0	6.3	0.0	0.0	0.0
	C. epigejos	0.0	0.0	0.0	0.0	0.0	0.0	0.0	9.2	0.0
		df	F	P	df	F	P	df	F	P
	Location	2	1.4	0.508	2	2.0	0.372	2	1.0	0.606
	Plant species	3	11.7	0.008**	3	3.6	0.308	3	11.7	0.009**
Soil	*E. farctus*	4.5	2.5	1.0	73.3	40.0	0.0	6.7	20.0	0.0
	vig. *A. arenaria*	4.7	4.7	0.3	0.0	20.0	0.0	0.0	20.0	0.0
	deg. *A. arenaria*	8.3	0.8	2.3	0.0	13.3	33.3	6.7	0.0	0.0
	C. epigejos	0.8	11.2	18.2	0.0	20.0	16.0	6.7	0.0	0.0
		df	F	P	df	F	P	df	F	P
	Location	2	0.1	0.953	2	4.0	0.138	2	3.9	0.140
	Plant species	3	4.9	0.180	3	2.4	0.499	3	1.6	0.657

Host specificity of H. arenaria

Aboveground biomass of *A. arenaria* and *F. rubra* ssp. *arenaria* was affected significantly by soil sterilisation (Table 3). Biomass in non-sterile soil was smaller than in sterilised soil (Fig. 1), although the comparison of the treatment means by Tukey's HSD test did not show significant differences in the case of *A. arenaria.* Root biomass of all plant species was enhanced significantly by soil sterilisation (Table 3). For *A. arenaria* and *C. epigejos*, the differences were apparently too small to result in significant differences between treatment means according to Tukey's test (Fig. 1). In sterilised soil, root production of *F. rubra* and *E. athericus* was reduced significantly by inoculation with *H. arenaria* (Fig. 1), although their shoot biomass was not affected. Root production of the other three plant species was not affected by inoculation with *H. arenaria.* In unsterilised soil, none of the plant species showed any significant response to inoculation with *H. arenaria.* Growth of *Carex arenaria* was very poor in all treatments, and pots were therefore harvested 8 weeks after inoculation. No differences were found between the treatments (data not shown).

Table 3. The effects of soil sterilisation and addition of *H. arenaria* on the growth of various plant species. The results of the two-way ANOVA's with soil sterilisation (S) and addition of 1700 *H. arenaria* juveniles (N) as factors are presented. Degrees of freedom, F- and P-values are presented for the shoot and root biomass per plant species. Root biomasses of *E. farctus* were log-transformed to achieve homogeneity of variances. Root biomasses of *F. rubra* were analysed by Kruskal-Wallis non-parametrical test. * P<0.05; ** P<0.01; *** P<0.001.

		Shoot			Root		
Plant species	Treatment	df	F	P	df	F	P
E. farctus	S	1	1.019	0.325	1	25.676	<0.001***
	N	1	0.403	0.533	1	1.648	0.214
	SxN	1	0.160	0.693	1	5.817	0.026*
A. arenaria	S	1	9.341	0.0062**	1	4.956	0.038*
	N	1	0.00003	0.996	1	0.160	0.693
	SxN	1	0.232	0.635	1	0.903	0.353
F. rubra	S	1	13.714	0.0014**	1	8.333	0.0039**
	N	1	0.272	0.608	1	2.613	0.106
	SxN	1	1.071	0.313			
E. athericus	S	1	0.028	0.869	1	17.667	<0.001***
	N	1	1.832	0.191	1	1.497	0.235
	SxN	1	2.077	0.165	1	13.872	0.0013**
C. epigejos	S	1	0.545	0.470	1	9.820	0.0057**
	N	1	0.022	0.883	1	0.037	0.849
	SxN	1	1.134	0.301	1	0.623	0.440

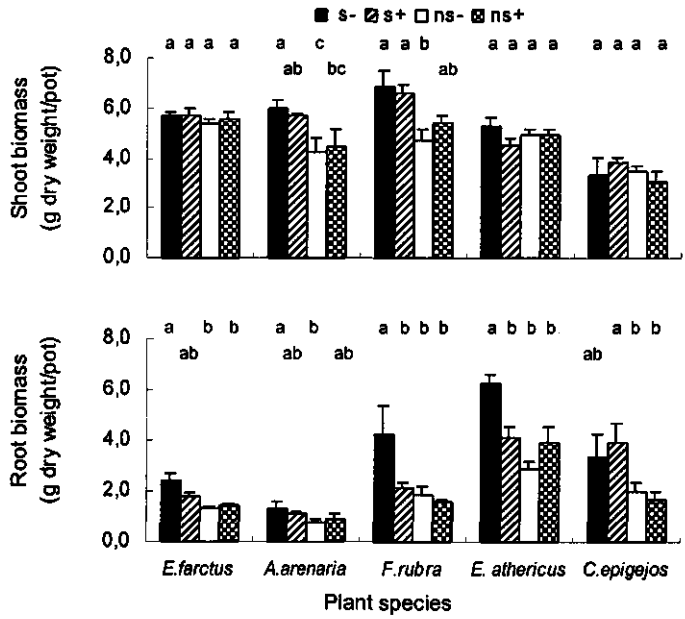

Figure 1. Shoot and root biomass of five successional foredune plant species grown for 13 weeks in pots containing soil of their specific root zone. The plants were grown on either sterilised soil without *H. arenaria* juveniles (s-), sterilised soil with *H. arenaria* juveniles (s+), non-sterile soil without added *H. arenaria* juveniles (ns-), or non-sterile soil with added *H. arenaria* juveniles (ns+). The error bars represent the standard error of the mean. Two-way ANOVA's with the factors 'soil sterilisation' and 'nematode addition' and subsequent Tukey's HSD tests have been carried out for each plant species. Bars with different letters indicate significantly differences within a plant species at $P < 0.05$.

Numbers of cysts per gram root dry weight were affected greatly by the plant species (Table 4). In sterilised soil, the numbers of cysts on the early successional plant species *E. farctus* and *A. arenaria* were significantly greater than on *F. rubra*, *E. athericus* and *C. epigejos*. This indicates a high degree of specificity of *H. arenaria* towards *E. farctus* and *A. arenaria*. Also in unsterilised soil, significantly greater numbers of cysts occurred on *E. farctus* and *A. arenaria* than on the other plant species, but their numbers were not significantly greater than those found on *F. rubra* ssp. *arenaria*.

The significant two-way interaction effect in the ANOVA between plant species and soil sterilisation was apparent mainly for *F. rubra*. On the roots of all plant species, numbers of cysts tended to be smallest in the non-sterile treatment. However, *F. rubra* showed significantly greater numbers on roots of the plants grown in inoculated non-sterile soil than in inoculated sterilised soil. No *Heterodera* spp. juveniles were found in the soils of the various origins at the start of the

experiment. The soil was not checked for the occurrence of cysts at the start of the experiment, but cysts were collected at the end of the experiment from non-inoculated pots with *F. rubra* on unsterilised soil (5.7 ± 2.0 (SE) per gram root dry weight; data not listed in Table 4). On all the other plant species a maximum of 0.5 cysts per gram root dry weight was found in the unsterilised soil not inoculated with *H. arenaria* (data not shown).

Table 4. The effect of addition of *H. arenaria* to sterile and non-sterile soil on the cyst production on the roots of various plant species. The mean number of cysts per gram root dry weight ± standard error on five plant species grown in pots with either sterilised or non-sterile soil. *H. arenaria* juveniles were added at the start of the experiment at a density of 1700/pot. ANOVA results (degrees of freedom, F- and P-values) of the log-transformed cyst numbers are presented in the lower part of the table. The results of the subsequent Tukey's HSD test are indicated by letters. Different letters indicate significant differences between the treatments at $P < 0.05$.

Soil treatment	*E. farctus*	*A. arenaria*	*F. rubra*	*E. athericus*	*C. epigejos*
Sterilised	19.8 (±5.3)^{ab}	37.3 (±10.3)^{a}	0.8 (±0.3)^{ef}	0.7 (±0.3)^{ef}	4.5 (±1.3)^{cde}
Non-sterile	15.2 (±3.6)^{abc}	30.1 (±7.1)^{ab}	7.2 (±1.4)^{bcd}	0.3 (±0.1)^{f}	2.6 (±1.0)^{de}

	df	F-value	P-value
Plant species	4	43.590	<0.001
Sterilisation	1	0.278	0.6006
Plant species x Sterilisation	4	6.218	0.0005

Response of A. arenaria to various inoculation densities of H. arenaria

Six weeks after inoculation, *H. arenaria* had influenced root biomass of *A. arenaria* negatively (Table 5). Root biomass was greatest when no nematode inoculum was added. At the lowest level of inoculation (1380 juveniles) root biomass was reduced significantly, but inoculation with more nematodes did not result in a further reduction of the root production. Later, 13 weeks after inoculation, root biomass was no longer affected significantly by the inoculation treatments. Shoot biomass of *A. arenaria*, at both harvests, was not affected by inoculation with *H. arenaria.*

In all three treatments where *Heterodera* juveniles had been inoculated cysts were produced. However, within the range applied, there was no effect of increasing inoculation densities on the number of cysts produced after 13 weeks.

Table 5. The effects of various densities of *H. arenaria* on the growth of *A. arenaria* and the production of cysts. Mean dry biomass (g.pot^{-1}) ± standard error of the harvested shoot and root biomass, at two harvest dates for four inoculation densities (0, 1380, 2760 and 4140 juveniles per pot). The number of cysts produced per gram root dry weight was determined 13 weeks after inoculation. Shoot and root biomass were statistically analysed by a one-way ANOVA (degrees of freedom, F- and P-values and Mean Square Error); cyst numbers were analysed by the Kruskal-Wallis non-parametric test (K-W). Treatment means were compared by Tukey's HSD. Different letters indicate significant differences at $P<0.05$.

Inoculation density (N.pot^{-1})	Harvest 6 weeks after inoculation		Harvest 13 weeks after inoculation		
	Shoot	Root	Shoot	Root	Cysts/g root dw
0	0.59 (±0.06)^a	0.76 (±0.13)^a	4.01 (±0.12)^a	1.64 (±0.12)^a	0.0 (±0.0)^b
1380	0.61 (±0.07)^a	0.32 (±0.07)^b	3.97 (±0.12)^a	1.19 (±0.17)^a	22.1 (±4.3)^a
2760	0.55 (±0.05)^a	0.23 (±0.04)^b	3.65 (±0.13)^a	1.32 (±0.11)^a	13.1 (±1.2)^a
4140	0.50 (±0.06)^a	0.37 (±0.11)^b	3.81 (±0.19)^a	1.33 (±0.05)^a	22.3 (±5.3)^a
df	3	3	3	3	3
F-value	0.648	6.010	1.320	2.548	13.117 (K-W)
P-value	0.596	0.006	0.303	0.092	0.004
MSE	0.0178	0.0458	0.1028	0.0711	

DISCUSSION

The present study addressed the hypothesis that the cyst nematode *Heterodera arenaria* is host specific for the early pioneer *Ammophila arenaria* and that it has the potential to reduce the growth of its natural host. The occurrence of *H. arenaria* on successional dominant monocotyledonous plant species in coastal foredunes corresponded well with the ability of the nematodes to complete their life cycle on those plant species. The nematode occurred on roots of two early successional plant species, *E. farctus* and *A. arenaria*. In the greenhouse, both *E. farctus* and *A. arenaria* allowed *H. arenaria* to complete its life cycle, so that these plant species were obviously susceptible hosts for *H. arenaria*. Only one later successional plant species, *C. epigejos*, allowed moderate reproduction in the experiment on host specificity, however, in the field *H. arenaria* did not occur on *C. epigejos*.

Cysts collected in the field from the root zone of *C. epigejos* were of *Heterodera hordecalis* (Clapp *et al.*, 2000). *H. hordecalis* has been found previously to parasitise various cereals and grasses (Andersson, 1975), such as *A. arenaria* in sand

dunes (Sturhan, 1996), *Festuca* spp. (Cook, 1982), and plants in salt marshes (Sturhan, 1982). It has therefore been suggested that *H. hordecalis* does not show profound host specificity to a particular plant species. In the present study, except for their occurrence on *C. epigejos*, *H. hordecalis* cysts were found also in the root zone of degenerating *A. arenaria*, but not on the roots themselves or in the vicinity of vigorous *A. arenaria*. It is, therefore, not clear from this study whether *A. arenaria* is a natural host to *H. hordecalis* as found by Sturhan (1996). Presuming that *A. arenaria* is, in fact, a host to both *Heterodera* spp., *H. hordecalis* and *H. arenaria* might not be expected to co-occur. Our results suggest that the *Heterodera* spp. are separated spatially by components of the abiotic environment (*e.g.* sand deposition). Further study is required to determine why *H. hordecalis* is absent in mobile dunes and why *H. arenaria* disappears when *A. arenaria* containing dunes become stabilised. One possibility may be that *H. hordecalis* is not able to survive the environmental conditions in the mobile dune area, whereas *H. arenaria* may be outcompeted by *H. hordecalis* when dunes become stabilised.

The cysts that were found on *F. rubra* in the non-sterile soil to which *H. arenaria* was added, most likely were due to the initial presence of *H. hordecalis* cysts in the soil. Two arguments support this suggestion. First, also the non-inoculated non-sterile soil collected from around *F. rubra* yielded cysts at the end of the experiment. Even though the unsterilised soil did not contain any juveniles of *Heterodera* spp. initially, the initial presence of cysts in the soil obviously cannot be excluded. Second, the identification of the cysts harvested at the end of the experiment from the inoculated pots with unsterilised *F. rubra* soil showed the presence of both *H. arenaria* and *H. hordecalis*, clearly indicating at the initial presence of *H. hordecalis*. Only few of the inoculated juveniles had actually been developed into new *H. arenaria* cysts, so that the reproduction of *H. arenaria* on *F. rubra* was not different from that on *E. athericus* and *C. epigejos*.

In the greenhouse trials, inoculation of the host species, *E. farctus* and *A. arenaria*, with *H. arenaria* did not cause growth reduction. Only at six weeks after inoculation in the dose-response experiment root biomass of *A. arenaria* showed a negative response to inoculation of *H. arenaria* juveniles. According to the dose-response experiment, the lower end of the range of initially inoculated *H. arenaria* densities was already above the maximum carrying capacity for the seedlings that were used as test plants. Within the range of nematode densities applied, there was no relationship of initial density of *H. arenaria* and final root biomass of *A. arenaria*. The highest two densities inoculated, exceeded the density in the field based on a rough estimation of 1 cyst per 100 gram of soil, which translates to 15 cysts per

pot, each estimated to contain approximately 150 eggs. Thus, when inoculated in isolation, field densities of *H. arenaria* appear to have little impact on its natural hosts. Thirteen weeks after inoculation, there was also no correlation between the initial inoculum density and the number of *H. arenaria* cysts produced.

In other studies on dose-response curves with similar endoparasitic nematodes on cereals, *e.g.* Seinhorst (1995) found a strong growth reduction with increasing inoculation density. De Rooij-Van der Goes (1995) found reduced growth of *A. arenaria* with increasing numbers of *T. ventralis*, an ectoparasitic nematode. However, significant growth reduction as caused by the ectoparasite occurred only at unnaturally high doses.

As *H. arenaria* does not negatively influence its natural host plant and reproduction may be regulated by the plant, it might be that the population development of *H. arenaria* is strongly regulated by the food supply, which keeps them in equilibrium with host growth (Cook and York, 1980). Furthermore, nematode species such as *H. arenaria*, which require living plant cells for their development and reproduction, may be regarded as biotrophic parasites (Jarosz and Davelos, 1995). Biotrophs usually show moderate pathogenic effects on hosts due to selection towards lower capacity to suppress resistance genes (Lenski and May, 1994; Jarosz and Davelos, 1995). Pathogens that are highly pathogenic may kill their host, thereby reducing their own food source (*e.g.* Burdon, 1993). In multi-parasite systems however, it has been hypothesised that biotrophic species may possess higher levels of pathogenicity then expected, because of the need to compete with other parasitic species (Lenski and May, 1994). Such levels of pathogenicity however, were not recorded for *H. arenaria* on its natural hosts. Apart from the selection for moderately pathogenic nematodes, the host plants may have developed tolerance to these nematodes.

In most of the agricultural systems, the interaction between plants and parasites might be different in two ways. Either the crop plant might not have had the time to develop tolerance to the parasite, because most agricultural crops are in the field only for a short period of time or the parasites might be more pathogenic because their food source is growing on the field only for a limited time during the year. In the system described by De Rooij-Van der Goes (1995) an ectoparasitic nematode had been used, which most probably did not have a highly specific relationship with the plant. In contrast to endoparasites, ectoparasites may be less influenced by selection and therefore not develop reduced levels of pathogenicity to the plant. So, in natural ecosystems there could be an analogy between

ectoparasitic nematodes and the aggressiveness of facultative pathogens as described by Jarosz and Davelos (1995).

In contrast to susceptible tolerant hosts that were not harmed by *H. arenaria* (*i.e. E. farctus* and *A. arenaria*), both *F. rubra* and *E. athericus* were negatively affected by the addition of *H. arenaria* in sterile soil. *F. rubra* and *E. athericus* were poor hosts for *H. arenaria*, and only very few cysts developed on their roots. As *F. rubra* and *E. athericus* seem non-susceptible to *H. arenaria* in the field, there may have been no opportunity for selection towards lower levels of pathogenicity to the plants (Jarosz and Davelos, 1995). In non-sterile soil, no effect could be measured from the addition of *H. arenaria* to *F. rubra* and *E. athericus*. The soil organisms of the rhizosphere of *F. rubra* and *E. athericus* may outcompete *H. arenaria*, or *H. arenaria* may contribute only slightly to the negative effect caused chiefly by other soil organisms in the rhizosphere of these plant species.

