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Studies on the biological and molecular variation among seven isolates of *Pratylenchus penetrans* from different geographical locations in Europe

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submitted by

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Dedicated to

MY MOTHER

Abebech Gebre

‘እናቴ! ኣበልዬ ! እምዬ !
የዘጠኝ ወር ጎጆ ምክኒያት መግኖዬ
የአፍ ቻንቻ መፍቻ እማ እማምዬ
እሹሩሩ ዜማ ቃናዬ
በጉያሽ ሙቀት ሽልብታዬ
እባክሽን ኣትርገፊ ! ኣብቢልኝ አበባዬ’

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Abstract

The root lesion nematode *Pratylenchus penetrans* is known to damage major crops of economic importance. For the development of environmentally friendly control practices, a good understanding of the biological and genetic diversity of this species is required. This study aimed to characterize the diversity among seven isolates of *P. penetrans* collected from different geographical regions based on morphometric and genetic characteristics, their level of reproduction and their pathogenicity on selected hosts. To establish pure cultures of *P. penetrans* different methods were compared and optimized. Best method in terms of establishment and maintenance of *P. penetrans* isolates was monoxenic culturing on carrot discs. The identities of *P. penetrans* isolates were confirmed by morphological and molecular analysis. To characterize the host status of cover crops, reproduction fitness and pathogenicity of the seven *P. penetrans* isolates, a standard test system was developed. The host status of the tested cover crops ranged from non-hosts (ribwort plantain) through maintenance hosts (daikon radish, rocket and black oat cultivars Panche and Silke) to excellent hosts (lentil and common vetch). The reproductive fitness of the seven *P. penetrans* isolates was tested *in vitro* on carrot discs and *in vivo* on lentil and common vetch. Pathogenicity of those *P. penetrans* isolates was studied on alfalfa, carrot, fodder radish and French marigold. Differences in reproductive fitness and pathogenicity were observed among *P. penetrans* isolates. In general, reproductive fitness correlated positively with plant damage, i.e. the higher the reproduction, the higher the damage. Morphometrics of *P. penetrans* isolates were within the range of the original descriptions, but showed differences in some features to populations reported from other countries, such as body length, a, b and c ratio, maximum body width, tail length and length of the post-vulval uterine sac. According to RAPD analysis, the seven *P. penetrans* isolates showed a high level of intraspecific genetic variability. Furthermore, genetic similarities between some *P. penetrans* isolates were reflected in similarities in reproductive fitness, pathogenicity and morphometrics. For example, isolates MN and UK were genetically and morphological similar and showed similar reproductive fitness and pathogenicity. In contrast, isolates BL and MN showing the largest genetic and morphological differences also showed the largest differences in reproductive fitness and pathogenicity.

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Chapter I

General introduction

1 Plant-parasitic nematodes of economic importance

Plant-parasitic nematodes (PPNs) are recognized as one of the greatest challenges to crop production throughout the world (Nicol *et al*., 2011). Global crop losses to PPNs are estimated to be over $159 billion annually (Burke *et al*., 2015). To date, over 4100 species of plant-parasitic nematodes have been described (Decraemer and Hunt, 2013). In a global survey conducted in 2012 Jones *et al*. (2013) listed the top ten damaging species. These included root-knot nematodes (RKNs) (*Meloidogyne* spp.), cyst nematodes (*Heterodera* and *Globodera* spp.), *Pratylenchus* spp., *Radopholus similis, Ditylenchus dipsaci, Bursaphelenchus xylophilus, Rotylenchulus reniformis, Xiphinema index, Nacobbus aberrans*, and *Aphelenchoides besseyi*. Among the PPNs, root-lesion nematodes of the genus *Pratylenchus* are considered one of the most economically important taxa worldwide. In terms of host range and distribution, they rank third to RKNs and cyst nematodes (Castillo and Vovlas, 2007). A survey conducted to evaluate the occurrence and economic importance of PPNs in organic farming in Germany showed *Pratylenchus* to be the most commonly detected species on vegetable and cereals with an incidence of over 90% (Hallmann *et al*., 2007).

Root-lesion nematodes are migratory endo-parasites that enter and migrate within the root and feed on various root tissues, resulting in necrotic lesions on the root surface (Townshend and Stobbs, 1981). The genus *Pratylenchus* comprises 97 valid species with worldwide distribution (Handoo *et al*., 2008). However, only a few species are responsible for significant yield and quality losses in many agronomic and horticultural crops. *Pratylenchus penetrans* is among these species considered to be of major economic importance (Castillo and Vovlas, 2007).

2 *Pratylenchus penetrans* (Cobb, 1917) Filipjev and Shuurmans Stekhoven, 1941

Pratylenchus penetrans belongs to the family Pratylenchidae, which belongs to the order Tylenchida and class Secernentea (Siddiqui, 2000). Information on differential diagnosis of *P. penetrans* has been published by several authors (Loof, 1960; Román and Hirschmann, 1969; Corbett, 1973; Handoo and Golden; 1989; Loof, 1991; Hernández *et al*., 2001; Ryss, 2002; Hunt *et al*., 2005; Castillo and Vovlas, 2007).

2.1 Economic importance, distribution and host range

Pratylenchus penetrans has a cosmopolitan distribution throughout the temperate regions, and is an important pathogen of a wide range of crops of economic importance (Corbett, 1973; Mai *et al*.**,** 1977; Loof, 1991; Castillo and Vovlas, 2007). *Pratylenchus penetrans* has been recorded on nearly 400 hosts mainly in temperate areas in Europe, America, Africa, Asia and Australia (Figure 1), (Corbett, 1973; Castillo and Vovlas, 2007).

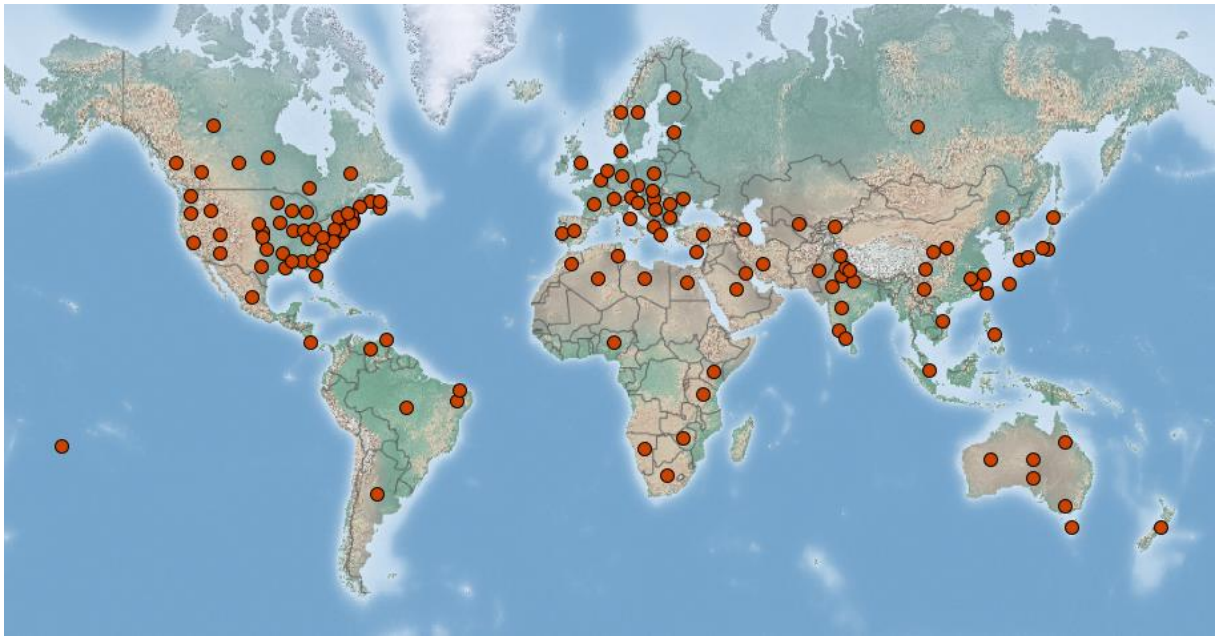

Figure 1: Geographical distribution of *Pratylenchus penetrans*. ● Present (CABI (2018)

Pratylenchus penetrans has been responsible for heavy losses in yield and quality of many crops including vegetables such as carrot, cauliflower, cabbage, onions and celery (Olthof and Potter, 1973; Vrain and Belair, 1981; Holgado *et al*., 2009; Teklu *et al*., 2016), but also of tobacco (Olthof, 1968), fruits (e.g. red raspberries, strawberries, peach and apple) (Mountain and Patrick, 1959; Pitcher *et al*., 1960; Townshend, 1963b; Potter *et al*., 1984; Zasada *et al*., 2015), maize (Dickerson *et al*., 1964; Olthof and Potter, 1973) and forage legumes (Willis and Thompson, 1969; Thies *et al*., 1995).

2.2 Biology and life cycle

Pratylenchus penetrans reproduces sexually (Thistlethwayte, 1970). All nematode stages can infest the plant. Infested plant organs include roots, tubers and rhizomes. The nematode migrates through the cortical tissue, where it feeds and causes extensive necrosis and destruction of root tissues. Once the root tissue is depleted or dead, nematodes move back to

the soil to find new roots to feed on (Figure 2). The life cycle of *P. penetrans* consists of the egg, four juvenile stages and adults. The adult female lays its eggs singly or in small groups within the roots or in the soil. The first-stage juveniles (J1) molt to second-stage juveniles (J2) within the egg and the J2 emerges from the egg into the soil. The J2 then undergoes three further molts with increase in size and sexual development, to become third (J3) and fourth (J4) stage juvenile, and adult. All the juvenile and adult stages are vermiform (Castillo and Vovlas, 2007).

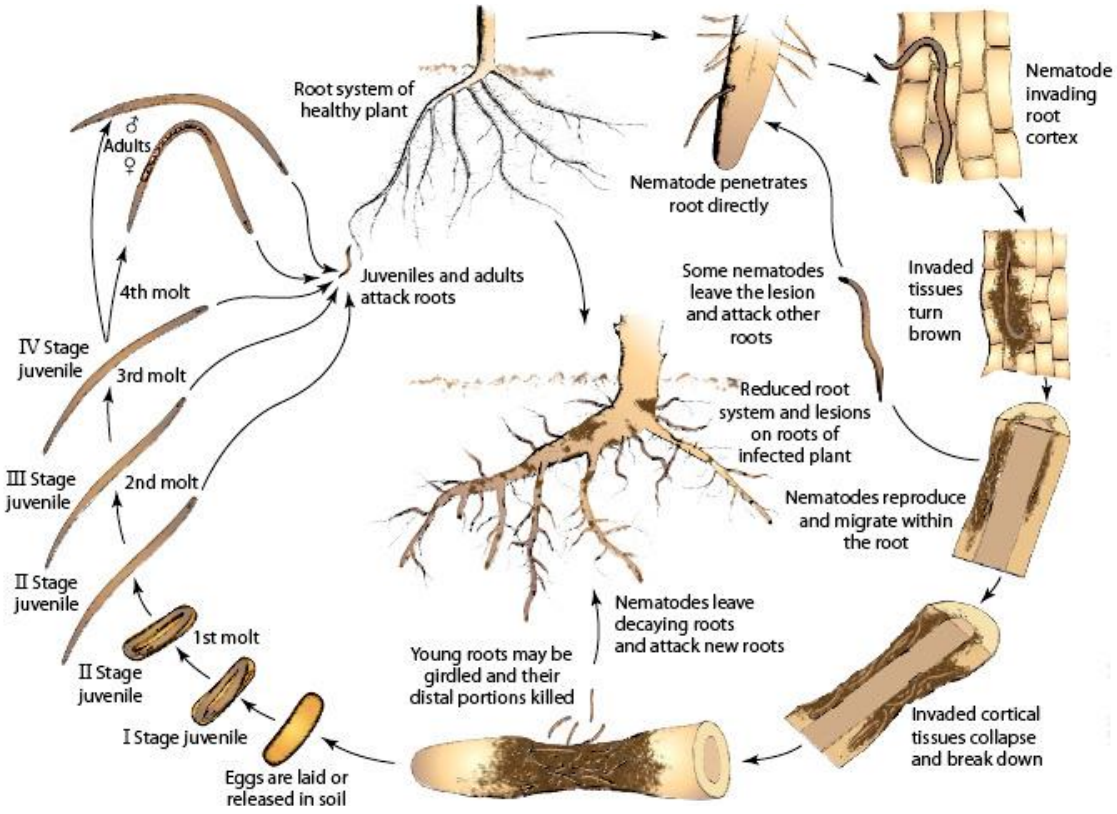

Figure 2: Life cycle and disease symptoms of *Pratylenchus* spp. (Agrios, 2005)

The life cycle and number of eggs deposited by the female varies depending on nematode species, temperature, moisture and host plant species and age (Acosta and Malek, 1979; Decker, 1989; Linsel *et al*., 2014). As is the case with most *Pratylenchus* species, information on the lifecycle of *P. penetrans* under field conditions is scarce. However, studies have been done on the duration of the *P. penetrans* life cycle in various nematode-host combinations under controlled conditions. According to Turner and Chapman (1972), *P. penetrans* completes its life cycle in red clover within 54-65 days and a total of 16-35 eggs are produced by a female at a rate of 1-2 eggs per day. The growth and development of *P. penetrans* is strongly affected by temperature. For instance, Mizukubo and Adachi (1997) demonstrated

that the life cycle of *P. penetrans* (egg deposition to egg deposition) is completed in 46 days at 17°C, in 38 days at 20°C, in 26 days at 27°C, and in 22 days at 30°C.

2.3 Histopathology

The invasion mechanisms of *P. penetrans* have been well-described (Oyekan *et al.*,. 1972; Kurppa and Vrain, 1985; Zunke, 1990a; Zunke, 1990b) and can be separated into root recognition, probing, penetration, ecto-parasitic and endo-parasitic feeding and reproduction.

2.3.1 Attraction, root exploration and penetration

It has been recognized that *Pratylenchus* and other nematodes are attracted to plant roots via root exudates and rhizosphere chemicals (Baxter and Blake, 1967; Prot, 1980; Reynolds *et al*., 2011). *Pratylenchus penetrans* preferably enters the roots in the region of root hair development (Zunke, 1990a) and the elongation zone (Troll and Rohde, 1966; Townshend, 1978). After contact with the roots, *P. penetrans* explores the root surface by rubbing with the lip and probing with the stylet to find a suitable penetration site. Afterwards the stylet is slowly inserted several times into the preferred root cell with increasingly deeper thrusts (Kurppa and Vrain, 1985; Zunke, 1990a). After the stylet is inserted about 2 µm, the nematode starts to salivate and subsequently ingests the content of the cell (Zunke, 1990a). The saliva may help to predigest the cytoplasmic material prior to ingestion (Kurppa and Vrain, 1985). After periods of salivation and ingestion adults of *P. penetrans* move into the root to feed endo-parasitically while the juveniles (J2 and J3) remain in the periphery of the roots feeding on the root hairs for extended period of times (Zunke, 1990a).

Factors affecting migration and penetration

The distribution, growth, reproduction, and development of *P. penetrans* are generally influenced by a number of biotic and abiotic factors, which in turn affect its pathogenic potential. Some of these factors include soil texture, soil temperature, soil moisture, soil pH and host plant (Heald and Robinson, 1990; Castillo and Vovlas, 2007). Townshend and Webber (1971) demonstrated maximum nematode movement of 4 cm in seven days for a low bulk density sandy loam at moisture tension between 10 and 3000 cm H_2O. The damage potential of *P. penetrans* also varies with soil type. For instance, on red raspberry, Zasada and Weiland (2015) stated that the damage potential of *P. penetrans* was greater on sandy loam soil than silt loam soils. Soil moisture may influence the activity and survival of the nematode. For instance, Kable and Mai (1968) demonstrated that very high or very low soil moisture conditions could suppress *P. penetrans* invasion and reproduction on alfalfa. Soil pH

plays an important role on survival and reproduction of the nematode. *Pratylenchus penetrans* reproduced well on alfalfa (Willis, 1972) and vetch (Morgan and MacLean, 1968) in the range of pH 5.2-6.4, but reproduction rate was inhibited when the pH rose to 7. However, based on Kimpinski and Willis (1981) the optimum pH for *P. penetrans* varies with the host plant. The authors found that raising the soil pH from 5.0 to 6.9 increased the number of *P. penetrans* on alfalfa but reduced the number on timothy.

Temperature influences the migration, root penetration, and reproduction of the nematode. Kimpinski and Willis (1981) recorded higher numbers of *P. penetrans* passing a two cm vertical soil column within four hours at 19°C than at either 29°C or 10°C. Regarding the effect of temperature on nematode reproduction, Townshend (1972; 1978) observed highest reproduction of *P. penetrans* on corn and alfalfa at 20°C. However, Dickerson (1964) noted that temperature limitation depends on the nematode host interactions and the author recorded the highest *P. penetrans* population on corn at 24°C and on potato at 16°C, but no reproduction occurred on either host at 35°C. Besides crop species, also the cultivar affects *P. penetrans* reproduction as shown for potato (Bird, 1977).

Root penetration by *P. penetrans* appears to be influenced by population composition, sex, and developmental stage. Sontirat and Chapman (1970) reported that females penetrated roots in greater numbers than males and fourth stage juveniles, while penetration by second stage juveniles was the lowest. Similarly, a study by Townshend (1978) showed that females were more infective than males or third stage juveniles as they penetrated the roots earlier, faster, in greater numbers, and over a wider range of soil temperatures. Townshend (1978) suggested that the greater ability of females to penetrate roots might be due to the larger size and activity of the glands in the posterior sub ventral lobe, which could allow them to produce greater quantities of enzymes at a faster rate than other life stages. Root penetration by *P. penetrans* is also affected by the age of the host plant at the time of inoculation. A study by Olthof (1982) showed that in alfalfa the number of *P. penetrans* at all life stages was inversely proportional to tissue age. Two-day-old root tissue was penetrated twice as much as 10 to 20 day old root tissue.

2.3.2 Cortical migration and symptoms of damage

Once inside the root, *P. penetrans* migrates intracellularly through the cortical tissue along the root by puncturing and penetrating adjacent cells for feeding. Migration and extended feeding of *P. penetrans* causes extensive tissue distraction and the invaded cells will then exhibit

shrunken tonoplasts, nuclear hypertrophy and granulation, and eventual cell death (Kurppa and Vrain, 1985; Zunke, 1990b). In addition, wounds made by the nematodes also provide entry points for other pathogenic organisms that could ultimately aggravate the damage (Kurppa and Vrain, 1985). The infested areas usually turn brown forming necrotic lesions that spread along the root surface leading to death of the affected root (Figure 3) (Acedo and Rohde, 1971). The formation of lesions have been associated with the presence of phenolic substances in the root tissues (Pitcher *et al*., 1960; Acedo and Rhode, 1971; Hung and Rhode, 1973). Mountain and Patrick (1959) found that browning and formation of necrosis in the roots of peach resulted from hydrolysis of the glycoside amygdalin by the nematode enzyme β-glucosidase to the phytotoxic compounds hydrogen cyanide and benzaldehyde.

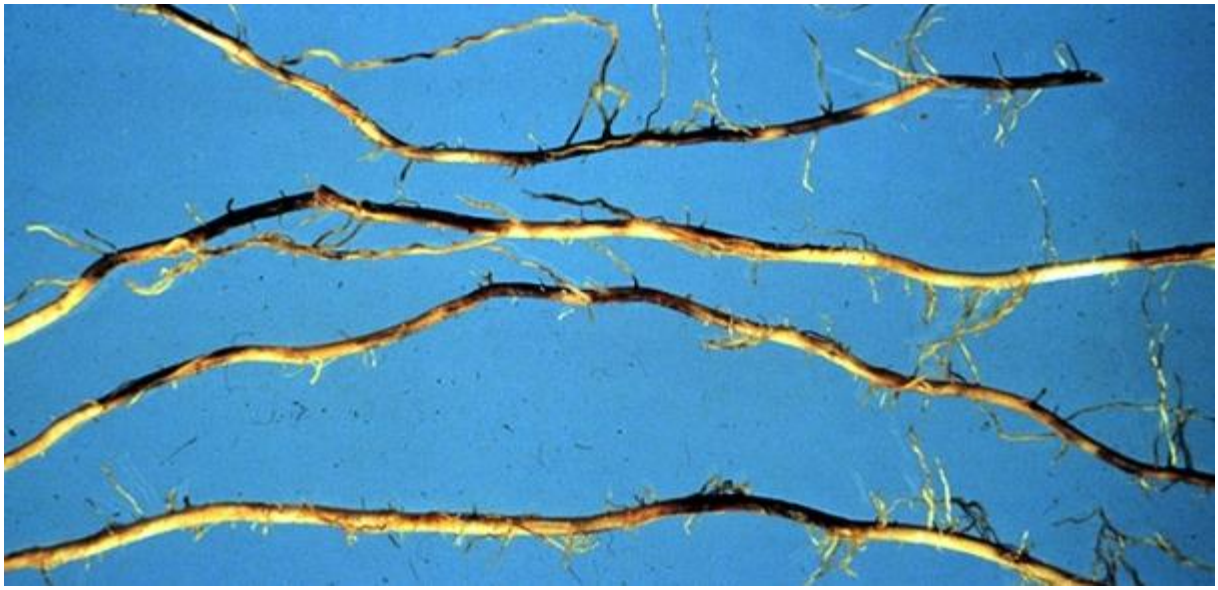

Figure 3: Reddish brown lesions on corn roots produced by *Pratylenchus* species (http://www.plantpath.wisc.edu/PDDCEducation/IPM Scout School alk/img71.Php).

In carrots, *P. penetrans* causes taproot stunting and formation of excessive lateral roots (Figure 4). Above ground symptoms, resulting from severe root infestations by *P. penetrans* often appear as round to oval patches of stunted chlorotic (yellowish) plants (Figure 5) (Duncan and Moens, 2013).

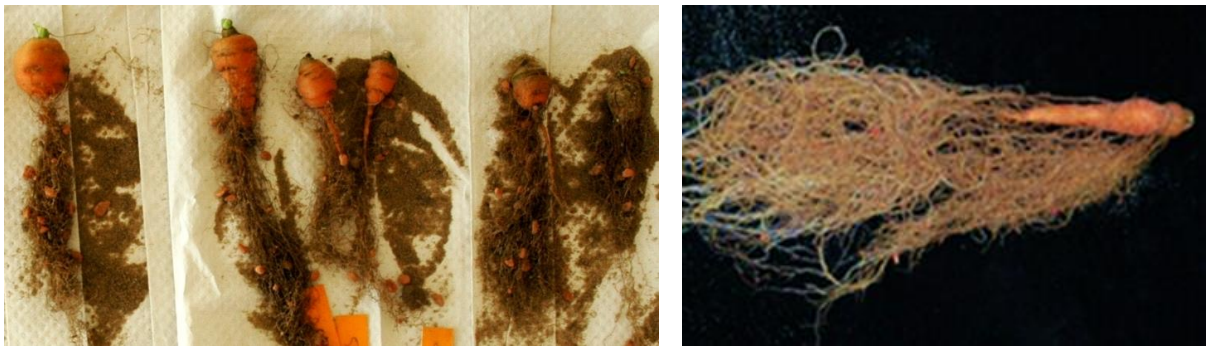

Figure 4: Stunted and stubby taproots caused by *Pratylenchus penetrans* infestation (own pictures).

Figure 5: Patches of stunted and chlorotic field peas infested with *Pratylenchus penetrans*. (https://www.agric.wa.gov.au/carrots/pratylenchus-penetrans-horticulturally-significant-root-lesion-nematode?page=0%2C1).

Histopathological studies showed that root infestation by *P. penetrans* causes different reactions in different hosts (Rohde, 1963; Castilio and Rohde, 1965; Acedo and Rohde, 1971). For instance, *P. penetrans* only penetrates the root cortex in crops like broad bean (Vovlas and Troccoli, 1990), celery (Townshend, 1963a), alfalfa (Townshend *et al.*, 1989), clover (Townshend and Stobbs, 1981), apple (Pitcher *et al*., 1960), peach, maize (Ogiga and Estey, 1975), strawberry (Townshend, 1963b; Ogiga and Estey, 1975), potato and tobacco (Zunke, 1990). In these hosts, the root endodermis prevented the nematode from invading the stele. However, in certain hosts such as carrot (Rohde, 1963), cabbage (Acedo and Rhode, 1971) and tomato (Hung and Rhode, 1973) *P. penetrans* was found to invade the endodermis and stele.

2.3.3 Biological diversity

Biological diversity within and between a nematode populations is an important factor determining the potential success of nematode management research or strategy. Biological variability within *P. penetrans* is known for long (Slootweg, 1956; France and Brodie, 1995). However, there are no recent studies on the variability in virulence among geographically different populations of *P. penetrans*. In case of intraspecific variability nematode populations are often referred to as biotype, pathotype, race or strain. Dropkin (1988) suggested to use the term 'race' for intraspecific variants that can reproduce on a set of host plants from different genera, whereas the term 'pathotype' should be used for populations that reproduces on a host species that otherwise is resistant to other populations of the same nematode species.

A number of techniques can be used for determining population variability, including morphological and morphometric characters, molecular markers such as RAPD or RFLP, and *in vitro* and *in vivo* reproduction on different hosts. Details on variability in reproductive fitness, pathogenicity, morphometric and DNA fingerprints among *P. penetrans* isolates of different geographical locations will be discussed in Chapter V and VI.

2.3.4 Interaction with other organisms

In addition to direct damage, *P. penetrans* is also involved in disease complexes with other soil borne pathogens (MacGuidwin and Rouse, 1990; Lamondia, 2003). The interactions of *P. penetrans* with the vascular wilt fungal pathogen, *Verticillium dahliae* results in a severe vascular wilt disease in potato called potato early dying (PED) (Rowe *et al*., 1985). The role of *P. penetrans* in the development of this syndrome has been demonstrated, as the nematode is capable of activating relatively low populations of *V. dahlia* that would otherwise not be biologically significant in causing disease (Martin *et. al*., 1982). *Pratylenchus penetrans* and *Pythium* spp. are also reported to be significant components of the diverse pathogen complex that incites apple replant disease (Mazola *et al*., 2009). The interaction of *P. penetrans* with other nematode species on different crops has also been studied. Pang *et al*. (2009) found that the reproduction of both *P. penetrans* and *M. hapla* on onion was reduced when nematodes were inoculated concomitantly compared to separate inoculations.

3 Control methods

A number of products and formulations of nematicides have been developed and proven to be effective in controlling nematode pests including *Pratylenchus*. However, due to human health, environmental issues, and costs there has been a marked shift from chemical based

nematode management to non-chemical tactics. Several alternative strategies are being applied in horticultural and field crops production systems, which include crop rotation, cover crops, host plant resistance, and biological control (Bakker *et al*., 1993; Noling and Becker, 1994; Barker and Koenning, 1998; Zasada, *et al*., 2010). Some specific cultural practices such as field sanitation, crop rotation, cover crops, and soil amendments to control *P. penetrans* and other root lesion nematodes are highlighted below.

Field sanitation

Several genera of broadleaf and grass weeds including many volunteer crops were shown to be good hosts for *Pratylenchus* species (Bellé *et al*., 2017; Bélair *et al*., 2007). Therefore, destroying them reduces *Pratylenchus* population buildup and thus damage to the current and subsequent crop. Furthermore, preventing the spread of infested plant materials and soil to uninfested field is also a vital management strategy.

Crop rotation

Root lesion nematodes can be controlled by rotating a susceptible host crop with non-host crops. For example, forage pearl millet (*Pennisetum glaucum*) and grain pearl millet were found to be more efficient at suppressing *P. penetrans* and improving crop yield and quality of the subsequent intolerant host tobacco (*Nicotiana tabacum*) than pearl millet, grain sorghum (*Sorghum bicolor*) and rye (*Secale cereale*) (Jagdale *et al*., 2000). Similarly, Bélair *et al*. (2010) demonstrated that one year of forage or grain pearl millet cultivation prior to potato decreased *P. penetrans* populations below the damage threshold of 1000 nematodes per kg of soil and improved the quality and yields of potato.

Cover crops

Several cover crops such as redtop (*Agrostis alba*), creeping red fescue (*Festuca rubra*), and ‘Saia’ oat (*Avena sativa*), perennial rye grass, French marigolds (*Tagetes patula* ssp. nana, and *T. erecta* cv. Crackerjack) were reported to provide efficient methods for *P. penetrans* management (Marks and Townshend, 1973; Miller, 1978; Vrain *et al*., 1996; Kimpinski *et al*., 2000; Belair *et al*., 2007).

