EDUARDO DE LA PEÑA

# INTERACTIONS BETWEEN MARRAM GRASS (*AMMOPHILA ARENARIA*), ROOT-LESION NEMATODES AND PLANT MUTUALISTS IN COASTAL DUNES

THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR (PHD) IN APPLIED BIOLOGICAL SCIENCES

In memory of my grandfather Fernando who from the summits of the Iberian mountains opened my eyes to Nature

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**COVER FIGURE:**

VIGOROUS STANDS OF *AMMOPHILA ARENARIA* SSP. *ARUNDINACEA* IN THE DUNES OF THE NATURE RESERVE OF COMPORTA, PORTUGAL.

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# Chapter 1

*General introduction*

Soil-borne pathogens, herbivores and plant-mutualists have a remarkable influence on the dynamics, temporal and spatial, of natural plant communities (Reinhart *et al.* 2003; De Deyn *et al.* 2004; Wardle *et al.* 2004). The interaction between plant roots, animals and microbes determine what, where and how things grow in plant communities (Copley 2000). On the other hand, plants selectively affect soil organisms, thereby establishing feed-back interactions between plants and the soil community (Bever *et al.* 1997; Viketoft *et al.* 2005). These feed-backs, either positive or negative, can have a direct impact on the further performance of the plant, the offspring or competing plant species. However, the knowledge on how belowground organisms interact is scarce when compared to the aboveground scenario. In order to have a good understanding of how soil biota affect plant community structure it is necessary to study the mechanisms of interaction between plants, their mutualists, herbivores and pathogens.

Herbivory is a key factor that affects the structure and composition of plant communities (Fraser & Grime 1999; Hamback & Beckerman 2003; Schadler *et al.* 2003). It acts either as a factor causing mortality of individual plants or as an agent influencing the competitive abilities among coexisting plant species (Verschoor *et al.* 2002). Albeit nematodes are the most abundant metazoan phylum on earth in terms of species richness and abundance, their effect on plant performance and its consequences on ecosystem functioning and structure has not been acknowledged until relatively recently (Hunt & Wall 2002; De Deyn *et al.* 2003; Bezemer *et al.* 2005). Plant-parasitic nematodes (PPN) are one of the major groups of belowground herbivores. This group is numerically abundant in terms of biomass and diversity in different natural systems; from mountainous habitats, prairies and grasslands till more extreme environments such as the sub-artic tundra and warm deserts (Steinberger *et al.* 2001; Talavera & Navas 2002; Hoschitz & Kaufmann 2004).

Some groups of PPN are notorious pests in economically important crops worldwide (Schomaker 2006). The presence of high densities of PPN can be linked to a decrease in crop yield, not only by the direct effect of nematode population density but also by their interactions with other soil-borne pathogens (McSorley & Gallaher 1993b; Wheeler *et al.* 1994; Taylor & Rodriguez-Kabana 1999). However, the effect, direct or indirect, of PPN on natural plant communities is not so clear. Some authors point at a decrease in primary production (Stanton *et al.* 1981) whereas some other indicate the contrary (Ingham & Detling 1990, 1991), depending on the density of PPN and root growth.

If herbivores are playing a major role in terrestrial ecosystems, the question what is controlling herbivores becomes relevant. Therefore, one of the main issues in ecology is to elucidate what kind of forces control herbivores (Hairston 1960; Hassell *et al.* 1998; Moon & Stiling 2002). The regulation of herbivores within a given system depends on the combination of abiotic and biotic factors. Leaving aside abiotic factors, herbivores are controlled by: (i) bottom-up forces or resource limitation, (ii) by top-down forces or natural enemies and (iii) by competition. Bottom-up forces control herbivores when the food source is limited either in terms of quantity, as a consequence of the size of the population and the amount of food available, or in terms of quality, by the action of secondary metabolites that alter the palatability of the plant for the herbivore. Top-down forces are the result of predation exerted by natural enemies upon the herbivore population. Finally, competition occurs when a set of species exploit the same food source and therefore interfere with each other.

Traditionally, the study of PPN (basically in Plant Nematology) has focused on understanding the effect of nematodes in agricultural systems. The main research goals are therefore: (i) to develop correct nematode identification techniques, (ii) to assess density-yield ratios for different species-crops combinations (iii) to obtain a better understanding of plant-nematode interactions and (iv) to develop strategies to control nematode pests by the use of chemicals, cultural practices, rotation and the use of resistant cultivars and biocontrol agents. However, the mechanisms of control of PPN in natural systems and its integration with the community aboveground have been only recently begun to be considered (Van der Putten *et al.* 1993; De Deyn *et al.* 2003; Wardle *et al.* 2005). New insights into these ecosystems might provide new approaches in biological pest control development and contribute to understanding soil-plant feed backs, nematode interactions and eventually, plant community structure.

Sand dunes act as a natural barrier to protect the inland from the forces of sea tides and waves. A succession, in time and space, of plant species is observed as a consequence of shifts in the biotic and abiotic factors from the seaside inwards. Coastal vegetation stabilizes wind-blown sand. *Ammophila arenaria* L. (Link) is the dominant plant species in coastal foredunes of the European Western Coast. A rich nematode diversity including sedentary endoparasitic nematodes (e.g. *Heterodera*, *Meloidogyne*) migratory endoparasitic nematodes (e.g. *Pratylenchus*, *Pratylenchoides*), ectoparasites (e.g. *Tylenchorhynchus*, *Telotylenchus*) in combination with arbuscular mycorrhizal fungi (AMF), fungal endophytes, fungal pathogens and antagonistic microorganisms (e.g. *Pasteuria penetrans, Pochonia chlamydosporia)* are

present in dunes soils associated with this pioneer grass. This complex of soil organisms is involved in the degeneration of *A. arenaria* in stabilized dunes and contributes to the natural process of primary succession within the system. In the last decade the relationship between *A. arenaria* and its rhizosphere organisms, including PPN has received considerably attention and two overlapping tendencies are noticed: (i) the interest of ecologists attempting to understand the effects of root symbionts, pathogens and herbivores on the vegetation (Van der Putten *et al.* 1993; Kowalchuk *et al.* 1997; Greipsson & El-Mayas 2002) (ii) the interest of field ecologists to preserve coastal sand dune ecosystems from either environmental or human induced stresses (e.g. biological invasions) (Seliskar & Huettel 1993; Hertling & Lubke 2000; Knevel *et al.* 2004). As a consequence, the composition and effect of the different groups of soil organisms, AMF, nematodes, bacteria, pathogenic fungi on the dominant vegetation has been analyzed in some cases. However, how all these groups interact has been acknowledged and suggested, but scarcely explored.

The natural densities of PPN associated with *A. arenaria* in foredunes reach levels below those observed in experimental conditions and agricultural systems (De Goede 1998); suggesting the occurrence of different mechanisms of control acting on the nematode community in foredunes. Plant defence, plant symbionts (AMF and fungal endophytes), and natural enemies (predatory mites and nematodes, bacteria and soil fungi) are the major candidates to be considered as the controlling agents of nematodes. However, the action of these control agents has been experimentally tested only in a few cases (Little & Maun 1996; Greipsson & El-Mayas 2002). In these studies the overall effect of several nematode species and AMF has been considered. However, in order to have a good understanding of how these soil organisms affect each other, a complementary approach in which the relationships between interacting organisms are singled out is needed.

Root-lesion nematodes (*Pratylenchus* spp.) are a frequent component of nematode communities in crops (Potter & McKeown 2003; Smiley *et al.* 2004), in temperate grassland ecosystems and also in coastal dunes (De Goede 1998). These nematodes multiply within root tissues of the host plant; causing root necrosis, secondary infections by pathogenic fungi and in consequence, growth reduction (yield loss) (Todd & Oakley 1996; Taylor *et al.* 1999). Different species of *Pratylenchus* such as *P. brzeskii* and *P. penetrans* (Van der Putten *et al.* 2005) are known to parasitize *A. arenaria* or related species (*A. breviligulata* Fern.). These species most likely present a variable degree of specificity as inferred from their distribution and host range. Modifying agents of plant (resource) quality and quantity such as the intrinsic genetic variation of host populations or by the mediation of plant mutualists might affect the

interaction between *Pratylenchus* and the host plant. Although, several aspects of the biology of this genus in coastal dunes have been studied in the past years (e.g. effect of interspecific competition, population dynamics) (Van der Stoel *et al.* 2002b; Brinkman 2004), different issues remain unknown such as the geographic distribution of the genus in coastal dunes, the effect and multiplication on the host plant and the interaction with other rhizosphere organisms.

The aim of this study was to analyze the interaction between *A. arenaria* and migratory endoparasitic nematodes using *Pratylenchus* spp. as model, and to explore the bottom-up control of these nematodes by the host plant or mediated by its plant mutualists. Hence, this study was undertaken with the following specific objectives:

- To asses the *Pratylenchus* species diversity and distribution associated to *A. arenaria* in Western Europe foredunes.
- To characterize morphologically and molecularly the different *Pratylenchus* populations associated with *A. arenaria* in Western European dunes.
- To study the effect and multiplication of *Pratylenchus* spp. on *A. arenaria*.
- To study the host suitability by comparing the multiplication of different *Pratylenchus* species on *A. arenaria* genotypes and other dune grasses.
- To analyze the interaction between *Pratylenchus* spp., *A. arenaria* with its symbionts: (i) arbuscular mycorrhizal fungi and (ii) fungal endophytes.

# Chapter 2

## **Ammophila arenaria** *in relation to soil organisms*

In the past years the interaction between dune plants (especially *Ammophila* spp.) and soil biota has become a prominent research field. As it has been briefly described in Chapter 1, the rhizosphere of dune sand contains a wide diversity of organisms. This review chapter analyzes the different ideas so far explored, the consequences and relationships with ecological research and also, in relation to this thesis.

## 2.1 Diversity and distribution of *Ammophila arenaria*

*Ammophila arenaria* (L.) Link or marram grass is a clonal species occurring naturally on foredunes along the European and North African coastline (Tutin 1980). It is commonly used as a sand fixing species in areas where coastal foredunes act as natural barriers against the forces of the sea tides. Therefore, it has been introduced for this purpose to North America (Knutson 1978), Australia (Mitchell 1974), New Zealand, the Falkland Islands (Wiedemann 1987) and South Africa (Hertling & Lubke 2000). However, *A. arenaria* becomes a highly invasive species outside of its natural range (Hertling & Lubke 1999; Beckstead & Parker 2003). Botanists recognize two subspecies of *A. arenaria* within the natural range based on the geographical distribution of the plant and differences in morphological characteristics (e.g. seed, leaf and root morphology). *Ammophila arenaria* ssp. *arenaria* is the subspecies present along the European North Atlantic coast, whereas at southern Atlantic latitudes (North and West coastline of the Iberian Peninsula) and in the Mediterranean, *A. arenaria* ssp. *arundinacea* is the subspecies present (Tutin 1980).

## 2.2 Ecology of *Ammophila arenaria*

In habitats with constant sand inundation such as coastal sand dunes, burial imposes a strong abiotic stress that, in combination with other intrinsic soil characteristics such as low nutrient content, high water drainage, sand blast and root aeration, impose forceful physiological constraints on dune plants (Maun 1998). In coastal dunes, plant species have adapted genetically, morphologically and physiologically to withstand these conditions. *Ammophila arenaria* not only is able to resist the adverse effects of sand burial, but it needs a regular level of sand accretion in order to maintain vigorous growth (Huiskes 1979). It can tolerate burial up to 1.5 m per year (Wiedemann 1987). Because the capacity of the plant to withstand sand burial and to form a long and dense root system (Maun, 1997) both *A. arenaria* subspecies, together with the North American homologous species *A. brevilugulata* Fern., are commonly used to stabilize coastal foredunes.

Sand deposition occurs usually throughout the autumn and early winter. During this period, *A. arenaria* grows upwards by means of stem elongation and forms nodes in the freshly deposited sand layers (Baye 1990) (Fig 2.1). During the spring, new roots develop and spread horizontally from the winter-formed nodes. Finally, during the summer, shoot (leaves) production increases. This growth pattern is repeated on a yearly basis. Nevertheless, once sand burial ceases, degenerated *Ammophila* stands that present shorter culms and a higher number of dead shoots appear (Huiskes & Harper 1979; Van der Putten 1988). Roots are then formed in old sand layers where root-pathogens and soil-herbivores accumulate in the rhizosphere. Eventually, *A. arenaria* declines and is gradually replaced by other competing plant species (Fig 2.2).

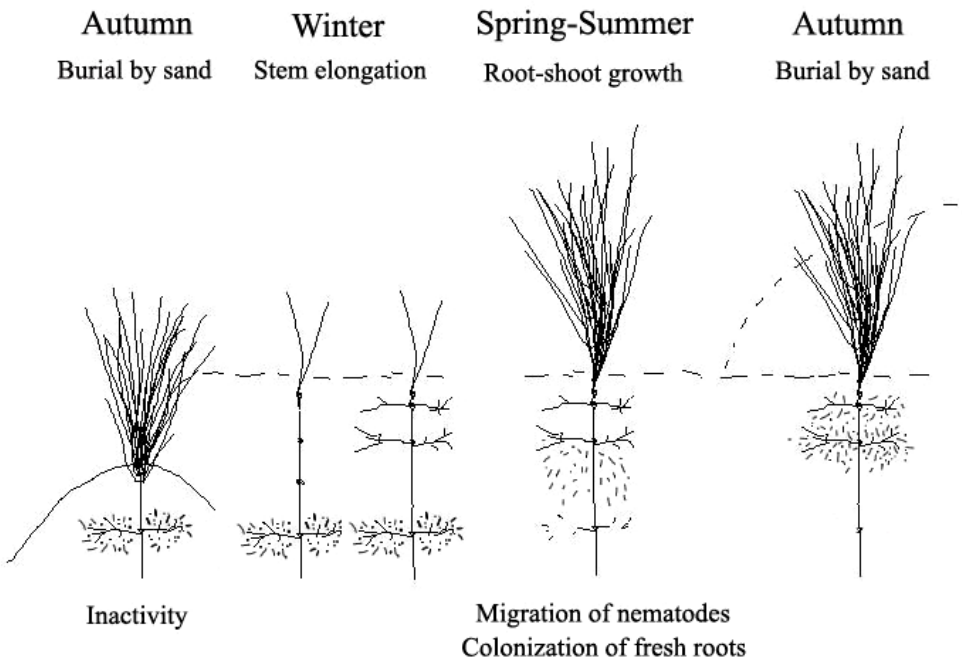


**Fig 2.1** Schematic representation of *Ammophila arenaria* growth as a function of the yearly sand burial and the migration and development of soil organisms. Adapted from De Rooij- Van der Goes (1996).

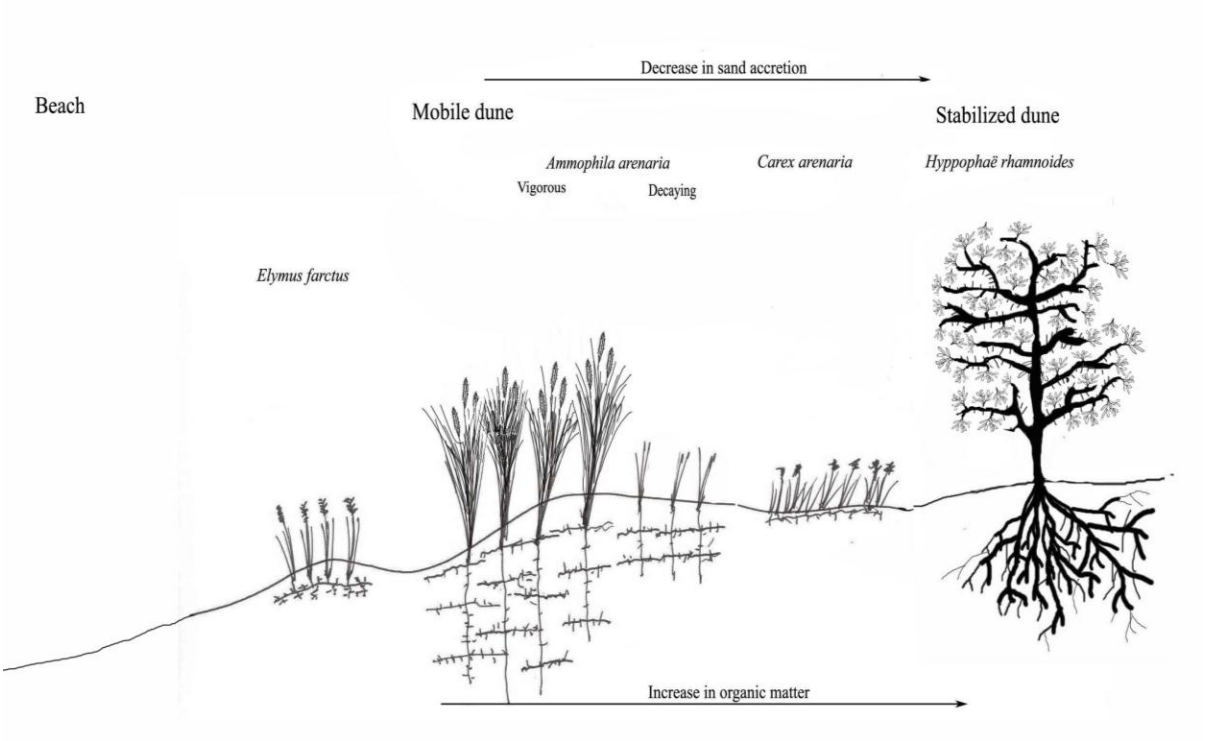


**Fig 2.2** Schematic view of vegetation succession on a North Atlantic coastal dune, changes in vegetation are linked to variation in soil properties and aboveground sand accretion.

## 2.3 The *Ammophila* problem

The dependency of *A. arenaria* on continuous sand deposition and the subsequent die-out in the lack of it, has been called the *Ammophila* problem (Little & Maun 1996; Wiedemann & Pickart 1996). Although a metanalysis of the different factors involved has not been considered yet, the underlying mechanism of the *Ammophila* problem has been the subject of active debate. Different hypotheses are proposed to explain this process:

i) Sand burial provides the plant with a new income of nutrients (Willis 1965; Day *et al.* 2004).

ii) Sand-burial increases the physiological activity of the plant and promotes elongation (Yuan *et al.* 1993; Voesenek *et al.* 1998).

iii) Sand burial allows *Ammophila* to avoid ageing by developing new roots in fresh sand layers because in the absence of sand accretion old roots can not be replaced by new ones or are less efficient (Wallen 1980).

iv) Sand burial provides the plant with a temporal escape from pathogens accumulating in the soil (Van der Putten 1988). Therefore, fresh layers of sand provide an enemy free environment beneficial for plant growth.

Whether these hypotheses fully explain the *Ammophila* problem or not, changes in dominant vegetation are a natural step in plant succession caused by the common action of biotic and biotic factors acting at different scales (Bach 1994; Jones *et al.* 2004; Munoz-Reinoso & de Castro 2005).

### 2.4 *Ammophila arenaria* and soil-borne organisms

The soil (sand) and the rhizosphere of plants in coastal dunes contain an ample variety of organisms that interact with the plant. Depending on the sign of these interactions, negative or positive, three groups can be categorized: plant mutualists that benefit the host plant, and soil-borne pathogens and root herbivores that affect plant growth negatively.

The presence/absence of plant mutualists affects the growth of *A. arenaria* and other plant species. In general terms, plant mutualists either help the host to overcome the deficiency of nutrients by the facilitation of nitrogen and phosphorous capture in this type of soil (Dalton *et al.* 2004; Hamel 2004). Endophytic bacteria help *A. arenaria* in its nitrogen uptake (Hassouna & Wareing 1964; Rudgers & Maron 2003; Dalton *et al.* 2004) and may also protect the plant from other soil-organisms (De Boer *et al.* 1998a). Another group of organisms that affect positively plant growth in coastal dunes are arbuscular mycorrhizal fungi (AMF). The relationship of AMF, plant succession (Gemma & Koske 1997) and the *Ammophila* die-out has been suggested (Little & Maun 1996) and differences in the AMF diversity and identity, between healthy and decaying stands of *A. arenaria*, have been observed (Kowalchuk *et al.* 2002). These differences in AMF can have consequences for plant health since the functional diversity of AMF taxa affects in different ways plant performance (Van der Heijden *et al.* 2003). However, plants might adapt their dependence on AMF according to soil nutrient properties. Consequently, it is not possible to link definitely shifts in the AMF community to the changes in vegetation.

Alternatively, the vigour of dune plants has been linked not only to plant mutualists, but also to the incidence of other soil-borne organisms, either plant pathogenic fungi or root-herbivores (e.g. plant-parasitic nematode (PPN)). Plant pathogenic fungi are present in the roots and rhizosphere of *A. arenaria* in both, vigorous and decaying stands (Dennis 1983; de Rooij-van der Goes *et al.* 1995b). However, the decline of the plant is not entirely explained by the direct inoculation of pathogenic fungi on *A. arenaria* plants (de Rooij-van der Goes *et al.* 1995a; De Boer *et al.* 1998b). In consequence, the effect of fungi can not be dissociated from other organisms and thus, must be enhanced by other soil components such as plant-parasitic nematodes (Greipsson & El-Mayas 2002).

## 2.5 Plant-parasitic nematodes and plant succession in coastal dunes

Plant-parasitic nematodes (Phylum Nematoda) are usually small 0.5mm-1.5mm, vermiform and translucent animals. They are well known organisms in agricultural systems because they are one of the major groups of pests causing yield loss in many important crops (Koenning *et al.* 1999; Belair 2005). They either induce yield reduction directly by damaging the roots, or induce secondary infections by other soil borne pathogens (e.g. pathogenic fungi) (Back *et al.* 2002). Plant-parasitic nematodes represent also a significant percentage of the soil herbivore fauna (Bongers & Ferris 1999) and, according to the different feeding habits and the degree of penetration in the root are classified into three categories (Yeates 1993): (i) sedentary endoparasitic nematodes, (ii) migratory endoparasitic nematodes and (iii) ectoparasitic nematodes.

Sedentary endoparasitic nematodes (e.g. *Heterodera, Meloidogyne*) show a very close relationship with the host plant and present a sessile stage in their life cycle strictly linked to the plant. This kind of nematodes has developed resistant structures to protect their offspring (e.g. cysts and egg masses). Migratory endoparasitic nematodes on the other hand (e.g. *Pratylenchus*) remain mobile throughout their entire life cycle and come in an out of the roots at any developmental stage. Finally ectoparasitic nematodes do not penetrate inside the roots and feed superficially with the aid of long stylets.

Dune areas, due to the changing conditions of soil, regular flooding and the particular vegetation, are an interesting habitat from a taxonomic and ecological point of view. Thereby, the relationship between nematode communities, soil factors and dominant plant species has been studied in this habitat (Goralczyk 1998; Verhoeven 2002; Wall *et al.* 2002). Moreover, several species of different groups of PPN have been described from *Ammophila* spp. or dune soils (s'Jacob 1966; Handoo *et al.* 1993; Robinson *et al.* 1996; Karssen *et al.* 1998b). Contrary to what happens with some nematode pests in agricultural systems (Trudgill 1997), PPN in coastal dunes seems to have a narrow host range (Van der Stoel 2001).

Coastal dunes provide also one the few examples that illustrate the influence of PPN in natural systems (Orion *et al.* 1982; Maas 1983; Zoon *et al.* 1993). In these pioneer papers the decline of plant species (*Hyppophäe rhamnoides*) in stabilized dune stages was linked to the effect of PPN. As a consequence, the decline of *A. arenaria* was also studied in relation to PPN (Van der Putten 1988; Van der Putten 1993). Soil-borne pathogens, PPN among them, colonize freshly deposited sand-layers following the growth of *A. arenaria* (Fig 2.1) and thus, the population dynamics of PPN is related to the yearly growth of the plant (De Rooij-Van der Goes 1998). The colonization of the new layer is sequential and a shift in nematode

species is observed throughout the growth of the plant. Sedentary endoparasitic nematodes (*Heterodera* and *Meloidogyne*) and then, migratory endoparasitic nematodes (*Pratylenchus*) are among the different PPN that colonize *Ammophila* roots at first instance. It was furthermore shown experimentally that this complex of PPN that colonize *A. arenaria*, negatively affects the host plant (Van der Stoel *et al.* 2002). This fact, points to a feed-back process by which, in the rhizosphere of *A. arenaria*, a complex of organisms develops that in the long term hampers the host plant and benefit competing plant species (Van der Putten & Van der Stoel 1998).

Obviously, the genus *Pratylenchus* is part of the nematode complex that follows *A. arenaria* root growth. As endoparasites they invade, move, feed and multiply within the root cortex of the host plant causing necrotic lesions in the roots and facilitate infections by soil-borne fungi (Zunke 1990; Back *et al.* 2002) (Fig 2.3). Surveys of the nematode community of *A. arenaria* and *A. breviligulata* have revealed the presence of several *Pratylenchus* species. *Pratylenchus brzeskii* was described from the coastal dunes of the Netherlands and the Baltic Sea (Karssen *et al.*, 2000). *Pratylenchus penetrans* has been reported on *A. breviligulata* (Seliskar & Huettel 1993) and *P. scribneri* has been cited in Polish coastal dunes (Van der Putten *et al.* 2005). These species most likely present different degrees of specificity as shown by the host range: *Pratylenchus penetrans* is a wide polyphagous species with more than 300 reported hosts (Corbett 1973)); and *P. brzeskii* however, seems to be restricted to coastal dunes and the presence of *A. arenaria* (Karssen *et al.* 2000).

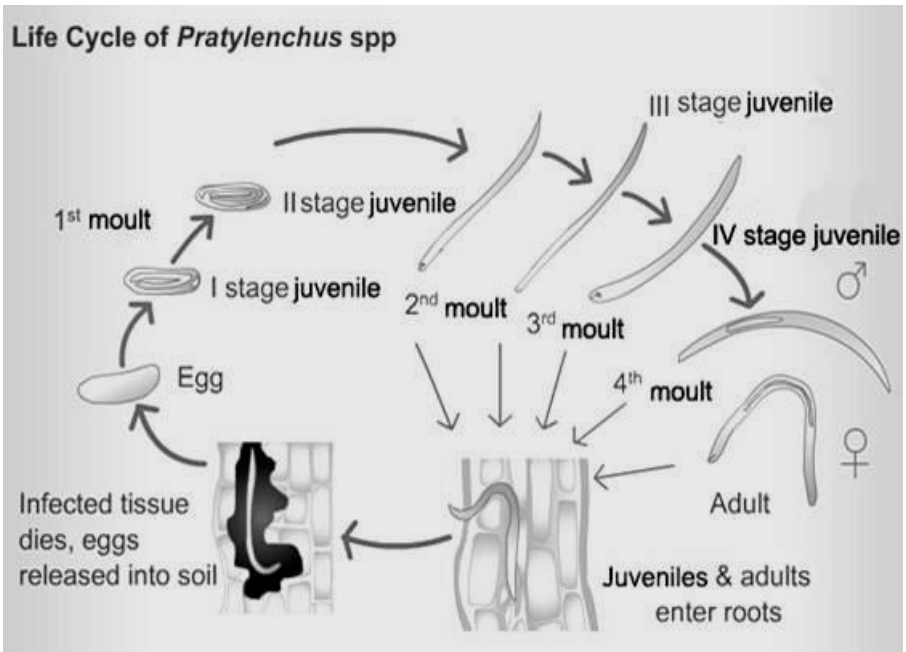


**Fig 2.3** Life cycle of *Pratylenchus* spp. adapted from www. rothamstead.ac.uk (March-2006).

Previous inoculation studies have shown variable impacts of PPN on the growth of *A. arenaria*. While inoculation with *Heterodera arenaria* did not affect plant growth (Van der Stoel, 2001), negative effects in the growth of *A. arenaria* were observed after inoculation with the ectoparasitic nematode *Tylenchorrhynchus ventralis* (De Rooij-Van der Goes, 1995). Also, inoculation with *P. penetrans* was not conclusive for the effect of this species on the growth of *A. arenaria,* aboveground biomass decreased with inoculation while belowground biomass was not affected (Brinkman 2004).

In field conditions, until now it has not been possible to demonstrate that nematodes alone result in the death of *Ammophila arenaria* (Brinkman *et al.* 2005a). Most probably succumb to a combination of several factors: (i) indicate that at natural densities PPN can be considered as biotrophic parasites and do not show powerful pathogenic effects on the host plant (ii) the effect of PPN must be understood in conjunction with other soil organisms such as pathogenic fungi or AMF affecting the growth of the plant (iii) the difficulty of relating the presence of species to their role within the system, which is a general problem in ecological studies (Harper 1977). In any case, the net effect of the soil-community is relevant in the spatio-temporal variation of plant communities in coastal dunes.

### 2.6 Invasiveness of *Ammophila arenaria* and enemy release hypothesis

As pointed out earlier, an ample variety of pathogens affects the growth of *A. arenaria* in its natural range. The invasiveness of *A. arenaria* outside its natural range has been studied testing the hypothesis of a release from its soil pathogens (enemy release hypothesis). This hypothesis states that invasive species escape from their natural enemies in newly occupied areas explaining their success. The development of a negative feed-back between soil organisms and *A. arenaria* keeps the expansion of the dune grass in its native area of distribution in balance, whereas in the introduced areas, free of soil-borne pathogens (e.g. PPN), nothing hampers its colonization abilities and growth (Hertling & Lubke 2000; Beckstead & Parker 2003). The success of *A. arenaria* outside its natural range correlates with escape from feeding specialist nematodes (Van der Putten *et al.* 2005) supporting partially this hypothesis (enemy release).

### 2.7 Mechanisms of control of PPN

The population dynamics of herbivores (e.g. PPN) is determined by both abiotic and biotic factors. Herbivores are controlled by bottom-up forces which limit resource availability

either quantitatively or qualitatively and top-down forces, natural enemies that reduce herbivore multiplication (Hassell *et al.* 1998). Separately, competition between species will alter the availability and the predation by antagonists, and therefore constitutes by itself another mechanism of control (Dezfuli *et al.* 2002; Diez *et al.* 2003; van Veen *et al.* 2006).

Plant-parasitic nematodes in dunes are likely controlled naturally in different ways (Fig 2.4). In the first place, nematodes are controlled directly by *A. arenaria* due to the amount of resources available. The phytoparasitic nematode community in coastal dunes is strictly linked to the growth pattern of *A. arenaria* and the multiplication depends of the quantity of roots available for the nematode community. This bottom-up control of nematodes has been observed for *H. arenaria* nematodes (Van der Stoel *et al.* 2006). Also the interaction between the plant and its mutualists might alter the quantity or quality of resources available inhibiting or enhancing nematode multiplication. Studies on *A. breviligulata* and *Leymus arenarius*, showed AMF to be to good candidates for nematode control in foredunes (Greipsson & El-Mayas 2002). However, up to now, clear evidence on *A. arenaria* and the mechanisms associated with it have not been provided. Besides AMF, other fungal symbionts (e.g. fungal endophytes) are present in *A. arenaria*. These types of fungi provide beneficial effects against abiotic stresses, but also against pathogens (Vicari *et al.* 2002; Arnold *et al.* 2003). The potential role of endophytes in the control of PPN in coastal dunes has not been analyzed.

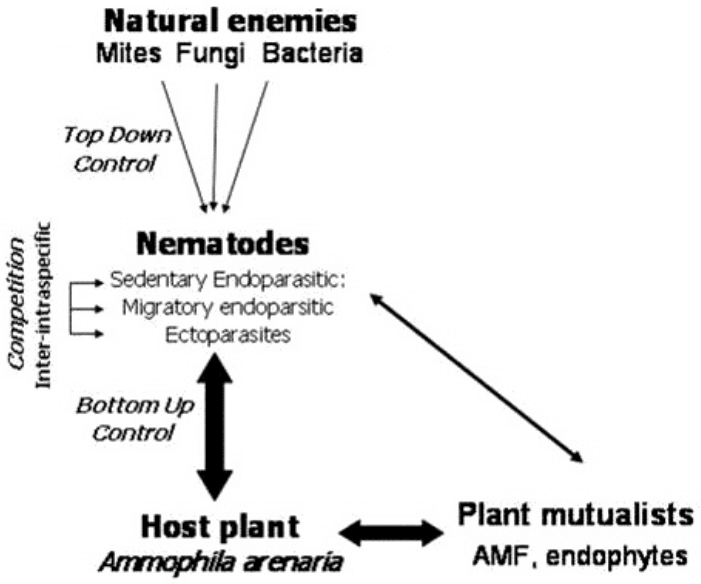


**Fig 2.4** Mechanisms of control of plant parasitic nematode in coastal dunes from (Van der Putten *et al.* in press).

Finally, PPN present in dune systems might also be controlled by bacterial and fungal antagonist (Top-down control) (Jaffee 1996). These antagonists, as shown in agricultural

systems, inhibit nematode reproduction and mobility, but also may interact with other fungal plant symbionts influencing the outcome between plant and nematodes (Hackenberg *et al.* 2000; Van Damme *et al.* 2005).

Plant-parasitic nematodes in agricultural systems are traditionally controlled by the use of pesticides (Cooke 1989; Browning *et al.* 2006), tolerant/resistant crop varieties or rational cropping practices (Trudgill *et al.* 1975; Scholte 2000). However, organisms that affect nematodes (*viz.* mites, collembola, predacious nematodes, fungi and bacteria) are getting increasing attention. Interactions between antagonistic organisms and PPN have been studied aiming at the development of potential biological control agents (Harrier & Watson 2004; Van Damme *et al.* 2005; Mennan *et al.* 2006). However, one disadvantage of those studies is that they do not take into account multitrophic interactions (interactions across many trophic levels). This conceptual framework has been primarily studied by entomologists (Price 1980) and the lack of knowledge on that subject for PPN and other soil herbivores is notorious. Most likely, a major reason for this is the complexity of the experiments needed when studying belowground multitrophic interactions (Van der Putten 2001). However, multitrophic approaches have been advocated for plant-parasitic nematodes (Sikora 1992; Kerry & Bourne 1996) and point at new strategies to understand ecosystem functioning and the development of new biocontrol strategies (Lewis 1997; Bennett *et al.* 2006).

From all the explained above, it becomes clear that coastal dunes might provide of a good system to study belowground multitrophic interactions. Understanding the forces that control nematodes in coastal dunes might yield new insights not only to understand the interaction between coastal plants and soil-biota but also, on the way nematodes, natural enemies, plant mutualists interact. The acquired knowledge from natural systems (coastal dunes) might be later implemented in biological control practices.

# Chapter 3

*General materials and methods*

### 3.1 Collection of samples

Samples were collected at different localities of the Western European coast (Fig 3.1). Mobile foredunes near the sea with vigorous *Ammophila arenaria* were chosen for the sampling surveys. Samples were collected about half way up in the seaward dune face. In that area, the sand was removed until the *A. arenaria* stems were visible. At the uppermost well-rooted node a sample was collected cutting out a volume of 15 x 15 x 15 cm of sand; roots within that volume were added to the sample. Four samples per locality were taken at each sampling. The samples were collected in plastic bags and stored at 4°C until used. Information on the sampling is summarized on Table 3.1.

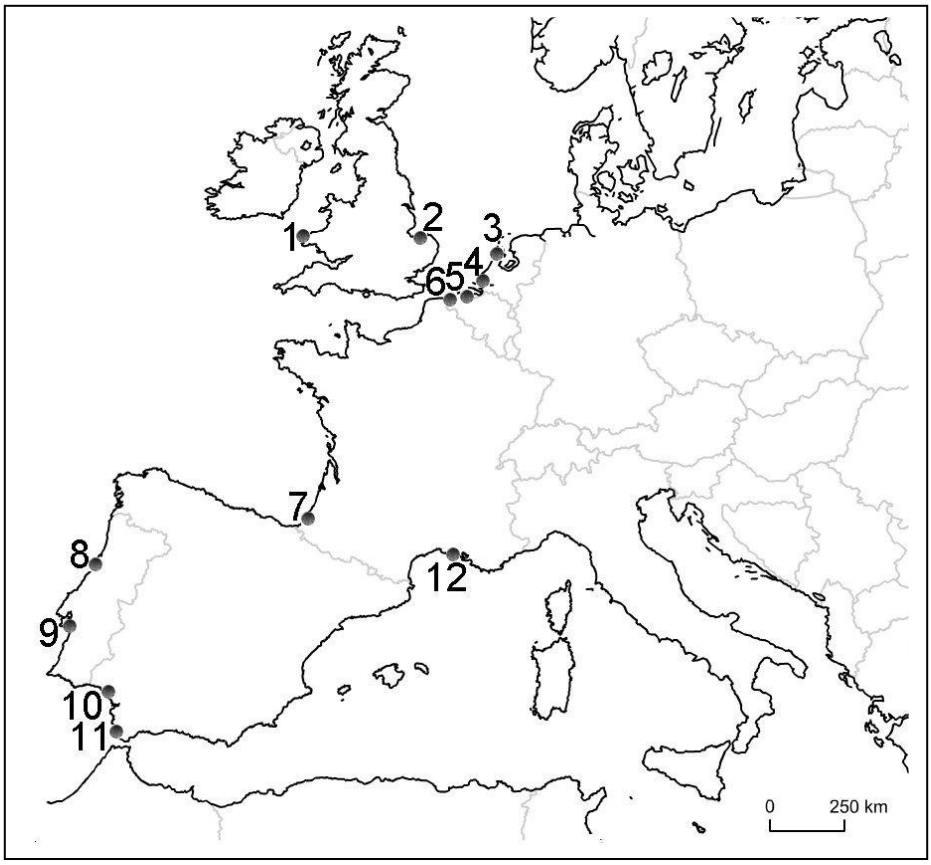


**Fig 3.1** Sampling points included in the study: 1. Ynyslas, Wales; 2. Blakeney Point, UK; 3. Groote Keeten, the Netherlands; 4. Oostvoorne, the Netherlands: 5. Het Zwin, Belgium; 6. De Panne, Belgium; 7. Biarritz, France; 8. São Jacinto, Portugal; 9. Comporta, Portugal; 10. Matalascañas, Spain; 11. Bolonia, Spain; 12. Carnon France.

**Table 3.1** Information related to the samples collected during surveys

| Sampling site | Sample type | Frequency | Coordinates | Collector |
|---|---|---|---|---|
| Ynyslas, WA | S, R, N, A, SE | 2 samples/year* | N5231W00403 | Hol, G. |
| Blakeney Point, EN | S, R, SE | 2 samples/year* | N5258 E0035 | Costa, S. |
| Groote Keeten, NL | S, R | 1 sample+ | N5254E0442 | de la Peña, E. |
| Oostvoorne, NL | S, R, FE, SE | 2 samples/year* | N5152 E0404 | Piskiewicz, A |
| Het Zwin, BE | S, R, N, A, SC, SE | 2 samples/year* | N5121W0032 | de la Peña, E. |
| De Panne, BE | S, R, N, SE | 2 samples/year* | N5105W0233 | de la Peña, E. |
| Biarritz, FR | S, R, N | 1 sample† | N4329W0133 | Maher, N. |
| Carnon, FR | S, R | 1 sample† | N4330 E0407 | Maher, N. |
| São Jacinto, PO | S, R, SE | 2 samples/year* | N4041 W0844 | Rodríguez-Echeverría, S. |
| Comporta, PO | S, R, SE | 2 samples/year* | N3823 W0848 | Rodríguez-Echeverría, S. |
| Matalascañas, SP | S, R | 1 sample‡ | N3654W0626 | de la Peña, E. |
| Bolonia, SP | S, R | 1 sample‡ | N3605W0547 | de la Peña, E. |

WA: Wales; EN: England; NL: the Netherlands; BE: Belgium; FR: France; PO: Portugal; SP: Spain.
S: Sand; R: Roots; N: *Pratylenchus* culture established; A: AMF; FE: Fungal endophyte; SC: sand for cultures, SE: seeds
*Samples collected in: December 2002, July 2003, Oct 2003, April 2004
+Samples collected in December 2004
†Samples collected in July 2004
‡Samples collected in April 2004

## 3.2 *Ammophila arenaria* propagation

*Ammophila arenaria* plants were used to maintain nematode cultures and in inoculation experiments.

### 3.2.1 Seed bank

Seeds were collected from healthy and vigorous *A. arenaria* stands. Spikes were cut from plant stems and stored in plastic bags. Once in the lab, fresh spikes were dried at room temperature for two to three weeks; afterwards seeds were removed manually from the spike and stored in paper bags in a cold (4°C) dark room until used.

### 3.2.2 Germination

Prior to germination, seeds were washed three times with demineralized water to remove debris and sand particles. Then, 25 to 50 seeds were deposited on top of a three to

four centimetre layer of 2 mm glass-beads inside a 400 ml plastic beaker. Demineralized water was added until glass-beads were just immersed. Germination trays were placed in the glasshouse under supplementary light to obtain night/day regime of 8/16 h and an average temperature of 18/25ºC. Once the seeds had germinated, they were maintained until further use in a latent status for up to three months in a growth chamber at 4ºC.

