A Thesis Submitted for the Degree of Doctor of Philosophy at

Harper Adams University

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Harper Adams University

The prevalence, detection and impact of root-lesion nematodes (*Pratylenchus* spp.) found in potato growing land in Great Britain

Thesis submitted for the award of the degree of Doctor of Philosophy by Harper Adams University

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2021

Director of studies: Dr. Matthew Back
Second Supervisor: Prof. Simon G. Edwards

DECLARATION

The study and results presented in this thesis have been conducted and written by the author. All information from other sources have been cited, referenced and fully acknowledged.

Valeria Orlando

20/07/2021

ACKNOWLEDGEMENTS

Many doors were closed before I could get the right PhD project at the right moment, for that I am very grateful to **AHDB potatoes** and **Harper Adams University** for funding my PhD project and give me this wonderful opportunity. The PhD journey is generally tough for everyone. I never met a PhD student saying the journey was easy and smooth. There are continuous up and down for all the path until the end. When something goes right, then there is something else going wrong in other parts of the project. It is like a ride with a bike: sometimes you need to stop for a breath, other times to fix the punctured wheels and others just enjoy the landscapes. I am not a cyclist, but I enjoy sometimes to go for a ride, and I enjoyed the English landscapes during one break and the other of my PhD, especially during lockdown. Metaphorically talking, I cycled a lot in the last five years, overcoming one obstacle to the other. When I started, I never thought I could deal with some personal issues and a pandemic due to COVID-19. Never! During my ride, the bike got broken and I had to stop. I pushed the bike uphill, and I managed to get it repaired and restart cycling faster than before. Forgive me for this unusual start of acknowledgments, it may sound selfish or haughty, but I firstly wish to thank **myself**, because this thesis could not be ended without my passion for Nematology, toughness, hard work, enthusiasm and wishes to win any battle in life. I thank myself that I never gave up and continue to believe in dreams, even when life turned against me. I always try to take the positiveness of any situation, and this allowed me to achieve my goals, including this thesis!

Of course, this thesis would not end in such good way without the constant help of my director of studies, **Dr. Matthew Back**. He guided me until the end of this ride, supporting me for any problem or joy achieved. I am sure it was not easy neither for him, but together we arrived at the end. I am very thankful for his constant presence. When I slipped off the bike, he helped me to get up and continue my ride, supporting me with “lots of bottles of water, boosts and cheers”. We enjoyed together the end of this journey and I am sure we will continue to collaborate in future or just be good friends as we became! - Thank you, Matt! I will be thankful to you for the rest of my life and career! -

Many thanks to my supervisor **Prof. Simon Edwards**, always keen to help me with preciousus suggestions and comments for statistical analysis and molecular diagnostics. - Thanks for all your support, Simon! I really appreciated it! –

Thanks also to **Dr. Ivan Grove**, **Tom Prior** and **Dr. Roy Nielson** for useful advice and constant guidance when requested. I learned a lot through their suggestions, comments and ideas. A special thanks also to **Dr. David Roberts** that delivered a fantastic training for the molecular

part at the James Hutton Institute (JHI). Without his help the fourth chapter of this thesis could not be like it is. - Thank you so much, Dave! My period at JHI has been one of the best parts of this journey! -

During my PhD, I travelled a lot, especially in autumn 2017 to take soil samples for my survey. I enjoyed many English landscapes that I will never forget. The sampling time was tough, but I enjoyed being in contact with farmers and agronomists. For that I would like to thank **UK farmers** and **agronomists**, especially **Martyn Cox**, **Jhon Keer** and **Peppa Blaylock** for helping me to find most of the potato fields for the survey. A special thank also to **CERC** (HAU), **Dr. Grace Milburn** and **Harriet Vinall** to arrange the sampling for me in 2019 and help to achieve the collection of 200 samples as we planned.

Thanks to all Nematology group – **Ahmed Mohammed**, **Musa Nasamu**, **Kasia Dybal**, **Alex McCormarck**, **Victoria Taylor**, **Bill Watts** and **Ana Morais Natalio** – for the nice chats and time spent together throughout all this journey. It was a fantastic experience to meet you all. Thanks also to other friends at HAU like **Michele Faralli**, **Fittonia Elgina**, **Dimitris Parapas**, **Andrea Labruzzo**, **Louise Wright**, **Emily Forbes**, **Annie Williams, Laura Shewbridge Carter**, **Francisca Sconce** and **Sarah Woods**. I will never forget our international dinners and many other nice activities organized all together. Thank you so much also to **Maria Chiara Rosace** that assisted me on the experiments for pathogenicity during summer 2018 and **Ahmed Mohammed** helping for some nematode extractions. I became friends of several PhD students and lecturers at HAU, but one is special for me: my best friend, **Rania Tremma**! She supported me in many ways during my PhD, cycling on my side between one stop and the other. She offered me a shelter where to get a rest and enjoy together any finish line achieved - Thanks, Rania, for all what you have done for me! You are my star, I am proud of our friendship and I know we will be friends for ever! -

I thank also **Dr. Zorka Besevic** for giving me the opportunity to arrange Italian classes for HAU staff members, it has been a funny and great experience that I will never forget. Thanks also to **Dr. Ed Harris** for all the comments and advice for statistics, especially the one at the last minute! - Thanks for your availability! -

Many thanks to **Nancy de Sutter** and **Dr. Nicole Viaene** (ILVO, Belgium) for providing carrot discs of *P. penetrans, P. crenatus* and *P. thornei* and precious advice for nematode cultures. Thanks also to **Dr. Francesca De Luca** (CNR of Bari, Italy) for providing culture of *P. vulnus* useful for the validation of qPCR. I also thank **Dr. Toon Janssen** and **Prof. Wim Bert** (Ghent University, Belgium) for providing slides of *Pratylenchus* spp and DNA samples for the validation of qPCR. Thanks to **Fera Science Ltd** and **The James Hutton Institute** for providing important trainings for morphological and molecular identification of root-lesion

nematodes that helped me to develop fundamental skills to achieve my next challenge: find a job in Nematology!

Finally, thank you so much to my friends and family that supported me during these years. Many thanks to my niece **Caterina Sciortino** and my nephew **Pasquale Sciortino**, to provide the best company during difficult times and the daily videocalls during my time away from home - Your smiles have given me the energy to go on! - Thank you so much to my mother, **Grazia Restivo**, example of hard worker and worrier, always supporting me for any decisions I made. Thanks to **Anna Maria** and **Laura Orlando**, the best sisters I could wish to have! - We are a dream team and your support has been essential to arrive at the end of this ride and start a new adventure for my career! I will be thankful to you both for the rest of my life! – Finally, thanks to my father **Pasquale Orlando**, certain that his invisible hand helped me to push the bike on the hardest climbs – Thanks for being always by my side!

"However difficult life may seem, there is always something you can do and succeed at!" - Stephen Hawking

Ad maiora!