In the present study, *H. arenaria* showed a high degree of specificity to the early successional plant species in the coastal foredunes. Although it was expected that a biotrophic parasite with a highly specific relation with its host plants would contribute to vegetation succession, the present results show that *H. arenaria*, even in a multi-parasite system, hardly affected host plant biomass. Therefore, this single species alone, through the moderate pathogenicity toward the host plant, is not able to affect the succession of foredune vegetation.

The effect of *H. arenaria* on non-host plants suggests that this species may negatively affect growth of non-host plant species. However, as reproduction of *H. arenaria* will be very low on non-host plants, and as in the field *H. arenaria* has hardly been found, this negative effect will not be very persistent. Considering the effects of *H. arenaria* on both host plants and non-hosts, it may not be the specificity of single species, but its interactions with other pathogens or the complex of parasites and pathogens as a whole, that determines the specificity of successional pathogen-complexes in coastal foredunes.

CHAPTER 5

WHAT CONTROLS THE POPULATION DYNAMICS OF PLANT-PARASITIC NEMATODES IN NATURAL ECOSYSTEMS? THE CYST NEMATODE *HETERODERA ARENARIA* AND THE CLONAL GRASS *AMMOPHILA ARENARIA* IN OUTER COASTAL DUNES AS A MODEL

with W. H. van der Putten and H. Duyts
submitted to *Phytopathology*

ABSTRACT

The population dynamics of plant-parasitic nematodes in natural ecosystems has hardly been given any attention, so that there is little, if any, knowledge about the factors that control plant-parasitic nematode densities in natural ecosystems. Here, the population dynamics of the cyst nematode *Heterodera arenaria* have been investigated in relation to the growth strategy of its natural host *Ammophila arenaria*. *Heterodera arenaria* is an endoparasitic nematode that occurs specifically in the mobile stage of outer coastal dunes. *Ammophila* is a clonal grass that thrives well in mobile dunes, and is an effective sand stabilising plant species.

We examined how important migration is for the nematode in order to persist in mobile dunes and analysed the possible roles of bottom-up and top-down processes for the control of nematode densities.

During two growing seasons, at monthly intervals, we collected root and soil samples at various depths in the soil. The depths represented the years of first formation of the roots. In the newly deposited and colonised sand layer the first *H. arenaria* cysts were found one month after formation of the first new roots. By then, the cysts produced in the previous year had already lost about 90 per cent of their eggs. At the sampling data between late autumn and spring of the following year, hardly any second stage juveniles were observed. The eggs or juveniles within or outside the cysts may have been parasitised, although there is no direct evidence to prove such top-down regulation. Only about 0.4 per cent of the hatched juveniles finally succeeded to develop into a new cyst. The relatively constant number of cysts per gram root throughout the year, suggests that, in spite of considerable mortality before parasitisation, bottom-up processes control this

specialist plant-parasitic nematode. This result is in contrast with many host-nematode studies known from agro-ecosystems.

We found no obvious direct positive effect of migration on the performance of individual nematodes. However, the development time in the newly colonised root layer and the contents of the newly formed cysts, showed that on the longer term migration provides a fitness advantage to *H. arenaria*.

INTRODUCTION

Few studies have concentrated on the ecology of plant-parasitic nematodes in natural ecosystems. This is in contrast to the numerous studies on plant-parasitic nematodes in agro-ecosystems. In non-agricultural systems most studies focus on the relative abundance of nematodes in different trophic groups (Magnusson, 1983; Hodda and Wanless, 1994), on the spatial distribution of various feeding types (Wasilewska, 1970, 1971; De Goede *et al.*, 1993; Popovici and Ciobanu, 2000), or on their distribution at a certain moment in time (Yeates, 1996). Other studies on nematodes in natural ecosystems have examined their taxonomy and concentrate on the morphological description of the nematodes (Sturhan, 1996; Karssen *et al.*, 1998a,b; Karssen *et al.*, 2000).

The population dynamics of plant-parasitic nematodes in natural ecosystems have hardly received any attention. In agricultural land, the population dynamics of endoparasitic nematodes have been studied extensively (*e.g.* Seinhorst, 1967, 1970, 1986a; Schomaker and Been, 1999; Been and Schomaker, 2000), because species that belong to this group of plant-parasites are involved in yield depressions of major crops (Stone, 1977; Baldwin and Mundo-Ocampo, 1991). In a natural sand dune, however, endoparasitic nematodes do not seem to be responsible for direct growth reduction (Van der Stoel and Van der Putten, see Chapter 4; Brinkman, in prep.). Therefore, the endoparasites behave like true obligate parasites (Lenski and May, 1994). This phenomenon makes it very interesting to examine the population dynamics of endoparasitic nematodes in a natural ecosystem, in order to determine what controls their population density and how individuals may maximise their fitness. As a model for our study, we used *Heterodera arenaria*, a cyst-forming endoparasitic nematode occurring in outer coastal sand dunes of north-western Europe.

Heterodera arenaria is specific to two pioneer foredune grasses that dominate the mobile area of the coastal foredunes: *Elymus farctus* (Sand twitch) and vigorous

Ammophila arenaria (Marram grass) (Van der Stoel and Van der Putten, Chapter 4). Both plant species occur on the seaward slope of the coastal foredunes, where the nematodes have to deal with yearly sand deposition in autumn and winter. Up to 80 cm may be deposited on top of *Ammophila* and plants still emerge (Van der Putten *et al.*, 1989). Later in the successional sere, in the more stabilised dunes, where *Ammophila* is heavily degenerated and later successional plant species, such as *Festuca rubra* ssp. *arenaria* have become established, *H. arenaria* is no longer observed (Van der Stoel and Van der Putten, Chapter 4).

In greenhouse inoculation trials, *H. arenaria* reproduced best on plants with which it was associated in the dunes. However, on its natural host *Ammophila* only a limited number of juveniles of *H. arenaria* was found to reproduce, and no growth reduction of the plants was observed (Van der Stoel and Van der Putten, Chapter 4). Bottom-up effects, *i.e.* that the survival and reproduction of an organism is controlled by the amount or availability of resources, seem to play a major role in the greenhouse. However, the bottom-up effects may not be the critical regulatory factor in the field, where migration, dispersal and predation may also affect the population densities. Therefore, it is of interest to quantify the population dynamics including the dispersal of this nematode species in its natural environment.

The question arises which properties allow *H. arenaria* to persist in mobile dunes and how it manages to deal with the yearly sand deposition. *Ammophila* requires burial with sand to keep its vigour (Huiskes, 1979). For *H. arenaria* the sand deposition implies that the juveniles, in order to reach the new root layer, need to migrate over large distances. In agricultural fields, plant-parasitic nematodes generally disperse only a few centimetres a year (Norton, 1978; Seinhorst, 1965), but this often has been measured in the horizontal direction. Prot (1980) argued that active movement of nematodes might also occur over larger distances. Vertical migration is known from *Anguina tritici*, a species that is able to disperse up to 30 cm to reach the roots of host plants (Leukel, 1962) and *Meloidogyne* spp. have been shown to bridge a vertical distance of 25 cm in 10 days (Prot, 1978).

In order to study the population dynamics of *H. arenaria*, we collected samples at monthly intervals during two growing seasons from root layers of *A. arenaria* at various depth layers. The depth layers each represent one sand burial event. After sand accretion, during autumn and winter storms, internodes elongate and new nodes are formed just underneath the sand surface. As the node-staples mark distinct root layers and the roots are formed horizontally, the root layers are

comparable to year-rings in trees, each layer produced at a different depth. After isolation from the roots and the soil, the numbers of individuals were quantified representing various stages of the life cycle (second stage juveniles, males, cysts and eggs).

In order to keep up with their host plant, nematodes continuously have to shift upwards. We tested the hypothesis that migration to a new root layer enhances the performance of individual nematodes in comparison to individuals that do not migrate. In order to test that hypothesis, we examined components of fitness of *H. arenaria* in both newly colonised and existing root layers of *A. arenaria*. This is the first detailed study on population dynamics and dispersal of a sedentary endoparasitic nematode in a natural and highly dynamic environment in the root zone of a clonal host plant. We discuss whether the upward migration of the nematodes closely following the root expansion of their host constitutes any fitness-related benefits to the nematode.

MATERIALS AND METHODS

Collecting soil samples from the field

Soil samples were collected from the coastal foredunes of Voorne, the Netherlands, at a site north of Haringvlietdam (51°52' N 4°04' E). This site has been used in preceding studies and has been described by Van der Putten *et al.* (1989) and De Rooij-Van der Goes *et al.* (1995a). At monthly intervals from April 1997 to December 1997 and from April 1998 to September 1998, samples were collected from the root zone of vigorous *Ammophila arenaria* growing on the seaward slope of the first dune ridge.

Roots and root zone sand were collected from root layers at different depths. In 1997, samples were collected from a layer that had been colonised in the summer of 1996 (indicated as L-96), and from a layer with sand deposited in the autumn/winter of 1996/1997 and colonised in the summer of 1997 (indicated as L-97). In 1998 also roots and root zone sand were collected from a layer with sand deposited during the autumn/winter of 1997/1998 and colonised in the summer of 1998 (indicated as L-98).

The sampling site consisted of an area of 150 m long, parallel to the coastline, and 10 m wide, covering the seaward slope of the outer foredune ridge. In 1997 and in April 1998, five random samples each of about 20x20x20 cm^3 were collected from each of the layers. For each replicate and each layer, a tussock of *A.*

arenaria was randomly chosen. Consequently, different year-layers were sampled independently. From May until September 1998, based on the power of the 1997 sampling data, we increased the number of replicates to 15. Per replicate a hole was dug in front of a randomly chosen tussock of vigorous *A. arenaria* exposing the three different root layers and vertical underground stems. From all three root layers (L-96, L-97 and L-98) a soil/root sample of about 15x15x15 cm^3 was collected.

Sample processing

Roots

Each sample was sieved (mesh size 0.5 cm) to remove coarse material, and the roots were separated from the soil. The roots were weighed fresh and divided into subsamples. One subsample was used for the extraction of free-living nematodes from the roots by the funnel-spray method (Oostenbrink, 1960). The extracted nematodes were identified using a reversed light-microscope (50-200x magnification) and counted. Another subsample was used to collect and count the number of *Heterodera arenaria* cysts by means of a binocular microscope (10-15x magnification). After the nematodes had been extracted from the roots, the subsamples used for nematode extraction and visual counting were dried at 70°C for 48 hours, and weighed.

Soil

After sieving, the soil was homogenised gently, and subsequently used for various purposes. For each purpose different fractions of the total sample were used. First, free-living nematodes were extracted from a subsample of 250 ml using the Oostenbrink elutriator (Oostenbrink, 1960). Nematodes were identified and counted as described before. Second, *H. arenaria* cysts were extracted from a subsample of 1 l of soil. After weighing the soil, 4 l of water was added, and stirred, whereafter the water with the floating cysts was decanted on a 180 µm mesh sieve. This procedure was repeated five times. Finally, about 50 g of soil was weighed, dried at 70°C for 48 hours, and weighed again to determine the soil moisture content of each sample.

Assessment of the number of eggs and juveniles in Heterodera cysts

To determine the contents of *H. arenaria* cysts, an image analysis system was used consisting of a reversed light microscope (50x magnification) that was connected via a camera to a computer. For each month and layer, approximately

20 cysts originating from the roots and approximately 20 from the sand were used to determine the number of eggs.

In Qwin, a special program was developed based on various parameters of the eggs and juveniles of *H. arenaria* (*e.g.* length, width, surface area) to analyse the pictures of the cyst contents sent to the computer. In each picture the real eggs and juveniles were translated into areas with a certain grey-intensity. These areas were measured and counted when they fitted within the given size. Each run of the program consisted of screening 25 wells of 1.4 cm^3 each, in a 5x5-grid pattern. In each well an individual cyst was placed into a droplet of tap water. The cyst was crushed, the cyst wall was removed and subsequently the well was filled up with water. Within each well, the total circular pattern of 8x10 pictures was screened on the presence and the number of juveniles and eggs. The results were automatically added in a Windows-Excel worksheet.

Data analyses

Root biomass

For each layer (two layers in 1997 and three in 1998), we carried out a one-way ANOVA to determine differences between months. The root biomass per kilogram of soil was considered as the dependent variable and month as the independent variable. Then, in order to determine the differences between layers, one-way ANOVA's were carried out for the different months with layer as the independent variable. Treatment means were compared using the Least Significant Difference (LSD) test ($P<0.05$).

Nematodes

The abundance of nematodes in samples was calculated by adding up the total numbers in the roots and in the soil. The number of nematodes per gram root dry weight was calculated by dividing the total number of nematodes by the total dry root biomass. This parameter was used for the statistical analyses. In order to achieve homogeneity, the data were log (x+1)-transformed, with x being the number of nematodes per gram root dry weight.

Three stages of the life cycle of *H. arenaria*, *i.e.* females/cysts, second-stage juveniles and males, were analysed separately. To determine whether there were any differences between months, a one-way ANOVA was carried out for each layer with month as independent variable. Treatment means were compared using the LSD-test ($P<0.05$). To determine whether nematode numbers differed

between layers, pair-wise comparisons between layers were carried out by comparing the means of corresponding months in a Wilcoxon matched pairs test.

Contents of the cysts

Differences between months in the numbers of eggs and juveniles within cysts in a particular layer were analysed using a one-way ANOVA for each selected layer with month as the independent variable. Differences between layers were compared exclusively based on the contents of cysts collected in corresponding months. Both one-way ANOVA's with layer as independent variable, and two-way analyses with layer and origin of the cysts (collected from the sand or from the roots) as independent variables were carried out. Treatment means were compared using Tukey's HSD test ($P<0.05$).

RESULTS

Root biomass

In 1997, in the newly deposited and colonised sand layer, the first new roots were observed in June, whereas in 1998 new roots were already found in April (Table 1). The later development in 1997 corresponds well with the low temperatures during the winter of 1996/1997 (Fig. 1) (KNMI-jaaroverzicht, 1996, 1997, 1998). In August of both years, the amount of root biomass per kg dry soil in the new layer was at the same level. In 1997, the peak root biomass was recorded in November. The peak root biomass of 1998 may have been missed, since sampling was stopped after September.

Early in the season root biomass per kg dry soil was significantly higher in the one-year-old root layer than in the newly formed root layer, whereas later in the season significantly more root biomass was present in the new root layer (Table 1). Following the root layers during two subsequent years, the root biomass tended to decrease with increasing age of the root layer (Table 1). In 1997, in the newly formed layer, root biomass dropped strongly between November and December. Since the biomass in December is in line with the biomass in the same root layer in April 1998, this sharp decline of root biomass does not seem to be due to an outlier. In already existing root layers the root biomass was more constant throughout the year than in newly formed root layers.

Table 1. Root biomass (average root dry weight per kg dry soil) in various root layers (L-96, L-97, and L-98) during two subsequent years, in the months April – December in 1997 and April – September in 1998. In the upper part of the table different letters indicate significant differences between months within a root layer. In the lower part of the table, for each of the two years individually, the letters indicate significant differences between root layers within a particular month. Treatment means were tested at $P<0.05$. (Data from 1997 were obtained from Van der Stoel *et al.*, see Chapter 2)

Month Root layer	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Within root layer; between months									
1997 L-97	0.000^{e}	0.000^{e}	0.036^{e}	0.150^{de}	0.395^{bcd}	0.701^{b}	0.483^{bc}	1.081^{a}	0.272^{cde}
1997 L-96	0.179^{cde}	0.405^{a}	0.173^{cde}	0.125^{de}	0.298^{b}	0.215^{bcd}	0.105^{e}	0.234^{bc}	0.108^{e}
1998 L-98	0.005^{c}	0.071^{c}	0.203^{b}	0.256^{b}	0.423^{a}	0.213^{b}			
1998 L-97	0.223^{ab}	0.263^{ab}	0.259^{ab}	0.310^{a}	0.206^{b}	0.200^{b}			
1998 L-96	0.128^{abc}	0.105^{bc}	0.150^{ab}	0.175^{a}	0.088^{c}	0.109^{bc}			
Between layers; within month									
1997 L-97	B	B	B	A	A	A	A	A	A
1997 L-96	A	A	A	A	A	B	B	B	B
1998 L-98	C	B	AB	AB	A	A			
1998 L-97	A	A	A	A	B	A			
1998 L-96	B	B	B	B	C	B			

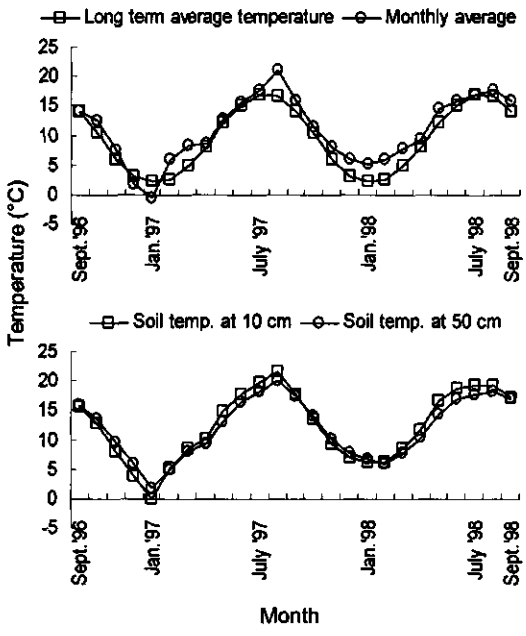

Figure 1. Average temperatures over the period September 1996 up to September 1998. (KNMI-jaaroverzicht, 1996, 1997, 1998)

Nematodes

Colonisation of the deposited sand layer

In both years, one month after the first roots were found, the first *H. arenaria* cysts appeared in the samples (Fig. 2A,C). In 1997, in L-97, the only significant difference between the numbers of cysts occurred between November and December, but not in other months (the months in which no cysts were found were excluded from the analysis). In 1998, in L-98, the only significant difference was found between July and May. In conclusion, in the new root layer the proportional occupation of roots with the cysts was fairly constant throughout the growing season, whereas the cyst density per 100 g dry soil (not shown) was steadily increasing. This implies that cyst formation is a continuous process once it has started.