Soil amendments

Soil amendments have been shown to suppress population densities of PPNs either by releasing substances toxic to nematodes or by stimulating soil-inhabiting microorganisms and antagonistic fungi. Application of agricultural waste compost resulted in a significant

reduction of *P. penetrans*, stimulated beneficial rhizosphere microorganisms, and improved growth of newly planted sweet cherry on replant diseased soil (Watson *et al*., 2017). Using *Brassica juncea* seed meal as a source of organic material suppressed the population density of *P. penetrans* and *Pythium* spp. in apple rootstocks grown in replant diseased soils (Mazola *et al*., 2009).

4 Objectives of the study

The overall objective of this study was to develop alternative strategies for *P. penetrans* management. The specific objectives were:

- To evaluate and optimize techniques for nematode culturing and resistance screening
- To establish pure cultures of *P. penetrans* isolates from different geographical locations
- To evaluate the host suitability of selected cover crops to *P. penetrans*
- To determine the *in vitro* and *in vivo* reproductive fitness and pathogenic variability among seven isolates of *P. penetrans*
- To evaluate morphometric and genetic variability among seven isolates of *P. penetrans*

The various steps of the research are shown in the flow diagram below (Figure 6).

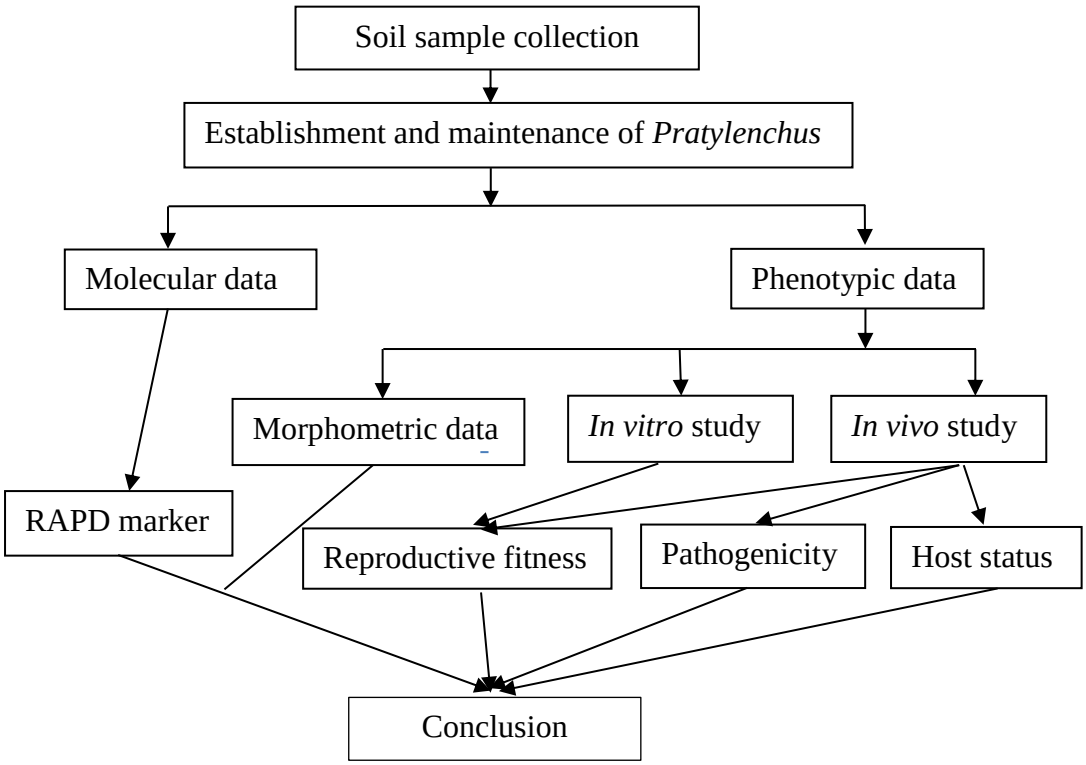

Figure 6: Flow diagram of the research program.

Chapter II
Optimization of methods for *Pratylenchus* culturing and resistance screening

1 Introduction

Evaluating host plant resistance to *Pratylenchus* requires efficient screening methods and precise test conditions. Main variables that need to be standardized are plant growth substrate, soil moisture, temperature, plant nutrition, time and density of nematode inoculation and harvest time (Peng and Moens, 2002; Asif *et al*., 2015; Kim *et al*., 2017). It also requires a standard nematode population which has a high infectivity and reproduction rate and can be reliably produced. Therefore, improving screening parameters such as soil type and plant nutrition, nematode extraction methods and other cultural conditions would be useful to produce reliable data and develop standard methods which could be used for further screening study. Therefore, the objective of this study was to improve methods for nematode culturing and greenhouse resistance.

2 Materials and methods

2.1 Optimization of carrot disc cultures

In order to improve *in vitro* nematode culturing on axenic carrot discs, a previous method for surface sterilizing of carrots and nematodes was compared with an optimized one. Details of these were as follows:

Surface sterilization of carrots

For the previous method, fresh carrots were thoroughly washed with tap water whereas for the optimized method, carrots were first soaked in 2.8% sodium hypochlorite (NaOCl) plus a drop of detergent (Pril, Henkel, Düsseldorf, Germany) for 5 min and then washed with sterile distilled water. The carrots of each treatment were placed separately on sterile paper towels and air dried under a laminar flow. Afterwards the carrots were sprayed with 96% ethanol and flamed. Next, the carrot skin was removed with a vegetable peeler. The carrots were cut in discs of approximately 5 mm thickness and placed in sterile 5 cm glass Petri dishes. All steps were performed in a laminar flow hood under aseptic conditions. Petri dishes with carrot discs remained under the laminar flow hood for two weeks to observe contamination, if any.

Surface sterilization of *Pratylenchus penetrans*

The nematode inoculum was obtained from a pure culture of *P. penetrans* that has been multiplied on carrot discs in an incubator at 25±1°C. Specimens were extracted with the Baermann funnel technique (EPPO, 2013) and nematodes were collected each day for up to seven days and stored at 4°C in a 50 ml measuring cylinder. Surface sterilization procedures of the nematodes was performed in a laminar flow hood under aseptic conditions. Therefore, 5 ml nematode suspension was passed through a 20 µm sieve (2.5 cm diam.). The sieve with the nematodes was then transferred in a second Petri dish containing the different treatments. For the previous method, nematodes in the sieve were rinsed with 5 ml 0.02% mercury chloride ($HgCl_2$) for 5 min followed by three washings with sterile distilled water. For the optimized method, nematodes were rinsed with 5 ml 0.1% streptomycin sulphate (SS) for 5 min followed by 5 ml 0.02% $HgCl_2$ for 1 min. Nematodes of both treatments were then washed off the sieve into a sterile 5 cm glass Petri dish.

Inoculation of the carrot discs

The Petri dishes containing the nematode suspension of each sterilization treatment were placed under a compound light microscope at 40-fold magnification. In total 50 mixed stages of *P. penetrans* were sucked up with a sterile micropipette and inoculated onto a carrot disc. For each sterilization treatment, 30 Petri plates were prepared. The Petri dishes were then sealed with Parafilm and incubated at 25 ±1°C in the dark.

Assessments of the carrot disc cultures for microbial contamination and nematode reproduction

The carrot disc cultures were weekly assessed for microbial contamination. Contaminated dishes were discarded. Ninety days after inoculation, nematode reproduction was assessed by infection symptoms, such as brown or dark-brown discoloration, and swarming of migrating nematodes at the bottom of the Petri dishes.

2.2 Optimization of efficiency of *Pratylenchus penetrans* extraction

2.2.1 Nematode extraction from carrot disc cultures

Two methods were compared regarding the efficacy of nematode extraction from carrot disc cultures. The first experiment compared the extraction efficacy of carrot disc maceration using an Ultra Turrax disperser TP 18/10 (Janke & Kunkel KG, IKA-Werk, Staufen, Germany) with a commercial blender (Karl Willers Laborbedarf e. K, Münster, Germany).

The second experiment was set up to define the optimum time for *P. penetrans* extraction from carrot disc cultures using the Ultra Turrax disperser.

For the first experiment carrot discs were inoculated with 50 mixed stages of *P. penetrans* and incubated for 2 months. The carrot disc was cut into two equal parts and each part was further cut into pieces and macerated using either the Ultra Turrax disperser or the commercial blender. For the Ultra Turrax disperser, the carrot pieces were placed in a 50 ml tube plus 20 ml tap water and macerated. For the commercial blender the carrot pieces were placed in the blender with 30 ml tap water and macerated. Maceration with both methods was continued until a homogenous suspension was obtained. The time required for a homogenous suspension was recorded. The macerated suspensions were then passed through a 100 µm sieve into a 200 ml glass beaker and allowed to settle for 24 h. Nematode suspensions were adjusted to 25 ml and the number of eggs, viable, and damaged juveniles and adults were counted in a 1 ml aliquot under a compound light microscope at 40-fold magnification. Comparison was made based on necessary maceration time to obtain a homogeneous suspension, total number of nematodes (eggs, juveniles and adults) and percentage of damaged vermiform nematodes.

The experiment 2 was performed with the Ultra Turrax method to find out if precutting of the carrot discs in smaller pieces would allow lower times of maceration and thus less damage to the nematodes. Therefore, the carrot disc pieces were either cut by hand into 4, 8 or 16 equal pieces. The pieces of each carrot disc were then placed in a 50 ml tube with 20 ml tap water and macerated for 7 sec each. The number of macerations required to obtain a homogeneous macerate were recorded.

2.2.2 Nematode extraction from roots

Two experiments were performed to determine the optimal extraction durations and methodology of Baermann extraction in a misting chamber. In each experiment, four pots with maize were inoculated with 700 mixed stages of *P. penetrans* and two pots each were harvested at six and nine weeks after inoculation, respectively. Maize roots were washed free from substrate and cut into pieces of about 1 cm length. In the first experiment roots were spread on Baermann funnels and placed in a misting chamber for five consecutive weeks. Nematode suspensions were collected daily and total numbers of extracted *P. penetrans* were counted after each week. The percentage of *P. penetrans* recovered at each week was

estimated by dividing the number of nematodes recovered at each week by the final cumulative after 5 weeks.

In the second experiment roots from each pot were divided in two parts of equal weight. One part each was incubated on a Baermann funnel and the other on a Baermann tray, both placed in a misting chamber. Nematodes were collected daily until the end of the second week. The total numbers of *P. penetrans* extracted by the two methods were compared.

2.3 Establishment of pure cultures of *Pratylenchus* isolates collected from different geographical locations

Pure cultures of *Pratylenchus* isolates from different geographical regions had to be established for characterization by biological and molecular means. This required an efficient and reliable method for the establishment and maintenance of pure nematode cultures starting from single females. Here, we compared two culturing methods, i. e. *in vitro* on carrot discs kept in an incubator and *in vivo* on maize grown in the greenhouse.

2.3.1 Collection of *Pratylenchus* isolates

Soil samples collected from different geographical locations were obtained from Joordens Zaden B.v., Kessel, The Netherlands and the Julius Kühn Institute (JKI), Münster, Germany. Nematodes were extracted from the soil samples using the centrifugal-flotation method (EPPO, 2013). The occurrence of *Pratylenchus* spp. in the obtained suspensions was examined under a compound light microscope at 40-fold magnification. Mixed stages of *Pratylenchus* were transferred with a micropipette to 5 cm glass Petri dishes. The nematode suspensions were stored at 4°C until required.

2.3.2 Nematode propagation on maize

Single maize plants were raised in 100 ml folded-boxes filled with steam sterilized sand and stacked in rectangular metal containers. After emergence a 3 cm deep hole was made near the base of the seedling in each pot. A single *Pratylenchus* female was hand-picked from the previously collected soil samples using a fine fishing stick and transferred into the hole by washing the nematode from the needle with a drop of water. Eight weeks after inoculation, root systems were harvested, washed, cut into 1 cm long pieces and placed on Baermann trays in a misting chamber for 2 weeks. Collected nematodes from roots of each plant were examined for reproduction of *Pratylenchus* under a compound light microscope at 40-fold magnification.

2.3.3 Nematode propagation on axenic carrot discs

Fresh carrots were surface sterilized with household bleach containing 2.8% sodium hypochlorite (DanKlorix, Colgate-Palmolive, New York, U.S.A.) and nematodes were surface sterilized with 0.1% streptomycin sulfate and 0.02% $HgCl_2$ as described above. A single female was sucked up with a sterile micropipette and transferred to a carrot disc. The Petri dishes were sealed with parafilm, labeled and then incubated at 25±1°C in the dark. Six weeks after inoculation the carrot disc cultures were monitored every two weeks for reproduction of *Pratylenchus.* If reproduction occurred, the progeny of the single *Pratylenchus* female was then either sterilized and inoculated or sub-cultured to new carrot discs and the procedure was continued.

2.4 Optimizing test conditions for growth of cover crops and *Pratylenchus penetrans* reproduction in the greenhouse

Growth substrate and plant nutrition are known to influence the development and reproduction of *Pratylenchus* spp. Thus, to determine the optimum conditions for the growth of cover crops and nematode development two experiments were conducted.

2.4.1 Comparison of different combinations of substrates and fertilizer rates on growth of fodder radish and reproduction rate of *Pratylenchus penetrans*

Plant material and growth conditions

The first experiment evaluated different growth substrates and nutrient concentrations. Therefore, five plants each of fodder radish (*Raphanus sativus* cv. RSAS1037) were grown in 700 ml plastic pots filled with one of three substrates: silver sand, silver sand-vermiculite mixture (4:1, v:v), and silver sand-perlite mixture (4:1, v:v). Plants were fertilized weekly with Wuxal Super liquid (Aglukon Spezialdünger GmbH, Düsseldorf, Germany) [N:P:K, 8:8:6 + microelements] at concentrations of either 0.1%, 0.3%, or 0.9% (v:v). In total, the experiment consisted of 3 growth substrates x 3 fertilizer rates x 10 replicates which ware arranged as a randomized complete block. Plants were grown in the greenhouse at a temperature range of 20-23°C and a photocycle adjusted to 16 h light using Pollux 0691XHIT 400 W lamps (Norka, Germany) and 8 h dark period. Plants were watered regularly as needed.

Nematode inoculum

Pratylenchus penetrans was reared on carrot disc cultures and mobile stages were extracted by the Baermann funnel method as described above. Before inoculation the nematode suspension was adjusted to 140 nematodes per ml of water. At the 2- to 3-true-leaf stage of the plants, five 3 cm deep holes were made near the base of the seedlings in each pot and 10 ml of the nematode suspension was inoculated into the holes with a dispenser so that each pot received a total of 1,400 mixed stages of *P. penetrans*. The holes were then covered with a thin layer of soil and moistened with a light spray of water.

Assessments of treatment effects

At harvest eight weeks after nematode inoculation, shoot fresh weight was measured. To determine nematode reproduction, root systems were removed from the pots, washed free from soil, weighed, cut into 1 cm long pieces and placed on Baermann trays in a misting chamber for 2 weeks. Soil from each pot was thoroughly mixed and nematodes were extracted from a 250 ml subsample using the centrifugal flotation method (EPPO, 2013). Collected suspensions from root and soil extraction were examined for *P. penetrans* by counting 1 ml aliquots under a compound light microscope at 40-fold magnification. *P. penetrans* reproduction was calculated by dividing nematode numbers from roots and soil at harvest with the initial density of 1,400 nematodes per pot.

2.4.2 Comparison of soil substrates to optimize aeration

The second experiment investigated if aeration of the substrate could be improved for better plant growth and nematode reproduction. Again fodder radish was grown in 700 ml pots. Two different substrates were compared: A mixture of silver sand, clay gravel (Seramis, Mogendorf, Germany) and field soil at the ratio of 4:1:1 (v:v:v) and a silver sand-vermiculite mixture (4:1, v:v). Plants were fertilized weekly beginning from one week after inoculation with Wuxal Super liquid fertilizer (N:P:K, 8:8:6 + microelements) at a concentration of 0.3% (v:v). *P. penetrans* inoculum was collected as described above and each pot was inoculated with 980 mixed stages. Pots were arranged as a randomized complete block design with 10 replicates per treatment. Plants were grown at a temperature range of 22-25°C. Plant growth and nematode reproduction were determined as described above.

Data were analysed using ANOVA (SPSS 20) and means were separated using Duncan's Multiple Range Test at $P \leq 0.05$. Nematode counts were log transformed prior to analysis.

3 Results

3.1 Optimization of carrot disc cultures

The modified method for surface sterilization of carrots and *P. penetrans* significantly ($P < 0.05$) reduced losses due to contamination of the carrot discs and failed nematode reproduction from 33.3% to 6.7% in comparison with the previous method (Table 1).

Table 1: Effects of sterilization methods on carrot disc culture contamination and nematode reproduction

Treatment	Number of carrot discs			Total loss (%)
	Per treatment	Contaminated	Without reproduction	
PM[1]	30.0	6.0	4.0	33.3[**]
MM[2]	30.0	1.0	1.0	6.7

[1]PM = previous method, [2]MM = modified method, [**]statistically significant ($P < 0.05$).

3.2 Optimization of efficiency of nematode extraction

3.2.1 Nematode extraction from carrot disc cultures

Experiment 1

The results obtained by comparing methods of nematode extracting from carrot disc culture showed differences between the Ultra Turrax disperser and commercial blender in extraction efficiency. The Ultra Turrax disperser method leads to higher numbers of recovered eggs and nematodes (17,000) than the commercial blender method (10,525). Moreover, Ultra Turrax disperser had lower percentages of damaged nematodes compared to the commercial blender as indicated Table 2. The Ultra Turrax disperser method required less time to obtain a homogenous macerate compared to the commercial blender method.

Table 2: Effects of using Ultra Turrax disperser (UTD) and commercial blender (CB) on the efficacy of *Pratylenchus penetrans* extraction from a 60 day old carrot disc culture

Method	*Pratylenchus penetrans*					Time
	Viable nematodes	Dead nematodes	Eggs	Total	Dead nematodes	
UTD	7,675	2,025	7,500	17,200	20.9%	20 s
CB	2,550	1,075	6,900	10,525	29.7%	30 s

Experiment 2

Cutting the carrot discs in smaller pieces before maceration will allow shorter times of maceration to receive a homogenous nematode suspension and at the same time decreases number of damaged nematodes. Thus, optimum homogeneous macerate was achieved when carrot disc was cut into eight pieces prior to extraction and macerated two times for seven seconds (Table 3).

Table 3: Effect of cutting carrot discs in smaller pieces on the number of maceration intervals required to receive a homogenous suspension

Number of carrot disc pieces	number of maceration intervals
4	3 x 7 s
8	2 x 7 s
16	1 x 7 s

3.2.2 Nematode extraction from roots

Experiment 1

Plant age affected the total numbers of extractable *P. penetrans* and also the rate of recovery over time (Figure 1). The total number of *P. penetrans* recovered during the entire extraction period was 4,568 and 29,269 for 6 and 9 weeks old maize roots, respectively. The recovery rate over time varied between the two treatments. For both 6 and 9 weeks old maize roots, the rate of nematode recovery after 1 week of extraction was similar with about 24.4% of extractable nematodes within the total 5 weeks period. However, after 2 weeks, the rate of *P. penetran*s recovery was higher from 9 weeks old maize roots (48.6%) than from 6 weeks old maize roots (34.3%). From week 3 onwards, the recovery rate of *P. penetrans* declined for both treatments. Nematodes were still recovered after 5 weeks of extraction, with slightly higher recovery rates for 6 weeks old maize roots than for 9 weeks old maize roots.

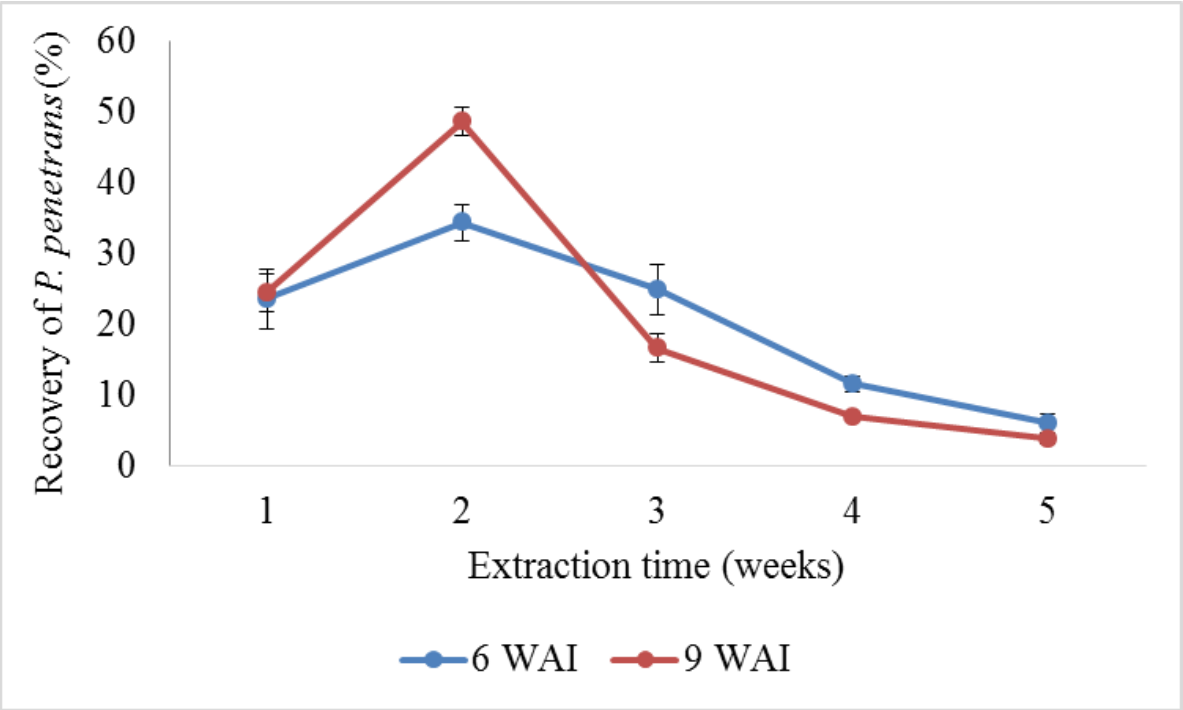

Figure 1: Percentage of *Pratylenchus penetrans* collected at 1 to 5 weeks from maize roots extracted at 6 and 9 weeks after inoculation (WAI) by Baermann funnel (BF) method (n = 2).

Experiment 2

The extraction efficiency of *P. penetrans* for roots incubated for 2 weeks on Baermann funnels and Baermann trays was compared. There were slightly higher numbers of *P penetrans* recovered from Baermann trays than Baermann funnels (Figure 2). Overall, more *P. penetrans* were extracted from 9 weeks old maize roots than from 6 weeks maize roots.

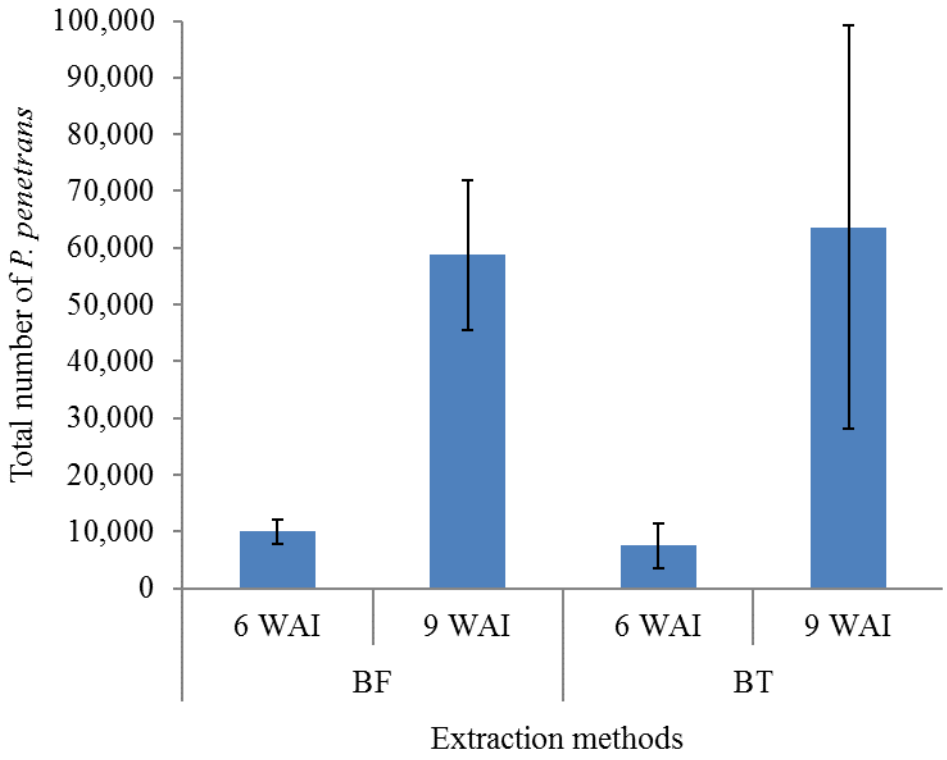

Figure 2: Final numbers of *Pratylenchus penetrans* after 2 weeks extraction of maize roots by Baermann funnel (BF) and Baermann tray (BT) methods. Maize roots were extracted 6 and 9 weeks after inoculation (WAI) with *P. penetrans* (n = 2).

3.4 Establishment of pure cultures of *Pratylenchus* isolates collected from different geographical locations

3.4.1 Nematode propagation on maize

The roots of 180 maize plants previously inoculated with 30 isolates of *Pratylenchus* were examined for reproduction of *Pratylenchus.* However, there was no reproduction of nematodes found on any of the tested roots of maize. Therefore, this method was replaced by the carrot disc technique.

3.4.2 Nematode propagation on axenic carrot discs

The monoxenic carrot disc culture technique yielded several single female cultures of *Pratylenchus* (Table 4). For soil samples infested with *Pratylenchus*, *P. neglectus* was the dominant species. A total of 30 isolates of *Pratylenchus* comprising four species (*P. mediterraneus, P. neglectus, P. penetrans* and *P. thornei*) originating from ten countries, namely Germany, Italy, The Netherlands, Slovenia, France, Belgium, United Kingdom, Sweden, Switzerland and Algeria, were established on axenic carrot discs.

Table 4: Isolates of *Pratylenchus* species cultured their geographical location and source of origin.

Species	Location	Country	Source
P. mediterraneus	unknown	Algeria	ENSA[1]
P. neglectus	Mere (UK-1)	United Kingdom	Joordens Zaden[2]
P. neglectus	Mere (UK-2)	United Kingdom	Joordens Zaden
P. neglectus	MFHI 363	The Netherlands	Joordens Zaden
P. neglectus	Groß Lüsewitz Schlag 4	Germany	Julius Kühn-Institut[3]
P. neglectus	Hohnhorst	Germany	Julius Kühn-Institut
P. neglectus	Nesch	Germany	Julius Kühn-Institut
P. neglectus	Biedesheim	Germany	DLR[4]
P. neglectus	Viterbo	Italy	Tuscia University[5]
P. neglectus	Lachadelle Onzerain	France	Joordens Zaden
P. neglectus	Gießen	Germany	Gießen University[6]
P. neglectus	Niederhummel	Germany	Julius Kühn-Institut
P. neglectus	Braunschweig, Schlag 9	Germany	Thünen-Institut[7]
P. neglectus	Braunschweig, Schlag 5	Germany	Thünen-Institut
P. neglectus	Tänikon	Switzerland	Agroscope[8]
P. neglectus	Uppsala	Sweden	SLU[9]
P. penetrans	Barnopf (UK-3)	United Kingdom	Joordens Zaden
P. penetrans	Bonn	Germany	Julius Kühn-Institut
P. penetrans	Vreedepel	The Netherlands	Joordens Zaden
P. penetrans	Münster	Germany	Julius Kühn-Institut
P. penetrans	Villampuy	France	Joordens Zaden
P. penetrans	Merelbeke	Belgium	ILVO[10]
P. penetrans	Witzenhausen	Germany	Kassel University
P. thornei	Villampuy	France	Joordens Zaden
P. thornei	unknown	Slovenia	KIS[11]
P. thornei	Barnopf (UK-3)	United Kingdom	Joordens Zaden
P. thornei	Lachadelle Onzerain	France	Joordens Zaden
P. thornei	Bonn	Germany	Julius Kühn-Institut
P. thornei	unknown	Algeria	ENSA
P. thornei	Viterbo	Italy	Tuscia University

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3.5 Comparison of different combinations of substrates and fertilizer rates on growth of fodder radish and reproduction rate of *Pratylenchus penetrans*

Average shoot and root fresh weights varied depending on the substrates and fertilizer levels used ($P < 0.001$). In general, shoot and root fresh weights increased as fertilizer level increased from 0.1% to 0.9% (Table 5). Plants fertilized with 0.9% Wuxal fertilizer and grown on sand + vermiculite or sand showed a significantly ($P < 0.05$) higher shoot fresh weight with 72.3 g and 69.7 g, respectively compared to plants grown on other substrate-fertilizer combinations. Sand + vermiculite fertilized with 0.3% and 0.9% Wuxal fertilizer significantly increased root fresh weight to 11.4 g and 12.2 g, respectively compared to other combinations. Increase in fertilizer level was not always associated with increase in root fresh weight. For example, root fresh weight of plants grown on sand decreased from 9.27 to 6.75 g as fertilizer level increased from 0.3% to 0.9%. Nematode numbers per gram of root fresh weight varied depending on substrate and fertilizer level, but were far below the maintenance level. However, the highest number of nematodes per gram fresh root weight (46.1) was found in sand fertilized with 0.3% Wuxal fertilizer, while sand-perlite with 0.9% Wuxal fertilizer resulted in the lowest number of nematode per gram root fresh weight (1.97). Overall, the increasing fertilizer levels had a positive correlation with shoot ($r = 0.0915$, $P < 0.001$) and root ($r = 0.519$, $P < 0.001$) fresh weights while the increasing fertilizer levels had a negative correlation with nematode numbers per gram of root fresh weight ($r = 0.305$, $P < 0.03$).