Valeria Orlando

RESEARCH OUTPUTS

Publications:

1) Orlando, V., Grove, G.I., Edwards, S.G., Prior, T., Roberts, D., Neilson, R., Back M., 2020. Root-lesion nematodes of potato: current status of diagnostics, pathogenicity and management. *Plant Pathology* 69, 405 - 417.
2) Orlando, V., Edwards, S.G., Neilson, R., Prior, T., Roberts, D., Back, M., 2020. Comparing the efficiency of six common methods for DNA extraction from root-lesion nematodes (*Pratylenchus* spp.). *Nematology* 23, 415 - 423.

Poster presentations

1) 69th ISCP symposium, Campus Coupure, Ghent (Belgium) – "Detection, distribution and impact of root lesion nematodes (*Pratylenchus* spp) in potato crops in England and Scotland" (23 May 2017)
2) AHDB crops PhD Studentship Conference 2017 - "Development and yield of potatoes cv. Maris Peer in soil infested with *Pratylenchus penetrans*" (7- 8 November 2017)
3) Student Colloquium Conference 2017, Harper Adams University - "Development and yield of potatoes cv. Maris Peer in soil infested with *Pratylenchus penetrans*" (1 December 2017)
4) AAB Advances in Nematology, Linnean Society, London - "Development and yield of potatoes 'Maris Peer' in soil infested with *Pratylenchus penetrans*" (12 December 2017)
5) The 33rd Symposium of Eurpean Society of Nematologists, Faculty of Bioscience Engineering, Ghent University, Coupure links 653, B-9000 Ghent, Belgium - "Development and yield of potato 'Maris Peer' in soil infested with *Pratylenchus penetrans* " (9-13 September 2018)

Oral presentations

1) AHDB crops PhD Studentship Conference 2016, Short oral presentation of the PhD project (16 - 17 November 2016)
2) Student Colloquium Conference 2016, Harper Adams University, short oral presentation of the PhD project (1 December 2016)
3) SpotWest Result Day, Harper Adams University - "Assessing the impact of root-lesion nematode (*Pratylenchus* spp.) infestations on the production of potatoes" (18 January 2018)
4) AHDB crops PhD Studentship Conference 2020 - "Assessing the impact of root-lesion nematode (*Pratylenchus* spp.) infestations on the production of potatoes" (January 2020)
5) Student Colloquium Conference 2020, Harper Adams University (webinar) – "Development and validation of four TaqMan qPCR methods for the identification and quantification of *Pratylenchus crenatus, Pratylenchus neglectus, Pratylenchus penetrans* and *Pratylenchus thornei*" (30 November 2020)
6) AHDB Agronomy Week 2020 (webinar) - "Detection and distribution of root-lesion nematodes in England and Scotland" (December 2020)
7) AAB Advances in Nematology, Linnean Society, London (online) - "Development and validation of four TaqMan qPCR methods for the identification and quantification of *Pratylenchus crenatus, Pratylenchus neglectus, Pratylenchus penetrans* and *Pratylenchus thornei*" (15 December 2020)

8) ESN’s virtual Nematology Conference (online) - “Detection and distribution of root-lesion nematodes in potato fields in England and Scotland” (26-28 May 2021)

Travel awards:

1) The 33rd Symposium of European Society of Nematologists, Faculty of Bioscience Engineering, Ghent University, Coupure links 653, B-9000 Ghent, Belgium. Poster presentation "Development and yield of potato ‘Maris Peer’ in soil infested with *Pratylenchus penetrans*"

2) BSPP (British society of plant pathologists) travel award to participate at ESN conference 2018. Poster presentation " "Development and yield of potato ‘Maris Peer’ in soil infested with *Pratylenchus penetrans*"

Teaching experience

1) The Big Bang Fair 2017, Birmingham, 18 March 2017: Promote careers in Entomology and routes from school/college. Promote HAU as institution offering higher education and research for the delivery of a sustainable food chain and rural economy.
2) Mini Beast Event, Dorothy Clive Garden: Promote Nematology and soil invertebrates to the public (17 - 18 July 2017)
3) CROPSS, Inspiring biology students to consider careers in crop protection, Harper Adams University: Practical class on the identification of soil nematodes, life stage and feeding type (6 - 7 July 2017 and 11 - 12 July 2019)
4) EntoSci18, Insect Ambassadors at Harper Adams University. Promote Entomology to school. Introduce students to the world of arthropods showing different insects, spiders, and other soil invertebrates (17 April 2018).
5) Italian short course in a voluntary basis to STAFF HAU members (From July 2019 to September 2019)

GENERAL ABSTRACT

Potato (*Solanum tuberosum*) is one of the most important crops in the world and yield can be negatively influenced by several pests, including plant-parasitic nematodes. Few studies have been conducted on root-lesion nematodes (*Pratylenchus* spp.) and their impact on the potato production in Great Britain. The main objectives of this study were: (1) to identify *Pratylenchus* spp. present in potato growing land in England and Scotland, and to develop molecular assays for their rapid confirmation and quantification; (2) to undertake a survey to determine the distribution and prevalence of *Pratylenchus* spp. in potato growing land in England and Scotland; (3) to determine pathogenicity and potato damage thresholds for *Pratylenchus* species in different soil types with Maris Peer cultivar, under controlled conditions.

Accurate identification and quantification of root-lesion nematodes (*Pratylenchus* spp.) is an important step for nematode management. Molecular diagnostics may provide an alternative to morphological identification that is time consuming and requires significant taxonomic experience. Efficient DNA extraction is the first important step for any molecular diagnostic. Six common DNA extraction protocols were compared to evaluate their efficiency to obtain quality DNA samples for *Pratylenchus penetrans*. Among all methods tested, the DNA extraction protocol with glass beads proved to be efficient for *P. penetrans* and it was selected to be tested for other *Pratylenchus* species (*P. crenatus, P. neglectus* and *P. thornei*). The method generated high quality DNA for molecular analysis, and it was used for the development of real-time quantitative PCR (qPCR). A TaqMan hydrolysis probe assay based on 28S rDNA D2-D3 expansion fragment was developed and validated for the identification and quantification of *P. crenatus, P. neglectus, P. penetrans* and *P. thornei*. Four standard curves were made showing a strong linear correlation ($R^2 = 0.99$) between Ct values and DNA copy numbers with no cross reaction with non-target species, demonstrating the specificity of primers and probes. Specificity, sensitivity and selectivity of the methods were confirmed also by three further experiments with different life stages, increasing numbers of target species and mixed samples. Finally, estimates obtained by qPCR methods were compared with counting done by microscopy showing a good correlation ($R^2 = 0.78$). The qPCR TaqMan assays here developed were specific, sensitive, fast and accurate for quantification of *P. crenatus, P. neglectus, P. penetrans* and *P. thornei*.