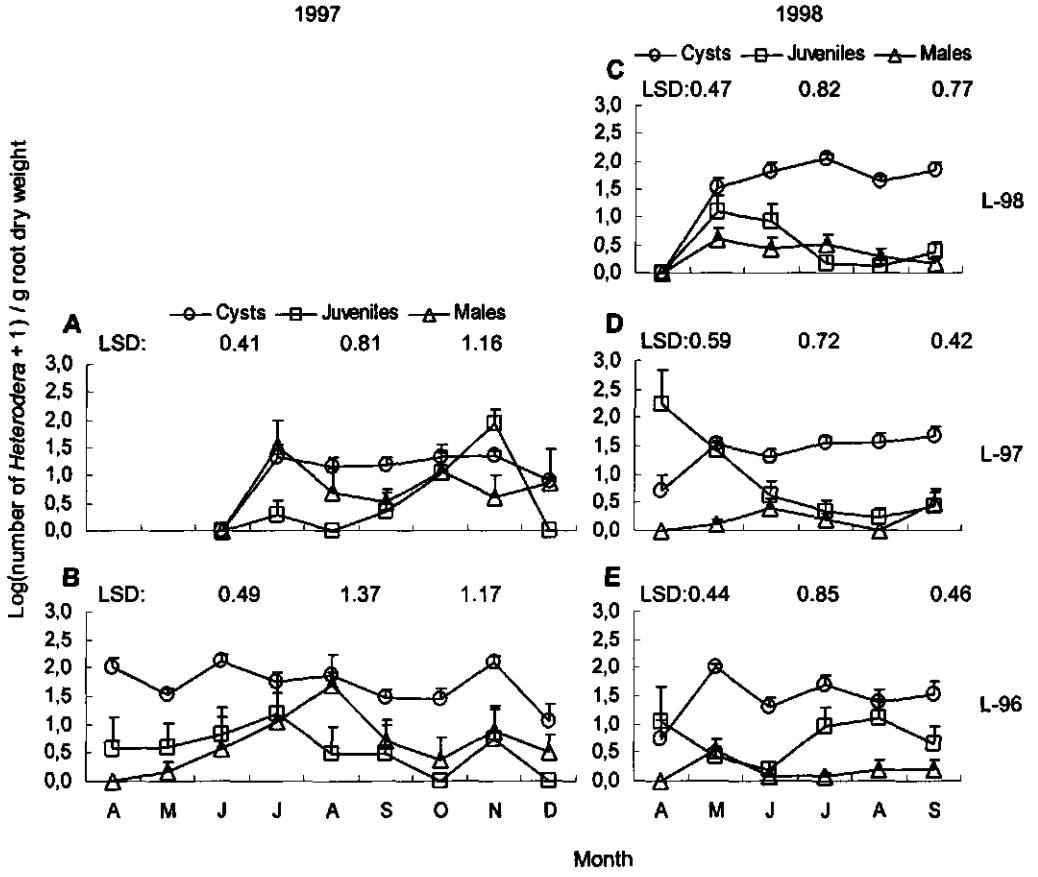

Figure 2. The development of cysts, juveniles, and males of *Heterodera arenaria* in various root layers (L-98, L-97, and L-96) of *Ammophila arenaria* during two subsequent years. Data are presented on a log(x+1)-transformed scale (+1 SE) per gram root dry weight. Above each graph the LSD-values for each developmental stage are presented at $P<0.05$.

In 1997 in October, the cysts in the newly formed layer contained significantly more eggs and juveniles than in the other months (Fig. 3A). In November, however, the contents of the cysts had decreased significantly. In 1997, there was no difference in contents between the cysts collected from the roots and those collected from the sand. In 1998, in the newly formed layer, the increase of the contents of the cysts up to September followed a pattern similar to that in the new layer in 1997 (Fig. 3C). However, in 1998, in the newly formed layer, the cysts collected from the sand contained significantly more eggs and juveniles than the cysts from the roots. This is most likely due to an incomplete development of the cysts still present on the roots, as in October 1998 when the contents of cysts were determined for other purposes a cyst contained on average 379 eggs and juveniles (data not shown).

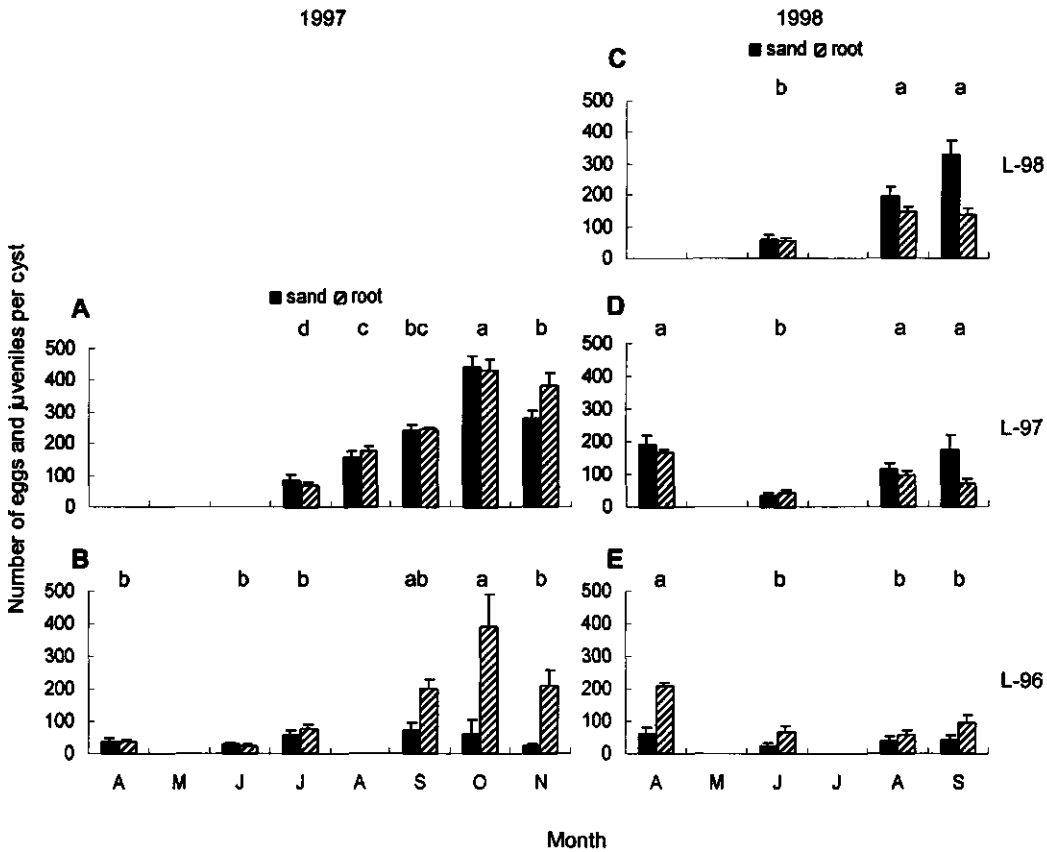

Figure 3. Average content (+1 SE) of *Heterodera arenaria* cysts collected from various root layers of *Ammophila* from the sand and the roots in two subsequent years. Different letters above the bars mean significant differences between months (for the cysts in the sand and in the roots together) within a root layer at $P<0.05$.

Based on the number of cysts and the content of these cysts, the total number of eggs was calculated per dry soil weight (Table 2). In both years, in the newly formed root layer, the total number of eggs per 100 gram dry soil increased during the growing season. In November 1997 and in September 1998, similar numbers of eggs were present. However, as in September 1998 fewer roots were present, the number of eggs expressed per gram roots was higher than in November 1997 (36086 in 1998 vs. 7707 in 1997 (data not shown)).

Heterodera males and second-stage juveniles also occurred one month after the first roots had been found in the newly colonised sand layer (Fig. 2A,C). The numbers of *Heterodera* juveniles per gram dry root varied between months. In 1997, significantly highest numbers of juveniles were observed in November, which may be due to hatching from cysts produced early in the growing season. In 1998, most juveniles were found in May, although it cannot be excluded that a possible peak later in autumn may have been missed (Fig. 2A,C).

Comparison between and within years

In the first sampling months of 1997, before *Heterodera* was present in the newly deposited sand layer, there were cysts, and, in lower numbers, juveniles and males present in the one-year-old root layer formed in 1996 (Fig. 2B). The cysts were brown and fully developed and appeared to have been formed in the previous growing season. The first new cysts in this root layer were observed in August, about one month later than in the newly formed root layer. In 1998, however, there was no time lag in the cyst formation between the newly formed and the one-year-old root layer. In both layers new cysts were found in May, but in the one-year-old root layer the number of new cysts was significantly lower (0.75/g root) than in the newly formed root layer (94.5/g root) (data not shown).

In 1997, it took at least two months (from April to later than June) for the juveniles to migrate to the newly formed root layer and to develop into young white females. The developmental time may be shorter, however, in the case that the juveniles observed in April did not succeed to develop into new females, and the later ones did succeed to develop into the females observed in July 1997. As the number of eggs in April 1997 in L-96 was already very low, juveniles most likely had already hatched from the cysts earlier than April. In the samples, the observed juveniles were only a small fraction of all eggs present in the cysts, suggesting that a large mortality occurred during the months when no samples had been collected. The sampling data do not show whether mortality is due to

parasitism of the eggs within the cysts or to predation or otherwise mortality of the juveniles.

As the number of cysts per soil dry weight in the newly formed root layer tended to increase during the growing season, and new white females were observed up to October, cyst formation is a continuous process once it has started. As also in the one-year-old root layer new cysts were produced from August onwards, obviously not all juveniles had migrated towards the newly formed root layer.

In 1997, in the one-year-old root layer, significantly more cysts per gram roots were present throughout the year (from July to December) than in the newly formed root layer (1.61 vs. 1.20 (Fig. 2A,B)). However, the total cyst density per kg soil in the newly formed root layer increased during the growing season, and from September onwards, cyst densities per unit of soil tended not to be different between the newly formed and the one-year-old root layer. In the one-year-old layer, most cysts were found in the sand and they contained significantly fewer eggs and juveniles than the cysts from the new root layer (Table 3A). Therefore, the total number of eggs in the cysts tended to be lower in the one-year-old root layer than in the new root layer (Table 2).

In 1998, the numbers of *Heterodera* cysts, juveniles and males per gram root were on average not different between the three root layers (Fig. 2C, D and E). However, as the biomass of roots on average tended to be lower in the oldest root layers (Table 1), the amount of cysts per unit of dry soil decreased with increasing age of the root layer. Furthermore, in the newly formed root layer, both the cysts collected from the sand and the roots contained more eggs and juveniles than the cysts from the older root layers (Table 3B). The larger contents of the cysts resulted in a higher total number of eggs that can form a new generation in the newly formed root layer than in the older root layers.

Similar to the comparison within a year, also between years particular root layers were compared to follow the development of the root layer in time. The cysts in the one-year-old root layer in 1998 contained fewer eggs than the cysts in the same layer in 1997 when it was newly formed (Table 3C). For the layer that has been formed in 1996, the cysts collected from the sand showed the same content during the years, but the cysts collected from the roots contained more eggs and juveniles when they were collected in 1997 than in 1998 (Table 3D). So, obviously the potential offspring in the cysts in a root layer decreased both with depth in the soil and in time.

Table 2. The absolute numbers of *Heterodera arenaria* per 100 g dry soil. For the months during which the contents of the *H. arenaria* cysts was determined, the total number of eggs was calculated. For each month, it was determined how many roots were present per 100 g dry soil (1.), and how many cysts were present on those roots (4.). Furthermore, the numbers of juveniles, males and cysts collected from the sand were expressed per 100 g dry soil. By multiplying the number of cysts on the roots by the average contents of those cysts, the number of eggs in the cysts on the roots was obtained. A similar calculation was carried out for the eggs in the cysts collected from the sand.

Layer	Month:	April '97	July	Aug.	Sept.	Oct.	Nov. '97	April '98	June	Aug.	Sept. '98
L-98	1. Roots/100 g dry soil								0.020	0.042	0.021
	2. Juveniles/100 g dry soil								1.43	0.15	0.20
	3. Males/100 g dry soil								0.19	0.50	0.09
	4. Cysts on roots/100 g dry soil								1.14	1.20	0.71
	5. Cysts in soil/100 g dry soil								1.17	1.35	2.39
	6. Average contents of cysts from root								52.7	149	137
	7. Average contents of cysts from soil								58.8	195	327
	8. Eggs (cysts on roots)/100 g dry soil								60.4	178	97.7
	9. Eggs (cysts in soil)/100 g dry soil								69.0	263	780
	10.Total eggs (=8+9)								129	440	878
L-97	1. Roots/100 g dry soil		0.015	0.040	0.070	0.048	0.108	0.022	0.026	0.021	0.020
	2. Juveniles/100 g dry soil		0.04	0.00	1.14	3.14	10.40	12.27	1.32	0.24	1.54
	3. Males/100 g dry soil		1.70	1.75	0.35	1.60	1.39	0.00	0.37	0.00	0.68
	4. Cysts on roots/100 g dry soil		0.43	0.67	1.14	0.76	1.19	0.06	0.20	0.21	0.33
	5. Cysts in soil/100 g dry soil		0.20	0.43	0.29	0.29	1.32	0.16	0.69	1.27	2.36
	6. Average contents of cysts from root		68.8	176	246	434	383	166	42.3	94.8	69.1
	7. Average contents of cysts from soil		82.2	159	240	443	278	189	34.7	112	178
	8. Eggs (cysts on roots)/100 g dry soil		29.6	118	281	329	457	9.6	8.5	19.6	22.8
	9. Eggs (cysts in soil)/100 g dry soil		16.6	68.4	68.6	129	366	29.4	23.8	142	420
	10.Total eggs (=8+9)		46	186	350	458	823	39	32	162	443
L-96	1. Roots/100 g dry soil	0.018	0.013		0.022	0.011	0.023	0.013	0.015	0.009	0.011
	2. Juveniles/100 g dry soil	2.05	4.34		2.34	0.00	2.27	2.55	0.06	1.23	1.37
	3. Males/100 g dry soil	0.00	1.15		0.25	0.28	1.18	0.00	0.02	0.21	0.51
	4. Cysts on roots/100 g dry soil	0.05	0.08		0.61	0.09	0.22	0.04	0.13	0.03	0.05
	5. Cysts in soil/100 g dry soil	2.10	0.92		0.13	0.33	2.73	0.15	0.39	0.52	1.14
	6. Average contents of cysts from root	37.0	77.2		202	392	210	208	66.3	55.1	94.9
	7. Average contents of cysts from soil	38.7	55.0		72.8	61.5	24.4	61.7	24.9	39.9	42.6
	8. Eggs (cysts on roots)/100 g dry soil	1.9	6.2		124	34.0	46.9	9.1	8.8	1.9	5.1
	9. Eggs (cysts in soil)/100 g dry soil	81.3	50.7		9.7	20.5	66.6	9.1	9.6	20.9	48.7
	10.Total eggs (=8+9)	83	57		133	55	114	18	18	23	54

Table 3. The average content of the *Heterodera* cysts. The main comparisons were: A. and B.) between different root layers within the same year; C. and D.) the same root layer sampled in two subsequent years. In all comparisons a two-way ANOVA was carried out with the layer and the cyst origin as independent factors. As often no homogeneity in the data was achieved, the non-parametric Kruskal-Wallis test was carried out. Different letters mean significant differences at $P<0.05$ in a Tukey's HSD test.

A. **1997L-97 vs. 1997L-96** (for the months July, September, October, and November)

	Cyst origin	
Layer	sand	root
1997L-97	291.3[a]	291.2[a]
1997L-96	49.0[b]	228.4[a]

	Independent variable	df	F	P
Kruskal-Wallis	Layer	1	107.037	<0.001***
	Cyst origin	1	20.586	<0.001***

B. **1998L-98 vs. 1998L-97 vs. 1998L-96** (for the months June, August and September)

	Cyst origin	
Layer	sand	root
1998L-98	203.7[a]	113.0[a]
1998L-97	113.7[b]	68.8[b]
1998L-96	37.1[c]	73.6[b]

Kruskal-Wallis with independent variable 'Layer'

	cysts from sand	cysts from roots
df	2	2
F	42.772	17.057
P	<0.001***	<0.001***

C. **1997L-97 vs. 1998L-97** (for the months July up to September)

	Cyst origin	
Layer	sand	root
1997L-97	159.9[a]	129.5[a]
1998L-97	113.7[ab]	68.8[b]

	Independent variable	df	F	P
Kruskal-Wallis	Layer	1	90.228	<0.001***
	Cyst origin	1	0.0019	0.9649 NS

D. **1997L-96 vs. 1998L-96**

	Cyst origin	
Layer	sand	root
1997L-96	43.5[c]	163.3[a]
1998L-96	39.6[c]	95.4[b]

	Independent variable	df	F	P
Kruskal-Wallis	Layer	1	1.001	0.317 NS
	Cyst origin	1	43.944	<0.001***

In April 1998, the cysts in the one-year-old root layer have already released the majority of the eggs that occurred in the cysts at the end of the growing season. In October 1997, in the newly formed root layer, there were on average about 430 eggs per cyst, whereas in June 1998, by then the one-year-old root layer, their content was reduced to about 40 eggs per cyst (Fig. 3A,D). This implies that already within a year after formation, over 90 per cent of the offspring had been released from the cysts. There are no data of 1996, but the low number of eggs and juveniles in the cysts in April 1997 suggests that this pattern is consistent. The juveniles may have hatched during winter or early spring before the new roots have been formed in the newly deposited sand layer. Eventually, in spite of the large numbers that have been released, only a small percentage of the juveniles survive and develop into a new cyst. Only 3.1 cysts per 100 gram of soil were observed in the new root layer in September 1998 out of the 823 eggs present in November 1997 (Table 2).