Table 5: Effects of substrates and fertilizer levels on plant growth and reproduction of *Pratylenchus penetrans* in fodder radish at 8 weeks after inoculation with 1.400 mixed stages.

Substrate + fertilizer	Shoot fresh weight (g)	Root fresh weight (g)	Nematodes/g	Pf/Pi
Sand + 0.1%	20.20[1] a[2]	5.99 ab	35.20 cd	0.20 cd
Sand + 0.3%	35.70 b	9.27 de	46.10 d	0.35 e
Sand + 0.9%	69.70 d	6.75 abc	23.50 bc	0.12 abcd
Sand-Vermiculite + 0.1%	25.50 a	5.89 ab	27.30 bcd	0.16 bcd
Sand-Vermiculite + 0.3%	44.30 b	11.40 ef	23.40 bc	0.23 d
Sand-Vermiculite + 0.9%	72.30 d	12.20 f	13.00 ab	0.14 bcd
Sand-Perlite + 0.1%	22.40 a	4.97 a	16.80 abc	0.07 ab
Sand-Perlite + 0.3%	36.30 b	7.5 bcd	16.30 abc	0.09 abc
Sand-Perlite + 0.9%	58.80 c	8.64 cd	1.97 a	0.01 a

[1]Mean (n = 10), [2]Different letters indicate significant differences according to Duncan's Multiple Range Test $P \leq 0.05$.

3.6 Comparison of soil substrates to optimize aeration

Our main aim in this experiment was to improve aeration of the soil substrate, which affects optimum plant growth particularly at early stage. Fodder radish plants were either cultivated in a mixture of silver sand, clay and field soil (SCF) (4:1:1, v:v:v) or silver sand and vermiculite (SV) (1:1, v:v). Thus, in addition to measurements of plant growth parameters and nematode reproduction, observations on plant growth conditions were made. As expected, plants grown in a mixture of silver sand and vermiculite (SV) showed foliage discoloration and stunted growth compared with plants grown in a mixture of silver sand, clay and field soil (SCF). Plant growth substrate affected plant growth parameters and nematode reproduction (Table 6). Shoot and root fresh weights were higher in plants grown on silver sand, clay and field soil mixture (SCF) compared to those grown on silver sand and vermiculite mixture (SV). Nematode reproduction also varied between the two substrate mixtures. Higher nematode numbers were recovered from the roots and soils of plants grown on silver sand, clay and field soil mixture (SCF) than those grown on silver sand and vermiculite (SV).

Table 6: Shoot and root fresh weight of fodder radish and final number of *Pratylenchus penetrans* grown on a mixture of silver sand, clay and field soil (SCF) or a mixture of silver sand and vermiculite (SV) at 8 weeks after inoculation with 980 mixed stages.

Treatment	Shoot fresh weight (g)	Root fresh weight (g)	Final population of *P. penetrans*		
			Root	Soil	Total
SCF	33.7 ± 2.6[1]	8.94 ± 0.7	511 ± 174	535 ± 174	1,045 ± 293
SV	26.9 ± 8.1	6.19 ± 2.2	465 ± 333	280 ±220	745 ± 478

[1]Mean and standard deviation (n = 10).

4 Discussion

4.1 Optimization of carrot disc cultures

The *in vitro* carrot disc culture is the most commonly used techniques for culturing *Pratylenchus* species. However, microbial contamination and chemical induced loss of nematode viability during nematode sterilization were the common challenges encountered in our attempt to culture nematodes *in vitro*. As a result, we had failure of approximately 15-20% of the cultures. Carrot disc culture loss due to microbial contamination can be as high as 50% (Kühnhold, 2011). However, a significant reduction in loss of carrot disc culture due to contamination and loss of nematode viability can be obtained when carrots and nematodes are surface disinfested by the modified method as revealed in this study.

A combination of sterilants which has been used for surface sterilization of nematode includes mercuric chloride ($HgCl_2$), streptomycin sulphate, chlorhexidine, bleach and antibiotics (Hay, 1994; Speijer and De Waele, 1997; Peng and Moens, 1999). Surface sterilization of *P. penetrans* in 0.01% $HgCl_2$ and 0.01% streptomycin sulphate for 5 min has been reported to provide viable axenic nematodes (Karakas, 2015). According to Peng and Moens (1999), 0.1% malachite green alone for 15 min or with 0.5% streptomycin sulphate were efficient to surface sterilize *P. penetrans*. However, streptomycin sulphate at 0.2% for 24 h and a split treatment of streptomycin sulphate at 0.2% for 24 h and malachite green at 0.1% for 10 min did not reduce surface contamination. Combinations based on $HgCl_2$ (0.05-0.1%, 1-1.5 min) affected the behaviour of juvenile and adult stages of *P. penetrans* (Peng and Moens, 1999).

Surface sterilization of several migratory endo-parasitic nematodes with various concentrations and exposure time of $HgCl_2$ and streptomycin sulphate have been reported (Speijer and De Waele, 1997; Verdejo-Lucas and Pinochet, 1992; Castillo, 1998). Nico (1999) surface sterilized *P. goodeyi* at 0.02% $HgCl_2$ and 0.1% streptomycin sulphate for 2 and 24 h respectively. In an *in vitro* multiplication study, Verdejo-Lucas and Pinochet (1992) sterilized 5 migratory endo-parasitic nematodes (*P. vulnus*, *P. neglectus*, *P. thornei*, *R. similis* and *Zygotylenchus guevarai*) in a solution of 0.01% $HgCl_2$ and 1% streptomycin sulphate for 5 min. These results suggest that concentration of nematode surface sterilients and exposure time are important factors and can vary with the species or races of nematode as sensitivities to chemicals for nematode species are different (Hooper, 1986).

Finally, the nematodes cultured on carrot discs in our study were able to infect, reproduce and damage plant tissues in greenhouse pot experiments.

4.2 Optimization of efficiency of *Pratylenchus penetrans* extraction

4.2.1 Nematode extraction from carrot disc cultures

Experiment 1

In this study, the Ultra Turrax disperser and commercial blender were compared for their efficiency to extract nematodes from carrot disc culture. The Ultra Turrax disperser was found to be more efficient; it provided a higher recovery of eggs and nematodes with lower percentage of damaged nematodes in less time compared to commercial blender.

Experiment 2

This experiment demonstrated that the required duration of maceration depends on the size of the carrot disc as reported previously (Speijer and De Waele, 1997). Best results were achieved by chopping carrot discs with a scalpel into eight pieces and macerating them in a 50 ml tube with 20 ml tap water two times for 7 seconds. Speijer and De Waele (1997) reported that blending carrot discs 3 times for 10 seconds worked best for the extraction of *R. similis* using a kitchen blender. The longer time required for maceration compared to our study is most likely attributed to differences in carrot disc size and extraction apparatus used.

4.2.2 Nematode extraction from roots

Experiment 1

Our study on the determination of plant age on *P. penetrans* extraction efficacy from maize roots using the misting chamber method showed higher rates of nematodes extracted 9 weeks after inoculation than 6 weeks after inoculation.

The *P. penetrans* recovery was highest at 2 weeks after extraction for both 6 and 9 weeks old maize roots. At this time, about 73% of the total *P. penetrans* extracted at 5 weeks were recovered from 9 old maize roots compared with 57% from 6 weeks old maize roots. Similarly, working with crop species of different ages, Taylor (2000) reported about 50 to 75% recovery of *P. neglectus* for 6 weeks old plants and over 90% for 16 weeks old plants after 2 weeks extraction. Using the misting chamber method, Merrifield (1990) recovered 90% of *P. penetrans* from peppermint roots at 2 weeks after extraction. Two weeks of

extraction has been considered the optimum time for identifying differences in plant resistance response towards *Pratylenchus* (Marks and Townshend, 1973; Bélair *et al*., 2002).

In our study, the weekly recovery rate from 6 weeks old maize roots was slightly higher than that of 9 weeks old roots from week 3 onwards. This may be due to increase in hatching of eggs or continued reproduction as the food source remains intact in roots cut from actively growing maize plants (6 weeks old) compared to senescing (9 weeks old) maize plants. A similar observation was reported by Taylor (2000) who received less *P. neglectus* from 16 week old plants than from 6 week old plants after 3 weeks of extraction.

Many species of endo-parasitic nematodes have been reported to multiply in the misting chamber and emerge for long periods if incubation is continued as the food source remains intact and the misting chamber offers a favorable environment for reproduction (McSorley *et al*., 1984; Taylor, 2000). This could not be confirmed in our study, as the weekly recovery of *P. penetrans* did not exceed 5 weeks.

Experiment 2

When Baermann funnels and Baermann trays were compared for the extraction efficiency of *P. penetrans* for maize roots of different ages incubated for 2 weeks in a misting chamber, there were slightly higher numbers of *P penetrans* recovered from Baermann trays than Baermann funnels. More nematodes were extracted from 9 weeks old maize roots than from 6 weeks old maize roots.

The efficiency of nematode extraction varies with nematode species, root type, plant age, life stage and extraction method (McSorley *et. al*., 1984; Prot *et. al*., 1993; Taylor, 2000; Behan, 2012). The observed difference in final nematode numbers extracted by the two methods may be attributed to variability in the misting chamber's mist coverage, temperature or distribution of roots on the sieves. Besides optimum temperature, also sufficient oxygen supply is an important parameter for nematode extraction that will affect the efficiency of endo-parasitic nematode extraction (Behan, 2012).

Using Baermann trays instead of Baermann funnels in the misting chamber had the advantage of accommodating larger sample numbers. However, Baermann trays have some disadvantages. First, they require additional time and materials (glass beakers) for processing the samples after extraction. Second, nematode suspensions had to be transferred into a beaker and allowed to settle before decanting excess water to have a manageable volume for

counting. In addition, there were accumulations of debris (e.g. algae) within the nematode suspensions, which made counting of nematodes more difficult. On the contrary, the Baermann funnel does not require additional time for processing after extraction and it provides a relatively clean and manageable volume of nematode suspension.

4.3 Establishment of pure cultures of *Pratylenchus* isolates collected from different geographical locations

4.3.1 Nematode propagation on maize

Our study showed that the establishment of pure *Pratylenchus* populations starting from single females on maize did not work under the conditions described here. This might be in part due to the fact that not all single females find proper penetration sites.

4.3.2 Nematode propagation on axenic carrot discs

According to our study, the monoxenic carrot disc culture technique was an efficient method to establish pure isolates of *Pratylenchus* starting from single females, provide reproduction of *Pratylenchus* in high numbers and allow long-term maintenance of viable cultures. A total of 30 isolates of *Pratylenchus* comprising four species (*P. mediterraneus*, *P. neglectus*, *P. penetrans* and *P. thornei*) originating from ten countries, namely Germany, Italy, The Netherlands, Slovenia, France, Belgium, United Kingdom, Sweden, Switzerland and Algeria, were established.

Challenges during culture initiation may occur if the chemicals used for surface sterilization of nematodes reduce the viability and infectivity of the nematodes or cause mortality. In the present study, observations were made only on nematode motility and change of their body shape in response to exposure time for each sterilizing chemicals used. When exposure times were too long nematodes exhibited sluggish movement and were banana-shaped or curly shaped. Exposure times during surface sterilization used in this study were considered to be short enough to avoid negative effects of chemicals for most, if not all, *Pratylenchus* isolates.

Depending on the *Pratylenchus* isolates, 5-12 weeks after inoculation, first nematodes appeared on the surface of the Petri dish around the edges of the carrot disc. This suggests that migration of nematodes away from the carrot disc is not necessarily a response to depleted food source (Ko *et al*., 1996). According to Coyne *et al*. (2014) the migration of nematodes from carrot disc varies with the aggressiveness of the nematode species or race being cultured. In our study, *P. thornei* was the most aggressive *Pratylenchus* species on carrot and nematode

swarms were observed on the surface of the Petri dish 5-6 weeks after inoculation. In addition, the Belgium (BL) isolate of *P. penetrans* was found to be the 2nd most aggressive and nematodes started to emerge 6-8 weeks after inoculation while for United Kingdom (UK) isolate, which was the least aggressive, it took at least 12 weeks before nematodes emerged.

Successful establishment of *Pratylenchus* on carrot discs starting from single females has also been reported by Mokrini *et al*. (2016) for four populations of *P. penetrans*, one population of *P. thornei* and one population of *P. pseudocoffeae*. Besides carrot discs, single female cultures of *P. penetrans* have also been successfully established on alfalfa callus (France and Brodie, 1996) and clover roots (Mizukubo, 1997).

Overall, the carrot disc method allowed us to multiply *Pratylenchus* isolates routinely and produce sufficient inoculum for the experiments conducted.

4.4 Optimizing test conditions for growth of cover crops and *Pratylenchus penetrans* reproduction in the greenhouse

4.4.1 Comparison of different combinations of substrates and fertilizer rates on growth of fodder radish and reproduction rate of *Pratylenchus penetrans*

Our study showed that the choice of growth substrate and fertilizer rates affected plant growth and reproduction of *P. penetrans*. In this study, nematode reproduction in general was far below the initial inoculum level. The resultant fewer nematodes obtained is mainly due to the low reproduction potential or low virulence of the *P. penetrans* population (isolate MN) used as evident in Chapter V, 3.1 and 3.2 in our study.

In this study, optimum plant growth was achieved in sand fertilized with 0.3% Wuxal-Super®-liquid fertilizer. Our findings are in line with other findings (Florini, *et al.* 1987; Chałańska *et al*. 2016) showing that *P. penetrans* prefers sandy soils. Peng and Moens (2002) did similar work but with different fertilizers and crops. They found highest *P. penetrans* reproduction and good plant growth when plants were grown in sand and fertilized with 0.75 g/dm^3 M77 (containing 20% N, 20% P_2O_5, 20% K_2O and balanced trace elements). Sandy soil have a light soil texture and its conditions affect positively on the infectivity and reproduction of *P. penetrans* (Castillo and Vovlas, 2007; Zasada and Weiland, 2015). Pinkerton (1983) found significant correlation between soil texture, root population of *P. penetrans* on peppermint and peppermint hay yield. Yield was negatively correlated with percentage of sand and positively correlated with percentage of silt. *Pratylenchus penetrans*

soil and root populations were positively correlated with sand and negatively correlated with silt (Pinkerton, 1983). Townshend (1972) studied the effect of edaphic factors on penetration of corn roots by *P. penetrans* and *P. minyus* in three soils. He found that lower bulk densities favoured root penetration by both *Pratylenchus* species, nematode penetration of roots in sandy loam was greater than in silt loam and loam.

The observation of the negative correlation between *P. penetrans* reproduction and fertilizer rate in this study suggests that nutrient rich environments may negatively influence *P. penetrans* reproduction. Previous studies have shown that host nutrition may cause changes in soil chemistry that adversely affect nematode mobility and pathogenicity (Rodriguez-Kabana, 1986; Seifi and Bide, 2013; Al-Hazmi and Dawabah, 2013). Melakeberhan *et al*. (1997) studied the effect of plant nutrition (N:P:K, 20:20:20) on *P. penetrans* infection of *Prunus avium* rootstocks. They found that the optimum nutrient regime resulted in greater soil nutrient levels and plant growth; but fewer *P. penetrans* than under the deficient nutrient regime. The impact of host nutrition also varies according to nematode species. For example, Miller (1976) found urea inhibited *P. penetrans* and *Hoplolaimus* spp. but did not affect *Tylenchorhynchus* spp.

4.4.2 Comparison of soil substrates to optimize aeration

Considerable effect of growth substrates was observed on early seedling growth and final nematode numbers. Plants grown in a mixture of silver sand, clay and field soil (4:1:1, v:v:v) showed optimum early seedling growth and higher final nematode numbers compared to plants grown on silver sand and vermiculite (1:1, v:v). This difference in results could most likely be attributed to the differences in soil texture which improved the soil porosity and hence favored aeration for plant and nematode growth. The mixture of growth substrate used in this study may also improve nematode movement and infectivity, optimum moisture and nutrient holding capacity as reported previously (Townshend, 1972; Pinkerton, 1983).

Chapter III

Host suitability of different cover crops for *Pratylenchus penetrans*

1 Introduction

In this chapter, cover crops refers to the crops planted between cycles of the main cash crops or intercropped with cash crops for multiple purposes namely improving the soil biological, physical and chemical properties (Dabney *et al*., 2001; Liua *et al*., 2005), protect loss of nutrients (Weinert *et al*., 2002), reduce soil erosion (Dabney *et al*., 2001), suppress weeds (Brust *et al*., 2014) and pathogens (Weibull *et al*., 2005). Cover crops are also reported to enhance nematode-antagonistic microorganisms by providing a more favorable environment for microbial activity (Kloepper *et al*., 1991). Besides these, cover crops suppress a number of plant parasitic-nematodes. Uses of cover crops that are poor or non-hosts have been shown to reduce *P. penetrans* population below damaging levels (Marks and Townshend, 1973; Miller, 1978; Kimpinski *et al*., 2000; Belair *et al*., 2007). Therefore, identification and evaluation of cover crops that have potential for management of *P. penetrans* is necessary. The objective of this experiment was to determine the host suitability of different cover crops to *P. penetrans*.

2 Materials and methods

Two separate experiments were performed to determine the effect of different cover crops on reproduction of *P. penetrans* under greenhouse conditions.

2.1 Experiment 1: Host suitability of different cover crops for *Pratylenchus penetrans*

Plant material and growth conditions

The eleven cover crops tested in the first experiment are listed in Table 1. All plants were grown in 700 ml pots filled with a mixture of steam sterilized silver sand and field soil, and heat sterilized clay gravel (4:1:1, v:v:v). To obtain a similar growth stage of plants at the time of nematode inoculation, cover crops were sown at different days before inoculation based on their growth characters (Table 1). Ten seeds of each cover crop were sown per pot, of which the five best developed plants were used for the experiment and the others were removed. The experimental design was a randomized complete block and each treatment was replicated eight times. Cover crops were grown in the greenhouse at a temperature range of 22-25°C and regularly watered as needed. Plants were fertilized weekly beginning from one week after inoculation with 0.3% Wuxal Super liquid fertilizer (N:P:K, 8:8:6 + microelements).

Table 1: Cover crops tested in the first greenhouse experiment for their host status for *Pratylenchus penetrans*. Maize and French marigold were included as positive and negative controls, respectively.

Common name	Botanical name	Cultivar name	GDBI[1]
Bird vetch	*Vicia cracca*	-	10
Bristle oat	*Avena strigosa*	AS08@	10
Bristle oat	*Avena strigosa*	Silke	10
Crimson clover	*Trifolium incarnatum*	Lexa	15
Daikon radish	*Raphanus sativus* var. *longipinnatus*	Structurator	10
Fodder radish	*Raphanus sativus* var. *oleiformis*	Doublet	10
Fodder radish	*Raphanus sativus* var. *oleiformis*	RSA S644	10
Fodder radish	*Raphanus sativus* var. *oleiformis*	RSA S1034	10
Oat	*Avena sativa*	JOB07-001	10
Maize	*Zea mays*	Padrino	8
French marigold	*Tagetes patula*	Single Gold	14

[1]GDBI = Growing days before inoculation

Nematode inoculum

The nematode inoculum was obtained from a pure culture of *P. penetrans* that has been reared on carrot discs in an incubator at 25±1°C. Specimens were extracted with the Baermann funnel technique. At the 2- to 3-true-leaf stage of plants, each pot was inoculated with 810 mixed stages of *P. penetrans* in 10 ml water at five holes around the stem.

Assessments of nematode reproduction

Ten weeks after inoculation, the root systems were removed from the pots, washed, weighed, cut into 1 cm long pieces and placed on Baermann dishes in a misting chamber for 2 weeks. Soil from each pot was evenly mixed and nematodes were extracted from a 250 ml subsample using the centrifugal flotation method (EPPO, 2013). Collected suspensions from root and soil extraction were examined for *P. penetrans* by counting 1 ml aliquots under a compound light microscope at 40-fold magnification. The final nematode population was calculated by adding nematode numbers from root and soil samples. The reproduction rates (Pf/Pi = final population/initial population) for each sample were determined. Based on the Pf/Pi the cover crops were categorized under 5 categories (modified after Mbiro and Wesemael, 2016) as

follows: non-host = (Pf/Pi < 0.15), poor host = (Pf/Pi < 1.0 ≥ 0.15), maintenance host = (Pf/Pi < 1.5 ≥1.0), Good host (Pf/Pi <10.0 ≥1.5) and excellent host (Pf/Pi ≥10.0).

2.2 Experiment 2: Host suitability of different cover crops for *Pratylenchus penetrans*

The cover crops tested in the second experiment are listed in Table 2. Growth conditions and procedures were the same as described for experiment 1 except that each pot was inoculated with 788 mixed stages of *P. penetrans*.

Table 2: Cover crops tested in the second greenhouse experiment for their host status for *Pratylenchus penetrans*. Lentil and French marigold were included as positive and negative controls, respectively.

Common name	Botanical name	Cultivar name	GDBI[1]
Buckwheat	*Fagopyrum esculentum*	Kora	10
Common bird's foot	*Ornithopus sativus*	Unknown	14
Common vetch	*Vicia sativa* subsp. nigra	Jose	10
Fodder radish	*Raphanus sativus* var. *oleiformis*	RSA S1037	10
Italian ryegrass	*Lolium italicum*	Tetraflorum	12
Rapeseed	*Brassica napus*	(Forage type)	11
Rapeseed	*Brassica napus*	(Summer oil type)	10
Sunflower	*Helianthus annuus*	(Oil type)	10
Lentil	*Lens culinaris*	(Forage type)	10
French marigold	*Tagetes patula*	Single Gold	14

[1]GDBI = Growing days before inoculation

Data analysis

Data were analysed using ANOVA (SPSS 20) and means were separated using Duncan's Multiple Range Test at $P \leq 0.05$. Nematode counts were log transformed prior to analysis.

3 Results

3.1 Experiment 1: Host suitability of different cover crops for *Pratylenchus penetrans*

Cover crops significantly ($P < 0.05$) affected reproduction of *P. penetrans* (Table 3). The reproduction rate varied between 0.0 in French marigold and 19.2 in bird vetch. Maize did not support nematode reproduction. Generally, more nematodes were extracted from the roots than from the soil. Nematode numbers recovered from soil under daikon radish and fodder radish (FR) cv. Doublet were significantly higher than those under the other cover crops. Bird vetch had the highest final nematode numbers with 15,583 nematodes followed by crimson clover and oat with 6,854 and 3,330 nematodes, respectively. Final nematode numbers of AS08@, Silke and daikon radish were not significantly different from each other but lower than for the other cover crops. Nematode reproduction rate was highest on bird vetch with Pf/Pi of 19.2 and thus bird vetch was rated as excellent host. Crimson clover, oat, FR cv. Doublet, FR cv. RSA S644 and FR cv. RSA S1034 were classified as good hosts. The bristle oat cultivars AS08@ and Silke and daikon radish were maintenance hosts with Pf/Pi of 1.08, 1.16, and 1.35, respectively. Overall, none of the cover crops tested except French marigold reduced reproduction of *P. penetrans*.

Table 3: Final numbers of *Pratylenchus penetrans* in root and soil and reproduction rate (Pf/Pi) 10 weeks after inoculation with 810 mixed stages, and host status of different cover crops. Maize and French marigold were included as positive and negative controls respectively.

Cover crops	Final numbers of *P. penetrans*			Pf/Pi	Host status
	Root	Soil	Total		
Bird vetch	15,483 ± 8,690[2] g[3]	100 ± 47 cde	15,583 ± 8,701 g	19.2	Excellent
Bristle oat AS08@	810 ±102 b	62 ± 29 d	872 ±109 b	1.08	Maintenance
Bristle oat Silke	926 ± 266 b c	16 ± 7 b	942 ± 263 b	1.16	Maintenance
Crimson clover	6,761 ± 3,477 f	93 ± 61 cd	6,854 ± 3,527 f	8.46	Good
Daikon radish	691 ± 194 b	403 ± 275 f	1,094 ± 296 bc	1.35	Maintenance
FR[1] Doublet	1,383 ± 822 cd	350 ± 139 f	1,733 ±751 d	2.14	Good
FR[1] RSA S644	1,498 ± 534 d	128 ± 63 de	1,626 ± 589 cd	2.01	Good
FR[1] RSA S1034	1,443 ± 641 d	179 ± 74 e	1,622 ± 640 cd	2.00	Good
Oat JOB07–001	3,243 ±1,165 e	87 ± 43 cd	3,330 ±1,163 e	4.11	Good
Maize	30 ± 9.3 a	21 ± 11 b	51 ± 15 a	0.06	Non-host
French marigold	0.0 a	0.0 a	0.0 a	0.00	Non-host

[1]FR = Fodder radish, [2]Mean and standard deviation (n = 8), [3]Different letters indicate significant differences according to Duncan's Multiple Range Test at $P \leq 0.05$.

3.2 Experiment 2: Host suitability of different cover crops for *Pratylenchus penetrans*

Cover crops significantly ($P < 0.05$) affected reproduction of *P. penetrans* (Table 4). The reproduction rate ranged from 0.0 in French marigold to 40.3 in lentil. Higher numbers of nematodes were extracted from the roots compared to the soils. The average number of nematodes recovered from roots ranged from 0 in French marigold to 20,680 in common vetch. The average number of nematodes per 700 ml soil was highest under buckwheat with 459 nematodes followed by fodder radish cv. RSA S1037 with 427 nematodes and lowest under Italian ryegrass with 42 nematodes. The susceptible control lentil had the highest final *P. penetrans* numbers with 31,792 nematodes followed by common vetch and 20,862 nematodes. Final *P. penetrans* numbers of common bird's foot and sunflower were not significantly different from each other but they were significantly higher than those of the other cover crops except lentil and common vetch. Final nematode numbers of buckwheat, fodder radish, and Italian ryegrass were not significantly different from each other and those of forage rape and rape seed, but final nematode numbers of the rape seed was significantly higher than that of forage rape. Lentil and bird vetch were excellent hosts for *P. penetrans* with Pf/Pi of 40.3 and 26.5, respectively, With the exception of French marigold, all other cover crops were good hosts with Pf/Pi values ranging from 2.43 for forage rape to 6.01 for common bird's foot.

Table 4: Final numbers of *Pratylenchus penetrans* in root and soil and reproduction rate (Pf/Pi) 10 weeks after inoculation with 788 mixed stages, and host status of different cover crops. Lentil and French marigold were included as positive and negative controls respectively.