### 3.2.3 Transplantation

*Ammophila arenaria* seedlings (2 to 4 cm shoots) were gently removed from the germination trays and washed carefully with demineralized water to remove remaining glass-beads. Seedlings were transplanted to pots filled with sterilized dune sand after resetting sand moisture at 10% ($ww^{-1}$). The sand was covered with aluminum to prevent desiccation and contamination. Pots were placed in the glasshouse or growth chamber at a night/day regime of 8/16h and additional illumination (250 µmol $m^{-2}h^{-1}$) from October to March.

### 3.2.4 Fertilization

Modified Hoagland's solution (Hewitt 1966) was added on regular basis to *A. arenaria* cultures and experiments according to the experimental design. Stock solutions 200 times concentrated of Ca $(NO_3)_2$, $KH_2PO_4$ and microelements+ $KNO_3$+$MgSO_4$• $7H_2O$, were prepared (Table 3.2).

**Table 3.2** Modification of Hoagland's solution.

| **Macroelements** | Concentration | $g \cdot l^{-1}$ | Stock solution $g \cdot l^{-1}$ |
|---|---|---|---|
| Ca $(NO_3)_2$ $4H_2O$ | 5mM | 1.180 | 236 |
| $KNO_3$ | 5mM | 0.505 | 101 |
| $KH_2PO_4$* | 1mM | 0.680 | 136 |
| $MgSO_4$ $7H_2O$ | 2mM | 1.230 | 246 |
| **Microelements** | | $mg \cdot l^{-1}$ | |
| FeNaEDTA | 24µM | 8.80 | 1.76 |
| $H_3B_3$ | 46µM | 14.30 | 2.86 |
| $MnSO_4$ $H_2O$ | 9µM | 6.90 | 1.38 |
| $ZnSO_4$ $7H_2O$ | 0.7µM | 1.10 | 0.22 |
| $CuSO_4$ $5H_2O$ | 0.3µM | 0.40 | 0.08 |
| $Na_2MoO_4$ 2 $H_2O$ | 0.1µM | 0,085 | 0.017 |

* For P free solution $KH_2PO_4$ was substituted by KCl

### 3.2.5 Sand sterilization

Dune sand was collected from the seaward slope of a foredune with vigorous *A. arenaria* growth. The upper 30 cm of sand was removed and put in plastic bags, and stored in the glasshouse until sterilization. Sand was divided in 5 kg portions and autoclaved at 120°C and 1 atm for at least 3 h.

## 3.3 Nematode cultures

*Pratylenchus* cultures were established from 50 to 100 identified specimens. Nematodes were inoculated on 1 month-old *A. arenaria* plants growing in 1.5 L pots filled with sterilized sand. Two months after inoculation, nematode infection was checked by extracting nematodes from a small root sample. When multiplication was observed, the whole pot was transplanted to a 15 L pot filled with sterilized sand. Pots were watered twice per week with 1000 ml of water and monthly fertilized with 500 ml half strength Hoagland's solution.

## 3.4 Nematode Extraction

### 3.4.1 Zonal centrifugation

Nematodes were extracted from soil and roots by zonal centrifugation (Hendrickx 1995). By using this method, nematodes were extracted in an automated zonal centrifugal machine. This machine separates nematodes from soil or macerated roots following the principles of conventional extraction by centrifugation; but the process is completely automated. Nematode suspensions, either with the macerated root fraction or with the mineral soil fraction are sub-sampled and automatically transferred along with water and $MgSO_4$ solution (density =1.2g·ml$^{-1}$) into a rotor. In this rotor the nematodes are separated from the other components and retained on the interface between the water and the $MgSO_4$ solution. After centrifugation, nematodes are eluted in 140 ml of $Mg_2SO_4$, kaolin and water. The eluted suspension is left in the 150 ml collection beaker for 8h. to allow nematodes to settle on the bottom. Prior to counting, 110 ml are gently removed from the beaker and nematodes were count using a dissecting microscope in a final volume of 40 ml. The extracted mobile stages of *Pratylenchus* (juveniles and adult females and males) were then counted.

Root extraction

Roots were obtained by sieving samples through a 0.5 cm aperture sieve. Roots were first chopped in 1-2 cm pieces and then macerated with a commercial mixer during 1 min in

100 ml of tap water. The obtained suspension was adjusted to 1000 ml. Five hundred ml of this suspension were used for zonal centrifugation. Nematodes were collected and counted as previously described.

Soil

One hundred ml of soil were stirred in 1000 ml of tap water. The same procedure as for root samples was followed.

### 3.4.2 Mistifier

To obtain nematodes from field samples and cultures a modification of the Seinhorst (1950) mistifier technique was used. Soil from field samples or cultures was sieved through a 0.5 cm aperture sieve. The collected roots were gently washed with tap water and subsequently cut into 1 cm. pieces. The root fragments were deposited on a cotton-wool filter lining a metal sieve placed on top of a funnel. The funnels were closed at the bottom using a clip. A regular mist water flow was given every 15 min for 5 min. Funnels were tapped-off every 24 h and the obtained nematode suspension was analyzed using the dissecting microscope.

## 3.5 Plant harvest and data collection

Plants from experiments were harvested according to the duration of the experiment. Fresh weight, from above and belowground plant parts, was obtained by direct weighting after belowground parts were cleaned with tap water. Per plant and according to the experimental design, the length of the longest leaf, the number of tillers and leaves was recorded A variable portion (according to root quantity) of each root was cut and weighed. The remaining plant material was dried for 48 h at 72°C in a hot-airflow oven. The dry weight (total, aboveground and root) was computed taking into account the excised root fragment. The total number of nematode in roots was calculated extrapolating the number of nematodes in the root fraction to the total fresh biomass. The same procedure was used to calculate nematodes·$g^{-1}$ dry root. Nematode numbers in soil were calculated based on the number of nematodes obtained from 100 ml of sand and extrapolated to the total volume of the pot.

# Chapter 4

## *Diversity and distribution of* Pratylenchus *spp. associated with* Ammophila arenaria *in Western Europe*[*]

[*] de la Peña, E., Van Aalst, A., Moens, M., Karssen, G. Description of *Pratylenchus dunensis* sp. n. (Nematoda: Pratylenchidae), a root-lesion nematode associated with the dune grass *Ammophila arenaria* (L.) Link. *Nematology*, 1, 2006

de la Peña, E., Karssen, G., Moens, M. Diversity and distribution of root lesion nematodes associated with *Ammophila arenaria* in Western Europe: morphological and molecular characterization *Nematology*, accepted.

## 4.1 Introduction

Coastal dunes are rich environments in terms of nematological fauna (Wall *et al.* 2002). Associated with *Ammophila arenaria* Link. and the North American *A. breviligulata* Fern. several species of sedentary and migratory endoparasitic nematodes have been described (Handoo *et al.* 1993; Robinson *et al.* 1996; Karssen *et al.* 1998b). A fact that suggests the incidence of adapted nematode species to the particular vegetation growing in coastal dunes.

The genus *Pratylenchus* Filipjev, 1936 has been studied in foredunes with *A. arenaria* in the North Sea (Karssen *et al.* 2001) and in the South West of Europe (Schreck-Reis 2005). Until now, three species have been reported from this host in different sites in areas of natural distribution of *Ammophila* spp. *Pratylenchus brzeskii* Karssen, Waeyenberge & Moens, 2000 was described from *A. arenaria* on the North and Baltic Sea coasts (Karssen *et al.* 2000). *Pratylenchus penetrans* (Cobb, 1917) Filipjev & Schuurmans Stekhoven, 1941 has been reported and linked to the die-out of *A. breviligulata* (Seliskar & Huettel 1993). Finally, *P. scribneri* Steiner 1943, occurs in some dune areas from Poland (Van der Putten *et al.* 2005) However, hitherto, there is a lack of knowledge about the species diversity and distribution of this genus over a wider geographical range in relation to the natural distribution of *A. arenaria*.

Morphological intraspecific variability within the genus *Pratylenchus* is well documented for most of the characters used in species identification (Loof 1992). The use of molecular diagnostic tools is a practical solution to overcome such a problem. The 28S rDNA has been used quite frequently to characterize different *Pratylenchus* populations and species of other genera of plant-parasitic nematodes (PPN) (Handoo *et al.* 2001; Subbotin *et al.* 2003; Madani *et al.* 2004); and therefore, it is a reliable region for species identification.

The purpose of the study was to characterize morphologically, morphometrically and molecularly different populations of *Pratylenchus* associated with *A. arenaria* along the European Atlantic and Mediterranean coast.

## 4.2 Material and Methods

### 4.2.1 Survey and morphological characterization

Roots from vigorous *A. arenaria* stands were collected in coastal foredunes (Chapter 3) from different localities of the Atlantic and Mediterranean European coast (Fig 4.1). Nematodes were extracted according to standard techniques (Chapter 3). They were heat

killed and fixed with formaline 4% and mounted in anhydrous glycerine using the slow method of (Hooper 1993). They were identified based on descriptions and identification keys (Loof 1960; Loof 1992; Brzeskii 1998). Morphometrics were taken using an Olympus BX50 compound microscope equipped with Leica image-capture IM500 system and software.

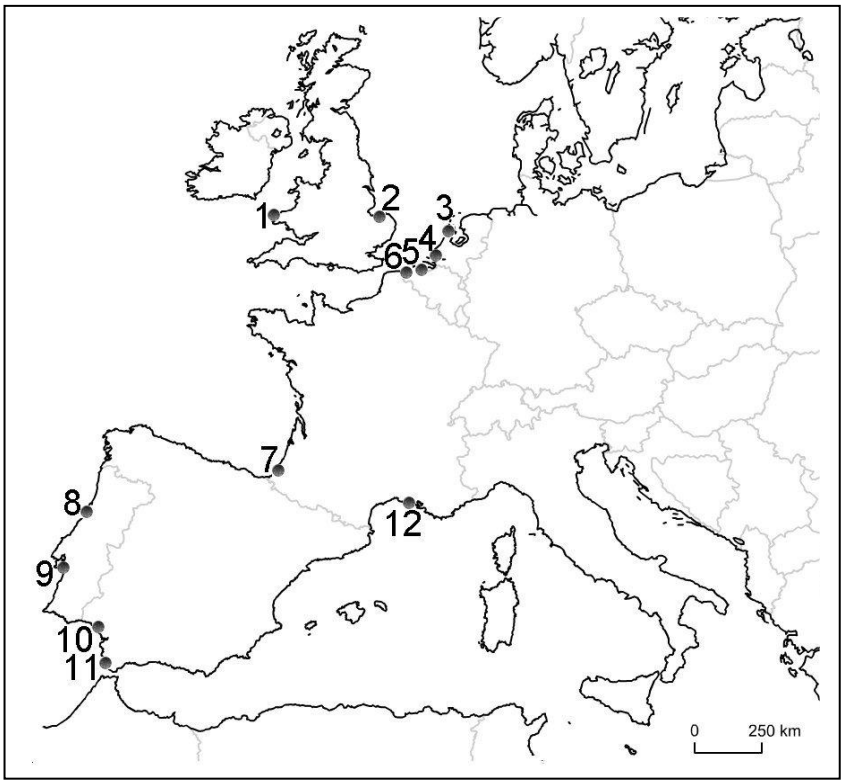


**Fig 4.1** Sampling points included in the study: 1. Ynyslas, Wales; 2. Blakeney Point, UK; 3. Groote Keeten, the Netherlands; 4. Oostvoorne, the Netherlands: 5. Het Zwin, Belgium; 6. De Panne, Belgium; 7. Biarritz, France; 8. São Jacinto, Portugal; 9. Comporta, Portugal; 10. Matalascañas, Spain; 11. Bolonia, Spain; 12. Carnon, France**.**

Morphological data of females and males from all the populations were analysed using a forward stepwise Canonical Discriminant Analysis. For this purpose the statistical software package Genstat 8.0 was used.

### 4.2.2 SEM

For scanning electron microscopy (SEM), fixed nematodes were coated with 4 to 5 nm platinum in a preparation chamber (CT-1500HT, Oxford Instruments, High Wycombe, UK) and observed with a Jeol 6300F field emission electron microscope at 3.5 kV.

### 4.2.3 Molecular characterization

For each of the populations, DNA was extracted from a single nematode that was previously identified. The nematode was placed in 8 $\mu$l of worm lysis buffer (100mM KCl, 20mM Tris-HCl pH 8.3, 3mM $MgCl_2$, 2mM DTT and 0.9% Tween 20), 10 $\mu$l double distilled water, 2 $\mu$l proteinase K (600µg·ml$^{-1}$) and homogenized using a vibromixer. The extract was incubated at 65°C for 2 h and then 5 min at 95°C to denaturise proteinase K. The crude extract was kept at –70°C until use. Five µl of the crude extract were used for PCR amplification. Primers and PCR conditions for both the D2D3 and the ITS (including 5.8S gene and flanking areas of the 18S and 28S genes of rDNA) amplification were as described by De Ley *et al.* (1999) and (Waeyenberge *et al.* 2000), respectively. After electrophoresis in 1% TAE buffered agarose gels (1h, 100V), the PCR product was visualised under UV-light.

#### 4.2.3.1 Cloning and Sequencing

DNA sequences were obtained after cloning PCR products. DNA was excised from 0.8% TAE buffered agarose gels using the QIAquick Gel Extraction Kit (Qiagen), ligated into the pGEM-T vector and transformed into JM109 High Efficiency competent Cells (Promega, Leiden, The Netherlands). One to three clones of each specimen were isolated using blue/white selection and submitted to PCR using vector primers with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA). The resulting products were purified using a Centriflex Gel Filtration Cartridge (Edge Biosystems Inc., Gaithersburg, MD, USA). DNA samples were sequenced using an ABI Prism 377 DNA Sequencer (PE Applied Biosystems).

#### 4.2.3.2 ITS-RFLP

DNA of three *Pratylenchus* species: *P. penetrans* isolated from *Wisteria sinensis* Sweet in Maaseik (Belgium), *P. brzeskii* collected on *A. arenaria* in Biarritz (France) and a *Pratylenchus* isolate from Groote Keeten (the Netherlands) were used for comparison of the ITS-RFLP band pattern. The ITS-PCR products of were first purified using a Qiagen Gel Purification Kit (Qiagen GmbH). After purification, one µl of purified PCR product was digested with one of five restriction enzymes, *viz. Cfo*I, *Hind*III, *Pst*I, *Eco*RI and *Msp*I according to the instructions of the manufacturer (Amersham Bioscience). Digested PCR products were checked by electrophoresis in a 1.5 % agarose-TAE gel run during 2h at 100V, 200mA, 15W.

#### 4.2.3.3 Sequence comparisons

All obtained sequences of *Pratylenchus* spp. were deposited in EMBL – Genbank (Table 4.1).

*Pratylenchus* spp. sequences for phylogenetic analysis of a 310 bp sequence of the D2D3 expansion region were obtained from genbank: *P. neglectus* (Rensch, 1924) Filipjev & Schuurmans Stekhoven, 1941 (AJ545023), *P. minyus* Sher & Allen, 1953 (U47548), *P. brachyurus* (Godfrey, 1929) Filipjev & Schuurmans Stekhoven, 1941 (U47553), *P. hexincisus* Taylor and Jenkins, 1957 (U47554), *P. pseudocoffeae* Mizukubo, 1992 (AF170444), *P. coffeae* (Zimmermann, 1898) Filipjev & Schuurmans Stekhoven, 1941 (U47552), *P. guterriezi* Golden, López, Vilchez, 1992 (AF170440), *P. scribneri* Steiner Sherbakoff and Stanley, 1943 (U47551), *P. loosi* Loof, 1960 (AF170437), *P. fallax* Seinhorst, 1968 (AF264181), *P. arlingtoni* Handoo, Carta and Skantar, 2001 (AF307328), *P. penetrans* (U47546) and *P. pinguicaudatus* Corbett, 1969 (AJ545014). DNA sequences were edited manually with Chromas 1.45 (Technelysium Pty, Helensvale, Australia) and aligned with Clustal X 1.64 (default options) (Thompson *et al.* 1994). Differences between sequences were estimated using the DNA distance option provided by BioEdit sequence alignment editor (Hall 1999). Equally weighted maximum parsimony (MP) analysis were conducted using PAUP (4.0 beta version) (Swofford 1998) in which a heuristic search procedure was used with the following settings: 10 replicates of random taxon addition, gaps were treated as missing data and a bootstrap analysis (BS) with 100 replicates was used to assess the degree of support for each branch on the tree using simple addition sequences with TBR swapping (tree-bisection connection). Trees were displayed with TREE view (Page 1996).

## 4.3 Results

### 4.3.1 Morphological identification of populations

From the localities sampled in this study, 19 populations were detected and characterised using molecular methods (Table 4.1); 18 of these populations were further identified based on morphology and morphometrics. Since no adult males/females were retrieved from the sample taken in Matalascañas (Spain) data on morphology and morphometrics are not available for this population. In total, six populations belonged to an undescribed *Pratylenchus* species, seven to *P. brzeskii*, five populations were identified as *P. pratensis* Filipjev 1936 (De Man, 1880) and one population was identified as *P. penetrans*.

#### 4.3.1.1 *Pratylenchus dunensis* de la Peña, Van Aalst, Moens, Karssen (2006) (*P. penetrans* apud Karssen *et al.* (2001))

(Figs 4.2 – 4.4)

Five *Pratylenchus* populations had the same characteristics as the *P. penetrans* population detected in *A. arenaria* roots from Groote Keeten, the Netherlands (Karssen *et al.* 2001). Although this population differed from the original description of *P. penetrans* by the number of lip annules, tail morphology, and some morphometrics, it was identified as *P. penetrans* because the ITS-RFLPs of the population were identical to those of *P. penetrans* as published by Waeyenberge *et al.* (2000). However, D2D3 LSU sequences of the five populations retrieved from *A. arenaria* showed further differences with those of *P. penetrans*. As a consequence the identity of these populations was reconsidered and comparisons, using the type population from Groote Keeten, of ITS–RFLPs were combined with extra observations on the SEM morphology and morphometrics. The additional data revealed that these populations belong to a new species, which was described as *Pratylenchus dunensis*.

**Measurements**

Females and males: Table 4.2 and Table 4.3

**Morphology**

Female

Body vermiform, slender and tapering towards both ends. Cuticle finely annulated with annules 1-1.3 µm wide at mid-body. Head slightly set-off, diameter three times its height, rounded with prominent cephalic sclerotisation and vestibule extension. Two lip annuli, often with one or two incomplete, transverse incisures, not visible with light microscope. Labial disc oval, elevated, fused with four submedial lips forming a smooth, dumb-bell shaped, headcap. Cephalic sensilla marked by a subtle depression on each submedial lip. SEM *en face* view showing oval prestoma. Stylet finely shaped, relatively short, with robust, anteriorly indented, set-off knobs. Stylet cone length equal or shorter than length of shaft plus knobs.

**Table 4.1** Occurrence of *Pratylenchus* spp. according to sampling locality and *Ammophila arenaria* subspecies. Genbank accession number indicates presence of the *Pratylenchus* species.

| Host Subspecies | Locality | *Pratylenchus* spp. | | | |
|---|---|---|---|---|---|
| | | *P. dunensis* | *P. brzeskii* | *P. pratensis* | *P. penetrans* |
| *A. arenaria* ssp *arenaria* | Ynyslas | | AM231925<br>AM231926<br>AM231927 | AM231929 | |
| | Blakeney Point | AM231950<br>AM231948 | | | |
| | Groote Keeten | AM231939<br>AM231940<br>AM231941 | | | |
| | Oostvoorne | AM231947<br>AM231949 | AM231916<br>AM231917<br>AM231918 | | |
| | Het Zwin | | AM231922<br>AM231923<br>AM231924 | AM231931 | |
| | De Panne | AM231944<br>AM231945<br>AM231946 | | | |
| | Biarritz | | AJ890463<br>AM231913<br>AM231914<br>AM231915 | | AM231936<br>AM231937 |
| *A. arenaria* ssp *arundinacea* | São Jacinto | | | AM231933<br>AM231934<br>AM231935 | |
| | Comporta | AM231942<br>AM231943 | AM231919<br>AM231920<br>AM231921 | AM231932 | |
| | Matalascañas | AM231938 | | | |
| | Bolonia | | AM231911<br>AM231912 | AM231930 | |
| | Carnon | | AM231928 | | |

Hemizonid near isthmus level, 2-3 µm in length, anterior to the oval shaped secretory-excretory pore located between the nerve ring and the pharyngeal junction. Pharyngeal gland

lobe three body diameters long. Vulva with well developed lips, often protruding. Lateral field with four lateral lines starting behind the level of stylet; at mid-body about one-third of body width. At pharyngeal-vulva region, middle ridge narrower than outer ones; at mid-body, width of inner ridge 60-80% of distance between outer lines. Outer incisures appear partially areolated, with striae crossing the outer lateral field ridges, in LM and SEM; presenting one stria every 1-1.5 µm. Lateral lines converging behind tail phasmid. Anterior gonad with single row of oocytes. Spermatheca round to slightly oval, filled with rounded sperm. Post uterine sac undifferentiated, short. Tail cylindrical with distinct annulations, narrowing in the posterior third with a dome-shaped smooth terminus; some specimens (1/5 of adult females) with one or two indentations next to the smooth tail tip. Hyaline part distinct. Anus round to oval shaped. Small, rounded, phasmid, located between inner lateral field lines, posterior to mid-tail.

Male

Occurring abundantly (nearly 50% of adults). Morphologically similar to females, but smaller for all non sexual characters. Head characters as in females, but more truncated in outline. Apical annules slightly raised. Incomplete transverse incisures and variation in lip annuli as described for females. One single testis anteriorly outstretched. Spicules and gubernaculum ventrally curved. Bursa enclosing tail. Ventral side of bursa coarsely crenate; phasmid orifice on bursa nearly at mid distance between cloaca and tail end.

**Type host and locality**

*Pratylenchus dunensis* was described from the *Pratylenchus* population detected in roots of *A. arenaria* growing in the fore dunes of Groote Keeten, Province Noord Holland, the Netherlands. The species was also detected in the roots of *Elymus farctus* Viv. present at the same area. The distribution of *P. dunensis* seems to be favoured by conditions in front dunes where there is considerable sand deposition and the two dune grasses grow vigorously. In older, more stabilised, dunes this species is less abundant.

**Table 4.2** Morphometric characters of adult females of *Pratylenchus dunensis* from different localities of Western European coast. Measurements in µm and in form: mean ± standard deviation (range).

| Population | Blakeney Point | De Panne | Oostvoorne | Comporta | Groote Keeten Paratypes | Groote Keeten Holotype |
|---|---|---|---|---|---|---|
| N | 10 | 10 | 15 | 16 | 15 | 1 |
| Head incisures | 1 | 1 | 1 | 1 | 1 | 1 |
| L | 494±50.1 (419-561) | 528±63.7 (459-610) | 575±73 (462-672) | 543±52.6 (446-637) | 505± 35.2 (454 - 579) | 487 |
| A | 29.1±1.4 (27.5-31.2) | 28.6±2.6 (24.9-32.4) | 29.4±2.2 (25.7-33.6) | 28.4±2.1 (24-32.2) | 28.3±1.9 (25 - 32) | 30.6 |
| B | 6.1±0.5 (5.3-7.2) | 7.2±0.8 (6.3-8.6) | 7.6±0.6 (7-8.6) | 7.9±0.7 (6.7-9.2) | 6.8±0.7 (5.8 - 8.3) | 6.6 |
| b' | 3.7±0.3 (3.3-4.3) | 4.4±0.6 (3.7-5.2) | 4.8±0.9 (3.8-6.4) | 4.4±0.5 (3.6-5.6) | 4.3 ± 0.4 (3.8 - 5.7) | 4.7 |
| C | 14.7±0.5 (13.9-15.5) | 15.9±1.7 (12.7-18.5) | 16±1.4 (13.2-18.4) | 16.7±1.4 (14.4-19.9) | 15 ± 1.2 (13 - 17) | 14.4 |
| c´ | 2.9±0.1 (2.7-3) | 2.8±0.2 (2.5-3.3) | 3±0.3 (2.5-3.5) | 2.7±0.3 (2.2-3.2) | 3.1 ± 0.2 (2.7-3.4) | 3.4 |
| V% | 77.2±1.39 (74.7-78.97) | 77±1 (74.9-78.4) | 78.2±1.9 (73.2-80.7) | 77.2±1.5 (74.2-79.5) | 78 ± 1.1 (76 - 79) | 76 |
| Greatest body diameter (GBD) | 16.9±1.1 (15-18) | 18.5±1.3 (16.5-21) | 19.2±1.3 (17-21) | 19.2±1.5 (17-22) | 18 ± 1.3 (15 - 20) | 16 |
| Body diameter at vulva (BDV) | 16.0±0.9 (15-17.3) | 18±1.5 (16.5-21) | 18.1±1.6 (16-21.1) | 17.8±2.4 (14-22) | 16 ± 1.0 (15 - 18) | 15 |
| Body width at anus | 11.4±0.7 (10.2-12.6) | 11.8±1.2 (10-14) | 12.1±1.1 (10-14) | 12±1.4 (10-16) | 11 ± 0.8 (10 - 12) | 10 |
| Head width (HW) | 7.9±0.13 (7.6-8.1) | 8±0.3 (7.5-8.5) | 8.2±0.5 (7.5-9) | 8.3±0.8 (7-9.5) | 8.4 ± 0.4 (8 - 9) | 8 |
| Head height (HH) | 2.9±0.3 (2.1-3.2) | 2.1±0.4 (1.8-3) | 2.4±0.4 (2-3) | 2.2±0.3 (2-2.8) | 2.5 ± 0.4 (2 - 3.2) | 2.0 |
| Stylet | 16.7±0.6 (16-17.6) | 16.4±0.5 (16-17) | 16.7±0.4 (16-17.2) | 16.4±0.7 (15-17.5) | 16.5 ± 0.4 (16 - 18) | 16.4 |

**Table 4.2 (Continued)**

| | | | | | | |
|---|---|---|---|---|---|---|
| Stylet knob width (SKW) | 4.2±0.41 (3.7-4.8) | 4.8±0.3 (4-5) | 4.3±0.7 (3-5) | 4.7±0.4 (3.8-5.2) | 5.3 ± 0.3 (5 - 6) | 5.1 |
| Stylet knob height (SKH) | 2.4±0.5 (1.9-3.1) | 2±0.2 (1.8-2.5) | 2.3±0.5 (1.8-3.1) | 2.5±0.5 (2-3.5) | 2.5 ± 0.4 (2 - 3) | 2.5 |
| DGO | 2.32±0.67 (1-3) | 1.8±0.2 (1.3-2) | 2.1±0.4 (1.3-3) | 1.8±0.2 (1.3-2.1) | 2 .1± 0.4 (1.3 - 2.8) | 2.1 |
| Ant. end to metacarpus | 54±4.16 (49-60) | 54.2±3.4 (48-59) | 49.1±3.9 (44-57) | 54.3±4.4 (46-59.5) | 50 ± 5.8 (35 - 57) | 48 |
| Ant. end to excret. pore | 81±6.77 (72-88) | 81±7 (64-88) | 81±5.7 (72-88.9) | 81±6.6 (69-89) | 80 ± 6.2 (63 - 88) | 63 |
| Ant. end to pharyngeal-intestinal junction | 81.1±5.2 (72-87) | 71±4.2 (64-78) | 73±9.1 (61-84) | 68±5.2 (60-77) | 75± 8.0 (56 - 86) | 73 |
| Ant. end to end of pharyngeal gland | 132±13.7 (115-151) | 121±5.5 (113-129) | 120±16.3 (98-153.2) | 123±12.2 (105-146) | 117 ± 10.5 (101 - 136) | 103 |
| Pharyngeal gland length | 50±9.2 (38-67) | 50 ±7.4 (37-63) | 47±12.8 (18.3-69.2) | 55±9.4 (39.2-69) | 42 ± 7.7 (32 - 54) | 43 |
| Post Uterine Sac Length | 20.2±2.8 (17-25) | 20.5±2.9 (15-24) | 21.4±2.1 (19-25) | 21.3±3.2 (15-27) | 19 ± 3.3 (12 - 25) | 20 |
| Lateral field width | 5.8±0.8 (5-7) | 5.5±0.8 (4-7) | 6±0.8 (5-7.2) | 6.4±1.4 (4-9) | 5.5 ± 0.5 (5 - 7) | 5.7 |
| Tail length | 33.4±2.46 (30-36) | 33.3±2.6 (30-37) | 35.9±2.5 (31-38) | 32.6±2.5 (29-38) | 33± 2.6 (30 - 38) | 34 |
| Tail annules | 31.7±2.11 (29-34) | 31.4±1.4 (29-34) | 31.6±1.8 (29-34) | 31.8±3.5 (23-38) | 34± 2.8 (31 - 39) | 33.0 |
| Hyaline part | 2.2±0.8 (1-3) | 1.8±0.4 (1-2.2) | 1.7±0.8 (1-3) | 1.7±0.8 (0.5-3.5) | 2.1± 0.5 (1.3 - 2.5) | 2.05 |
| HW / HH | 2.7±0.3 (2.4-3.7) | 3.9±0.5 (2.8-4.7) | 3.7±0.7 (2.9-4.5) | 3.8±0.6 (2.8-4.8) | 3.5 ± 0.5 (2.8 - 4.7) | 3.6 |
| SKW/SKH | 1.8±0.3 (1.32-2.4) | 2.4±0.2 (2-2.8) | 2±0.4 (1.3-2.5) | 2±0.4 (1.4-2.5) | 2.3 ± 0.4 (1.7 - 2.9) | 2.0 |

**Table 4.3** Morphometric characters of adult males of *Pratylenchus dunensis* from different localities of Western European coast. Measurements in µm and in form: mean ± standard deviation (range).

| Population | De Panne | Groote Keeten |
|---|---|---|
| N | 5 | 15 |
| Head incisures | 1 | 1 |
| L | 473±18.3 (447-492) | 447±34 (387-480) |
| A | 28.4±1.2 (26.9-29.8) | 28.7±2.2 (23-31) |
| B | 6.9±0.3 (6.6-7.4) | 6.2 ± 0.6 (5-7.5) |
| b' | 4.4±0.2 (4.1-4.6) | 4.1 ± 0.4 (3.8-4.8) |
| C | 15.6±0.5 (14.9-16.2) | 15 ± 1.3 (12-17) |
| c´ | 2.6±0.1 (2.5-2.7) | 2.6 ± 0.3 (2-3.1) |
| Greatest body diameter | 16.7±1 (15-17.5) | 16 ± 0.7 (14-17) |
| Body width at anus | 11.6±0.5 (11-12) | 11.3 ± 0.6 (10-12) |
| Head width | 7.7±0.6 (7-8.3) | 7.3 ± 0.3 (7-8) |
| Head height | 2.1±0.1 (2-2.2) | 2.5 ± 0.2 (2.2-3.2) |
| Stylet | 15.9±0.7 (15-17) | 14.6 ± 0.5 (14-15) |
| Stylet knob width | 4±0.3 (3.8-4.5) | 3.8 ± 0.4 (3-4.5) |
| Stylet knob height | 1.8±0.2 (1.5-2) | 1.7 ± 0.4 (1.3-2.5) |
| DGO | 2.3±0.4 (2-3) | 2.4 ± 0.4 (1.9-2.5) |
| Ant. end to metacorpus | 50±2.1 (48-53) | 50 ± 2.3 (46-56) |
| Ant. end to excretory-pore | 68±6.6 (62-78) | 75 ± 6.9 (63-87) |
| Ant. end to pharyngeal-intestinal junction | 68.2±0.8 (67-69) | 72 ± 4.1 (63-78) |
| Ant. end to end of pharyngeal gland | 108±6.9 (103-120) | 109 ± 7.2 (99-120) |
| Pharyngeal glands length | 41±6.8 (35-53) | 36 ± 5.9 (32-48) |
| Lateral field width | 5.9±0.4 (5.3-6.2) | 4.4 ± 0.5 (4-5) |
| Tail length | 30.4±1.1 (29-32) | 29.9 ± 3.0 (23-35) |
| Spicules | 15.8±1.5 (14-18) | 15.1 ± 0.8 (14.5-16.4) |
| Gubernaculum | 4.8±0.8 (4-6) | 5.0 ± 0.5 (4.5-6) |

**Table 4.3 (Continued)**

| | | |
|---|---|---|
| Testis | 210±19.5<br>(191-240) | 194 ± 29.3<br>(143-237) |
| HW / HH | 3.6±0.3<br>(3.2-3.9) | 2.9 ± 0.2<br>(3-3.5) |
| SKW/SKH | 2.2±0.3<br>(1.9-2.7) | 2.3 ± 0.5<br>(1.5-3.4) |

**Other localities and hosts**

*Pratylenchus dunensis* has also been found in Oostvoorne, The Netherlands, parasitizing *A. arenaria* and *E. farctus*. roots. This species was also detected on *A. arenaria* roots in Blakeney Point, Norfolk (UK), in De Panne (Belgium), in Comporta (Portugal) and in Matalascañas (Spain).

**Type material**

One holotype female, six female and seven male paratypes were deposited at the Nematode Collection of Wageningen (collection numbers WT3399 and WT3400), University and Research Centre, Wageningen, the Netherlands. Eight male and eight female paratypes were deposited at the Museum voor Dierkunde (collection numbers UGMD 104068 and UGMD 104069), University of Gent, K.L. Ledeganckstraat 35, 9000 Gent, Belgium.

**Diagnosis and relationships**

*Pratylenchus dunensis* is characterised by two lip annuli with incomplete transverse incisures, a stylet (*ca* 16 µm) with robust knobs anteriorly indented and set off, vulva at 78% of body length, four parallel lateral lines unequally spaced from oesophagus to vulva, the inner ridge covering 60-80% of the distance between outer incisures, lateral field partially areolated through whole body, rounded to oval spermatheca filled with round sperm, conical tail with 31-39 annules, tail-tip rounded and smooth with conspicuous short hyaline part (*ca* 2 µm), phasmid between inner lateral field incisures in posterior half of tail. *Pratylenchus dunensis* is further characterised by the abundant presence of males (nearly 50% of adults).

As *P. dunensis* the following species have two lip annules and a non-crenate tail: *P. acuticaudatus* Braasch & Dekker, 1989, *P. agilis* Thorne & Malek, 1968, *P. alleni* Ferris, 1961, *P. angulatus* Siddiqi, 1994, *P. brachyurus* (Godfrey, 1929) Filipjev & Schuurmans Stekhoven, 1941, *P. brzeskii* Karssen 2000, *P. coffeae* (Zimmermann, 1898) Filipjev & Schuurmans Stekhoven, 1941, *P. hexincisus* Taylor & Jenkins, 1957, *P. jordanensis* Hashim, 1984, *P. loosi* Loof, 1960, *P. macrostylus* Wu, 1971, *P. neglectus* (Rensch, 1924) Filipjev &

Schuurmans Stekhoven, 1941, *P. neobrachyurus* Siddiqi, 1994, *P. pseudocoffeae* Mizukubo, 1992, *P. scribneri* Steiner *in* Sherbakoff and Stanley, 1943 and *P. silvaticus* Brzeski, 1998.

Morphologically, *P. acuticaudatus, P. agilis, P. angulatus, P. brachyurus, P. hexincisus, P. jordanensis, P. macrostylus, P. neglectus* and *P. scribneri* differ from *P. dunensis* in having an empty spermatheca (no males or very rarely). *Pratylenchus alleni* differs by a small stylet (<15 *vs* 15.8-17.7 µm) and a bluntly rounded tail with phasmids in the anterior half of the tail (*vs* conical tail with phasmids posterior to mid-tail). *Pratylenchus coffeae* and *P. pseudocoffeae* differ in a generally more posterior vulva position (V= 78-83 *vs* 76-79), a truncate tail (*vs* conical), elongated spermatheca (*vs* round to oval) and a well differentiated post uterine branch (*vs* undifferentiated and short). *Pratylenchus loosi* differs from *P. dunensis* in having a more posterior vulva position (V = 79-85 *vs* 76-79), a broad lateral field with four to six incisures, and a shorter ratio tail length to total body length (c = 18-25 *vs* 13-17). *Pratylenchus neobrachyurus* is shorter (310-410 *vs* 454-579 µm) and wider (a = 20-31 *vs* 25-32) with a more posterior vulva position (V = 79.5-83 *vs* 76-79), a smaller number of tail annules (19-23 *vs* 31-39) and a non-areolated lateral field. *Pratylenchus silvaticus* differs from *Pratylenchus dunensis* in head morphology (head no set-off), a compact pharyngeal lobe (*vs* relatively long) and tail tapering before terminus. The ratio between body length and greatest body diameter is also different between the two species (a = 21-27 *vs* 25-31). The vulva position in *P. silvaticus* is lightly more posterior than in *P. dunensis* (V = 80-83 *vs* 76-79).

*Pratylenchus brzeskii* shares the habitat with *P. dunensis* and therefore is likely that this species co-occur however, they can be separated morphologically since *P. brzeskii* is longer than *P. dunensis* (627-737 *vs* 454-579 µm), has a greater body diameter (23-25 *vs* 15-20 µm), longer pharyngeal glands (66-101 *vs* 33-54 µm), a longer stylet (18-19 *vs* 16-18 µm) and tail length (41-51 *vs* 30-37 µm). More difference is found in the lateral lines. In both species the lateral field at mid-body form three ridges, the middle one smaller than the outer ones. However, in *P. brzeskii* the inner ridge is considerably smaller than in *P. dunensis* (ridge occupying 40-50 *vs* 60-80% of the distance between the outer lines).

The most common morphotype of *P. penetrans* has three lip annuli. However, some populations of *P. penetrans* have been reported with two lip annuli (Tarté & Mai 1976). Compared with *P. dunensis*, *P. penetrans* is generally more slender (a = 17-34 *vs* 25.4-31.6) and shows a larger range in the pharyngeal gland/body length ratio (b = 4.1-8.1 *vs* 3.8-5.7). Some tail characteristics are further useful characters to distinguish the two species: the tail of *P. penetrans* is 21-38 µm long whereas the tail of *P. dunensis* is 31-39 µm, and the tail

annulation is greater in *P. dunensis* than in *P. penetrans* (31-39 *vs* 15-27). The lateral field is a further useful character to separate the species (*P. penetrans* with four equidistant incisures *vs* four unequidistant incisures in *P. dunensis*).

Within the genus *Pratylenchus*, three species are described with two lip annules and a crenate tail, *viz P. flakkensis* Seinhorst, 1968, *P. estoniensis* Ryss, 1982 and *P. gibbicaudatus* Minawa & Nozuma, 1982. These species show an obvious crenation of the tail differing from *P. dunensis* that has a smooth tail tip. *Pratylenchus flakkensis* has an elongated spermatheca (*vs* round to oval), a smaller number of tail annules (14-26 *vs* 31-39) and males are uncommon. *Pratylenchus estoniensis* presents six lateral lines (*vs* four) and an empty spermatheca (*vs* filled with round sperm) and *P. gibbicaudatus* also has an empty spermatheca and no, or rarely present males.

#### 4.3.1.2 *Pratylenchus brzeskii* Karssen, Waeyenberge, Moens (2000)

A total of seven *P. brzeskii* populations were detected in the survey (Table 4.2). The overall morphological characteristics did not differ from the population described by Karssen *et al.* (2000). However, the morphometrics for all characters but a, b, c showed a smaller range than the original described populations.

**Measurements**

Females and males: Table 4.4 and Table 4.5

**Morphology**

Female

Body vermiform, straight, slightly tapering at body ends and relatively long compared to other *Pratylenchus* species. Annulation of the cuticle conspicuous. Lateral field with four incisures not aerolated and not equidistant, the distance between the two inner incisures being closer than the outer. In the anterior and posterior body end the two inner incisures merge to one line. Head low showing set off. Head width approximately three times its height. Heavily sclerotized cephalic framework and distinct vestibule extension. Two head annules (one incisure). Apical lip flattened with rounded edges. Stylet long and robust. Straight cone and cylindrical shaft showing at its end robust knobs. Dorso-pharyngeal gland orifice close to the stylet base. Metacorpus well developed and round. Pharyngeal glands long and ventrally overlapping the intestine. Three gland nuclei present. Vulva positioned posteriorly (75-78%), well developed, lips slightly protruding. Spermatheca always faint from round to oval and

filled with small round sperm. Posterior uterine branch short. Tail conical with distinct annulations, tail tip always smooth with distinct and long hyaline part.