DISCUSSION

The potential offspring produced by a population of *Heterodera arenaria* cyst nematodes is highest in the newly formed root layer of their host plant *A. arenaria*. The population density may be affected both by bottom-up effects, given the relatively constant number of cysts per gram root biomass, and by top-down effects or natural mortality in the winter. Ultimately, however, the amount of new cysts formed seems to be controlled at the root surface or inside the roots, which is a bottom-up process. However, the differences between numbers of cysts and eggs per gram root dry weight varied considerably between years. In order to explain this variation, the mechanism of bottom-up control needs further study.

The question arises whether there is an immediate benefit at the individual level to migrate, and what the relative contribution is of top-down and bottom-up effects in regulating the cyst density. Variation in the developmental time in different layers, the emergence of the second-stage juvenile, the contents of the produced cysts, and the chance of survival will be discussed to elucidate the possible advantages of migration.

Development time: Rapid development in the new root layer offers the possibility of having more generations during the same period of time, and could thus provide a selective advantage (Kozlowski and Wiegert, 1986). However, the development time from a second-stage juvenile to the production of a young

female was found to differ only slightly between root layers at different depths in the soil. In 1997, in July the first young females were found in the new root layer, whereas in the one-year old root layer the first were found in August. This suggests that the development time in the new root layer is shorter. However, in 1998 in both root layers new young cysts were recovered in the same month. Therefore, evidence on developmental advantages of vertical migration was not consistent between years, although shorter development time only once every several years could already be a selective advantage of migration.

Juvenile emergence: In November 1997, large numbers of juveniles emerged, which suggests that a second generation may start to be formed within one year. The number of generations per year varies strongly between *Heterodera* species (Mulvey, 1959; Von Mende and McNamara, 1995). For example, juveniles of *Heterodera avenae*, a cyst nematode that is closely related to *H. arenaria* (Clapp *et al.*, 2000), also were present in highest numbers during late autumn and winter (Meagher, 1970), although *H. avenae* is known to produce only one generation per year (Cook, 1982; Mor *et al.*, 1992). The juveniles of *H. mani*, on the other hand, immediately hatch and reinvade the roots after the cysts have been formed (Cook, 1982). The strategy observed for *H. arenaria* could lead to three generations in two years. On the other hand, cysts that had been produced in late summer may not be able to release juveniles already in November, and may produce only one generation in one year.

Apart from a possible advantage of emergence before the winter starts, there is also the potential risk of emergence before the winter, as juveniles are less well protected to survive harsh conditions than cysts. As large numbers of eggs disappear during the winter period, the survival during winter indeed seems to be low. However, during summer, desiccation in the upper layer of sandy soil may cause a high juvenile mortality as well. The release of juveniles from cysts in autumn was observed in both root layers in 1997, which suggests that the cysts are unable to actively keep the juveniles inside their cyst wall. In conclusion, there are no indications that migration to the upper root layer directly affects emergence.

Reproduction: The cysts collected from the sand in older root layers were less well filled than those in the new root layer. However, the older root layers contained a mixture of old and young cysts. The cysts on the roots are more likely to have been newly formed than those in the sand. In 1998, in the old root layer, cysts on the roots contained significantly lower numbers of eggs and juveniles than those in the new root layer. In general, a reduced content of the cyst is related to unfavourable conditions, such as reduced plant metabolism, death of the roots, or

fungal infection of the feeding site (Cook, 1977; Perry and Gaur, 1996). So, although we cannot completely exclude that older cysts were present on the roots, the reduced numbers of eggs per cyst indicates a disadvantage for individual nematodes when they do not migrate to a new root layer. However, the results from 1997 indicate only a slight, but insignificant trend towards lower numbers of eggs per cyst collected from the roots in the one-year-old root layer. Therefore, evidence on improved individual performance following vertical migration in terms of achieving higher reproduction is inconsistent between the two years of sampling. Improved individual performance could, therefore, be a factor in some years but not in each year.

Survival: Although hard to measure, as in the old root layer cysts of older generations interfere, in spring 1998, about 90 per cent of the contents of the cysts, as was observed in October 1997, was released. No data are available of autumn 1996, but the contents of the cysts in spring 1997, suggest that the strong reduction of the number of eggs and juveniles in cysts over the winter period is a more general phenomenon. From agricultural systems much lower percentages of hatching have been reported (Sharma and Nene, 1992). However, the hatching percentages greatly depend on the absence or presence of a suitable host, as Den Ouden (1963) also found hatching percentages from 84 up to 95 when placing the cysts in root diffusates of a suitable host. The dune system is covered by permanent vegetation of a perennial host plant. The release pattern of juveniles from the cysts may be different in the dune system than in the case of an annual host plant that is only incidentally present, such as in a crop in rotation. It is, therefore, of interest to perform population studies of cyst nematodes in natural annual plant species as well in order to compare the release patterns of eggs from cysts.

The decrease in the number of eggs, between November 1997 and April 1998, has an important effect on the population dynamics, since no new roots had been formed in the new root layer. In the cysts in the one-year-old root layer, only a small percentage of the eggs were left, and only very few juveniles were recovered from the soil. As we did not take samples during the winter period, it is not clear what has happened to the contents of the cysts. There seems to be a severe mortality over winter. Except for possible inaccuracy in detection and natural mortality, which is not likely to be 90 per cent of the total potential offspring, mortality may be caused by a number of factors besides natural mortality. Even before the juveniles hatch from the cysts, the eggs inside the cyst may be parasitised by micro-organisms or fungi (Kerry, 1993, 1995; Chen and Dickson,

1996; Chen *et al.*, 1996; Rao *et al.*, 1997). Furthermore, juveniles may die during their migration to the new root layer or predators may cause mortality of juveniles when leaving the cysts. In conclusion, excluding major recovery inaccuracy, there is considerable mortality over winter, to which natural antagonists may contribute. Several species of soil micro-organisms with antagonistic potential have been isolated from the root zone of *A. arenaria* (P.C.E.M. De Rooij-Van der Goes, unpubl. results), but quantification of top-down effects is open for further studies.

When trying to answer fitness-related questions on nematodes, there are a number of constraints. In spring, hatched juvenile nematodes either migrate to the newly deposited sand layer, or they stay in the same root layer where they hatched. Both the hatching of the juveniles and the formation of new cysts are processes that continue for several months. The inability to follow individual juveniles makes it impossible to estimate the exact amount of time required for the moment of hatching until the juvenile enters the root, and from the entering of the root to the presence of a new female. Furthermore, it was not possible to determine from what root layer the juveniles originate, in which layer they enter the root and whether the juveniles develop into males or females. Therefore, we cannot determine the chance of survival in the different root layers, which may largely influence the individual performance of the nematode.

Another constraint is the establishment of the longevity of males. In another cyst nematode species, the longevity of a male cyst nematode was, on average, 9 or 10 days (Evans, 1970). As we did not check this for *H. arenaria*, the monthly samplings do not allow to make assumptions on the real total number of males produced per year. Furthermore, it is hard to make a proper assessment of the increasing number of females during the year, and in the one-year old root layer a mixture of newly formed cysts and cysts of a former generation may be present. Single generations cannot be separated and the plant, as well as the resource availability may affect the sex ratio of the nematode (Yeates, 1987).

As we have been able to only measure small direct advantages of migration on the level of the individual nematode performance, there may be cues that induce a juvenile to migrate, which are not a component of fitness but may work proximately. Root exudates often play an important role in the attraction and orientation of cyst nematodes (Klingler, 1965). But similar to *H. major* (Hesling, 1957), *H. arenaria* juveniles continued to hatch over a period of several months, whereas, when root exudates are the only cue for hatching, juveniles may emerge from cysts within a period of four to five weeks (Fenwick and Reid, 1953). On the

other hand our results showed a strong correlation between the formation of the roots and the occurrence of the first cysts, suggesting that juveniles are capable of tracking newly formed roots in an early stage. Whether this is an active or passive process has not been investigated, but physical factors such as the soil moisture and the soil temperature may be involved in timing (*e.g.* Sharma and Sharma, 1998; Meagher, 1970; Clarke and Perry, 1977). Another factor that may positively affect a juvenile's individual performance following migration, is the competitive advantage of an early migrating juvenile over the later arriving individual of the same or another nematode species (Eisenback, 1985).

In conclusion, for the population as a whole the roots formed in the new root layer of *Ammophila* are qualitatively superior to the roots in the lower layer. For individual nematodes, based on the field data, it is hard to discern any direct fitness-related advantages of migration although on a longer time-scale even small effects as a slightly faster development and a higher content of the cysts may positively affect the migrated individual nematode. The highest mortality seems to take place during winter, when eggs disappear from cysts whereas no accumulation of juveniles is observed. Whether this is due to egg-parasites or predation of juveniles in the soil is an open question. Top-down effects may influence the population density. However, bottom-up effects by the plant are probably more important in finally controlling the density of the *H. arenaria* population. Further studies are needed to elucidate which factors affect decisions made by nematodes, as well as what signals are perceived that affect the processes of decision-making.

CHAPTER 6

DISPERSAL-RELATED PERFORMANCE OF THE CYST NEMATODE *HETERODERA ARENARIA* AFTER SAND DEPOSITION IN OUTER COASTAL FOREDUNES

with W.H. Van der Putten
submitted to *Functional Ecology*

ABSTRACT

The costs and benefits of the dispersal of cryptic organisms, such as plant-parasitic nematodes, have received little attention. Dispersal is an important aspect of the life history, but to study the dispersal of a cryptic species is highly complicated. In outer coastal dunes, yearly up to 80 cm freshly windblown sand is deposited. In this layer the majority of new roots of *Ammophila arenaria* is formed and plant-parasitic nematodes have to migrate upwards to keep up with their host. A previous field study has shown that part of the population of the cyst nematode *Heterodera arenaria* migrates upwards to the new root layer, whereas another part remains in the older root layer, where also some new roots are formed. Depending on the year of study, the nematodes that had migrated to the new root layer had a fitness advantage due to an earlier start of development and a higher production of eggs in the newly formed cysts.

In the present study, we tested the hypothesis that juveniles from cysts that are formed in the new root layer after migration perform better than juveniles in cysts present in a one-year-old root layer. To test this hypothesis, cysts were collected from the newest root layer of *A. arenaria*, and from the one-year-old root layer. In two experiments under controlled conditions, we compared the rate of emergence of the second-stage juveniles, their survival and their reproductive success.

The first juveniles from the mother-cysts of the new root layer emerged faster than the juveniles from the mother-cysts of the one-year-old root layer. After the early start the further development resulted in equal proportions of males and females for both origins. Furthermore, the chance of survival and the reproductive success were not different for juveniles of both origins, although the daughters of

the cysts collected from the new root layer appeared to better adjust the numbers of eggs to unfavourable conditions.

In conclusion, on the short term, the daughters of the cysts formed after dispersal to the new root layer of *A. arenaria* did not show obvious fitness advantages. Fast emergence may lead to a second generation in favourable years, but most responses suggest an ultimately determined development irrespective of the origin of the cysts. However in the long run, dispersal will enable the nematodes to keep up with the root formation of *A. arenaria* in the new sand layer, which is a clear fitness advantage. The observed fixed development after release of the juveniles in combination with results from a previous field study suggests that there are few specific cues used by *H. arenaria* in the timing of emergence. The strategy of dispersal, possibly with the help of more general cues that stimulate migration upwards, seems to be effective for *H. arenaria* in order to encounter new roots.

INTRODUCTION

In studies on life history theory and the optimisation of the life history strategy, considerable attention has been paid to dispersal (*e.g.* Stearns, 1992; Roff, 1992; Van der Pijl, 1969). An obvious advantage of dispersal is the increase in the chance of finding new resources, which is necessary for survival when the resources in a patch become depleted. Another advantage may be the chance of meeting new mating partners, which lowers the chance of inbreeding (Silvertown *et al.*, 1997). On the other hand, there are also costs, as dispersal requires energy and may involve a higher exposure to natural enemies and, consequently, a higher chance of mortality.

Very few studies have focused on dispersal as a component of the life history strategy of cryptic organisms. Siepel (1994, 1995) studied the life history strategy of soil microarthropods, but to our knowledge hardly anything is known on dispersal as a component of fitness in soil inhabiting plant-parasitic nematodes. Most long-distance dispersal of nematodes occurs passively by wind (White, 1953; Orr and Newton, 1971). Human activities, such as the use of agricultural machinery or planting-material (Von Mende, 1985), also contribute considerably to nematode dispersal. Active dispersal of nematodes is limited, as nematodes may spread horizontally about 10 cm per year in agricultural soils (Wallace, 1963). In the vertical soil profile, the nematode distribution may passively follow the root

distribution of the host plant deeper into the soil (Barker and Nussbaum, 1971). Nematodes even may be found at several meters depth in fruit tree root zones (O'Bannon and Tommerlin, 1969).

On active vertical dispersal of plant-parasitic nematodes only few studies are known. Plant-parasitic nematodes that occur in the outer coastal dunes have to disperse vertically in the upward direction over considerable distances in order to keep up with the yearly sand deposition and the root formation in this sand layer. Sand deposition occurs mostly during autumn and winter storms, and may be up to 80 cm annually (Van der Putten *et al.*, 1989). In subsequent spring and summer, the nematodes migrate upwards and colonise the new root zone (De Rooij-Van der Goes *et al.*, 1998). There is, however, little known about the costs and benefits of nematode dispersal, fitness consequences and on cues involved.

One of the major plant species in the outer coastal dunes, the clonal grass *Ammophila arenaria*, is the natural host of the cyst nematode *Heterodera arenaria* (Cook, 1982). The dune grass needs sand deposition to produce new roots on top of old root layers, thereby maintaining its vigour (Huiskes, 1979). In the field, most of the new *H. arenaria* cysts are formed after dispersal of juveniles to the newly deposited sand layer, although some cysts of the new generation are formed in one of the already existing root layers deeper in the soil (Van der Stoel *et al.*, see Chapter 5).

In the field, some differences were observed between cysts originating from the migrated versus the non-migrated juveniles, which suggest a benefit of migration. First, in the newly formed root layer, at least in one out of two growing seasons development started earlier in the growing season (Van der Stoel *et al.*, see Chapter 5). This may enlarge the chances of survival or the production of a second generation within one growing season. Secondly, the higher numbers of eggs in the *H. arenaria* cysts produced in the new root layer (Van der Stoel *et al.*, see Chapter 5) may also constitute a fitness advantage to the migrated juveniles most likely related to the higher resource-quality (*e.g.* Koenning and Sipes, 1998). As with increasing depth and age of a root layer both the root biomass and the *H. arenaria* population density gradually decline, it is disadvantageous to remain in one of the older root layers. In the field study, however, it could not be tested whether a higher potential offspring (*i.e.* the numbers of eggs per mother-cyst) in the cysts formed in the newest root layer would lead to a higher actual offspring (*i.e.* the number of daughter-cysts).

In the present study, the hypothesis is tested that juveniles emerging from cysts collected from the newest root layer of the dune grass *A. arenaria* perform

better than juveniles emerging from cysts collected in a one-year-old root layer. Based on a previous field study (Van der Stoel *et al.*, see Chapter 5), a better performance is here defined as a faster emergence (*i.e.* the exit of the hatched juvenile from the cyst (Sharma and Sharma, 1998)) of the juveniles leading to an earlier start of development, and a higher chance of survival and development into new cysts filled with eggs. One experiment was conducted in order to test the success of reproduction of the cysts originally collected from the field (mentioned as mother-cysts) into new cysts (mentioned as daughter-cysts) on test plants in a growth chamber. We compared newly formed cysts of a new root layer with cysts of a one-year-old root layer of *A. arenaria*. The one-year-old layer consisted of a mixture of one-year-old and newly formed cysts. A second experiment was conducted by sequentially harvesting a pot trial, in order to establish the start of emergence and development of the juveniles from the cysts of both root layers.

MATERIALS AND METHODS

Soil

Early October 1998, soil was collected from the coastal foredunes of Voorne, the Netherlands, at a site north of Haringvliet (51°52' N 4°04' E). Around vigorous *A. arenaria* soil was collected that, after sieving on a 0.5 cm-mesh sieve, was sterilised by means of gamma irradiation (average 25kgray). The soil was stored until the start of the experiment at 4°C in the dark.

H. arenaria cysts

Simultaneously, at the same site, roots and rhizosphere soil were collected from vigorous *A. arenaria* for the extraction of *H. arenaria* cysts. From two distinct root layers of vigorous *A. arenaria*, the roots and the rhizosphere sand were collected separately. One batch of cysts was extracted from the root layer that was newly formed in the growing season of 1998 and only contained cysts also being formed in 1998 (the 1998-cysts). The other batch of cysts was extracted from the root layer that was formed first during the summer of 1997, and contained a mixture of cysts formed in the growing seasons of both 1997 and 1998 (the 1997-cysts) (Van der Stoel *et al.*, see Chapter 5). Roots and rhizosphere sand were washed in a bucket in order to extract the cysts. The water and the cysts were decanted onto a 180 µm-mesh sieve. From the sieve, the cysts were washed in a

filterpaper-cone and left to dry for 2 days. Thereafter, the cysts were handpicked from the filter using a binocular microscope (10-15x magnification).

Before the start of the experiments, the numbers of eggs and juveniles within the cysts were determined. From each root layer 25 cysts were randomly chosen and the number of eggs and juveniles inside these cysts was determined by automatic image-analysis as described by Van der Stoel *et al.* (see Chapter 5).

Culturing A. arenaria seedlings

Seeds of *A. arenaria* had been collected from the same dune area during the summer of 1995. Prior to the start of the experiment, seeds were germinated for 21 days on glass beads at a 16/8 hour dark/light regime with corresponding temperatures of 25/15°C. Every three weeks a new amount of *A. arenaria* seeds from the same batch was germinated for the same period of time until use. After germination, seedlings were pre-cultured for 14 days in cones filled with 30 ml of the gamma-irradiated foredune soil in a greenhouse with a 16/8 hour light/dark regime at 23/19 (±2)°C.