Cover crops	Final number of *P. penetrans*			Pf/Pi	Host status
	Root	Soil	Total		
Buckwheat	1,495 ± 232[1] b[2]	459 ± 245 f	1,954 ± 284 bc	2.48	Good
Common vetch	20,680 ± 6,068 d	182 ± 116 cd	20,862 ± 6,105 e	26.5	Excellent
Common bird's foot	4,638 ± 1,423 c	98 ± 45 c	4,736 ± 1,422 d	6.01	Good
Fodder radish	1,631 ± 335 b	427 ± 230 ef	2,058 ± 419 bc	2.61	Good
Forage rape	1,823 ± 806 b	95 ± 39 c	1,918 ± 793 b	2.43	Good
Italian ryegrass	1,993 ± 217 b	42 ± 21 b	2,035 ± 222 bc	2.58	Good
Rapeseed	2,934 ± 2,742 b	238 ± 140 de	3,172 ± 2,747 c	4.03	Good
Sunflower	3,504 ± 1,163 c	242 ± 163 de	3,746 ± 1,068 d	4.75	Good
Lentil	31,543 ± 6,704 e	249 ± 139 de	31,792 ± 6,634 f	40.3	Excellent
French marigold	0.0 a	0.0 a	0.0 a	0.00	Non-host

[1]Mean and standard deviation (n = 8), [2]Different letters indicate significant differences according to Duncan's Multiple Range Test at $P \leq 0.05$.

4 Discussion

4.1 Experiment 1: Host suitability of different cover crops for *Pratylenchus penetrans*

Based on nematode reproduction it was possible to classify the host status of cover crops into three groups. Bird vetch turned out to be an excellent host. Good hosts were crimson clover, oat JOB07–001, Doublet, RSA S644, RSA S1034. Maintenance hosts were AS08@ and Silke and Daikon radish. Maize and French marigold which were included as positive and negative control respectively, turned out to be non-hosts.

Maize was used as susceptible control in this study. Unfortunately, maize did not reproduce *P. penetrans*, which contradicts other studies showing maize being an excellent host of *P. penetrans* (Bélair, *et al*. 2002; Mbiro and Wesemael, 2016; Kutywayo and Been, 2006). This difference in results may be due to differences in cultivars or unknown environmental factors. However, different *Pratylenchus* species react differently to maize. Al-Rehiayani and Hafez (1998) reported maize cv. Hybrid 'AP622' as being a maintenance host for *P. neglectus*. In Australia, maize is listed as moderately resistant to *P. neglectus* and *P. thornei* (www.dpi.qld.gov.au) allowing its use to control those species.

In our study, bird vetch was found to be an excellent host for *P. penetrans* allowing 19.2-fold reproduction of the nematode. Those results are in contrast to greenhouse trials by Bélair *et al*. (2007) who achieved a reproduction rate of *P. penetrans* on bird vetch of 0.39 and 0.94 in two separate experiments. Those differences in *P. penetrans* reproduction are most likely be attributed to differences in cultivars used, variation in environmental and experimental conditions or different virulence of the selected *P. penetrans* population.

Crimson clover and oat are described as hosts for *P. penetrans* (Westerdahl *et al*., 1998; Bélair, *et al*., 2002). Those results agree with our findings that crimson clover and oat were good hosts of *P. penetrans* with reproduction rates of 8.5 and 4.1, respectively.

Both black oat cultivars, AS08@ and Silke, were categorized as maintenance hosts with a reproduction rate of 1.0 and 1.2, respectively. However, our results differed from that of Uesugi *et al*. (2014) showing that black oat cultivar 'Hay Oats' was a poor host with a reproduction rate below 1. As discussed before, those differences are most likely be explained by differences in cultivar and nematode population used. Different black oat cultivars have been shown to vary in their response to nematode species and between populations of a given species (Lima *et al*., 2009). In our study fodder radish cultivars Doublet, RSA S644 and RSA

S1034 were good hosts of *P. penetrans*. These results confirm previous findings showing fodder radish as good hosts of *P. penetrans* (Bélair *et al*., 2007; Grabau *et al.,* 2017). In contrast, the closely related daikon radish turned out to be a maintenance host for *P. penetrans*.

4.2 Experiment 2: Host suitability of different cover crops for *Pratylenchus penetrans*

Cover crops were grouped according to *P. penetrans* reproduction. Common vetch turned out to be an excellent host. Good hosts were common bird's foot, forage rape, rapeseed, Italian ryegrass, common vetch, Sunflower, buckwheat and fodder radish. Lentil and French marigold which were included as positive and negative control respectively turned out to be excellent and non-hosts, respectively.

In this study, the highest number of nematodes in the soil was found under buckwheat and the lowest was found besides French marigold under Italian ryegrass. In their study, Marks and Townshend (1973) found the highest and the lowest number of nematodes in the soil under buckwheat and perennial rye grass, respectively. The differences in the number of nematodes in the soil may be attributed to the differences in the rate of degradation of roots of the two hosts (Marks and Townshend, 1973).

Among the cover crops tested lentil and common vetch were rated as excellent hosts with reproduction rate of 40.3 and 26.5, respectively, which is in agreement with previous findings describing lentil (Di Vito *et al.* 2002) and common vetch (Westerdahl *et al*. 1998) as good hosts for *P. penetrans*. Although lentil is a good host for *P. penetrans*, it is a poor host for the often co-occurring *P. neglectus* (Collins, 2013).

Rapeseed was found to be a good host for *P. penetrans*. This is in line with results achieved by Forge (2000), Bélair *et al.* (2002) and Lamondia (2006) describing rapeseed as host for *P. penetrans*. If rapeseed cultivars differ in their host status for *P. penetrans* is not known. For *P. neglectus* no such differences in cultivars was observed (Smiley, 2014). The effect of different rapeseed cultivars on nematode reproduction was tested with *P. neglectus*. In this regard, all four tested rapeseed cultivars 'Dwarf Essex', 'Salut', 'Hyola 401' and 'Goldrush' turned out to be good hosts for *P. neglectus* (Smiley, 2014). Quite interestingly, all four cultivars were minor to non-hosts for *P. thornei* (Smiley, 2014). In field trials, Tailor *et al.* (2000) screened 81 cultivars of field crops and pasture species including 7 cultivars of rape seed for their

resistance against *P. neglectus*. The results showed that all cultivars of *B. napus* tested were susceptible to *P. neglectus*.

The good host status of buckwheat, Italian ryegrass and oilseed radish for *P. penetrans* shown here is in line with previous studies describing buckwheat (Dunn and Mai, 1973; Markus and Townshend, 1973; Bélair, *et al*. 2002), Italian ryegrass (Markus and Townshend, 1973; Tateishi *et al*. 2014), and oilseed radish (Miller, 1978; Grabau *et al.* 2017) as good hosts of *P. penetrans*.

Research results indicated that some crops supported nematode reproduction as a cover crop in greenhouse experiments, but incorporation of their green manure suppress nematode population density. For instance, brown mustard cultivar 'Pacific Gold' was reported to be a good host for *P. penetrans* (Rudolph *et al*. 2017) and *P. neglectus* (Smiley, 2014), but a poor host for *P. thornei* (Smiley, 2014). On the other hand, a field study in Germany showed that incorporation of another cultivar of brown mustard into soil as a green manure crop reduced the density of a mixture of *P. neglectus* and *P. penetrans* (Daub *et al*., 2008). Similarly, incorporation of cover crops such as buckwheat, oilseed radish and rapeseed, which were confirmed to be good hosts of *P. penetrans* in our study, as a green manure prior to potato has been reported to reduce *P. neglectus* and *M. chitwoodi* population density and increased tuber yield (Hafez and Sundaraj, 2000).

Sunflower was found to be a good host for *P. penetrans.* This is in agreement with previous work by Kaplan *et al*. (1976) showing that sunflower cultivar 'Mamoth' was a good host of *P. penetrans*. However, Di Vito *et al*. (2002) came to contrary conclusions. In their study, sunflower cultivar 'Isoleic' turned out to be a non-host for *P. penetrans*. Those authors tested the host status of sunflower also for other *Pratylenchus* species and found that it was a non-host for *P. pinguicaudatus*, a poor to non-host for three populations of *P. thornei*, but a good host for *P. neglectus*. However, contrary to Di Vito *et al*. (2002) a study by Smiley *et al*. (2014) showed Sunflower cultivar '2PD08' being a poor hosts for *P. neglectus*. Given those contrary and variable results, the host status of sunflower for different *Pratylenchus* species is still unsolved.

Common bird's foot was found to be a good host for *P. penetrans*. Due to the lack of available information, the host status of common bird's foot could not be discussed any further. For *P. neglectus*, Collins *et al*. (2013) showed that common bird's foot cultivars 'Margurita' and 'Cudiz' were resistant and moderately resistant to *P. neglectus*.

Chapter IV

Susceptibility of different cultivars of green manure crops against *Pratylenchus penetrans*

1 Introduction

Plant resistance to plant-parasitic nematodes is an economical and environment friendly control method. However, there are only few sources of resistance in crop species or related wild relatives, which limits this approach in praxis (Boerma and Hussey, 1992). This limitation in sources of resistance in crop species appears to be more pronounced for migratory endo-parasites such as *Pratylenchus* than for sedentary endo-parasites. Generally, very little is known about crop plant resistance to *Pratylenchus* (Roberts, 1982; Boerma and Hussey, 1992). Hence, efficient and continuous screening of existing crop cultivars for their resistance to plant-parasitic nematodes is a key step for identification of resistance sources and their exploitation through breeding.

Several studies indicated that different cultivars of various cover crop species have the ability to suppress certain nematode species (Lindsey and Cairns, 1971; Miller 1978). For instance, different cultivars of black oat have been shown to react differently to different *Meloidogyne* species (Moritz *et al*., 2003; Oka *et al*., 2003; Carneiro *et al*., 2006; Borges *et al*., 2009; Lima *et al*., 2009). Similar information on cover crops for *Pratylenchus* is still lacking. Thus the objective of this study was to determine the susceptibility of cultivars of different cover crops against *P. penetrans*.

2 Materials and methods

Four greenhouse experiments were performed to investigate the susceptibility of different cover crops and for the cover crops fodder radish, lentil, common vetch and black oat also different cultivars against *P. penetrans*.

Growth conditions and procedures were the same as described in Chapter III, materials and methods for Experiment 1. Based on the Pf/Pi (final population/initial population) the cover crops were categorized in 5 categories (modified after Mbiro and Wesemael, 2016) as follows: non-host = (Pf/Pi < 0.15), poor host = (Pf/Pi < 1.0 ≥ 0.15), maintenance host = (Pf/Pi < 1.5 ≥1.0), Good host (Pf/Pi <10.0 ≥1.5) and excellent host (Pf/Pi ≥10.0).

2.1 Experiment 1: Susceptibility of fodder radish cultivars (*Raphanus sativus*) and other cover crops against *Pratylenchus penetrans*

The tested cover crops in this experiment are listed in Table 1. Plants were inoculated with 670 mixed stages of *P. penetrans* per pot.

Table 1: Fodder radish cultivars and other cover crops tested in the first greenhouse experiment for susceptibility against *Pratylenchus penetrans*. Lentil and French marigold were included as positive and negative controls, respectively.

Common name	Botanical name	Cultivar name	GDBI[1]
Camelina	*Camelina sativa*	Deder	20
Ribwort plantain	*Plantago lanceolata*	Trio	15
Rocket	*Eruca sativa*	-	20
Fodder radish	*Raphanus sativus* var. *oleiformis*	Edwin	10
	R. sativus var. *oleiformis*	RSA S1035	10
	R. sativus var. *oleiformis*	Angus	10
	R. sativus var. *oleiformis*	Contra	10
	R. sativus var. *oleiformis*	Colonel	10
	R. sativus var. *oleiformis*	Defender	10
	R. sativus var. *oleiformis*	Siletina	10
Lentil	*Lens culinaris*	(Forage type)	10
French marigold	*Tagetes patula*	Single Gold	14

[1]GDBI = Growing days before inoculation

2.2 Experiment 2: Susceptibility of common vetch (*Vicia sativa*) and lentil (*Lens culinaris*) cultivars against *Pratylenchus penetrans*

The tested cultivars of common vetch and lentil are listed in Table 2**.** Plants were inoculated with 900 mixed stages of *P. penetrans* per pot.

Table 2: Common vetch and lentil cultivars tested in the second greenhouse experiment for susceptibility against *Pratylenchus penetrans*. Common vetch cv. Jose, lentil (forage type), and French marigold were included as positive and negative controls, respectively.

Common name	Botanical name	Cultivar name	GDBI[1]
Common vetch	*Vicia sativa*	Ahelia	10
		Libia	10
		Rada	10
		Wellunger	10
	V. sativa subsp. nigra	Jose	10
Lentil	*Lens culinaris*	Gestreifte Linse	10
		Marmorierte Linse	10
		Linse Pisarecka perla	10
		Schwarze Linse	10
		Forage type	10
French marigold	*Tagetes patula*	Single Gold	14

[1] GDBI = Growing days before inoculation

2.3 Experiment 3: Susceptibility of black oat (*Avena strigosa*) cultivars against *Pratylenchus penetrans* – part A

A total of 14 black oat cultivars were evaluated for their susceptibility against *P. penetrans* in experiment 3 and experiment 4.

The black oat cultivars tested in experiment 3 are listed in Table 3. Plants were inoculated with 630 mixed stages of *P. penetrans* per pot.

Table 3: Black oat cultivars tested in the third greenhouse experiment for susceptibility against *Pratylenchus penetrans*. Oat and French marigold were included as positive and negative controls, respectively.

Common name	Botanical name	Cultivar name	GDB[1]
Black oat	*Avena strigosa*	AS08@	10
		Bajex (HS 53	10
		Iendex	10
		Luxurial	10
		Panache	10
		Pratex	10
		Silke	10
		Tradex (HS 61)	10
Oat	*Avena sativa*	JOB 07-001	10
French marigold	*Tagetes patula*	Single Gold	14

[1] GDBI = Growing days before inoculation

2.4 Experiment 4: Susceptibility of black oat (*Avena strigosa*) cultivars against *Pratylenchus penetrans* – part B

The black oat cultivars tested in experiment 4 are listed in Table 4. Plants were inoculated with 800 mixed stages of *P. penetrans* per pot.

Table 4: Black oat cultivars tested in the fourth greenhouse experiment for susceptibility against *Pratylenchus penetrans*. Oat and French marigold were included as positive and negative controls, respectively.

Common name	Botanical name	Cultivar name	GDBI[1]
Black oat	*Avena strigosa*	Biotex	10
		Codex (HS 52)	10
		Duplex	10
		Lunex	10
		Otex	10
		Pratex	10
		Xerex	10
Oat	*Avena sativa*	JOB 07-001	10
French marigold	*Tagetes patula*	Single Gold	14

[1] GDBI = Growing days before inoculation

3 Results

3.1 Experiment 1: Susceptibility of fodder radish (*Raphanus sativus*) cultivars and other cover crops against *Pratylenchus penetrans*

Considerable differences in reproduction of *P. penetrans* were found between the different cover crops tested but not between fodder radish cultivars (Table 5). Reproduction rates ranged from 0.0 for French marigold to 25.0 for lentil. Final nematode numbers of those two cover crops were 0.70 and 19,724, respectively. Final nematode numbers of ribwort plantain and French marigold were significantly lower than for all other cover crops, but not significantly different from each other. Similar, reproduction rate on ribwort plantain was almost as low as for French marigold and reproduction rate of both cover crops was significantly lower than for all other cover crops tested. *Pratylenchus penetrans* reproduced poorly on camelina and was only able to maintain the total nematode number on rocket. Reproduction rates on fodder radish cultivars ranged from 1.59 for FR Defender to 2.43 for FR Siletina. All fodder radish cultivars were considered good hosts. Final nematode numbers of fodder radish cultivars were not significantly different between each other and from rocket, but significantly higher than for camelina and ribwort plantain. In general, numbers of *P. penetrans* recovered from the root were higher than numbers extracted from the soil with the exception of rocket and FR RSA S1035, where nematode numbers in the soil were higher than in roots. On lentil, which was considered an excellent host, more than 99% of the total *P. penetrans* counts originated from the roots.

Table 5: Final numbers of *Pratylenchus penetrans* in root and soil, reproduction rate (Pf/Pi) and host status of different cover crops and fodder radish (FR) cultivars 10 weeks after inoculation with 670 mixed stages. Lentil and French marigold were included as positive and negative controls, respectively.

Cover crops and fodder radish (FR) cultivars	Final numbers of *P. penetrans*			Pf/Pi	Host status
	Root	Soil	Total		
Camelina	354 ± 346[1] c[2]	189 ± 144 c	543 ± 390 b	0.69	Poor
Rocket	409 ± 276 c	448 ± 211 cd	857 ± 380 bc	1.09	Maintenance
Ribwort plantain	30 ± 33 b	0.4 ± 1.0 a	30 ± 32 a	0.04	Non-host
FR Angus	1,171 ± 398 d	655 ± 395 de	1,825 ± 670 c	2.32	Good
FR Colonel	1,095 ± 299 d	697 ± 257 de	1,792 ± 306 c	2.27	Good
FR Contra	1,054 ± 585 d	448 ± 226 cd	1,502 ± 590 c	1.91	Good
FR Defender	931 ± 487 d	319 ± 186 cd	1,249 ± 597 c	1.59	Good
FR Edwin	871 ± 480 d	399 ± 142 cd	1,270 ± 388 c	1.61	Good
FR RSA S1035	715 ± 424 d	879 ± 297 e	1,594 ± 488 c	2.02	Good
FR Siletina	1,184 ± 569 d	735 ± 618 de	1,919 ± 1,028 c	2.43	Good
Lentil	19,696 ± 2,766 e	28 ± 20.0 b	19,724 ± 2,776 d	25.00	Excellent
French marigold	0.0 a	0.7 a	0.7 a	0.00	Non-host

[1]Mean and standard deviation (n = 8), [2]Different letters in each column indicate significant differences according to Duncan's Multiple Range Test at $P \leq 0.05$.

3.2 Experiment 2: Susceptibility of common vetch (*Vicia sativa*) and lentil (*Lens culinaris*) cultivars against *Pratylenchus penetrans*

Pratylenchus penetrans reproduced well on common vetch and lentil. However, nematode numbers and reproduction rate differed between crop species and cultivars (Table 6). The negative control French marigold did not support nematode reproduction. On common vetch, final nematode numbers were highest on Libia with 44,097 nematodes followed by Ahelia with 35,011 and lowest on Jose with 10,116 nematodes. Final nematode numbers on cultivar Libia was not significantly ($P > 0.05$) different from that of Ahelia, but significantly higher than those on the other common vetch cultivars. Reproduction rates on common vetch ranged from 11.2 on Jose to 49.0 on Libbia. On lentil, the final nematode numbers varied from 17,192 on cultivar Gestreifte to 30,128 on Pisarecka. Corresponding reproduction rates were 19.1 on Pisarecka perla and 33.5 on Gestreifte. Final nematode numbers on cultivars Gestreifte and Forage type were significantly higher than on Pisarecka perla. In both common vetch and lentil about 99% of the total numbers of nematodes were extracted from the root

fraction. With the exception of cultivar Jose, *P. penetrans* reproduced better on common vetch than on lentil. Nevertheless, both plant species are considered excellent hosts of *P. penetrans*.

Table 6: Final numbers of *Pratylenchus penetrans* in root and soil, reproduction rate (Pf/Pi) and host status of different common vetch and lentil cultivars 10 weeks after inoculation with 900 mixed stages. Common vetch cv. Jose, lentil (forage type), and French marigold were included as positive and negative controls, respectively.

Cover crops cultivars	Final numbers of *P. penetrans*			Pf/Pi	Host status
	Root	Soil	Total		
Common vetch cultivars					
Ahelia	34,759 ± 11,888[1] ef[2]	252 ± 227 bcd	35,011 ± 11,873 ef	38.90	Excellent
Libia	43,788 ± 8,310 f	309 ± 176 de	44,097 ± 8,389 f	49.00	Excellent
Rada	29,123 ± 10,267 e	331 ± 104 e	29,454 ± 10,357 e	32.70	Excellent
Wellunger	26,622 ± 15,345 de	343 ± 160 e	26,965 ± 15,409 de	30.00	Excellent
Jose	9,983 ± 3,125 b	133 ± 61 bc	10,116 ± 3,114 b	11.20	Excellent
Lentil cultivars					
Gestreifte	30,023 ± 8,192 e	105 ± 42bcd	30,128 ± 8,189 e	33.50	Excellent
Marmorierte	18,588 ± 4,307 cd	196 ± 120 bcd	18,784 ± 4,309 cd	20.90	Excellent
Pisarecka perla	17,066 ± 4,158 c	126 ± 123 b	17,192 ± 4,148 c	19.10	Excellent
Schwarze	18,823 ± 790 cd	175 ± 63 bcd	18,998 ± 810 cd	21.10	Excellent
(Forage type)	24,678 ± 4,530 de	168 ± 123 bcd	24,846 ± 4,514 de	27.60	Excellent
French marigold	0.0 a	0.7 a	0.7 a	0.00	Non-host

[1]Mean and standard deviation (n = 8), [2] Different letters in each column indicate significant differences according to Duncan's Multiple Range Test at $P \leq 0.05$.

3.3 Experiment 3: Susceptibility of black oat (*Avena strigosa*) cultivars against *Pratylenchus penetrans* – part A

All tested black oat cultivars significantly suppressed the reproduction of *P. penetrans* compared to the oat cultivar JOB 07-001. The reproduction rate on oat was 6.70, while in the black oat cultivars the reproduction rate varied from 0.59 for cultivar Jendex to 1.33 for cultivar Panache (Table 7). French marigold did not support *P. penetrans* reproduction. Numbers of *P. penetrans* were mainly collected from the root but not the soil fraction. Panche showed a significantly higher final number of *P. penetrans* (837) than the other black oat cultivars. Except for Panche and Silke all black oat cultivars reduced the nematode

population. Silke and Panche were maintenance hosts with Pf/Pi of close to 1, while the rest of black oat cultivars tested were poor hosts with Pf/Pi of < 1.

Table 7: Final numbers of *Pratylenchus penetrans* in root and soil, reproduction rate (Pf/Pi) and host status of different black oat cultivars 10 weeks after inoculation with 630 mixed stages. Oat and French marigold were included as positive and negative controls respectively.

Cover crops	Final numbers of *P. penetrans*			Pf/Pi	Host status
	Root	Soil	Total		
AS08@	480 ± 99.0[1] b[2]	15.8 ± 9 b	496 ± 98.4 b	0.79	Poor
Bajex (HS 53)	399 ± 122 b	15.8 ±12 b	415 ± 130 b	0.60	Poor
Index	365 ± 53.2 b	7 ± 8 ab	372 ± 59.5 b	0.59	Poor
Luxurial	369 ± 95.5 b	22.8 ± 17 b	386 ± 96.0 b	0.61	Poor
Panache	814 ± 209 c	22.8 ± 17 b	837 ± 213 c	1.33	Maintenance
Pratex (HS 1)	395 ± 94.1 b	10.5 ± 7 ab	406 ± 92.6 b	0.64	Poor
Silke	617 ± 576 b	21 ± 15 b	638 ± 570 b	1.01	Maintenance
Tradex (HS 61)	22.8 ± 21.1 b	469 ± 126 b	492 ± 134 b	0.78	Poor
Oat	4,153 ± 1,917 d	70 ± 34 c	4,223 ± 1,920 d	6.70	Good
French marigold	1.75 ± 4.9 a	0.0 a	1.75 ± 4.9 a	0.00	Non-host

[1]Mean and standard deviation (n = 8), [2] Different letters in each column indicate significant differences according to Duncan's Multiple Range Test at $P \leq 0.05$)

3.4 Experiment 4: Susceptibility of black oat (*Avena strigosa*) cultivars against *Pratylenchus penetrans* – part B

All black oat cultivars significantly reduced the numbers of *P. penetrans* below the initial nematode density (Table 8). Reproduction rates ranged from 0.37 for cultivar Otex to 0.64 for cultivar Biotex. Reproduction rate was highest on the susceptible control common oat with Pf/Pi of 3.61. The final number of nematodes was mainly derived from the root but not the soil fraction. No *P. penetrans* was found in the roots and soils of French marigold. From the total number of *P. penetrans* extracted the number of nematodes extracted from the roots of black oat cultivars ranged between 94% (Xerex) and 99% (Lunex and Duplex). The total nematode numbers on black oat cultivars ranged from 293 on Otex to 511 on Biotex. Nematode numbers of black oat cultivars were not significantly different from each other but they were significantly lower than for the susceptible control common oat with 2,885

nematodes. All black oat cultivars were poor hosts with Pf/Pi values ranging from 0.37 (Otex) to 0.64 (Biotex).

Table 8: Final number of *Pratylenchus penetrans* in root and soil with their respective reproduction rate (Pf/Pi) and host status on different black oat cultivars extracted 10 weeks after inoculation with 800 mixed stages. Oat and French marigold were included as positive and negative controls respectively.

Black oat cultivars	Final number of *P. penetrans*			Pf/Pi	Host status
	Root	Soil	Total		
Biotex	483 ± 71[1] c[2]	28 ± 40 a	511 ± 81 b	0.64	Poor
Codex (HS 52)	352 ± 140 bc	7 ± 13 a	359 ± 140 bc	0.45	Poor
Duplex	354 ± 118 bc	4 ± 10 a	357 ± 118 bc	0.45	Poor
Lunex	338 ± 78 bc	4 ± 10 a	341 ± 80 b	0.43	Poor
Otex	283 ± 79 b	11 ± 21 a	293 ± 78 b	0.37	Poor
Pratex	351 ± 123 bc	7 ± 13 a	358 ± 129 bc	0.45	Poor
Xerex	373 ± 94 bc	25 ± 35 a	397 ± 74 bc	0.50	Poor
Oat	2,791 ± 355 d	95 ± 85 b	2,885 ± 326 d	3.61	Good
French marigold	0.0 a	0.0 a	0.0 a	0.00	Non-host

[1]Mean and standard deviation (n = 8), [2]Different letters in each column indicate significant differences according to Duncan's Multiple Range Test at $P \leq 0.05$.

4 Discussion

4.1 Experiment 1: Susceptibility of fodder radish (*Raphanus sativus*) cultivars and other cover crops against *Pratylenchus penetrans*

Cover crops were categorized into 3 out of 5 possible groups according to *P. penetrans* reproduction. All fodder radish cultivars were found to be good hosts. Maintenance host was rocket. Poor hosts were camelina and ribwort plantain. Lentil and French marigold which were included as positive and negative control respectively turned out to be excellent and non-hosts, respectively**.**

The good host status of all fodder radish cultivars for *P. penetrans* shown here is in agreement with previous findings describing fodder radish as a good hosts for *P. penetrans* (Grabau *et al.*, 2017 and Miller, 1978). In contrast, Mbiro and Wesemael (2016) reported that another fodder radish cultivar, line RsV79/80, was a poor host for *P. penetrans*. This suggests that susceptibility of fodder radish to *P. penetrans* can vary between cultivars, although such variation was not observed in the present study. The effect of different fodder radish cultivars on nematode reproduction was tested with *P. neglectus*. In this regard, 2 oilseed radish cultivars ‘Melodie’ and ‘Trez’ were classified as maintenance hosts for *P. neglectus* with reproduction rates of 1.8 and 1.7 respectively (Al-Rehiayani and Hafez, 1998). According to our rating scheme, these cultivars would have been rated as good hosts.

In our study, rocket was considered a maintenance host for *P. penetrans* with a reproduction rate of 1.09. This is in contrast with previous work by Mbiro and Wesemael (2016) showing that rocket cultivar Nemat was a poor host of *P. penetrans* with a reproduction rate of 0.52. This suggests that rocket cultivars might vary in response to *P. penetrans*.

Camelina and ribwort plantain were poor hosts with reproduction rates of 0.69 and 0.04, respectively. Due to the lack of available information, the host status of camelina and ribwort plantain could not be discussed any further. In a study involving another species of *Pratylenchus*, three cultivars of camelina, i.e. Yellowstone, Blaine Creek and Calena, turned out to be minor to non-host for *P. neglectus* and poor hosts for *P. thornei* (Smiley *et al*. 2014).

4.2 Experiment 2: Susceptibility of common vetch (*Vicia sativa*) and lentil (*Lens culinaris*) cultivars against *Pratylenchus penetrans*

All common vetch and lentil cultivars evaluated were found to be excellent hosts for *P. penetrans.* French marigold which was included as a negative control turned out to be a non-host. Comparing the nematode numbers recovered from roots and soils, about 99% of *P. penetrans* were extracted from the root fraction of both cover crops cultivars. This points out the importance of the root fraction for evaluation of *P. penetrans* numbers.

Our results describing common vetch and lentil as excellent hosts for *P. penetrans* confirm previous findings showing common vetch (Yorston, 1970; Di Vito *et al*. 2002) and lentil (Di Vito *et al.*, 2002) as good hosts of *P. penetrans*. Due to the lack of available information, the host status of common vetch and lentil could not be discussed any further. Also not for *P. penetrans*, but for *P. neglectus* and *P. thornei* differences in cultivar reaction have been shown for common vetch and lentil. In South Eastern Australia, common vetch cultivars Languedoc, Popany and Blanchefleur turned out to be susceptible for *P. negelectus* (Tailor *et al*. 2000) and with the exception of Popany, the other two cultivars plus cultivar 'Morava' were listed as susceptible host for *P. thornei* (Hollaway, 2000).