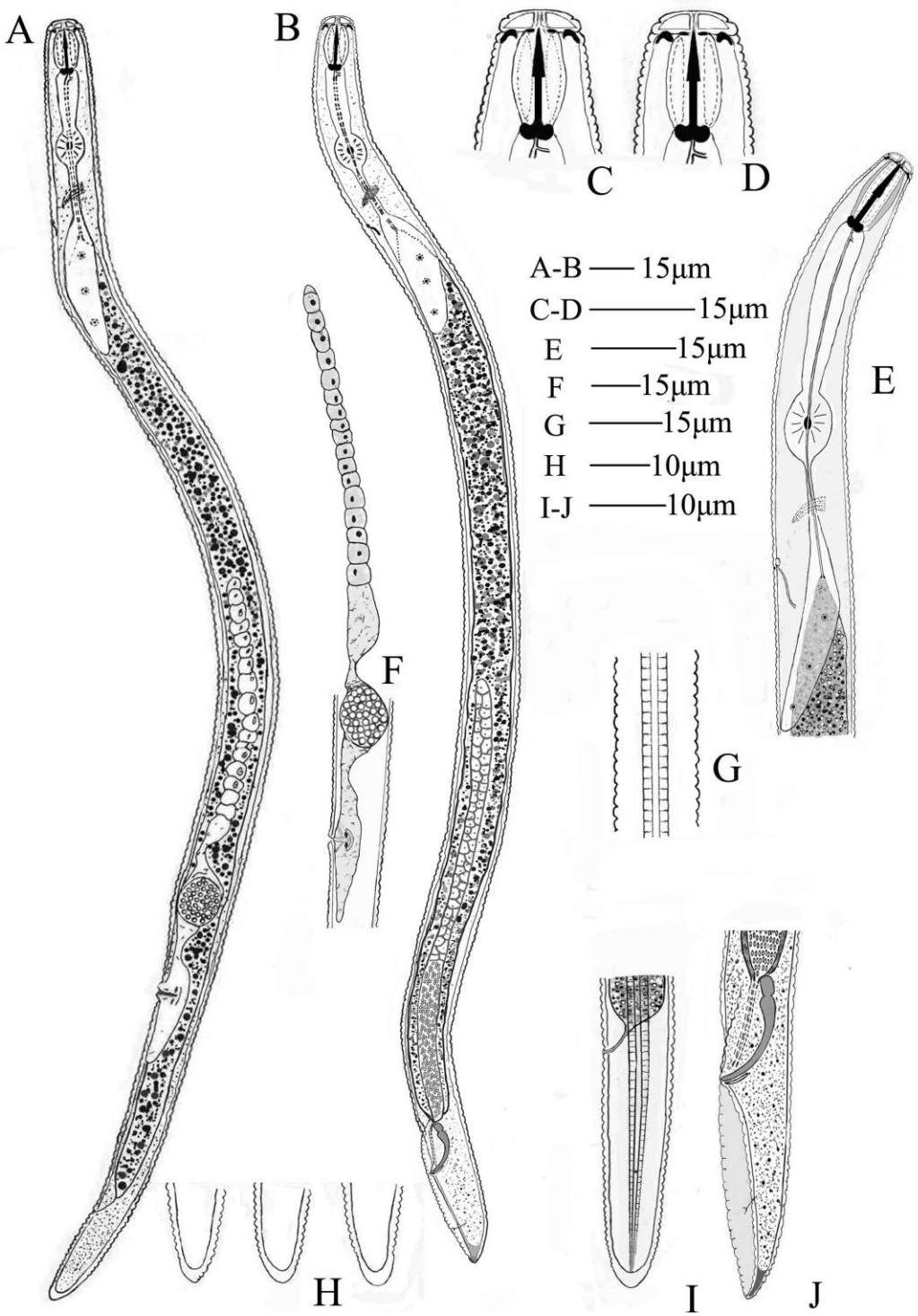


**Fig 4.2** *Pratylenchus dunensis* A: Entire female body, lateral view; B: Entire male body, lateral view; C: Male cephalic region; D: Female cephalic region; E: Female anterior end including pharyngeal gland; F: Female vulval region, ovary and spermatheca; G: Lateral field at mid-body; H: Female tail tips; I: Female posterior end; J: Male posterior end.

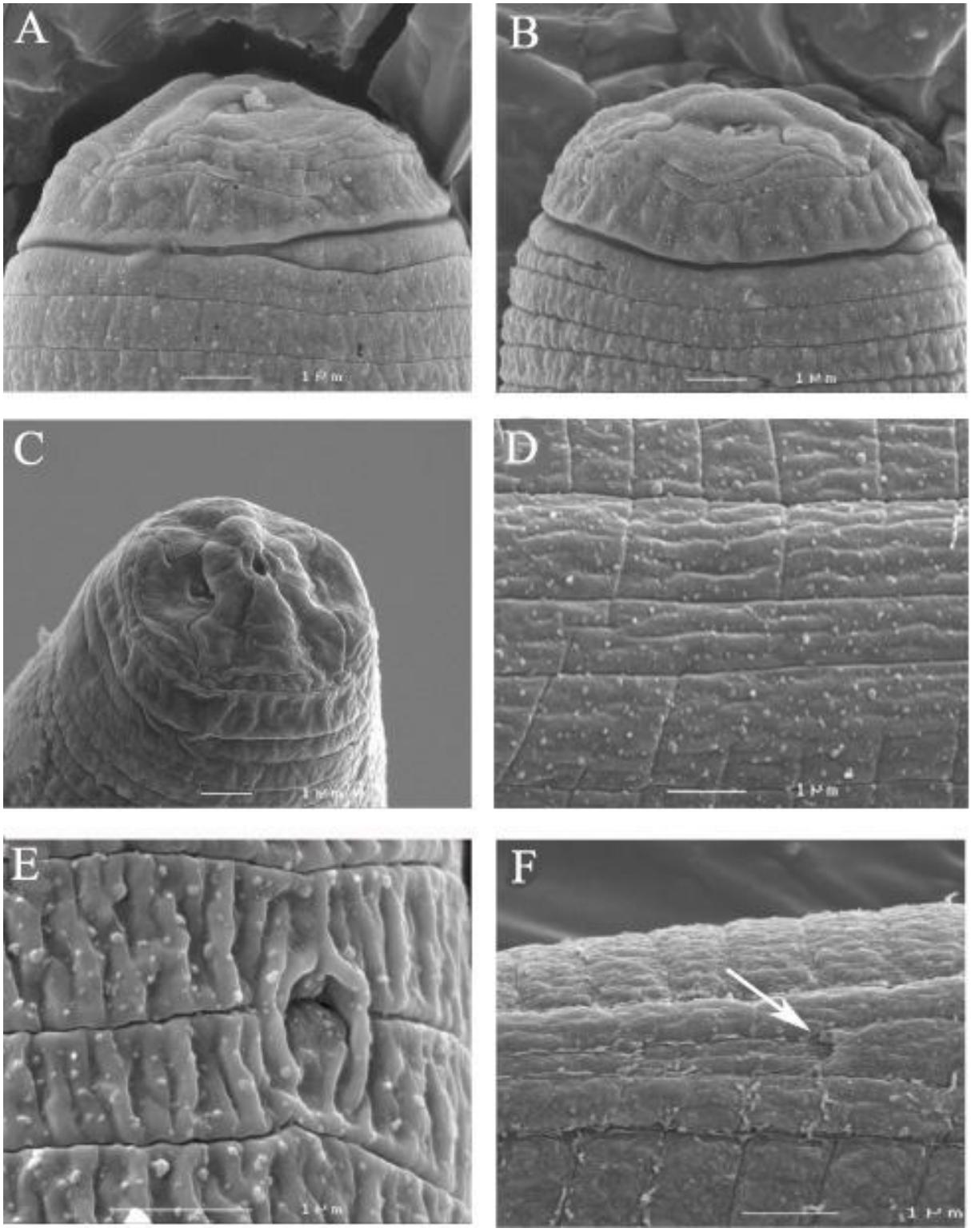


**Fig 4.3** *Pratylenchus dunensis* SEM A: Lateral view of female head region; B: Lateral view of male head region C: En face view of female lip region; D: female lateral field view at mid-body; E: Excretory / Secretory pore; F: lateral field and phasmid at female tail.

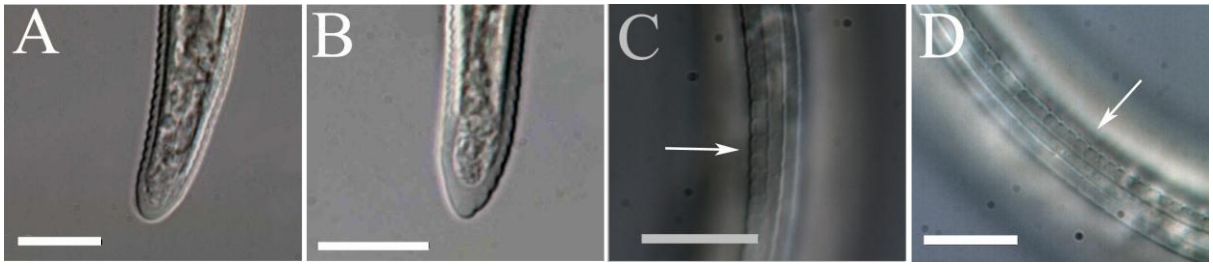


**Fig 4.4** *Pratylenchus dunensis* A: Female tail terminus; B: Female tail terminus with indentations next to tail tip; C-D: Female lateral field at mid-body and areolations (Scale bar= 10µm).

Males

Males occur frequently (1-2 males per 5 females). Except for sexual characters, males are morphologically comparable to females but slightly smaller morphometrically. Single testis anteriorly stretched. Spicules finely shaped, long and ventrally curved. Bursa enclosing tail tip. Gubernaculum short and slightly curved.

### 4.3.1.3 *Pratylenchus pratensis* de Man (1880), Filipjev (1936)

Five populations of *P. pratensis* were detected in the samples (Table 4.1) Morphology and morphometrics fitted the description of previously described populations.

**Measurements**

Females and males: Table 4.6 and Table 4.7

**Morphology**

Female

Body vermiform, slender. Cuticle showing fine striations, Lateral field with four longitudinal lines. Lip region forms almost a continuous with body. Head bearing three annules (with two incisures). Spear compact, moderately developed with two anteriorly intended and flattened knobs. Metacorpus broadly oval. Pharyngeal gland overlapping intestine ventrally. Hemizonid immediately anterior to extretory/secretory pore which is generally anterior to pharyngeo-intestinal junction. Spermatheca elongated when filled with sperm, ranging from quadrangular to oval. Ovary with oocytes set in a single row except for a multiplication zone near the anterior end. Post uterine sac short, slightly longer than body diameter. Tail annulated from fine to coarsely annulated, with annulations observed also around tail terminus. Tip varies in shape from obliquely and narrowly rounded to broadly rounded.

Male

Similar to female for all non sexual characters.

#### 4.3.1.4 *Pratylenchus penetrans* Cobb (1917)

Only one population of *P. penetrans* was detected in the samples analysed. This population occurred in Biarritz (France).

**Measurements**

Females and males: see Table 4.8

**Morphology**

Female

Body slender and vermiform, straight and slightly curved ventrally. Lateral field with four lines. Head slightly offset, bearing three annules (two incisures). Stylet knobs anteriorly flattened or rounded, sometimes cupped anteriorly. Oesophagus overlapping intestine ventrally in a lobe about 1.5 body-widths long. Excretory/secretory pore about opposite to pharyngeo-intestinal junction with hemizonid occupying about two body annules immediately in front of it. Post-uterine sac short. Spermatheca mostly rounded, sometimes only slightly longer than wide. Vulva position 78-82%. Tail tip smooth, with small hyaline part.

Male

Similar to female for all non sexual characters.

### 4.3.2 Canonical discriminant analysis (CDA)

Morphometrics of adult females and males from the 18 populations identified morphologically were used in a Canonical discriminant analysis. The morphometrics of ten characters were used for the analysis of the females; and eight characters in the case of males (Table 4.9).

Using the selected characters, the CDA clearly separated the four *Pratylenchus* species. Three morphological traits of females (*viz.* b, stylet length, hyaline part) provided the most useful taxonomic characters for identification and discrimination of *Pratylenchus* spp. females. The CDA for male morphometrical characters equally allowed the separation of the species; however, due to the reduced size of the data matrix a reduced number of characters had to be used. In this case c', spicule length, pharyngeal gland length and b, were the best characters for species separation.

**Table 4.4** Morphometric characters of adult females of *Pratylenchus brzeskii* from different localities of Western European coast. Measurements in µm and in form: mean ± standard deviation (range).

| Population | Ynyslas | Oostvoorne | Het Zwin | Biarritz | Carnon | Comporta | Bolonia | (after Karssen *et al*. 2000) |
|---|---|---|---|---|---|---|---|---|
| n | 28 | 10 | 10 | 6 | 15 | 5 | 10 | 20 |
| Head incisures | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Length | 568±69.3 (403-685) | 604±60.9 (539-683) | 617±59.1 (541-708) | 598±34.5 (555-640) | 566±60.7 (480-656) | 696±51.8 (636-756) | 554±56.32 (487-625) | 691±37 (624-682) |
| a | 29±2.1 (24.9-31.6) | 28.1±2 (24.5-31) | 29.1±2.4 (24.4-31.2) | 31.1±1.9 (27.9-33.1) | 26.5±1.1 (25.1-28.9) | 30.3±1.8 (28.2-32.8) | 25.9±2.6 (23.2-30.8) | 28.3±1.6 (25.8-30.7) |
| b | 7.7±0.5 (6.7-8.9) | 7.7±0.5 (7-8.5) | 7.7±0.6 (7.1-9) | 8.8±1 (7.5-9.8) | 8±0.6 (7-9.3) | 7.7±0.5 (7.1-8.6) | 7.5±0.2 (7.1-7.9) | 7.7±0.5 (7.2-8.4) |
| b' | 3.7±0.5 (2.5-4.6) | 4.1±0.5 (3.6-4.9) | 3.8±0.3 (3.4-4.2) | 4.1±0.4 (3.7-4.7) | 4.4±0.4 (3.6-4.9) | 3.8±0.3 (3.4-4.3) | 3.9±0.4 (3.5-4.9) | - |
| C | 15±1.3 (13-17.1) | 14.2±1.2 (12.1-15.4) | 14.6±1.3 (12.9-16.5) | 14.4±0.8 (13.6-15.6) | 15.8±1.6 (12.8-17.9) | 17.1±0.7 (15.8-17.5) | 13.5±1 (11.8-14.7) | 14.3±1 (13.2-16.5) |
| c’ | 3±0.5 (2.2-3.8) | 3.1±0.3 (2.6-3.6) | 3.2±0.5 (2.4-3.7) | 3.2±0.3 (2.8-3.5) | 2.8±0.3 (2.4-3.3) | 2.87±0.19 (2.53-3) | 3.1±0.3 (2.7-3.7) | - |
| V | 77.4±2.5 (70.5-80.4) | 77.1±3 (73.8-81.3) | 78.7±1.3 (76.1-81) | 77.7±2.2 (73.3-79.3) | 79.3±1.4 (77.7-81.4) | 76.1±2.9 (72.22-80) | 77.4±1.5 (75.1-80.2) | 77±1.0 (75-78) |
| Greatest body diameter | 19.6±2.5 (15-25) | 21.5±1.8 (18-23.5) | 21.4±3.8 (18-29) | 19.3±0.8 (17.9-20) | 21.4±2.4 (18-25.1) | 22.9±0.4 (22.4-23.5) | 21.44±1.69 (19.99-25) | 24.3±0.6 (23.4-25.6) |
| Body diameter at vulva | 18±2.9 (12-24) | 21.1±2.2 (17.5-25) | 20.3±4.3 (17-29) | 19.3±0.8 (17.9-20) | 19.7±1.3 (18-21) | 20.5±1.8 (18-22) | 20.3±2.59 (18-25) | 23.4±1.1 (21.5-24.5) |
| Body width at anus | 12.8±1 (11.7-16) | 13.8±1.4 (12-16) | 13.3±2.4 (11.3-17.9) | 13.1±0.8 (12.2-14.3) | 12.7±0.5 (12-13.6) | 14.1±0.4 (13.6-14.7) | 12.9±0.9 (11.5-14) | 15±1.1 (13.3-16.4) |
| Head width | 8.6±1.3 (3-10) | 9.3±0.8 (8-10.3) | 9.1±0.5 (8.6-10) | 9±0.4 (8.6-9.5) | 8.7±0.4 (8-9) | 9.8±0.71 (9.1-10.6) | 8.6±0.4 (7.6-9.0) | 10±0.4 (9.5-10.7) |
| Head height | 2.8±0.5 (2-4) | 2.5±0.5 (2-3.1) | 3±0.5 (2.2-3.5) | 2.8±0.5 (2.1-3.4) | 2.6±0.2 (2.3-3) | 2.5±0.1 (2.5-2.6) | 2.8±0.43 (2.09-3.28) | 3±0.3 (2.5-3.2) |
| Stylet | 18.1±0.6 (17-19) | 17.6±0.5 (17-18.4) | 18.2±0.4 (17.6-18.8) | 17.8±0.3 (17.5-18.2) | 17.1±0.3 (17-18) | 18.26±0.19 (18.1-18.6) | 17.58±0.5 (17.01-18.4) | 18.9±0.3 (18.3-19.0) |

**Table 4.4 (Continued)**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Stylet knob width (SKW) | 4.3±0.7 (3-6) | 5.1±0.1 (4.9-5.3) | 4.6±0.4 (4-5) | 4.5±0.2 (4.2-4.8) | 4.4±0.4 (4-5) | 4.76±0.42 (4.4-5.3) | 4.53±0.47 (4.05-5.6) | 5.6±0.3 (5.1-5.7) |
| Stylet knob height (SKH) | 2.2±0.5 (1.5-4) | 2.2±0.2 (1.9-2.5) | 2.3±0.5 (1.5-3.1) | 2.4±0.2 (2.1-2.6) | 2.6±0.5 (2-3.4) | 2.1±0.4 (1.8-2.7) | 2.6±0.2 (2.38-3.2) | 2.7±0.3 (2.5-3.2) |
| DGO | 2.4±0.6 (1.8-4) | 2.2±0.3 (1.9-2.9) | 2.5±0.6 (1.9-3.5) | 2.4±0.4 (1.8-2.9) | 2.1±0.2 (1.9-2.4) | 2±0.2 (1.67-2.36) | 2.6±0.4 (2.02-3.2) | 2.5±0.5 (1.9-3..2) |
| Ant. end to metacorpus | 55.3±3.9 (50-64) | 57.5±4.4 (50-63) | 59.9±4.2 (52-65) | 60±4.3 (53.1-64.5) | 57.9±5.2 (49-64) | 71.2±1.12 (70-72.9) | 58.7±2.1 (55.4-61.3) | 64±2.6 (61-69) |
| Ant. end to excretory-pore | 90±8.1 (69-104) | 86±8.1 (68-93) | 89±6.6 (77-96) | 78±13.8 (65-100) | 75±4.1 (69-82) | 96.4±3.44 (91-99) | 80.1±9.3 (70-95) | 94±7.7 (82-111) |
| Ant. end to pharyngeal-intestinal junction | 74±7.7 (53-84) | 78.8±5.3 (72-87) | 80±7.2 (71-90) | 69.2±12.3 (56.4-85.9) | 70.8±8.7 (61-83) | 90.2±2.3 (87.9-93) | 73.2±7.8 (63.4-87.1) | - |
| Ant. end to end of pharynx | 153±12 (131-175) | 149±13.5 (124-172) | 164±8.2 (144-171) | 147±21.2 (123-170) | 129±14.5 (110-161) | 181±10.9 (167-195) | 141±14.7 (126-167) | 168±15.6 (145-199) |
| Pharyngeal gland length | 73.2±12.5 (56-115) | 64.9±13.1 (49-85) | 79.3±9.8 (63-94) | 78.2±12.6 (63.5-97.2) | 59.4±9.9 (47-78) | 100.2±7.12 (95-108) | 56.97±12.5 (45.3-75) | 79±10 (66-101) |
| Post uterine sac | 23.8±4 (18-36.5) | 25.9±3.1 (19-28.5) | 22.5±0.6 (21-23.2) | 21.9±3.9 (18.1-28.1) | 24.2±2.4 (21-27.7) | 21±1.1 (20-22) | 24±2 (21-28) | 23.5±5.4 (19-34) |
| Lateral field width | 5.4±1.2 (4-8) | 5.9±0.7 (4.9-6.7) | 5.8±0.6 (5-6.8) | 6.2±0.8 (4.9-7) | 5.8±0.9 (4.5-7.2) | 6.6±0.6 (5.9-7.3) | 6.2±1 (4.6-7.6) | 8.4±0.6 (7.6-9.5) |
| Tail length | 38.1±4.5 (30-46) | 42.7±2.9 (36-46.3) | 42.2±2.7 (39.2-46.2) | 41.5±1.7 (39-44.1) | 36±2.6 (33-39) | 40.7±2.2 (37-43) | 40.7±1.9 (38-43.3) | 48.3 ± 2.2 (38 - 43.3) |
| Tail annules | 35.5±2.7 (29-39) | 32.9±2.2 (29-36) | 32.1±4.7 (20-36) | 30.7±4.1 (27-38) | 32.4±2.4 (28-36) | 33±1.4 (32-35) | 34.6±3.4 (27-39) | 30.7 ± 1.8 (29 – 34) |
| Hyaline part | 4.8±1.2 (2-7) | 4.4±0.9 (3.5-6) | 4.2±0.7 (3.4-5.2) | 3.8±0.7 (2.9-4.8) | 4.4±0.6 (3.6-5.5) | 5.6±1.2 (4.8-7) | 4.7±1.6 (3-7) | 5.2 ± 0.7 (4.5 – 6.5) |
| HW / HH | 3.2±0.8 (1-4.6) | 3.9±0.7 (2.9-5) | 3.1±0.5 (2.5-3.9) | 3.3±0.7 (2.5-4.4) | 3.4±0.3 (2.7-3.8) | 4.1±0.8 (3.3-5.1) | 3.1 ± 0.4 (2.4 - 3.6) | 3.5 ± 0.4 (3.0- 4.3) |
| SKW/SKH | 2±0.4 (1-3.3) | 2.3±0.2 (2-2.6) | 2.1±0.6 (1.5-3.3) | 1.9±0.1 (1.7-2.1) | 1.8±0.4 (1.3-2.5) | 2.2±0.4 (1.8-2.7) | 1.7 ± 0.1 (1.5 - 1.9) | 2.1 ± 0.2 (1.6 – 2.3) |

**Table 4.5** Morphometric characters of adult males of *Pratylenchus brzeskii* from different localities of Western European coast. Measurements in µm and in form: mean ± standard deviation (range).

| Population | Ynyslas | Oostvoorne | Bolonia | (after Karssen *et al*., 2000) |
|---|---|---|---|---|
| N | 15 | 15 | 3 | 10 |
| Head incisures | 2 | 2 | 2 | 2 |
| L | 559±44.2 (485-637) | 591±47.8 (520-653) | 617±67.5 (514-682) | 656±22 (624-682) |
| A | 30.9±1.9 (27.5-33.4) | 30±2.1 (27.5-33) | 31.2±1.5 (29.1-32.5) | 30.4±1.4 (28.1-31.7) |
| B | 7.3±0.8 (6.2-8.7) | 7.1±0.6 (6.5-7.9) | 6.6±0.8 (5.4-7.5) | 7.8±0.5 (7.2-8.3) |
| b' | 4±0.4 (3.4-4.8) | 4.1±0.3 (3.8-4.7) | 4.2±0.3 (3.7-4.4) | - |
| C | 14.7±1.9 (12-19.1) | 16±0.6 (15.5-17) | 15.5±1 (14.6-17) | 14.1± 0.7 (12.8-14.8) |
| c´ | 3.1±0.7 (2.2-4.7) | 2.8±0.2 (2.6-3.1) | 2.9±0.2 (2.6-3) | - |
| Greatest body diameter | 18.2±1.9 (15.2-22) | 19.8±1.8 (18-22) | 19.8±2.1 (17-22) | 21.6±0.9 (20.2-22.8) |
| Body width at anus | 12.8±2 (10-18) | 13.4±1.9 (12-16) | 13.9±1.3 (12-15.1) | 14.6±0.7 (13.9-15.8) |
| Head height | 3.1±0.7 (2-4) | 2.5±0.5 (2-3) | 2.5±0.3 (2.1-2.8) | 2.3±0.3 (1.9-2.5) |
| Head width | 8±0.6 (7-9) | 8.6±0.5 (8-9) | 8±0.3 (7.6-8.4) | 8.9±0.7 (7.6-9.5) |
| Stylet | 18±0.7 (17-19) | 17.4±0.5 (17-18) | 17.3±0.1 (17.1-17.5) | 17.9±0.3 (17.7-18.3) |
| Stylet knob width | 3.8±0.7 (3-5) | 4.1±0.8 (3.2-5) | 4.2±0.3 (4-4.8) | 5.3±0.5 (4.4 -5.8) |
| Stylet knob height | 2±0.5 (1.5-3) | 2.1±0.5 (1.5-3) | 2.3±0.2 (2.1-2.6) | 2.3±0.3 (1.9-2.5) |
| DGO | 2±1 (1-4) | 1.7±0.7 (1-2.4) | 2.6±0.6 (2-3.5) | 2.3±0.3 (1.9-2.5) |
| Ant. end to metacorpus | 47±13.2 (1-58) | 50.4±5 (43-57) | 61±3.9 (57-67) | 60±2.2 (55-63) |
| Ant. end to excretory-pore | 87.4±7 (74-96) | 86±8.4 (74-95) | 99±11.7 (82-111) | 91±8.8 (82-104) |
| Ant. end to pharyngeal-intestinal junction | 77.5±6.6 (70-91) | 83.7±7.2 (75-91) | 93.8±4.5 (88-100) | - |
| Ant. end to end pharynx | 139±7.1 (128-150) | 145±8.8 (135-156) | 146±8 (138-156) | 150± 7.2 (138-158) |
| Pharyngeal glands length | 64±7.2 (57-78) | 61±3.4 (57.5-65) | 55±8.8 (48-70) | 65±5.7 (57-73) |
| Lateral field width | 5.3±1.2 (4-8) | 5.4±0.9 (4.9-7) | 5.5±0.8 (5.1-6.9) | 6.7±0.6 (6.3-7.6) |
| Tail length | 38.6±4.9 (30-47) | 37±3.8 (32-42) | 40±5 (34.5-45.9) | 46.4±3.4 (43.0-51.8) |
| Spicules | 19.1±2.2 (16-22) | 19.8±1.5 (18-22) | 18.7±0.8 (18-19.7) | 22±0.7 (21.5-22.8) |
| Gubernaculum | 6.1±1.3 (4-8) | 5.9±0.4 (5.3-6.5) | 7.3±0.7 (6.6-8.1) | 7±0.4 (6.3-7.6) |

**Table 4.5 (Continued)**

| | | | | |
|---|---|---|---|---|
| Testis | 226±38.6 (168-313) | 245±44.6 (186-310) | 202±21.1 (179-234) | 300±27.7 (253-344) |
| HW / HH | 2.7±0.8 (2-4.5) | 3.6±0.9 (2.7-4.5) | 3.2±0.3 (2.9-3.7) | 3.2±0.5 (2.8-3.8) |
| SKW/SKH | 2±0.5 (1.3-3) | 2.1±0.6 (1.6-3) | 1.9±0.2 (1.6-2) | 2.4±0.4 (2.0-3.0) |

**Table 4.6** Morphometric characters of adult females of *Pratylenchus pratensis* from different localities of Western European coast. Measurements in µm and in form: mean ± standard deviation (range).

| Population | Ynyslas | Het Zwin | Comporta | São Jacinto | Bolonia | (after Loof, 1960) |
|---|---|---|---|---|---|---|
| N | 15 | 15 | 25 | 10 | 3 | - |
| Head incisures | 2 | 2 | 2 | 2 | 2 | 2 |
| L | 524±27 (478-572) | 468±41.2 (400-537) | 548±63.6 (419-645) | 508±66.8 (438-655) | 573±83.6 (483-649) | 380-690 |
| A | 27.2±2.2 (23.9-32.1) | 24.8±2.4 (20-29.7) | 27.8±3.7 (20.7-33.7) | 26.5±3.7 (20.5-32.7) | 28.9±6.6 (21.3-32.8) | 21-32 |
| B | 6.8±0.6 (5.8-7.7) | 5.8±0.7 (4.3-7.5) | 6.9±0.7 (4.8-7.9) | 7.1±1.1 (5.79-8.53) | 6.2±0.4 (5.7-6.5) | 4.7-7.1 |
| b' | 4.1±0.2 (3.6-4.4) | 3.6±0.4 (2.9-4.2) | 4.1±0.4 (3.4-4.8) | 3.9±0.5 (3.3-5.04) | 3.9±0.4 (3.5-4.2) | 3.4-4.6 |
| C | 15.7±1.5 (12.4-18.5) | 14±1.9 (10.9-17) | 15.3±2.2 (11.9-19.1) | 15.2±2.2 (11.9-19.9) | 14.8±1.9 (13.7-17) | 12-21 |
| c’ | 2.8±0.3 (2.2-3.5) | 2.9±0.4 (2.1-4.1) | 3.1±0.5 (2-3.7) | 2.8±0.3 (2.1-3.5) | 3.1±0.3 (2.9-3.4) | 1.8-3.1 |
| V | 75±3.4 (71.4-79.9) | 75±3.2 (70-80.4) | 74±2.7 (70.2-79.4) | 76.5±4.47 (71.4-82.4) | 73.7±1.6 (71.9-75) | 72-81 |
| Greatest body diameter | 19.4±2 (17-24) | 19±2.2 (14-24) | 19.9±2.3 (17-24.2) | 19.3 ±2.3 (17-24) | 20.2±2.4 (18-22.8) | |
| Body diameter at vulva | 17.9±1.8 (15-22) | 18.1±2 (14-22) | 19.1±2.6 (14.7-24.2) | 18±2 (16-22) | 20.2±2.4 (18-22.8) | |
| Body width at anus | 11.9±1.3 (10-14) | 11.6±1 (10-13) | 11.7±0.8 (10-14) | 11.72±0.7 (10.5-13) | 12.4±1.4 (11.3-14) | |
| Head width (HW) | 8.3±0.7 (7-9) | 7.4±0.6 (6.8-9) | 8±0.7 (6.8-9.6) | 7.75±0.6 (7-8.5) | 8.8±0.5 (8.4-9.4) | |
| Head height (HH) | 2.8±0.5 (2-3.5) | 2.4±0.4 (2-3.6) | 2.2±0.4 (1.5-3.2) | 1.9±0.4 (1-2.8) | 2.5±0.2 (2.3-2.7) | |
| Stylet | 16.9±0.6 (16-18) | 15.9±0.7 (14.4-17.5) | 16.9±0.7 (15.1-18) | 15.9±0.7 (15-17) | 17.1±0.6 (16.5-17.6) | 13-17 |
| Stylet knob width (SKW) | 4.5±0.8 (4-6.1) | 3.9±0.3 (3-4.5) | 4.5±0.5 (3.6-5) | 3.7±0.7 (3-5) | 4.5±0.4 (4.1-4.8) | |
| Stylet knob height (SKH) | 2.2±0.3 (2-3) | 2±0.2 (1.5-2.5) | 2.2±0.3 (1.8-3) | 2.1±0.4 (1.8-3) | 2.2±0.2 (2-2.3) | |
| DGO | 2.4±0.7 (1-3) | 2.1±0.5 (1.2-3.1) | 2.2±0.7 (1-3.2) | 1.92±0.4 (1-2.8) | 2.8±0.2 (2.6-2.9) | 2.5 |
| Ant. end to metacorpus | 49±5.9 (42-60) | 49±5.5 (40-60) | 60±4.3 (51-69) | 54±4.7 (48-61) | 56±2.4 (54-58) | |

**Table 4.6 (Continued)**

| | | | | | | |
|---|---|---|---|---|---|---|
| Ant. end to excretory-pore | 82±6.6 (66-93) | 76±9.6 (55-94) | 81±10.3 (60-105) | 87±5.3 (80-98) | 83±14.8 (73-94) | |
| Ant. end to pharyng.-intestinal junction | 77.9±7.4 (70-91) | 80.1±7.5 (64-92) | 80±5.6 (70-88) | 72.8±6.8 (60-79) | 92.7±7.5 (85-100) | |
| Ant. end to end of pharynx | 129.7±8.5 (119-148) | 130±11.9 (112-158) | 135.4±8.9 (112-154) | 129.4±6.4 (119-137) | 147±14.7 (138-164) | |
| Pharyngeal gland length | 51.8±3.2 (47-58) | 47.6±8 (33-64) | 54.9±5.5 (37-64) | 56.6±4.7 (46-63) | 55.7±7.4 (50-64) | |
| Post uterine sac | 22.9±2.7 (17.8-26) | 18.6±2.8 (14-23) | 19.8±4.1 (12.9-29.4) | 22.5±3.0 (20-30) | 22±1 (21-23) | |
| Lateral field width | 5.1±0.7 (3.5-6) | 5.3±0.8 (4-6.8) | 5.8±1.1 (3.8-8) | 4.9±1.12 (3-6.6) | 5.9±0.7 (5.1-6.5) | |
| Tail length | 33.5±2.8 (29-39) | 33.9±4.5 (25.1-45) | 36.3±5.2 (24.4-42) | 36.3±5.2 (24.4-42) | 39.1±7.2 (34.5-47.4) | 3 times body width/anus |
| Tail annules | 25±2.4 (21-28) | 26.2±2.2 (21-29) | 27.9±3.7 (21-35) | 27.9±3.7 (21-35) | 23.7±2.1 (22-26) | 20-28 |
| Hyaline part | 2.3±0.7 (1-3.5) | 2.3±0.7 (1-4) | 3.7±1 (2-6) | 2.2±1.21 (1-5) | 4±0.7 (3.2-4.7) | |
| HW / HH | 3.1±0.4 (2.4-4) | 3.2±0.5 (2.2-4.1) | 3.7±0.7 (2.5-5.3) | 4.26±1.1 (3-7) | 3.6±0.4 (3.1-3.9) | |
| SKW/SKH | 2.1±0.4 (1.7-3.1) | 2±0.3 (1.4-2.7) | 2.1±0.3 (1.4-2.5) | 1.7±0.2 (1.5-2) | 2.1±0.3 (1.8-2.4) | |

**Table 4.7** Morphometric characters of adult males of *Pratylenchus pratensis* from Het Zwin, Belgium. Measurements in µm and in form: mean ± standard deviation (range).

| Population | Het Zwin | (after Loof, 1960) |
|---|---|---|
| N | 10 | |
| Head incisures | 2 | |
| L | 496±82.5 (415-580) | 440-630 |
| A | 29.8±2.4 (27.4-32.2) | 28-36 |
| B | 6.3±1.3 (5.4-7.8) | 5.3-6.6 |
| b' | 3.8±0.6 (3.2-4.4) | - |
| C | 19.5±0.6 (18.8-20) | 17-21 |
| c´ | 1.9±0.1 (1.8-2) | |
| Greatest body diameter | 16.7±2.3 (14-18) | |
| Body width at anus | 13.3±1.5 (12.2-15) | |
| Head width | 7.1±0.5 (6.8-7.7) | |

**Table 4.7 (Continued)**

| | | |
|---|---|---|
| Head height | 2.6±0.5<br>(2-3) | |
| Stylet | 15.6±0.6<br>(15-16.3) | 13-14 |
| Stylet knob width | 3.6±0.4<br>(3.3-4) | |
| Stylet knob heigth | 1.6±0.1<br>(1.5-1.8) | |
| DGO | 1.7±0.4<br>(1.3-2) | |
| Ant. end to metacorpus | 51±6.7<br>(46-59) | |
| Ant. end to excretory-pore | 82±10<br>(75-94) | |
| Ant. end to pharyngeal-intestinal junction | 79.7±9.8<br>(74-91) | |
| Ant. end to end pharynx | 131±4<br>(128-136) | |
| Pharyngeal glands length | 52±6.2<br>(45-57) | |
| Lateral field width | 4.8±0.3<br>(4.5-5) | |
| Tail length | 25.4±3.5<br>(22.1-29) | |
| Spicules | 17.7±2<br>(16.2-20) | 17-19 |
| Gubernaculum | 6±0.1<br>(6-6.1) | 6-7 |
| Testis | 139±33.2<br>(116-163) | |
| HW / HH | 2.9±0.7<br>(2.1-3.5) | |
| SKW/SKH | 2.3±0.3<br>(2.1-2.7) | |

**Table 4.8** Morphometric characters of adult females and males of *Pratylenchus penetrans* from Biarritz, France. Measurements in µm and in form: mean ± standard deviation (range).

| Population | Females | (after Loof, 1960) | Males | (after Loof, 1960) |
|---|---|---|---|---|
| N | 21 | | 4 | |
| Head incisures | 2 | 2 | 2 | 2 |
| L | 460±24 (421-501) | 343-811 | 467±14.8 (449-486) | 305-574 |
| A | 24.7±2.1 (22-28.6) | 19-32 | 25.9±1.4 (23.4-27) | 23-34 |
| B | 6.5±0.6 (5.9-7.7) | 5.3-7.9 | 7.5±0.6 (7.1-8.5) | 5-8 |
| b' | 3.7±0.2 (3.3-3.9) | | 4±0.3 (3.6-4.4) | |
| C | 18±2.3 (15.3-21.9) | 15-24 | 18.2±1 (17.4-19.9) | 16-22 |
| c’ | 2±0.2 (1.8-2.4) | | 2±0.1 (1.8-2.1) | |
| V | 80±3 (72.6-83.1) | 75-84 | 18.1±1.5 (16.8-20.8) | |
| Greatest body diameter | 18.4±1.2 (15.9-19.6) | | 12.9±0.3 (12.6-13.3) | 13-16 |
| Body diameter at vulva | 18±2.1 (12.8-19.6) | | - | |
| Body width at anus | 12.5±1.3 (9.9-14.1) | | - | |
| Head width (HW) | 8±0.4 (7-8.5) | | 7.3±0.4 (6.8-7.6) | |
| Head height (HH) | 2.6±0.6 (1.2-3.1) | | 2.3±0.7 (1.2-2.9) | |
| Stylet | 15.3±0.8 (14-16.5) | 15-17 | 14.6±0.7 (14-15.3) | |
| Stylet knob width (SKW) | 4.1±0.3 (3.6-4.6) | | 3.4±0.3 (3-3.8) | |
| Stylet knob height (SKH) | 2.3±0.2 (2.1-2.7) | | 2.2±0.1 (2-2.3) | |
| DGO | 2±0.6 (1.1-3.1) | | 1.4±0.1 (1.3-1.6) | |
| Ant. end to metacorpus | 46±3.6 (40-51) | | 47±5.8 (41-52) | |
| Ant. end to excretory-pore | 71±6.1 (61-78) | 74-101 | 72±5.2 (64-78) | 66-79 |
| Ant. end to pharyngeal-intestinal junction | 61±6.7 (48-70) | | 62.7±5.6 (53.1-67.5) | |
| Ant. end to end of pharynx | 114±9.6 (105-139) | | 117±10.6 (103-128) | |
| Pharyngeal gland length | 54.1±7.1 (45-69) | | 54.5±10.6 (38.5-63.2) | |
| Post uterine sac | 18.6±1.5 (16-21) | | - | |

**Table 4.8 (Continued)**

| | | | | |
|---|---|---|---|---|
| Lateral field width | 6.5±1.1 (5-8.8) | | 6.1±0.9 (5.2-7.1) | |
| Tail length | 25.5±2.6 (20.8-29.3) | | 25.8±1.9 (22.6-27) | |
| Spicules | | | 15.6±2.2 (11.8-17.2) | 14-17 |
| Gubernaculum | | | 4±0.5 (3.6-4.8) | 3.9-4.2 |
| Testis | | | 211±11.3 (199-222) | |
| Tail annules | 19.4±5.1 (15-31) | 15-27 | | |
| Hyaline part | 2.5±0.7 (1.3-3.5) | | | |
| HW / HH | 3.3±1 (2.5-6) | | | |
| SKW/SKH | 1.8±0.2 (1.5-2) | | | |

**Table 4. 9** Standardized coefficients for canonical variates of *Pratylenchus* spp. for adult males and females

| Females | | | Males | | |
|---|---|---|---|---|---|
| | Root 1 | Root 2 | | Root 1 | Root 2 |
| % of variation | 62.80 | 28.26 | | 65.18 | 28.29 |
| Selected characters | Vector Loadings | | Selected characters | Vector Loadings | |
| A | 0.0159 | 0.1051 | A | -0.1244 | 0.2314 |
| B | -0.4441 | -0.1276 | B | 0.2091 | -0.4121 |
| C | 0.1802 | 0.1038 | C | 0.0142 | -0.3281 |
| c’ | -0.1323 | 0.9998 | c’ | -1.0840 | 0.4102 |
| Vulva position (V%) | -0.1011 | -0.1057 | Stylet length | -0.0285 | -0.1071 |
| Stylet length | -0.5910 | -0.0378 | Pharyngeal gland length | -0.2215 | 0.5723 |
| Pharyngeal gland length | -0.0233 | -0.0316 | Spicule length | -0.1777 | 0.0709 |
| Post Uterine Sac length | -0.1048 | -0.0427 | Gubernaculum length | -0.0209 | 0.0102 |
| Tail annules | -0.1754 | 0.2179 | | | |
| Hyaline part of tail | -0.3195 | -0.7620 | | | |

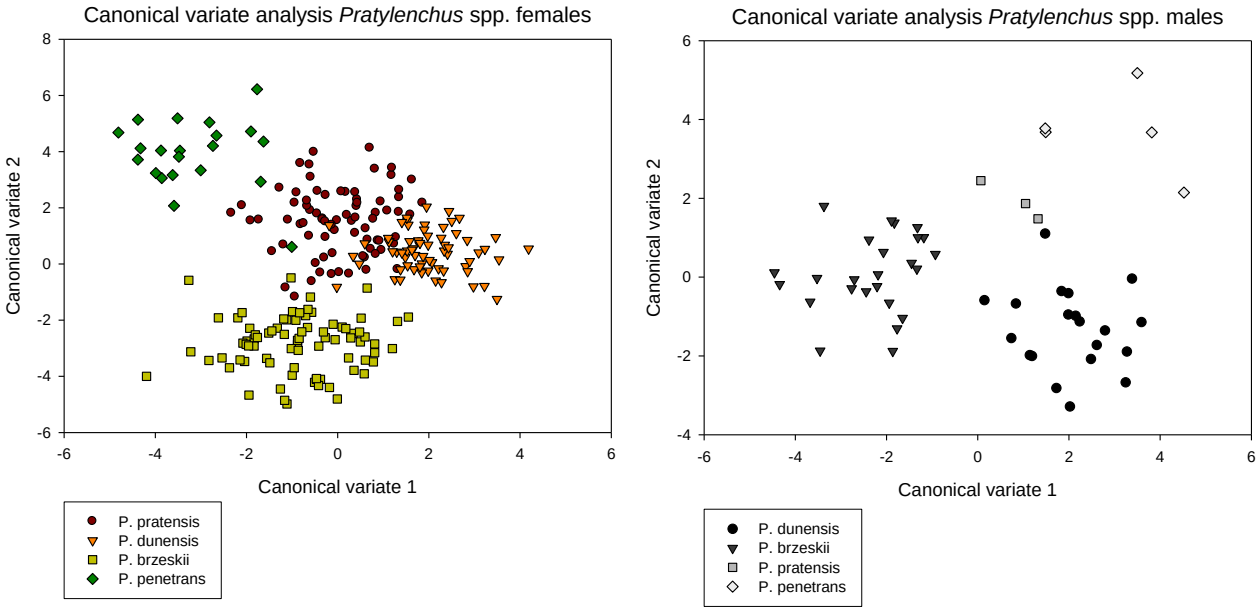


**Fig 4.5** Canonical Discriminant Analysis of *Pratylenchus* spp. isolated from *Ammophila arenaria* in Europe for females (A) and males (B) performed with ten and seven morphometric variables (Table 4.9), respectively.