Little is known regarding the presence and distribution of *Pratylenchus* spp. in potato fields in Great Britain. Two hundred potato fields from the top fifteen counties in England with highest area of potato production were sampled between September and November, in 2017 and 2019. Samples from eighteen fields in Scotland were examined in a separate study. Root-lesion nematodes were detected in 88% of soil samples from England and 94% from Scotland,

revealing a high presence in agricultural land in both countries. Positive detections were higher in the East and North East of England, with *Pratylenchus neglectus* and *P. thornei* being the most widely distributed species in England*;* *P. crenatus* and *P. penetrans* were also detected but were less abundant. *Pratylenchus thornei* was the most abundant species in South East and South West England, *P. neglectus* was mainly found in the East and *P. penetrans* in North East and South East of England. Biotic factors that may influence the presence and prevalence of each species such as soil type, previous crop, crop at sampling, irrigation, nematicide application did not have significant effects on the presence and abundance of the genus as a whole or the individual species. In Scotland, the most present and abundant species were represented by *P. neglectus* and *P. crenatus*, but also *P. penetrans* and *P. thornei* were recorded.

Three controlled environment experiments investigated the impact of *P. penetrans* and *P. thornei* on the yield and quality loss of the Maris Peer potato variety. The first experiment assessed the impact of mixed juveniles and adult populations of *P. penetrans*, ranging from 0.125 to 4 nematodes g^{-1} soil, on growth and yield of Maris Peer. Different proportions of coarse sand and compost (John Innes No. 2) were mixed to form three different sandy based soils: ST1 (10% compost and 90% coarse sand), ST2 (20% compost and 80% coarse sand) and ST3 (40% compost and 60% coarse sand). Nematode density had no effect on potato yield at six weeks after inoculation and planting, although the yield was significantly affected by soil type with ST1 giving the lowest yield. Root-lesion nematodes were detected within the roots of potatoes grown in each soil type, highlighting that *P. penetrans* was able to infect this cultivar without inducing yield loss at these population densities. Two further controlled experiments using a broader range of nematode densities, from 2 to 32 nematode g^{-1} soil were conducted with *P. penetrans* and *P. thornei*, respectively. As with the first experiment, the nematode densities investigated were found to have no effects on potato yield, but both species were detected within the roots of potatoes, confirming invasion occurred. Final population density (P_f) were less than the initial population density (P_i) in all treatments and experiments, suggesting that reproduction was limited on Maris Peer and may indicate partial resistance by this cultivar. However, the length of experiment and other factors such as temperature, moisture or soil matrix under pot trials may have influenced the reproduction of nematodes. Further studies using other GB potato cultivars, other common root-lesion nematode species like *P. neglectus* or *P. crenatus*, considering also stress factors such as drought conditions, may help provide a better understanding of the nematode's pathogenicity and impact on yield in GB potato cultivars. Consequently, further strategies may be considered for root-lesion nematode management to help farmers to limit the potential impact of these nematodes on potato production.

LIST OF ABBREVIATION

AHDB	Agricultural and Horticultural Development Board
COX1	Cytochrome c oxidase subunit 1
Ct	Cycle thresholds
CV	Coefficient of variation
D2-D3	D2-D3 expansion fragment
DNA	Deoxyribonucleic acid
FLN	Free living nematodes
GB	Great Britain
GPS	Global positioning system
ITS	Internal transcribed space
PCN	Potato cyst nematodes
PCR	Polymerase chain reaction
Pf	Final population density
PI	Initial population density
PPN	Plant-parasitic nematodes
qPCR	Quantitative polymerase chain reaction
Rf	Reproduction factor
RLN	Root-lesion nematodes
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SEM	Standard error of mean
UK	United Kingdom

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Chapter 1

1. LITERATURE REVIEW

A literature review on the diagnostics, pathogenicity and management of root-lesion nematodes on potato production in the U.K. and in the world.

Chapter modified from: Orlando, V., Grove, I.G., Edwards, S.G., Prior, T., Roberts, D., Neilson, R. and Back, M. (2020). Root-lesion nematodes of potato: current status of diagnostics, pathogenicity and management. *Plant Pathology* 69, 405-417. DOI:10.1111/ppa.13144.

1.1 ABSTRACT

Root-lesion nematodes of the genus *Pratylenchus* are migratory endoparasites with worldwide economic impact on several important crops including potato, where certain species reduce the yield and quality of potato tubers. Morphological identification of *Pratylenchus* spp. is extremely challenging; however, recent advancements in molecular techniques are providing the opportunity to develop robust and rapid diagnostics to differentiate species without the need of specialist skills. In general, root-lesion nematodes are difficult to manage once introduced into agricultural land and damage can be related to pathogenicity and population densities; for this reason, management interventions are often focused on limiting nematode reproduction before planting crops. This review discusses the current status of the diagnosis, pathogenicity and management of the main species of root-lesion nematodes reported to infect potatoes worldwide.

1.2 INTRODUCTION

Potato (*Solanum tuberosum* L.) is an herbaceous annual plant, ranked as the fifth most important staple food crop in the world (FAOSTAT, 2018). Potatoes are subject to a plethora of different pests and diseases including plant-parasitic nematodes that reduce the yield and quality of tubers, which impact their marketability (Mugniéry and Phillips, 2007). Root-lesion nematodes of the genus *Pratylenchus* are migratory endoparasites with worldwide economic impact on several important crops such as cereals, coffee, vegetables, and potato (Sasser and Freckman, 1987; Castillo and Vovlas, 2007; Mokrini *et al.*, 2019). To date, according to taxonomic studies, there are 101 valid species of root-lesion nematodes described (Janssen *et al.*, 2017a), but only *P. alleni, P. andinus, P. brachyurus, P. coffeae, P. crenatus, P. flakkensis, P. neglectus, P. penetrans, P. scribneri*, and *P. thornei* are associated with potatoes (Oostenbrink, 1958, 1961; Brodie *et al.*, 1993; Ingham *et al.*, 2005; Scurrah *et al.*, 2005; Castillo and Vovlas, 2007; Yan *et al.*, 2016).

In general, root-lesion nematodes are difficult to manage once introduced into agricultural land and damage can be related to pathogenicity and population densities. In addition, root-lesion nematodes interact with fungi such as *Verticillium dahliae* and *Rhizoctonia solani*, resulting in disease complexes that enhance the damage inflicted on the potato crop. Management interventions are often focused on limiting nematode reproduction before planting crops and include the application of nematicides, and cultural practices such as crop rotation, cover crops, biofumigation, and biological control. Understanding the limitations of the available crop protection strategies is important and there are many gaps for further study.

This review discusses the status of the diagnosis, distribution, pathogenicity, and management of the main species of root-lesion nematodes, reported to infect potatoes worldwide, and highlights areas for potential future research.