Experimental conditions

At the start of the experiments, the soil was set at 10% ($w \cdot w^{-1}$) soil moisture. Twice a week the moisture content was reset at 10% by adding demineralised water. Once a week a full strength Hoagland nutrient solution was added. In both experiments 12.5 ml was added to each pot during the first three weeks, whereas later 25.0 ml was added. In the climatised growth chamber where the plants grew during the first three weeks after planting (Fig.1; pots and rings indicated with A), conditions were set at a 16/8 hour light/dark regime at 20/16°C. In the larger climatised room, where the plants were placed for the last 14 weeks of the repetitive attraction experiment (Fig.1; pots and rings indicated with B), the temperature was set at 23/19°C during a 16/8 hour light/dark regime.

Testing the study system

A small pilot experiment was carried out to test the experimental procedure. Cysts were added to a 15-cm high, 1.5 l pot, filled with sterilised dune soil. A ring, 10-cm high, with the same diameter as the pot was placed on top, filled with sterilised dune soil and planted with four *A. arenaria* seedlings. We tested whether it was possible to attract juveniles to the plant roots without the roots growing from the ring into the pot within an experimental time long enough to allow ample nematode migration into the root zone. As roots were not found to have grown

into the pot after three weeks, this seemed an appropriate time for the experiment to separate the two parts.

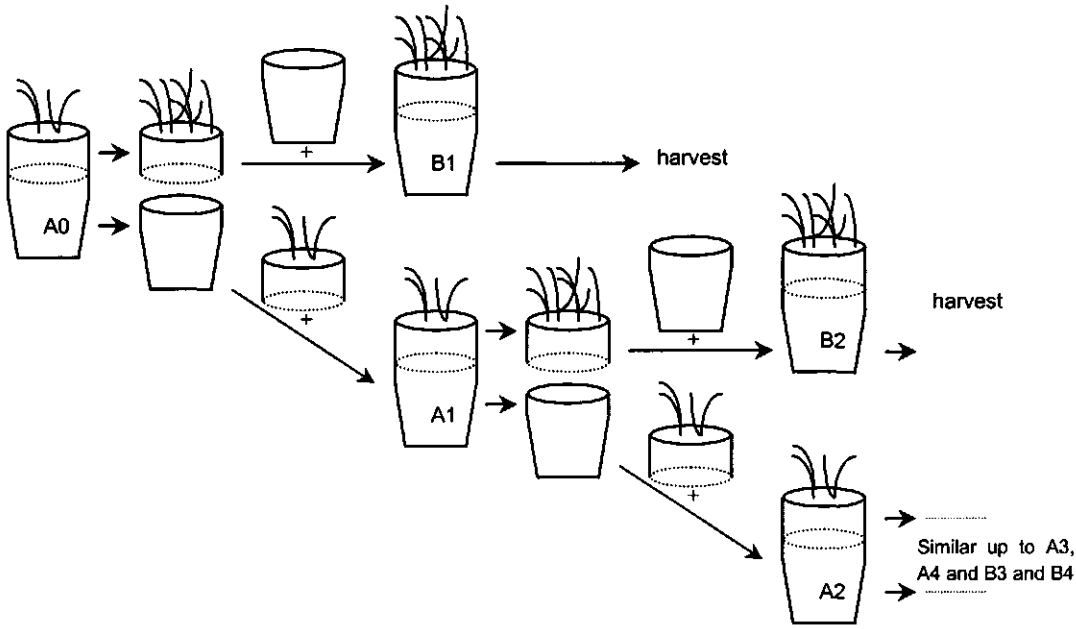

Figure 1. Repetitive attraction experiment: experimental procedure. Pot and ring (A0) were separated 3 weeks after the start of the experiment. Underneath the original ring a new pot was placed with sterilised soil without *H. arenaria* cysts. This new combination (B1) was harvested 14 weeks later. On top of the original pot (containing the mother-cysts) a new ring was placed with four pre-cultured *A. arenaria* seedlings. Three weeks later, the separation and the formation of new combinations (A1) was repeated. In total, the separation of the pot and the ring was carried out four times: 3, 6, 9, and 12 weeks after the first planting of the pre-cultured seedlings.

Repetitive attraction experiment

The repetitive attraction experiment started with thirty 1.5 l pots. Each pot was filled with a layer of about 3 cm of sterilised soil (foredune sand) on top of which thirty cysts were placed (the mother-cysts), originating from the 1997-layer (15 pots) or from the 1998-layer (15 pots). With about 12 cm of sterilised soil, the pots were then filled up to the brim and set at 10% ($w \cdot w^{-1}$) soil moisture. A 10-cm high ring was placed on top of the pot and also filled up with sterilised sand and set at 10% ($w \cdot w^{-1}$) soil moisture. Four 5-week-old *A. arenaria* seedlings were placed in each ring. The soil surface was covered with aluminium foil to prevent desiccation of the soil. The pots with the ring and plants on top of them were placed in a climatised growth chamber (see experimental conditions) for three

weeks (Fig.1, pot and ring indicated with A0). During this period, juveniles were allowed to emerge from the cysts and migrate towards the roots. Water and nutrient solution were added as described before.

After three weeks, the ring and the pot were carefully separated by placing a sharp blade between these two parts. The ring with the plants including the juveniles that had migrated during the previous three weeks, was placed on top of another 1.5 l pot filled with sterilised foredune sand and set at 10% ($w \cdot w^{-1}$) soil moisture. These combinations of a pot and a ring were placed in the climatised room for another 14 weeks to allow daughter-cysts to develop out of the attracted juveniles before being harvested (Fig.1, pot and ring indicated with B1).

On top of the original pots containing the mother-cysts, a new ring was placed, filled with sterilised soil, set at 10% soil moisture, and planted with four new 5-weeks-old *A. arenaria* seedlings. The new combination was placed back in the climatised growth chamber, again for three weeks (Fig.1, pot and ring indicated with A1). In total, the procedure of placing and separating rings and pots was carried out four times, so that the whole experiment included 4x2x15 (time interval x cyst origin x replicates) pots.

After 12 weeks, when the rings of the fourth series had been placed on new pots, the pots with the mother-cysts were harvested. The soil was washed in a 10-l bucket, and the juveniles, males and mother-cysts were extracted by decanting (see harvest details). The remaining contents (eggs and hatched but non-emerged juveniles) of the mother-cysts were established by automatic image-analysis as described by Van der Stoel *et al.* (see Chapter 5). The difference in content between the start and the end of the experiment gave an estimate of the maximum number of juveniles that had emerged from the cysts during the period of 12 weeks.

Continuous attraction experiment

In addition, a continuous attraction experiment parallel to the repetitive attraction experiment was carried out. In the continuous attraction experiment, pots with rings were harvested sequentially, and we allowed one set of plants to develop roots and to attract nematodes for a period of 12 weeks in order to study the emergence of juveniles from the *H. arenaria* mother-cysts and the formation of daughter-cysts.

Simultaneously with the start of the repetitive attraction experiment, forty 1.5 l pots were filled with sterilised soil and set at 10% ($w \cdot w^{-1}$) soil moisture. In a pot we placed twenty cysts from the 1997-layer or twenty cysts from the 1998-layer. There were 20 pots of each cyst origin. Similar to the repetitive attraction

experiment, an additional ring was placed on top of the 1.5 l pot and filled with sterilised sand and set at 10% ($w \cdot w^{-1}$) soil moisture. Per ring four 5-weeks-old *A. arenaria* seedlings were planted. The soil surface was covered with aluminium foil to prevent desiccation of the soil and the pots with the rings were placed in the climatised growth chamber. Every three weeks 10 pots and their rings were harvested: 5 replicates of each cyst origin. At each harvest, plant biomass and the numbers of juveniles and males were determined. After 12 weeks, when the last replicates were harvested, also the cysts, both the mother- and the daughter-cysts, were harvested. From the mother-cysts the remaining numbers of eggs and juveniles were determined by using an automatic image-analysis system (Van der Stoel *et al.*, see Chapter 5).

Harvests

In both experiments, at harvest, the soil was washed from the roots into a 10-l bucket. To extract the juveniles, males and cysts from the soil, the suspension was stirred and the water with the floating nematodes was decanted on a set of sieves (1.0 mm, 180 μm, 75 μm, and 3x45 μm mesh). Shoot and root biomass was dried at 70°C for at least 48 hours, and weighed. The numbers of juveniles and males were counted by reversed light-microscopy (50-200x magnification). The numbers of daughter-cysts were counted, using a binocular microscope (10-15x magnification), and their content was quantified by automatic image-analysis system.

Data analysis

A one-way ANOVA was used to test for differences between the initial content of the 1998- and 1997-mother-cysts, with the origin of the cysts as independent variable and the numbers of eggs and juveniles as the dependent variable. In the repetitive attraction experiment, it was tested whether there was any effect of the origin of the mother-cysts and of the period in which juveniles had migrated on the number of daughter-cysts, the root biomass of the plants, and the number of cysts formed per gram of root biomass. To this end, three two-way ANOVA's were carried out with the origin and the period of migration as the two independent variables. Tests were carried out with the number of daughter-cysts, the root biomass, or the number of daughter-cysts per gram root dry weight as the dependent variables. Treatment means were compared in an LSD-test ($P<0.05$).

In a one-way ANOVA the origin of the mother-cysts was used as independent variable and the proportion of emerged juveniles as the dependent

variable. In order to calculate this proportion, the number of eggs within a cyst at the end of the experiment was subtracted from the average initial number of eggs in the cysts at the start of the experiment, and divided by the average initial number of eggs in the cysts. This analysis allowed to test for differences between 1997- and 1998-cysts irrespective of their different initial contents at the start of the experiment (a number of the 1997-cysts had already released most of their juveniles before the start of the experiment). A similar one-way ANOVA, with the proportion of daughter-cysts formed after 12 weeks related to the initial content of the mother-cysts as dependent variable, was carried out to test whether the origin of the mother-cysts affected the formation of daughter-cysts.

In a one-way ANOVA with the origin of the mother-cysts as the independent variable and the contents of the daughter-cysts as the dependent variable, it was tested for each time interval whether the origin of the mother affected the number of eggs and juveniles inside the daughter-cysts. Furthermore, a Wilcoxon matched pairs test was carried out, including data of all four time-intervals, to test for differences in the contents of the daughter-cysts between the two origins of the mother-cysts.

In the continuous attraction experiment, a Wilcoxon matched pairs test was used to test whether different numbers of juveniles had emerged from the mother-cysts from both origins. Therefor, for all four harvests, the average number of juveniles that had emerged from the 1997-mother-cysts was compared to the average number of juveniles that had emerged from the 1998-mother-cysts. In order to determine the emergence during the 12-week period for both cyst origins, data of the continuous attraction experiment of males, juveniles and newly formed cysts were expressed in a cumulative way. To check whether cysts of a different origin had a different strategy in releasing their juveniles, the cumulative numbers were divided by the initial numbers of eggs and juveniles inside the mother-cysts. This was necessary because there had already been more natural release from the 1997-cysts than from the 1998-cysts before being collected in 1998.

In a one-way ANOVA with the origin of the mother-cysts as independent and the proportional number of emerged juveniles after three weeks of attraction as the dependent variable, it was tested whether cysts of a different origin had a different strategy of releasing their juveniles. In order to determine whether the numbers of daughter-cysts formed after 12 weeks was different between the origins, a one-way ANOVA was carried out with the origin of the mother-cysts as independent variable and the proportion of daughter-cysts, related to the initial content, as the dependent variable.

In a two-way ANOVA with plant biomass as the dependent variable, it was tested whether the date of harvesting and the origin of the parent cysts affected the dry weight of the *A. arenaria* plants.

RESULTS

Repetitive attraction experiment

In all four sequential time-intervals of three weeks, juveniles had migrated to the rings with *A. arenaria* plants, and developed into cysts in the next 14 weeks (Fig. 2A). Both the origins of the mother-cysts and the time-interval, during which juveniles had migrated, significantly affected the number of new cysts that were formed. Significantly more daughter-cysts were formed after hatching of the juveniles from 1998-mother-cysts than from cysts of the one-year-old root layer. The third time interval was the only exception. For both cyst origins, the peak of attraction seemed to be at the same time as most cysts were formed by juveniles attracted in the last time-interval. The low number of cysts that had been produced after the third time-interval correlated with an aphid infestation in the climate chamber. The aphids most likely caused the interaction-effect between the factors 'time-interval' and 'origin of the cysts'.

The origin of the added cysts had no effect on the root biomass of *A. arenaria* plants harvested 14 weeks after the ring and pot were separated (Fig. 2B). The number of daughter-cysts expressed per gram root dry weight (Fig. 2C) resulted in a significantly higher occupation of the roots in the pots to which 1998-mother-cysts were added, except for the third time interval, when there was an aphid infestation.

The cysts collected from the 1997- and 1998-layers initially contained significantly ($P<0.001$) different numbers of eggs and juveniles (132 in cysts from the 1997-layer and 378 in the cysts from the 1998-layer, respectively). In order to determine what has happened with the offspring, the numbers of produced males and females over all four time-intervals were added and compared to the initial numbers of eggs and juveniles in the mother-cysts (Table 1). A significantly higher fraction of the initial numbers of eggs and juveniles had emerged from the 1997-cysts than from the 1998-cysts ($P=0.0018$). Also the number of daughter-cysts as a proportion of the average initial number of eggs in the mother-cysts was significantly highest for the cysts originating from the 1997-layer. However, in absolute numbers, more eggs had hatched from the 1998-mother-cysts (Table 1).

The further development of the juveniles that had actually emerged from the 1997- and 1998-mother-cysts, resulted in similar percentages that developed into males and females, causing a similar female-biased sex ratio for both cyst origins (Table 1).

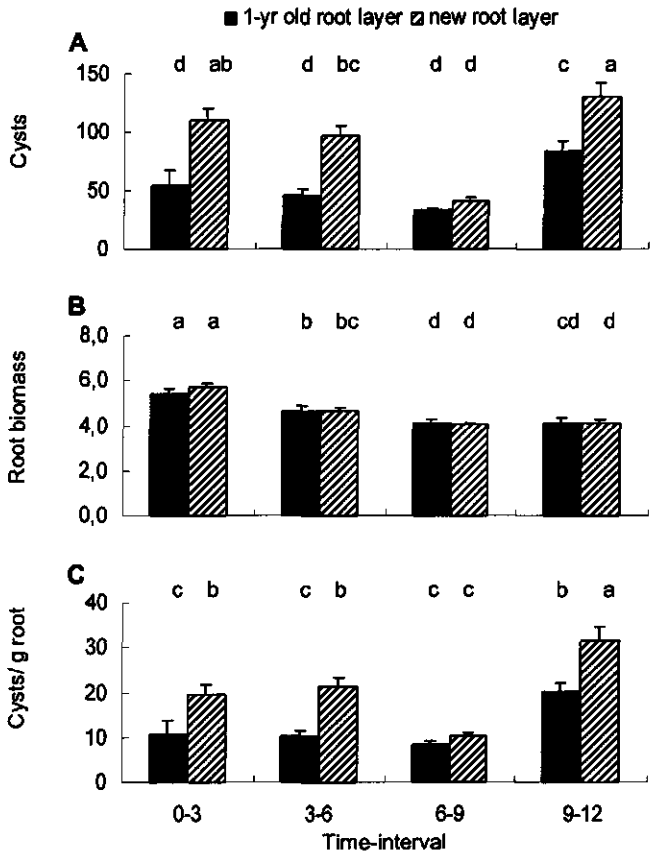

Figure 2. Repetitive attraction experiment. A.) Numbers of newly formed *H. arenaria* cysts (+1 SE) harvested 14 weeks after juveniles had migrated to the roots. 0-3, 3-6, 6-9, and 9-12 represent four time-intervals. The mother-cysts originated from a one-year-old and a newly formed root layer of an *A. arenaria* stand in mobile foredunes. B.) Root biomass at the moment of harvesting. C.) The newly formed cysts expressed per gram root dry weight. Different letters indicate significant differences at $P<0.05$, and the comparison includes both cyst origins and the time-intervals.

As also the potential reproduction of the next generation may be a measure for the fitness of the nematode, it was tested whether the daughter-cysts of the 1997- and 1998-mother-cysts showed a different potential offspring (as indicated by the number of eggs in the daughter-cysts). For each time interval, a one-way ANOVA was carried out to test whether the origin of the mother-cysts affected

the final content of their daughters (Table 2). Only after the third time interval the cysts showed significantly different numbers of eggs and juveniles. The 1998-daughter-cysts contained most eggs. Over all, however, comparing the averages over the four time intervals, no significant difference was found between the contents of the daughter-cysts of both origins (Table 2).

Table 1. Repetitive attraction experiment. Per pot the initial content, the final content, and the percentage of emerged eggs of the mother-cysts of the two root layers are presented. The total number of produced males and females of the newly formed generation are added up for all four time-intervals, and calculations on these numbers are presented in the lower part.

	1-year-old root layer	new root layer
Mother-cysts		
Initial content	3951	11355
Final content	1803	7821
Number emerged (percentage of initial)	2148 (54.4%)	3534 (31.1%)
Newly formed generation		
Males		
absolute	45.4	88.7
as % of initial	1.15	0.78
as % of emerged	2.1	2.5
Females (daughter-cysts)		
absolute	217	378
as % of initial	5.49	3.33
as % of emerged	10.1	10.7
Sex ratio (males:females)	0.21	0.23

Table 2. Repetitive attraction experiment. Results of one-way ANOVA's for each time-interval with the origin of the mother-cyst as the independent variable and the total amount of eggs and juveniles in the daughter-cysts as the dependent variable. Different letters indicate significant differences within the comparison of treatment means at $P<0.05$.

Factor		df	*F*	*P*	Treatment mean	
	Time-interval				1-year-old	new
Cyst origin	0-3	1	3.846	0.054	366[a]	454[a]
	3-6	1	2.311	0.132	347[a]	417[a]
	6-9	1	6.194	0.013*	456[b]	545[a]
	9-12	1	3.007	0.087	514[a]	440[a]
	Overall (Wilcoxon)	1	1.095	0.273	421[a]	464[a]

Continuous attraction experiment

As in the repetitive attraction experiment, 1997- and 1998-mother-cysts did not affect plant growth differently (Fig. 3). The total amount of juveniles emerged from the 1998-cysts was almost significantly higher (P=0.068) than from the 1997-cysts. The weak significance is most likely due to the low number of pairs in the test. At the end of the experiment, the mother-cysts originating from both 1997- and 1998-root layers had released 25% of the initial numbers of eggs (data not shown).