### 4.3.3 Molecular characterization

D2D3 LSU

Using the primers described by de Ley *et al.* (1999) the polymerase chain reaction amplified a single DNA product of about 790 bp for each of the 19 *Pratylenchus* populations detected. Between one and three sequences were obtained per population (Table 4.1); they were subsequently used for the molecular analysis of this DNA region.

The comparison of the D2D3-LSU sequences at intraspecific level showed very low sequence divergences, ranging between 0% and 1.7% for *P.* dunensis, 0% and 1.3% for *P. brzeskii* and 0% and 1.4% for *P. pratensis* (Table 4.11-4.13). The interspecific variation was much greater with conspicuous divergences between species. *Pratylenchus dunensis* and *P. penetrans* were the closest species with 9.8% nucleotide divergence for the compared sequence. *Pratylenchus brzeskii* and *P. pratensis* showed a nucleotide divergence of 23.8%. Finally, *P. penetrans* and *P. brzeskii* showed a divergence of 26.1% (Table 4.14).

The relationship of the detected populations with other *Pratylenchus* species was measured throught MP analysis. The sequence of 11 populations obtained from genbank were aligned and compared with the sequences of the 19 *Pratylenchus* populations isolated from *A.*

*arenaria*. Only a partial sequence of the D2D3 region was used since sequences from genbank did not present the full length of the D2D3 region. A partial sequence of *Meloidogyne arenaria* Chitwood, 1949 (Neal, 1889) was used as an out-group. The pair wise alignment that was obtained presented 311 characters of which 65 were parsimony informative. The topology of the consensus tree obtained (Fig 4.7) positioned *P. dunensis* clustering as a separate clade with the group of *P. penetrans* (including the sequence from the population isolated from *A. arenaria*), *P. arlingtoni* and *P. fallax*. *Pratylenchus brzeskii* clustered in a separate group with *P. neglectus* and *P. minyus*. *Pratylenchus pratensis* composed a cluster on its own. The position of the different clusters containing the sequences of the 19 dune populations was supported by high bootstrap values, except for the group of *P. penetrans* that was supported by a 66.6 bootstrap value.

The size of the ITS-PCR product was different for the three species examined. For *P. dunensis* and *P. penetrans* a product of nearly 700bp (695 and 705 bp, respectively) was obtained, whereas for *P. brzeskii* the PCR yielded a slightly smaller fragment (680 bp). The ITS-RFLP patterns obtained after the restriction of the ITS-region, allow the three species to be differentiated (Table 4.10 and Fig 4.6). *Pratylenchus brzeskii* can be separated from the two other species by *Cfo*I, *Hind*III, *Msp*I and *Pst*I. *Pratylenchus penetrans* can be separated from the two other species by *Eco*RI. *Pratylenchus dunensis* cannot be separated with a single digestion, but can be distinguished by comparing the RFLP patterns obtained with *Eco*RI and *Msp*I; *Eco*RI only digested the PCR product of *P. penetrans* and *Msp*I only yielded restriction fragments for *P. brzeskii*.

**Table 4.10** Length (bp) of restriction fragments of rDNA Internal Transcribed Spacer regions for *Pratylenchus dunensis*, *P. brzeskii* and *P. penetrans*.

| *Enzyme* | *Pratylenchus dunensis* | *P. penetrans* | *P. brzeskii* |
|---|---|---|---|
| *Cfo*I | 415, 280 | 425, 275 | 610, 70 |
| *Eco*RI | No restriction | 405, 295 | No restriction |
| *Hind*III | 400, 295 | 410, 290 | No restriction |
| *Msp*I | No restriction | No restriction | 425, 255 |
| *Pst*I | 400, 295 | 395, 305 | 300, 380 |

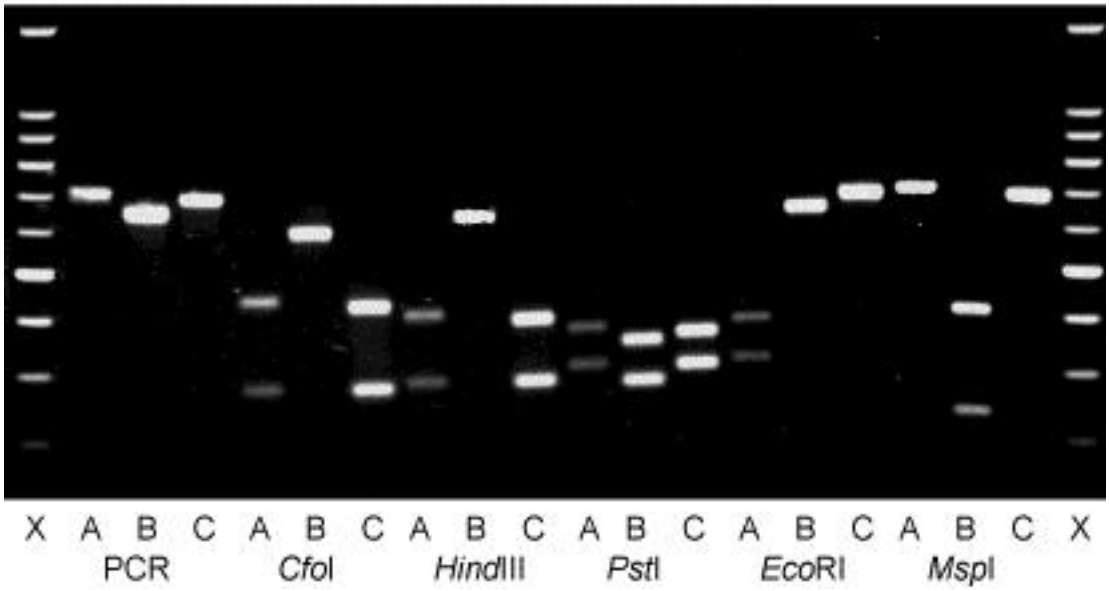


**Fig 4.6** Restriction fragments of amplified internal transcribed spacers of: (A) *Pratylenchus penetrans*; (B) *P. brzeskii*; (C) *P. dunensis*; (X) DNA ladder 100bp (Promega).

### 4.4 Discussion

The survey yielded a total of 19 populations associated with *A. arenaria* in its native distribution area. Four *Pratylenchus* species *viz. P. dunensis, P. brzeskii, P. pratensis* and *P. penetrans* were identified.

The CDA of morphometrical data enabled to discriminate the four species. The morphometrical characters used for this analysis correspond partially with the important taxonomic characters for the genus *Pratylenchus viz.* a, b, c, pharyngeal, gland length, tail length and tail annules (Brzeskii 1998). The characters that allowed discrimination of the species were b, c', tail annules and hyaline part. For this genus males do not provide many valid taxonomical characters. Nevertheless, it was possible to separate the different species using a total of eight morphometrical characters, from which c', b and spicule length yield the most of the variation between the populations.

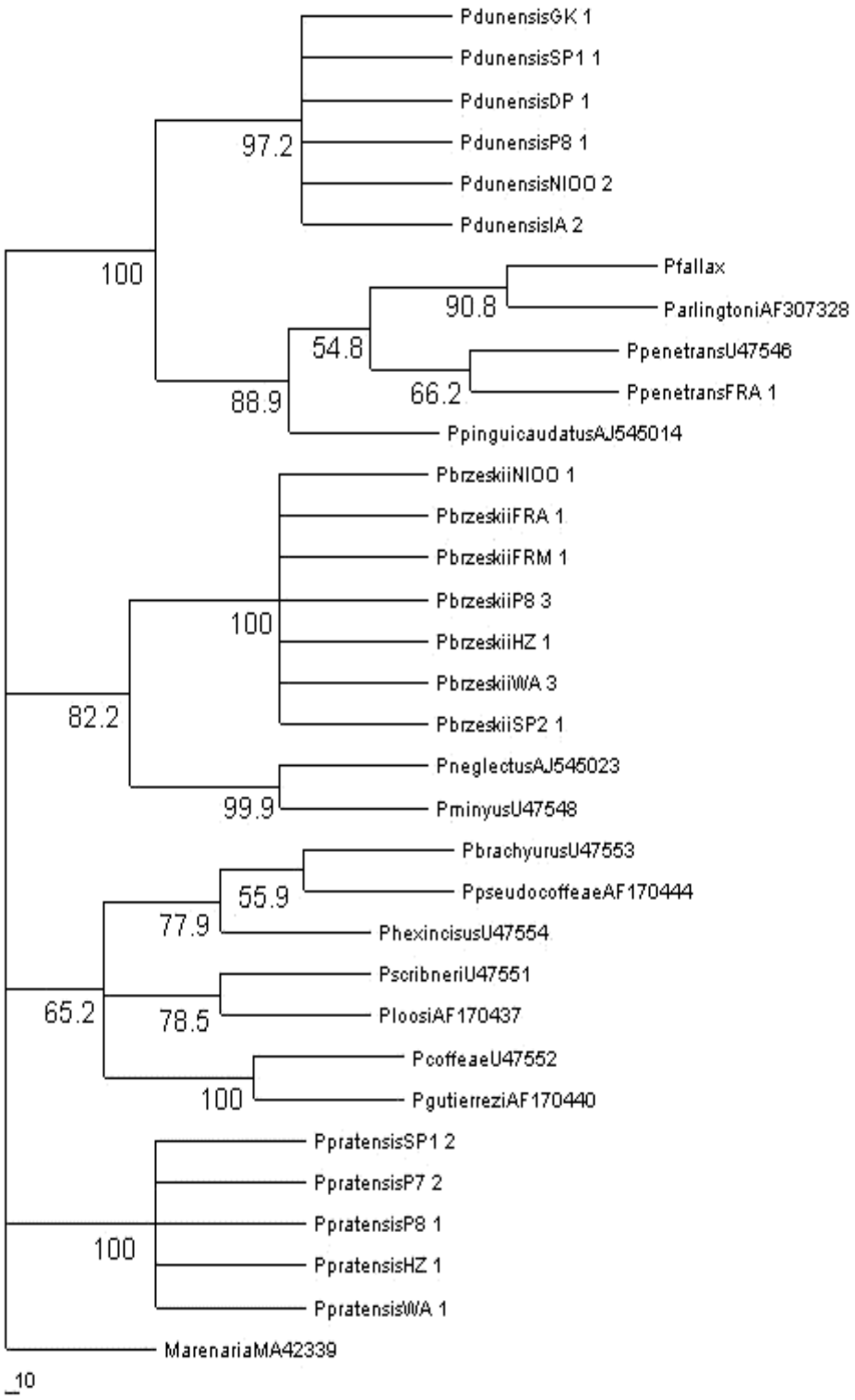


**Fig 4.7** Maximum parsimony tree (50% majority rule) obtained from the analysis of the alignment of 19 populations of *Pratylenchus dunensis, P. brzeskii, P. pratensis* and *P. penetrans* isolated from *Ammophila arenaria* with sequences of other *Pratylenchus* spp. GK: Groote Keeten, SP1: Matalascañas; DP: De Panne; P8: Comporta; P7: São Jacinto; NIOO: Oostvoorne; IA: Blakeney Point; FRA: Biarritz; FRM: Carnon: WA: Ynyslas; SP2: Bolonia.

**Table 4.11** Pairwise D2D3 sequence (750bp) alignment of *Pratylenchus dunensis* population.

| | % Similarity | | | | | |
|---|---|---|---|---|---|---|
| Populations | *P. dunensis* De Panne | *P. dunensis* Comporta | *P. dunensis* Blakeney Point | *P. dunensis* Oostvoorne | *P. dunensis* Groote Keeten | *P. dunensis* Matalascañas |
| *P. dunensis* De Panne | 100 | 99.5 | 99 | 98.7 | 99.1 | 99.3 |
| *P. dunensis* Comporta | | 100 | 98.6 | 98.3 | 99 | 99.4 |
| *P. dunensis* Blakeney Point | | | 100 | 99.7 | 99 | 98.3 |
| *P. dunensis* Oostvoorne | | | | 100 | 98.7 | 98.1 |
| *P. dunensis* Groote Keeten | | | | | 100 | 98.7 |
| *P. dunensis* Matalascañas | | | | | | 100 |

**Table 4.12** Pairwise D2D3 sequence (750bp) alignment of *Pratylenchus brzeskii* populations.

| | % Similarity | | | | | | |
|---|---|---|---|---|---|---|---|
| Populations | *P. brzeskii* Comporta | *P. brzeskii* Het Zwin | *P. brzeskii* Ynyslas | *P. brzeskii* Carnon | *P. brzeskii* Oostvoorne | *P. brzeskii* Bolonia | *P. brzeskii* Biarritz |
| *P. brzeskii* Comporta | 100 | 99.8 | 99.6 | 99.2 | 99.3 | 99.2 | 98.8 |
| *P. brzeskii* Het Zwin | | 100 | 99.7 | 99.3 | 99.2 | 99 | 98.7 |
| *P. brzeskii* Ynyslas | | | 100 | 99.6 | 99.3 | 99 | 98.8 |
| *P. brzeskii* Carnon | | | | 100 | 98.9 | 98.9 | 98.7 |
| *P. brzeskii* Oostvoorne | | | | | 100 | 98.8 | 98.9 |
| *P. brzeskii* Bolonia | | | | | | 100 | 98.5 |
| *P. brzeskii* Biarritz | | | | | | | 100 |

**Table 4.13** Pairwise D2D3 sequence (750bp) alignment of *Pratylenchus pratensis* populations.

| | % Similarity | | | | |
|---|---|---|---|---|---|
| Populations | *P. pratensis* Ynyslas | *P. pratensis* Bolonia | *P. pratensis* Het Zwin | *P. pratensis* Comporta | *P. pratensis* São Jacinto |
| *P. pratensis* Ynyslas | 100 | 99.7 | 99.8 | 99.8 | 99.6 |
| *P. pratensis* Bolonia | | 100 | 99.8 | 99.8 | 99.6 |
| *P. pratensis* Het Zwin | | | 100 | 100 | 99.7 |
| *P. pratensis* Comporta | | | | 100 | 99.7 |
| *P. pratensis* São Jacinto | | | | | 100 |

**Table 4.14** Pairwise D2D3 sequence alignment (750bp) of *Pratylenchus dunensis*, *P. brzeskii*, *P. pratensis* and *P. penetrans.*

| | % Similarity | | | |
|---|---|---|---|---|
| Populations | *P .brzeskii* Oostvoorne | *P. pratensis* Ynyslas | *P. penetrans* Biarritz | *P. dunensis* Groote Keeten |
| *P .brzeskii* Oostvoorne | 100 | 76.2 | 73.9 | 76 |
| *P. pratensis* Ynyslas | | 100 | 79.1 | 79.1 |
| *P. penetrans* Biarritz | | | 100 | 90.2 |
| *P. dunensis* Groote Keeten | | | | 100 |

The morphometrics of all the populations fitted the descriptions and earlier observations with the exception of *P. brzeskii* for which the obtained measurements showed a smaller range for all the characters with respect to the type population. Since the fixation procedure was different from the one used to describe the type population (Karssen *et al.* 2000) it is possible that that had an influence on the morphometrics. Also the period in which the sampling was made in relation to the host growth period, might have an influence on the morphometrics of the specimens. However, the populations included in this study were obtained at different sampling periods and localities, it is likely that the range presented in this study complement rather than dispute the data presented by Karssen *et al.* (2000).

The type population described in this paper as *P. dunensis* was originally identified as *P. penetrans*. However, further studies of additional molecular data, morphological characters and morphometrics, indicated that the differences compared with *P. penetran*s were too important to be ignored. *Pratylenchus dunensis* was therefore described from *A. arenaria* in Groote Keeten, The Netherlands. In addition to the morphology and morphometrics the sequence comparison of the D2D3 rDNA expansion region clearly separates the new species not only from the other species associated with *A. arenaria* (*viz. P. brzeskii, P. pratensis* and *P. penetrans*) but also from other *Pratylenchus* spp. isolated from other parts of the world and hosts*;* as revealed by the MP analysis and the direct sequence comparison.

rDNA D2D3 sequences have been commonly used to separate different groups of nematodes at species level, including pratylenchids (AlBanna *et al.* 1997; Powers *et al.* 1997; Duncan *et al.* 1999; Handoo *et al.* 2001; Spiridonov 2004). Our results confirm this fact but demonstrate that this region cannot be used for the study of intraspecific variation. The results indicate that this region is highly conserved with an intraspecific variation usually smaller than 1% (0-0.3%) and only within *P. dunensis* a higher percentage of variation (~1.7%) was found. Variation of the D2D3 region between isolates of the same species has been acknowledged (Duncan *et al.* 1999).

The phylogeny inferred from the D2D3 data sets *P. dunensis* in a clade with *P. fallax, P. arlingtoni, P. convalliarae* and *P. pinguicaudatus. Pratylenchus brzeskii* is grouped with *P. minyus* and *P. neglectus.* The topology of the consensus tree obtained in this study is relatively similar to the one obtained previously for a partial sequence of the D2D3 rDNA (De Luca *et al.* 2004), However, *P. pratensis* was not included anteriorly, and in this case forms a separate cluster containing the different populations of the species detected during this survey.

The use of ITS-RFLP for species identification has been widely used for different groups of nematodes (Orui & Mizukubo 1999; Subbotin *et al.* 2003; De Luca *et al.* 2004; Madani *et al.* 2004). The type population of *P. dunensis* was originally identified as *P. penetrans* mainly because its ITS-RFLP revealed a similar pattern to the reported by Waeyenberge *et al.* 2000 (Karssen *et al.* 2001). The difference between *P. dunensis* and *P. penetrans* is now further confirmed by differences in patterns obtained after restriction with more enzymes. The RFLP profiles I obtained with C*f*oI, *Hind*III and *Msp*I were the same as those presented by Karssen *et al.* 2001; however; *EcoR*I yielded a different pattern for both species (no restriction *vs* two bands) and separates both species. Obviously, the number of restriction enzymes used was not sufficient in the observations of Karssen *et al.* (2001). It is clear that the variability of DNA fragments is better estimated when comparing their sequences rather than restriction patterns. As a consequence, my results reject the previously identification and also justifies the description as a new species.

Previous studies have demonstrated the presence of *P. brzeskii* in dunes along the North Sea and the Baltic Sea (Karssen *et al.* 2000). *Pratylenchus brzeskii* was found in the North Sea in Koksijde and Bray-Dune in Belgium, in Oostvoorne, the islands of Texel and Terscheling in the Netherlands but, also in Cabourg at the Atlantic coast in France. My results support previous observations, and confirm that this species show a wider distribution than previously expected, occurring not only in the indicated areas, but also in the British Isles (Ynyslas), in more southern latitudes of the Atlantic such as the South-West coast of the Iberian Peninsula (São Jacinto, Comporta and Bolonia) and the Mediterranean basin (Carnon). According to this wide distribution is it reasonable to assume that this species is linked to the distribution of *A. arenaria*, not only in the Northern part of Europe, but also at more Mediterranean latitudes.

*Pratylenchus pratensis,* until now, has never been reported on *Ammophila arenaria.* My results show that this species is a common member of the root-feeding nematode community in foredunes with *A. arenaria* and is found from the North Atlantic (Ynyslas, Het Zwin) up to the South West of the Iberian Peninsula (Comporta and Bolonia). I did not detect *P. pratensis* in Carnon. Previously, Schreck-Reis *et al.* (2005) analyzed the dynamics of colonization by PPN of *A. arenaria* at different locations of the Portuguese coast. One of the sampling points of that study was the front dunes of São Jacinto, which are also included in my study. *Pratylenchus* spp. was found in the nematode community at this locality; however, the identity of this Portuguese population was not confirmed. Because in my survey I

detected only *P. pratensis* in São Jacinto, it could be inferred that the population from this previous work in São Jacinto belongs to *P. pratensis.*

*Pratylenchus dunensis* has also a very wide distribution, from the North Sea and the North Atlantic coast line (Groote Keeten, Oostvoorne, De Panne) until the South-West of the Iberian Peninsula (Comporta and Matalascañas). However, I did not detect this species in the Mediterranean sampling spot (Carnon). My sampling survey comprised four localities with a strict Mediterranean climate *viz.* São Jacinto, Matalascañas, Bolonia and Carnon (Rivas-Martínez 1987). However, only the latter was located in the Mediterranean Basin and the other three were in the Atlantic coastline but having a Mediterranean climate. It is not possible to be sure that this species is really absent in this area (since the climate conditions are similar) or my results are biased by having only one sampling point in the Mediterranean coastline.

*Pratylenchus penetrans* has been reported associated with the North American dune grass *A. breviligulata* (Seliskar & Huettel 1993). In Western Europe, previous studied indicated the occurrence of this species associated with stands of *A. arenaria* at different dune stages and *Hyppophäe rhamnoides* in stabilized dunes (Zoon *et al.* 1993). In this survey, restricted to foredunes with vigorous *A. arenaria, P. penetrans* was detected only in one sampling point of the Atlantic coast, in Biarritz (France). Thereby, this species does not seem to be the most dominant species in front dunes; however, as shown previously, it probably occupies a more inner sector of the dune with presence of decaying *A. arenaria* stands and other species of the plant succession.

*Pratylenchus scribneri* associated with *A. arenaria* has been reported from a locality on the North Sea area (Kampinos dunes, Poland) (Van der Putten *et al.* 2005). At none of the sampling points included in my study I have detected the presence of this species. Its distribution in relation to *A. arenaria* needs also consideration. It might be interesting to asses whether it occurs in inner dune stages or whether its natural range of distribution is located at more northern localities than those included in my sampling survey.

*Ammophila arenaria* presents two different subspecies in the natural area of distribution, *A. arenaria* ssp. *arenaria* at the North Atlantic and *A. arenaria* ssp. *arundinacea* at Mediterranean latitudes (South-West Iberian Peninsula and Mediterranean Basin). The region where the shift in subspecies is observed corresponds with the North coast of the Iberian Peninsula (S. Rodríguez-Echeverría, pers. com). My sampling survey demonstrated that three of the four *Pratylenchus* species that occur in coastal dunes (*P. dunensis, P. pratensis* and *P. brzeskii*) are found associated with the two recognized

subspecies of the host plant. *Pratylenchus penetrans* on the contrary, was only found associated with *A. arenaria* ssp. *arenaria* in Biarritz (France). Whether or not the multiplication of these nematode species is affected by the plant subspecies needs to be addressed in order to verify if the presence/absence depends on the type of host; or should be explained by other factors.

From the data obtained, it is striking that a complex of two to three *Pratylenchus* species seem to occur in most of the sampling sites analyzed. The sampling survey was designed to be qualitative (detect absence/presence) rather than quantitative. Unfortunately, it was not possible to get samples from all sampling points at the same time and with the same intensity and regularity. In consequence, it was not possible to precise whether seasonal variation in the *Pratylenchus* species composition occurred within a sampling site; which can be an interesting point to analyze.

The results of the survey reveal that the diversity of *Pratylenchus* spp. associated with *A. arenaria* is richer than expected. The distribution of *P. dunensis, P. brzeskii* in coastal foredunes with vigorous *A. arenaria* and *E. farctus*, suggests the adaptation of the species to this habitat and thus, might play a role in the dynamics and structure of the plant community in coastal dunes. The relationship between the different groups of nematodes and the host plant are not yet clear and therefore, to have a clearer picture of the ecological processes involving plant-parasitic nematodes in coastal dunes, the effect of the *Pratylenchus* species reported in this study should be considered.

# Chapter 5

## *Damage and multiplication of* **Pratylenchus** *spp. on dune plants*[¥]

[¥] de la Peña, E., Vandegehuchte, M., Bonte, D., Moens, M. Analysis of the specificity of three root-feeders towards grasses in coastal dunes. Submitted to *Plant and Soil*

## 5.1 Introduction

Plant yield losses are influenced by the pathogenicity of nematode species involved, by the nematode population density at planting, by the susceptibility and tolerance of the host and by a range of environmental factors (Trudgill *et al.* 1996). The relationship between initial nematode densities and the effect on plant growth has been studied for economic important crops (Ingham & Detling 1991; Cook *et al.* 1992; McSorley & Gallaher 1993a; Sarah *et al.* 1993; De Ruijter & Haverkort 1999). One of the models that relate these variables is the Seinhorst's damage function (1965) which is expressed as:

$$y = y_m \text{ , for } x \leq t, \quad (1)$$

$$y = y_m \bullet m + y_m \bullet (1 - m) \bullet z^{(x-t)} \text{ , for } x > t \quad (2)$$

where y = plant biomass, x = nematode population density (P); t = the nematode population density below which biomass reduction can not be measured (= also known as the tolerance limit or damage threshold density (T)); $y_m$ = mean plant biomass where the nematode density is below the tolerance limit (t); m = a constant, usually between zero and one. Therefore, $y_m \bullet m$ is the biomass at the highest possible nematode density, and z = the slope determining parameter (value between zero and one).

The Seinhort's function does not account for how the increase of initial nematode densities determines the multiplication of a given nematode species. Different models derived from the competition-resource equation (Nicholson 1933) try to explain the connection between these two variables; Ferris (1985) proposed a model derived from a negative exponential decay function in which the relationship between nematode multiplication and initial nematode density is expressed as:

$$Pf/Pi = ce^{-bLnPi} \quad (3)$$

Where Pf/Pi is the nematode multiplication, Pi is the initial nematode density, b is the rate determining variable, c= the scaling factor and e is the base of the natural logarithm. Since $e^{LnPi} = Pi$ the model can also be expressed as:

$$Pf/Pi = cPi^{-b} \text{ or } Pf = cPi^{1-b} \quad (4)$$

Understanding how and at which population densities nematodes affect negatively different crops and how the initial densities determine nematode multiplication has important practical and economical consequences in pest management and crop protection (Schomaker 2006). However, the effect of plant-parasitic nematodes (PPN) on plant growth and how is affected the multiplication by different natural hosts is scarcely addressed in natural systems.

In *A. arenaria* the effect of PPN on growth has been analyzed only in a few cases. For *P. penetrans* previous studies used a small range of densities and showed detrimental effects on aboveground biomass but did not affect root parts Brinkman (2005).

Another important aspect of plant-nematode interactions is the study of the way different hosts affect nematode multiplication. The ability of a nematode to multiply on a given host will influence not only that particular host but also the plants that will follow in crop rotation (or in later stages of natural succession) (McSorley & Gallaher 1993b; Ehwaeti *et al.* 2000). Also, the multiplication of a nematode species will affect the access to roots (food sources) by other coexisting nematode species. Therefore, is an important factor in the competition between nematode species (Eisenback 1993).

As explained in Chapter 2, *A. arenaria* occupies early stages in the plant succession that occurs in coastal dune and thereby is preceded or replaced by other species. *Elymus farctus* is the preceding species of *A. arenaria* in foredunes (van der Laan 1985); in stabilized dunes *A. arenaria* is replaced by other grass species and shrubs such as *Carex arenaria, Festuca rubra, E. athericus, Hyppophäe rhamnoides,* etc (Rodwell 2000). However, the multiplication of *Pratylenchus* spp. on different dune hosts, up to now has not been studied.

The separation of *A. arenaria* in two subspecies (Chapter 2) is based on different morphological traits reflecting adaptation to different environmental conditions. The phenotypic plasticity of *A. arenaria* occurring in dunes and recent molecular observations suggest that genetic differences in *A. arenaria* populations could also be linked to different ecological requirements (Gray 1985). The multiplication abilities of PPN (including *Pratylenchus* spp.) depend on the quality of the host plant; which is determined by its tolerance and resistance as a function of genetical and physiological attributes. Based on observations of other pathogen-host associations the prediction is that pathogens multiply better on their sympatric hosts (from the same locality) which reflect an adaptation to the natural host populations (Lively 1999; Dybdahl & Storfer 2003). However, until now it has not been explored whether the genetic diversity of *A. arenaria* has consequences on the multiplication of PPN.

The present chapter addresses two fundamental aspects of the interaction between *A. arenaria* and *Pratylenchus* spp. On first place, the effect on *A. arenaria* growth of different *Pratylenchus* species found in coastal dunes was studied. Secondly, the multiplication of *Pratylenchus* spp. on different hosts was investigated. Three related experiments were performed. A classical nematode density-growth experiment, in which the effect of different nematode densities on the host plant growth and on the multiplication of *Pratylenchus* spp., was conducted. To address the effect of the host plant on the multiplication of *Pratylenchus* spp. two cross inoculation experiments were performed. One experiment compared the multiplication on different host species (*A. arenaria* and *Elymus farctus* two dune grass species and *Lolium perenne*, a typical species of inland grasslands). The second experiment compared the multiplication of several *Pratylenchus* spp. on different *A. arenaria* populations from different geographical origin.

## 5.2 Materials and Methods

### 5.2.1 Nematode cultures and inocula

A total of five nematode cultures were used in the experiments, *Pratylenchus* spp. were multiplied in *A. arenaria* plants from their own origin when possible (Table 5.1). The maintenance of nematode cultures and the extraction of *Pratylenchus* inocula were described in Chapter 3. Prior to the experimental set up, the different nematode stages (adult females, males and juveniles) of all inocula were counted. No significant differences (data not shown) were observed between the juvenile/adult ratio for the cultures used except for the *P. brzeskii* (Biarritz) (Table 5.1).

**Table 5.1** *Pratylenchus* spp. cultures and *Ammophila arenaria* populations used for experiments

| Nematode populations | Plant populations |
|---|---|
| [1]*P. dunensis* (Oostvoorne)$^{54/17/29+}$ | *A. arenaria* (Oostvoorne)‡ |
| [1]*P. dunensis* (Comporta)$^{49/21/30+}$ | *A. arenaria* (Blakeney Point)‡ |
| [1]*P. brzeskii* (Ynyslas)$^{56/16/28+}$ | *A. arenaria* (Ynyslas)‡ |
| [2]*P. brzeskii* (Biarritz)$^{69/12/19+}$ | *A. arenaria* (Comporta)† |
| [2]*P. penetrans* (Sandhoven*)$^{59/21/20+}$ | *Elymus farctus* Viv. (De Panne) |
| - | *Lolium perenne* L. |

[1]: Nematode populations multiplied on *A. arenaria* plants from the same origin [2]: Nematode populations multiplied on *A. arenaria* from Oostvoorne (the Netherlands); [+]Average percentage of juveniles/males/females; ‡ *Ammophila arenaria* ssp. *arenaria*; †*Ammophila arenaria* ssp. *arundinacea*; * Nematode culture donated by L. Waeyenberge (ILVO Nematode collection)

### 5.2.2 Experiment 1: Nematode density-*Ammophila arenaria* growth

Two-week old seedlings (germination procedure described in Chapter 3) of *A. arenaria* (Oostvoorne) growing in 500 ml pots filled with 600 g sterilized sand collected in Het Zwin (Belgium) were inoculated with either *Pratylenchus dunensis* (Oostvoorne), *P. brzeskii* (Ynyslas) or *P. penetrans* (Sandhoven). For each nematode species 3000, 1500, 750, 375, 190, 90 or 45 nematodes per pot were inoculated to obtain seven nematode densities (*viz.* 5, 2.5, 1.2, 0.6, 0.3, 0.15 and 0.075 nematodes·$g^{-1}$sand). Six replicates were used per inoculation density and six control pots (no inoculation) were used per nematode species. Pots were distributed randomly on the bench of a growth chamber. The experiment was run for 16 weeks (June-September 2005) with a day/night illumination (250 µmol $m^{-2}h^{-1}$) regime of 16/18 h and a temperature of 22/18°C. Plants were fertilized with 50 ml of half strength Hoagland solution (Chapter 2) every 20 days and water content was reset at 5-10% every 2 days using 100-150 ml of demineralized water.

At harvest, plant growth related parameters (fresh total biomass, aboveground biomass and root biomass) were recorded. The dry weight of above and belowground plant parts and nematode numbers were calculated as described in Chapter 3. The multiplication was computed by dividing the final obtained number of nematodes (Pf) by the initial inoculation density on that pot (Pi).

### 5.2.3 Experiment 2: Host Suitability experiment

In a cross inoculation experiment three plant species *viz. A. arenaria* (Oostvoorne), *E. farctus* (De Panne) and *Lolium perenne* were inoculated with *P. dunensis* (Oostvoorne), *P. brzeskii* (Wales) or *P. penetrans* (Sandhoven). Two-week old seedlings from each host were inoculated with 150 nematodes of each of the *Pratylenchus* species separately. Pots with un-inoculated seedlings were used as control for each of the plant species. Seven replicates were used for the different plant-nematode combinations or controls. Pots were placed randomly on benches in a glasshouse. The experiment ran for 12 weeks (July-September 2005) under ambient light conditions and 25/18°C mean day/night temperatures. Plants were fertilized three times during the experiment (once every four weeks) with 50 ml of half-strength Hoagland's solution. Water content was reset to 5-10% every two days adding by 100-120 ml of demineralized water. At harvest plant growth parameters and nematode numbers were assessed (see above).

### 5.2.4 Experiment 3: *Ammophila arenaria*-host suitability

In a cross-inoculation experiment five *Pratylenchus* populations (of *P. dunensis*, *P. brzeskii* and *P. penetrans*, Table 5.1) were inoculated on four *A. arenaria* populations (*A. arenaria* ssp. *arenaria* and *A. arenaria* ssp. *arundinacea*, Table 5.1). Pots and seedlings were prepared as explained above. Each *Pratylenchus* population was inoculated in each of the *A. arenaria* populations; 150 nematodes were inoculated per pot and pots with un-inoculated seedlings were used as controls. Six replicates were used for each plant species-nematode combination or control pots. Pots were randomly distributed on the bench of a glasshouse with environmental and fertilization conditions as in previous experiment.

At harvest plant growth related parameters were assessed as described previously. However, for this experiment only nematodes from roots were extracted.

### 5.2.5 Statistical analysis

#### 5.2.5.1 Experiment 1

To analyze the effect of initial nematode densities on plant growth related parameters a Spearman rank test (SPSS 11.0.1) was used to detect possible correlations of plant growth (total dry biomass, aboveground biomass and belowground) and Pi.

The SeinFit computer program (Viaene 1997) was used for the estimation of the Seinhorst's equation. The value of z was set at 0.95 based on Seinhorst's calculations for different crops (1998). The 'Grid Method" implemented by the program was used to obtain the equation variables; by this method the minimum residual sum of squares is calculated for a range of *t*, *z* and *m* values. Once, the equation was obtained with Seinfit, the model curve for each of the species was constructed by iterating 3000 different Pi values.

The relationship between nematode multiplication and initial nematode density was estimated by fitting the data to the negative exponential decay function proposed by Ferris (1985).

#### 5.2.5.2 Experiment 2

The statistical analysis was performed with the ANOVA General Linear Model (SPSS). All data were checked for normality with the Kolmogorov-Smirnov test and homogeneity of variance with Levene's test. Variables that did not meet ANOVA model assumptions were Log (x+1) transformed. Nematode multiplication, total dry biomass, aboveground dry biomass and root dry biomass were first compared with two-way ANOVA and Tukey's multiple range comparisons using inoculation (I) (control, *P. dunensis*, *P.*

*brzeskii* and *P. penetrans*) and plant identity (P) (*A. arenaria*, *E. farctus* and *L. perenne*) as factors. When ANOVA assumptions were not achieved (root dry biomass for all plant comparisons) a non-parametric Kruskal-Wallis test was performed using either plant identity or inoculation as grouping factors. Afterwards, one way ANOVA (with inoculation as single factor) and Tukey's multiple range test for overall comparison were conducted to estimate differences in nematode multiplication and biomass (total, root dry and aboveground) in each host separately.

#### 5.2.5.3 Experiment 3

Normality and homogeneity of variances were checked as previously explained. Normality was not achieved for plant growth parameters (total dry biomass, aboveground dry biomass, root dry biomass) and therefore, a non-parametric Kruskal-Wallis test using plant identity or inoculation as grouping factor was performed for those variables. One-way ANOVA and Tukey's multiple range comparison were used (with plant population as factor) to estimate differences in plant growth parameters (in this case normality was achieved), nematodes·$g^{-1}$ dry root, total number of nematodes for each nematode population, separately.

## 5.3 Results

### 5.3.1 Experiment 1

The results obtained in the experiment clearly show a negative effect on *A. arenaria* growth by high densities of the three species studied. Negative correlations according to the Spearman rank test were found between nematode density and *A. arenaria* total dry biomass, root dry biomass and aboveground dry biomass for the three species analyzed. The slope variable (rho coefficient) shows that the correlation was stronger in aboveground biomass with values ranging from -0.744 to -0.572 while for root biomass the values ranged between -0.381 and -0.510 (Table 5.2). The relationship between initial inoculation density and plant growth was moderately supported by the Seinhorst equation (Table 5.3, Fig 5.1). Although the $r^2$ values obtained in each of the three equations were low, the fitted Seinhorst functions shows differences in tolerance limit (population density (Pi) at which damage first became apparent) for each of the three species (Table 5.3). *Ammophila arenaria* has a similar t value for *P. dunensis* (300) and *P. penetrans* (360) (0.5 and 0.6 nematodes·$g^{-1}$ soil), whereas for *P. brzeskii* the t value was higher (600) (1 nematode·$g^{-1}$soil). Also, the lowest plant growth (biomass) is different for each of the species compared, 1.32, 1.45 and 1.16 g respectively

(Table5.3), These values correspond with a reduction of plant growth at high nematode densities ranging between 35 to 55%.

**Table 5.2** Spearman's rho coefficients for correlations between initial nematode density and total biomass, root biomass, aboveground biomass for *Pratylenchus* spp. ** *P≤0.01, *P≤0.05*

| | Total dry biomass | Root dry biomass | Aboveground dry biomass |
|---|---|---|---|
| *P. dunensis* | - 0.528** | - 0.381** | - 0.616** |
| *P. brzeskii* | - 0.628** | - 0.395** | - 0.667** |
| *P. penetrans* | -0.616** | - 0.510* | -0.510** |

**Table 5.3** Parameter estimates for the Seinhorst equation that relates initial *Pratylenchus* spp. density (Pi) and growth (total dry biomass).

| | $y_{max}$ | Z | m | t | SS | $r^2$ | Equation* |
|---|---|---|---|---|---|---|---|
| *P. dunensis* | 2.40 | 0.95 | 0.550 | 300 | 19.965 | 0.39 | $y = 1.325 + 2.40 \bullet (1\text{-}0.55) \bullet 0.95^{(x-300)}$ |
| *P. brzeskii* | 2.24 | 0.95 | 0.650 | 600 | 26.501 | 0.22 | $y = 1.458 + 2.24 \bullet (1\text{-}0.65) \bullet 0.95^{(x-600)}$ |
| *P. penetrans* | 2.59 | 0.95 | 0.450 | 360 | 29.873 | 0.41 | $y = 1.168 + 2.59 \bullet (1\text{-}0.55) \bullet 0.95^{(x-360)}$ |

*The Seinhorst model is of the form: $Y = y_{max}$ for $Pi \leq t$, and $Y = y_{max} \bullet m + Y_{max} \bullet (1\text{-} m) \bullet z^{(Pi-t)}$ for $Pi > t$
$Y_{max}$= total dry biomass (g) without nematode damage, m = a constant so $y_{max} \bullet m$ equals the minimum biomass, z = parameter determining the slope of the curve, t = damage threshold density (nematode per plant at inoculation)

*Pratylenchus dunensis* and *P. brzeskii* multiplied at all densities examined; at the highest Pi *P. penetrans* hardly increased in number almost reaching the equilibrium point (Pf=Pi), (Fig 5.2). The relationship between initial inoculation density and nematode multiplication fitted the negative exponential model proposed by Ferris (1985) with $r^2$ ranging between 0.73 and 0.88 (Table 5.4 and Fig 5.3). The maximum multiplication calculated based on the lowest inoculation density used (45 nematodes, 0.075nematodes$\cdot g^{-1}$soil) in the experimental set-up was different for each of the species compared. *Pratylenchus dunensis* and *P. penetrans* showed the highest multiplication at this density. However, the decay in multiplication at higher nematode densities was more marked for *P. penetrans* than for the other two species.