1.3 THE POTATO PLANT

1.3.1 Introduction

The potato plant (*S. tuberosum*) grows in over 130 countries, in almost all climatic conditions (FAO, 2008). The potato plant grows about 60 cm high, producing the characteristic tubers called “potatoes”. In general, potatoes can be multiplied using “seed potatoes”, which are the daughter tubers produced or pieces of potato (tubers) that develop from 2 to 10 buds on their surface. Each of the buds forms a shoot that develops into a new plant with below-ground parts including roots, stolons and tubers, and above-ground parts including stems, foliage and flowers (Figure 1.1) (Wohleb *et al.*, 2014). The potato is a plant that grows in a range of soils but prefers good drainage and aeration. The most suitable soil to grow potatoes is a well-drained medium loam, with a soil pH ranging from 5.5 to 6 (FAO, 2008). Potatoes require constant water management to obtain high yield and quality of tubers, so the soil moisture content needs to be retained close to field capacity, especially from tuber initiation onwards (Levy and Coleman, 2014). For instance, a crop of c.140 days requires from 500 to 700 mm of water, from rain or irrigation, to obtain the best yield (FAO, 2008). Regular irrigation avoids the malformation of tubers or tuber cracking when there is a period of drought followed by irrigation (Levy *et al.*, 2014). Temperature is another factor that limits potato yield, with 18-20°C being optimal. In the UK potatoes are usually planted from March and harvested from June until October (Struik, 2007).

There is a wide range of potato varieties available which differ in tuber properties such as size, shape, colour, texture and taste. There are about 4000 different varieties available in the world (Bond, 2014), and they are divided in three main groups called “first earlies”, “second earlies” and “main crops”. First earlies are potatoes that grow the fastest, maturing after approximately 100 days from planting. Usually the tubers are small and white. They include UK varieties like, for example, Accord, Premiere, Maris Bard, Annabelle, Home Guard and Ulster Prince. In the UK, they are usually planted during spring, from the end of February until early April, and harvested in June (NIAB TAG, 2016). Second earlies mature later than the first earlies, requiring 16-17 weeks to mature after planting before being harvested from July until August. They include, for instance, Marfona, Arran Pilot, Charlotte, Maris Peer and Mimi (NIAB TAG, 2016). Marfona potatoes are short, oval shape potatoes with a smooth texture, good to mash, wedges and boiled potatoes, whereas Maris Peer potatoes have a medium to low dry matter and it has good boiling quality. Maincrops are considered the potatoes that take approximately 20 weeks to mature and are ready to be harvested from August until October. Maris Piper, Markies, Estima, Lady Rosetta, Desiree, King Edward, and Majestic are the most common varieties and they produce large potatoes for baking and roasting (NIAB TAG, 2016;

AHDB, 2016). Moreover, depending on the growth habit, potatoes can be classified as "determinate" and "indeterminate". Determinate potatoes are the early potatoes plants that, after tuber initiation, stop growing flowers and foliage, like for example Estima, while indeterminate potatoes, like Royal, produce flowers and foliage during the whole growing season (Wohleb *et al*., 2014).

Potatoes have become a staple food all over the world. Being rich in carbohydrates and low in fat, they can be cooked in several ways and combined with other vegetables or food in different dishes. Each potato contains 80% water and 20% dry matter of which approximately 70% is starch. Potatoes are also rich in protein, vitamins such as vitamin C, B1, B3 and B6, and minerals such as potassium, phosphorus and magnesium. However, not all potatoes are used for cooking. Indeed, potatoes can be used also as food ingredients, starch for industry, and feed for cattle, pigs and chickens. Moreover, seed tubers are useful to produce other potatoes in the next planting season (FAO, 2008).

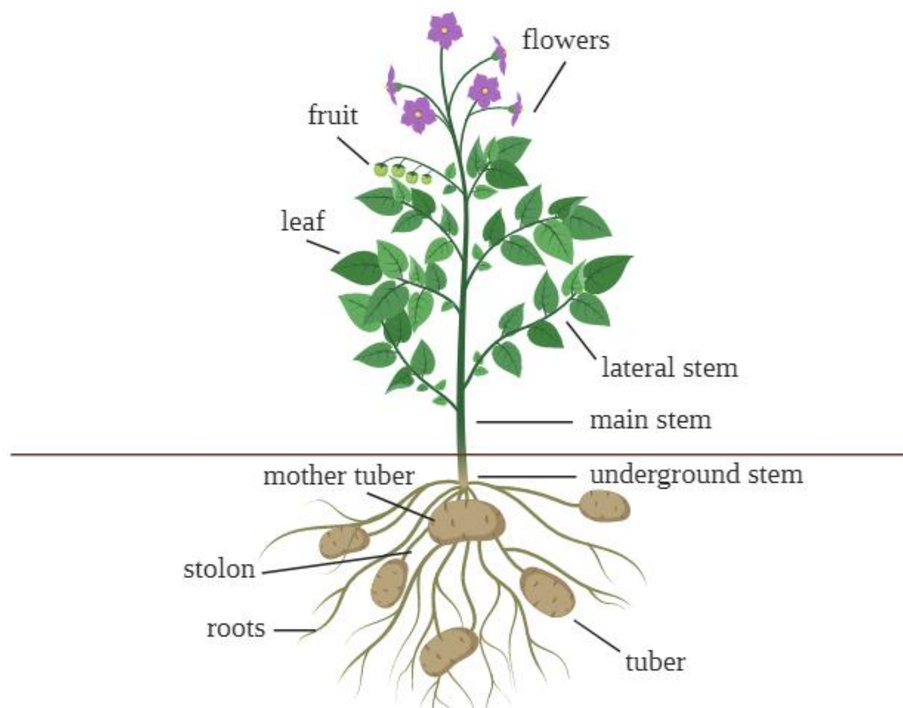

Figure 1.1: The anatomical features of the potato plant. Diagram is the authors own, created in BioRender.com

1.3.2 History of potato crops in the UK

The potato originated 8,000 years ago in in the Andes mountain of South America and was introduced to Europe in the 16th century by the Spanish (Brown *et al*., 2007). It became an important crop with a world-wide total production of 368,096 thousand tonnes estimated in 2013 (AHDB, 2015). To date, China is the largest producer with 96 million tonnes of

production, followed by India and Russia (AHDB, 2017), whereas Germany, France and Belgium are the major European producers with 8 million, 6,3 million and 4 million tonnes, respectively (AHDB, 2019). Potato has an important value also in the UK's agricultural industry, representing one of the most important crops. In 1940, the total area of GB potatoes was estimated to be in the region of 280,000 ha before increasing to 520,000 ha during the Second World War due to the higher demand for food (AHDB, 2017). Potato was very important during the industrial revolution because it was an inexpensive source of nutrients and very easy to cultivate, especially for poor families. However, after the Second World War, the total area decreased by 23 %, with 118,953 ha of total planted area in 2018 (AHDB, 2019). The reduction in area may be due to the decline in the number of potato growers during the last 55 years from approximately 80,000 in 1960 to 1,998 in 2015. Price of production and imports from other countries at lower price can be other reasons of this decline (AHDB, 2019).