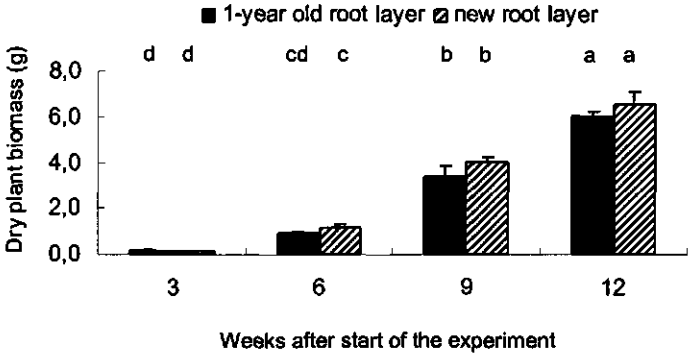

Figure 3. Continuous attraction experiment. The total plant biomass (+ 1 SE) of *A. arenaria* per pot, 3, 6, 9, and 12 weeks after addition of cysts. N=5.

In the continuous attraction experiment, as a result of the destructive way of harvesting, the pattern of emergence from mother-cysts and the nematode development could be quantified. Three weeks after the start of the experiment, juveniles were found to have hatched from the cysts (Fig. 4). From week 6 onwards, males were present, so that the second juvenile stage had gone through three more moults via the third and fourth juvenile stage into adult males. The emergence of juveniles was a continuous process as at all harvests juveniles were observed, which confirmed the results of the repetitive attraction experiment.

At the final harvest, 12 weeks after the start of the experiment, the amount of newly formed cysts was quantified. As the final number of daughter-cysts and males was considerably lower than the estimated numbers of eggs that had disappeared from the mother-cysts in the total experimental period, many eggs or juveniles either had died in the course of the experiment, or juveniles were still in the roots as immobile stage. On average 53% of the expected emerged juveniles

from the 1997-mother-cysts and 46% of the expected emerged juveniles from the 1998-mother-cysts could not be recovered as a male or a daughter-cyst.

The emergence of juveniles from the 1998-cysts started earlier than the emergence from the cysts of the one-year-old layer (Fig. 4B), whereas the pattern of development of emergence over the rest of the 12-week period was similar for both cyst origins. The proportion of juveniles released during the first three weeks of the experiment was significantly (P=0.009) higher for the 1998-mother-cysts than for the cysts originating from the one-year-old layer. After the early start in the first three weeks, the percentages that had hatched at week 6 were already similar for both cyst origins.

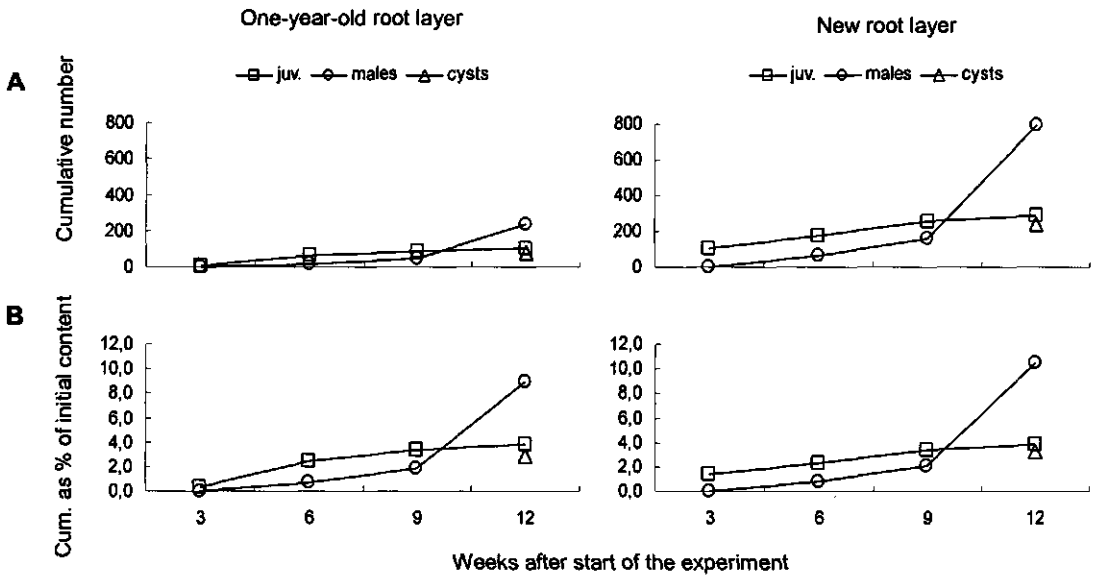

Figure 4. Continuous attraction experiment. Numbers of *H. arenaria* juveniles, males, and cysts extracted at 3, 6, 9, and 12 weeks after the start of the experiment to which cysts were added originating from a one-year-old root layer (left) and from a newly formed root layer (right). In A.) the data are presented as cumulative numbers. In B.) the cumulative numbers are expressed as a percentage of the initial content of the mother-cysts. For each data point N=15.

The 1997 layer will have contained a mixture of cysts formed in 1998 and in 1997, which was indicated by the distribution of the mother-cysts based on their initial contents over various categories (Fig. 5A). In the 1997 layer, 60% of the cysts contained 0-100 eggs. Only about 30% of the cysts contained more than 200 eggs, which may be the cysts that have been formed in 1998. In the 1998 layer,

about 70% of the cysts contained more than 200 eggs. These cysts may have contained more 'fast-hatching' juveniles, which probably had disappeared already from the cysts collected from the 1997 layer. Furthermore, the cysts of the 1997 layer that contained most eggs still contained fewer eggs than the best-filled cysts of the 1998 layer (Fig. 5B). So, even though a number of cysts of the 1997 layer may have been formed in 1998, they contained fewer eggs than the best-filled cysts of the 1998 layer.

Although early emergence of juveniles was more apparent for the cysts of the 1998 layer, it finally did not result in a significantly higher proportion of daughter-cysts, as related to the initial contents of the mother-cysts (P=0.656). 3.21% of the initial content of the mother-cysts from the 1998 root layer had developed into daughter-cysts *vs.* 2.93% of those from the one-year-old root layer.

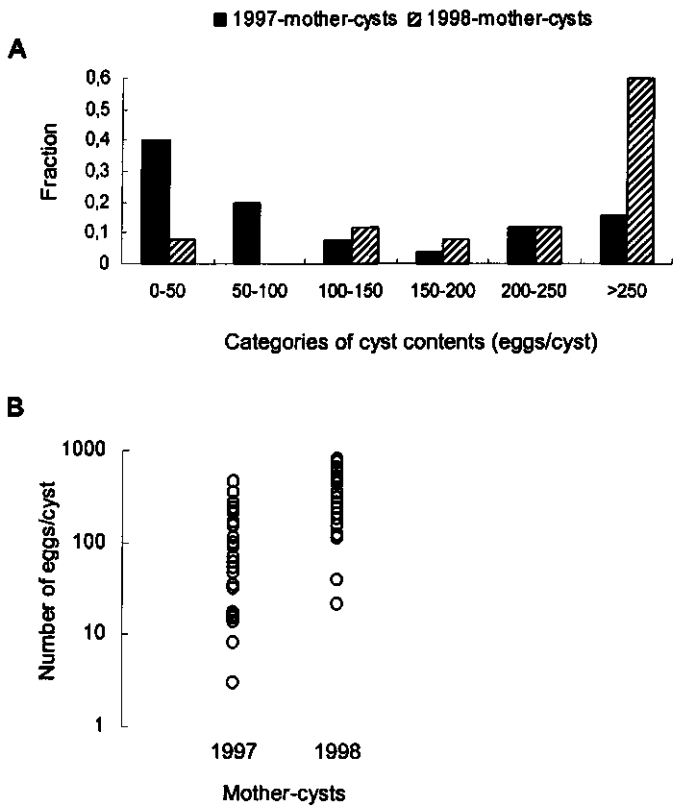

Figure 5. A.) The distribution of the 1997- and 1998-mother-cysts over various categories of cyst contents, based on the initial contents of the mother-cysts. B.) For 25 cysts of both 1997- and 1998-mother-cysts the content per cyst is presented on a log-scale.

DISCUSSION

We studied whether *H. arenaria* juveniles emerging from cysts formed in the expansion zone of the clonal grass *A. arenaria* may perform better than juveniles from cysts in a more established part of the clone. In this study, the expansion zone corresponded with the root layer formed in 1998 and the more established zone with the one-year-old root layer formed in 1997. The performance of *H. arenaria* from the different root zones is discussed based on the rate of emergence of the juveniles, the number of eggs produced by the daughter-cysts, and the mortality of the juveniles.

Rate of emergence: In the first phase of the continuous attraction experiment, more juveniles emerged from the 1998-cysts than from the cysts originating from the one-year-old root layer. Fast emergence of the first juveniles implies a fitness advantage as it enables the fast individuals to benefit most from the resources or feeding sites. Crowding and competition for feeding sites enhances the numbers of males (*e.g.* Trudgill, 1967; Mugniéry and Fayet, 1981; Von Mende *et al.*, 1998), whereas a higher food availability for the fast emerging juveniles may result in higher numbers of females (Yeates, 1987).

In spring, in the dune system early emerging nematodes could take advantage of the newly formed root layer, but as observed in a field study (Van der Stoel *et al.*, see Chapter 5), *H. arenaria* juveniles already emerge from the newly formed cysts at the end of the same growing season in which they have been produced. During winter, large numbers of eggs and juveniles were found to disappear (Van der Stoel *et al.*, see Chapter 5), so that the fitness advantage of the fast emergence at the end of the growing season may be questioned. The most likely possibility is that fast emergence enables the new juveniles to produce a second generation, which also had been found in herbage crops (Cook and York, 1980) and suggested for other *Heterodera* species, such as *H. humuli* (Von Mende and McNamara, 1995) and *H. avenae* (Meagher, 1970). However, possibilities for producing a second generation will strongly depend on the start of the root development and the length of the growing season (Van der Stoel *et al.*, see Chapter 5), so that the advantage of fast hatching will only become apparent in some years. Previous field observations have shown that new cysts were (in one out of two years of field observations) produced earlier after dispersal to a new root zone than in an existing one (Van der Stoel *et al.*, see Chapter 5). Therefore, selection in favour of fast emergence will be stronger in newly formed than in older root layers.

The cysts collected from the one-year-old 1997-root layer consisted of a mixture of one-year-old cysts, formed in 1997 that contain low numbers of eggs, and new cysts, formed in 1998 that are most likely the cysts that are better filled. The juveniles that emerge from the latter group of cysts may possibly achieve the same rate of emergence as the juveniles from the cysts of the same age, collected from the 1998-layer. We were, however, not able to separate new and one-year-old cysts collected from the 1997-layer. The difference in rate of emergence between juveniles from new (1998) cysts and largely emptied (1997) cysts may have been larger than the average difference between the cysts originating from the 1997 and the 1998 root layers. If all newly formed cysts (from both the 1998 and 1997 layers) contain fast emerging juveniles, the first-hatched juveniles from cysts in the newly formed root layer may have an advantage over first-hatched juveniles in the older root layer. The latter have less roots available (Van der Stoel *et al.*, see Chapter 5), or first need to migrate upwards in order to get access to the new root layer. However, when there is no possibility to form a second generation, fast emergence may even be disadvantageous, because these juveniles will most likely not survive the winter period (Van der Stoel *et al.*, see Chapter 5).

Eggs produced by daughters: In a previous field survey, daughter-cysts in the new root layer contained more eggs than the average cyst present in older root layers, at least in one out of two growing seasons (Van der Stoel *et al.*, see Chapter 5). As a higher number of eggs per cyst may be due to favourable feeding conditions (Cook, 1977; Seinhorst, 1986a; Koenning and Sipes, 1998), a higher numbers of eggs in the daughter-cysts indicate a selective advantage. The repetitive attraction experiment, however, did not support the idea that daughter-cysts from a new root layer may produce more eggs than daughter-cysts from an older root layer. The higher number of eggs in the daughter-cysts from 1998-mothers in the third time interval may have been caused by the aphid infestation of the young plants. Masters *et al.* (1993) suggested that above-ground insect herbivory may have an indirect negative effect on root feeders. The aphid infestation, as observed in the present study, may therefore have reduced the larval establishment in the roots. The aphid infestation was under control after the three-week period of attraction. As there was no reduction of the root biomass at harvest as compared to the root biomass at the fourth harvest, there are no indications that the root biomass had been reduced after the three-week period of attraction as a consequence of the aphid infestation. As lower numbers of juveniles had established on the roots as 1998-daughter-cysts after the third time-interval, the unplanned treatment of aphid infestation may have reduced intra-specific competition for resources. The

observed difference in the contents of the daughter-cysts suggests that the daughter-cysts of 1998-mother cysts were more plastic in their response to reduced intra-specific competition than daughters of cysts from the 1997 layer.

In these experiments, we cannot completely exclude the possibility that the numbers of produced cysts may have been limited by the amount of roots. The limitation may have lead to a higher production of males (Trudgill, 1967) or the competition for food may have resulted in lower numbers of eggs per cyst (Seinhorst, 1986a). However, as in the repetitive attraction experiment significantly most 1998-daughter-cysts per gram of root biomass were produced after the fourth time-interval, and as no such numbers had been found in earlier time intervals, only after the fourth time-interval for the 1998-cysts the amount of roots may have been limiting. Also in the continuous attraction experiment root limitation may have reduced the final number of 1998-daughter-cysts. A possible limitation of the cyst production may result in an underestimation of the total production of daughter-cysts, but most likely the emergence has not been affected, still allowing the previously discussed suggestions on the rate of emergence of both cyst origins. Furthermore, to achieve the same proportional success of reproduction as the 1997-mother-cysts, 1998-mother-cysts additionally had to produce 250 daughter-cysts, which is 1.66 times as many cysts as have been formed in the experiment.

The contents of the daughter-cysts produced by the 1997-mother-cysts were similar for all four time-intervals. Even though the number of juveniles emerged from the 1997-mother-cysts will not have provided a limitation for cyst formation, the numbers of 1997-daughter cysts per gram root dry weight was still lower than of the daughter-cysts originating from the 1998-mothers. The higher numbers of 1998-daughter-cysts and the similar availability of roots for mother-cysts of both origins indicate that root availability has not been limiting for 1997-daughter-cysts.

Obviously the formation of daughter-cysts from the 1997-mother-cysts was limited by another factor. As the experiments were carried out in sterilised soil, it is not likely that natural predators negatively affected the survival of the emerged juveniles, unless the mother cysts may have been parasitised prior to the experiment. In coastal dune soil, De Rooij-Van der Goes (unpubl. results) had observed fungal egg-parasites, but it is not clear whether those parasites were restricted to older cysts only. Some of the *H. arenaria* cysts were filled with large fungal spores, and these always concerned older cysts (data not shown). The fungal spores have not been identified, and could therefore not be confirmed as possible parasite. In agricultural systems parasitism of eggs and cysts has often been

reported (*e.g.* Chen and Dickson, 1996; Kim *et al.*, 1998). Chen *et al.* (1994) found a higher frequency of fungal colonisation in older brown cysts of *Heterodera glycines* than in younger females. This may indicate the possibility of an age-restricted parasite in *H. arenaria* limiting the formation of 1997-daughter-cysts.

Juvenile mortality: Except for the possibility of parasitism causing mortality and limiting the number of 1997-daughter-cysts being formed, there was a high overall mortality. Based on the initial and final contents of the cysts and the observed numbers of females and males, many juveniles seem to die after emergence. Whether the mortality is related to the age of the mother-cysts has still to be proven, but juveniles from older cysts could have had less energy reserves so that a larger percentage of juveniles may fail to reach the roots (Reversat, 1981).

The high mortality in combination with the continuous emergence of the juveniles and the equal percentages of produced males and females from the emerged juveniles suggests that the release of juveniles is not regulated by any cue. Juvenile release seems a fixed, ultimately determined process, which has not often been observed in agricultural systems. Many cyst nematodes show a clear response to a plant-related stimulus (*e.g.* Wallace, 1958; Clarke and Perry, 1977; Hashmi and Krusberg, 1995). However, also in agricultural systems, either in absence of the host (Seinhorst, 1986b; Sharma and Nene, 1992; Devine *et al.*, 1999) or due to a period of unfavourable conditions such as occurs during winter time (Sipes *et al.*, 1992), the population density declines each year (Ferris and Ferris, 1998).

The question rises how this strategy of fixed emergence that continues in winter can be effective for a plant-parasitic nematode. Possibly, gradual release of juveniles over a longer period of time is effective because of the large and unpredictable variation in the start of root formation in spring (Van der Stoel *et al.*, see Chapter 5). Although a temperature or moisture gradient may be helpful in orientation over larger distances (Wallace, 1958; Rode, 1969) temperature fluctuations may not be easily sensed in deep soil layers. In that case, random release seems the best strategy when specific cues are lacking or not recognised. The juveniles that are released later, that are too late to successfully produce a second generation in the same year, will at some stage encounter the roots formed in the newly deposited sand layer.

In conclusion, on the short term, none of the factors, rate of emergence, survival of the juveniles, and contents of the produced daughter-cysts indicates an advantage for the juveniles from the cysts of the 1998-layer. However, on the long run it may be an advantage having the ability to form a second generation in the case that it is allowed by the length of the growing season. Moreover, there is a

benefit of being closest to the root layer to be formed in the next year or having the possibility to adjust the number of eggs in the newly formed cysts. As *H. arenaria* was found to release juveniles in an ultimately determined way, it is suggested that this species may not be capable to use or recognise a plant-stimulus for the timing of the emergence of the juveniles.