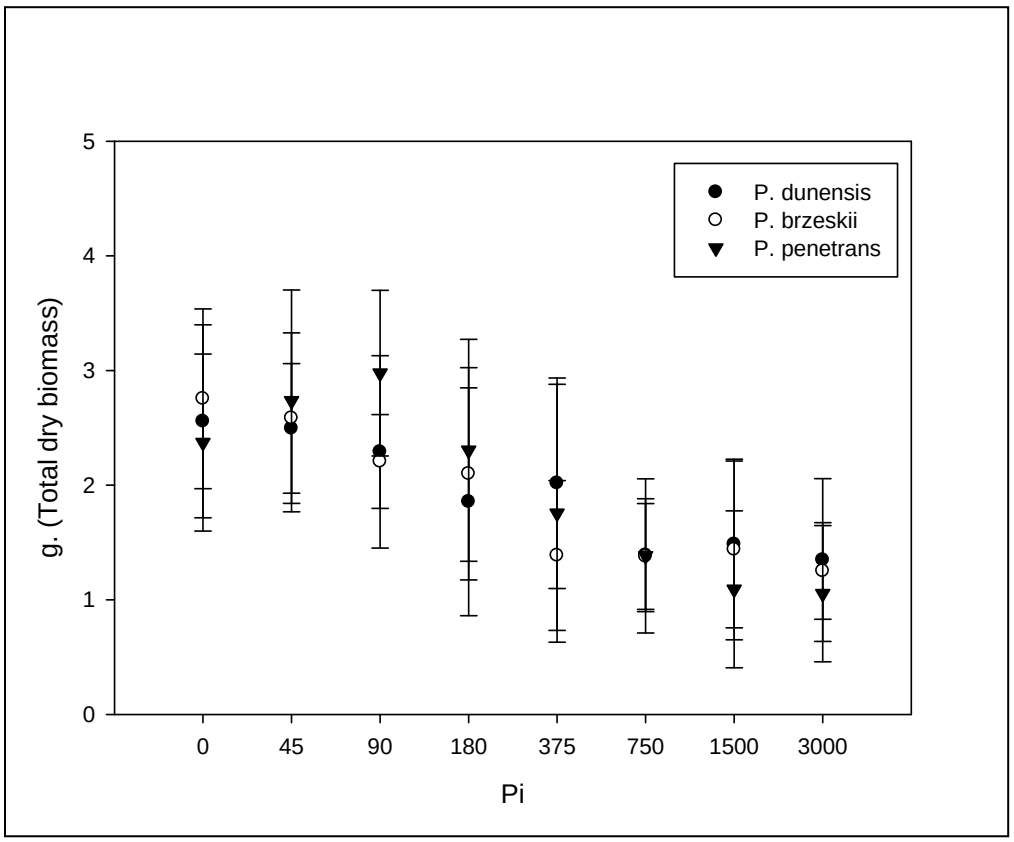


**Fig 5.1** Relationship between initial *Pratylenchus* spp. density (Pi, expressed in nematodes·pot$^{-1}$) and total plant dry biomass (g).

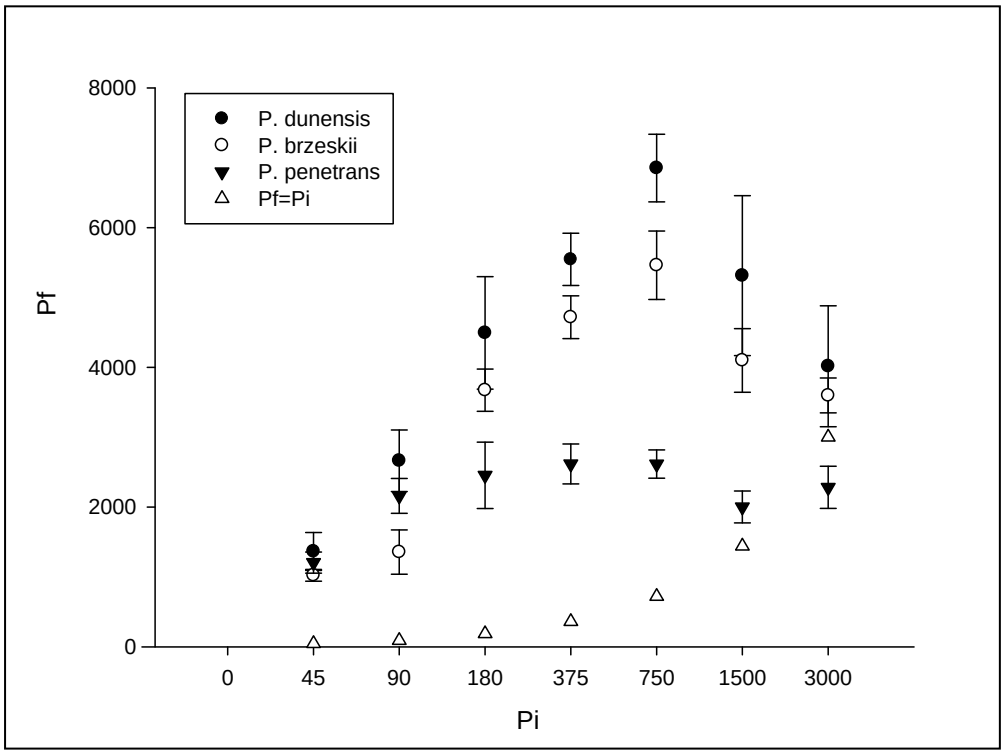


**Fig 5.2** Relationship between initial *Pratylenchus* spp. density (Pi) and final number of nematodes (Pf); both parameters expressed as nematodes·pot$^{-1}$.

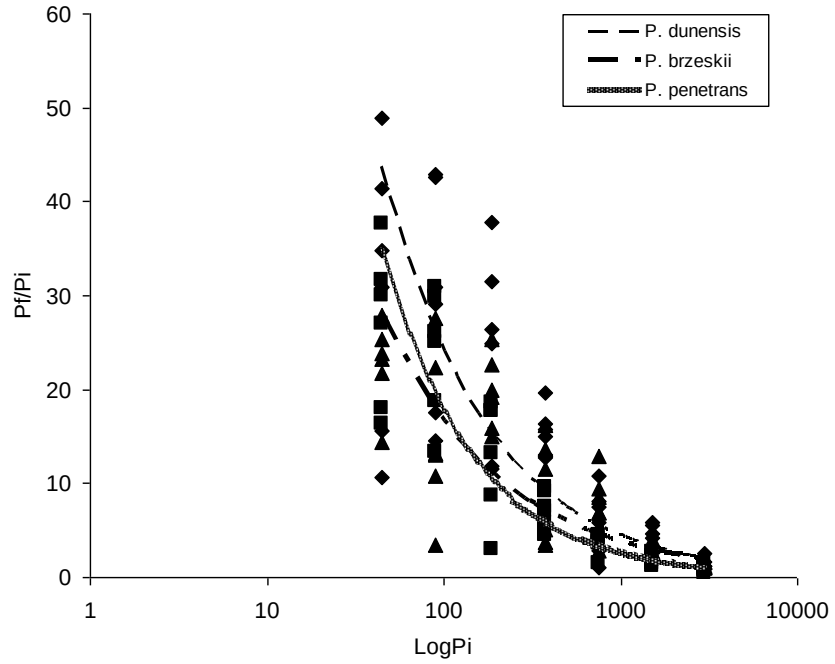


**Fig 5.3** The relationship between initial *Pratylenchus* spp. inoculation density nematodes·pot$^{-1}$ (LogPi) and nematode multiplication (Pf/Pi) for experimental data *P. brzeskii* (▲), *P. penetrans* (■), *P. dunensis* (♦) and for the adjusted model (Ferris equation: $Pf/Pi = cPi^{-b}$ see Table 5.4).

**Table 5.4** Relationship between multiplication rate (Pf/Pi) and initial *Pratylenchus* spp. densities (Pi) expressed by the Ferris equation. MM is the maximum multiplication at the lowest Pi (45 nematodes or 0.075nematodes·g$^{-1}$of sand)

| Species | c | b | Equation* | MM | $r^2$ |
|---|---|---|---|---|---|
| *P. dunensis* | 709.58 | -0.73 | $Pf/Pi= 709.58 \bullet Pi^{-0.73}$ | 44.07 | 0.75 |
| *P. brzeskii* | 325.92 | -0.64 | $Pf/Pi= 325.92 \bullet Pi^{-0.64}$ | 28.51 | 0.73 |
| *P. penetrans* | 844.95 | -0.83 | $Pf/Pi= 844.95 \bullet Pi^{-0.83}$ | 35.86 | 0.88 |

*The Ferris equation is of the form $Pf/Pi=cPi^{-b}$; Pf/Pi= the nematode multiplication, Pi is the initial nematode density, b = rate determining variable, c= the scaling factor and e = base of the natural logarithm.

### 5.3.2 Experiment 2

The two-way ANOVA and Tukey's HSD test showed significant differences in total dry biomass ($P<0.01$, $F_{2,\ 67}=54.183$) for the three plant species that were compared (Fig 5.4 and Table 5.5). The growth of *L. perenne* was greater than the other two species; however, no significant differences for total dry biomass were observed between *A. arenaria* and *E. farctus* (Fig 5.4). The same pattern is also observed for aboveground dry biomass ($P\leq0.01$, $F_{2,\ 67}= 4.589$, Table 5.5). With respect to the root dry biomass, normality was not achieved after transformation. The significant differences were detected with the Kruskal-Wallis non-parametrical test ($P\leq0.05$, $\chi^2=30.171$, Table 5.5).

No significant differences in total dry biomass and aboveground dry biomass were found between inoculation treatments (I), neither for the interaction between plant identity

and inoculation (PxI) (Table 5.5). Also, the non-parametric Kruskal-Wallis test using inoculation as grouping variable did not show differences for root dry biomass (Table 5.5).

With respect to nematode multiplication (Pf/Pi), the two-way ANOVA indicated significant differences between plant species ($P \leq 0.01$, $F_{2, 52}=34.794$, Table 5.5), inoculations (I) and in the interaction between plant and nematode species (PxI) ($P \leq 0.01$, $F_{2, 52}=5.206$ and $F_{2, 52}=12.145$ respectively, Table 5.5). To detect differences between nematode species multiplication a one-way ANOVA was conducted for each host plant separately using inoculation as factor.

According to one-way ANOVA and Tukey's HSD significant differences in multiplication (Pf/Pi) were found between nematode species for each of the host plant species (Table 5.6). On *L. perenne*, *P. penetrans* showed the highest multiplication, whereas *P. dunensis* and *P. brzeskii* presented a similar and lower multiplication ($P \leq 0.01$, $F_{2, 18}=6.842$, Table 5.6 and Table 5.7). On *E. farctus* the inverse situation was observed; the lowest multiplication was obtained for *P. penetrans* whereas *P. dunensis* and *P. brzeskii* had a higher multiplication factor ($P \leq 0.05$, $F_{2, 17}=14.795$, Table 5.6, 5.7 and Figure 5.5). For *A. arenaria* a significant difference ($P \leq 0.05$, $F_{2, 17}=4.278$) was observed between *P. penetrans* and *P. dunensis*, however *P. brzeskii* did not differ from either of the other species (Fig 5.4 and Table 5.6 and 5.7).

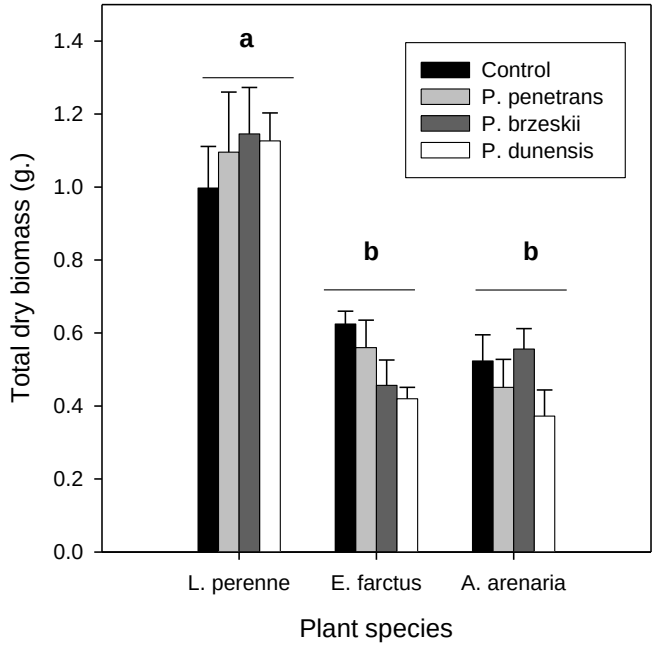


**Fig 5.4** Total dry biomass (g·plant$^{-1}$) produced by *Lolium perenne, Elymus farctus* and *Ammophila arenaria* in controls (no inoculation) or after inoculation with *Pratylenchus penetrans, P. brzeskii* and *P. dunensis*. Data are mean ± SE. Different letters indicate significant differences according to ANOVA Tukey's test.

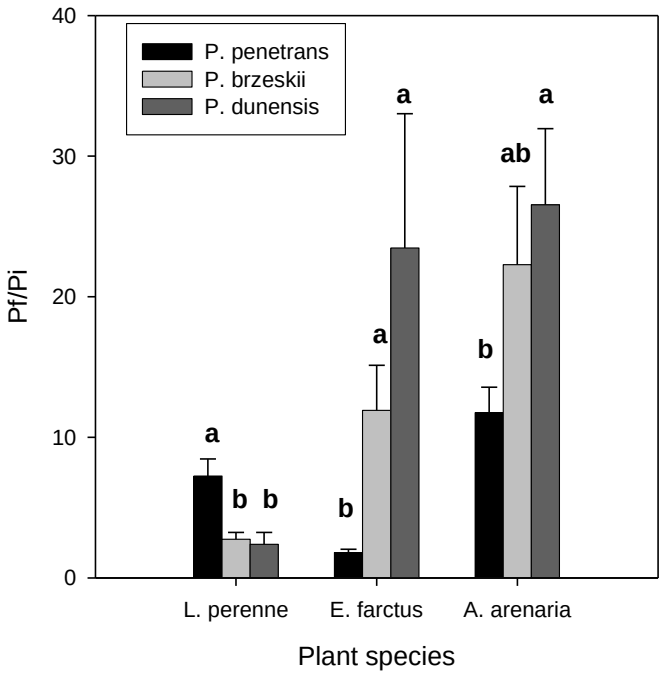


**Fig 5.5** *Pratylenchus* spp. (*P. penetrans, P. dunensis* and *P.* brzeskii) multiplication (Pf/Pi) on three different hosts *Lolium perenne, Elymus farctus* and *Ammophila arenaria*. Data are mean ± SE. Significant differences according to oneway ANOVA and Tukey's HSD test within a given species are indicated by different letters.

No differences in total dry biomass, root dry biomass and aboveground dry biomass were found between nematode inoculations for the different plant species (Table 5.6 and 5.7).

### 5.3.3 Experiment 3

Because either normality or homogeneity of variances were not achieved for the variables: total dry biomass, aboveground biomass and root dry biomass, it was not possible to perform a two-way ANOVA using *A. arenaria* populations (origin) and inoculation (control and five *Pratylenchus* populations) as factors. A Kruskal-Wallis non-parametric test was used instead using plant population (P) and inoculation (I) as grouping variables. Significant differences in total dry biomass and aboveground dry biomass were found between *A. arenaria* populations ($P \leq 0.05$, $\chi^2=9.021$, Table 5.8); using inoculation as grouping variable, significant differences were found in root dry biomass ($P \leq 0.05$, $\chi^2=13.485$, Table 5.8).

In order to detect differences between nematode inoculations and *A. arenaria* populations, a one-way ANOVA for the five different *Pratylenchus* populations was performed using plant population as single factor. For *P. brzeskii* (Biarritz) the highest number of nematodes·g$^{-1}$ dry root were found in plants from Ynyslas which were

significantly different from the number found in plants from Comporta ($P \leq 0.05$, $F_{3, 20}=3.111$, Fig 5.6a, Table 5.9 and 5.10). *Ammophila arenaria* plants from Oostvoorne and Blakeney Point showed intermediate values for that variable; no significant differences were found with respect to plants from Comporta or Ynyslas (Fig 5.6 a, Table 5.9 and 5.10). In the case of *P. brzeskii* (Ynyslas), the highest number of nematodes·g$^{-1}$ root was found in *A. arenaria* from Ynyslas and that was significantly different to the other three *A. arenaria* populations ($P \leq 0.01$, $F_{3, 20}=6.941$, Fig 5.6b, Table 5.9 and 5.10). No significant differences were found for nematodes·g$^{-1}$ root in *Pratylenchus dunensis* (Comporta) between plants from Ynyslas, Blakeney Point and Oostvoorne; however for this variable the number found in the plat population from Comporta was significantly lower. ($P \leq 0.01$, $F_{3, 20}=14.322$, Fig 5.6d, Table 5.9 and 5.10). For *P. dunensis* (Oostvoorne) and *P. penetrans* (Sandhoven) no significant differences were found in nematodes·g$^{-1}$ root between the different *A. arenaria* populations compared (Fig 5.6c and Fig 5.6e).

To avoid bias in the interpretation of nematode numbers due to differences in root biomass for the different plant-nematode combinations. Differences in plant growth related parameters (total dry biomass, aboveground biomass and root dry biomass) were checked with one-way ANOVA (plant origin as single factor). Significant differences ($P \leq 0.05$) in total dry biomass and root dry biomass were found between plants from Ynyslas and Oostvoorne when inoculation was done with *P. brzeskii* (Ynyslas) ($P \leq 0.05$, $F_{3, 20}= 4.558$, Table 5.11 and 5.12). The inoculation of *P. penetrans* yielded significant differences ($P \leq 0.05$) in total dry biomass and root dry biomass when comparing plants from Blakeney Point and Comporta ($P \leq 0.05$, $F_{3, 20}= 3.650$ and $F_{3, 20}= 3.464$, Table 5.11 and 5.12).

**Table 5.5** Statistics of the host suitability experiment: Mean Squares (MS), degrees of freedom (d.f.), F-ratios and $X^2$ (Chi-Square) values of different measured variables for two-way ANOVA and Kruskal-Wallis. The analysis compared the effect of plant identity (P) (*Lolium perenne*, *Elymus farctus*, *Ammophila arenaria*), and inoculation (I)(Control, *Pratylenchus penetrans*, *P. brzeskii* and *P. dunensis*) and the interaction of the two factors (PxN). Significant differences according to ANOVA are indicated by ***P*≤*0.01*, **P*≤*0.05* or ‡ *P*≤*0.01* according to Kruskal-Wallis non-parametric test.

| Factor | Dependent Variable | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Multiplication (Pf/Pi) | | | Total nr. of nematodes (roots+ soil) | | | Total dry biomass | | | Aboveground dry biomass | | | Root dry biomass | |
| | MS | F | d.f. | MS | F | d.f. | MS | F | d.f. | MS | F | d.f. | $X^2$ | d.f. |
| P | 2.000 | 34.794** | 2 | 2.966 | 33.210** | 2 | 0.184 | 54.183** | 2 | 0.119 | 4.589* | 2 | 30.171 ‡ | 2 |
| I | 0.299 | 5.206** | 2 | 0.324 | 3.625* | 2 | 2.301 E-03 | 0.705 | 3 | 7.89E-03 | 0.305 | 3 | 2.815 | 2 |
| P x I | 0.691 | 12.145** | 4 | 1.154 | 12.924** | 4 | 3.88 E-03 | 1.144 | 6 | 3.99 E-02 | 1.542 | 6 | - | - |

**Table 5.6** Statistics of the host suitability experiment: Mean Squares (MS), degrees of freedom (d.f.) and F-ratios of different measured variables for one-way ANOVA using inoculation as single factor for each of the three plant species (*Lolium perenne*, *Elymus farctus* and *Ammophila arenaria*). Significant differences at ***P*≤*0.01*, **P*≤*0.05*.

| Plant | Variables | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Multiplication (Pf/Pi) | | | Total nematodes (roots+ soil) | | | Total dry biomass | | | Aboveground dry biomass | | | Root dry biomass | | |
| | F | MS | d.f. | F | MS | d.f. | F | MS | d.f. | F | MS | d.f. | F | MS | d.f. |
| *L. perenne* | 6.842** | 0.333 | 2 | 5.497* | 0.664 | 2 | 0.385 | 0.157 | 3 | 1.069 | 0.030 | 3 | 0.507 | 0.014 | 3 |
| *E. farctus* | 14.795* | 1.162 | 2 | 18.641** | 1.744 | 2 | 0.680 | 2.931 | 3 | 2.508 | 0.027 | 3 | 2.135 | 0.005 | 3 |
| *A. arenaria* | 4.278* | 0.196 | 2 | 4.193* | 0.217 | 2 | 0.883 | 0.028 | 3 | 0.820 | 0.017 | 3 | 1.741 | 0.002 | 3 |

**Table 5.7** Mean ± S.E (Standard Error) for different measured variables in the host suitability experiment. Different letters indicate significant differences in the variable within a plant species for the different inoculations according to one-way ANOVA and Tukey's HSD test.

| Plant | Variables | | | | | |
|---|---|---|---|---|---|---|
| | Inoculation | Multiplication (Pf/Pi) | Total nr. nematodes (roots+soil) | Total dry biomass (g) | Aboveground dry biomass (g) | Root dry biomass (g) |
| *Lolium perenne* | Control | - | - | 0.99±0.11 | 0.45±0.05 | 0.54±0.06 |
| | *P. penetrans* | 7.23±1.22$^{a}$ | 1086±183.92$^{a}$ | 1.09±0.06 | 0.53±0.10 | 0.55±0.08 |
| | *P. brzeskii* | 2.74±0.48$^{b}$ | 411±71.90$^{ab}$ | 1.14±0.12 | 0.54±0.07 | 0.62±0.03 |
| | *P. dunensis* | 2.38±0.84$^{b}$ | 354±125.63$^{b}$ | 1.12±0.07 | 0.60±0.03 | 0..52±0.05 |
| *Elymus farctus* | Control | - | - | 0.62±0.03 | 0.51±0.02 | 0.10±0.01 |
| | *P. penetrans* | 1.79±0.23$^{b}$ | 268.79±35.35$^{b}$ | 0.56±0.07 | 0.45±0.06 | 0.10±0.02 |
| | *P. brzeskii* | 11.91±3.20$^{a}$ | 1786.96±481.11$^{a}$ | 0.45±0.06 | 0.37±0.06 | 0.08±0.02 |
| | *P. dunensis* | 23.46±9.55$^{a}$ | 3521.01±1434.20$^{a}$ | 0.41±0.03 | 0.37±0.02 | 0.04±0.01 |
| *Ammophila arenaria* | Control | - | - | 0.52±0.07 | 0.46±0.07 | 0.06±0.01 |
| | *P. penetrans* | 11.17±1.44$^{b}$ | 1678.67±217.55$^{b}$ | 0.45±0.07 | 0.39±0.06 | 0.05±0.01 |
| | *P. brzeskii* | 22.27±5.57$^{ab}$ | 3341.42±836.02$^{ab}$ | 0.55±0.05 | 0.46±0.05 | 0.09±0.01 |
| | *P. dunensis* | 26.53±5.42$^{a}$ | 3961±61±816.15$^{a}$ | 0.37±0.07 | 0.32±0.05 | 0.04±0.02 |

**Table 5.8** Statistics of *Ammophila arenaria*-host suitability: Degrees of freedom (d.f.) and $X^2$ (Chi-Square) values of different measured variables according to the Kruskal-Wallis non-parametric test in which the effect of plant origin (P) (*Ammophila arenaria* from Oostvoorne, Blakeney Point, Ynyslas or Comporta) or inoculation (I) (Control, *P. brzeskii* (Biarritz), *P. brzeskii* (Ynyslas), *P. dunensis* (Oostvoorne), *P. dunensis* (Comporta), *P. penetrans* (Sandhoven) was compared. Significant differences at *P≤0.05

| Grouping Variable | Dependent Variable | | | | | |
|---|---|---|---|---|---|---|
| | Total dry biomass | | Aboveground dry biomass | | Root dry biomass | |
| | $X^2$ | d.f. | $X^2$ | d.f. | $X^2$ | d.f. |
| P | 9.021* | 3 | 8.604* | 3 | 4.271 | 3 |
| I | 1.919 | 5 | 1.036 | 5 | 13.485* | 5 |

**Table 5.9** Statistics of *Ammophila arenaria*-host suitability: Mean Squares (MS), degrees of freedom (d.f.) and F-ratios values of different measured variables for one-way ANOVA for each of the nematode inoculations (inoculation with *P. brzeskii* (Biarritz), *P. brzeskii* (Ynyslas), *P. dunensis* (Oostvoorne), P. dunensis (Comporta) and *P. penetrans* (Sandhoven) using plant origin as single factor. Significant differences at ***P≤0.01*, **P≤0.05*.

| Nematode Population | Variables | | | | | |
|---|---|---|---|---|---|---|
| | Nematodes·$g^{-1}$ dry root | | | Total nr. of nematodes (roots) | | |
| | F | MS | d.f. | F | MS | d.f. |
| *P. brzeskii* (Biarritz) | 3.111* | 444302.623 | 3 | 3.357* | 192129.761 | 3 |
| *P. brzeskii* (Ynyslas) | 6.941** | 13336682.04 | 3 | 6.449** | 7207589.061 | 3 |
| *P. dunensis* (Oostvoorne) | 1.641 | 1712701.501 | 3 | 2.538 | 1329185.528 | 3 |
| *P. dunensis* (Comporta) | 14.322** | 0.480 | 3 | 5.154** | 1487606.693 | 3 |
| *P. penetrans* (Sandhoven) | 1.485 | 32217.862 | 3 | 0.448 | 448.433 | 3 |

**Table 5.10** Mean ± S.E (Standard Error) number of nematodes per g dry root an total number of nematodes per root after inoculation of four populations of *Ammophila arenaria* with five *Pratylenchus* populations. Different letters indicate significant differences in the variable within a nematode inoculation for the different plant populations according to one-way ANOVA and Tukey's HSD test.

| Plant Origin | Variables | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Nematodes·g$^{-1}$dry root | | | | | Total nematodes in root (fresh) | | | | |
| | *P. brzeskii* (Biarritz) | *P. brzeskii* (Ynyslas) | *P. dunensis* (Oostvoorne) | *P. dunensis* (Comporta) | *P. penetrans* (Sandhoven) | *P. brzeskii* (Biarritz) | *P. brzeskii* (Ynyslas) | *P. dunensis* (Oostvoorne) | *P. dunensis* (Comporta) | *P. penetrans* (Sandhoven) |
| Oostvoorne | 606±93$^{ab}$ | 1768±668$^{b}$ | 1643±389 | 1111±271$^{a}$ | 400±49 | 426±58$^{a}$ | 1207±492$^{a}$ | 961±269 | 743±178$^{ba}$ | 206±40 |
| Blakeney Point | 772±164$^{ab}$ | 2034±672$^{b}$ | 2829±596 | 1851±300$^{a}$ | 408±64 | 461±83.94$^{ab}$ | 1375±450$^{a}$ | 2012±438 | 1385±264$^{a}$ | 187±38 |
| Ynyslas | 1107±227$^{a}$ | 4681±555$^{a}$ | 1928±325 | 1660±301$^{a}$ | 469±85 | 720±155$^{ab}$ | 3398±509$^{a}$ | 1182±187 | 1219±298$^{a}$ | 238±51 |
| Comporta | 479±88$^{b}$ | 1422±270$^{b}$ | 1788±286 | 424±40$^{b}$ | 293±22 | 292±67$^{b}$ | 1077±201$^{b}$ | 1124±220 | 281±43$^{b}$ | 179±21 |

**Table 5.11** Statistics of *Ammophila arenaria*-host suitability experiment: Mean Squares (MS), degrees of freedom (d.f.) and F-ratios of different measured variables for one-way ANOVA for each of the nematode inoculations (inoculation with *P. brzeskii* (Biarritz), *P. brzeskii* (Ynyslas), *P. dunensis* (Oostvoorne), P. dunensis (Comporta) and *P. penetrans* (Sandhoven)) using plant origin as single factor. Significant differences at $^{**}P \leq 0.01$, $^{*}P \leq 0.05$

| Plant | Variables | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Total dry biomass | | | Aboveground biomass | | | Root dry biomass | | |
| | F | MS | d.f | F | MS | d.f. | F | MS | d.f. |
| *P. brzeskii* (Biarritz) | 0.222 | 0.06 | 3 | 0.295 | 0.005 | 3 | 0.95 | 0.001 | 3 |
| *P. brzeskii* (Ynyslas) | 4.558* | 0.047 | 3 | 2.669 | 0.02 | 3 | 4.249 | 0.033 | 3 |
| *P. dunensis* (Oostvoorne) | 1.347 | 0.020 | 3 | 0.912 | 0.009 | 3 | 2.642 | 0.003 | 3 |
| *P. dunensis* (Comporta) | 1.232 | 0.038 | 3 | 0.927 | 0.022 | 3 | 2.551 | 0.04 | 3 |
| *P. penetrans* (Sandhoven) | 3.650* | 0.023 | 3 | 2.372 | 0.013 | 3 | 3.464* | 0.003 | 3 |

**Table 5.12** Mean (± S.E) of *Ammophila arenaria* dry biomass (g) after inoculation with different *Pratylenchus* spp. Different letters indicate significant differences in the variable within a nematode inoculation for the different *A. arenaria* origins (Oostvoorne, Blakeney Point, Ynyslas, Comporta) according to one-way ANOVA and Tukey's HSD test.

| Nematode populations | Total dry biomass (g) | Aboveground dry biomass (g) | Root dry biomass (g) |
|---|---|---|---|
| *P. brzeskii* (Biarritz) | | | |
| Oostvoorne | 0.56±0.07 | 0.48±0.06 | 0.07±0.015 |
| Blakeney Point | 0.49±0.08 | 0.42±0.06 | 0.07±0.014 |
| Ynyslas | 0.56±0.03 | 0.48±0.03 | 0.08±0.013 |
| Comporta | 0.53±0.05 | 0.45±0.04 | 0.08±0.012 |
| *P. brzeskii* (Ynyslas) | | | |
| Oostvoorne | 0.67±0.02$^{a}$ | 0.58±0.02$^{a}$ | 0.09±0.013 |
| Blakeney Point | 0.51±0.04$^{ab}$ | 0.45±0.04$^{ab}$ | 0.06±0.016 |
| Ynyslas | 0.47±0.05$^{b}$ | 0.40±0.04$^{b}$ | 0.06±0.013 |
| Comporta | 0.53±0.03$^{ab}$ | 0.48±0.03$^{ab}$ | 0.059±0.012 |
| *P. dunensis* (Oostvoorne) | | | |
| Oostvoorne | 0.57±0.06 | 0.46±0.05 | 0.10±0.018 |
| Blakeney Point | 0.56±0.05 | 0.49±0.04 | 0.07±0.014 |
| Ynyslas | 0.66±0.03 | 0.54±0.02 | 0.12±0.014 |
| Comporta | 0.52±0.03 | 0.44±0.03 | 0.07±0.01 |
| *P. dunensis* (Comporta) | | | |
| Oostvoorne | 0.68±0.05 | 0.57±0.04 | 0.11±0.017 |
| Blakeney Point | 0.50±0.06 | 0.43±0.05 | 0.076±0.013 |
| Ynyslas | 0.53±0.07 | 0.48±0.07 | 0.051±0.01 |
| Comporta | 0.55±0.08 | 0.47±0.07 | 0.08±0.02 |
| *P. penetrans* (Sandhoven) | | | |
| Oostvoorne | 0.55±0.04$^{ab}$ | 0.053±0.010 | 0.50±0.044$^{ab}$ |
| Blakeney Point | 0.45±0.027$^{a}$ | 0.045±0.005 | 0.40±0.02$^{b}$ |
| Ynyslas | 0.55±0.03$^{ab}$ | 0.069±0.014 | 0.49±0.020$^{ab}$ |
| Comporta | 0.59±0.02$^{b}$ | 0.094±0.013 | 0.50±0.019$^{a}$ |

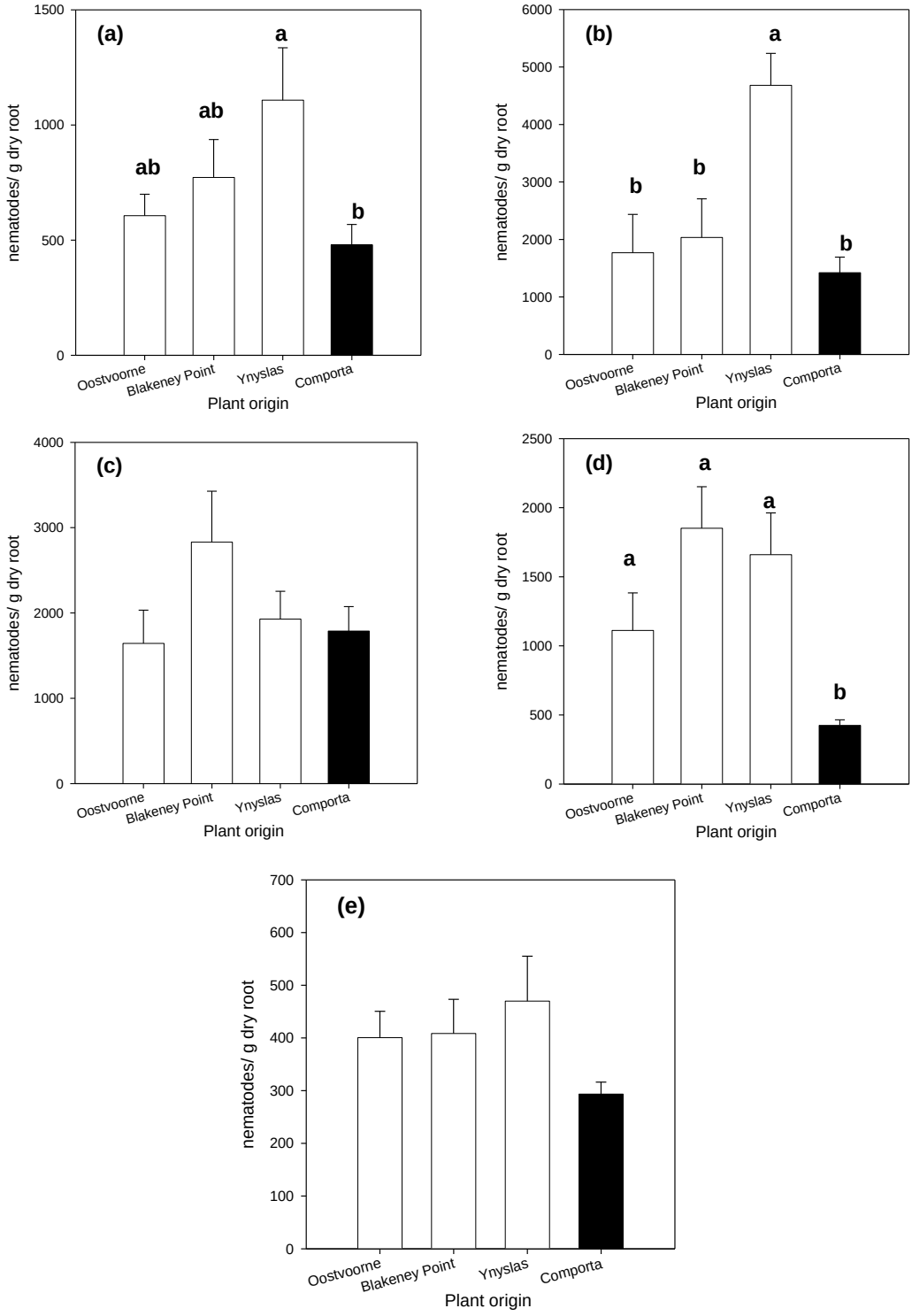


**Fig 5.6** Nematode reproduction on *Ammophila arenaria* from different origins and inoculated with different *Pratylenchus* spp.: (a) *P. brzeskii* (Biarritz); (b) *P. brzeskii* (Ynyslas); (c) *P. dunensis* (Oostvoorne); (d) *P. dunensis* (Comporta); (e) *P. penetrans* (Sandhoven). Data are mean ± SE. Different letters indicate significant differences according to one-way ANOVA and Tukey's HSD test.

## 5.4 Discussion

The results on which I report in this chapter confirm the detrimental effect of *P. dunensis, P. brzeskii* and *P. penetrans* on the growth of *A. arenaria*. The parameters m, t, $y_{max}$ of the Seinhorst's models obtained for each of the three species, point at differences in the tolerance level and susceptibility of *A. arenaria*. While in *P. dunensis* and *P. penetrans* the tolerance level corresponds with nematode densities around 0.2 nematodes·$g^{-1}$ soil (initial inoculation density of 300 nematodes), in *P. brzeskii* the tolerance level is 1 nematodes·$g^{-1}$ soil (600 nematodes). Previous inoculation studies have shown variable impact of PPN on the growth of *A. arenaria*. Either inoculation of *A. arenaria* seedlings with *Heterodera arenaria* did not affect plant growth (Van der Stoel 2001) or negative effects in the growth of *A. arenaria* were observed after inoculation with the ectoparasitic nematode *Tylenchorrhynchus ventralis* (de Rooij-van der Goes *et al.* 1995a); however, the nematode densities used in this experiment were considerably higher than natural *T. ventralis* densities in coastal dunes. More recent experiments using *P. penetrans* were not conclusive for the effect of this species on the growth of *A. arenaria*. Initial inoculation densities of 0.2 and 1 nematodes·$g^{-1}$ reduced aboveground biomass while root-biomass was not affected (Brinkman 2004).

The differences demonstrated by my results should be taken with caution. Firstly, the Seinhorst model although support the detrimental effect of nematodes on plant growth, the low $r^2$ values do not allow to compare t, m values between species and should not be directly extrapolated to field conditions. Secondly, as pointed for *T. ventralis*, natural densities in dune soils of *Pratylenchus* spp. do not reach the high densities used in my experimental design. Sampling surveys of *Pratylenchus* spp. and other PPN in coastal dunes of Western Europe have shown that the density of this group range between 0.003-0.15 nematodes·$g^{-1}$ soil in foredunes with vigorous *A. arenaria* (de Rooij-van der Goes *et al.* 1995a; Schreck-Reis 2005) and 0.15-1 nematodes·$g^{-1}$ soil in the rhizosphere of *A. arenaria* and *Hyppophäe rhamnoides* in inner dunes (Zoon *et al.* 1993). For the range of natural densities detected in foredunes no significant detrimental effect on plant biomass is observed for the three species in my experiment. Only in inner dunes with densities beyond 0.2 nematodes per gram of soil negative effect might be found. One of the hypotheses to explain the *Ammophila* problem states that when deposition of sand ceases, PPN and other soil pathogens accumulate in the rhizosphere of the plant and affect negatively plant growth (Van der Putten & Van der Stoel 1998). The results presented here support the idea of a detrimental effect of high densities of nematodes in plant growth, whether or not these densities can be reached in natural conditions deserves further investigation.

The Jarosz and Davelos hypothesis states that biotrophic parasites (as PPN) show mild pathogenicity to their hosts because they require living plant cells as food supply (Jarosz & Davelos 1995). Previous studies in which *A. arenaria* was inoculated with increasing densities of *H. arenaria* did not show a strong pathogenicity, and detrimental effect on plant growth was not observed (Van der Stoel 2001); which was in concordance with the hypothesized mild pathogenicity of biotrophic parasites. However, in the case of *Pratylenchus* spp. my observations indicate a different situation, with a considerable detrimental effect at mid-high initial densities.

*Pratylenchus* species are part of a complex of nematode species that interact with *A. arenaria*. In dune soils *Pratylenchus* numbers are much lower than other endoparasitic nematodes such as *Heterodera* or *Meloidogyne*. It has been hypothesized that biotrophic parasites would show higher pathogenicity in multispecies complexes to be able to compete with other parasitic species (Lenski 1994). In pot and field experiments *Pratylenchus penetrans* is a strong competitor of the endoparasitic nematode (Van der Stoel 2001; Brinkman 2004). The results presented here showing the negative effect of *Pratylenchus* could be more in agreement with this hypothesis.