In a field trial, Hollaway *et al*. (2000) screened 93 crop and pasture cultivars including nine cultivars of lentil for their resistance against *P. thornei*. The results showed that all cultivars of lentil tested were resistant to *P. thornei*. For *P. neglectus*, Collins (2013) and May *et al*. (2016) listed lentil as being a poor host of *P. neglectus.* On the other hand, a study by Smiley (2014) showed that three lentil cultivars ('Skyline', 'Granger' and 'Journey') were minor to good hosts for *P. neglectus* and good to very good hosts for *P. thornei*. In conclusion, there seems to be quite some variability regarding the host status of common vetch and lentil cultivars for different *Pratylenchus* species.

4.3 Experiment 3: Susceptibility of black oat (*Avena strigosa*) cultivars against *Pratylenchus penetrans* – part A

Based on nematode reproduction black oat cultivars could be categorized as maintenance hosts, i.e. Silke and Panche, and poor hosts, i.e. AS08@, Pratex, Jendex, Bajex (HS 53), Tradex (HS 61) and Luxurial. Oat and French marigold, which were included as positive and negative control, turned out to be good host and non-host, respectively**.**

The black oat cultivar AS08@ was found to be a poor host for *P. penetrans*. This is in contrast to our previous findings in Chapter III, 3.1 showing AS08@ as a maintenance host for *P. penetrans*. A possible explanation could be variability in experimental conditions. Our results on the poor host status of AS08@, Pratex, Jendex, Bajex (HS 53), Tradex (HS 61) and Luxurial are in line with previous findings by Uesugi *et al*. (2014) showing that the black oat cultivar 'Hay oats' was a poor host of *P. penetrans*. For other *Pratylenchus* species, bristle oat exhibited the greatest reduction in the population of *P. brachyurus* in greenhouse and field studies (Chiamolera, *et al*., 2012). On the other hand, Inomoto and Asmus (2010) found other black oat cultivars to be maintenance host for *P. brachyurus*. Black oat cultivar Hay oats (*A. strigosa*) was reported as being a poor host of *P. coffeae* (Uesugi *et al*., 2014; Tateishi *et al*., 2016).

In our study, oat was a good host for *P. penetrans* with a reproduction rate of 6.7. This result confirms previous findings showing oat as a good host of *P. penetrans* (Florini and Loria, 1990; LaMondia, 1999; LaMondia *et al*. 2002; Lima *et al*. 2009). Oat has also been reported to be a good host for *P. neglectus* and *P. thornei* (Smiley, 2014).

4.4 Experiment 4: Susceptibility of black oat (*Avena strigosa*) cultivars against *Pratylenchus penetrans* – part B

In this experiment, all seven black oat cultivars tested turned out to be poor hosts of *P. penetrans*. Oat and French marigold which were included as positive and negative control were good and non-hosts, respectively**.**

The poor host status of black oat cultivars in this experiment is in line with our previous results (Experiment 3) showing the majority of black oat cultivars as poor hosts of *P. penetrans*. Black oat cultivar Pratex which was included as a reference poor host in this experiment exhibited poor host status and this confirms our previous finding (Experiment 3) showing Pratex as poor hosts of *P. penetrans*. In this experiment oat was a good host with a reproduction rate of 3.6. This result is not consistent with our previous result (Experiment 3) showing oat as good host with a reproduction rate of 6.7. This difference in result may be due to difference in temperature because the first experiment was conducted in summer with higher temperature reaching 30℃ as opposed to the second experiment, which was conducted during winter.

Chapter V

In vitro and *in vivo* reproductive fitness and pathogenicity of seven *Pratylenchus penetrans* isolates from different geographical locations

1 Introduction

The pathogenicity of an organism, i.e. its ability to cause disease, is determined by the combination of two major factors: reproductive fitness which is defined as the ability of a species or population to multiply on a specific host plant and virulence, which is defined as the damage potential (Shaner *et al*., 1992; Sarah *et al*., 1993; Fallas *et al*., 1995). Reproductive fitness together with virulence have often been used to discriminate biological variants of a species (Inomoto *et al*., 2007).

Several authors reported about the existence of biological variation within and among populations of *Pratylenchus* species (Griffin, 1991; Pinochet *et al*., 1993; France and Brodie, 1996). For example, Slootweg (1956) found that certain populations of *P. penetrans* attacked lilies but not narcissi and suggested to refer to them as biological races. Olhaf (1968) later distinguished two races of *P. penetrans* on tobacco and celery based on their pathogenicity and reproductive fitness. France and Brodie (1995) differentiated two populations of *P. penetrans* (Cornell and Long Island pathotypes) based on their emergence and reproduction. Furthermore, France and Brodie (1996) also examined the extent of variability among 10 populations of *P. penetrans* from diverse geographical locations of South America and found 4 populations distinct based on their emergence and reproduction on selected potato clones.

One option to compare the reproductive fitness of *Pratylenchus* species under standardized conditions is the axenic carrot disc technique. This technique is commonly used for monoxenic culture of *Pratylenchus* species (Moody *et al*., 1973; Kagoda *et al*., 2010; Santos *et al*., 2012) and has been successfully applied to describe intraspecific variability in reproductive fitness for a number of *Pratylenchus* species (Pinochet *et al*., 1994; Castillo *et al*., 1998; Tuyet *et al*., 2013). Several studies have shown that differences in reproductive fitness on carrot disc cultures were related to differences in pathogenicity on plant roots in pot experiment (Sarah *et al*., 1993; Pinochet *et al*., 1994; Fallas *et al*., 1995). Moreover, the correlation between biological and genetic variability in several *Pratylenchidae* has also been established (Hahn *et al*., 1994; Fallas *et al*., 1996; Sarah and Fallas; 1996; Hafez *et al*., 1999;

Agudelo *et al*., 2005). However, information on intraspecific differences in reproductive fitness on carrot discs and established relationship between biological and genetic diversity in *P. penetrans* is scarce.

The reproductive fitness of *P. penetrans* populations is of key importance when it comes to non-chemical nematode management, such as growing poor host crops or resistant cultivars (Roberts, 1982; Starr *et al*., 2002; Agudelo *et al*., 2005). Hence, understanding the biological and genetic diversity within nematode populations is an important requirement for choosing the optimum control measure. Therefore, the objective of this study was to determine the reproductive fitness and pathogenicity of seven isolates of *P. penetrans* collected from different geographical locations in Europe. Here we use the term 'isolate' to describe *P. penetrans* collected from soils of different geographical locations that were multiplied from single females on carrot disc culture.

2 Materials and methods

2.1 Establishment of *Pratylenchus penetrans* isolates

As described in Chapter II materials and methods for Experiment 2.3.3 *Pratylenchus* isolates were raised from single females extracted from soil samples of different geographical locations. Isolates were maintained by repeated propagation on carrot discs. For this study, seven isolates of *P. penetrans* were selected that had been previously identified by morphological and molecular means (Table 1).

Table.1: Geographical origin of *Pratylenchus penetrans* isolates

Geographical origin	Isolate code
Germany (Münster)	MN
Germany (Witzenhausen)	WZ
Germany (Bonn)	BN
Belgium	BL
United Kingdom	UK
France	FR
The Netherlands	NL

2.2 *In vitro* reproductive fitness of *Pratylenchus penetrans*

Nematode inoculum

To compare the reproductive fitness of those seven *P. penetrans* isolates, for each isolate ten carrot disc cultures in 5 cm sterile glass Petri dishes were prepared. Isolates were surface sterilized using the optimized method described in Chapter II, Experiment 2.1. Each carrot disc was inoculated with ten surface sterilized females of the respective isolate. The Petri dishes were sealed with parafilm, arranged in a randomized complete block design and incubated at 25±1°C in the dark.

Assessment of the nematode reproduction

The reproduction of *P. penetrans* isolates on carrot discs was determined 80 days after inoculation. Nematodes were collected from the Petri dishes and carrot discs by rinsing them off with 5 ml water. Based on the results from experiments in Chapter II, 3.2.1 Experiment 2, the carrot discs were chopped with a scalpel in 8 pieces and transferred into a 50 ml tube with 20 ml tap water. The suspension was then macerated using an Ultra Turrax disperser for two times of 7 seconds each. The macerate was passed through a 100 µm sieve into a 200 ml glass beaker. The nematode suspension in the beaker was left to settle for 24 h. Then, the nematode suspension was adjusted to 30 ml and the number of eggs, juveniles, males and females in 1 ml aliquot was counted under a compound light microscope at 40-fold magnification. Carrot residues retained on the 100 µm sieve were further extracted for 72 h using Baermann funnels. Suspensions collected from the Baermann funnels were counted in the same way and nematode counts were added to the results obtained from the filtrate suspensions.

2.3 *In vivo* reproductive fitness of *Pratylenchus penetrans*

In vivo reproductive fitness of the seven *P. penetrans* isolates was evaluated in a repeated greenhouse experiment using lentil (*Lens culinaris*) and common vetch (*Vicia sativa* subsp. *nigra*) as test plants. Those two cover crops were selected as good hosts based on results of preliminary greenhouse trials. The experiment included 7 *P. penetrans* isolates x 2 cover crops x 8 replicates. Pots were set up as a randomized complete block. Growth conditions and procedures were the same as described in Chapter III, materials and methods for Experiment 1. Pots were inoculated with 800 mixed mobile stages of the respective *P. penetrans* isolate.

2.4 Pathogenicity of *Pratylenchus penetrans* isolates

Plant material and growth conditions

The pathogenicity of the seven *P. penetrans* isolates was tested on four crops. Carrot (*Daucus carota* cv. Nerac) and alfalfa (*Medicago sativa*) were selected as susceptible/intolerant control plants based on literature and preliminary greenhouse experiments. Fodder radish (*Raphanus sativus* var. *oleiformis* cv. RSA S1037) represented a susceptible cover crop with unknown tolerance status. French marigold (*Tagetes patula* cv. Single Gold) was used as non-host control. Pots were filled each with a 215 ml mix of steam-treated silver sand, field soil and clay gravel (4:1:1, v:v:v). Five seeds per pot of carrot, alfalfa, fodder radish or French marigold were sown at 17, 20, 10 or 14 days before inoculation. Different sowing dates were chosen to obtain similar growth stages at the day of *P. penetrans* inoculation. Seedlings were thinned to one plant per pot after emergence. The experimental design was 7 isolates of *P. penetrans* x 4 test plants x 3 inoculum levels x 5 replicates. Thus, the total experiment consisted of 420 pots. Pots were set up as a randomized complete block design. Growth conditions in the greenhouse were the same as described in Chapter III, materials and methods for Experiment 1. Soil temperature was recorded using a Em50 data logger (UMS AG, Munich, Germany).

Nematode inoculum

The nematode inoculum was obtained from pure cultures of the seven *P. penetrans* isolates that had been reared on carrot discs in an incubator at 25±1°C. Specimens were extracted with the Baermann funnel technique. Nematode suspensions were adjusted to the intended inoculum levels. At the 2- to 3-true-leaf stage of plants, pots were inoculated with mixed stages of *P. penetrans* at the level of 0, 5,000 or 10,000 nematodes/plant. Inoculum was given in 10 ml water at five holes around the stem.

Assessments of plant damage and nematode reproduction

The plants were grown for ten weeks after nematode inoculation. At harvest shoots were cut off near the ground and dry weights were measured after oven drying at 105ºC for 48 h. Root systems were removed from the pots, washed and root fresh weights were measured. Carrot taproot fresh weights were measured and quality damages on carrots caused by *P. penetrans* were visually examined and graded on a 0-5 scale: 0 = no damage, 1 = taproot with shortened tips, 2 = moderate stunting, 3 = severe stunting, 4 = small round shaped taproot, 5 = taproot very thin and hairy, stubby and deformed. To extract the nematodes from the roots, the

washed root systems were cut into 1 cm long pieces and placed on Baermann dishes in a misting chamber for 2 weeks. For carrots, instead of using the entire root system, roots collected during sieving of the soil and washing the roots as well as the peel of tap roots were used for Baermann extraction as suggested by Teklu *et al*. (2016).

Following sieving, soil from each pot was evenly mixed and nematodes were extracted from a 100 ml subsample using the centrifugal flotation method (EPPO, 2013). Collected suspensions from root and soil extraction were examined for *P. penetrans* by counting 1 ml aliquots under a compound light microscope at 40-fold magnification. The final nematode population was calculated by adding nematode numbers from root and soil samples. The reproduction rate (Pf/Pi = final population/initial population) for each sample was determined.

Data analysis

Nematode counts were normalized by log transformation prior to analysis. Data were analysed using ANOVA (SPSS 20) and means were separated using Duncan's Multiple Range Test at $P \leq 0.05$.

3 Results

3.1 *In vitro* reproductive fitness of *Pratylenchus penetrans*

Pratylenchus reproductive fitness was measured as the final number of nematodes 80 days after inoculation. All *P. penetrans* isolates reproduced, however, marked differences between populations were observed in total numbers (Figure 1) and relative occurrence of developmental stages (Figure 2). The significantly highest number was received by the Belgium (BL) isolate with 41,149 *P. penetrans* followed by those from Germany (WZ: 8,004 and BN: 4,836) (Figure 1). The lowest number of *P. penetrans* was received from the United Kingdom (UK) isolate with 1,020 nematodes. Isolate UK was significantly different from all other isolates except for isolate MN with 1,384 *P. penetrans*.

Regarding developmental stages, eggs were the most numerous stage for most tested *P. penetrans* isolates, ranging between 49% (WZ) and 62% (BL) (Figure 2). Exceptions were isolates MN and UK which were dominated by juvenile stages reaching 47% and 45% of the total population, respectively. Adult stages, i. e. females and males together, reached between 8% (BL) and 22% (MN) of the population. The juvenile/adult ratio varied between 1.82% (FR) and 3.58% (BL). The male/female ratio varied between 0.56% (BL) and 1.89% (UK).

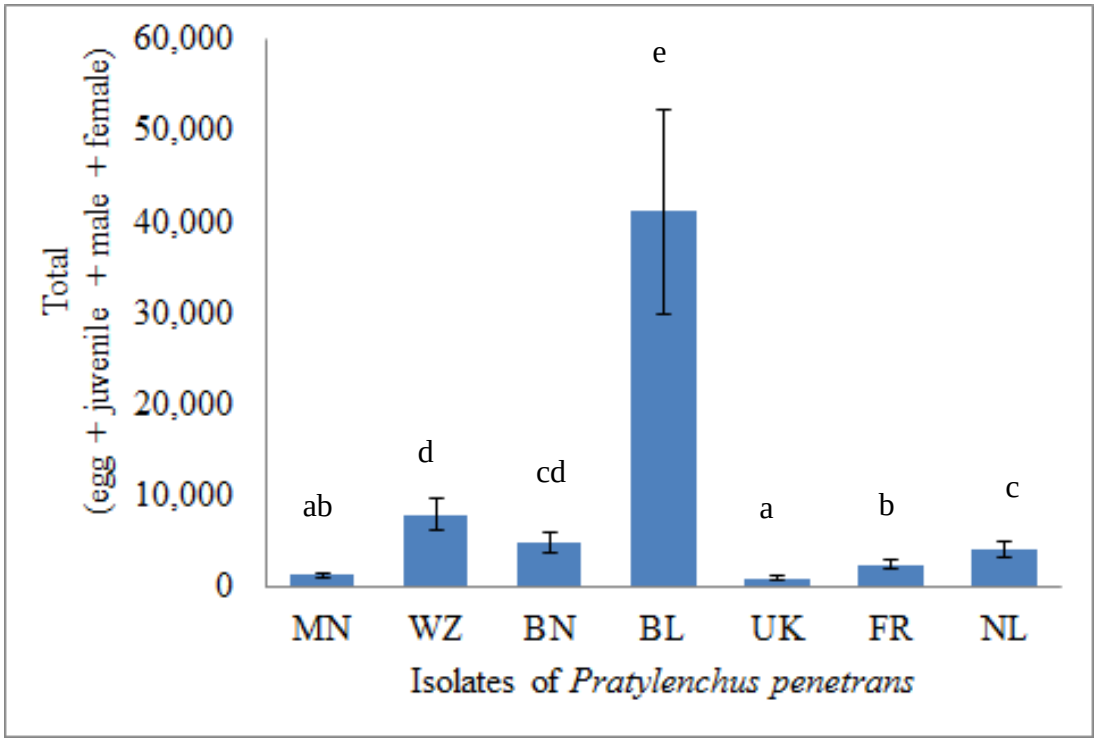

Figure 1: Total number of nematodes of seven *Pratylenchus penetrans* isolates after 80 days on carrot disc culture (n = 10). Different letters indicate significant differences according to Duncan's Multiple Range Test at $P \leq 0.05$.

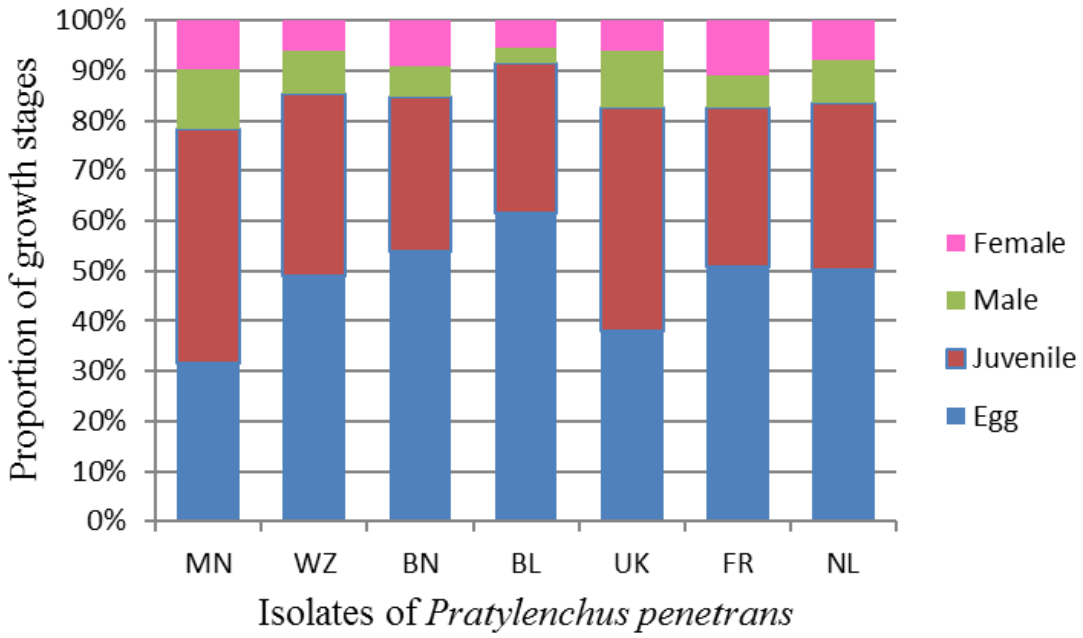

Figure 2: Relative occurrence of developmental stages of seven *Pratylenchus penetrans* isolates following 80 days culturing on carrot discs (n = 10).

3.2 *In vivo* reproductive fitness of *Pratylenchus penetrans*

In vivo reproductive fitness of the seven *P. penetrans* isolates was assessed as final nematode numbers recovered from the root and soil. All *P. penetrans* isolates reproduced on the two tested species common vetch and lentil. However, nematode numbers (Table 2) and reproduction rate (Figure 3) varied depending on *P. penetrans* isolate and crop species.

Final *P. penetrans* numbers on common vetch ranged from 1,830 nematodes for isolate UK to 43,250 nematodes on isolate BL (Table 2). Nematode numbers of those two isolates were significantly different from all other *P. penetrans* isolates which showed intermediate numbers. Quite similar, nematode numbers on lentil were also lowest for isolate UK with 1,810 nematodes and highest for isolate BL with 31,311 nematodes. On lentil, final nematode numbers of the isolates MN, WZ, BN and NL were not significantly ($P > 0.05$) different from each other; however, nematode numbers of all those four isolates were significantly higher than for isolate UK and significantly lower than for isolate BL. Overall, nematode numbers on common vetch were higher than on lentil. For example, nematode numbers of isolates NL and WZ were 2.55 and 2.19 times higher on common vetch than on lentil, whereas for isolate UK, nematode numbers on common vetch were only marginally higher than on lentil. With the exception of isolates BL and UK nematode numbers were significantly different between both crops.

Table 2**:** Final nematode numbers of seven isolates of *Pratylenchus penetrans* on common vetch and lentil extracted 8 weeks after inoculation with 800 mixed stages.

Isolates	Final numbers of *P. penetrans*	
	Common vetch	Lentil
MN	7,660 ± 1,152[1] b[2] E[3]	4,000 ± 444 b B
WZ	12,720 ± 1,597 c FG	5,788 ± 3,152 bc BC
BN	9,115 ± 1,587 b EF	6,150 ± 2,602 bc BCD
BL	43,250 ± 21,016 d H	31,311 ± 6,008 d H
UK	1,830 ± 452 a A	1,810 ± 1,071 a A
FR	13,575 ± 807 c FG	8,510 ± 3,957 c E
NL	17,080 ± 4,434 c G	6,690 ± 1,842 bc CD

[1]Mean and standard deviation (n = 8), [2]Means followed by different small letters are significantly different between isolates within each cover crop ($P < 0.05$). [3]Means followed by different capital letters are significantly different between cover crops ($P < 0.05$).

Besides total nematode numbers, reproduction rate (Pf/Pi) was calculated. The highest reproduction rate on common vetch was achieved by isolate BL with a Pf/Pi of 54.1, which was 23.6 times higher than the lowest Pf/Pi of 2.29 for isolate UK (Figure 3). On lentil, the highest Pf/Pi was 39.1 for isolate BL, which was 17.3 times higher than the lowest Pf/Pi of 2.26 for isolate UK. Average final nematode numbers of WZ, FR, and NL isolates were not significantly ($P > 0.05$) different from each other but differed significantly ($P < 0.05$) from MN and BN.

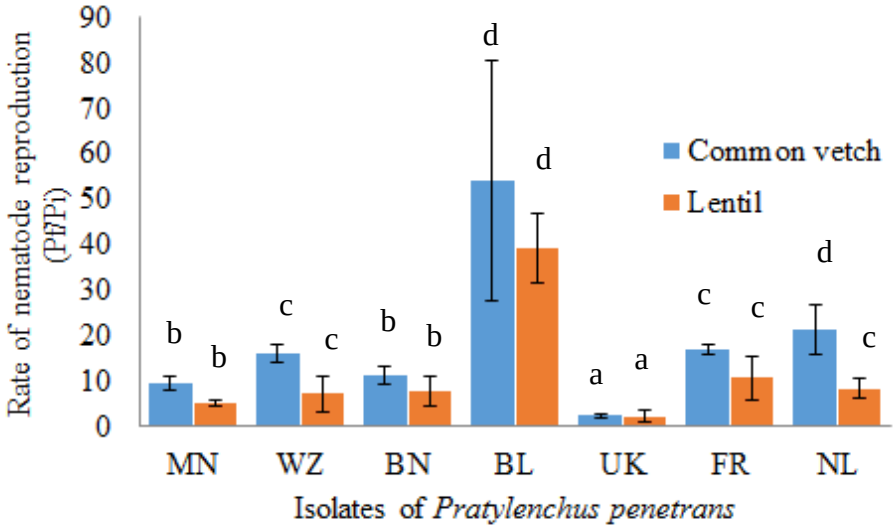

Figure 3: Reproduction rate (Pf/Pi) of seven *Pratylenchus penetrans* isolates on common vetch and lentil (n = 8). Different letters indicate significant differences according to Duncan's Multiple Range Test at $P \leq 0.05$.

3.3 Pathogenicity of *Pratylenchus penetrans* isolates

Pathogenicity of the seven *P. penetrans* isolates was tested in separate experiments on alfalfa, carrot, fodder radish and French marigold. Analysis of variance (ANOVA) was performed for each test plant separately. Differences of nematode numbers between inoculum levels of the same isolate were analyzed by t-test.

Alfalfa

The average root fresh weight and shoot dry weight of alfalfa varied depending on the isolate of *P. penetrans* and inoculum level (Table 3). All plants inoculated with *P. penetrans* showed a lower root fresh weight than the non-inoculated control plants. However, differences were not significant for plants inoculated with isolates MN and UK at low and high inoculum levels (Pi = 5,000 and Pi = 10,000) and isolate WZ at low inoculum level. Plants inoculated with isolates BL and BN showed a significantly higher reduction in root fresh weight compared to plants inoculated with the other isolates of *P. penetrans*. At low nematode inoculum level, the reduction in root fresh weight was highest for isolate FR with 28.4% followed by isolate BN with 27.3%. At high nematode inoculum level, reduction in root fresh weight was highest for isolate BL with 71.3%. Isolates BL, BN, FR and NL reduced root fresh weight significantly at both low and high inoculum levels, whereas isolate WZ reduced root fresh weight only at high inoculum level significantly. Differences in root fresh weight between low and high inoculum levels were significant for all isolates of *P. penetrans* except for isolate BL. Overall, the relation between root fresh weight and inoculum level of *P. penetrans* resulted in a significant negative correlation ($r = -0.538$, $P < 0.001$).

Regarding shoot dry weight, plants inoculated with isolates BL, BN and FR showed a significantly higher reduction in shoot dry weight compared to plants inoculated with the other isolates of *P. penetrans* (Table 3). At low nematode inoculum level, the reduction in shoot dry weight was highest for isolate BL with 14.7% followed by isolate FR with 12.5%. At high nematode inoculum level, reduction in shoot dry weight was highest for isolate BN with 36.7% followed by isolate BL with 33.3%. Isolate BL significantly reduced shoot dry weight at both low and high inoculum levels; whereas isolates WZ, BN, NL and FR reduced shoot dry weight only at high inoculum level significantly. As was the case for root fresh weight, plants inoculated with isolates MN and UK did not show any significant reduction in shoot dry weight. Differences in shoot dry weight between low and high nematode inoculum levels were only significant for isolates BN, BL and NL. Overall, the relation between shoot

dry weight and inoculum level of *P. penetrans* resulted in a significant negative correlation (r = -0.632, $P < 0.001$).

Table 3: Effect of inoculum level of seven *Pratylenchus penetrans* isolates on root fresh weight and shoot dry weight of alfalfa.

Isolate	Inoculum level	Root fresh weight (g)	Reduction (%)	Shoot dry weight (g)	Reduction (%)
	0	4.56 ± 0.51[1] a[2] FG[3]		3.55 ± 0.24 a D	
MN	5,000	4.55 ± 0.64 a FG	0.13	3.20 ± 0.57 a BCD	9.75
	10,000	4.89 ± 0.56 a G	-7.15	2.98 ± 0.48 a BC	16.1
	0	4.56 ± 0.51 b FG		3.55 ± 0.24 b D	
WZ	5,000	3.76 ± 0.98 ab DEF	17.5	3.40 ± 0.38 b CD	4.11
	10,000	3.48 ± 0.50 a CDE	23.8	3.05 ± 0.32 a BCD	14.1
	0	4.56 ± 0.51 b FG		3.55 ± 0.24 b D	
BN	5,000	3.31 ± 1.20 a CD	27.3	3.26 ± 0.62 b BCD	8.17
	10,000	2.15 ± 1.00 a AB	52.9	2.25 ± 0.23 a A	36.7
	0	4.56 ± 0.51 c FG		3.55 ± 0.24 c D	
BL	5,000	3.64 ± 0.74 b CDEF	20.2	3.03 ± 0.28 b BC	14.7
	10,000	1.31 ± 0.38 a A	71.3	2.37 ± 0.17 a A	33.3
	0	4.56 ± 0.51 a FG		3.55 ± 0.24 a D	
UK	5,000	4.44 ± 0.58 a FG	2.54	3.38 ± 0.32 a CD	4.90
	10,000	4.41 ± 0.60 a EFG	3.38	3.32 ± 0.23 a CD	6.54
	0	4.56 ± 0.51 b FG		3.55 ± 0.24 b D	
FR	5,000	3.27 ± 0.86 a CD	28.4	3.11 ± 0.35 ab BCD	12.5
	10,000	2.82 ± 1.02 a BCD	38.2	2.82 ± 0.51 a B	20.7
	0	4.56 ± 0.51 b FG		3.55 ± 0.24 b D	
NL	5,000	3.41 ± 0.85 a CD	25.2	3.36 ± 0.51 b CD	5.24
	10,000	2.73 ± 1.25 a BC	40.2	2.82 ± 0.47 a B	20.5

[1]Mean and standard deviation (n = 5) at 10 weeks after inoculation, [2]Means followed by different small letters are significantly different between inoculum levels within each isolate ($P < 0.05$). [3]Means followed by different capital letters are significantly different between all treatments in a column ($P < 0.05$).