CHAPTER 7

GENERAL DISCUSSION

Species identification

In previous studies on the occurrence of soil-borne pathogens in coastal foredunes, the presence of various *Heterodera* spp. had been observed, and it was suggested that they might contribute to the degeneration of *A. arenaria* (De Rooij-Van der Goes, 1995; De Rooij-Van der Goes *et al.*, 1995a). More than one species of *Heterodera* was thought to be present, however, their exact identity was not known.

In the present study various species of *Heterodera* have been observed in the rhizosphere of different grass species that dominate sequential stages of vegetation succession in the coastal dune area. With the use of PCR-SSCP the species were identified to the species level based on the ITS2 sequence of their ribosomal RNA. *Heterodera arenaria* occurred in the root zone of *Elymus farctus* and *Ammophila arenaria* in the mobile area of the outer coastal foredunes. At the more stabilised sites, later in the successional sere, *Heterodera hordecalis* and in one occasion *Heterodera mani* were recorded (Chapter 3).

Heterodera arenaria is known to occur on *A. arenaria* (Cooper, 1955; Robinson *et al.*, 1996), but *H. arenaria* could not be distinguished from *Heterodera avenae* on the basis of ITS2 PCR-SSCP (Chapter 3). Also on the basis of restriction enzyme analysis, no enzymes were found that allowed discrimination between European populations of *H. arenaria* and *H. avenae* (Subbotin *et al.*, 1999). The proposition that *H. arenaria* may be a polyploid of *H. avenae*, resembling large *H. avenae* but having very long juveniles and longer eggs (Cooper, 1968), could not be confirmed as no difference in ploidy level has been detected thus far (Karssen and Van der Beek, pers. comm.). Further studies are needed to establish whether *H. arenaria* and *H. avenae* are actually different species or whether it is one species with populations that are adapted to coastal foredunes and others that are adapted to agricultural conditions.

Specificity

In the outer coastal dunes, *Heterodera arenaria* showed considerable host specificity (Chapter 4). The occurrence of *H. arenaria* was limited to *Elymus farctus*

and *Ammophila arenaria* in the mobile area of the outer coastal dunes. Only at the island of Texel, *H. arenaria* was found to occur also in the samples collected from degenerating *A. arenaria*. This may have been due to site characteristics. At the site where samples had been collected at Texel, the trajectory of degeneration occurs over a range of several hundred metres, whereas at Walcheren and Haringvliet *A. arenaria* is heavily degenerated within 50 metres from the vigorous stands. At the latter sites rhizosphere samples had been collected from heavily degenerated stands, whereas at the island of Texel samples were collected from *A. arenaria* that had just started to degenerate. In addition to the high degree of specificity found to occur in the field, in the greenhouse *E. farctus* and *A. arenaria* were also the only two plants species that allowed the development from second stage juveniles of *H. arenaria* to adult cysts, whereas hardly any cysts were produced on other foredune grasses that naturally occur more inland of the coastal foredunes (Chapter 4).

In the present study, *Heterodera hordecalis* was found to occur in the rhizosphere of degenerating stands of *A. arenaria* and in stands with *Calamagrostis epigejos*. *H. hordecalis* is known to parasitise various cereals and grass species (Andersson, 1975). At the end of an experiment in which *H. arenaria* was inoculated to sterilised or non-sterilised soil with different dune grass species (Chapter 4), cysts of *H. hordecalis* were found in the pots with non-sterilised soil collected from the rhizosphere of *Festuca rubra* ssp. *arenaria*. As *Heterodera* spp. were not observed on the roots of severely degenerated *A. arenaria* stands, but only in the soil, it is still possible that *A. arenaria* is not the actual host to *H. hordecalis*, but that *F. rubra* ssp. *arenaria* is the actual host (Chapter 4). More inland, when *A. arenaria* degenerates, the species is succeeded by *Festuca rubra* ssp. *arenaria*, leading to a mixture with *A. arenaria*. Therefore, it can be concluded that *H. arenaria* occurs on grasses in mobile dunes, whereas *H. hordecalis* is found on grass species more inland, when coastal foredunes become stabilised.

As it had been suggested that specific complexes of soil pathogens are involved in the species succession within the vegetation in coastal foredunes, specific pathogens or parasites are most likely to be key species in these vegetation processes (Van der Putten and Van der Stoel, 1998). The specificity of *H. arenaria*, and its presence in vigorous and early declining stands of *A. arenaria* suggest that, of the observed *Heterodera* spp., *H. arenaria* may be the most likely species involved in the actual degeneration of *A. arenaria*.

Pathogenicity

Within a month after the formation of the first roots of *A. arenaria* in the newly deposited sand layer, colonisation of this layer by soil organisms already resulted in the development of pathogenicity in the bioassay (Chapter 2). As addition of a nematicide to unsterilised soil compensated for most of the growth reduction, the bioassay results suggest that plant-parasitic nematodes were involved in the poor growth of *A. arenaria* in the unsterilised soil. The colonisation of the soil by *H. arenaria* and *Pratylenchus* spp. parallel to the first development of pathogenicity suggested that these species might be involved in the growth reduction in the bioassays and, possibly, also in the degeneration of *Ammophila* in the field. However, in the greenhouse addition of *H. arenaria* to sterilised soil planted with seedlings of *A. arenaria* did not lead to a reduction in plant growth (Chapter 4). Therefore, it could not be concluded that *H. arenaria* as a single species has a major direct role in the poor growth in the bioassays, and in the degeneration of *A. arenaria* in the field. Furthermore, in the field, the root biomass in the newly deposited sand layer continued to increase throughout the growing season (Chapter 2). Therefore, the results from the bioassays may not be directly extrapolated to the field situation (Troelstra *et al.*, 2001). This has important consequences for the view on the length of the period during which *A. arenaria* may escape from its soil pathogens.

With the resulting low expectations of the contribution of *H. arenaria* to the degeneration of *Ammophila*, the role of this nematode as a keystone species in directing vegetation succession, as had been hypothesised by Van der Putten and Van der Stoel (1998), may be small.

In conclusion, the host range of *H. arenaria* is limited to *E. farctus* and *A. arenaria* out of six monocotyledonous species tested, but the nematode only occurs in the mobile dunes. In contrast to many sedentary endoparasitic species that cause severe damage in agricultural crops (Stone, 1977; Baldwin and Mundo-Ocampo, 1991), *H. arenaria* may be considered to be a biotrophic parasite. Instead of reducing their own food source, biotrophic parasites usually show moderate pathogenicity, possibly due to selection towards a lower capacity to suppress resistance genes (Lenski and May, 1994; Jarosz and Davelos, 1995). In order to play a role in the degeneration of *A. arenaria*, *H. arenaria* also should possess a more distinct pathogenicity towards its host.

From the nematode's point of view, two main questions arise, related to these findings. The first question is how *H. arenaria* is able to deal so well with the

yearly sand deposition and how this nematode can keep up with the root growth of *Ammophila*. Secondly, it may be questioned what mechanisms control the population density of *H. arenaria* in the field. In order to answer these questions, the population dynamics of *H. arenaria* were studied in the field and in the greenhouse.

Population development

Both in the field (Chapter 5) and in the greenhouse (Chapter 6) second stage juveniles of *H. arenaria* were capable of migrating to the roots of *A. arenaria*. In the field, already one month after the first roots had been formed in the newly deposited sand layer cysts and males were observed, suggesting that the roots were colonised by juveniles immediately after formation.

In the field, *H. arenaria* cysts were produced both in the new root layer and in the one-year-old root layer. Based on the estimation of the numbers of eggs per 100 gram soil in autumn, higher numbers were observed in the new root layer than in the one-year-old and the two-year-old root layers (Chapter 5). However, it could not be established whether the chance of success was larger for the juveniles that had migrated, since it was not possible to calculate which proportion of the emerged juveniles migrated to the new root layer and which proportion remained in the layer where the juveniles had emerged. In the two-year-old root layer, no new cyst-formation has been observed and it may be questioned whether the remaining contents of the cysts present in this root layer (about 10% of the original contents) still contribute to the population development in the newly formed root layer.

Possible advantages of migration

As the majority of the population increase of *H. arenaria* takes place in the newly formed root layer, the possible advantages of migration were examined. It was discussed whether the possible advantages on the population level were also beneficial for an individual nematode. In the field, the development of new cysts in the newly colonised sand layer tended to start earlier than in the one-year-old layer (Chapter 5). Also in the greenhouse, the juveniles from the mothers that had migrated prior to cyst formation emerged earlier than the juveniles from the cysts of the one-year-old root layer (Chapter 6). Early development may enhance the possibility of the formation of a second generation within the same year, which has been found to occur in other *Heterodera* species (Cook, 1982; Von Mende and McNamara, 1995). Another possible advantage of early migration may occur in the

competition between early migrating *H. arenaria* juveniles and later migrating individuals of the same species or of another nematode species for favourable sites on the roots (Eisenback, 1985). This will be the case if the availability of such sites is limited.

The cysts that were formed on the roots in the newest root layer contained more eggs than the cysts in the one-year-old root layer (Chapter 5). Although it was not possible in the one-year-old root layer to distinguish between the newly formed cysts and the one-year-old ones, the contents of the best filled cysts collected from the one-year-old root layer, were smaller than the contents of the best filled cysts of the new root layer (Chapter 6). The daughter-cysts produced from mothers originating from the two different layers did not contain different numbers of eggs (Chapter 6). However, the contents of the daughters produced by the mother-cysts that were formed after migration seemed to be more plastic in their response to unfavourable conditions *i.e.* when aphids had infested the *A. arenaria* plants. Cysts containing more eggs may potentially produce more offspring, so also the better filling of the cysts points at an advantage of migration.

Apart from the possible advantages of migration, it may also be costly as dispersal requires energy and involves a higher exposure to natural enemies. Furthermore, desiccation of the upper layer of sandy soil may increase the risk of mortality. Non-migration and formation of cysts in the one-year-old root layer (Chapter 5) may, therefore, also be an evolutionary stable strategy, resulting from opposite selection pressure.

Winter mortality

As no field samples have been collected from January to March, it is not clear what exactly happens during the winter period (Chapter 5). The root biomass in the newest layer strongly decreased and within one year after the cysts were formed, 90 per cent of the contents had been released from the cysts. The final number of newly formed cysts at the end of the growing season (September 1998) was only about 0.4 per cent of the numbers of eggs present the year before (October 1997). As already 60 per cent of the contents of the cysts had emerged between October 1997 and April 1998, and no newly formed cysts had been found yet in April 1998, there should be a high mortality during the winter period. A high natural mortality may be a possible explanation for the high mortality during winter, but also top-down effects for instance due to parasitism on the nematodes may affect the population density. From other systems, it is known that fungal or bacterial organisms may parasitise the eggs inside the cysts (Kerry, 1993; Chen and

Dickson, 1996; Rao *et al.*, 1997), which causes a reduction of the numbers of eggs. Outside the cyst, emerged juveniles may die during migration or may die after being predated. Although there is considerable potential for top-down effects on nematode numbers (P.C.E.M. De Rooij-Van der Goes, unpubl. results), there is, so far, no direct evidence that such effects limit the population size.

Life history strategy

The development of *H. arenaria* after emergence from the cysts was found to be an ultimately determined process (Chapter 6). Although in absolute numbers more juveniles emerged from cysts originating from the new root layer, the sex ratio of the developed males and females was equal to the sex ratio of the adults developed from juveniles of cysts collected from the one-year-old root layer. In the field, the formation of cysts in the new root layer was found to be a continuous process from about late spring to late autumn (Chapter 5). When root exudates are involved as a cue for hatching, juveniles may emerge from cysts within a period of four to five weeks (Fenwick and Reid, 1953). The continuous formation of cysts, therefore, suggests that the hatching of juveniles is not directly stimulated by, *e.g.*, root exudates. Also the response as observed in Chapter 6, and the emergence of juveniles in November suggest that no specific cue is involved in the emergence of *H. arenaria* juveniles.

In coastal dunes, this life history strategy of continuous formation and an ultimately determined response may be effective because of the relatively high variability in the formation of *A. arenaria* roots in the newly deposited sand layer between years.

Population density control

In contrast to the lack of evidence for top-down effects regulating the population density, there seem to be indications in favour of bottom-up control. In the inoculation experiment in which various densities of *H. arenaria* were added to *A. arenaria* plants, *H. arenaria* did not reduce growth of its host plant (Chapter 4). The lowest inoculation density was already above the maximum carrying capacity for new cysts to be formed, resulting in equal numbers of cysts developed per gram root in all inoculation densities. In the field, during the growing season, the number of cysts per unit of roots was relatively constant (Chapter 5), which suggests that a bottom-up process is the main regulatory factor controlling the population density of *H. arenaria.*

Various mechanisms may explain the results. First, the availability of feeding sites may be limiting. With a given size of the plant, above a certain threshold level of second stage juveniles, a maximum number of cysts may be formed because of intraspecific competition (Eisenback, 1985). Secondly, the juveniles may induce resistance in the plant, a mechanism that has mostly been studied in sedentary endoparasites (Ogallo and McClure, 1996; Huang, 1998). As an effect of induced resistance later juvenile nematodes may still penetrate the root, but fail to develop and reproduce (Huang, 1998). This mechanism may also lead to a certain limited number of cysts produced per unit of root biomass.

In conclusion, given the very low percentage of the content of the *H. arenaria* cysts that is needed to produce new offspring, the control near or inside the plant root, either by the mechanism of resource competition or other plant-related mechanisms, seems to be the most likely way of regulating the population density of *H. arenaria.*

Conclusions

- In outer coastal dunes, *H. arenaria* is specific to *E. farctus* and *A. arenaria*, but its occurrence is limited to the mobile dunes.
- In stabilised foredunes, *H. hordecalis* is associated with *C. epigejos* and occurs in the mixture of degenerated *A. arenaria* and *F. rubra* ssp. *arenaria*, where *H. mani* was also found to occur in one occasion.
- *H. arenaria* has not been found to be pathogenic to *A. arenaria.*
- *H. arenaria* is not a keystone species in the degeneration of *A. arenaria* by direct activity.
- *H. arenaria* can keep up with the growth of *A. arenaria* by migrating to the newly formed root layer.
- An earlier start of development and a higher number of eggs in the cysts in the newly deposited sand layer are indicative of the benefits of migration of *H. arenaria* on the longer run.
- The population density of *H. arenaria* seems to be bottom-up controlled by *A. arenaria*, but considerable mortality takes place even before the plant may affect the nematode density.

Suggestions for further research

In future research it would be interesting to test the effect of other nematode species and combinations with *H. arenaria*, in trying to explain the

degeneration of *A. arenaria*, and the role of plant-parasitic nematodes in foredune vegetation succession. Do nematodes compete with each other or do they have a synergistic effect on the plant? Also other groups of organisms such as pathogenic soil fungi, natural antagonists and arbuscular mycorrhizal fungi have to be included to tackle questions on degeneration and vegetation succession. Can the nematodes serve as a vector for bacteria or fungi enabling these species to infest the plant roots? Or do mycorrhizal fungi protect the roots against penetration of plant-parasitic nematodes or pathogenic fungi? These are all questions that remain to be answered.

In order to understand the population dynamics of *H. arenaria* more completely, the development has to be quantified also throughout the winter period. There do not seem to be plant-originating cues used by the nematodes to start hatching. However, subsequent tests need to be carried out to determine whether attraction over decimetres can be excluded, *e.g.*, by varying the distance in experimental attraction studies and doing tests with and without the presence of the host. In order to study the control mechanisms, the addition of other groups of organisms such as microbial antagonists and predators, as suggested previously, may give insight in their role as control agents. To obtain insight in the mechanisms of bottom-up control of the population density of *H. arenaria*, experiments discriminating between density dependent regulation and regulation by induced resistance have to be carried out.

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SUMMARY

In natural ecosystems hardly any attention has been given to the population dynamics of plant-parasitic nematodes in relation to the development of their host plant. In the present thesis the population dynamics and dispersal of *Heterodera arenaria*, as well as its specificity and pathogenicity on the dominant sand-fixing coastal foredune grass *Ammophila arenaria* have been studied.

Plant-parasitic nematodes of the genus *Heterodera* are sedentary endoparasites that generally have a high degree of host specificity. In outer coastal dunes of northwestern Europe, *Heterodera* spp. have been supposed to be involved in the degeneration of *A. arenaria*. As *A. arenaria* is the most important sand-fixing plant species in the coastal foredunes, degeneration may negatively affect the stability and the functioning of coastal foredunes as natural sea walls.

Specificity and pathogenicity

At various sites along the Dutch coast, samples were collected from the root zones of successional dominant foredune plant species, in order to establish the occurrence and relative abundance of *Heterodera* species. *Heterodera* spp. were found to occur mainly on *Elymus farctus*, *Ammophila arenaria* and on *Calamagrostis epigejos* (Chapter 4). Species identification based on the ITS2 region of the ribosomal DNA by using the molecular technique PCR-Single-Strand Conformational Polymorphism (PCR-SSCP), showed that *Heterodera arenaria* specifically occurred in the mobile dunes in stands of *E. farctus* and of vigorous *A. arenaria*. *Heterodera hordecalis* was observed later in the successional sere, in degenerated stands of *A. arenaria* and near *C. epigejos*, (Chapter 3). On the basis of the ITS2, *H. arenaria* could not be distinguished from *Heterodera avenae*.

It was decided to concentrate the present study on *H. arenaria*, because this species was present when the degeneration of *A. arenaria* starts, usually when sand deposition diminishes or suddenly stops. The specificity of *H. arenaria* as observed in the field was confirmed by an inoculation experiment in the greenhouse with six dominant monocotyledonous foredune species. The nematode was only able to complete its life cycle on *E. farctus* and *A. arenaria* and not on later successional plant species (Chapter 4).