The East of England is the major production area of Great Britain (55%) with 33,189 ha of potato planted area, followed by Scotland (21%) and the rest of England and Wales (24%) (AHDB, 2020). To date, Maris Piper is the variety with the largest area grown (13,920 ha) in the UK followed by Markies (5,870 ha), Melody (4,730 ha), Taurus (3,860 ha), Sagitta (3,740 ha), Innovator (3,580 ha), Maris Peer (3,540 ha), Royal (3,430 ha), Lady Rosetta (3,310 ha) and Nectar (2,480 ha) (AHDB, 2020). Maris Piper represents 15% of the total planted area; it is an early maincrop, usually giving high yield producing many tubers per plant, violet flowers, and tubers that are oval with cream colour (NIAB TAG, 2016). Markies is also characterized by oval tubers with cream/light yellow colour in the skin and flesh, and they usually have an excellent fry quality (NIAB TAG, 2016). Melody has high yield producing tubers with oval shape with light yellow skins and flesh (NIAB TAG, 2016). Lady Rosetta is an early maincrop producing round tubers with red skin and light-yellow flesh; it has a moderate to high yields and it has good qualities for crisp production (NIAB TAG, 2016). Maris Peer is a second early potato presenting moderate yields with potatoes that have good boiling quality (NIAB TAG, 2016).

1.3.3 Pests and diseases of potato

Potato plants are subject to different pests and diseases represented by fungi, oomycetes, bacteria, phytoplasma, viruses, viroids, insects, molluscs and nematodes. The different varieties have different resistances or susceptibility to each of them. In general, pests and diseases are difficult to control once introduced into the plant and they have a negative impact on the total yield and quality of tubers.

The most common and damaging pathogen of potatoes worldwide is represented by the oomycete, *Phytophtora infestans*, which causes potato late blight with prolific development of irregular lesions in the foliage and a dry brown rot affecting the tubers. It was introduced in Europe from Mexico in 1830 and since then it has spread all over the world. In Ireland, late blight caused the potato famine in 1845-52, which had a cruel and significant impact on the Irish population, with approximately one million people dying of starvation (Brown *et al.*, 2007). Fungal pathogens can also cause problems to potatoes such as *Verticillium dahliae* which causes potato early dying and *Fusarium* spp. causing dry rot and wilt of potatoes. Also, *Rhizoctonia solani* causes stem, stolon and root canker and black scurf symptoms on the daughter tubers (Wale *et al.*, 2008).

There are fewer bacterial species that cause problems to potatoes. One of the most known species is *Streptomyces scabiei* causing common scab lesions on the tubers, which is an important blemishing disease (Wale *et al.*, 2008). Instead, there are over 50 different viruses that are pathogens of potatoes such as *Potato Leaf Roll Virus, Potato Virus A, Potato Virus X, Potato vVirus Y* and *Potato Yellow Vein Virus.* Potato viruses can be vectored by insects, such as aphids, thrips and whiteflies, and nematodes.

There are a variety of insect species that are potato pests, mainly attacking the above-ground parts or below-ground tissue, with only a few species causing direct damage to tubers. Indeed, insects feed on the leaf tissues causing destruction of foliage and consequently yield loss. Some examples of insects infesting potato plants are represented by the european corn borer (*Ostrinia nubilatis*), colorado potato beetle (*Liptinotarsa decemlineata*), potato flea beetles (*Epitrix* spp.), potato leafhoppers (*Empoasca fabae*), leafminer fly (*Liriomyza huidobrensis*), potato tuber moth (*Phthorimaea operculella*) and different aphids such as the peach potato aphid (*Myzus persicae*) and the potato aphid (*Macrosiphum euphorbiae*) (Brown *et al.*, 2007).

1.3.4 Nematodes infesting potato crops

Plant-parasitic nematodes (PPN) are one of the major pests of potatoes, affecting the yield and the quality of the tubers which then impact on their marketability. Damage can be related to population densities, and for this reason, control measures are focused on the limitation of nematode damage, before infection of the crop. Globally, it is estimated that plant nematodes cause crop losses in the region of $US80 billion per year (Jones *et al.*, 2013), so it is of paramount importance to adopt preventative control measures. It can be difficult to determine economic damage thresholds from specific PPN due to their microscopic size, mixture of species within single fields, where the symptoms are often non-species specific. There is also

a lack of expertise on the diagnosis of nematode species (Palomares-Rius *et al.*, 2014 b). In addition, soil analysis (assessment of nematodes) carried out by different laboratories can vary greatly, possibly due to differences in the equipment used, adaptations of the nematode extraction method or differences in expertise for diagnosis (EPPO, 2013).

Generally, the symptoms caused by PPN on the above-ground parts may consist of stunted plant growth and yellowing and wilting foliage and usually tuber yield and quality is reduced. There are plant parasitic nematodes infesting potato plants that are polyphagous such as *Meloidogyne, Nacobbus* and *Pratylenchus*, while other species are highly host specific to *S. tuberosum* like such as potato cyst nematodes, *Globodera* spp. and the tuber rot nematode, *Ditylenchus destructor.* Moreover, nematodes such as *Trichodorus* spp. and *Paratrichodorus* spp., cause damage due to feeding on the roots and by transmitting Tobacco Rattle Virus (TRV) to the plant (Brodie *et al.*, 1993).

Potato cyst nematodes (PCN) are the major nematode pests of potato, widely distributed around the world. In the UK, PCN (*Globodera pallida* and *G. rostochiesis*) cause annual yield losses of around £50 million (Wale *et al.*, 2008). They are sedentary endoparasites nematodes; the J2 penetrates the roots and produces secretions thought the stylet that form the syncytium (feeding cell). Feeding damage caused by PCN causes a reduction of the root system, which consequently leads to stunted growth, yield loss and lower quality of tubers (EPPO, 1997; Palomares-Rius *et al.*, 2014 b). Root-knot nematodes (*Meloidogyne* spp.) are the second important crop damaging PPN in the world, causing the formation of galls on the roots. Root-knot nematodes have a wide range of host plants, and six species are considered pests of potatoes: *M. incognita, M. fallax, M. chitwoodi, M. javanica, M. hapla* and *M. minor* (Brodie *et al.*, 1993; Palomares-Rius *et al.*, 2014 b). They cause stunting, chlorosis or wilting of the haulm and they may affect also the tubers with wart-like protuberances. *Meloidogyne hapla, M. fallax* and *M. minor* are the most common species present in the UK (EPPO, 2006; 2016) that can cause problems on potatoes. False root-knot nematodes (*Nacobbus* spp.) are also pests of potatoes, which invade and feed upon on the roots and tubers, causing lesions, necrosis and cavities. Females produce also galls on the roots. They can cause severe damage to potato crops and are classified as quarantine organisms in many countries (Brodie *et al.*, 1993). *Nacobbus aberrans* has been reported mainly in South and North America, it is absent in Europe, but it has been found once in England, although it has been eradicated since this recording (EPPO, 2009). Another important quarantine nematode causing severe damage of potato plants is the potato rot nematode, *Ditylenchus destructor*, which has a wide distribution in temperate regions, including Europe and the UK (EPPO, 2017). The nematode penetrates the tubers through the lenticels, feeds and reproduces, completing its life cycle in 3 weeks, and causes additional damage during tuber storage (Palomares-Rius *et al.*, 2014 b).