Final nematode numbers varied depending on the isolate of *P. penetrans* and inoculum level (Table 4). Isolates BL, BN and WZ showed significantly higher final nematode numbers compared to the other isolates of *P. penetrans*. At low nematode inoculum level, final nematode numbers were highest for isolate BL with 18,088 nematodes followed by isolate

BN and WZ with 11,546 and 11,175 nematodes, respectively. At high nematode inoculum, final nematode numbers were highest for isolate WZ with 15,281 nematodes followed by isolate BN with 13,451 nematodes. Differences in final nematode numbers between low and high inoculum levels were significant for plants inoculated with isolates MN, WZ, FR, and NL but not for the others. Overall, the relation between reproduction rate and inoculum level of *P. penetrans* resulted in a significant negative correlation (r = -0.587, $P < 0.001$).

Table 4: Effect of inoculum level of seven *Pratylenchus penetrans* isolates on final numbers and reproduction rate on alfalfa.

Isolate	Inoculum level	Final number of *P. penetrans*			Pf/Pi
		Root	Soil	Total	
MN	5,000	3,653 ± 1,945[1] a[2]AB[3]	43.0 ± 26.5 a ABC	3,696 ± 1,953 a A	0.74
	10,000	7,790 ± 1,428 b C	26.0 ±17.9 a A	7,816 ± 1,429 b BC	0.78
WZ	5,000	11,136 ± 1,321 a E	39.0 ±17.9 a ABC	11,175 ± 1,314 a DE	2.24
	10,000	15,128 ± 3,112 b F	153 ± 111 b CDE	15,281 ± 3,048 b F	1.53
BN	5,000	11,240 ± 966 a E	306 ± 105 a FGH	11,546 ± 1,081 a DE	2.31
	10,000	12,432 ± 6,466 a E	1,019 ± 717 b H	13,451 ± 5,876 a EF	1.35
BL	5,000	18,088 ± 1,379 a G	245 ± 134 a FGH	18,333 ± 1,492 b G	3.67
	10,000	4,800 ± 968 b B	564 ± 42 a GH	5,716 ± 1,009 a AB	0.57
UK	5,000	7,504 ± 2,499 a C	48.0 ± 27.9 a BCD	7,552 ± 2,498 a BC	1.51
	10,000	8,006 ± 1,063 a C	104 ± 61.6 b DEF	8,109 ± 1,073 a C	0.81
FR	5,000	8,426 ± 1,438 a CD	129 ± 52.7 a DEFG	8,555 ± 1,488 a C	1.71
	10,000	11,278 ± 1,425 b E	185 ± 106 a EFG	11,463 ± 1,395 b DE	1.15
NL	5,000	10,439 ± 2,450 a DE	331 ± 97.9 a GH	10,770 ± 2,407 a D	2.15
	10,000	10,626 ± 3,112 a DE	235 ± 73.9 a FG	10,861 ± 3,073 a D	1.09

[1]Mean and standard deviation (n = 5) at 10 weeks after inoculation, [2]Means followed by different small letters are significantly different between inoculum levels within each isolate ($P < 0.05$). [3]Means followed by different capital letters are significantly different between all treatments in a column ($P < 0.05$).

Carrot

The average taproot fresh weight varied depending on the isolate of *P. penetrans* and inoculum level (Table 5). All isolates except MN reduced the taproot fresh weight. However, plants inoculated with isolates NL, BN and BL showed a significantly higher reduction in taproot fresh weight compared to plants inoculated with the other isolates of *P. penetrans*. At low nematode inoculum level, the reduction in taproot fresh weight was highest for isolates BL and BN with 31.7%, followed by isolate NL with 19.3%. At high nematode inoculum level, reduction in taproot fresh weight was highest for isolate NL with 68.6% followed by isolates BN and BL with 65.6% and 54.7%, respectively. Plants inoculated with isolates WZ and UK did not show a significant decrease in taproot fresh weight compared with the non-inoculated plants. Isolates BN, NL and BL reduced taproot fresh weight significantly at both low and high inoculum levels, whereas isolate FR reduced taproot fresh weight only at high inoculum level significantly. Differences in taproot fresh weight between low and high inoculum levels were significant only for isolates BN, BL and NL.

Besides decreasing taproot fresh weight, *P. penetrans* infestation caused major quality losses. Infested taproots showed symptoms such as shortened tips, small round shaped tips, very thin and hairy roots, stubby roots and root deformation. Those symptoms were rated according to a quality loss index with a 0-5 scale. Plants inoculated with *P. penetrans* isolates BN, BL and NL showed the highest quality loss index compared to plants inoculated with the other four isolates of *P. penetrans* and the non-inoculated plants. At low nematode inoculum level, taproot quality loss index was highest for isolates NL with 3.0 followed by isolate BL with 2.0. At high nematode inoculum level, taproot quality loss index was highest for isolates NL and BL with 3.4, followed by isolate BN with 3.0. Overall, taproot fresh weight was negatively correlated with inoculum level ($r = -0.548$, $P < 0.001$) and quality loss index ($r = -0.847$, $P < 0.001$). Furthermore, the relation between taproot quality loss index and inoculum level of *P. penetrans* resulted in a significant positive correlation ($r = 0.753$, $P < 0.001$).

Shoot dry weight was not significantly affected by the inoculum level or the *P. penetrans* isolate (Table 5). At low inoculum level, plants inoculated with isolates BL, BN and FR showed a slightly lower shoot dry weight than the non-inoculated plants. At high inoculum level, plants inoculated with WZ, BN and BL showed a slightly higher shoot dry weight than the non-inoculated plants. Isolates MN and UK caused a slight decrease in shoot dry weight at high inoculum level whereas isolate NL caused a slight decrease in shoot dry weight at both low and high inoculum levels.

Table 5: Effect of inoculum level of seven *Pratylenchus penetrans* isolates on taproot fresh weight, quality loss index (QLI) and shoot dry weight of carrot.

Isolate	Inoculum Level	Taproot fresh weight (g)	Reduction (%)	QLI (0-5)	Shoot dry weight (g)	Reduction (%)
	0	14.5 ± 1.36[1] a[2] DE[3]		0.00	2.55 ± 0.07 a A	
MN	5,000	14.5 ±1.21 a DE	-0.32	0.80	2.55 ± 0.12 a A	0.00
	10,000	15.7 ± 3.91 a F	-8.34	1.00	2.54 ± 0.09 a A	0.31
	0	14.5 ± 1.36 a DE		0.00	2.55 ± 0.07 a A	
WZ	5,000	12.8 ±1.84 a CD	11.2	1.60	2.55 ± 0.14 a A	0.08
	10,000	13.2 ± 2.20 a DE	8.79	1.80	2.56 ± 0.16 a A	-0.55
	0	14.5 ± 1.36 c DE		0.00	2.55 ± 0.07 a A	
BN	5,000	9.89 ± 2.74 b B	31.7	3.00	2.48 ± 0.12 a A	2.90
	10,000	4.99 ± 4.05 a A	65.6	3.00	2.50 ± 0.30 a A	1.96
	0	14.5 ± 1.36 c DE		0.00	2.55 ± 0.07 a A	
BL	5,000	9.90 ± 2.33 b B	31.7	2.40	2.51 ± 0.05 a A	1.65
	10,000	6.57 ± 2.86 a A	54.7	3.40	2.57 ± 0.05 a A	-0.78
	0	14.5 ± 1.36 a DE		0.00	2.55 ± 0.07 a A	
UK	5,000	13.9 ± 1.72 a DE	4.14	1.20	2.55 ± 0.08 a A	0.00
	10,000	12.8 ± 2.13 a CD	11.9	1.20	2.46 ± 0.07 a A	3.45
	0	14.5 ± 1.36 b DE		0.00	2.55 ± 0.07 a A	
FR	5,000	12.8 ± 2.04 b CD	11.9	1.80	2.53 ± 0.15 a A	0.86
	10,000	10.3 ± 2.52 a BC	28.9	2.00	2.58 ± 0.17 a A	-1.10
	0	14.5 ± 1.36 c DE		0.00	2.55 ± 0.07 a A	
NL	5,000	11.7± 3.23 b BCD	19.3	1.80	2.48 ± 0.11 a A	2.59
	10,000	4.56 ± 1.89 a A	68.6	3.40	2.41 ± 0.12 a A	5.65

[1]Mean and standard deviation (n = 5) at 10 weeks after inoculation, [2]Means followed by different small letters are significantly different between inoculum levels within each isolate ($P < 0.05$). [3]Means followed by different capital letters are significantly different between all treatments in a column ($P < 0.05$).

Final nematode numbers varied depending on the isolate of *P. penetrans* and inoculum level (Table 6). For all isolates, the percentage of nematodes obtained from the root fraction compared to the total population ranged from 89% for UK to 98% for WZ and BN at low inoculum level and from 75% for UK to 99% for MN and WZ at high inoculum level. Isolates BL, BN and NL showed significantly higher final nematode numbers compared to the other isolates of *P. penetrans*. At low nematode inoculum level, final nematode numbers were highest for isolate BL with 28,369 *P. penetrans* followed by isolate BN with 19,806 *P.*

penetrans. At high nematode inoculum, final nematode numbers were highest also for isolate BL with 30,290 *P. penetrans* followed by isolates BN and NL with 22,522 and 15,414 *P. penetrans,* respectively. Differences in final nematode numbers between low and high inoculum levels were only significant for isolate MN. Isolates MN, WZ and UK showed the lowest reproduction rate with Pf/Pi ranging from 0.14 for UK to 0.47 for WZ at low inoculum level and from 0.11 for UK to 0.34 for WZ at high inoculum level. Overall, the relation between the nematode reproduction rate and inoculum level *P. penetrans* resulted in a significant negative correlation (r = -0.286, $P = 0.016$).

Table 6: Effect of inoculum level of seven *Pratylenchus penetrans* isolates on final numbers and reproduction rate on carrot.

Isolate	Inoculum level	Final number of *P. penetrans*			Pf/Pi
		Root	Soil	Total	
MN	5,000	1,174 ± 1,943[1] a[2] B[3]	31 ± 13 a AB	1,205 ± 200 a A	0.24
	10,000	2,958 ± 1,072 b C	43 ± 15 a B	3,001 ± 1,059 b B	0.30
WZ	5,000	2,320 ± 1,291 a C	53 ± 40 a AB	2,373 ± 1,310 a BC	0.47
	10,000	3,328 ± 302 a C	48 ± 38 a AB	3,376 ± 288 a B	0.34
BN	5,000	19,384 ± 5,387 a EF	422 ± 251 a CDE	19,806 ± 5,498 a E	3.96
	10,000	21,930 ± 9,043 a FG	592 ± 373 a CDE	22,522 ± 9,036 a F	2.59
BL	5,000	27,212 ± 9,883 a FG	1,157± 450 b E	28,369 ± 9,776 a G	5.67
	10,000	29,400 ± 2,086 a G	890 ± 552 a DE	30,290 ± 1,902 a H	3.02
UK	5,000	642 ± 228 a A	82 ± 60 a AB	724 ± 216 a A	0.14
	10,000	850 ± 440 a AB	284 ± 104 b CD	1,134 ± 503 a A	0.11
FR	5,000	10,992 ± 1,911 a D	361 ± 203 a CD	11,353 ± 1,981a C	2.27
	10,000	12,160 ± 2,615 a D	228 ± 172 a C	12,388 ± 2,586 a D	1.23
NL	5,000	12,416 ± 3,454 a D	538 ± 371 a CDE	12,954 ± 3,310 a D	2.59
	10,000	14,826 ± 6,134 a DE	588 ± 155 a CDE	15,414 ± 6,250 a E	1.15

[1]Mean and standard deviation (n = 5) at 10 weeks after inoculation, [2]Means followed by different small letters are significantly different between inoculum levels within each isolate ($P < 0.05$). [3]Means followed by different capital letters are significantly different between all treatments in a column ($P < 0.05$).

Fodder radish

Average root fresh weight and shoot dry weight varied depending on nematode inoculum level but not by different isolates of *P. penetrans* (Table 7). All plants inoculated with *P. penetrans* showed a lower root fresh weight than the non-inoculated control plants. However, differences were not significant for plants inoculated with isolates MN, WZ and BN at both low and high inoculum levels. Plants inoculated with isolates NL, BL, UK, and FR showed a significantly higher reduction in root fresh weight compared to plants inoculated with the other isolates of *P. penetrans*. At low nematode inoculum level, the reduction in root fresh weight was highest for isolate NL with 28.3% followed by isolate BL with 18.6%. At high nematode inoculum level, reduction in root fresh weight was highest for isolate NL with 39.7% followed by isolates UK and BL with 29.6% and 27.0%, respectively. Isolates NL, UK and BL reduced root fresh weight significantly at both low and high inoculum levels, whereas isolate FR reduced root fresh weight only at high inoculum level significantly. Overall, the relation between root fresh weight and inoculum level *P. penetrans* resulted in a significant negative correlation ($r = -0.46$, $P < 0.001$).

Shoot dry weight was not significantly affected by inoculum level or *P. penetrans* isolate (Table 7). At high inoculum level plants had a higher shoot dry weight compared to low inoculum level except for isolate FR; however, differences were not significant.

Table 7: Effect of inoculum level of seven *Pratylenchus penetrans* isolates on root fresh weight and shoot dry weight of fodder radish.

Isolate	Inoculum level	Fresh root weight (g)	Reduction (%)	Dry shoot weight (g)	Reduction (%)
	0	3.83 ± 0.47[1] a[2] C[3]		3.75 ± 0.19 a AB	
MN	5,000	3.73 ± 1.63 a BC	2.51	3.60 ± 0.37 a AB	4.05
	10,000	3.10 ± 0.59 a ABC	18.9	3.86 ± 0.41 a AB	-2.99
	0	3.83 ± 0.47 a C		3.75 ± 0.19 a AB	
WZ	5,000	3.26 ± 1.20 a ABC	14.9	3.61 ± 0.38 a AB	3.79
	10,000	2.92 ± 0.46 a ABC	23.8	3.87 ± 0.22 a AB	-3.09
	0	3.83 ± 0.47 a C		3.75 ± 0.19 a AB	
BN	5,000	3.70 ± 1.41 a BC	3.50	3.81 ± 0.56 a AB	-1.65
	10,000	3.07 ± 0.69 a ABC	19.9	3.80 ± 0.21 a AB	-1.44
	0	3.83 ± 0.47 b C		3.75 ± 0.19 ab AB	
BL	5,000	3.12 ± 0.46 a ABC	18.6	3.68 ± 0.15 a AB	6.03
	10,000	2.76 ± 0.38 a ABC	27.8	3.98 ± 0.28 b B	-6.40
	0	3.83 ± 0.47.b C		3.75 ± 0.19 a AB	
UK	5,000	3.24 ± 0.42 a ABC	15.5	3.75 ± 0.09 a AB	-0.05
	10,000	2.70 ±1.36 a AB	29.6	3.97 ± 0.21a B	-5.97
	0	3.83 ± 0.47 b C		3.75 ± 0.19 a AB	
FR	5,000	3.37 ± 0.81 ab BC	11.9	3.70 ± 0.32 a AB	1.23
	10,000	2.87 ± 0.41 a ABC	25.0	3.69 ± 0.47 a AB	1.60
	0	3.83 ± 0.47 b C		3.75 ± 0.19 ab AB	
NL	5,000	2.75 ± 0.50 a AB	28.3	3.52 ± 0.25 a A	1.81
	10,000	2.31± 0.62 a A	39.7	3.99 ± 0.44 b B	-6.03

[1]Mean and standard deviation (n = 5) at 10 weeks after inoculation, [2]Means followed by different small letters are significantly different between inoculum levels within each isolate ($P < 0.05$), [3]Means followed by different capital letters are significantly different between all treatments in a column ($P < 0.05$).

Final nematode numbers varied depending on the isolate of *P. penetrans* and inoculum level (Table 8). Average numbers of *P. penetrans* recovered from soil ranged from 17.6 for WZ to 3,523 for BL at low inoculum level and from 25.0 for MN to 4,053 for BL at high inoculum level. Average numbers of *P. penetrans* recovered from roots ranged from 56.0 for UK to 7,264 for BN at low inoculum level and from 48.0 for MN to 11,851 for FR at high inoculum level. Isolates BL, BN and FR showed significantly higher final *P. penetrans* numbers compared to the other isolates. At low nematode inoculum level, final nematode numbers

were highest for isolate BL with 10,547 nematodes followed by isolates BN and FR with 8,528 and 7,590 nematodes, respectively. At high nematode inoculum, final nematode numbers were highest for isolate FR with 14,792 nematodes followed by isolate BN and BL with 11,957 and 11,021 nematodes, respectively. Isolates MN, WZ, and UK showed the lowest final nematode numbers at both low and high inoculum levels and were not significantly different from each other, but differed from the other isolates. Differences in final nematode populations between low and high inoculum levels were significant for all isolates except for isolates MN and BL. All isolates except isolates WZ and UK showed a slightly lower reproduction rate at high inoculum level compared to low inoculum level. The relation between reproduction rate and inoculum level of *P. penetrans* resulted in a non-significant correlation ($P > 0.05$).

Table 8: Effect of inoculum level of seven *Pratylenchus penetrans* isolates on final numbers and reproduction rates on fodder radish.

Isolate	Inoculum level	Final numbers of *P. penetrans*			Pf/Pi
		Roots	Soil	Total	
MN	5,000	64.0 ± 35.8[1] a[2] A[3]	34.4 ± 19.2 a A	98.4 ± 36.1 a A	0.02
	10,000	48.0 ± 33.5 a A	25.8 ± 23.6 a A	73.8 ± 53.7 a A	0.01
WZ	5,000	156 ± 26.1 a A	17.6 ± 9.84 a A	174 ± 30.9 a A	0.03
	10,000	216 ± 31.3 b A	138 ± 12.1 b A	354 ± 31.5 b A	0.04
BN	5,000	7,264 ± 862 a C	1,264 ± 303 a B	8,528 ± 1,115 a C	1.71
	10,000	10,056 ± 678 b D	1,901 ± 141 b C	11,957 ± 791 b E	1.20
BL	5,000	7,024 ± 608 a BC	3,523 ± 445 a E	10,547 ± 347 a D	2.11
	10,000	6,968 ±2,749 a BC	4,053 ± 219 b F	11,021 ± 2,766 a DE	1.10
UK	5,000	56.0 ± 21.9 a A	73.2 ± 11.7 a A	129 ± 26.4 a A	0.03
	10,000	168 ± 30.3 b A	172 ± 26.5 b A	340 ± 17.9 b A	0.03
FR	5,000	6,328 ± 609 a BC	1,262 ± 130 a B	7,590 ± 486 a BC	1.52
	10,000	11,851 ± 488 b E	2,941 ± 582 b D	14,792 ± 914 b F	1.48
NL	5,000	5,904 ± 573 a B	985 ± 437 a B	6,889 ± 662 a B	1.38
	10,000	6,880 ± 505 b BC	3,221 ± 223 b DE	10,101 ± 382 b D	1.01

[1]Mean and standard deviation (n = 5) at 10 weeks after inoculation, [2]Means followed by different small letters are significantly different between inoculum levels within each isolate ($P < 0.05$). [3]Means followed by different capital letters are significantly different between all treatments in a column ($P < 0.05$).

French marigold

Root fresh weight was not significantly affected by inoculum level or *P. penetrans* isolate (Table 9). However, at both low and high inoculum levels plants showed a slight increase in root fresh weight. An exception was isolate UK, which caused a slight decrease in root fresh weight at high inoculum level. Similar to root fresh weight, shoot dry weight was not significantly affected by inoculum level or *P. penetrans* isolate (Table 9). At both low and high inoculum levels plants showed a slight decrease in shoot dry weight. An exception was isolate FR, which caused a slight increase in shoot dry weight at low inoculum level.

Table 9: Mean root fresh weight and shoot dry weight of French marigold

Isolate	Inoculum level	Fresh root weight (g)	Reduction (%)	Dry shoot weight (g)	Reduction (%)
	0	2.25 ± 0.34[1]		3.31 ± 0.10	
MN	5,000	2.53 ± 0.37	-12.4	3.15 ± 0.08	4.77
	10,000	2.50 ±0.47	-11.1	3.19 ± 0.17	3.69
	0	2.25±0.34		3.31 ± 0.10	
WZ	5,000	2.56±0.12	-13.9	3.23 ± 0.14	2.54
	10,000	2.64±0.31	-17.4	3.30 ± 0.17	0.24
	0	2.25 ± 0.34		3.31 ± 0.10	
BN	5,000	2.51 ± 0.20	-11.6	3.13 ± 0.07	5.38
	10,000	2.46 ± 0.40	-9.33	2.94 ± 0.44	11.3
	0	2.25 ± 0.34		3.31 ± 0.10	
BL	5,000	2.41 ± 0.38	-7.29	3.27 ± 0.27	1.27
	10,000	2.53 ± 0.26	-12.4	3.05 ± 0.17	7.98
	0	2.25 ± 0.34		3.31 ± 0.10	
UK	5,000	2.44 ± 0.56	-8.36	3.20 ± 0.25	3.32
	10,000	1.97 ± 0.27	12.4	3.03 ± 0.06	8.46
	0	2.25 ± 0.34		3.31 ± 0.10	
FR	5,000	2.59 ± 0.25	-15.1	3.39 ± 0.14	-2.54
	10,000	2.54 ± 0.32	-12.9	3.30 ± 0.25	0.30
	0	2.25 ± 0.34		3.31 ± 0.10	
NL	5,000	2.40 ± 0.50	-4.44	3.11 ± 0.24	6.10
	10,000	2.30 ± 0.40	-1.87	3.22 ± 0.11	2.78

[1]Mean and standard deviation (n = 5) at 10 weeks after inoculation.

Regarding *P. penetrans* reproduction, none of the isolates were found to reproduce on French marigold (Table 10)

Table 10: Effect of inoculum level of seven *Pratylenchus penetrans* isolates on final numbers and reproduction rates on French marigold.

Isolate	Inoculum level	Final numbers of *P. penetrans*			Pf/Pi
		Roots	Soil	Total	
MN	5,000	0.0[1]	0.0	0.0	0.0
	10,000	0.0	0.13	0.13	0.0
WZ	5,000	0.0	0.0	0.0	0.0
	10,000	0.0	0.0	0.0	0.0
BN	5,000	0.0	0.0	0.0	0.0
	10,000	0.0	0.25	0.25	0.0
BL	5,000	0.0	0.0	0.0	0.0
	10,000	0.0	0.25	0.25	0.0
UK	5,000	0.0	0.0	0.0	0.0
	10,000	0.0	0.0	0.0	0.0
FR	5,000	0.0	0.0	0.0	0.0
	10,000	0.0	0.13	0.13	0.0
NL	5,000	0.0	0.0	0.0	0.0
	10,000	0.0	0.13	0.13	0.0

[1]Mean (n = 5) at 10 weeks after inoculation.

4 Discussion

4.1 *In vitro* reproductive fitness of *Pratylenchus penetrans*

The reproductive fitness of the seven isolates of *P. penetrans* from different geographical locations was compared using monoxenic carrot disc cultures. In this study, all *P. penetrans* isolates tested completed their life cycle and reproduced at 25°C. This is in accordance with previous observations by Verdejo-Lucas and Pinochet (1991), Castillo *et al*. (1995), Gonzaga and Santos (2010) and Kagoda *et al*. (2010) showing that *Pratylenchus* species reproduced successfully between 20°C and 30°C. In general, *P. penetrans* reproduces over a wide range of temperature between 15°C and 30°C (Acosta and Malek, 1979; Mizukubo and Adachi, 1997).

In the present study, depending on the isolate, a 102 to 4,115-fold reproduction of the nematode was obtained at 80 days after inoculation. Karakas (2015) recorded a 82-fold reproduction for *P. penetrans* following 80 days at 24°C. Mokrini *et al*. (2016), working with four populations of *P. penetrans*, observed nematode reproduction between 350 and 459-fold when cultures were maintained for 8 weeks on carrot discs at 20°C. Depending on the isolates, reproduction rates in this study were within the range or above of what have been reported for *P. penetrans* and other root lesion nematodes. Our results on the relative occurrence of developmental stages are in accordance with previous findings by Verdejo-Lucas and Pinochet (1991), Pinochet *et al*. (1994), France and Brodie (1995) and Mokrini *et al*. (2016) showing that the percentage of a developmental stage varies depending on nematode species and isolate. Eggs were the most numerous stage of all tested *P. penetrans* isolates with the exception of MN and UK isolates, in which juvenile stage had the highest percentage of occurrence. Only a low proportion of the population were adult stages (females + males), which was between 8% (BL) and 22% (MN) of the population. Our results showed that isolates with the highest reproductive fitness had a higher female:male ratio than isolates with a lower reproductive fitness. For example, isolate BL had the highest reproductive fitness and the highest female:male ratio (1.79), while isolate UK had the lowest reproductive fitness with the lowest female:male ratio (0.53). This confirms findings of Elbadri *et al*. (2001) showing that isolates with a high reproduction rate also had a higher ratio of females:males than isolates with a low reproduction rate. According to Townshend (1978) the higher female:male ration is explained by the fact that *P. penetrans* females are more infective than males or third stage juveniles as they penetrated the roots earlier, faster, in a greater number, and over a wider range of soil temperatures. Overall, relative composition of developmental

stages and total population numbers have been reported to be influenced by initial inoculum density, temperature, incubation time and nematode species or race (Pinochet *et al*., 1994; Feijoo, 1996; Mudiope *et al*, 2004; Tuyet *et al*., 2013). Verdejo-Lucas and Pinochet (1991) studied the effect of incubation time on reproduction of the root lesion nematode *Zygotylenchus guevarai* on carrot discs. They recorded a high proportion of females (52% of the population) when reproduction was assessed at 45 days, but when reproduction was assessed at 85, 107 or 125 days, the population was dominated by eggs (40-60% of the population).

Because a common host under similar growth conditions was used in this study, the differences observed in final nematode numbers among isolates of *P. penetrans* can be largely attributed to the intrinsic reproduction rate.

Regarding *in vitro* reproductive fitness of *P. penetrans* on carrot discs, a recent study showed that 4 geographic populations of *P. penetrans* differed in their reproductive fitness on carrot discs (Mokrini *et al*., 2016). Similar has been reported for other *Pratylenchus* species. Variability in *in vitro* reproductive fitness on carrot discs has been shown for *P. neglectus* (Esteves *et al*., 2018), *P. sudanensis* (Mudiope *et al.*, 2004), *P. vulnus* (Pinochet *et al*., 1994) and *P. coffeae* (Stoffelen *et al.*, 1999).

Overall, based on reproductive fitness of *P. penetrans* isolates on carrot discs, our results demonstrated intraspecific differences among the seven geographic isolates of *P. penetrans*.

4.2 *In vivo* reproductive fitness of *Pratylenchus penetrans*

All *P. penetrans* isolates examined reproduced well on common vetch and lentil. However, reproduction rates varied depending on isolate and cover crop species. In our study, all isolates reproduced more on common vetch than on lentil. These results are consistent with our previous findings (Chapter IV results for Experiment 2) showing that *P. penetrans* isolates reproduced more on common vetch than on lentil.

Lentil was found to be an excellent host for isolates BL and FR with reproduction rates of 39.1 and 10.6, respectively and a good host for the other isolates with reproduction rates ranging from 2.3 to 8.4. In comparison, common vetch turned out to be an excellent host for all isolates except UK with reproduction rates ranging from 9.6 to 54.1. Isolate UK turned out to be a good host with a reproduction rate of 2.29. Overall, results of *in vivo* reproductive fitness clearly demonstrated the occurrence of intraspecific differences among the *P.*

penetrans isolates. These results support earlier studies (Olthof, 1968; Brodie, 1995; France and Brodie, 1995; France and Brodie, 1996) describing that geographic isolates of *P. penetrans* behaved differently.