Pathogenicity development against *A. arenaria* was assessed by repetitive sampling of soil from subsequent year-layers of the root zone in the field, and testing the soil in a series of bioassays in the greenhouse (Chapter 2). In the freshly deposited sand layer, initially not containing any harmful soil pathogens,

pathogenicity developed within a month after the layer had been colonised by plant roots and corresponded with the colonisation of the layer by the nematode species *H. arenaria* and *Pratylenchus* spp. In the first phase of the growing season, addition of the nematicide Vydate counteracted the reduced growth of *A. arenaria*, suggesting that *H. arenaria* and *Pratylenchus* spp. were involved in the observed growth reduction. However, direct addition of different densities of *H. arenaria* juveniles to sterilised soil did not cause any growth reduction of the host *A. arenaria* even when the field density was exceeded (Chapter 4).

Later in the growing season, addition of nematicide no longer completely counteracted the growth reduction. The incomplete but positive effect of the nematicide was also observed when *A. arenaria* seedlings were grown in soil collected from a one-year-old root layer and from a degenerating stand of *A. arenaria*. Although the effects of Vydate have not been studied in more detail, the pathogenicity of the complex of soil organisms seemed to build up and possibly also change during the growing season. The results suggest that not only plant-parasitic nematodes, but also other biotic factors, such as pathogenic fungi may be involved in the complex of soil organisms that cause pathogenicity to *A. arenaria*.

In conclusion, *H. arenaria* was found to behave as a biotrophic parasite. In spite its specificity in the mobile dunes on *E. farctus* and *A. arenaria, H. arenaria* is not a keystone species directly involved in the degeneration of *A. arenaria*.

Population dynamics

In the field, soil and root samples were collected at monthly intervals during two growing seasons. The samples were collected from different root layers of vigorous *A. arenaria* in order to study the population dynamics of *H. arenaria* and its dispersal towards newly formed root layers (Chapter 5). Numbers of second-stage juveniles, males and cysts, as well as eggs in the cysts were determined. Both in newly formed and in one-year-old root layers new cysts were formed, albeit that in both growing seasons the highest numbers were observed in the newest root layer. Each year the largest part of the population that produced new cysts appeared to have migrated upwards to the new root layer. In the newly deposited layer, juveniles tended to be present earlier in the growing season and the cysts that developed from the migrated juveniles contained more eggs. Possibly, early release of juveniles could enable the (start of) formation of a second generation in a growing season. These findings suggest that, especially on the long run, it is beneficial for an individual to migrate.

The cysts that were formed in the field originating from a new and from a one-year-old root layer were used in an experiment to study the emergence and migration of juveniles (chapter 6). It was tested whether the next generation, produced by the cysts that were formed after migration to a new root layer, had a better performance than the daughters of the cysts collected from a one-year-old root layer. In these experiments the juveniles from the cysts of the new root layer emerged earliest. The daughters of both groups contained equal numbers of eggs. However, the daughters from the mothers of a new root layer seemed to have a higher plasticity in egg formation.

Although migration seems to be beneficial, only 0.4 per cent of the offspring in a cyst was found to succeed to develop into the next generation (Chapter 5). Furthermore, the process of releasing juveniles and the further development into males and females appeared to be an ultimately determined process (Chapter 5 and 6). Juveniles emerged already in November, which causes a risk of not surviving the winter (Chapter 5). The proportions of the emerged juveniles that developed into males and females were not different between layers (Chapter 6).

The results of chapters 4, 5 and 6 have been used to discuss the natural control of *H. arenaria* in the root zone of *A. arenaria*. The population density of *H. arenaria* is most likely controlled by bottom-up processes. This is suggested by the levelling off pattern of the numbers of cysts of *H. arenaria* cysts formed on the roots of *A. arenaria* that were exposed to an increased number of larvae added (Chapter 4). In addition, the number of cysts per gram root present in the field was fairly constant (Chapter 5) However, it remains an open question what mechanisms are involved.

Many eggs and juveniles did not seem to survive the winter period. The programmed process of release of juveniles from the cysts still may be an effective strategy. The distance between release of the juveniles from the cysts and root formation by the plant in newly deposited sand could be too large for specific cues. As the start of formation of roots in the newly deposited sand layer was found to highly vary in the two years of study, random release of juveniles from the cysts, therefore, seems to be the most efficient strategy when there are no or insufficient specific cues for timing. The relatively early release of the first juveniles from cysts originating from the newest root layer may be indicative of a potential to produce a second generation when the growing season starts early and ends late.

In conclusion, *H. arenaria* is not a keystone species directly involved in the degeneration of *A. arenaria*, so that it is not likely that *H. arenaria* will negatively

affect the stability and functioning of the outer coastal dunes as natural sea walls. The present study on the population dynamics of *H. arenaria* as a specific endoparasitic nematode on *A. arenaria* contributes to the general understanding of relations between plant-parasitic nematodes and their hosts in natural plant communities.

In onderzoek aan natuurlijke plantengemeenschappen is relatief weinig aandacht besteed aan de populatiedynamica van bodemorganismen, zoals plantenparasitaire nematoden. Over de relatie tussen plantenparasitaire nematoden en hun natuurlijke waardplant is ook relatief weinig informatie bekend. In dit proefschrift is de populatiedynamica, de verspreiding, de specificiteit en de pathogeniteit bestudeerd van de nematode *Heterodera arenaria* ten opzichte van mogelijke waardplanten. Deze plantenparasitaire nematode komt o.a. voor bij *Ammophila arenaria* (helm), de belangrijkste natuurlijke zandvastleggende grassoort in de buitenduinen langs de kust van noordwest Europa.

Plantenparasitaire nematoden die tot het geslacht *Heterodera* behoren, zijn sedentaire endoparasieten die behoren tot de zogenaamde cystenvormende nematoden. Door hun grote afhankelijkheid van en hun veelal nauwe relatie met de plant zijn *Heterodera* soorten over het algemeen specifiek en hebben ze weinig waardplanten. In eerder onderzoek naar de oorzaken van vermindering van de vitaliteit van helm (degeneratie) in buitenduinen werden *Heterodera* soorten gevonden, waardoor werd verondersteld dat deze nematoden betrokken zijn bij de degeneratie van helm als de aanvoer van vers strandzand stagneert. Aangezien helm belangrijk is bij het vastleggen van zand, kan degeneratie van helm de stabiliteit van de duinen verminderen, wat een gevaar zou betekenen voor de veiligheid van de Nederlandse kust.

Specificiteit en pathogeniteit

Om vast te stellen of *Heterodera* algemeen voorkomt langs de Nederlandse kust zijn grondmonsters en plantenwortels verzameld op verschillende plaatsen langs de kust. Op elk van de locaties zijn de belangrijkste in successie voorkomende plantensoorten in de buitenduinen bemonsterd. Op alle locaties werden *Heterodera* soorten aangetroffen, die hoofdzakelijk voorkwamen op *Elymus farctus* (biestarwegras), helm en *Calamagrostis epigejos* (duinriet) (hoofdstuk 4). Met behulp van de moleculaire techniek PCR-SSCP (PCR-Single-Strand Conformational Polymorphism) zijn de voorkomende *Heterodera* soorten geïdentificeerd en vergeleken met bekende *Heterodera* soorten (hoofdstuk 3). *Heterodera arenaria* komt alleen voor op biestarwegras en op vitale helm in het dynamische deel van de duinen. *Heterodera hordecalis* werd later in de successie aangetroffen, rondom gedegenereerde helm en duinriet. Ondanks het goede onderscheidende vermogen van PCR-SSCP, bleek het niet mogelijk te zijn

onderscheid te maken tussen *H. arenaria* en *Heterodera avenae*, een soort die voorkomt op *Avena sativa* (haver).

In de huidige studie werd besloten verder te gaan met *H. arenaria*, omdat deze soort in het veld aanwezig is op de plaats waar de vitaliteit van helm begint af te nemen, terwijl *H. hordecalis* alleen aanwezig is als helm nauwelijks meer groeit en wordt verdrongen door de opvolgende plantensoort.

De in het veld aangetroffen specificiteit van *H. arenaria* werd eveneens waargenomen in een experiment waarin *H. arenaria* werd toegevoegd aan zes plantensoorten, allen grasachtigen, die allen in een deel van de successie van de buitenduinen dominant zijn. Het bleek dat *H. arenaria* alleen op biestarwegras en op helm in staat was zich te ontwikkelen van juveniel tot een volwassen cyste. Op de overige plantensoorten was de ontwikkeling van *H. arenaria* niet volledig (hoofdstuk 4).

De ontwikkeling van pathogeniteit tegen helm werd getoetst door maandelijks monsters te verzamelen uit de wortelzone van helm. Uit wortellagen van verschillende leeftijden, gekoppeld aan de groeiseizoenen, werden de monsters verzameld die vervolgens gebruikt werden in een maandelijks terugkerende biotoets in de kas (hoofdstuk 2). In de vers overstoven zandlaag waarin zich in eerste instantie nauwelijks bodemorganismen bevinden, die gedurende de laatste winterperiode was vastgelegd door helm, ontwikkelde de pathogeniteit in de kas zich binnen een maand nadat de eerste wortels in het veld gevormd waren. Het moment van optreden van pathogeniteit correspondeerde met het moment waarop *H. arenaria* en *Pratylenchus* spp. (wortellesienematode) deze zandlaag in het veld koloniseerden. In de eerste maanden van het groeiseizoen kon de groeireductie worden opgeheven door toediening van het nematicide Vydate. Dit suggereert dat plantenparasitaire nematoden een negatief effect hebben op de groei van helm. Echter, in een experiment waarin juvenielen van *H. arenaria* in verschillende dichtheden werden toegediend aan helmplanten in steriele grond, trad er geen groeireductie op in helm, terwijl de hoogste inoculatiedichtheid de velddichtheid ruim overschreed (hoofdstuk 4).

Later in het groeiseizoen werd de groeireductie in het niet-gesteriliseerde zand niet meer volledig gecompenseerd door de toediening van het nematicide. Ondanks het feit dat er geen volledige compensatie optrad, verminderde de toevoeging van het nematicide de groeireductie. In de potten met zand verzameld in de andere wortellagen (een 1-jaar-oude wortellaag rondom vitale helm en de bovenste wortellaag rondom gedegenereerde helm) werden dergelijke resultaten gedurende het hele groeiseizoen gevonden. De resultaten suggereren dat gedurende

het groeiseizoen de pathogeniteit van het complex aan bodemorganismen toeneemt en dat niet alleen plantenparasitaire nematoden groeireductie veroorzaken, maar dat ook andere biotische factoren, zoals bijvoorbeeld pathogene bodemschimmels, betrokken kunnen zijn in het complex aan bodempathogenen dat een rol speelt in de degeneratie van helm.

Naar aanleiding van de voorgaande veldwaarnemingen en experimenten wordt geconcludeerd dat *H. arenaria* specifiek voorkomt op biestarwegras en vitale helm in het dynamische gebied van de duinen, maar dat *H. arenaria* door de geringe pathogeniteit niet direct betrokken is bij de degeneratie van helm.

Populatiedynamica

In het veld zijn gedurende twee groeiseizoenen maandelijks grondmonsters en plantenwortels verzameld uit verschillende wortellagen van helm, die ieder het jaar vertegenwoordigen waarin ze in eerste instantie zijn gevormd. Aan de hand van deze monsters is de populatiedynamica van *H. arenaria* bestudeerd en is nagegaan hoe *H. arenaria* zich over de verschillende wortellagen verspreidt (hoofdstuk 5). Hiertoe zijn de aantallen juvenielen in het tweede juveniele stadium, de volwassen mannetjes, de cysten en de aantallen eieren en juvenielen in de cysten bepaald. In de nieuwste en in de 1-jaar-oude wortellaag werden nieuwe cysten gevormd, waarbij de grootste aantallen waargenomen zijn in de nieuwste wortellaag. In beide jaren vond migratie plaats naar de nieuwste wortellaag waar, afhankelijk van het jaar waarin de bemonstering is uitgevoerd, het groeiseizoen vroeger of later op gang kwam. De cysten die na de migratie van de juvenielen naar de nieuwste laag werden gevormd, bleken beter gevuld te zijn met eieren. De resultaten suggereren dat het, zeker op langere termijn, voor de individuele nematode gunstig is om naar de nog te koloniseren zandlaag te migreren.

In twee experimenten zijn vervolgens cysten gebruikt die afkomstig waren uit de nieuwste wortellaag of uit de 1-jaar-oude laag. Met deze experimenten werd beoogd het uitkomen van de juvenielen uit de cysten en hun verspreiding te bestuderen (hoofdstuk 6). Getoetst werd of de juvenielen van de cysten verzameld uit de nieuwste laag (gevormd na migratie) eerder uit de cyste kwamen, en of de dochtercysten uit de gemigreerde juvenielen zelf ook grotere aantallen eieren bevatten dan de dochters van de cysten uit de 1-jaar-oude wortellaag. Het bleek dat de juvenielen eerder uit de moedercysten tevoorschijn kwamen die verzameld waren in de nieuwste wortellaag. Echter, het aantal eieren in beide groepen dochtercysten verschilde niet. Het leek er wel op dat de dochters van de moeders

uit de nieuwste wortellaag beter in staat waren hun aantallen eieren aan te passen aan omgevingsfactoren.

Slechts 0,4 procent van de nakomelingen ontwikkelt zich uiteindelijk tot volwassen cyste in het veld (hoofdstuk 5). Het uitkomen van de juvenielen uit de cysten en de verder ontwikkeling tot mannetje of cyste bleek volgens een vast patroon te verlopen (hoofdstukken 5 en 6). Ondanks de reële kans om de winter niet te overleven als in het juveniele stadium, werden in november in het veld juvenielen waargenomen (hoofdstuk 5). Daarnaast bleek dat een vast percentage van de uitgekomen juvenielen zich tot mannetjes en cysten ontwikkelden, ongeacht de wortellaag waaruit de moeders afkomstig waren (hoofdstuk 6).

De resultaten uit de hoofdstukken 4, 5 en 6 zijn vervolgens gebruikt om de manier waarop de natuurlijk voorkomende aantallen *H. arenaria* op helm gecontroleerd worden te bediscussiëren. De min of meer gelijke aantallen cysten die op de wortels van helm gevormd werden, ondanks het toedienen van verschillende dichtheden (hoofdstuk 4) en de min of meer constante aantallen cysten per gram wortel in het veld (hoofdstuk 5) suggereren dat de populatiedichtheid van *H. arenaria* gecontroleerd wordt door de plant. Het is echter niet duidelijk welke mechanismen hierbij een rol spelen.

Ondanks de grote sterfte gedurende de winter kan het continue proces van uitkomen van juvenielen een effectieve overlevingsstrategie zijn. De afstand die de juvenielen moeten overbruggen om bij de nieuwe wortels te komen, kan zodanig groot zijn dat eventuele specifieke plantenstoffen niet worden waargenomen. Bovendien lijkt de start van de vorming van nieuwe wortels in de vers overstoven laag sterk te variëren tussen opeenvolgende jaren. Hierdoor lijkt het continu en verspreid in de tijd uitkomen een betere kans te bieden aan juvenielen om de gastheer te vinden dan proberen af te gaan op signaalstoffen die wortelvorming aanduiden. Het vroege uitkomen van de juvenielen uit de cysten, die verzameld zijn in de nieuwste wortellaag, zou kunnen duiden op de mogelijkheid om een tweede generatie binnen een jaar te vormen als de lengte van het groeiseizoen dat toestaat.

Geconcludeerd kan worden, dat *H. arenaria*, alleen, geen directe negatieve effecten veroorzaakt die zullen leiden tot de degeneratie van helm, waardoor *H. arenaria* ook geen directe negatieve gevolgen zal hebben voor de veiligheid van de Nederlandse kust. Daarnaast draagt deze studie aan de populatiedynamica van *H. arenaria* als specifieke endoparasitaire nematode rondom helm bij aan het begrip over de relatie tussen plantenparasitaire nematoden en hun gastheer in natuurlijke plantengemeenschappen.

Daar zit ik dan..... De klus is geklaard, en ik ben daar op z'n zachtst gezegd erg blij mee. Zover was ik niet gekomen als ik het allemaal op eigen kracht had moeten doen. Een aantal mensen wil ik dan ook zeker bedanken!

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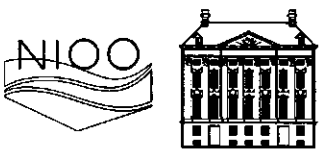
CURRICULUM VITAE

Christine Dieuwke (Ineke) van der Stoel werd geboren in Barendrecht op 16 juli 1971. Zij behaalde haar Gymnasium diploma in 1989 aan de scholengemeenschap 'Johannes Calvijn' te Rotterdam. In dat zelfde jaar begon zij aan de opleiding Plantenziektekunde aan de Landbouwuniversiteit Wageningen. Binnen de specialisatie ecologie en epidemiologie verrichte zij drie afstudeervakken en een buitenlandse stage voor de vakgroepen Entomologie en Nematologie. Voor Entomologie hield zij zich bezig met onderzoek naar de bestrijding van spintmijt en de geurrespons van roofmijten op de door spintmijt aangetaste planten. Vervolgens verbleef zij een half jaar in Nieuw Zeeland waar ze de mogelijkheid onderzocht om sluipwespen in te zetten ter bestrijding van wespen. Aan de vakgroep Nematologie wijdde zij haar eerste nematologische afstudeervak aan de bestrijding van het aardappelcysteaaltje met behulp van een bodemschimmel. Het tweede nematologische onderzoek werd uitgevoerd aan het toenmalige Proefstation voor de Akkerbouw en Groenteteelt in de Vollegrond in Lelystad (nu Praktijkonderzoek Plant en Omgeving). Tijdens dat onderzoek werd getoetst of nematoden ingezet konden worden om naaktslakken te bestrijden.

Direct na het voltooien van haar universitaire opleiding, in september 1995, trad zij in dienst bij het Nederlands Instituut voor Oecologisch Onderzoek in Heteren, bij de werkgroep Plant Micro-organisme Interacties. De resultaten van het promotieonderzoek zijn beschreven in dit proefschrift.



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