Trichodorus spp. and *Paratrichodorus* spp., commonly known as stubby-root nematodes, are ectoparasites feeding on the roots of plants including potatoes. However, they are well known due to their ability vector of viruses. The species considered most important for vectoring tobacco rattle virus (TRV), are *T. primitivus, T. similis, P. anemones* and *P. pachydermus* (AHDB, 2015). The needle nematodes, *Longidorus* spp., are also pest of potatoes and may vector tomato blackring virus. The most abundant species in the UK is *L. elongatus* and it is prevalent in lighter soils in Scotland and England (AHDB, 2015).

1.4 ROOT-LESION NEMATODES (RLN):

1.4.1 Introduction

The root-lesion nematodes of the genus *Pratylenchus* Filipjev, 1936 (Table 1.1) are migratory endoparasites with worldwide economic impact on several important crops such as cereals, coffee, potato, vegetables and fruit trees (Castillo and Vovlas, 2007). To date, according to taxonomic studies, there are 101 species described (Janssen *et al.*, 2017 a, b).

The identification of *Pratylenchus* species is usually based on female morphology as they have more diagnostic characters than the male, which in any case is rare or unknown for a substantial number of species (Loof, 1991). The diagnosis of root-lesion nematodes is a challenging task, mainly because there is a low interspecific variation and a high intraspecific variation for certain important morphological characters to confound the delimitation of species (Tarte and Mai, 1976; Castillo and Vovlas, 2007; Geraert, 2013). In general, nematodes present a considerable genetic complexity, and in several species, races, pathotypes and geographic variants are known (Abebe *et al.*, 2011). Moreover, it seems that cryptic species are common within nematodes (Palomares-Rius *et al.*, 2014 a, b). Cryptic species are defined as two or more distinct species that are wrongly classified under a single species name; basically, they are species that are morphologically similar but genetically distant. The existence of cryptic species or species complexes have been demonstrated for *P. coffeae* (Duncan *et al.*, 1999), *P. hippeastri* (De Luca *et al.*, 2010) and *P. penetrans* (Janssen *et al.*, 2017b).

Like many nematode taxa, the identification of *Pratylenchus* with microscopy is time consuming and require expertise that is not commonly available in every laboratory. Fortunately, recent molecular techniques are giving the opportunity to develop robust and fast diagnostics useful for differentiating each nematode species from the others and without the need for experienced taxonomists. However, it is important also that care must be taken to consider the sensivity of molecular diagnostics by excluding potentially false positive results

(Roberts *et al.*, 2016), and this might be the case of some molecular diagnostics developed in the past for *Pratylenchus*, as discussed in the section 1.4.3.

Table 1.1: Classification of root-lesion nematode, *Pratylenchus*

CLASSIFICATION	
Phylum	Nematoda
Class	Secernentea
Subclass	Diplogasteria
Order	Tylenchida
Superfamily	Tylenchoidea
Family	Pratylenchidae
Subfamily	Pratylenchinae
Genus	***Pratylenchus***
Common name	Root-lesion nematodes

1.4.2 Morphological identification of root-lesion nematode

The genus *Pratylenchus* is characterized by a lip region that is flattened anteriorly, usually continuous with the body contour and with a strong sclerotization (Geraert, 2013) (Figure 1.2). The lateral incisures are typically four to six, deirids are absent, whilst phasmids are near to the middle of the tail. The stylet is typically around 15–20 µm in length with basal knobs that can vary in shape according to species, and between individuals (Tarte and Mai, 1976; Mizukubo and Minagawa, 1991). The pharyngeal glands overlap the intestine ventrally and the metacorpus is muscular, and oval to round. The reproductive system is pseudo-monoprodelphic, with only the anterior genital tract having a spermatheca with a functional ovary, and a post-vulval uterine sac present (Figure 1.3). The vulva is situated posteriorly, usually at 70%–80% of the total body length (Loof, 1960, 1991; Geraert, 1983; Luc, 1987; Handoo and Golden, 1989; Ryss, 2002 a, b). The female tail is subcylindrical to conoid with smooth or annulated terminus (Castillo and Vovlas, 2007; Geraert, 2013). Morphological identification of *Pratylenchus* species is usually based on female morphology as they have more diagnostic characteristics than males, which are rare or unknown for a substantial number of species (Loof, 1991). Certain characteristics have taxonomic validity such as number of lip region annuli, number of lateral incisures, vulva position, length of post-vulval uterine sac, tail shape, and presence/absence of males (Table 1.2). According to Corbett and

Clark (1983), the head pattern, formed by the first annule and oral disc, is a feature that allows the classification of the species in 3 main groups: undivided (group 1), divided into sub-median and large (group 2) or small (group 3) lateral segments. However, head patterns can only be accurately studied using scanning electron microscopy (SEM). There are other key features typical of each species that are useful for the identification. For example, a clavate tail with annulated tip shape and the presence of a strong musculature at the secretory-excretory (SE) pore are features generally observed only with *P. crenatus* (Karssen and Blok, 2000). *Pratylenchus neglectus* is similar to *P. crenatus*, but generally has a smooth tail tip and poorly developed musculature at the SE pore in addition to fewer head annules. However, *P. neglectus* has large variability between some populations in stylet knob and tail shape, complicating the identification (Mizukubo and Minagawa, 1991). Indeed, the diagnosis of root-lesion nematodes is challenging, mainly due to the low interspecific variation and conversely the high intraspecific variation that exists for certain important morphological characters, such as shape of the spermatheca, vulva position, and tail shape, that confound species discrimination (Castillo and Vovlas, 2007; Geraert, 2013; Janssen *et al.*, 2017a). For example, populations of *P. penetrans* from different geographical locations have a high level of morphological variation, mainly in the tail shape (Tarte and Mai, 1976). The tail tip is useful in distinguishing *P. penetrans* from *P. fallax,* as this is usually smooth in *P. penetrans* and usually annulated for *P. fallax*, however they remain difficult to distinguish morphologically from each other (Tarte and Mai, 1976). Despite their similarities, several molecular studies have demonstrated they are separate taxonomic entities (Ibrahim *et al.*, 1995; Waeyenberge *et al.*, 2000; Carta *et al.*, 2001; Handoo *et al.*, 2001; Janssen *et al.*, 2017b), highlighting the importance of molecular diagnostics to overcome the issues of overlapping morphological characters. Further problems to consider are the presence of more than one species in the same soil sample.