The physiological responses of *A. arenaria* to nematode attack have not been studied yet. In general terms, the primary damage caused by endoparasitic nematode to the attacked roots can be attributed to mechanical damage associated with feeding or invasion and to withdrawal of nutrients. The negative effect is usually observed as a reduction in the rate of root extension which also reduces the rate of uptake of nutrients and water and, if any of those factors become limiting growth rates aboveground and belowground are reduced (Trudgill 1991). Interestingly, the data presented in this experiment confirm growth (biomass) reduction of both the aboveground parts and root dry biomass; however, the correlation was stronger for aboveground biomass. The physiology of *A. breviligulata* has been studied in relation to plant burial (Voesenek *et al.* 1998). According to this study, sand burial triggers the production of ethylene in the plant and that is reflected in more shoot production and stem elongation. Endoparasitic nematodes alter different physiological routes within the plant responsible for biomass allocation (Mateille 1994; Bird & Koltai 2000). Whether *Pratylenchus* spp. are able to modify the host hormone metabolism and in consequence shoot/root production deserves further attention.

The variability of the plant (*A. arenaria*) response to nematode inoculation in earlier studies can be explained by the high fertilization regimes applied to plants; they were considerably higher than those used in the experiments presented here. As pointed by

Troelstra *et al.* (2004) the fertilization regime is an important factor to interpret the effect of soil fauna on dune grasses. Therefore, the effect of soil nutrients and nematodes in *A. arenaria* should be reconsidered since the nutrient supply modifies directly the response of the host plant to different densities of nematodes in soil (Trudgill *et al.* 1975; De Deyn *et al.* 2004).

Data obtained in the experiment, confirmed the density dependent character of the multiplication for the three species of *Pratylenchus* compared. The higher is the initial density, the less root biomass is available for nematodes and that is translated in a reduction of multiplication. These data, in combination with previous works analyzing the population dynamics of *Pratylenchus* spp. in relation to *A. arenaria* growth support the bottom-up control of root-lesion nematodes in costal dunes. The three *Pratylenchus* species are directly dependent on the quantity of food caused by the combination of intraspecific competition and the nematode damage to the roots that will further reduce the amount of food available for nematodes. Other nematode species from coastal dunes have shown a similar pattern of multiplication being limited by the amount of resources (quantity of roots) (Van der Stoel *et al.* 2006).

Interestingly, the maximum multiplication was different for each of the nematode species. Between the species the maximum multiplication was found for *P. dunensis* and *P. penetrans.* However, the effect of both biomass reduction (less food source available) and competition (higher initial densities) was stronger in *P. penetrans.* For the same range of high initial densities the Pf for *P. brzeskii* and *P. dunensis* was near the equilibrium point, but in the case of *P. penetrans* was reached at Pi=3000. This suggests that *A. arenaria* is more tolerant for more specific host nematodes, than for the very polyphagous *P. penetrans.* This might have consequences for the development of nematode populations and since the three species occur in *A. arenaria* and often together, should be considered when studying interspecific competition between these species.

The results from the second experiment demonstrated differences in nematode multiplication as a function of the host. I used a low initial nematode density to obtain high multiplication. In *L. perenne* only *P. penetrans* multiplied, whereas in *E. farctus* and *A. arenaria*, the typical dune species, *P. dunensis* and *P. brzeskii* presented the highest multiplication. These data are in agreement with field observations in which *P. brzeskii* and *P. dunensis* were extracted from *E. farctus* roots (Karssen *et al.* 2000). In my experiment, *P. penetrans* hardly multiplied on *E. farctus* and therefore, this observation might support that this species occupying inner sites of the dune area where other plant species (including *A.*

*arenaria*) are better hosts. In any case, the identity of the host plant, is a fundamental factor in the multiplication of the three nematode species. A certain degree of specificity has also been observed for other PPN in coastal dunes. Different species of *Meloidogyne* occur in dunes of Western Europe such as *M. duytsi* and *M. arenaria*, and seem to multiply on different hosts (*E. farctus* and *A. arenaria* respectively) (Karssen *et al.* 1998b). Even though the range of hosts tested for the three *Pratylenchus* species is rather limited, this result might agree with previous observations in which a high diversity of hosts is more suitable for polyphagous (generalists) herbivores (Steffan-Dewenter & Tscharntke 2000; Otway *et al.* 2005). In the fore dunes the diversity of hosts is reduced to only a few species of pioneer grasses; on the other hand in inner dunes a richer plant community is found. Therefore, a polyphagous species as *P. penetrans* would have more chances of survival and multiplication in these environments. To confirm this hypothesis, experiments with the three *Pratylenchus* species and the whole set of dune hosts (i.e. *A. arenaria, Carex arenaria, Calamagrostris epigijeos, Leymus arenarius, Festuca rubra, E. athericus, etc*) should be undertaken.

The density I used in the host suitability experiment did not hamper the growth of any of the hosts used. In order to verify whether different *Pratylenchus* species of foredunes may drive plant succession, density-growth experiments should be conducted not only with *A. arenaria* but also with other plant species preceding or replacing *A. arenaria* in the succession. Similar experiments have been conducted wit *H. arenaria* and demonstrated the detrimental effect on late succession species whereas *A. arenaria* was not affected (Van der Stoel 2001).

As pointed in the host-suitability experiment the multiplication of *P. dunensis*, *P. brzeskii* and *P. penetrans* depends on the host identity. Up to date, whether the multiplication of a nematode species is also influenced by differences in the *A. arenaria* genotype has not been addressed. This has been observed in crops, different varieties show a different degree of tolerance/resistance for nematode species including *Pratylenchus* spp. (Brodie & Plaisted 1993; Castillo *et al.* 1998; Taylor *et al.* 1999) The results of the third experiment show that different genotypes of *A. arenaria* affect the multiplication of *Pratylenchus* spp. Similarly the multiplication of different populations of a given *Pratylenchus* spp. (i.e. *P. dunensis* from Comporta *vs.* Oostvoorne), showed different multiplication potential on the same host. I used two *A. arenaria* subspecies that present a different distribution and different adaptation traits in relation to the ecology of the plant in different climates. In general terms it seems that for all *Pratylenchus* spp. compared in general *A. arenaria* ssp. *arundinacea* (Comporta) is a worst host than *A. arenaria* ssp. *arenaria* from Oostvoorne, Ynyslas and Blakeney Point).

Local adaptation occurs when the multiplication of a parasite is higher in the sympatric host combination that in the allopatric (Gandon 1998; Lively & Dybdahl 2000). Unfortunately, I could only test three sympatric host-parasite combinations which showed different scenarios. In the case of *P. brzeskii* (Ynyslas) the highest multiplication was found on plants from the same origin (Ynyslas), with respect to *P. dunensis* (Oostvoorne) no differences according to the host origin were observed, whereas for *P. dunensis* from Comporta, the multiplication was higher in plants from allopatric origin. The data confirm an influence of the plant origin on the *Pratylenchus* multiplication for different species but a biogeographical pattern or local adaptation should not be directly assumed. This complex scenario suggests an idiosyncratic dependence in the multiplication for combinations between *Pratylenchus* spp. and *A. arenaria* populations as shown for other plant parasitic nematodes in coastal dunes (Brinkman *et al.* 2005a)

One also should be aware of the fact that the multiplication of a nematode species on *A. arenaria* from a given origin during a long period of time (in cultures) might have exerted a certain selection pressure affecting the outcome of the experiment. Similarly maternal effects, since seeds for experiments were collected in the field, might be another cause affecting the outcome of the experiment (Agrawal 2002; Adler & Kittelson 2004). However, the fact that different combinations of *Pratylenchus* spp. were used for each host, and that some of the populations were cultured in allopatric hosts, are arguments that support differences in multiplication beyond selection biases by culture methods or maternal effects from *A. arenaria* seeds used in the experiments.

The results presented on this chapter support a bottom-up control of three different *Pratylenchus* species. The effect of the host plant in nematode multiplication is not only due to the amount of food resources as shown in the density-growth experiment, but it is also influenced by differences in the quality of the host.

# Chapter 6

## Mechanisms of control of *Pratylenchus penetrans by* AMF *in Ammophila arenaria*[*]

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

## 6.1 Introduction

Belowground plant pathogens, parasites, herbivores and mutualists influence the performance and competitive ability of plant species and their offspring (Klironomos 2002; Reinhart *et al.* 2003; De Deyn *et al.* 2004). Plants also selectively affect the soil biota associated with their rhizosphere (Wardle 2002) and as a consequence, feedback interactions are established between plants and soil organisms. These interactions are crucial for the spatial and temporal composition of natural plant communities (Gange & Brown 2002; Bever 2003). The sign of these feedbacks (positive or negative) can also change depending on the multitrophic interactions between different rhizosphere organisms. However, belowground interactions that include more than one group of soil organisms have been largely ignored in ecological studies.

As explained in Chapter 3 coastal foredunes are highly dynamic ecosystems characterized by severe wind-driven sand accretion. Sand burial allows *A. arenaria* to avoid ageing by developing new roots (Marshall 1965) and provides the opportunity to escape temporarily from root-pathogens and herbivores (Van der Putten *et al.* 1990). Although plant-parasitic nematodes gradually colonize the new deposited sand layers, there is a lag of 4-5 weeks in which the new roots grown in a freshly deposited layer of wind-blown beach sand are in an enemy-free environment (de Rooij-van der Goes *et al.* 1998; Van der Stoel *et al.* 2002a).

*Pratylenchus penetrans* as seen in Chapter 4 is one of the species that accumulate in the rhizosphere of *A. arenaria*; this species also occurs at relatively high densities in later stages of dune succession where decaying stands of *A. arenaria* are in combination with other host plants (*Hyppophäe rhamnoides*) (Zoon *et al.* 1993).

The deleterious effect of PPN on plant growth is dependent on the density of nematodes in the rhizosphere (Seinhorst 1998). However, in dune soils the density of PPN is considerably lower than that observed when nematodes are added to plants growing in sterilized soil (de Rooij-van der Goes 1995; Brinkman *et al.* 2004). Such nematode control in natural systems may be explained by bottom-up mechanisms (exerted by the host-plant), top-down control (by natural enemies) and control by plant mutualists (e.g. arbuscular mycorrhizal fungi and endophytes).

The role of arbuscular mycorrhizal fungi (AMF) as protective agents against PPN has been tested in crop plant species with highly variable results (Hol & Cook 2005). One major

limitation of those studies is the use of commercial strains of AMF which had not co-evolved with the crop and the nematodes. The diversity of AMF found in natural communities might be important for the outcome of the interaction because of the functional diversity of different AMF taxa ((Klironomos 2003)). In coastal sand dunes, AMF account for 30% of the total soil microbial biomass (Olsson & Wilhelmsson 2000). It is therefore reasonable to assume that they play an important role in these systems. Based on studies with *A. breviligulata* and *Leymus arenarius*, arbuscular mycorrhizal fungi are considered to be a major candidate for nematode control in foredunes (Little & Maun 1996; Greipsson & El-Mayas 2002), but no data are available neither for other sand dune plant species nor for the putative mechanisms involved in nematode control. Plant protection by AMF might be caused by physical and physiological plant responses to the fungal infection (Graham 2001). Alternatively, AMF could have a direct suppressive effect on PPN if both organisms compete for root space and feeding sites (Francl 1993). My objectives were to determine whether AMF can suppress nematode infection and reproduction and to explore the mechanisms of nematode control by AMF. A sequential inoculation experiment and a split-root experiment were designed respectively to analyse the importance of plant tolerance and resistance and of direct competition between AMF and *P. penetrans* for the root herbivore and the plant. I designed two experiments to study the mechanisms by which AMF may control PPN, using *A. arenaria* and *P. penetrans* as model organisms. In the first experiment I examined whether pre-inoculation with AMF makes plants more tolerant to herbivory or provides an increase in plant resistance to the herbivores. In the second experiment, I analysed the importance of the presence of arbuscular mycorrhizal fungi and *P. penetrans* in the same root compartment of *A. arenaria* for the outcome of the interaction.

## 6.2 Materials and Methods

### 6.2.1 Plants and soil

Two *A. arenaria* populations, from Het Zwin (Belgium) and Ynyslas (Wales, U.K.), were used on this experiment. Seeds were germinated as described in Chapter 3. Seedlings of Belgian and Welsh origin were used for experiments 1 and 2 respectively.

Sand from Het Zwin (Belgium) for the experiments was collected and sterilized as described in Chapter 3.

### 6.2.2 Nematodes

*Pratylenchus penetrans* was originally isolated in Sandhoven, (Belgium) and was maintained (as described in Chapter 3) in *A. arenaria* for 8 months before the establishment of the experiments. Nematodes for inoculation experiments were extracted from *A. arenaria* roots using the mistifier technique (Chapter 3). In both experiments, 900 nematodes (mobile stages) were added per pot.

### 6.2.3 Arbuscular mycorrhizal fungi

In November 2003, soil was collected from the rhizosphere of four different *A. arenaria* plants in Het Zwin (Belgium) and Ynyslas (Wales) and used to set up trap cultures of the AMF community with *Zea mays* L. as host plant. The trap cultures were maintained in a plant growth chamber at 24/16 ºC and 16/8 h photoperiod and watered regularly. After 5 months, plants were harvested and roots examined to confirm AMF colonization. (1998). A portion of the roots was stained with ink (Blue Quink, Parker) following a modification of the protocol from Vierheilig *et al.* (1998); roots were cleared in 2.5% (wt/vol) KOH for 1h at 90ºC, rinsed with tap water and immersed in 1% HCl overnight and stained with 1% (vol/vol) ink in 1% HCl for 30 min at 60ºC. Root colonization was estimated using a stereoscopic microscope (Leica MZ 8) using the grid-line intersect method (Giovannetti & Mosse 1980). After verifying root colonization, the remaining corn roots were cut in 2 cm pieces, and disinfected by immersion in 2 % Chloramin T for 3 minutes and in an antibiotic solution (Streptomycin 200 mg $l^{-1}$ + Penicillin 100 mg $l^{-1}$) for 3 h. Then, roots were rinsed with autoclaved water and air-dried (Little & Maun 1996).

Spores were extracted from the trap cultures by wet sieving. The material retained in the 0.250-mm, 0.100-mm and 0.045-mm aperture sieves was collected and assessed using a stereoscopic microscope (Leica MZ 8). Spores of *Scutellospora castanea* and several *Glomus* spp. were observed in the trap cultures from Belgium, while in the trap cultures from Wales the spores were mainly from *Glomus* spp. Healthy spores of both cultures were collected, washed and re-suspended in autoclaved distilled water to a final concentration of 100 spores·$ml^{-1}$.

For the first experiment, 550 spores and 0.5 g of dried corn roots from the Belgian trap cultures were used to inoculate each pot containing four *A. arenaria* seedlings. For the split-root experiment, 50 spores and 0.3 g of corn roots from the trap cultures of Wales were used to inoculate each plant. Corn roots were mixed with the autoclaved sand and spores

were inoculated by adding the appropriate volume of spore suspension to the rhizosphere of each *A. arenaria* seedling.

### 6.2.4 Experiment 1: Sequential inoculation

Four two-week old seedlings of *A. arenaria* were planted in 1.5 L pots filled with 1800 g of sterilized dune sand. Pots were covered with aluminium foil to prevent desiccation and watered every second day to keep the moisture content at 5-10 % based on pot weight. Every two weeks all treatments received 120 ml half-strength modified (P-free) Hoagland's nutrient solution. There were six treatments with six replicates per treatment: a non-inoculated control (C), inoculation with arbuscular mycorrhizal fungi (AMF), inoculation with *P. penetrans* (Nem), simultaneous inoculation with AMF and nematodes (FN), inoculation with AMF and two weeks later with nematodes (FN2), and inoculation with AMF and five weeks later with nematodes (FN5). In addition, we included four pots inoculated with AMF for infection assessment after two and five weeks. Root colonization was only detected in plants harvested after five weeks. The pots were placed on a bench in the glasshouse in a randomised design and repositioned every two weeks, after each fertilizer application. The experiment was conducted from June 2004 until September 2004 under ambient light conditions and 25/18°C mean day/night temperatures.

### 6.2.5 Experiment 2: Split root experiment

Two-week old seedlings were transferred to 1.5 L pots with sterilized soil and grown for six additional weeks to obtain roots big enough to be split. Afterwards, the roots from each plant were split in half and each fraction was placed into a separate pot with 800 ml of sterilised dune sand. Pots were covered with tin foil to avoid desiccation and prevent contamination and placed in a completely randomized design on a bench in a growth chamber. The experiment ran from June 2004 until September 2004 under 16/8 h day/night artificial illumination (250 µmol $m^{-2}h^{-1}$) at 24/18 °C day/night temperatures and a constant relative humidity of 80 %. Pots were watered weekly to maintain 5-10 % moisture and were fertilized every two weeks with 100 ml of half-strength modified (P-free) Hoagland's solution. AMF and nematodes were inoculated either together or alone in each root subsystem when plants were transferred to the split root systems. The experiment included five treatments with nine replicates per treatment: Non-inoculated plants (C); inoculation with nematodes (Nem); inoculation with AMF (AMF); AMF and nematodes inoculated

separately (Split), and nematodes and AMF inoculated together in each root sub-subsystem (FN).

### 6.2.6 Harvest and data collection

Plants from both experiments were harvested after 14 weeks. The fresh weight of shoots and roots, the number of tillers and leaves, and the length of the longest leaf were measured for each plant. A portion of each root was weighed and stained using acid fuchsin for nematode and AMF assessment (Baker & Gowen 1996). Using a compound microscope, root infection by AMF and nematodes was estimated as the percentage of 1 cm root fragments containing structures of each or both organisms (Fig 6.1). Nematodes were also counted in each root fragment and the mean number of nematodes present in each infected 1 cm root fragment was calculated. The total number of nematodes per gram of root was estimated using the weight of the root portion used in the staining process. Nematodes were extracted from 100 ml soil by zonal centrifugation following Hendrickx (Hendrickx 1995) (Chapter 3). Nematodes in any developmental stage were taken as a positive count. Because in experiment 1 the nematodes were added at different times, we calculated the rate of nematode multiplication rate per day **(**$Nr \cdot t^{-1}$**)** by computing the ratio between the total number of nematodes (in roots and soil) at the end of the experiment and the initial number of nematodes added to each pot, and dividing this value by the number of days that the roots were exposed to nematodes (98 for Nem and FN, 84 for FN2 and 63 days for FN5). After taking the root fraction for assessing colonization, the remaining plant material was dried at 72ºC for 48 h to estimate plant biomass. Subsequently, leaves and roots were separated manually and ground using an electric mill (Culatti MFC, Zürich, Switzerland). Plant carbon and nitrogen contents were measured by combustion using an automatic elemental analyser FlashEA 1112 coupled with gas chromatographic (GC) separation and thermal conductivity detection (TCD) systems (ThermoFinnigan, CA, USA). Phosphorus analyses could not be performed because of lack of plant material.

### 6.2.7 Analysis of AMF diversity

Total DNA was extracted from the roots of plants inoculated with AMF in both experiments. In experiment 1, DNA was extracted from all replicates of the treatment AMF whereas in experiment 2, DNA extraction was done from the plants in the three treatments that included AMF inoculation. DNA was extracted from 1 cm root fragments by crushing

them in sterile 1.5 ml tubes using a micro-pestle in 60 µl of TE buffer pH 8.0 (10 mM), adding 40 µl of 20 % Chelex 100 and incubating the extract at 95 ºC for 10 min. After cooling on ice for 15 minutes the extract was centrifuged at 12000 g for 4 min and the supernatant transferred to a sterile tube (van Tuinen *et al.* 1998).

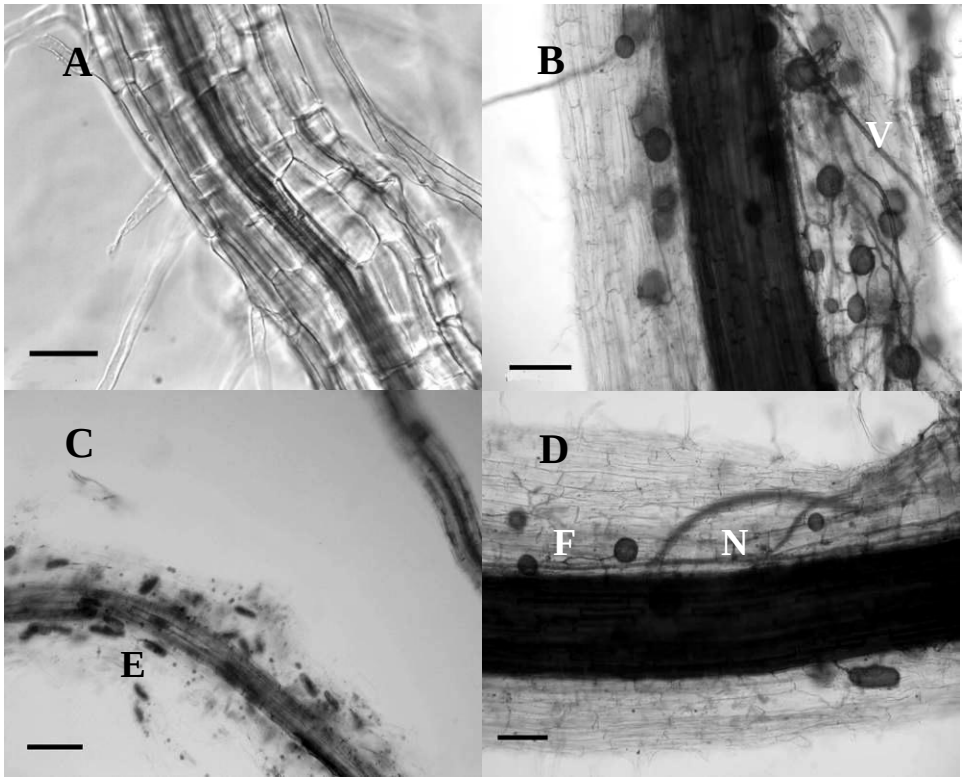


**Fig 6.1** *Ammophila arenaria* roots stained with acid fuchsin to detect infection by AMF and nematodes. A. non-infected root; B. AMF colonization, vesicles (V) and hyphae (H); C. necrotic root, infested with nematode eggs (E); D. root infected with AMF (F) and adult nematodes (N) Scale bar: 100µm.

A nested-PCR was used to selectively amplify fungal DNA from the extracts. All reactions were carried out in a GeneAmp PCR 9700 system (Perkin Elmer, CA, USA). The first PCR used the forward primer NS1 in combination with the reverse primer ITS4, covering the region from the beginning of the 18S rRNA gene through the 5' end of the 25S rRNA gene (White *et al.* 1990). PCRs were performed in a final volume of 20 µl using 1 µl of a 1:10 dilution of the DNA extract, 200 µM of each dNTP (Amersham-Pharmacia), 1.5 mM $MgCl_2$, 0.4 µM of each primer, and 1 U of the Taq DNApolymerase (Amersham-Pharmacia). The conditions for the PCR were 94 °C for 4 min; 30 cycles of (94 °C, 30 s; 55 °C, 40 s; 68 °C 2 min + 5 s per cycle); and 68 °C for 7 min. The product of this first PCR was diluted and used in the second PCR with the primers NS31 (Simon *et al.*, 1992) and AM1 (Helgason *et al.*, 1998) targeting at the region V3-V4 of the 18S rRNA gene and

designed to specifically amplify AMF sequences. Thermocycling used the following program: 94°C for 2 min; 35 cycles of (92 °C, 30 s; 61 °C, 60 s; 68 °C 50 s + 1 s per cycle); and 68 °C for 5 min. The products from the second PCR were examined by standard 1 % (w/v) agarose gel electrophoresis with ethidium bromide staining, to confirm product integrity and estimate yield. Afterwards, they were purified using the QiaQuick PCR purification kit (Qiagen) with a final elution volume of 30 µl. Cloning of the purified products was done using the pGEM-T Easy Vector System from Promega according to the manufacturer protocol. Three colonies from each cloning reaction were grown overnight at 37 °C with shaking at 200 rpm in 3 ml of Luria-Bertani medium supplemented with 100 mg $ml^{-1}$ ampicillin and plasmids were purified using the Qiagen Kit following the manufacturer's protocol. Positive clones were sequenced using ABI PRISM Dye Terminator Cycle Sequence Ready Reaction Kit (Perkin-Elmer, CA, USA).

All sequences were compared to sequences available in internet databases using BLAST to check for similarities with previously described species (Altschul *et al.* 1997). Sequences of *Glomus* sp and two outgroup taxa (*Endogone pisiformis* Link (X58724), *Mortierella polycephala* Coem. (X89436) were acquired from GenBank/EMBL databases and used in the phylogenetic analyses. Sequences were aligned using BioEdit (Hall 1999) and neighbour-joining analyses were performed with Kimura parameters (Kimura 1980) using PHYLYP 3.5 (Felsenstein 1993). The input order of species was randomised and analyses were bootstrapped. Trees were visualized with TreeView 1.6.6 (Page 2001).

### 6.2.8 Statistical analysis

The statistical analysis was performed with the ANOVA General Linear Model (SPSS). All data were checked for normality with Kolmogorov-Smirnov and homogeneity of variance with Levene's test and log X, log (X+1) or square-root transformed when needed to meet ANOVA model assumptions. Data on biomass, nutrient content, AMF colonization and nematode infection and multiplication were analysed with one-way ANOVA and Tukey's multiple range test for overall comparisons. When ANOVA assumptions were not achieved (tiller, longest leaf length and leaf number) a non parametric Kruskal-Wallis test and pair wise comparisons using Mann-Whitney test were performed to detect differences among treatments.

## 6.3 Results

### 6.3.1 Experiment 1: Sequential inoculation

Root infection by AMF ranged from 43 % (FN) to 55 % (AMF) (Fig 6.2a). However, significant differences for root infection by AMF were not found between treatments. Root colonization by nematodes ranged between 2 and 29 % again depending on the treatment (Fig 6.2b). Nematode infection was lower when AMF were also present in the root, and significant differences were found ($F_{3,\ 18}$=21.21, $P$<0.005) between the treatments previously inoculated with AMF (FN2, FN5) and the other two treatments (Nem, FN). The total number of nematodes per pot was also drastically reduced by the presence of AMF (Fig 6.2c) from 2863 (Nem) to 1516 (FN). A further reduction to less than 1000 in FN2 and FN5 was, at least partly, due to shorter reproduction time of the nematodes that were inoculated two and five weeks later. Significant differences ($F_{3,\ 18}$=37.71, $P$<0.001) were found between all treatments except when comparing FN2 and FN5. To avoid misinterpretation due to the differences in inoculation times between FN, FN2 and FN5 in the sequential experiment, we calculated nematode multiplication per day and the number of nematodes per unit of infection; i.e. 1 cm root pieces (Fig 6.3). The ratio of nematode multiplication per day (Nr $t^{-1}$) decreased with the presence of AMF (Fig 6.3a). Significant differences ($F_{3,\ 18}$=28. 81; $P$<0.001) were found between the plants inoculated only with nematodes and those inoculated with both AMF and nematodes. The ratio was significantly lower in the treatment FN5 when compared to FN, showing that the lower number of nematodes was not only due to shorter multiplication time. The average number of nematodes per infected root was more than twice higher in Nem than in each of the other treatments (Fig 6.3b). This value was significantly different ($F_{3,\ 18}$=7.78; $P$<0.005) from the treatments that included AMF. No significant differences were found between FN, FN2 and FN5.

Plant biomass was significantly higher ($F_{5,\ 124}$=5.02; $P$<0.001) in the treatment AMF than in Control, Nem and FN (Table 6.1). At the nematode density used in the experiment, a negative effect on plant biomass was not observed, but the inoculation with nematodes at the same time as AMF and two weeks after AMF inoculation suppressed the beneficial effect of mycorrhizal fungi on plant biomass. The proportion of biomass allocated belowground (root/total biomass ratio) was significantly higher in the treatments AMF and FN than in control plants ($F_{5,\ 124}$=6.68; $P$<0.001) (Table 6.1). The colonization by AMF significantly increased the number of leaves and tillers produced by *A. arenaria* plants, whereas nematode infection significantly reduced the number of tillers (Table 6.1).

The inoculation with AMF and nematodes affected plant nutrient content and allocation (Table 6.2). The plants inoculated only with nematodes had the lowest nitrogen content, which was significantly different ($F_{5,\ 25}$=13.94; $P$<0.01) from the other values. The same was observed for the proportion of nitrogen allocated belowground and for total carbon content ($F_{5,\ 25}$=16.75, $F_{5,\ 25}$=7.41*;* $P$<0.01), although no differences were found in total carbon between Nem and FN. The proportion of carbon allocated belowground was significantly higher in the treatment Nem than in the control ($F_{5,\ 25}$=6.56; $P$<0.001) (Table 6.2).

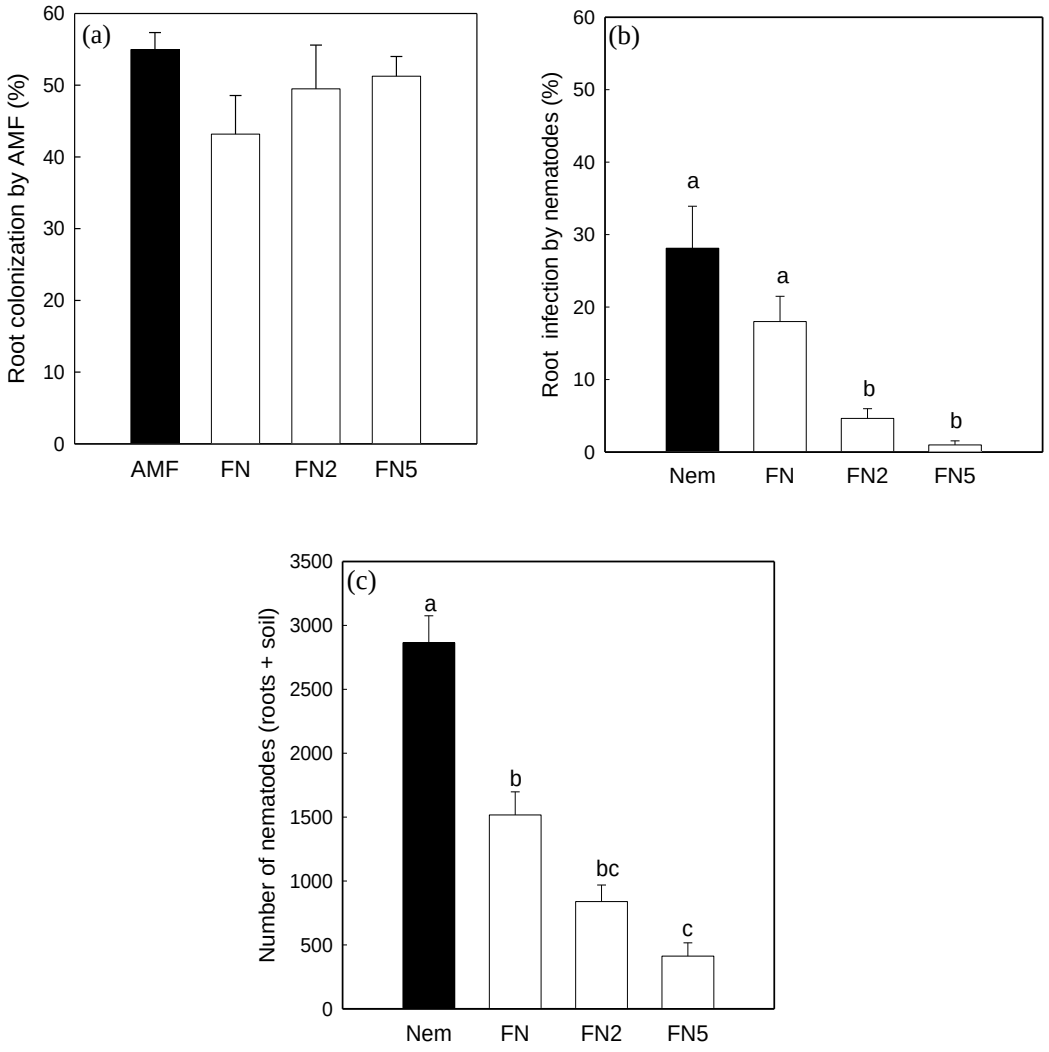


**Fig 6.2** Sequential inoculation experiment: (a) Percentage of *Ammophila arenaria* roots infected by AMF, (b) Percentage of *A. arenaria* roots infected by *Pratylenchus penetrans* and (c) total final number of nematodes. Data are mean ± SE. AMF: Inoculation with AMF; Nem: Inoculation with nematodes, FN: inoculation with AMF and nematodes, FN2: Nematode inoculation two weeks after AMF, FN5: nematode inoculation five weeks after AMF. Different letters above the bars indicate significant differences ($P$<0.005) between treatments after one-way ANOVA and Tukey's HSD test.

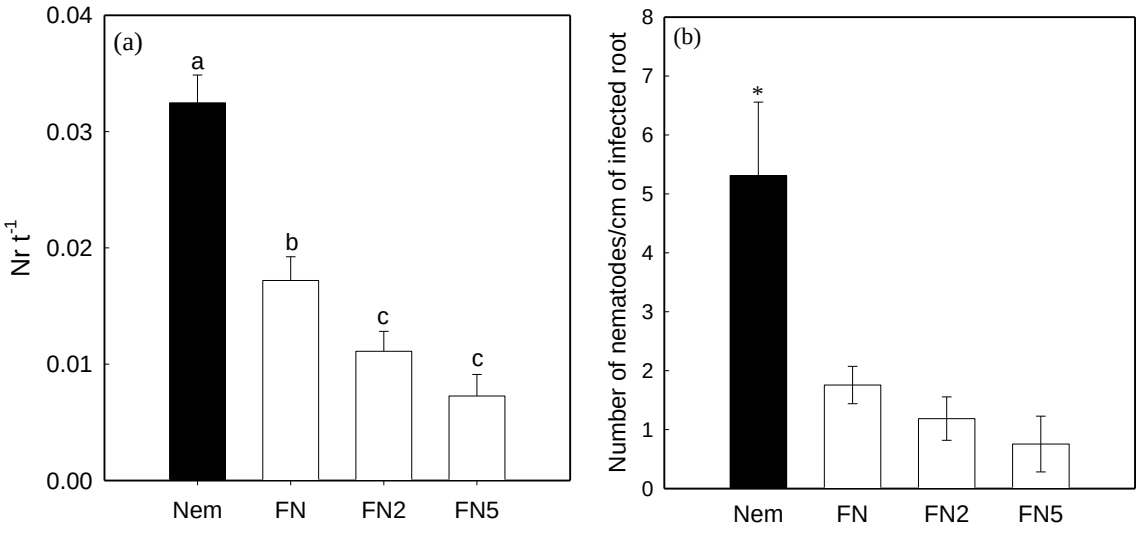


**Fig 6.3** Sequential inoculation experiment: (a) Ratio of nematode multiplication per day (Nr $t^{-1}$) ((Final number of nematodes/Initial number of nematodes)/days) and (b) number of nematodes per fragment of root infected. Data are mean ± SE. Nem: Inoculation with nematodes, FN: inoculation with AMF and nematodes, FN2: Nematode inoculation two weeks after AMF, FN5: nematode inoculation five weeks after AMF. Different letters above the bars indicate significant differences ($P$<0.001) between treatments after one-way ANOVA and Tukey's HSD test.

### 6.3.2 Experiment 2: Split root experiment

Root colonization by AMF was lower in this experiment with values around 30 % (Fig 6.4a). Significant differences in the percentage of root colonized by AMF were not found between treatments. Root colonization by nematodes ranged between 15 and 26 % and it was significantly reduced when nematodes and AMF were inoculated together (Fig 4b, $F_{2, 22}$=6.44, $P$<0.01). The total number of nematodes and the number of nematodes per infected unit were also significantly lower ($F_{2, 22}$=3.94, $P$<0.05; $F_{2, 22}$=6.05, $P$<0.005, respectively) when nematodes and AMF were inoculated together (Figs. 6.4c, d). The average final number of nematodes per pot was 2152 in treatment Nem and 795 in treatment FN. The inoculation of AMF and nematodes in different subsystems of the root did not reduce root colonization by nematodes, but a slight reduction in the total number of nematodes was observed.

No significant differences between the treatments were observed for plant biomass, ratio of biomass allocated belowground and for the number of tillers and leaves (Table 6.3). However, plants inoculated with AMF and nematodes in the same root (FN treatment) were significantly shorter than the control plants and plants inoculated with nematodes or AMF ($\chi^2$ = 19.82, $P$<0.05). In the split-root experiment, significant differences in nutrient content and allocation were found between the inoculation treatments (Table 6.4). Plants inoculated only

with nematodes had a significantly higher N content than plants inoculated with both AMF and nematodes ($F_{4,\ 24}$=5.46; $P$=0.003) (Table 6.4). Significant differences in total carbon content were found between the plants inoculated only with nematodes and those inoculated with nematodes and AMF in the same root subsystem ($F_{4,\ 24}$=3.48; $P$=0.022). The highest proportion of nitrogen and carbon allocated belowground were observed in the plants inoculated only with nematodes and the lowest in the SPLIT treatment. Significant differences were found between these two values ($F_{4,\ 24}$=3.08, $P$=0.035 for belowground nitrogen; $F$=4.01, $P$=0.013 for belowground carbon).

AMF diversity

The diversity of the AMF associated to *A. arenaria* in both experiments was analysed because different AMF genera have morphological and functional differences that could be important for the interaction with the nematodes. Thirty-one different sequences were obtained from the analysis of DNA extracted from *A. arenaria* roots and are deposited in GenBank (accession numbers DQ090845-DQ090875). All the sequences displayed a strong homology with sequences of *Glomus* spp. available in GenBank. The phylogenetic tree constructed using data of AMF species from GenBank showed that all my sequences clustered in the clade *Glomus*-group A within the order Glomerales (Schüßler *et al.* 2001) (Fig 6.5). The closest described *Glomus* species were *G. fasciculatum* (Y17640), *G. intraradices* (AY635831, AJ301859, X58125) and *G. vesiculiferum* (L20824). All the sequences obtained from the first experiment and six sequences obtained from the split-root experiment clustered in a sub-group with sequences obtained in previous studies in grasslands (Vandenkoornhuyse *et al.* 2002; Wirsel 2004). The remaining sequences obtained from AMF in the split-root experiment clustered in another sub-group within the *Glomus*-group A with other *Glomus* sequences obtained from studies in grasslands and northern forests (Vandenkoornhuyse *et al.* 2002; Öpik *et al.* 2003).