The *in vivo* reproductive fitness of different *P. penetrans* isolates on common vetch and lentil in this study correlated with those of the *in vitro* reproductive fitness on carrot discs. In both, *in vitro* and *in vivo* experiments, isolates BL and UK showed the highest and lowest reproductive fitnesss, respectively, while the other isolates showed intermediate fitness. Due to the lack of available information, the relationship between *in vitro* reproductive fitness on carrot discs and reproductive fitness in greenhouse pot experiment for *P. penetrans* could not be discussed any further. However, this relationship has been established for other *Pratylenchidae.* Pinochet *et al*. (1994) investigated the variability in reproductive fitness among seven isolates of *P. vulnus* from different geographical areas and hosts on *in vitro* carrot disc culture and in greenhouse pot experiments. An isolate from rose showed the highest reproduction rates on both carrot disc and plum. Another isolate from walnut also showed a similar reproductive fitness in both *in vitro* culture and in the pot experiment with plum, but was the fittest on sour orange. Castillo *et al*. (1998) studied differences in reproductive fitness among four populations of *P. thornei* from different locations. However, they did not find any differences in reproductive fitness either on carrot discs or in a pot experiment with four chickpea genotypes. Dochez (2004) studied differences in reproductive fitness among four populations of *R. similis* from Uganda. He found that for the four populations of *R. similis* the reproductive fitness on banana in pot experiments correlated with those in the *in vitro* carrot disc experiments. Moens (2004) observed differences in reproductive fitness among 11 *R. similis* populations from Costa Rica but did not correlate the results from pot experiments and *in vitro* carrot discs.

4.3 Pathogenicity of *Pratylenchus penetrans* isolates

The results of the pathogenicity test showed considerable differences between *P. penetrans* isolates regarding their reproductive fitness and damage potential towards alfalfa, carrot and fodder radish. On alfalfa both shoot dry weight and root fresh weight were affected by *P. penetrans* and thus are good indicators for nematode damage. This is in line with a study by Griffin (1994) showing that both shoot and root growths were affected by *P. penetrans*. However, Santo (1980) reported that *P. penetrans* significantly reduced the shoot, but not the root weight of the alfalfa cultivar Vernal. Based on the observed reduction of both shoot and root growth, alfalfa can be termed intolerant to *P. penetrans*. In this study, depending on

isolates, significant reductions in shoot dry weight and root fresh weight occurred both at inoculum levels of 5,000 and 10,000 *P. penetrans* per plant. In other studies inoculum levels of 425 *P. penetrans* per pot (Townshend, 1984), 540 *P. penetrans* per plant (Griffin, 1993) or 200 *P. penetrans* per 2 plants (Mauza and Webster, 1982) were reported to already reduce shoot and root growth of alfalfa. A similar effect on alfalfa plant growth has also been reported for *P. neglectus*. Griffin and Gray (1990) showed that shoot and root weights of alfalfa were reduced when plants were inoculated with 5,000 or 10,000, but not 1,000 *P. neglectus* per plant. Overall, *Pratylenchus penetrans* has been reported to be more virulent to alfalfa than *P. neglectus* (Griffin, 1994).

In our study, infestation with *P. penetrans* caused plant growth reductions ranging from 4% to 36% for shoot dry weight and from 0% to 71% for root fresh weight when compared with non-inoculated plants. However, reductions in shoot and root weights of alfalfa were not as high as those reported by Griffin (1993) showing shoot weight reductions from 13% to 69% and root weight reductions from 17% to 75% at 120 days after inoculation with 540 to 10,800 *P. penetrans* per plant. Differences in plant growth reduction between our experiments and the one conducted by Griffin (1993) can most likely be attributed to the different duration of both experiments, i.e. 70 days in our study compared with 120 days in the study by Griffin (1993). Experimental length was found to influence the magnitude of plant growth reduction (Aalders *et al*., 2009). Furthermore, other parameters such as inoculum level, plant cultivar, experimental conditions or nematode isolate also contribute to variability between experiments. For example, in our study, isolates BL and BN were more pathogenic to alfalfa compared to isolate FR, NL and WZ, and isolates MN and UK caused no damage at all to alfalfa. Intraspecific differences in pathogenicity to alfalfa have also been reported for *P. neglectus* populations from different geographic origin (Griffin, 1991). Griffin (1993) on the other hand did not find difference in damage potential among four populations of *P. penetrans* on Lahontan alfalfa.

Reduced root fresh weights were not always correlated with high final nematode numbers. For example, isolate BL at high inoculum level caused the highest damage but showed a lower final nematode population compared to the remaining isolates except MN. The lower final nematode numbers in isolate BL may be explained by the extensive damage of nematodes on the roots resulting in less availability of food and competition for space and nutrition, which in turn limits nematode reproduction (Seinhorst, 1970). On the other hand, isolate BN was more pathogenic to alfalfa than isolate WZ, though final nematode numbers of

isolate WZ were comparable at both inoculum levels. This suggest that the pathogenicity may not always depend on reproductive fitness but also on the inherent damaging potential of a particular nematode population or isolate as previously reported for other plant-nematode systems (Hahn *et al*., 1996a; Costa *et al*., 2008).

A relationship between reproductive fitness on carrot discs and pathogenicity on pot experiments has been found for different *Pratylenchidae* (Sarah *et al*., 1993; Pinochet *et al*., 1994; Fallas *et al*., 1995). In this study, a correlation was found between reproductive fitness *in vitro* on carrot discs, *in vivo* on lentil, common vetch and alfalfa and pathogenicity on alfalfa for almost all isolates.

On carrot, shoot dry weights were not affected by *P. penetrans*, which contradicts previous findings by Coosemans (1975) and Vrain and Belair (1981) showing a reduction of carrot shoot growth by *P. penetrans*. A possible reason could be that plants in the present study were inoculated 17 days after seeding compared with at seeding in case of the other two studies. As showed by Santo *et al*. (1988) carrots with later inoculation did not show reduction in shoot growth in comparison to carrots with immediate inoculation. In contrast, carrot root system was significantly affected by most *P. penetrans* isolates. The root system has been reported to be the most intolerant to nematode infestation and used as a good indicator for pathogenicity (Sarah *et al*., 1993; Fallas *et al*., 1995). This could be explained by the fact that the root is more early and directly affected by the nematode than the shoot and thus the resultant effect at harvest appears to be more pronounced.

In this study, *P. penetrans* infestation was also associated with quality losses. Taproots showed shortened tips, were small round shaped, very thin and hairy, stubby and deformed. However, forking of the taproots and death of seedlings were not noticed. Coosemans (1975) reported that an initial density of 10 *P. penetrans* per 100 ml soil caused 75% of the carrots to be forked with multiplication of rootlets, while 100 *P. penetrans* per 100 ml soil killed 40% of the plants. Vrain and Belair (1981) and Teklu *et al*. (2016) who used the same cultivar Nerac, also reported forking of taproots as one of the major quality losses associated with *P. penetrans* but they did not notice death of seedlings. The lack of seedling death and forking of taproots in this study could be mainly attributed to the differences in inoculation time, which was 17 days after sowing compared to sowing. Kayani *et al*. (2018) suggested that the magnitude of damaging effects of nematode decreased as the age of plants increased at the time of inoculation. This implies that the main damaging effect of *P. penetrans* may be initiated at the early stages of seedling emergence. Previous studies showed that the quality

losses associated with *P. penetrans* infestation were inoculum level dependent (Vrain and Belair, 1981; Teklu *et al*., 2016). These are in accordance with our findings showing that the intensity of damage or quality loss index was positively correlated with inoculum level. In comparison to *P. penetrans*, other *Pratylenchus* species seem to be less damaging on carrot. Hay (2005) did not find any relationship between number of *P. crenatus* and carrot quality losses.

At high inoculum level the reproduction rate of *P. penetrans*, the taproot fresh weight and the taproot quality were reduced, which confirms previous observations by Vrain and Belair (1981) and Teklu *et al*. (2016). Reproductive fitness and damage potential were closely linked in two different groups of *P. penetrans* isolates. Isolates MN, WZ and UK did neither reproduce nor cause significant root damage, while isolates BN, BL, FR and NL showed higher reproduction as well as higher damage to the root system compared to others. However, reproductive fitness and damage to the root system were not always correlated. For example, the final nematode numbers of isolate BL was significantly higher compared to BN and NL, but no considerable differences in taproot fresh weight or quality loss index were found between the three isolates. This suggests that other factors apart from reproductive fitness could influence the pathogenicity of a specific isolate.

Pathogenicity of *P. penetrans* isolates on carrot correlated with the reproductive fitness in *in vitro* carrot discs culture and in pot experiments. An exception was the isolate WZ, which was able to reproduce *in vitro* and in pot experiments on lentil and common vetch but not on carrot in pot experiment.

On fodder radish, shoot dry weights were not affected but root fresh weights were reduced at inoculum levels of 5,000 and 10,000 *P. penetrans* per plant. Reductions in root growth averaged around 20% to 30% for most isolates, with the exception of isolate NL, which caused up to 40% root growth reduction. Damage potential was not correlated with reproductive fitness. While for isolates MN, WZ and UK fodder radish seemed to act as a non-host, in isolates BN, BL, FR and NL the final nematode numbers ranged from 6,889 to 14,792 depending on inoculum level. Due to the lack of available information in the literature, the damage potential of *P. penetrans* on fodder radish could not be discussed any further. The observation that the final nematode numbers was not correlated with the amount of damage may suggest that pathogenicity depends not only on the reproductive fitness, but also on the nematode capacity to overcome defence responses from the plants (Hahn *et al*., 1996a; Ali *et al*., 2018). Similar observations were reported for *P. penetrans* on potato (Bernard and

Laughlin, 1976) and for other plant-nematode systems (Hahn *et al*., 1996a). Working on four potato cultivars, Bernard and Laughlin (1976) found that *P. penetrans* caused losses in the cultivar with the lowest final population density. Conversely, no significant yield losses were found in the cultivar with the highest final population of *P. penetrans*. Hahn *et al*. (1996a) studied reproductive fitness and pathogenicity of different *R. similis* populations on banana plants under controlled experimental conditions. They reported no linear correlation between final numbers of *R. similis* per gram of root and the decrease of root weight.

In our study, isolates with the highest reproductive fitness in *in vitro* carrot discs also revealed the highest final population numbers on fodder radish in the pot experiment with the exception of isolate WZ, which reproduced well *in vitro* and *in vivo* on lentil and common vetch but not on fodder radish.

Finally, our study confirmed previous studies that *P. penetrans* was neither able to reproduce on French marigold, nor do any damage, and therefore can be considered as a non-host (Kimpinski *et al*., 2000; Lamondia, 2006). French marigold contains secondary compounds in the root known as thiophene α-terthyenil, which are known to have a nematicidal effect on *P. penetrans* and many other species of plant parasitic nematodes (Gommers, 1972; Bakker *et al*., 1979). French marigold is commonly used as an antagonisitic crop (Pudasaini *et al*., 2008).

Chapter VI

Morphometric and molecular variability among seven isolates of *Pratylenchus penetrans*

1 Introduction

The genus *Pratylenchus* has a worldwide distribution and comprises some of the most economically important species (Castillo and Vovlas, 2007) including species with quarantine status (Ryss, 2002). Accurate species identification is crucial for efficient nematode management and quarantine inspection. In *Pratylenchus* taxonomy, species identification was traditionally done according to morphological and morphometric characters of females and males (Ryss, 2002; Castilo and Vovlas, 2007). Main diagnostic characters are presence/absence of males, body length, de Man ratio's, shape of head, number of lip annules, stylet length, shape of stylet, shape of stylet knobs, length of pharyngeal overlap, number of lateral field lines at vulval region, presence/absence of areolated bands on the lateral fields at vulval region, length and structure of post-vulval uterine sac, shape of spermatheca, shape of female tail and tail tip (Román and Hirschmann,1969; Hernández *et al*., 2001; Hunt *et al*., 2005; Castilo and Vovlas, 2007; Handoo and Golden, 2008).

Identification and determination of species within the genus *Pratylenchus* using morphological and morphometric characters alone can be difficult due to morphological similarity, limited number of distinguishable diagnostic features and intraspecific variability of some of these characters (Corbett and Clark, 1983; Castillo and Vovlas, 2007; Handoo *et al*., 2008). In recent years, different molecular techniques have been developed for species identification and assessment of genetic variation within and between species of *Pratylenchus.* Commonly used techniques are: amplified fragment length polymorphism (AFLP) (Jung *et al*., 2010; Correa *et al*., 2014), restricted fragment length polymorphism (RFLP) (Waeyenberge *et al*., 2000; Širca *et al*., 2010), random amplified polymorphic DNA (RAPD) (Pinochet *et al.,* 1994; Hahn *et al.,* 1996b; Fallas *et al.,* 1996), sequence characterized amplified region (SCAR) (Correa *et al*., 2014; Tigano *et al*., 2010), single nucleotide polymorphism (SNP) (Figueiredo *et al.,* 2013) and simple sequence repeats or variable number of tandem repeats (VNTR)/SSRs) (De Luca *et al*., 2002; Arias *et al*., 2009).

In this study, we used traditional morphometric analysis and RAPD-PCR to analyze the variability among seven *P. penetrans* isolates of different geographical regions.

2 Materials and methods

Nematode isolates

The seven *P. penetrans* isolates listed in Table 1 of Chapter V were used for morphometric and molecular analysis.

2.1 Morphological identification of *Pratylenchus penetrans* isolates

Killing and fixing of nematodes

Killing and fixing were done following the method described by Hooper *et al*. (2005). Nematode suspensions from each *P. penetrans* isolate were transferred into 10 ml glass vials and concentrated in a volume of about 2 ml water. Next, a double strength TAF fixative stock solution consisting of 10 ml formalin (35% formaldehyde), 1 ml triethanolamine and 56 ml aqua dest was prepared and heated to 70℃ in a water bath. The vials containing the nematode suspension were filled with approx. 2 ml hot fixative and left for 24 h. By the next day, the TAF fixative was sucked out to about 1 ml solution, which was then transferred on a sterile 5 cm plastic Petri dish. The Petri dishes containing the fixed specimens were filled with a solution consisting of 30% ethanol (96%), 67% aqua dest and 3% glycerin. Afterwards, the Petri dishes were covered partly and placed in a wooden cabinet at room temperature for 5-7 weeks to allow evaporation of the ethanol and water and specimen remained in pure glycerine.

Mounting of nematodes

Microscopic glass slides (26 x 76 mm) with cover slip (18 mm diam.) (Diagonal GmbH & Co. KG, Münster, Germany) were used for mounting. To make a paraffin ring, paraffin wax was heated in a ceramic bowl on a heating plate at 100℃. A copper tube was heated in a gas flame, dipped into the melted paraffin and then gently stamped onto the centre of a glass slide. As mounting agent a small drop of glycerine was placed in the centre of the paraffin ring and 5 to 6 female *P. penetrans* were fished and transferred to each slide. Paraffin rings were covered with a cover slip and the slide was gently heated to 65-70℃ on the heating plate until the paraffin ring melted and then allowed to cool down. Nematode specimens were sealed within the drop of glycerol and were ready for microscopic examination.

Measurement of morphometrics

The selection of morphometrical characters studied were in accordance with those described by Decraemer and Hunt (2013) and Castillo and Vovlas (2007) (Table 1). Ten females of each *P. penetrans* isolate were evaluated. Measurements were performed with a Nikon ECLIPSE Ni-U microscope at 100× magnification with the aid of a Nikon DS Fi-2 camera and exclusive NIS-Elements image analysis software (Nikon, Tokyo, Japan).

Table 1: Morphological and morphometric parameters used for comparing seven isolates of *Pratylenchus penetran*s.

Characters	Abbreviation
Body length (µm)	L
Body length divided by maximum body width	a
Body length divided by the length from anterior end to pharyngeal-intestinal junction	b
Body length divided by pharynx length from anterior end to posterior end of pharyngeal gland	b'
Body length divided by tail length	c
Tail length divided by body width at anus	c'
Distance of vulva from anterior end divided by total body length (%)	V
Stylet length (µm)	Stylet
Stylet knob height (µm)	SKH
Stylet knob width (µm)	SKW
Distance from basal knobs of stylet to dorsal pharyngeal gland orifice (µm)	DGO
Lip height (µm)	LH
Lip width (µm)	LW
Distance from anterior end to end of pharyngeal gland (pharynx length) (µm)	Ph-L
Distance from pharyngeal-intestinal junction (anterior end of pharyngeal gland) to posterior end of pharyngeal gland (pharyngeal overlap) (µm)	Ph-O
Distance from anterior end to excretory pore (µm)	EP
Maximum body width (µm)	MBW
Distance from vulva to anterior most part of ovary (µm)	Ovary
Length of post-vulval uterine sac (µm)	PUS
Distance from vulva to anus (µm)	V-A
Length of post-vulval uterine sac divided by body width at vulva (µm)	P
Tail length (µm)	Tail

2.2 Molecular analysis of *Pratylenchus penetrans* isolates by RAPD

DNA extraction

DNA extraction was performed as previously described by Holterman *et al.* (2009). Ten nematodes (4^{th}-stage juveniles and adults) of each *P. penetrans* isolate were transferred with 25 µl sterile water into a 1.5 ml Eppendorf tube using a micropipette. Four replicates of each isolate were prepared and stored at -20°C until used. An equal volume of lyses buffer (25 µl) consisting of 0.2 M NaCl, 0.2 M Tris-HCl (PH 8.0), 1% v/v ß-Mercaptoethanol, 0.8 mg/ml Proteinase K was added to each sample. Then the samples were mixed thoroughly by vortexing for 1 min followed by centrifugation for 5-7 s at 16,000 rpm and incubated overnight in a Thermomixer (Eppendorf, Hamburg, Deutschland) at 65°C and 750 rpm for digestion. Following this, the samples were incubated for 5 min at 100°C and stored at -20°C until used.

RAPD analysis

The RAPD-PCR was performed with a total PCR reaction mixture of 25 µL containing 2 µL of genomic DNA, 5 µL of 5x Go *Taq* buffer, 2.5 µL of 25 mM $MgCl_2$, 2.5 µL of 2 mM dNTP, 1 µL of 10 µM primer (SC10-30), 0.25 µL of 5u μL^{-1} Go *Taq* Flexi polymerase (Promega, Mannheim, Germany) and 11.75 µL distilled water. PCR amplification was performed in a PCR cycler (Eppendorf Mastercycler, Munich, Germany) programmed for the following conditions: an initial stage of 5 min at 95°C, followed by 45 cycles of 1 min at 92°C, 1 min at 38°C, 2 min at 72°C and a final stage of 10 min at 72°C. Amplified PCR products were stored at 4°C.

Because of limitations in gel chamber size number of samples were split and run in parallel in two electrophoresis. Therefore, each gel received fourteen samples. Eight µL of each of the RAPD-PCR products was loaded and run on 2% agarose gels in 0.5x TBA buffer at 50 V for 3 h. On each gel a 1 kb plus DNA ladder was added to the left, middle and right of the samples. *Heterodera carotae* DNA was included as a positive control. Following electrophoresis, the gel was stained in 0.5 µg/ml ethidium bromide for 30 min and photographed with an UV transilluminator (INTAS GmbH, Göttingen, Germany). The scanned electrophoresis gels were analyzed using the software GelCompar II 6.6 (Applied Maths, Ghent, Belgium). Lanes were normalized with common bands as internal standard.

Data analysis

For morphometric analysis, data were analysed using ANOVA (SPSS 20) and means were separated using Duncan's Multiple Range Test at $P \leq 0.05$. Coefficient of variation (CV) was calculated for all characters measured. For RAPD analysis, pairwise comparison of RAPD-PCR profiles was performed using software GelCompar II 6.6. Cluster analysis was done using pairwise Pearson similarity coefficients based on the unweighted pair group method with arithmetic averages (UPGMA). The permutation tests for significant differences (d-values) between isolates were performed using pairwise Pearson similarity coefficients according to Kropf *et al*. (2004).

3 Results

3.1 Morphological identification of *Pratylenchus penetrans* isolates

Significant differences in morphometric characters of the seven *P. penetrans* isolates were found (Table 2). The body length ranged from 381 to 625 µm. Isolate WZ had the shortest average body length (437 µm) and isolate FR had the longest average body length (545 µm). The average body lengths of isolates FR, NL, BL and BN were not significantly different from each other, but they were significantly higher than those of isolates MN, WZ and UK. The a ratio ranged from 22.1 to 30.8. Isolate WZ had the lowest average a ratio (25.0 µm) while isolate FR had the highest (27.7 µm). The average a ratios of isolates FR, BN, NL and MN were not significantly different from each other but they were significantly higher than WZ and UK isolates. The b ratio ranged from 5.3 to 10.3. Isolate MN had the lowest average b ratio (6.88) while isolate NL had the highest (8.00). The average b ratios of isolates NL, FR, UK and WZ were not significantly different from isolates BL and BN but significantly higher than that of isolate MN. The c ratio ranged from 14.1 to 23.3. Isolate BN had the lowest average c ratio (17.4) while isolate FR had the highest (19.9). The average c ratios of isolates FR and MN were not significantly different from each other and from those of isolates WZ, BL, UK and NL. However, the average c ratios of isolates FR and MN were significantly higher than that of isolate BN. The V value ranged from 73.3% to 85.9%. The average V value was lowest for isolate UK (78.8%) and highest for isolate BL (80.9%). The average V values of these two isolates were significantly different from each other but did not differ significantly from those of the other isolates. The stylet length ranged from 14.5 to 16.8 µm. The stylet length was on average shortest for isolates WZ, FR and NL (15.0 µm) and longest for isolate UK (16.0 µm). The average stylet lengths of isolates UK and BL were not significantly different from each other but significantly higher than those of the other isolates. The distance of the excretory pore from the anterior end ranged from 63.3 to 98.5 µm. This distance was on average shortest for isolate WZ (68.0 µm) and longest for isolate BL (81.8 µm). Measurements of the isolates FR and NL were not significantly different from that for isolates BN and BL. However, the distance of the excretory pore from the anterior end for isolates FR and NL was significantly higher than that of the other isolates. The pharynx length ranged from 89.0 to 161 µm. The pharynx length was on average shortest for isolate UK (97.0 µm) and longest for isolate BL (121 µm). The average pharynx lengths of isolates WZ and UK were not significantly different from each other and from isolate MN. However, the average pharynx lengths of isolates WZ and UK were significantly lower than those for the

remaining 4 isolates. The length of the pharyngeal overlap ranged from 30.9 to 58.7 µm. The length of pharyngeal overlap was on average shortest for isolate UK (37.0 µm) and longest for isolate BL (46.3 µm). The average length of the pharyngeal overlap for isolate BL was not significantly different from those of isolates WZ, BN and NL but it was significantly higher than those of the other isolates. The length of ovary ranged from 114 to 244 µm. The length of ovary was on average shortest for isolate MN (151 µm) and longest for isolate BL (191 µm). The average length of ovary for isolates UK and FR were not significantly different from each other and from those for isolates MN, WZ, BN and NL but they were significantly shorter than that for isolate BL.The tail length ranged from 20.3 to 36.2 µm. The tail length was on average shortest for isolate MN and WZ (23.0 µm) and longest for isolates BL, BN and NL (29.0 µm). The average tail lengths of isolates MN and WZ were significantly lower than those of the remaining of the isolates.

Coefficient of variation (CV) values for the various characters measured ranged from 2.2 to 14.9% (Table 2). Among the morphometric characters measured the coefficient of variation was lowest for V value (2.2%) and stylet length (2.4%). The coefficient of variation was highest for length of ovary (14.9%), length of post-vulval uterine sac (14.6%) and ratio of the length of post-vulval uterine sac to body width at vulva (14.3%).

Table 2: Morphometrics of the females of seven *Pratylenchus penetrans* isolates.

Characters	*P. penetrans* isolates							CV[4] (%)
	MN	WZ	BN	BL	UK	FR	NL	
L	448 ± 11[1] ab[2] (431 – 462)[3]	437 ± 33 a (381 – 492)	506 ± 39 c (465 – 578)	525 ± 42 cd (443 ± 594)	470 ± 28 b (428 – 517)	545 ± 34 d (505 – 625)	527 ± 30 cd (465 – 572)	6.23
a	25.9 ± 1.1 ab (24.8 – 28.6)	25.1 ± 2.0 a (22.2 – 28.9)	27.6 ± 2.2 b (24.4 – 31.2)	26.5 ± 2.6 ab (22.1 – 29.7)	25.2 ± 1.3 a (22.2 -27.3)	27.7 ± 1.5 b (25.9 – 30.2)	27.2 ± 1.9 b (24.6 – 30.8)	6.93
b	6.88 ± 1 a (5.84 – 9.22)	7.96 ± 0.8 b (6.63 – 9.55)	7.72 ± 1.3 ab (6.32 – 10.3)	7.06 ± 0.9 ab (5.62 – 8.44)	7.93 ± 0.4 b (6.45 – 9.23)	7.96 ± 1.3 b (5.33 ± 9.17)	8.0 ± 0.5 b (6.92 – 8.5)	12.4
b’	4.34 ± 0.3 a (3.8 – 5.08)	4.39 ± 0.3 a (3.98 – 4.81)	4.53 ± 0.3 ab (4.04 – 5)	4.35 ± 0.4 a (3.69 - 5.02)	4.86 ± 0.4 bc (4.08 – 5.49)	4.9 ± 0.7 c (3.88 -7.79)	4.87 ± 0.2 bc (4.55 – 5.21)	7.92
c	19.3 ± 1 b (17.1 ± 20.5)	19.1 ± 1.4 ab (16.4 -21.1)	17.4 ± 2.1 a (14.1-20.3)	18.3 ± 1.8 ab (14.4 – 20.7)	18.2 ± 1.9 ab (14.6 – 21.7)	19.9 ± 2.1b (16.9 ± 23.3)	18.4 ± 1.7 ab (16.0 ± 21.0)	9.48
c’	2.2 ± 0.1 ab (2.0 – 2.37)	2.02 ± 0.2 a (1.71 – 2.43)	2.3 ± 0.4 ab (1.69 – 2.88)	2.36 ± 0.4 bc (1.66 – 3.25)	2.0 ± 0.2 ab (1.78 – 2.5)	2.13 ± 0.3 ab (1.86 – 2.57)	2.61 ± 0.3 c (2.09 – 3.0)	12.3
V	79.2 ± 0.9 ab (77.9 – 80.8)	79.9 ± 0.9 ab (78.6 – 81.9)	79.7 ± 2.5 ab (73.3 – 82.9)	80.9 ± 2.4 b (76.8 – 85.9)	78. 8 ± 1.9 a (76.7 – 81.6)	79.8 ± 1.6 ab (77.3 – 82.7)	79.6 ± 1.9 ab (76.0 – 82.3)	2.18
Stylet	15.1 ± 0.3 a (14.6 – 15.6)	15.0 ± 0.5 a (14.5 – 16)	15.3 ± 0.2 a (15.0 – 15.8)	15.8 ± 0.6 b (15.1 – 16.8)	16.04 ± 0.4 b (15.2 – 16.4)	15.0 ± 0.3 a (15.0 – 16.2)	15 ± 0.3 a (14.6 – 15.6)	2.40
SKH	2.0 ± 0.1 bcd (1.82 - 2.45)	2.0 ± 0.2 cd (1.83 – 2.4)	1.8 ± 0.2 a (1.48 – 2.04)	2.2 ± 0.3 d (1.75 – 2.6)	2.0 ± 0.2 bcd (1.67 – 2.4)	2.0 ± 0.3 abc (1.57 – 2.31)	2.0 ± 0.3 ab (144 – 2.28)	11.3
SKW	4.23 ± 0.4 b (3.7 - 4.86)	4.0 ± 0.4 b (3.23 – 4.78)	4.05 ± 0.3 b (3.72 – 4.7)	3.59 ± 0.4 a (2.86 – 4.23)	4.0 ± 0.2 b (4.01 – 4.75)	3.0 ± 0.3 a (2.99 – 3.87)	3.0 ± 0.4 a (2.67 4.08)	9.33
DGO	2.2 ± 0.1 ab (2.04 –2.41)	2.2 ± 0.6 ab (2.09 – 2.3)	2.3 ± 0.2 bc (2.07 – 2.59)	2.3 ± 0.2 bc (2.09 ± 2.49)	2.1 ± 0.1 a (2.06 – 2.26)	2.4 ± 0.1 c (2.17 – 2.59)	2.4 ± 0.1 c (2.13 – 2.5)	5.26
LH	2.19 ± 0.2 ab (1.81 – 2.38)	2.0 ± 0.4 a (1.14 – 2.49)	2.13 ± 0.2 ab (1.95 – 2.57)	2.35 ± 0.4 b (1.95 – 2.9)	2.0 ± 0.3 a (1.56 – 2.46)	2.0 ± 0.2 a (1.75 – 2.28)	2.0 ± 0.3 ab (1.61 – 2.8)	12.6
LW	7.35 ± 0.5 a (6.62 – 7.94)	7.0 ± 0.5 a (6.28 – 7.94)	7.12 ± 0.4 a (6.65 – 7.73)	7.07 ± 1.8 a (2.03 – 8.6)	7.0 ± 0.3 a (6.52 – 7.6)	7.0 ± 0.4 a (6.5 – 7.6)	8.0 ± 0.5 a (6.87 – 8.62)	5.91
Ph - L	104 ± 6.9 abc (89.0 – 115)	100 ± 6.9 ab (89.8 – 112)	112 ± 11 cd (96.8 – 133)	121 ± 11 d (100 – 136)	97 ± 6.4 a (94.5 – 111)	111 ± 20 cd (90.2 – 161)	108 ± 7.6 bc (97.6 – 119)	9.21
Ph - O	37.6 ± 3.4 a (30.9 – 40.2)	44 ± 3.3 bc (37.6 – 50)	45.4 ± 9.3 bc (32.6 – 58.7)	46.3 ± 5.3 c (35.9 – 52)	37 ± 2.8 a (33.1 – 41.5)	40 ± 4.9 ab (30.5 – 45.8)	42 ± 4.7 abc (34.8 – 50.4)	11.3
EP	70.6 ± 1.7 a (67.1 – 72.4)	68.0 ± 4.3 a (58.6 – 72.6)	76.4 ± 4.3 b (70.5 – 83.3)	81.8 ± 6.9 c (74.3 – 94.7)	72 ± 1.7 a (69.5 – 74.1)	79 ± 4.6 bc (68.2 – 84.3)	78 ± 3.0 bc (73.5 – 82)	4.98
MBW	17.3 ± 0.6 a (16.0 – 18.0)	17 ± 0.9 a (16.2 – 19.4)	18.4 ± 1.2 ab (16.1 – 20.2)	19.9 ± 1.7 c (17.4 – 23.4)	19 ± 0.8 bc (17.8 – 20)	20 ± 1.7 c (18 – 24)	19 ± 1.5 bc (16.6 – 21.2)	6.29
Ovary	151 ± 14 a (134 – 174)	182 ± 20 bc (155 – 218)	172 ± 31 abc (137 – 242)	191 ± 39.7 c (114 – 244)	163 ± 30 ab (131 – 221)	155 ± 19.9 ab (122 – 184)	177 ± 25 abc (142 – 220)	14.9
PUS	23.6 ± 3.9 b (18.5 – 29.3)	20.5 ± 3.7 ab (17.4 – 28.5)	19.6 ± 3.6 a (15.6 – 26.9)	23.1 ± 0.9 b (21.3 – 24.4)	19.7 ± 2.4 a (17.2 – 23.7)	20.7 ± 4.7 ab (15.7 – 29.3)	22.6 ± 2.2 ab (18.6 – 26.7)	14,6
BWV	14.7 ± 1.1 a (11.9 – 15.7)	15.7 ± 1.1 a (13.0 – 16.7)	18.2 ± 0.9 b (16.6 – 19.6)	17.8 ± 1.7 b (15.2 – 20.0)	17.8 ± 0.7 b (16.7 – 19.0)	18.1 ± 1.9 b (14.9 – 21.3)	17.9 ± 1.0 b (15.6 – 19.4)	7.07
P	1.62 ± 0.4 b (1.18 – 2.47)	1.3 ± 0.2 b (1.06 – 1.72)	1.07 ± 0.2 a (0.88 – 1.42)	1.31 ± 0.1 b (1.14 – 1.55)	1.1 ± 0.2 ab (0.94 – 1.41)	1.14 ± 0.2 ab (0.83 – 1.45)	1.26 ± 0.1 ab (1.0 – 1.5)	14.3
V - A	70.9 ± 2.2 ab (68.3 – 74.5)	66 ± 6.1 a (59.8 – 73.3)	71.3 ± 6.1 abc (64.7 – 80.6)	75.2 ± 7.7 bc (64.9 – 93)	77 ± 9.0 bc (65.5 – 96.5)	85 ± 11 d (70.2 – 107)	79 ± 7.4 cd (68.2 – 87.8)	9.34
Tail	23.4 ± 1.1 a (22.0 – 25.4)	23 ± 0.8 a (21.9 – 24.7)	29.3 ± 2.4 c (26.4 – 33.4)	28.9 ± 3.6 c (25.6 ± 36.2)	26 ± 2.6 b (20.3 – 30.4)	28.2 ± 3.8 c (23.7 – 31.6)	29 ± 1.9 c (24.7 – 32.1)	8.07