Table 1.2: Main morphological characters used for the identification of the common root lesion nematodes species on potato (adapted from Castillo and Volvas, 2007; Duncan and Moens, 2013; Geraert, 2013)

Species	**Head annuli**	**Lateral fields**	**Spermatheca**	**Post-vulval uterine sac**	**Tail**	**Male present**
P. brachyurus	2	4	Empty	Short	Conical with rounded, truncate or spiculate tip	No
P. coffeae	2	4-5	Large, broadly oval to nearly rounded, usually with sperm	Long	Bluntly rounded, trucante or indented	Yes
P. crenatus	3	6	Empty	Long	Clavate with annulated tip	No
P. neglectus	2	4	Empty	Short	Conical with rounded smooth tip	No
P. penetrans	3	4	Round	Short	Round and smooth tip	Yes
P. scribneri	2	5-6	Oblong, empty	Short	Smooth tip	Yes
P. thornei	3	4	Small, empty	Slightly longer than body width	Broad with truncate tip	No

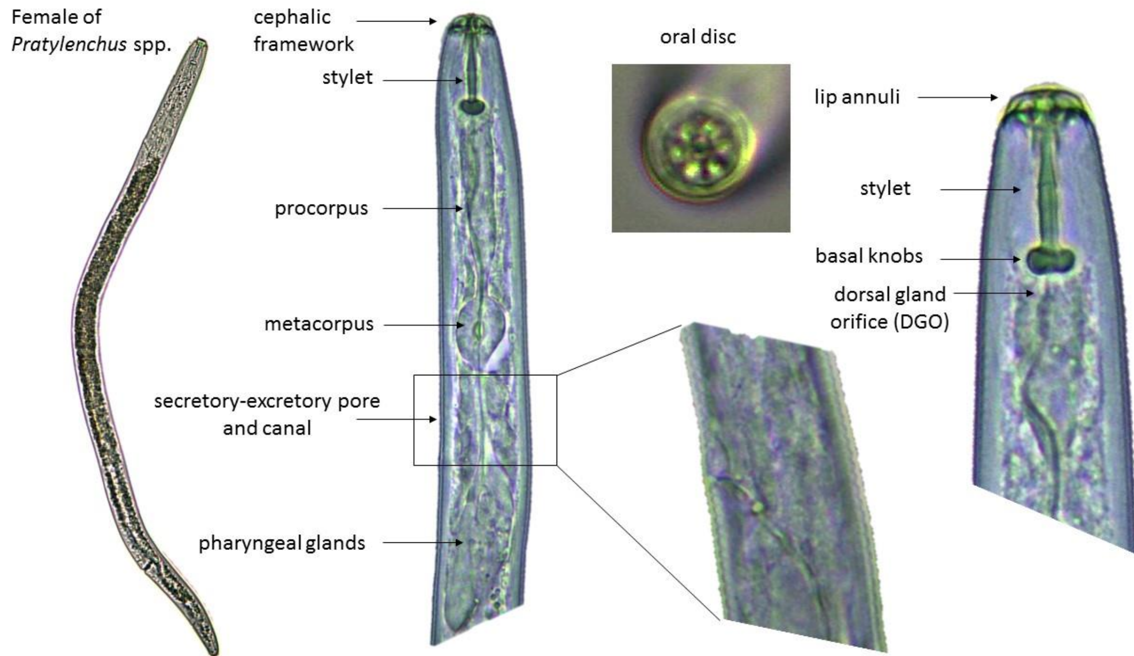

Figure 1.2: General morphology of *Pratylenchus*. Whole nematode and details of the anterior part from the head until the pharyngeal glands. Photographs are the authors own.

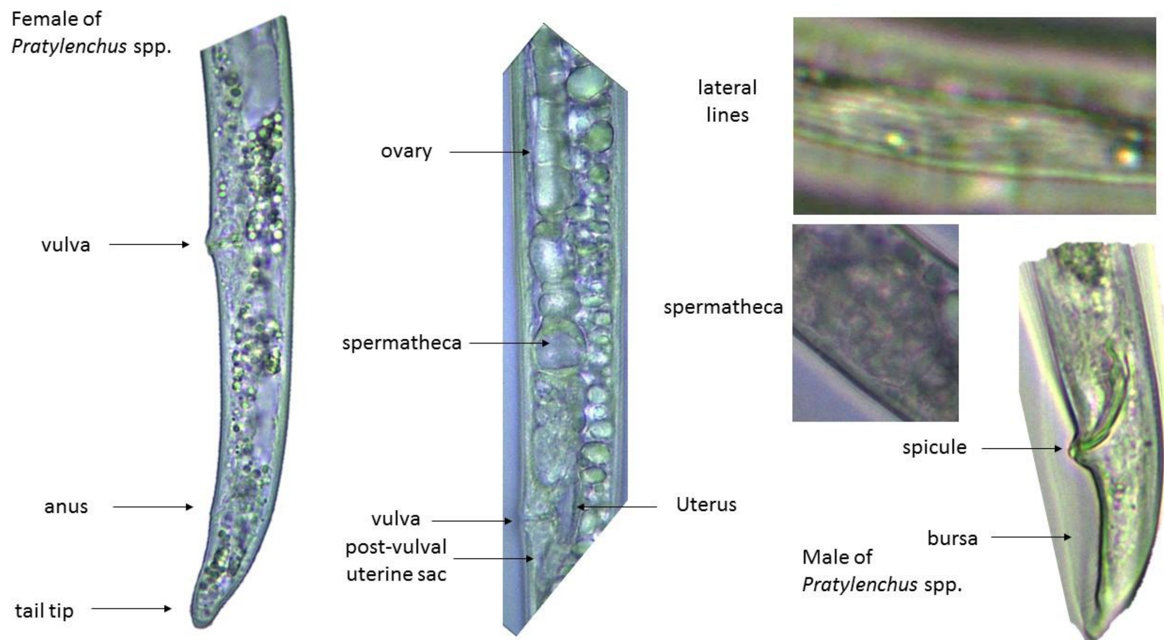

Figure 1.3: General morphology of *Pratylenchus*. Details of the posterior part of female (on the left) with reproductive system and male (on the right). Photographs are the authors own.

1.4.3 Molecular characterization of root-lesion nematode

New methods of molecular analyses are becoming increasingly important for nematode diagnostics. Indeed, the polymerase chain reaction (PCR) has improved the diagnosis of different plant parasitic nematodes. Molecular tools can give the possibility to detect a specific species within a mixture of nematodes in one sample and to distinguish closely related species (Powers *et al.*, 2004; Oliveira *et al.*, 2011) and cryptic species (Palomares-Rius *et al.*, 2014 a). To obtain a robust identification, it is important to combine classical taxonomy with molecular diagnostics using different molecular markers from single specimens (Palomares-Rius *et al.*, 2014 a; Janssen *et al.*, 2017a). However, only a fraction of the existing species can be characterised with molecular sequences. Also, misidentification due to the use of sequences that have been incorrectly identified is a major problem for molecular analysis (Subbotin *et al.*, 2008; Luc *et al.*, 2010; Oliveira *et al.*, 2011; Janssen *et al.*, 2017a). Indeed, before the submission of gene sequences to GenBank, it should be mandatory to provide an extensive morphological description of the corresponding specimen linked to that sequence. Ideally each sequence should be linked to measurements, photographs, information about the collection data and voucher material (Powers, 2004; Janssen *et al.*, 2017a).