**Table 6.1** Experiment 1 (mean ± SE). Control (C), inoculation with nematodes (Nem), inoculation with AMF and nematodes (FN), nematode inoculation two weeks after AMF (FN2), nematode inoculation five weeks after AMF (FN5). Different letters indicate significant differences between treatments according to one-way ANOVA and Tukey's HSD test (†) or non-parametric tests (‡) ***$P$<0.001; ns: non-significant.

| Variable | Treatments | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | C | Nem | AMF | FN | FN2 | FN5 | $F$-Values | df | $X^2$ |
| Biomass | 0.35 ± 0.03$^{b}$ | 0.36 ± 0.02$^{b}$ | 0.56 ± 0.04$^{a}$ | 0.37 ± 0.03$^{b}$ | 0.40 ± 0.02$^{ab}$ | 0.49 ± 0.05$^{ab}$ | 5.02*** † | 5 | - |
| Root/Total | 0.18 ± 0.01$^{c}$ | 0.22 ± 0.01$^{abc}$ | 0.26 ± 0.01$^{a}$ | 0.24 ± 0.01$^{ba}$ | 0.20 ± 0.01$^{cb}$ | 0.18 ± 0.02$^{c}$ | 6.68*** † | 5 | - |
| Longest leaf | 60.32 ± 2.37 | 62.57 ± 2.23 | 66.46 ± 1.83 | 62.19 ± 1.76 | 66.71 ± 1.80 | 65.74 ± 2.23 | - | 5 | 9.96ns ‡ |
| Tillers | 1.64 ± 0.12$^{cb}$ | 1.17 ± 0.08$^{d}$ | 2.38 ± 0.15$^{a}$ | 1.52 ± 0.12$^{c}$ | 1.90 ± 0.14$^{b}$ | 2.07 ± 0.16$^{abc}$ | - | 5 | 42.96*** ‡ |
| Leaves | 3.95 ± 0.17$^{b}$ | 3.57 ± 0.12$^{b}$ | 4.96 ± 0.20$^{a}$ | 4.04 ± 0.17$^{b}$ | 4.85 ± 0.29$^{a}$ | 3.93 ± 0.16$^{b}$ | - | 5 | 37.44*** ‡ |

**Table 6.2** Plant nitrogen and carbon content (% w/w) and percentage of nitrogen and carbon allocated belowground in experiment 1 (mean ± SE). Control (C), inoculation with nematodes (Nem), inoculation with AMF and nematodes (FN), nematode inoculation two weeks after AMF (FN2), nematode inoculation five weeks after AMF (FN5). Different letters indicate significant differences between treatments according to one-way ANOVA and Tukey's HSD (***$P$<0.001).

| Variable | Treatments | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | C | Nem | AMF | FN | FN2 | FN5 | df | $F$-value |
| Plant N | 1.29 ± 0.04$^{a}$ | 0.75 ± 0.09$^{b}$ | 1.43 ± 0.13$^{a}$ | 1.51 ± 0.04$^{a}$ | 1.49 ± 0.05$^{a}$ | 1.47 ± 0.09$^{a}$ | 5 | 13.94*** |
| Percentage of belowground N | 35.11 ± 0.86$^{a}$ | 23.39 ± 2.75$^{b}$ | 40.44 ± 1.80$^{a}$ | 34.92 ± 1.20$^{a}$ | 40.79 ± 1.01$^{a}$ | 41.00 ± 1.62$^{a}$ | 5 | 16.75*** |
| Plant C | 39.32 ± 0.62$^{a}$ | 30.78 ± 1.16$^{b}$ | 38.07 ± 0.77$^{a}$ | 35.30 ± 1.47$^{ab}$ | 38.55 ± 1.56$^{a}$ | 37.60 ± 0.90$^{a}$ | 5 | 7.41*** |
| Percentage of belowground C | 56.49 ± 0.94$^{b}$ | 70.83 ± 3.14$^{a}$ | 58.90 ± 1.05$^{b}$ | 63.19 ± 2.19$^{ab}$ | 58.53 ± 2.58$^{b}$ | 58.54 ± 1.22$^{b}$ | 5 | 6.56*** |

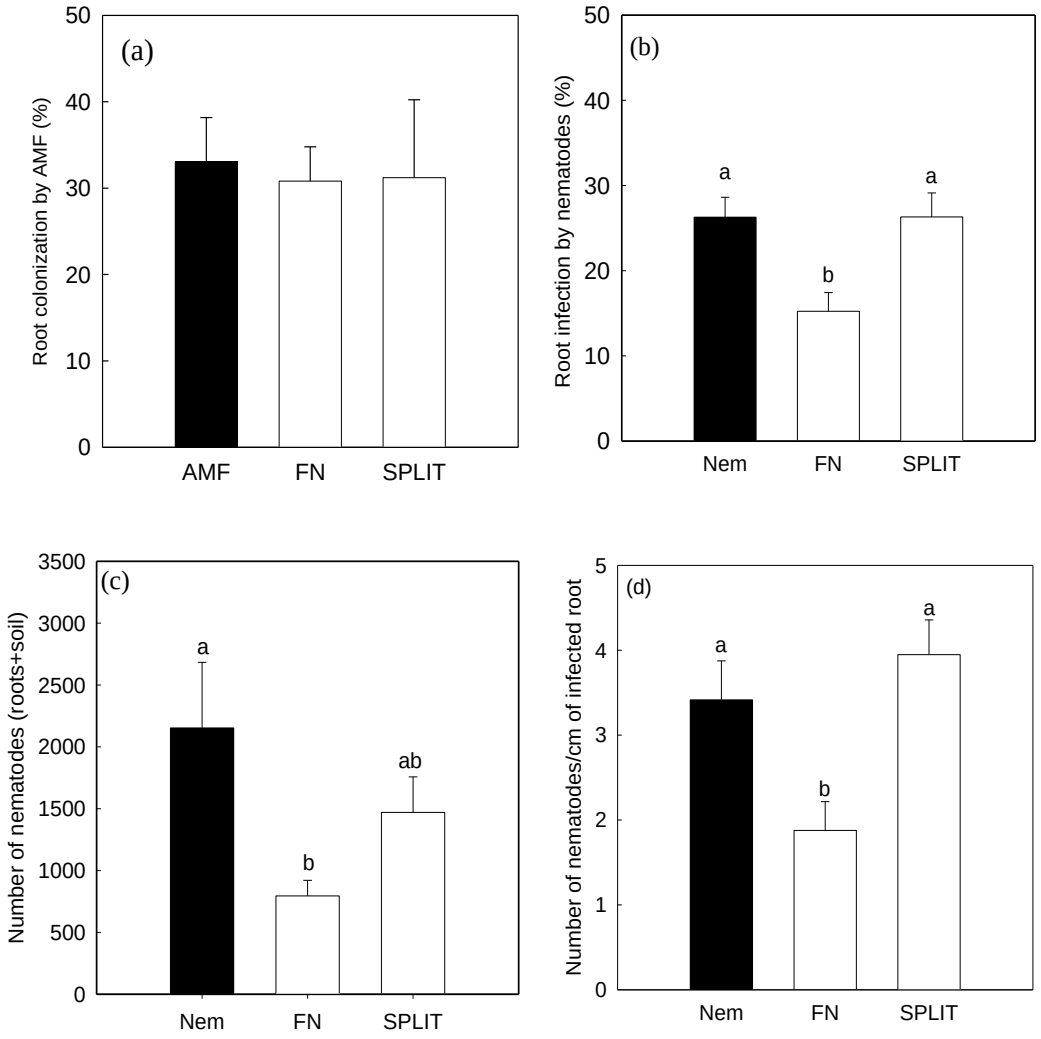


**Fig 6.4** Split root experiment: (a) Percentage of *Ammophila arenaria* roots infected by AMF; (b) Percentage of *A. arenaria* roots infected by *Pratylenchus. penetrans*; (c) total number of nematodes and (d) Ratio of nematode multiplication per day Nr $t^{-1}$) ((Final number of nematodes/Initial number of nematodes)/days). Data are mean ± SE. AMF: Inoculation with AMF, Nem: Inoculation with nematodes, FN: inoculation with AMF and nematodes, SPLIT: split inoculation of AMF and nematodes. Different letters above the bars indicate significant differences ($P$< 0.05) between treatments after one-way ANOVA and Tukey's HSD test.

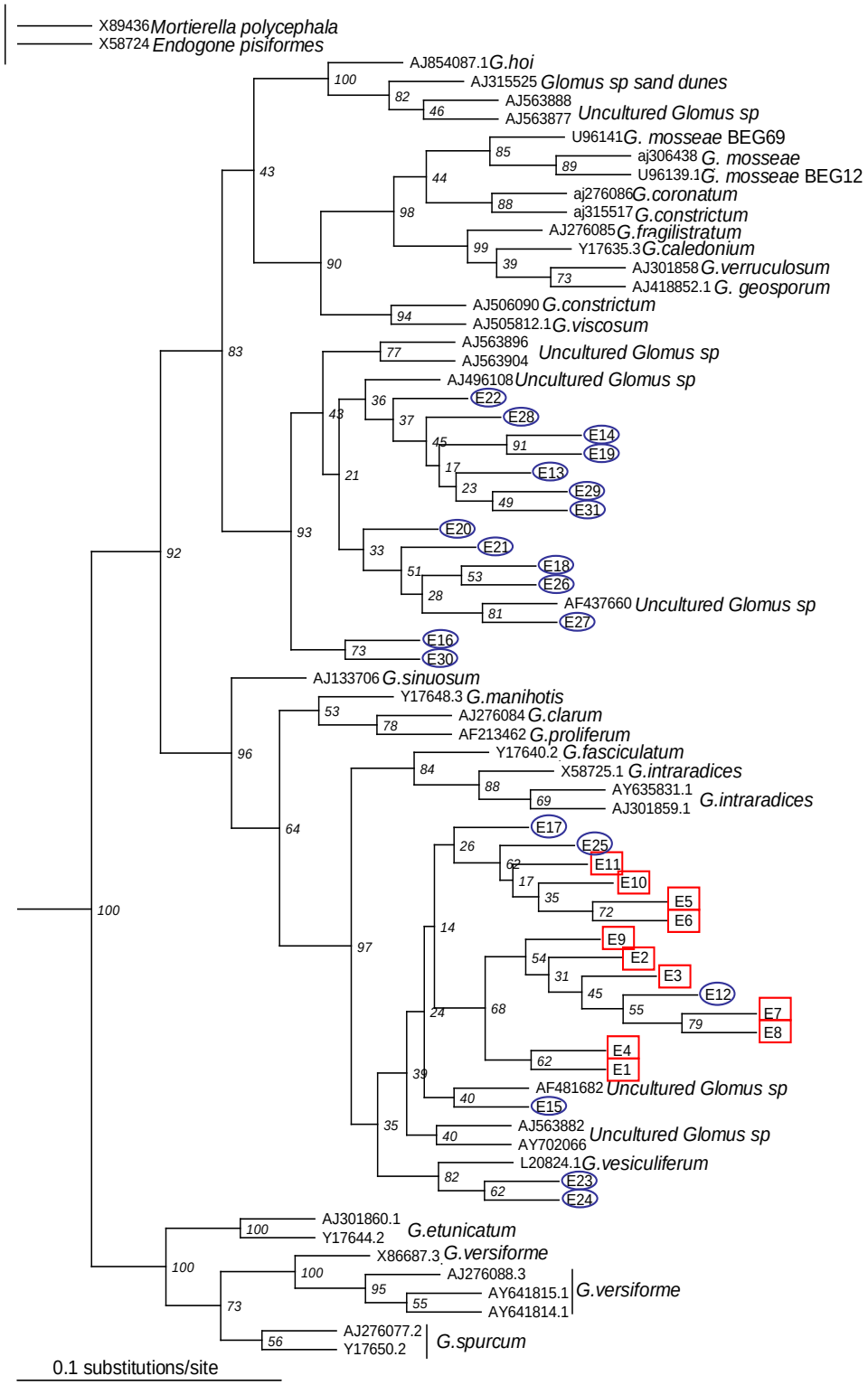


**Fig 6.5** Neighbour-joining tree inferred from partial SSU rDNA sequences obtained from *A. arenaria* roots and other described and undescribed *Glomus* spp. In squares, E1-E11: Sequences obtained from experiment 1 (Trap cultures from Het Zwin, Belgium). In circles, E12-E31: Sequences obtained from experiment 2 (Trap cultures from Ynyslas, Wales.)

**Table 6.3** Total biomass (d. wt) (g), root/total biomass ratio (Root/Total), length of longest leaf (cm), number of tillers and number of leaves in experiment 2 (mean ± SE). Control (C), inoculation with nematodes (Nem), inoculation with AMF and nematodes (FN), split inoculation of AMF and nematodes (SPLIT). Different letters indicate significant differences between treatments according to one-way ANOVA and Tukey's HSD (†) or non-parametric tests (‡). **P*<0.05; ***P*<0.01; ****P*<0.005; ns: non-significant.

| Variable | Treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | C | Nem | AMF | FN | SPLIT | *F*-Values | *df* | $X^2$ |
| Biomass | 3.05 ± 0.29 | 2.66 ± 0.33 | 3.54 ± 0.73 | 2.52 ± 0.56 | 2.62 ± 0.24 | 1.06ns † | 4 | - |
| Root/Total | 0.37 ± 0.03 | 0.40 ± 0.03 | 0.36 ± 0.04 | 0.44 ± 0.04 | 0.48 ± 0.05 | 1.69ns † | 4 | - |
| Longest leaf | 67.66 ± 2.64$^{ab}$ | 61.17 ± 1.80$^{b}$ | 70.20 ± 2.65$^{a}$ | 54.80 ± 1.80$^{c}$ | 56.31 ± 1.54$^{bc}$ | - | 4 | 19.82 * ‡ |
| Tillers | 6.11 ± 1.14 | 5.11 ± 0.53 | 5.75 ± 1.37 | 6.25 ± 0.61 | 5.25 ± 0.70 | - | 4 | 1.632 ns‡ |
| Leaves | 14.00 ± 2.12 | 12.44 ± 1.66 | 17.25 ± 4.38 | 15.62 ± 2.06 | 12.00 ± 1.42 | - | 4 | 2.947 ns‡ |

**Table 6.4** Plant nitrogen and carbon content (% w/w) and percentage of nitrogen and carbon allocated belowground in experiment 2 (mean ± SE). Control (C), inoculation with nematodes (Nem), inoculation with AMF and nematodes (FN), split inoculation of AMF and nematodes (SPLIT). Different letters indicate significant differences between treatments according to one-way ANOVA and Tukey's HSD test. * *P*<0.05; ****P*<0.005; ns: non-significant.

| Variable | Treatment | | | | | | |
|---|---|---|---|---|---|---|---|
| | C | Nem | AMF | FN | SPLIT | *df* | *F*-value |
| Plant N | 0.97 ± 0.01$^{ab}$ | 1.24 ± 0.08$^{a}$ | 0.78 ± 0.09$^{ab}$ | 0.87 ± 0.06$^{b}$ | 0.93 ± 0.10$^{b}$ | 4 | 5.46*** |
| Percentage of belowground N | 31.18 ± 4.47$^{ab}$ | 34.19 ± 2.49$^{a}$ | 34.99 ± 4.75$^{ab}$ | 30.16 ± 1.76$^{ab}$ | 22.89 ± 2.31$^{b}$ | 4 | 3.48* |
| Plant C | 36.28 ± 1.77$^{ab}$ | 36.87 ± 1.35$^{a}$ | 33.77 ± 0.37$^{ab}$ | 31.24 ± 1.01$^{b}$ | 37.95 ± 1.68$^{ab}$ | 4 | 3.09* |
| Percentage of belowground C | 39.39 ± 2.47$^{ab}$ | 39.46 ± 2.36$^{a}$ | 39.37 ± 4.48$^{ab}$ | 36.39 ± 0.81$^{ab}$ | 28.07 ± 2.43$^{b}$ | 4 | 4.01* |

## 6.4 Discussion

These results show that native AMF can protect *A. arenaria* through the suppression of *P. penetrans* colonization and reproduction. In other studies on coastal dune systems, Greipsson and El-Mayas (2002) found that a commercial AMF inoculum protected the dune grass *Leymus arenarius* against migratory endoparasitic nematodes. Also, Little and Maun (1996) showed that mycorrhizal protection of *Ammophila brevigulata* against *Pratylenchus* and *Heterodera* spp. was effective when sand burial occurred simultaneously. The majority of studies on the interaction between AMF and *Pratylenchus* spp. have been done with perennial crops and the results are quite inconsistent. Some showed increases in plant tolerance or resistance to *Pratylenchus* spp. as a consequence of plant inoculation with AMF, but others did not find any protective effect of AMF (Roncadori 1997; Forge *et al.* 2001; Elsen *et al.* 2003b).

The data presented here show for the first time that AMF can outcompete migratory endoparasitic nematodes when they occur together in the same root compartment; in contrast with previous studies with migratory endoparasitic nematodes in which AMF seemed to enhance nematode multiplication (Borowicz 2001). On the other hand, root colonization by AMF was not affected by the migratory endoparasitic nematodes, so I did not detect mutual inhibition between AMF and nematodes as proposed previously (Francl 1993). The detailed mechanisms of suppression of nematodes were not analysed; however, the results suggest that direct competition with AMF hyphae in the root or local changes in root chemistry or exudates may have been responsible for the inhibition of nematode reproduction (Graham 2001).

Some authors have hypothesized that AMF protection is only effective if plants are colonized by the mycorrhizal fungi before the attack by pathogens and/or herbivores. This hypothesis is based on the improved nutritional and health status of mycorrhizal plants which allow them to support higher densities of PPN (Azcón-Aguilar & Barea 1996). I did not find a higher concentration of nitrogen and carbon in the plants that were pre-inoculated with AMF two and five weeks before nematode inoculation, but plant biomass was significantly higher in the treatment FN5 than when nematodes and AMF were inoculated simultaneously. However, this positive effect of AMF pre-inoculation occurred through nematode suppression and not through increased plant tolerance because the effect of pre-inoculation with AMF was a further reduction in nematode reproduction and infection. Therefore, plant pre-inoculation with AMF increased plant resistance to the PPN.

Increases in plant growth through an improved plant nutrient uptake are considered the main benefits that plants obtain from the symbiosis with AMF (Jeffries *et al.* 2003). A significant increase in plant growth was observed in the first experiment but not in the second one. This disparity might be caused by differences in the AMF species between both experiments, but also, and more likely, by the different age of the plants used in the two experiments, two *vs* eight weeks, because younger *A. arenaria* plants display a greater response to AMF (Rodríguez-Echeverría *et al.* 2004). Thus, changes on plant biomass and nutrient content between treatments were not as severe in the split-root experiment as in the first experiment. The biomass allocated belowground was 20 % in the sequential inoculation experiment and 40 % in the split-root experiment. The proportion of biomass allocated above and belowground by a plant species depends on environmental factors, plant age and growing time (Klepper 1991). Because pot size was different in both experiments, the variation in the percentage of belowground biomass could be explained not only by plant age but also by the greater sand volume that each plant had in the split-root experiment. It is noteworthy that in both experiments the proportion of biomass allocated belowground increased with the inoculation of nematodes and/or AMF.

The presence of nematodes did not have a negative impact on plant growth. However, nematodes wiped out the beneficial effect of AMF and affected plant nitrogen and carbon content. These differences were again greater in the sequential inoculation experiment, probably because young plants are more sensitive to the attack by root-feeding herbivores (Van der Putten *et al.* 1990). Plants in the sequential inoculation experiment also reallocated nitrogen and carbon aboveground when attacked by the nematodes, a common reaction in plants subjected to important root damage (Masters & Brown 1997). In the split-root experiment the highest nitrogen content was found in plants infected only by nematodes. Although these plants were probably more tolerant to herbivory than the 2-week old seedlings, this increase in nitrogen content can be considered an indicator of plant stress (Whittaker 2003), also observed for *A. arenaria* when growing in non-sterilized soil (Van der Putten *et al.* 1988). Root colonization by AMF did not increase nutrient content in the plants of the split-root experiment, a fact that could be explained by the lower responsiveness of older seedlings combined with the lower values of root colonization by AMF.

The AMF communities associated to the roots of *A. arenaria* were very similar in both experiments, containing mainly *Glomus* sp. from the *Glomus*-group A (Schüßler *et al.* 2001). The genus *Glomus* comprises the majority of species within the phylum

Glomeromycota. *Glomus* species are also more resistant to disturbances than other genera of AMF (Dodd *et al.* 2000). Therefore, the AMF communities detected on the roots probably represent the fraction of field inoculum that can survive and grow in our experimental conditions. As in other molecular studies of AMF colonising plant roots, our sequences did not correspond to previously described AMF species suggesting a higher natural AMF diversity than acknowledged from culture collections. Our understanding of the importance of AMF diversity for the symbiosis is still limited; however, the interactions of plants with complex natural AMF communities are probably richer than with commercial AMF inocula. Studies addressing ecological issues should not underestimate the importance of the natural high diversity of AMF.

The ability of AMF to control *P. penetrans* in the rhizosphere of *A. arenaria* could be crucial under natural field conditions. A study by Van der Stoel et al. (2002a) showed that PPN, including *Pratylenchus* spp., accumulate in 4-5 weeks after the growth of new roots in the new fresh sand layer. They found that the density of nematodes after a month of the sand deposition could significantly reduce plant growth in greenhouse trials. However, they also observed that *A. arenaria* can overcome that negative effect of nematodes in the foredunes. Arbuscular mycorrhizal fungi were excluded from their greenhouse trials, but my results demonstrate that the interaction between *A. arenaria* and nematodes can not be fully understood without AMF.

The diversity of organisms involved in belowground interactions makes it difficult to single-out the direct implications and effects of different groups, however, this work shows that AMF can control root herbivores associated to the grass *A. arenaria*. This mechanism can be added to the bottom-up and interspecific competition processes that have been previously reported as regulatory of nematode populations in coastal dunes (Brinkman *et al.* 2004). The role of nematode antagonists, the effect of AMF in other nematode genera and the consequences of this interaction for nematode competition needs further consideration to completely understand nematode control in natural systems.

# Chapter 7

## *Interaction between* Ammophila arenaria, *the fungal endophyte* Acremonium strictum *and* Pratylenchus *spp*.*

*Hol, G., de la Peña, E, Moens, M, Cook, R. Beneficial effect of a fungal endophyte on *Ammophila arenaria* growth and herbivore tolerance. *Basic and Applied Ecology*, accepted.

## 7.1 Introduction

The strategies and mechanisms developed by plants to overcome environmental stress remain a keystone in plant ecology (Stanton *et al.* 2004; Escudero *et al.* 2005). The interactions between hosts and their mutualists affect the competing abilities of the host plant with other plants and therefore affect the dynamics of plant communities (Reynolds *et al.* 2003; Rodríguez-Echeverría & Pérez-Fernández 2005).

In habitats with regular sand deposition such as coastal sand dunes, burial in combination with other abiotic (e.g. high water drainage and low nutrient content in soils) and biotic factors (e.g. herbivory) impose a strong physiological constraint on plant establishment (Maun & Sun 2002). *Ammophila arenaria* harbours different symbiotic organisms: vesicular-arbuscular mycorrhizal fungi (Kowalchuk *et al.* 2002) and nitrogen fixing bacteria (Dalton *et al.* 2004), which enhance nutrient uptake by the plant and help the plant to overcome the harsh abiotic conditions of dune soils. However, interactions between endophytic fungi and *A. arenaria* have been overlooked.

Fungal endophytes represent an ubiquitous and still largely unknown component of plant communities that may contribute to endure environmental stress and herbivory by the hosts. In Europe, no endophytic fungi for *A. arenaria* have been reported until recently. Hol, Nash and Cook (in press) isolated different *Acremonium* spp. from the stems of *A. arenaria* from the Netherlands, United Kingdom and Portugal. Unlike the widely researched and more specialized *Neotyphodium* spp., the genus *Acremonium* comprises a large group of unspecialized fungi. The nature of the association between *Acremonium* spp. and *A. arenaria* is unknown. The question whether this plant symbiont affects plant performance directly or act as a mediator of plant-herbivore interaction remains unanswered. Previous studies, however, suggested that *Acremonium* species could act as plant-herbivore mediators (Raps & Vidal 1998; Jallow *et al.* 2004).

The potential of endophytic fungi, as a control agent of root-feeding nematodes has never been explored in coastal dunes, although endophytic fungi are well known for affecting nematodes (Cook *et al.* 1991).

In this Chapter, I test whether *Acremonium strictum*, a native endophytic fungus of the grass *A. arenaria*, affects plant growth. In addition, whether this symbiont acts as a plant-herbivore mediator affecting the resistance or tolerance to two root-lesion nematode species, *P. dunensis* and *P. penetrans*, that occur in coastal foredunes was explored.

## 7.2 Materials and methods

### 7.2.1 Seedling establishment

*Ammophila arenaria* seed from Oostvoorne (the Netherlands) were used for the two experiments established. To remove any endophytic fungi, seeds were heat treated at 57°C for 15 min (Siegel *et al.* 1987) prior to germination. Seeds were germinated as described in Chapter 3. Two weeks after germination seedlings were transferred to 600 ml pots containing 600 g of autoclaved (121ºC for 2 h) dune sand.

Sand from Het Zwin (Belgium) was collected and sterilized as described in Chapter 3.

### 7.2.2 Endophyte isolation and maintenance

The endophytic fungus was isolated from an *A. arenaria* stem collected in Oostvoorne, the Netherlands. The isolate showed high homology with *Acremonium strictum*, based on ITS sequencing (Hol et al., in press). Morphologically it was identified as an isolate from the *Acremonium strictum* complex. The isolate is deposited in the public collection of the Fungal Biodiversity Centre, the Netherlands (Accession: CBS 118929). The isolate was maintained on full strength potato dextrose agar (PDA) at 25°C in the dark and propagated on several plates to produce enough inoculum to be used in the experiments.

### 7.2.3 Nematode cultures and inoculation

Two nematodes cultures were used for the experiments described on this chapter: *Pratylenchus dunensis* from Oostvoorne (the Netherlands) and *P. penetrans* from Sandhoven (Belgium). Nematode inocula were obtained from the cultures by using a modification of the mistifier technique (Chapter 3).

### 7.2.4 Experiment 1. The effect of inoculation of *Ammophila arenaria* stems with *Acremonium strictum* on plant parameters and root-lesion nematodes

Fifty-six three-week old *A. arenaria* seedlings were planted separately in 0.6 L plastic pots filled with autoclaved dune soil. The experiment comprised six treatments: C: plants without endophyte and nematodes (n=14), E: plants with endophyte but without nematodes (n=14), P: plants + 100 *P. penetrans* (n=7), PE: plants + 100 *P. penetrans* and endophyte (n=7), D: plants + 100 *P. dunensis* (n=7), DE: plants + 100 *P. dunensis* and endophyte (n=7). Where appropriate, 100 mobile nematode stages were added to the pots nine days after planting. Twenty six days after planting half of the plants were inoculated with *A. strictum*.

The other half was inoculated with PDA homogenate only. Inoculation of the fungus was done by inserting a homogenate of PDA and fungus in a superficial cut made by a sterile razor blade in the coleoptile. Plants were placed in a randomized block design with seven blocks and eight pots per block (2 C, 2 E, 1 P, 1 PE, 1 D, 1 DE). All pots received 60 ml and 40 ml half strength Hoagland solution 16 and 26 days after planting, respectively. Plants received tap water when necessary. Pots were placed on a bench in the glasshouse. The experiment ran in for 13 weeks from May to August 2005 under ambient light conditions and 25/18°C mean day/night temperatures.

### 7.2.5 Experiment 2. The effect of inoculation of *Ammophila arenaria* roots with *Acremonium strictum* on plant parameters, reproduction and competition of root-lesion nematodes

Forty three-week-old seedlings of *A. arenaria* were dipped for three h in a suspension of mycelium and conidia of *A. strictum*, PDA and sterilized water. Forty other seedlings were placed in a similar suspension but without the fungus. These latter plants are considered endophyte-free. After the root dip, all seedlings were planted in 600 ml pots filled with autoclaved dune soil. Eight treatments were compared: control (C), endophyte only (E), *P. penetrans* (P), *P. penetrans* on endophyte plants (PE), *P. dunensis* (D), *P dunensis* on endophyte plants (DE), *P. penetrans* with *P. dunensis* (PD), and *P. penetrans* with *P. dunensis* on endophyte plants (PDE). Twenty-one days after planting of the seedlings, 100 nematodes were added to the pots in the nematode treatments. Twenty pots received 100 *P. penetrans*, 20 pots received 100 *P. dunensis*, and 20 pots received a mixture of *P. penetrans* and *P. dunensis*, containing 50 individuals of each. Plants were placed in a randomized block design with ten blocks and eight pots per block, one of each treatment. All pots received 50 ml Hoagland solution seven days after planting. Plants were further watered three times a week to 10% soil moisture. Plants were kept in a climate chamber at a regime of 24/18ºC and 16/8 hr day/night, and 70% humidity. After 13 weeks plants were harvested and nematodes extracted.

### 7.2.6 Data collection

At harvest, the number of leaves and tillers were counted and the length of the longest leaf/tiller was measured. Fresh weight of roots and shoots was determined. The roots were divided into two parts: one part for nematode extraction and the other to estimate the water content, the remaining plant material was dried at 72°C for 48°C in a hot air-flow oven.

Shoots were also divided into two: (i) the lower three cm of the basal stem part was used to extract endophytic fungi (ii) the remainder was dried at 70°C during three days. Fresh and dry weights were used to estimate the percentage moisture in the shoot: the difference between fresh weight and dry weight was divided by fresh weight and multiplied by 100. Fungal extraction was done after surface sterilization (70% MeOH (30s) and 1.3% NaClO (10 min)) of the basal stem part by placing stem pieces on full strength PDA. After three weeks, plates were examined for presence of *A. strictum*. Nematodes were extracted from roots and soil by centrifugal flotation (Chapter 3). For the extraction of nematodes from soil 100 ml of each port were used. The total number of nematode in roots was calculated extrapolating the number of nematodes in the root fraction to the total fresh biomass. Nematode numbers in soil were calculated based on the number of nematodes obtained from 100 ml of sand and extrapolated to the total volume of the pot.

### 7.2.7 Data analysis

Experiment 1. Dry weight of biomass did not meet the ANOVA assumptions and therefore the effects of endophyte and nematodes were tested with Wilcoxon signed rank test. Total number of nematodes in the roots were compared with the same test.

Experiment 2. Dry weight of shoot and root biomass was tested with two-way ANOVA with blocks, after testing the assumptions for normality and homogeneity of variance. Main treatments were 'endophyte' (two levels) and 'nematode' (two levels). Pairwise comparisons between endophyte-infected and endophyte-free plants per nematode treatment were made with paired t-tests. For all tests SPSS was used.

## 7.3 Results

### 7.3.1 Experiment 1. Inoculation of *Ammophila arenaria* stems with *Acremonium strictum*

The endophytic fungus was successfully re-isolated from 16 out of the 25 plants that had been inoculated with *A. strictum*. Shoot biomass was significantly reduced by nematodes ($Z = -3.29$, $n = 23$, $P = 0.001$; Fig 7.1a), but not affected by endophytes ($Z = -1.00$, $n = 25$, $P = 0.339$; Fig 7.1b). This reduction in biomass was associated with fewer leaves per plant; plant height was not significant affected by nematodes or endophytes (Table 7.1). Root biomass was reduced by nematodes ($Z = -2.43$, $n = 23$, $P \leq 0.005$; Fig 7.1a) but increased by endophytes ($Z = -2.597$, $n = 25$, $P \leq 0.01$; Fig 7.1b). The endophyte effect on root biomass was

strongest in the presence of nematodes as it caused a 32% increase of the average root biomass, while this was only 7% in the absence of nematodes (data not shown). Consequently, the average nematode damage to the root biomass was reduced from 42% in the absence of endophyte to only 18% in the presence of endophyte. However, this did not cause a significant increased shoot biomass as well. The total number of nematodes in the roots was variable and not significantly affected by the presence of endophyte ($Z = -1.54$, $n = 14$, $P = 0.124$), nor by the nematode species ($Z = -1.54$, $n = 14$, $P = 0.124$). Total numbers of nematodes per root system were similar between the two nematode species in the absence of endophyte ($Z = -0.169$, $n = 7$, $P = 0.866$; table 7.1). Plants inoculated with *A. strictum* and *P. penetrans* had on average nearly three times as much nematodes in the root system then plants inoculated with *A. strictum* and *P. dunensis*, but this was not statistically significant ($Z = -1.859$, $n = 7$, $P = 0.063$; Table 7.1). Plants inoculated with *P. penetrans* produced a significantly greater total biomass when inoculated with *A. strictum*; this was not the case for *P. dunensis* (Fig 7.3).

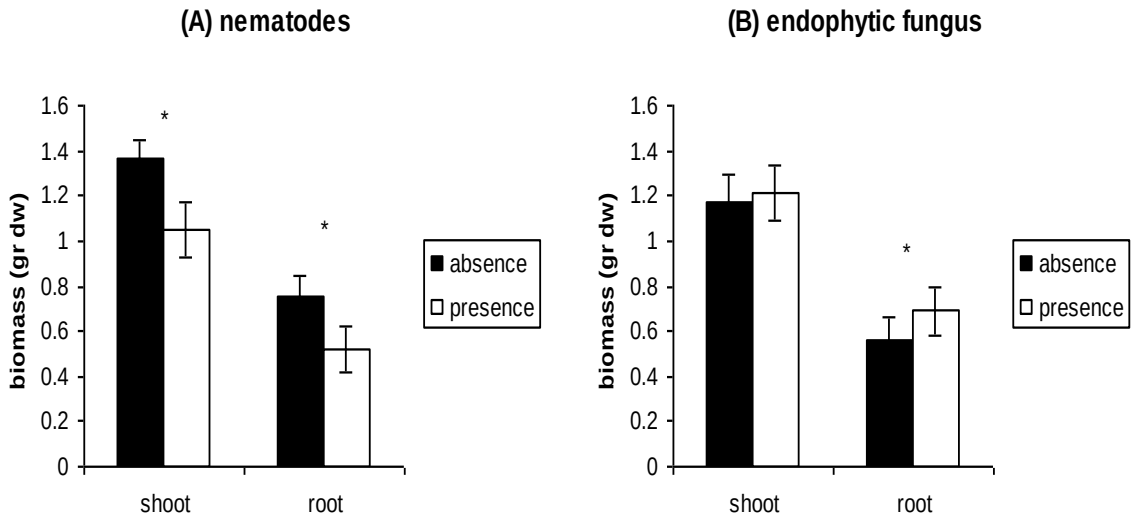


**Fig 7.1** *Ammophila arenaria* shoot and root dry biomass (g) after inoculation with *Acremonium strictum* and the nematode species *Pratylenchus penetrans* or *Pratylenchus dunensis*. A: combined data for the two nematode species B: endophytic fungus. An asterisk indicates a significant difference between two adjacent bars; based on Wilcoxon's signed Rank Test. Error bars indicate 95% confidence levels.

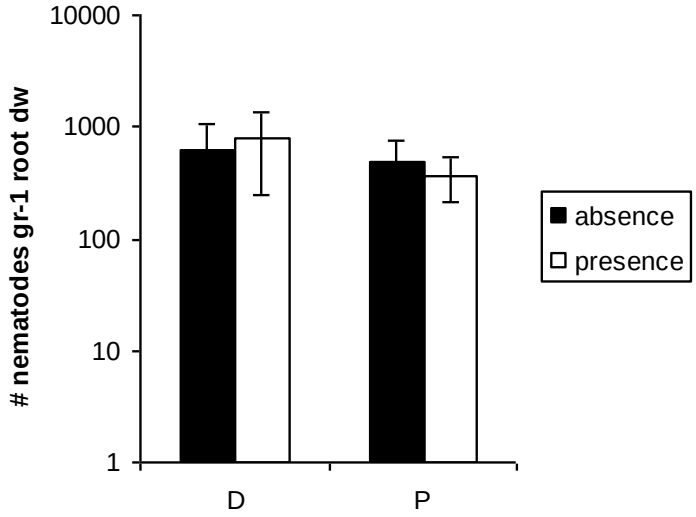


**Fig 7.2** Final number of nematodes (nematodes·g$^{-1}$ root) for *Pratylenchus penetrans* (P) and *P. dunensis* (D) after inoculation of *Ammophila arenaria* plants with *Acremonium strictum*. Error bars indicate 95% confidence levels.

**Table 7.1** The effect of the nematodes *Pratylenchus dunensis* and *Pratylenchus penetrans* on plant parameters of *Ammophila arenaria* plants inoculated at the stems with the endophyte *Acremonium strictum*. E- = no *A. strictum;* E+ = *A. strictum* inoculation. In a row, different letters indicate significant differences between treatments. Friedman test (n = 7, df = 5), followed by Wilcoxon tests for pairwise comparisons.

| Variable | Control (no nematodes) | | *Article I. . dunensis* | | *Article II. . penetrans* | | $X^2$ | *P* |
|---|---|---|---|---|---|---|---|---|
| | E- | E+ | E- | E+ | E- | E+ | | |
| Height (cm) | 86a | 96a | 92a | 82a | 84a | 79a | 8.93 | 0.115 |
| Leaves (N) | 10.3ab | 10.5a | 7bc | 6c | 7bc | 9ab | 13.199 | 0.022 |
| Tillers (N) | 2.8a | 2.8a | 1.7a | 1.3a | 2.3a | 2.4a | 10.156 | 0.071 |
| Nematodes (N in root system) | | | 171a | 165a | 159a | 446a | 5.229 | 0.156 |

**Table 7.2** The effect of the nematodes *Pratylenchus dunensis* and *Pratylenchus penetrans* (alone or in combination) on plant parameters of *Ammophila arenaria* plants inoculated at the roots with the endophyte *Acremonium strictum*. E- = no *A. strictum*; E+ = *A. strictum* inoculation. In a row, different letters indicate significant differences between treatments. Friedman test (n = 7, df = 5), followed by Wilcoxon tests for pairwise comparisons.

| Variable | Control (no nematodes) | | *P. dunensis* | | *P. penetrans* | | *P. dunensis* + *P. penetrans* | | $X^2$ | *P* |
|---|---|---|---|---|---|---|---|---|---|---|
| | E- | E+ | E- | E+ | E- | E+ | E- | E+ | | |
| Height (cm) | 38a | 41a | 36a | 35a | 37a | 39a | 37a | 36a | 5.389 | 0.613 |
| Nr. of leaves | 6a | 7a | 6a | 7a | 6a | 7a | 7a | 7a | 4.655 | 0.702 |
| Nr. of tillers | 2.4a | 2.9a | 2.5a | 2.9a | 2.4a | 3a | 2.8a | 2.8a | 5.012 | 0.659 |
| Nematodes ( in roots) | | | 2320a | 1738ab | 128a | 148a | 852b | 802b | 37.829 | 0.000 |

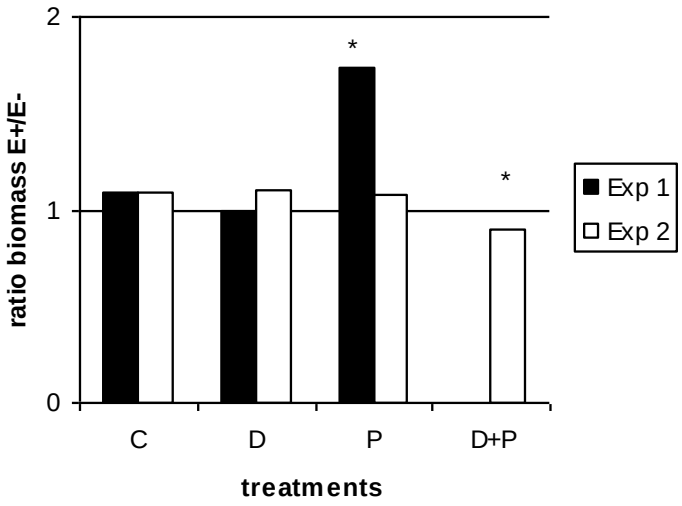


**Fig 7.3** Ratio obtained by dividing the total biomass of *Ammophila arenaria* plants inoculated with *Acremonium strictum* and the total biomass of endophyte-free plants inoculated with *Pratylenchus* spp. Exp 1 = *A. arenaria* stems inoculated with *A. strictum*, Exp 2 = *A. arenaria* roots inoculated with *A. strictum*, C = control; D = *Pratylenchus dunensis*; P = *Pratylenchus penetrans*; D+P = *P. dunensis* and *P. penetrans*. An asterisk indicates a significant difference between endophyte-infected plants and endophyte-free plants ($p<0.05$, t-test).

### 7.3.2 Experiment 2. Inoculation of *Ammophila arenaria* roots with *Acremonium strictum*

Neither shoot nor root biomasses were significantly affected by endophytes or nematodes (Fig 7.4a and 7.4b). Overall, plant parameters were different from those registered in the first experiment (Tables 7.1, 7.2). *Acremonium strictum* inoculation had a significant effect on plant parameters. Firstly, the moisture content of the shoot (difference between fresh and dry weight divided by fresh weight) of infected plants (62%) was less than that in endophytic-free plants (64%; GLM F = 8.461, *P* = 0.005, df = 1,68), regardless of the presence of nematodes. Secondly, the endophyte affected plant tillering; plants with endophyte had on average 2.9 tillers per pot, while endophyte-free plants had 2.6 tiller per pot (Z = -1.941, n = 40, *P* = 0.052; Table 7.2). The total number of root-lesion nematodes extracted from *A. arenaria* was significantly affected by the nematode species (Fig 7.5), but not by endophyte infection of the plant. In the absence of *A. strictum* the number of *Pratylenchus* in plants inoculated with a mixture of species deviated from the expected numbers calculated on basis of the individual species (expected = 0.5 * numbers in D + 0.5 * numbers in P). In endophyte-free plants, an average of 4301 nematodes·$g^{-1}$ root were expected, but only 2172 were found (paired t-test, n = 10, *P* = 0.051). In the presence of the

endophyte, the calculated expected number of nematodes per g root was not different from the observed number ($2659g^{-1}$: paired t-test, n = 10, $P > 0.10$). Compared to endophyte-free plants, plants inoculated simultaneously with both *P. dunensis* and *P. penetrans* were the only treatment with decreased total biomass in the presence of endophytic fungus (Fig 7.3).

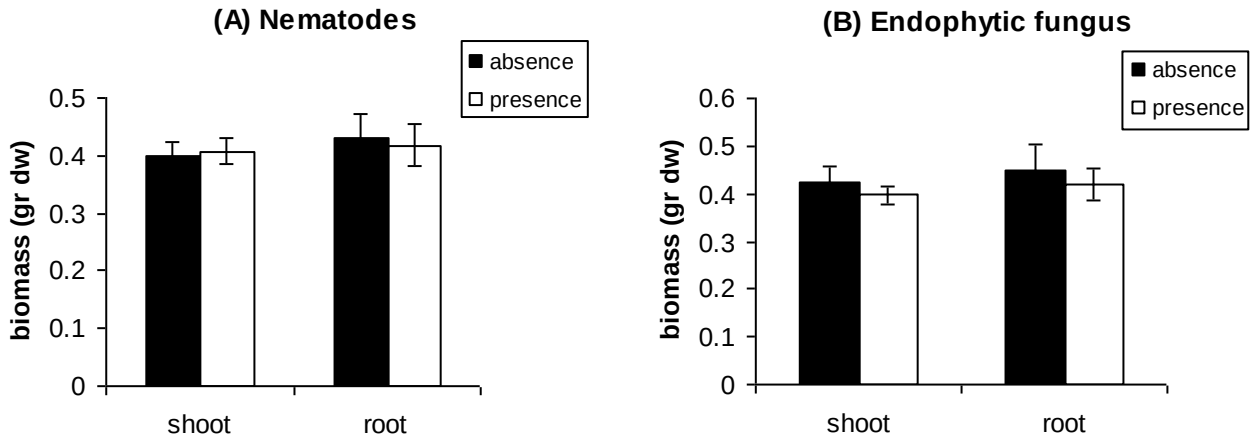


**Fig 7.4** *Ammophila arenaria* shoot and root dry biomass (g) after inoculation with *Acremonium strictum* and *Pratylenchus penetrans* or *Pratylenchus dunensis* A: combined data for the nematodes B: endophytic fungus. Error bars indicate 95% confidence levels.