[1]Average and standard deviation (n = 10), [2]Different letters between column in the same row indicate significant differences according to Duncan’s Multiple Range Test at $P \leq 0.05$, [3]Range, [4]Coefficient of variation.

3.2 Molecular analysis of *Pratylenchus penetrans* isolates by RAPD

The primer SC-1030 used in this study produced RAPD profiles that distinguished the seven *P. penetrans* isolates from each other as well as from the positive control *H. carotae* (Figure 1). The average Pearson similarity coefficient for pairwise comparison of the seven *P. penetrans* isolates ranged from 11.4 to 66.3% (Table 3). The lowest similarity was found between isolates BL and MN (11.4%) followed by isolates FR and WZ (14.4%). The highest similarity was observed between isolates MN and UK (66.3%). Regarding the distance between geographic regions of *P. penetrans* isolates, the longest distance was between isolates WZ and UK (861 km) and the shortest was between BL and NL (127 km) (Table 3). However, genetic distance was not always correlated with geographic distance. For example, isolates MN and UK showed the highest genetic similarity, but the geographic distance between them was among the highest distances recorded (693 km).

To determine the relatedness among the seven *P. penetrans* isolates, a cluster analysis was performed based on the unweighted pair group method with arithmetic averages (UPGMA) using pairwise Pearson similarity coefficients. The resulting dendrogram grouped the RAPD fingerprints of the different *P. penetrans* isolates into seven clusters according to the seven *P. penetrans* isolates (Figure 1).

Permutation test based on Pearson correlation showed highly significant differences between all geographical isolates ($P < 0.001$). RAPD profiles were highly similar among samples of the same isolate rather than between isolates (d = 45). Overall, there was a high level of intraspecific variability between isolates of *P. penetrans* from different geographic regions.

Table 3: Average percentage genetic similarity (below diagonal) and approximate geographic distance (km; above diagonal) among seven geographic isolates of *Pratylenchus penetrans*.

Isolates	MN	WZ	BN	BL	UK	FR	NL
MN	_	169	143	288	693	616	129
WZ	24.0[1]	_	206	428	861	704	278
BN	36.0	28.0	_	237	712	501	127
BL	11.4	23.0	23.0	_	493	366	159
UK	66.3	23.0	46.0	23.0	_	650	594
FR	26.1	14.4	29.0	31.2	30.0	_	499
NL	29.0	37.0	28.0	16.0	23.0	20.4	_

[1]Average of four replicates per *P. penetrans* isolate.

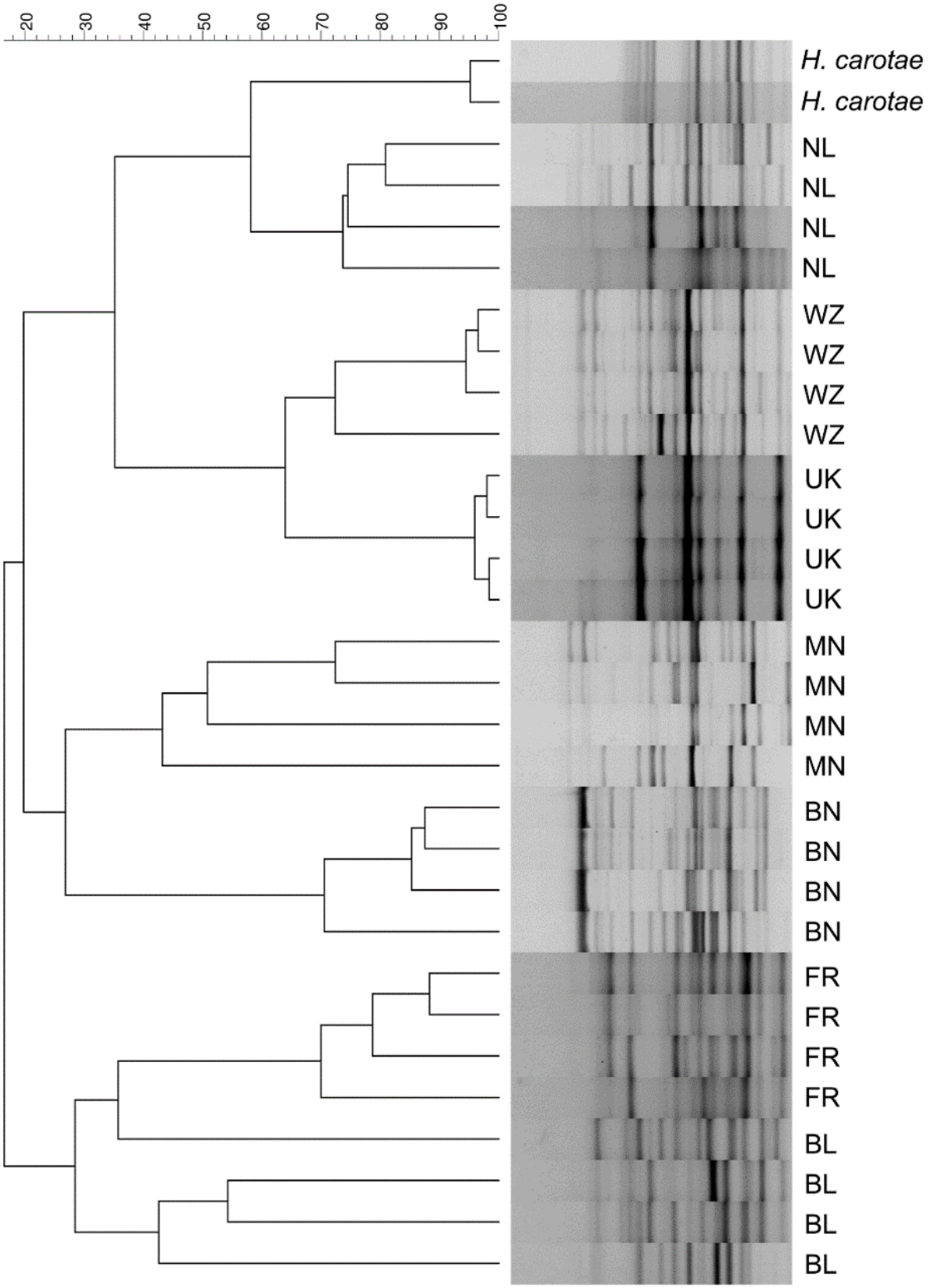

Figure 1: Dendrogram generated from the UPGMA cluster analysis of RAPD profiles of 7 *Pratylenchus penetrans* isolates using the primer SC-1030.

4 Discussion

4.1 Morphological identification of *Pratylenchus penetrans* isolates

The majority of morphometric characters of the seven isolates of *P. penetrans* studied here were within the range of the original description (Loof, 1960; Corbett, 1973) and population of *P. penetrans* described from China (Wu *et al*., 2002; Chen *et al*., 2009), Colombia, Ethiopia, France, Japan, Rwanda, The Netherlands, USA (Janssen *et al*., 2017) and Morocco (Mokrini *et al*., 2016). However, remarkable differences were found for a number of morphometric comparisons.

The average a ratio observed in *P. penetrans* isolates examined here ranged from 25.1 to 27.7. These values were higher than those described by Janssen *et al*. (2017) (24.0), but lower than those described by Chen *et al*. (2009) (29.9 - 32.0) and Mokrini *et al*. (2016) (29.2 - 33.0). The average b ratios in *P. penetrans* isolates examined (6.88 - 8.00) were comparable to those described by Mokrini *et al*. (2016) (6.66 - 7.70) but higher than those of Wu *et al* (2002) (5.60) and Janssen *et al*. (2017) (4.50). The average c ratios in *P. penetrans* examined ranged from 17.0 to 19.9. Most of these values were lower than those measured by Wu *et al*. (2002) (21.4), Chen *et al*. (2009) (20.2 - 22.1) and Janssen *et al*. (2017) (25.0). The average body lengths in *P. penetrans* isolates examined (437 - 545 µm) were shorter than in the populations described by Wu *et al*. (2002) (666 µm), Chen *et al*. (2009) (540 - 610 µm) and Janssen *et al*. (2017) (689 µm). The average V value observed in *P. penetrans* isolates ranged from 78.8 to 80.9%. Most values were similar to those described by Chen *et al*. (2009) (79.0 - 79.7%), Wu *et al*. (2002) (78.7%) and Mokrini *et al*. (2016) (75.0 - 80.0%), but higher than those measured by Janssen *et al*. (2007) (77.0%). The average distance of the excretory pore from the anterior end (72 - 81 µm), maximum body width (17 - 20 µm) and tail length (23 - 29 µm) observed in our study were comparable to those reported by Mokrini *et al*. (2016) (74.0 - 81.5 µm, 18.0 - 20.6 µm and 27.0 - 30.5 µm, respectively). Except maximum body width, which was considerably higher in our study, the average distance of the excretory pore from the anterior end and tail length were also comparable to Chen *et al*. (2009) (69.0 - 80.0 µm, 9.40 - 10.4 µm and 25.0 – 28.0 µm, respectively). However, the measurements of those 3 morphometrical features were shorter than described by Wu *et al*. (2002) (91.9 µm, 25.4 µm and 31.4 µm, respectively) and Janssen *et al*. (2017) (97 µm, 28 µm and 31 µm, respectively). The length of the post-vulval uterine sac (19.6 - 23.6 µm) observed in our study was shorter than reported by Mokrini *et al*. (2016) (26.2 - 30.9 µm) and Wu *et al*. (2002) (24.9 µm).

A comparison of the coefficient of variation of the morphometric characters examined showed that the position of the vulva and stylet length were the least variable characters. This supports previous reports on *P. penetrans* (Román and Hirschmann, 1969; Wu *et al*., 2002) and other *Pratylenchus* species (Tarjan and Frederick, 1978; Tuyet *et al*., 2012) showing that these characters are very stable allowing a clear demarcation between different isolates. On the contrary, the coefficient of variation of the length of the ovary (14.9%) and the length of the post-vulval uterine sac (14.6%) were very high, confirming previous studies by Román and Hirschmann (1969), Tarjan and Frederick (1978) and Wu *et al*. (2002). Other morphometrical characters of high variability in this study included b ratio (12.0%), length of stylet knob (11.3%), length of lip (12.6%), length of the pharyngeal overlap (11.3%), and the ratio of length of the post-vulval uterine sac to body width at vulva (14.3%).

Townshend *et al*. (1978) proposed for *P. penetrans* a relationship between pathogenicity and female tail characteristics. In our study, we found similar relationships between morphometric characters on one side and reproductive fitness and pathogenicity on the other side. For example, *P. penetrans* isolates FR, NL, BL and BN, which were highly damaging on alfalfa and carrot, all had a long body (545 µm, 527 µm, 525 µm and 506 µm, respectively), a long distance of excretory pore from anterior end (79.0 µm, 78.0 µm. 81.8 µm and 76.4 µm, respectively) and a long tail (28.2 µm, 29.0 µm, 28.9 µm and 29.3 µm, respectively). In contrast, isolates UK, MN and WZ showed lower damage potential on alfalfa and carrot and had a shorter body (470 µm, 448 µm and 437 µm, respectively), a shorter distance of excretory pore from anterior end (72.0 µm, 70.6 µm and 68.0 µm, respectively) and a shorter tail (26.0 µm, 23.4 µm and 23.0 µm, respectively). But there were not only differences between groups of isolates, but there were also many differences at the level of individual isolates. For example, isolate BL not only showed a high reproductive fitness and high damage potential but also a long pharyngeal overlap (46.3 µm) and ovary (191 µm), while isolates MN showed a low reproductive fitness and damage potential and a short length of pharyngeal overlap (37.6 µm) and ovary (151 µm).

4.2 Molecular analysis of *Pratylenchus penetrans* isolates by RAPD

RAPD-PCR analysis of the seven *P. penetrans* isolates from different geographical locations provided sufficient polymorphisms to reveal significant intraspecific genetic differences. The seven *P. penetrans* isolates clustered into seven distinct groups according to their origin. Our results contradict observations made by Pinochet *et al*. (1994) on *Pratylenchus vulnus*. Those authors studying seven populations of *P. vulnus* from different geographical areas (USA,

Argentina, France, Spain and Italy) were not able to separate the populations by RAPD-PCR. Those differences are probably best explainable by the different primer system used and different *Pratylenchus* species studied. Although our own study showed a clear clustering of RAPD profiles of the seven *P. penetrans* isolates according to their geographical origin, the proximity of RAPD profiles did not correspond with the proximity of geographic regions. For example, the shortest geographic distance was between isolates BL and NL (127 km) but these two isolates were not clustered together. This corresponds with a study by Tuyet *et al*. (2014) who examined 10 *Pratylenchus coffeae* populations isolated from different host plants and regions of Vietnam using RAPD-PCR analysis and concluded that genetic similarity did not correspond either with geographic proximity or the host plants.

RAPD-PCR has also been used to correlate genetic diversity of plant-parasitic nematodes with pathogenicity (Pinochet *et al*., 1994; Hahn *et al*., 1995; Fallas *et al*., 1996; Costa *et al*., 2008). In our study, a correlation between pathogenicity, morphometrics and genetic similarity was found for some *P. penetrans* isolates, but not all. The two isolates with the largest genetic difference (BL *vs* MN), also showed the largest difference in body length (525 µm *vs* 448 µm), stylet length (15.8 µm *vs* 15.1 µm), maximum body width (19.9 µm *vs* 17.3 µm), tail length (28.9 µm *vs* 23.4 µm) and several other parameters. Similar, isolates FR and WZ showed the second highest genetic difference (14.4%), which was reflected in their morphometric divergence regarding body length (545 µm *vs* 437 µm), a ratio (27.7 *vs* 25.0), maximum body width (20.0 µm *vs* 17 2 µm) and distance between vulva and anus (85 2 µm *vs* 66 2 µm). On the other side, the genetically two most similar *P. penetrans* isolates (MN *vs* UK) also shared similar morphometric features, like a ratio (25.9 *vs* 25.2), V value (79.2% *vs* 78.8%), distance of excretory pore from anterior end (70.6 µm *vs* 72 µm), pharyngeal length (104 µm *vs* 97 µm) and length of pharyngeal overlap (37.6 µm *vs* 37 µm). However, on the contrary, isolates BL and MN, which were ranked among the highest and the lowest regarding reproductive fitness and pathogenicity, showed the lowest genetic similarity (11.3%). Furthermore, reproductive fitness and pathogenicity of isolate UK was at a similar low level like isolate MN, and showed the highest average genetic similarity (65.3%).

In this study a high intraspecific genetic variability was observed among *P. penetrans* isolates from different geographical origin, which suggests that they were reproductively isolated. Various factors are likely to contribute to intraspecific variability. For example, genetic drift and selection by various environmental parameters may influence the genetic structure of a nematode population (Star and Spencer, 2013; McGaughran *et al*., 2014). Due to missing

information regarding the environmental conditions of the seven *P. penetrans* isolates studied here, the reasons for the observed intraspecific genetic variability between those *P. penetrans* cannot not be discussed any further. Maybe methods other than RAPD-PCR are better suited to describe intraspecific variability within *P. penetrans*. Within this respect, Janssen *et al*. (2017) studied the genetic diversity and phylogeny of several populations of *P. penetrans* and other *Pratylenchus* species using the Generalized Mixed Yule Coalescent (GMYC), Automatic Barcode Gap Discovery (ABGD) and D2-D3 sequences. They also reported high intraspecific variability for *P. penetrans* populations from different locations in The Netherlands. On the contrary, Mokrini *et al*. (2016) and Anders *et al*. (2000) using D2-D3 sequences and isozyme markers, respectively found very low diversity between *P. penetrans* populations from Morocco and Canada, and between France and Germany, respectively.

In conclusion, RAPD is a powerful tool for the assessment of intra- and interspecific genetic diversity in plant parasitic nematodes such as *Pratylenchus* spp. (Pinochet *et al*., 1994; Ouri, 1996: Ouri and Mizukubo, 1999; Tuyet, *et al*., 2014), *Meloidogyne* spp. (Cenis, 1993; Block, *et al*., 1997), *Globodera rostochiensis*, (Chacón *et al*., 1994) and *R. similis* (Fallas *et al*., 1996; Hahn *et al*., 1996b). Our results confirm the suitability of this technique in discriminating isolates of *P. penetrans* at intraspecific level.

Summary

The root lesion nematode, *Pratylenchus penetrans* is regarded as one of the most economically destructive plant-parasitic nematodes worldwide. It is widely distributed and causes damage to a wide range of annual and perennial crops. In Europe, several *Pratylenchus* species are known to harm a wide spectrum of vegetables and field crops and of these, *P. penetrans* is the most important (Hooker, 1981; Castillo and Vovlas 2007). In recent years, there has been increasing interest to employ nonchemical nematode management strategies such as the use of resistant cultivars, crop rotation, cover cropping and biological control. The application and efficient utilization of such nonchemical nematode management methods requires a good understanding about the biology and genetic diversity of the *P. penetrans* isolates present in the field. The present study was conducted to investigate the variability among seven *P. penetrans* isolates collected from different geographical regions based on their reproductive fitness and pathogenicity on selected hosts and their morphometric and genetic characteristics. The study also assessed the host status of several cover crop species and cultivars for *P. penetrans*.

In Chapter I the economic importance, the biology, ecology and histopathology of the *P. penetrans* were explained. Then some cultural control methods were highlighted. Finally the objectives of the study were explained and the flow diagram showing various steps of the research was provided.

In Chapter II, pure cultures of *Pratylenchus* isolates were generated from 10 soil samples originating from Germany, Italy, The Netherlands, Slovenia, France, Belgium, United Kingdom, Sweden, Switzerland and Algeria. Different methods for nematode culturing were compared and optimized. Under our conditions, the monoxenic carrot disc culture technique proved to be the best for the establishment and maintenance of *Pratylenchus* isolates and to provide sufficient inoculum for the experiments. The method involves surface sterilization of fresh carrots by soaking them in 2.8% sodium hypochlorite (Klorox) solution with a drop of detergent (Pril) for 5 min followed by washing with sterilized water. Nematodes were then surface sterilized with 0.1% streptomycin sulphate for 5 min followed by 0.02% mercury chloride for 1 min and then washed three times in sterilized water. Regarding nematode extraction from carrot discs, cutting the carrot discs in 8 pieces followed by maceration with an Ultra turrax disperser was found to give best results in terms of number of extractable nematodes and eggs, and cleanness of the suspension. The isolated *Pratylenchus* isolates were

identified by morphological and molecular means as *P. mediterraneus, P. neglectus*, *P. penetrans*, and *P. thornei*. The most prevalent species was *P. neglectus*.

The *P. penetrans* isolates were then studied in several pot experiments. For best plant growth and nematode infection conditions, different combinations of growth substrates and fertilizer levels were compared in greenhouse experiments. A mixture of sand, clay (Seramis) and field soil (4:1:1, v:v:v) fertilized with 0.3% Wuxal Super liquid fertilizer (N:P:K, 8:8:6 + microelements) was found to be best for plant growth and nematode multiplication. Optimum extraction of mobile nematodes from roots was achieved by two weeks extraction in a misting chamber. Using Baermann trays instead of Baermann funnels for extraction of *P. penetrans* resulted in slightly higher nematode numbers.

In Chapter III, two separate experiments were performed to determine the effect of different cover crop species and cultivars on the reproduction of *P. penetrans* under greenhouse conditions. In the first experiment, bird vetch was found to be an excellent host for *P. penetrans*. Crimson clover and three cultivars of fodder radish were good hosts while two cultivars of bristle oat and daikon radish were maintenance hosts for *P. penetrans*. In the second experiment, lentil and common vetch were found to be excellent hosts. Buckwheat, common bird's foot, fodder radish, forage rape, Italian ryegrass, rapeseed and sunflower all were good hosts for *P. penetrans*. None of the tested cover crops were poor or non-hosts for *P. penetrans*. Thus, none of the tested cover crops can be recommended if management of *P. penetrans* is the primary objective.

In Chapter IV, the susceptibility of different cover crop species plus different cultivars of fodder radish, lentil, common vetch and black oat against *P. penetrans* was tested in four consecutive greenhouse experiments. In the first experiment, all fodder radish cultivars tested were found to be good hosts. Furthermore, rocket turned out to be a maintenance host, camelina and ribwort plantain being poor and non-hosts for *P. penetrans*, respectively. In the second experiment, all common vetch and lentil cultivars were found to be excellent hosts for *P. penetrans*. In the two following experiments, out of 14 cultivars of black oat, two were found to be maintenance hosts while the remaining cultivars were poor hosts of *P. penetrans*.

In Chapter V seven geographic isolates of *P. penetrans* were compared for their in *vitro* and *in vivo* reproductive fitness and pathogenicity. In the *in vitro* carrot disc assay, *P. penetrans* isolates differed in their final population size and relative occurrence of developmental stages at 80 days after inoculation. Isolates BL and UK showed the highest and the lowest

reproductive fitness, respectively, whereas the remaining isolates showed intermediate reproduction. The *in vivo* reproductive fitness was tested on common vetch and lentil. Final nematode numbers at 10 weeks after inoculation differed among the seven isolates of *P. penetrans*. Results on reproductive fitness achieved *in vitro* on carrot discs and *in vivo* on common vetch and lentil were highly correlated. The pathogenicity of the seven *P. penetrans* isolates was tested on alfalfa, carrot, fodder radish and French marigold. Differences in pathogenicity varied depending on the isolate of *P. penetrans* and the inoculum level. On alfalfa, both root and shoot growth was significantly affected by *P. penetrans*. Isolates BL and BN were more pathogenic compared to isolates FR, NL and WZ. Isolates MN and UK reproduced poorly and showed only little effect on plant growth. On carrot, only taproot growth and quality was significantly affected by *P. penetrans*. Isolates NL, BN, BL and FR were pathogenic to carrot, whereas isolates MN, WZ and UK did neither reproduce nor cause any significant damage on taproots. On fodder radish, isolates NL, BL, UK, and FR caused a significantly higher reduction in root fresh weight, compared to isolates MN, WZ and BN. However, damage potential was not correlated to the reproductive fitness. Isolates MN, WZ and UK reproduced poorly whereas isolates BN, BL, FR and NL reproduced well on fodder radish. Reproductive fitness and pathogenicity of *P. penetrans* isolates on alfalfa and carrots and reproductive fitness on fodder radish correlated with the reproductive fitness on common vetch and lentil and *in vitro* on carrot discs for all isolates except isolate WZ. Our study confirms that *P. penetrans* was neither able to reproduce nor to cause any damage on French marigold.

In the last Chapter VI, the seven geographical isolates of *P. penetrans* were compared for their variability in morphometric parameters and genetic characteristics using RAPD method. Morphometrics of *P. penetrans* isolates were within the range of the original descriptions, but showed differences in some features to populations reported from other countries, such as body length, a, b and c ratio, maximum body width, tail length and length of the post-vulval uterine sac.

Pratylenchus penetrans isolates BL, BN, NL and FR sharing morphometric similarities showed the highest reproductive fitness and pathogenicity whereas the morphological similar isolates MN and UK showed the lowest reproductive fitness and pathogenicity. RAPD analysis indicated a high level of genetic variability between the seven *P. penetrans* isolates examined. All seven isolates could be clearly distinguished from each other according to their geographical origin. A correlation between pathogenicity, morphometrics and genetic

similarity was found for some *P. penetrans* isolates. Isolates MN and UK were genetically and morphological similar and showed similar reproductive fitness and pathogenicity. In contrast, isolates BL and MN with the largest genetic and morphological differences also showed largest differences in reproductive fitness and pathogenicity. In conclusion, the seven isolates of *P. penetrans* could be clearly distinguished based on morphological and genetic characterization, as well as biological parameters such as reproductive fitness and pathogenicity.

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Declaration / Erklärung

I declare: this dissertation submitted is a work of my own, written without any illegitimate help by any third party and only with materials indicated in the dissertation. I have indicated in the text where I have used texts from already published sources, either word for word or in substance, and where I have made statements based on oral information given to me. At any time during the investigations carried out by me and described in the dissertation, I followed the principles of good scientific practice as defined in the "Statutes of the Justus Liebig University Giessen for the Safeguarding of Good Scientific Practice".

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Place, date**:** Giessen, September 25, 2019 Signature_____

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