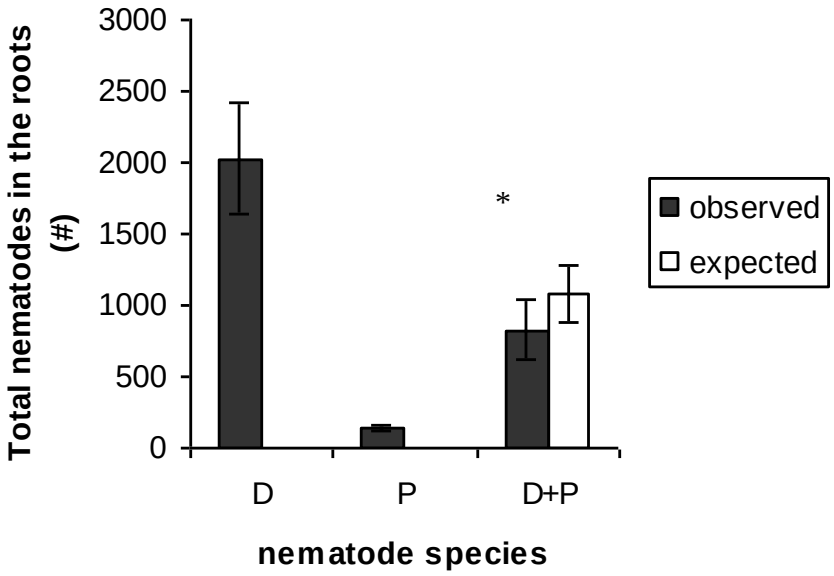


**Fig 7.5** Number of nematodes (mean ± SE) in the roots of *Ammophila arenaria* after inoculation with *Pratylenchus dunensis* (100 individuals; D), *Pratylenchus penetrans* (100 individuals; P), or *P. dunensis* (50 individuals) + *P. penetrans* (50 individuals) (D+P). The asterisk indicates a significant difference between the observed and expected numbers for the combined inoculation. Expected number is calculated as 0.5 * D + 0.5 * P.

## 7.4 Discussion

The results obtained in this study show for the first time that a native fungal endophyte, *Acremonium strictum*, isolated from *Ammophila arenaria,* had a positive effect on plant growth. They provide new insights in the ecology of this grass species in coastal dunes and also indicate new plausible interactions between *A. arenaria* and its rhizosphere organisms. The effect of fungal endophytes as protective agents against root herbivores has been acknowledged for different *Neotyphodium* species, in economically important grasses or in natural systems (Schardl *et al.* 2004); however, this is the first time that a positive effect on *A. arenaria* is demonstrated for *A. strictum*. Endophyte infection increased root biomass. This effect was also observed when root herbivores had been inoculated, thus reducing the negative effect of root-lesion nematodes. Endophyte infection also increased the number of tillers per plant. Endophyte-infected plants further showed a lower water content than endophyte-free plants and that was most likely the result of increased transpiration since the total water applied did not differ between endophyte and non-endophyte plants (data not shown). These data support the concept of a positive effect of the endophyte-plant symbiosis in the water balance of the plant (Hesse *et al.* 2005). The effect on the transpiration of *A. arenaria* might be of major importance since water availability is a limiting factor in the establishment, seedling survival and leaves' photosynthetic rate in several dune colonizing plant species (Gagne & Houle 2001; Alessio *et al.* 2004).

*Ammophila arenaria* is commonly used as a sand stabilizing species. The increased root growth of endophyte-infected plants, especially in the presence of nematodes, might present consequences for dune stabilization. Faster root growth means faster spread and thus more retention of sand, in combination with increased tillering allowing faster clonal spread. The enhancement of clonal properties of the host plant might have consequences in the competition ability against other dune grasses. Other endophytes (*Neotyphodium lolii*) also caused increased tillering in *Lolium perenne* (Ravel *et al.* 1997). The effect of *A. strictum* on seed production, which can be very different from the vegetative growth (Hesse *et al.* 2003), is unknown and therefore deserves further consideration. However, establishment through seed dispersal seems to be of minor importance for *A. arenaria* spread (Huiskes & Harper 1979).

The effects of both the endophyte and the root-lesion nematodes on plant growth varied between the experiments. This difference can be attributed to the completely different experimental conditions that most probably affect the outcome of the fungi-host plant symbiosis and interactions with herbivores (Bultman & Bell 2003). Nevertheless, common

patterns can be derived from the two experiments. A direct reduction of nematode multiplication by the endophyte was not found in both experiments. On the contrary, fungal endophytes might be beneficial for the nematodes due to the higher root biomass of endophyte-infected plants. Obviously, the increased root growth provides more resources for the herbivores. In the first experiment *P. penetrans* seemed to benefit from the endophyte infection, while this was certainly not the case for *P. dunensis*. In the second experiment, competition between *P. dunensis* and *P. penetrans* was relieved on the endophyte-infected plants, again indicating a positive effect on nematode multiplication. This bring me to reject the hypothesis that in *A. arenaria*, the endophytic fungus *A. strictum* contributes to the control of *Pratylenchus* spp. However, this does not automatically imply that endophytes would be completely unimportant for nematode control in *A. arenaria*. There is a large variability in nematode-endophyte interaction (Cook *et al.* 1991) and therefore, more nematode species with different parasitic strategies need to be tested before it can be concluded that *A. strictum* has no effect on nematode reproduction. Moreover, the fact that *Pratylenchus* spp. might take advantage of endophyte-infected plants would have implications for the subsequent colonization by other root-feeding species (Brinkman *et al.* 2005a).

*Ammophila arenaria* establishes interactions with endophytic fungi and mycorrhizal fungi. Previous studies indicated that endophytic fungi may inhibit or enhance mycorrhizal fungi activity (Muller 2003). Moreover, mycorrhizal fungi protect *A. arenaria* plants from root-lesion nematodes (Chapter 6) and the combined effect of the two fungal symbionts should be considered. Meanwhile, the positive effect of *A. strictum* on *A. arenaria* growth parameters and its wide distribution along European coasts justifies further research into the fungal endophytes of *A. arenaria*. Currently it is unknown how *A. strictum* is transmitted; it is possible that the fungal endophyte can survive as a saprophytic fungus in soil. *Acremonium* spp. were already found in the rhizosphere of *A. arenaria* (de Rooij-van der Goes 1995).

The effect of interspecific competition between different species of root-feeding nematodes in *A. arenaria* has recently been analyzed, pointing at *Pratylenchus* as a good competitor when compared to other sedentary endoparasitic nematodes (Brinkman *et al.* 2005b). However, the competition between two species within the same genus has not been explored until now. My results shows interspecific competition between *Pratylenchus* spp., a point deserve further consideration.

In the past decade, the dynamics and feed-back processes between soil-biota and the aboveground plant communities in coastal dunes have been extensively studied, either from a

conservational point of view or as a model for multi-trophic interactions in soil. My results point at *A. strictum* as a new component in this complex and therefore the positive or negative effects of fungal endophytes against mycorrhizal fungi and root-feeding nematodes needs to be further explored in order to get a more comprehensive view.

# Chapter 8

*General discussion*

*Ammophila arenaria* L. (Link), marram grass, is a sand-stabilizing species typical of coastal dunes in the Atlantic and Mediterranean coast line in Europe. A complex formed by plant-parasitic nematodes and soil fungi is involved in the degeneration of this species in stabilized dunes; in consequence, this complex contributes to the process of primary succession within the system. The aim of this study was to analyze the interaction between *A. arenaria* and migratory endoparasitic nematodes (*Pratylenchus* spp.) and to explore the bottom-up control of these nematodes by the host plant or mediated by its plant mutualists. Therefore, several aspects of these interactions have been addressed.

The diversity and distribution of the genus *Pratylenchus* was studied in relation to the natural distribution of *A. arenaria* in Western Europe (Chapter 4). Previous studies indicated that this genus is an important component of the rhizosphere of plants in coastal foredunes. *Pratylenchus brzeskii* was previously found in different localities along the North Sea and the Atlantic Ocean (Karssen *et al.* 2000). Earlier, *P. penetrans* was recognized as the causing agent of root necrosis and symptoms associated with the die out of *A. breviligulata* in North American coastal dunes (Seliskar & Huettel 1993). The results of the survey along the European coastline revealed a complex scenario in which four *Pratylenchus* species are associated with *A. arenaria*, *viz. P. dunensis*, a new described species, *P. brzeskii*, *P. pratensis* and *P. penetrans*. The sampling survey also revealed the incidence of two to three *Pratylenchus* species occurring concurrently at a sampling site. However, it was designed to be qualitative (detect absence/presence) rather than quantitative and in consequence, it was not possible to precise whether seasonal variation in the *Pratylenchus* species composition occurred within a sampling site; an aspect that deserves further consideration.

The survey was limited to the foredunes with vigorous *A. arenaria* stands. As reported in Chapter 2 other grass species precede, coexist with or replace *A. arenaria* in the natural succession that takes place in coastal dunes. Previous studies have further shown a shift in the phytoparasitic nematodes in relation to the dominant plant species at different stages of the succession. For example, at different sampling points along the North Sea, *Heterodera arenaria* was found associated with vigorous *A. arenaria* stands while *H. hordecalis* was only detected in association with degenerating *A. arenaria* and *Calamagrostis epigejos*, a species typical for later dune stages (Clapp *et al.* 2000). Also the root-knot species *Meloidogyne maritima* and *M. duytsi* have a different host preference; the first species is usually associated with *A. arenaria*, whilst the latter is frequently found with *Elymus farctus* (Karssen *et al.* 1998b). The data obtained in my study combined with the available literature

might point at a similar pattern for some of the *Pratylenchus* species detected. In the case of *Pratylenchus brzeskii* and *P. dunensis* both species are found in foredunes with *A. arenaria* and *E. farctus* roots while are less frequent at interior dune sites (Karssen *et al.* 2000). Alternatively, *Pratylenchus penetrans* was found in only one locality in foredunes with *A. arenaria*; whereas Zoon *et al* (1993) positioned this species in association with decaying stands of *A. arenaria* and *Hyppophäe rhamnoides* in inner dune stages.

The *Pratylenchus* species were found to be distributed differently over the sampling points. Whilst the subspecies *A. arenaria* ssp. *arenaria* harboured all four species detected, *P. penetrans* was absent on *A. arenaria* ssp. *arundinacea*. However, as mentioned earlier (Chapter 2) a geographical bias in the sampling scheme should be acknowledged because only one sampling spot was located in the Mediterranean Basin. As a consequence, to have an overall picture of the *Pratylenchus* distribution in Mediterranean dunes, nematode surveys should consider additional sampling points along the continental Mediterranean coastline, the Mediterranean archipelagos (e.g. Balearic Islands, Hellenic archipelago) and in the North African coast line.

The distribution of *P. dunensis* and *P. brzeskii* in West European coastal dunes is quite similar to that of other sedentary endoparasitic nematode genera. *Meloidogyne duytsi* and *M. maritima* have been found in coastal dunes of the British Isles and at different spots of the North Sea but also in Portugal (Karssen *et al.* 1998a; Karssen *et al.* 1998b; Schreck-Reis 2005); *H. arenaria* most likely shows a similar distribution (Maher 2004). However, although the distribution of ectoparasitic nematodes has not yet been studied deeply, there are some indications (e.g. for *Helicotylenchus* and *Tylenchorhynchus*) suggesting that the ectoparasitic species associated with *A. arenaria* in Northern Europe are different from those found at Southern latitudes (Schreck-Reis 2005). The change in species composition, when comparing endoparasitic *vs.* ectoparasitic nematodes, might be explained by the adaptation in different regions of ectoparasitic nematodes to differences in soil abiotic factors (Yeates 2003). In addition, the dispersal abilities of different nematode groups can be also account for the observed pattern in species composition. Populations of aquatic and beach plants may undergo shifts in location and density due to the destruction of a part or all of their populations (Johansson & Nilsson 1993). Both, *A. arenaria* and *A. breviligulata* suffer from rhizome fragmentation caused by the action of sea waves. As observed for other clonal species, root fragments of *A. arenaria*, can be spread by sea water and some buds of these fragments are able to germinate and produce new stands (Maun 1984). Therefore, *A. arenaria* fragments that are spread by the action of the waves are potential agents of dispersion of

endoparasitic nematodes. This group of nematodes could be transported inside root pieces remaining protected from the sea water while the direct exposure of ectoparasitic nematodes to the sea water would limit their spread.

*Pratylenchus* species were easily discriminated by their morphological characters and morphometrics (CDA). The molecular characterisation confirmed the separation; however, relationships between populations could not be inferred from the molecular data since only a very low intraspecific variation was observed for all the species. More powerful molecular tools such as AFLP, mtDNA sequences or microsatellites would make possible to analyse the genetic relationship between geographically separated populations of the same species and allow to infer colonization and spreading patterns eventually linked with the genetic structure of *A. arenaria* (Blouin 1998; Blouin 2003).

Preceding studies of the population dynamics of plant-parasitic nematodes in coastal dunes indicated that *Pratylenchus* species follow the root growth of *A. arenaria* through out the year and suggest their direct dependence on root availability for multiplication (Van der Stoel *et al.* 2002b). I was able to demonstrate that high initial densities of *P. penetrans, P. dunensis* and *P. brzeskii* are detrimental for *A. arenaria* growth and that the multiplication of the three species is density dependent As discussed on Chapter 5, these findings suggest a bottom-up control (by limitation of the amount of food resources) of *Pratylenchus* spp. This decrease of the nematode multiplication in response to the reduction of the availability of functional roots (the nematode damage decreases root size and increases nematode competition) is common for plant-parasitic nematodes (Trudgill 1991; Trudgill *et al.* 1996). Since damage producing densities of *Pratylenchus* are not detected in the foredunes it is unlikely that reduction in multiplication takes place due to root damage. Therefore, the bottom-up control should originate from the action of plant mutualists (Chapter 6 and Chapter 7) or by interspecific and intraspecific competition (Brinkman *et al.* 2005a).

Differential multiplication as a function of the host it is a general pattern in plant-parasitic nematodes (Trudgill 1991). Multiplication of plant-parasitic nematodes is influenced by the host plant physiology and the availability of food resources both in quantity and quality; it is clear that these factors change with the host. As demonstrated in Chapter 5, the multiplication of the three *Pratylenchus* species differs with the host. *Lolium perenne*, a frequent species in temperate grasslands, is a good host for *P. penetrans*, which is a very polyphagous species frequently found in temperate areas (Brzeskii 1998). However, rye grass is a bad host for the typical 'dune species' *P. dunensis* and *P. brzeskii*. The opposite situation

was observed for *Elymus farctus*, a pioneer plant that grows at the basis of foredunes. On this host, *P. penetrans* hardly multiplies. Finally *Ammophila arenaria* was a good host for the three species. Interestingly, *L. perenne* produced the highest root biomass of the three plant species compared and no differences were observed between *A. arenaria* and *E. farctus*, thereby the differences in nematode multiplication should be related with quality of food sources and not with the amount of resources. The density I used in the host suitability experiment did not hamper the growth of any of the hosts used. In order to verify whether different *Pratylenchus* species of foredunes may drive plant succession, density-growth experiments should be conducted not only with *A. arenaria* but also with other plant species preceding or replacing *A. arenaria* in the succession.

Multiplication of *Pratylenchus* spp. not only differs between host species, differences in reproduction are also observed between *A. arenaria* populations. This might have consequences when looking at the effect of plant parasitic nematodes in different geographical areas (i.e. when comparing the effect on *A. arenaria* populations from native areas with those on introduced) but also when comparing the effect of plant-parasitic nematodes on vigorous and decaying stands since foredune and inland *A. arenaria* populations seem to differ genetically (Gray 1985).

The tendency of herbivores to evolve in specialisation to one or a small range of plant species is probably a major reason for the enormous diversity of herbivorous traits. This general trend towards specialisation includes a range of evolutionary mechanisms (Ballabeni *et al.* 2003). Local adaptation or specialisation is one the main mechanisms. It takes place when a parasite, herbivore or pathogen, multiplies better in sympatric hosts than in allopatric (Gandon 1998; Dybdahl & Storfer 2003). The effect of different genotypes of *A. arenaria* was tested on different *Pratylenchus* populations. The results obtained put forward different scenarios in the relationship between sympatric and allopatric host-nematode pairs (*Pratylenchus* spp. and *A. arenaria)* and suggest local adaptation, local mal-adaptation or no differences according to the host. This complex scenario indicates idiosyncratic effects in the combinations between *Pratylenchus* spp. and *A. arenaria* populations. Previous studies have shown idiosyncratic responses to soil fauna in relation to plant identity (De Deyn *et al.* 2004; Brinkman *et al.* 2005a) and between nematodes from same functional groups within nematode communities in relation to litter decomposition processes in soil (Porazinska *et al.* 2003).

When comparing *Pratylenchus* multiplication from both temperate and Mediterranean climates, I did not take into account the effect of temperature on nematode multiplication. The experiment was done in a glasshouse with a single temperature regime for all populations. Temperature is an important factor for the development and multiplication of nematodes (Trudgill & Perry 1994; Trudgill *et al.* 2005). In order to ensure that differences in interactions are strictly due to the *A. arenaria* origin (and not caused by the difference in temperature between the origin of the population and that during the experiment) further experiments in climate chambers at different temperatures should be considered.

Arbuscular mycorrhizal fungi (AMF) are well known plant mutualists that not only contribute in the plant nutrient uptake, but also can act as a protective agent against nematode infection and multiplication (Elsen *et al.* 2003c; Hol & Cook 2005). In coastal dunes, the potential benefit of AMF against root herbivores has been acknowledged and tested for some dune plant species(Little & Maun 1996; Greipsson & El-Mayas 2000). The experiments reported on in Chapter 6 show that native AMF from coastal dunes can reduce infection and multiplication of *Pratylenchus* populations. However, several aspects of the relationship between *A. arenaria, Pratylenchus* spp. and AMF remain unclear. In my experiments I used only *P. penetrans* and entire AMF communities from different localities. To have a broader view on how AMF affect *Pratylenchus* spp., single AMF species should be examined. AMF are not always beneficial to the plant; different AMF species might affect in a different way the host plant and, as a consequence, its associated nematodes (Elsen *et al.* 2003a; Van der Heijden *et al.* 2003). Also as mentioned repeatedly in this thesis, the rhizosphere of *A. arenaria* is occupied by different plant-parasitic nematode genera. The effect of AMF on those nematodes might be completely different from that observed for *P. penetrans.*

Differences in AMF communities were found associated with vigorous or decaying stands of *A. arenaria* (Kowalchuk *et al.* 2002). Several questions might appear from this observation. As pointed out by Borowicz (2006), nematodes might determine shifts in AMF communities along the plant succession or inversely, shifts in AMF communities may enhance or mitigate the effects of nematodes. Both possibilities remain to be analysed in the future. I could demonstrate that *P. penetrans* is controlled by AMF. However, the question whether other *Pratylenchus* species or other plant-parasitic genera that occur in coastal dunes respond in the same way remains open.

The success of *A. arenaria* outside its natural range can be explained partially by its release from its natural enemies (e.g. plant-parasitic nematodes) (Chapter 2). Therefore, the

effect of different AMF and their interaction with plant-parasitic nematodes must be taken into account to understand the invasion of not only *A. arenaria* but also of other dune species.

The interaction between *A. arenaria*, *Pratylenchus* spp. and the fungal endophyte *Acremonium strictum* did not show a reduction of the multiplication of any of the *Pratylenchus* species. Other fungal endophytes occur in association with *A. arenaria*; their potential effect on the multiplication of *Pratylenchus* spp. cannot be completely excluded (White *et al.* 1992). Fungal endophytes might affect the secondary metabolism of the host plants and produce different alkaloids in the host plant (Wilkinson *et al.* 2000; Blankenship *et al.* 2001). These alkaloids affect the palatability of the plant for different herbivores and in consequence protect the plant against these groups. However the production of alkaloids depends directly of other environmental conditions (Bultman & Bell 2003). In the shoots of *Ammophila* spp. the presence of different alkaloids has been detected (White *et al.* 1992; Gilbert 2002). Whether *A. strictum* is able to produce and in which conditions need to be addressed.

Further considerations

The effect of plant-parasitic nematodes on natural systems has until relatively recently not been considered in detail. However, besides a better view on the functioning of such ecosystems, this type of research also provides information on interactions of its components that can be applied in practical situations. In the EU Habitat Directive (92/43/EEC) coastal dunes are included as preferential conservation areas due to the singular characteristics of the flora, the high degree of erosion produced by humans and the natural risks associated with habitat loss. Some of the results obtained on this thesis might show new approaches for coastal dune management and preservation. *Ammophila arenaria* is planted every year along hundreds of kilometres of the North Sea and the Atlantic Coast. Inoculation of replants of *A. arenaria* with beneficial AMF (and eventually with fungal endophytes) might stimulate the early establishment and perdurability of those revegetation events.

This thesis has been developed within the framework of the EU research project, EcoTrain (HPRN-CT 200200210) "Ecology of plant parasitic nematodes, their host plants and antagonists in European coastal sand dunes: Training opportunity for ecologists and agricultural biocontrol researchers". One of principal aims of this project was stimulate

biological control of plant-parasitic nematodes in agriculture by learning how this works in nature.

Current research in (applied) plant nematology involves different research lines such as the development of correct nematode identification techniques; the assessment of density-yield ratios for different species-crops combinations and the development of new strategies to control nematode pests by the use of chemicals, cultural practices, rotation and the use of resistant cultivars and biocontrol agents.

With respect to the latter area of research, nematologists tend to develop tactics beyond the bullet approach of nematode control in which nematodes are treated locally in favour of a management system in which the impact of nematodes is reduced in time and in space. One may ask the question of whether a more comprehensive approach treating the agricultural field as an ecosystem might not be an interesting path to explore. In this multitrophic approach the underlying principle is that components of agricultural ecosystems interact and through a set of feedback mechanisms maintain equilibrium. This kind of approach is not innovative in an ecological background but it is scarcely implemented in crops. The basis of pest (and nematode) management in agricultural systems should take into account plant defence, plant mixtures, soil, natural enemies and other components of the system.

Consideration of the crop as a component of an ecological system where multitrophic interactions occur is crucial for pest management. It will be recognised that multitrophic level interactions (plant, herbivores, antagonists, mutualists) are very complicated processes to study experimentally but in order to have better idea of how processes occur, natural systems, as shown in this thesis, provide a good system to test this kind of interactions without the urge an necessity of astonishing results directly applicable, but on mid-long term might provide knowledge and mechanisms suitable for pest management.

## Summary

Plant-parasitic nematodes can affect plant performance and the composition of natural plant communities, but there is little information about the mechanisms that control this group of organisms in natural ecosystems. *Ammophila arenaria* L. (Link), marram grass, is a sand-stabilizing species typical of coastal dunes in the Atlantic and Mediterranean coast line in Europe. A complex formed by plant-parasitic nematodes and fungi is involved in the degeneration of this species. The aim of this study was to analyze the interaction between *A. arenaria* and migratory endoparasitic nematodes (*Pratylenchus* spp.) and to explore the bottom-up control of these nematodes by the host plant or mediated by its plant mutualists.

A survey was performed along the European Atlantic and Mediterranean coastline. From the localities sampled in the survey, 19 *Pratylenchus* populations were detected. Eighteen of these populations were identified based on their morphology and morphometrics; 19 populations were molecularly characterised (rDNA D2D3 sequences). Six populations belonged to an undescribed *Pratylenchus* species, seven to *P. brzeskii* and five populations were identified as *P. pratensis*. One population was identified as *P. penetrans*. The type population of *Pratylenchus dunensis*, the new described species, was isolated from *A. arenaria* in Groote Keeten, the Netherlands. It is characterised by medium sized (454-579 µm) vermiform and slender females and males, with two lip annuli (sometimes 3-4; incomplete incisures only visible with scanning electron microscopy), medium to robust stylet (*ca* 16 µm), with robust stylet knobs slightly set off and long pharyngeal glands (*ca* 42 µm). The lateral field is composed of four parallel non-equidistant lateral lines, in which the middle ridge is narrower than outer ones; lateral lines present partial areolation and converge at tail after tail phasmid, which is located between the two inner lines of the lateral field in the posterior half of the tail. The round spermatheca are filled with round sperm, the vulva is at 78% of total body length with protruding vulval lips, the posterior uterine sac is relatively short (*ca* 19 µm). The cylindrical tail (*ca* 33 µm) is narrowing in the posterior third with smooth tail tip and presenting conspicuous hyaline part (*ca* 2 µm). Males occur abundantly and present similar characteristics, but have smaller dimensions for all morphological characters. The head region is more truncated in outline than the female one, the spicule length is *ca* 15 µm and testis length is *ca* 195 µm. Nucleotide sequence comparisons of the rDNA expansion region D2D3 allowed clear discrimination between the four species detected in the roots of *A. arenaria* and between other *Pratylenchus* species isolated from

other hosts and regions. *Pratylenchus dunensis* can be separated from *P. penetrans* and *P. brzeskii* by PCR-RFLPs patterns of the ITS-rDNA.

The four *Pratylenchus* species were distributed differently. *Pratylenchus dunensis* was found along the North Sea and the Atlantic coast. However, this species was not detected in the Mediterranean Basin. A similar distribution was observed for *P. pratensis*. *Pratylenchus brzeskii* was found along both, the Atlantic and the Mediterranean coastline. *Pratylenchus penetrans* was only found at one sampling point in the Atlantic coast. All four *Pratylenchus* species were detected on *A. arenaria* ssp. *arenaria* whereas *P. penetrans* was not isolated from *A. arenaria* ssp. *arundinacea*.

The effect of *P. dunensis, P. brzeskii* and *P. penetrans* on the growth of *A. arenaria* was studied in pot experiments in the glasshouse. For each nematode species 3000, 1500, 750, 375, 190, 90 or 45 nematodes were inoculated per pot to obtain seven nematode densities (*viz.* 5, 2.5, 1.2, 0.6, 0.3, 0.15 and 0.075 nematodes·$g^{-1}$soil). After 16 weeks the plants were uprooted and the plant growth parameters (total, aboveground and belowground biomass) measured and the number of nematodes in plant and soil estimated. The relationship between plant growth (biomass) and initial nematode densities fitted partially the Seinhorst equation, and pointed at differences in the tolerance level for each of the species compared; for *P. dunensis* and *P. penetrans* the tolerance level was approximately 0.5 nematodes·$g^{-1}$soil, *P. brzeskii* showed a higher value one nematode·$g^{-1}$soil. The relationship between the initial nematode densities and the final nematode density was fitted to an exponential decay model which indicated that the multiplication of the three species was density dependent and therefore determined by the quantity of root available and the intraspecific competition at high initial densities. The highest multiplication (i.e. multiplication obtained at the lowest initial density) was different for each of the species. *Pratylenchus dunensis* showed the highest multiplication, followed by *P. penetrans* and *P. brzeskii*. At the highest initial density *P. penetrans* reached the equilibrium density, whereas *P. dunensis* and *P. brzeskii* did not.

The effect of differences in host species on *P. dunensis, P. brzeskii* and *P. penetrans* multiplication was studied using a cross inoculation experiment in which seedlings of *A. arenaria, Elymus farctus* or *Lolium perenne* growing in 500 ml pots were inoculated with 150 nematodes of each *Pratylenchus* species. The experiment lasted for 12 weeks and at harvest plant growth and nematode numbers were estimated. The multiplication of the three nematode species depended on the identity of the host species. *Lolium perenne* was a good host for *P. penetrans* but not for the other two species. In *Elymus farctus*, multiplication of *P. penetrans* was almost absent, whereas *P. brzeskii* and *P. dunensis* multiplied equally well.

*Ammophila arenaria* was a good host for the three species. On this host *P. dunensis* showed the highest multiplication, *P. penetrans* the lowest and the multiplication of *P. brzeskii* did not defer from either of the other species. The effect of different *A. arenaria* genotypes (origins) on the multiplication of *Pratylenchus* spp. was also studied in a cross inoculation experiment in which seedlings of four different *A. arenaria* populations were inoculated separately with 150 *Pratylenchus* of different populations. After 12 weeks the plant growth and the number of nematodes in roots was estimated. Differences were observed according to the *Pratylenchus* identity and the origin of the *A. arenaria* population. Local adaptation nor maldaptation could be concluded. Therefore, the relationship between *A. arenaria* and *Pratylenchus* spp. seems to be idiosyncratic (depends on the identity of both organisms).

The interactions between *A. arenaria*, arbuscular mycorrhizal fungi (AMF) and *P. penetrans* was studied to determine whether AMF can suppress both nematode infection and reproduction and to explore the mechanisms of nematode control by AMF. A sequential inoculation experiment in which nematodes and AMF were inoculated at different times and a split-root experiment in which nematodes and AMF were inoculated separately or in combination were designed to analyse the importance of plant tolerance and resistance and of direct competition between AMF and *P. penetrans* for the nematode and the plant. After 14 weeks the experiment was harvested plant growth, AMF infection and nematode infection and multiplication estimated. Root infection and multiplication of *P. penetrans* were significantly reduced by the native inoculum of AMF. Plant pre-inoculation with AMF further decreased nematode colonization and reproduction. Nematode suppression by AMF did not occur through a systemic plant response but through local mechanisms. These results suggest that AMF are crucial for the control of root-feeding nematodes in natural systems and illustrate the mechanisms that are involved in this process.

The effect of the endophytic fungus, *Acremonium strictum* complex, on plant-growth related parameters of *A. arenaria* and its potential as protective agent against *P. dunensis* and *P. penetrans* was investigated in two inoculation experiments under different conditions. Endophyte-inoculated plants showed increased plant development in terms of root biomass and number of tillers and suffered less damage from the nematodes than the endophyte-free plants. In neither experiment did the endophyte reduce multiplication of the nematodes. On the contrary, endophyte-inoculated plants seemed to increase nematode multiplication. In terms of total biomass, plants infected with *P. penetrans* benefited more from the endophytic fungus than those with *P. dunensis*. The effect of the endophyte on interspecific competition was also analysed by plant inoculation with both nematode species. In endophyte-free plants

with mixed inoculum (nematodes and fungal endophyte), the total number of nematodes, with respect to numbers observed in one-species inoculation, was less than expected, suggesting that interspecific competition took place. Plants inoculated with *P. dunensis*, *P. penetrans* and endophyte showed decreased total biomass compared to endophyte-free plants inoculated with the same nematodes.

## Samenvatting

Plant-parasitische nematoden kunnen de prestaties van planten en de samenstelling van natuurlijke plantengemeenschappen beïnvloeden. Er is echter weinig informatie over de mechanismen die deze groep organismen in natuurlijke ecosystemen beheersen. *Ammophila arenaria* L. (Link), helmgras, is een zandstabiliserende soort, typisch voor kustduinen aan de Atlantische en Middellandse kust in Europa. Een complex van plantenparasiterende nematoden en schimmels is verantwoordelijk voor de degeneratie van deze soort. Het doel van deze studie was de interactie tussen *A. arenaria* en migrerende endoparasiterende nematoden (*Pratylenchus* spp.) te analyseren en om de bottom-up controle van deze nematoden door de waardplant of door interventie van plantmutualisten te onderzoeken.

Duinen langs Europese Atlantische en Middellandse kustlijn werden op verschillende plaatsen bemonsterd. In de onderzochte plaatsen werden 19 *Pratylenchus* populaties gevonden. Achttien van deze populaties werden geïdentificeerd op basis van hun morfologie en morfometrie; 19 populaties werden moleculair gekarakteriseerd (rDNA D2D3 sequenties). Zes populaties behoorden tot een onbeschreven soort van het geslacht *Pratylenchus*, zeven tot *P. brzeskii* en vijf populaties werden herkend als *P. pratensis*. Eén populatie werd geïdentificeerd als *P. penetrans*. De typepopulatie van *Pratylenchus dunensis*, de nieuw beschreven soort, werd geïsoleerd van *A. arenaria* in Groote Keeten, Nederland. Ze wordt gekenmerkt door middelgrote (454-579 µm) wormvormige en slanke wijfjes en mannetjes, beide met twee lipannuli (soms 3-4; onvolledige insnijdingen alleen zichtbaar met scanning electron microscopie), een middelgrote tot robuuste stekel (*ca* 16 µm), met robuuste, lichtjes omhoogstekende stekelknoppen en lange speekselklieren (*ca* 42 µm). Het laterale veld bestaat uit vier parallelle, niet op gelijke afstand gelegen laterale lijnen, waarvan de middelste kam smaller is dan de buitenste; laterale lijnen tonen gedeeltelijke areolatie en komen samen in de staart achter de fasmide, die tussen de twee binnenste lijnen van de laterale kant in het achterste deel van de staart ligt. De ronde spermatheek is gevuld met rond sperma. De vulva is gesitueerd op 78% van de totale lichaamslengte met uitstekende schaamlippen; de achterste uterinezak is tamelijk kort (*ca* 19 µm). De cylindrische staart (*ca* 33 µm) wordt smaller in het achterste gedeelte met een gladde staartpunt en heeft een opmerkelijk hyalien deel (*ca* 2 µm). Mannetjes komen veelvuldig voor en vertonen dezelfde kenmerken, maar hebben kleinere afmetingen voor alle morfologische kenmerken. De mannelijke kopzone is meer afgeknot in omtrek dan de vrouwelijke, de lengte van de spicula is *ca* 15 µm en de lengte van de testis is *ca* 195 µm.

Nucleotide sequentievergelijkingen van de rDNA expansiezone D2D3 maakte het mogelijk de vier soorten die in de wortels van *A. arenaria* gedetecteerd werden, te onderscheiden van andere *Pratylenchus* soorten, geïsoleerd van andere gastplanten en regio's. *Pratylenchus dunensis* kan onderscheiden worden van *P. penetrans* en *P. brzeskii* door PCR-RFLPs patronen van de ITS-rDNA. De vier *Pratylenchus* soorten waren op verschillende wijze verspreid in de duinen. *Pratylenchus dunensis* werd langs de Noordzee en de Atlantische kust gevonden, maar niet in het Middellandse Zeegebied. Eenzelfde verspreiding werd genoteerd voor *P. pratensis*. *Pratylenchus brzeskii* werd zowel langs de Atlantische als de Middellandse kustlijn gevonden. *Pratylenchus penetrans* werd maar op één onderzochte plaats aan de Atlantische kust gevonden. Alle vier de *Pratylenchus* soorten werden gedetecteerd op *A. arenaria* spp. *arenaria*, terwijl *P. penetrans* niet werd geïsoleerd van *A. arenaria* ssp. *arundinacea*.

Het effect van *P. dunensis, P. brzeskii en P. penetrans* op de groei van *A. arenaria* werd bestudeerd in potexperimenten in de kas. De zaailingen gekweekt in 500 ml potten gevuld met duinzand, werden geïnoculeerd met steeds hogere hoeveelheden nematoden om acht verschillende dichtheden te verkrijgen. Dit experiment duurde in totaal 16 weken. Aan het einde van die periode werden de planten opgebroken en de parameters voor plantengroei (totale, bovengrondse en ondergrondse biomassa) gemeten. Terzelfder tijd werd de nematodenpopulatie in de wortels en het zand geextraheerd en geteld. De relatie tussen plantengroei (biomassa) en geïnoculeerde dichtheden kwam gedeeltelijk overeen met de Seinhorst (1965) vergelijking en wees op verschillen in tolerantieniveau voor elk van de vergeleken soorten; voor *P. dunensis* en *P. penetrans* bedroeg het tolerantieniveau ongeveer 0.5 nematoden $\cdot g^{-1}$ grond, *P. brzeskii* vertoonde een hogere waarde (1 nematoden $\cdot g^{-1}$ grond). De relatie tussen de initiële nematodendichtheden en de dichtheid geregistreerd op het einde van de groei kwam overeen met het Ferris model (1985), dat aantoonde dat de vermenigvuldiging van de drie soorten afhing van de dichtheid en bijgevolg bepaald werd door de kwantiteit van de beschikbare wortels en de intrasoortelijke competitie bij hoge initiële dichtheden. De hoogste vermenigvuldiging (d.w.z. de vermenigvuldiging verkregen bij de laagste initiële dichtheid) was verschillend voor elke soort. *Pratylenchus dunensis* vertoonde de hoogste vermenigvuldiging, gevolgd door *P. penetrans* en *P. brzeskii*. Bij de hoogste initiële dichtheid bereikte *P. penetrans* de evenwichtsdichtheid, maar dat was niet zo bij *P. dunensis* en *P. brzeskii*. Het effect van verschillen in gastsoorten op de vermenigvuldiging van *P. dunensis, P. brzeskii en P. penetrans* werd bestudeerd door middel van een kruisinoculatie-experiment waarbij zaailingen van *A. arenaria, Elymus farctus* of

*Lolium perenne*, groeiend in 500 ml potten, werden geïnoculeerd met 150 individuen van elke *Pratylenchus*soort. Het experiment duurde 12 weken. De vermenigvuldiging van de drie soorten nematoden hing af van de identiteit van de gastsoort. *Lolium perenne* was een goede gastheer voor *P. penetrans* maar niet voor de twee andere soorten. In *E. farctus* was er geen vermenigvuldiging van *P. penetrans*, terwijl *P. brzeskii* en *P. dunensis* zich beide even goed vermenigvuldigden. *Ammophila arenaria* was een goede waardplant voor alle drie de soorten: *P. dunensis* vertoonde de hoogste vermenigvuldiging, *P. penetrans* de laagste; de vermenigvuldiging van *P. brzeskii* week niet af van de andere soorten. Het effect van verschillende *A. arenaria* genotypes (origines) op de vermenigvuldiging van *Pratylenchus* spp. werd ook bestudeerd in een kruisinoculatie-experiment. Er werden verschillen genoteerd naargelang de *Pratylenchus*identiteit en de origine van de *A. arenaria* populatie. Goede noch slechte lokale aanpassing kon worden geobserveerd. Bijgevolg lijkt de relatie tussen *A. arenaria* en *Pratylenchus* spp. idiosyncratisch te zijn, afhankelijk van de identiteit van beide organismen.

De interacties tussen *A. arenaria*, arbusculaire mycorrhiza schimmels (AMS) en *P. penetrans* werden bestudeerd om na te gaan of AMS zowel nematodeninfectie als –reproductie kan onderdrukken en om de mechanismen van nematodencontrole door AMS te onderzoeken. Een sequentieel inoculatie-experiment en een splitwortel-experiment werden ondernomen om het belang te analyseren van planttolerantie en –resistentie en van directe competitie tussen AMS en *P. penetrans* voor de nematode en voor de plant. Wortelinfectie en vermenigvuldiging van *P. penetrans* werden significant verminderd door de natuurlijke inoculatiestof van AMS. Pre-inoculatie van de plant met AMS deed de kolonisatie en reproductie van nematoden verder afnemen. De onderdrukking van nematoden door AMS gebeurde niet door een systemische plantreactie maar door lokale mechanismen. Deze resultaten suggereren dat in natuurlijke systemen AMS cruciaal zijn voor de beheersing van wortelparasiterende nematoden en ze illustreren de mechanismen die bij dit proces betrokken zijn.

Het effect van het endofytische schimmelcomplex *Acremonium strictum* op parameters verbonden met de groei van *A. arenaria* en zijn potentieel als beschermende agent tegen *P. dunensis* en *P. penetrans* werd onderzocht in twee inoculatie-experimenten onder verschillende condities. Planten geïnoculeerd met de endofytische schimmel vertoonden een verhoogde ontwikkeling van de wortelbiomassa en het aantal uitlopers en leden minder schade door de nematoden dan endofyt-vrije planten. In geen enkel van de experimenten reduceerde het endofyt de vermenigvuldiging van nematoden. In tegendeel, planten

geïnoculeerd met endofytische schimmel leken de vermenigvuldiging van nematoden te verhogen. Wat betreft de totale biomassa haalden planten geïnfecteerd met *P. penetrans* meer voordeel uit de endofytische schimmel dan die met *P. dunensis*. Het effect van de endofyt op intersoortelijke nematodencompetitie werd geanalyseerd door inoculatie met beide soorten nematoden. In endofyt-vrije planten met gemengde inoculatie (nematoden en endofyt) was het totale aantal nematoden kleiner dan verwacht wat betreft aantallen geobserveerd bij inoculatie van één soort, wat suggereert dat intersoortelijke competitie plaatsvond. Vergeleken met endofyt-vrije planten geïnoculeerd met dezelfde nematoden, vertoonden planten geïnoculeerd met *P. dunensis, P. penetrans* en endofyt een verminderde totale biomassa.

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