The polymerase chain reaction (PCR) is a routine technique in molecular biology that allows the amplification of any nucleic acid sequence. It is used to amplify a single copy of a segment of DNA producing thousands to millions copies of the same DNA fragment with an enzymatic reaction carried out in a single tube in a thermal cycler. It requires one pair of oligonucleotides, called forward and reverse primers, deoxyribonucleotide triphosphates (dNTP), a buffer with magnesium ions ($MgCl_2$), a DNA polymerase and a DNA template with the fragment to amplify. There are three basic steps at different temperatures: denaturation of the double strand at 94°C during which the hydrogen bonds are broken and the two strands are separated each other; an annealing step at 50-60°C that allow the binding of the primers at specific position of each DNA strand; and then the temperature is raised to 72°C and the DNA Polymerase attach at the 5' end of each primer and synthesizes new strand of DNA. The PCR consists of 30-40 cycles, with a final extension at 72°C. Once the PCR is terminated, the PCR products can be separated by their size on an agarose or polyacrylamide gel and visualized by ethidium bromide or DNA loading gel buffer under ultraviolet (UV) light. It is important to remember before performing any molecular assays that many compounds, used for DNA extraction and present in some soil samples, can inhibit the PCR (Roberts *et al.*, 2016). As congruence of PCR inhibition, the sensitivity of any molecular assay will be decreased and there is more possibility of false-negative results. Some examples of PCR inhibitors are represented by calcium ions and organic compounds like phenol, ethanol,

polysaccharides, sodium dodecyl sulphate (SDS), as well as proteins like collagen, haemoglobin and proteinases. However, some protocols have been developed to remove some of these PCR inhibitors, depending the nature of each (Schrader *et al.*, 2012).

Different molecular approaches have been developed to identify a wide range of *Pratylenchus* species, which included analysis of rRNA genes such as 18S (Subbotin *et al.*, 2008; Palomares-Rius *et al.*, 2010), internal transcribed spacer regions ITS1 and ITS2 (Uehara *et al.*, 1998; Waeyenberge *et al.*, 2000, 2009; Palomares-Rius *et al.*, 2010; De Luca *et al.*, 2011; Troccoli *et al.*, 2016; Oliveira *et al.*, 2016; Janssen *et al.*, 2017a, b), and 28S rDNA (Al-Banna *et al.*, 1997, 2004; Handoo *et al.*, 2001; De Luca *et al.*, 2004; Palomares-Rius *et al.*, 2010; Troccoli *et al.*, 2016; Janssen *et al.*, 2017a, b). Recently, more attention has been given to the *cytochrome coxidase subunit* 1 (COI) gene fragment (Janssen *et al.*, 2017a, b) and Hsp90 (Fanelli *et al.*, 2018).

In 2011, a phylogenetic analysis of ITS fragments of 18 root lesion nematode species showed that these sequences present a wide variation in length, distinguishing 12 highly or moderately supported major clades. Whilst these authors supported the usefulness of the ITS genes for the identification of *Pratylenchus* species, they confirmed the high variability which can cause confusion on the relationships among the species of the genus (De Luca *et al.*, 2011). Janssen *et al.* (2017 b) confirmed that intra-individual variability of ITS regions in root lesion nematodes is more common than expected, and hypothesized that ITS variation is due to single nucleotide mutations and large insertions and deletions, meaning that different copies of the ribosomal gene in a single genome can have a wide variation in ITS1 and ITS2 sequences. However, the authors did not completely exclude this variation is due to exogenous induced variation from the cloning process.

In order to obtain a reliable identification, it is important to identify a suitable DNA-target with high interspecific variation and low or null intra-specific and intra-individual variability. In this regard, several studies highlighted that D2-D3 expansion segment of 28S rRNA gene sequence seems a suitable target for this purpose because it possesses a higher degree of interspecific genetic variability and low intra-specific variation (Al-Banna *et al.*, 2004; De Luca *et al.*, 2004; Subbotin *et al.*, 2008). Al-Banna *et al.* (2004) were able to identify and describe six species of *Pratylenchus* common in California (*P. brachyurus, P. neglectus, P. scribneri, P. penetrans, P. thornei,* and *P. vulnus*) based on the D3 expansion region of the 28S rDNA, suggesting that this region can be an important tool for diagnostics. De Luca *et al.* (2004) investigated several populations of *P. thornei, P. neglectus* and *P. penetrans* from different geographic origins using sequence analysis of the D3 fragment of the 28S gene. *Pratylenchus neglectus* showed a high intraspecific variability, while several specimens of *P. thornei* did not present any variation, suggesting that this molecular fragment was more applicable for the

identification of *P. thornei*. These authors supported the hypothesis that *P. penetrans* belongs to a species complex consisting of *P. penetrans*, *P. dunensis*, *P. fallax, P. arlingtoni*, *P. pinguicaudatus*, and *P. convallariae*, a concept that was later adopted also by other authors (Subbotin *et al.*, 2008; Palomares-Rius *et al.,* 2014 b; Janssen *et al.*, 2017 a,b). Also, Subbotin *et al.* (2008) produced a phylogenetic analysis based on the partial 18S rRNA and D2–D3 expansion segments of 28S rRNA genes using several populations of *Pratylenchus* species from geographically distinct sites. This analysis separates all *Pratylenchus* species in six major clades that correspond to the clades obtained with phylogenetic analysis with morphological characters such as number of lip annuli and head patterns. Considering these two different molecular markers, D2-D3 expansion fragment seemed to recognize a higher degree of interspecific variation than the partial 18S r RNA (Subbotin *et al.*, 2008). However, recently Pereira and Baldwin (2016) reported a hight intra-genomic variability for 28S rDNA in the genus *Cephalenchus*, indicating that possibly this intra-individual variability might be associated with ribosomal genes, so this might be possible to find also for *Pratylenchus* spp. in future.

Recently, nematologists have focussed their attention on mitochondrial DNA (mtDNA) and in particular, the protein coding gene, cytochrome c oxidase subunit I (COI) of mtDNA. Mitochondrial DNA is maternally inherited, the recombination is limited, it is present in hundreds of copies per cell and it has been shown to evolve much more quickly than rRNA genes, resulting in low intra-specific and high inter-specific variability (Palomares-Rius *et al.*, 2014 a). Such characteristics make mtDNA a highly suitable target for the identification of closely related species (Subbotin *et al.*, 2013; Palomares-Rius *et al.,* 2014 a; Janssen *et al.*, 2017 a). Consequently, mtDNA is a promising molecular marker to solve the taxonomy of different nematodes (Power *et al.*, 2004; Derycke *et al.*, 2010 a, b; Prosser *et al.*, 2013; Palomares-Rius *et al.*, 2014a; Toumi *et al.*, 2015; Orlando *et al.*, 2016; Palomares-Rius *et al.*, 2014 a) and specifically root-lesion nematodes (Palomares-Rius *et al.*, 2014b; Janssen *et al.*, 2017 a,b). Although Janssen *at el.* (2017 a) demonstrated that cytochrome c oxidase subunit I (COI) of mtDNA is a useful gene for the identification of root-knot and root-lesion nematodes, some limitations are also reported like for example the presence of nuclear mitochondrial pseudogenes (NUMTs) that might be a problem for DNA barcoding studies and the difficulty to find conserved regions to design universal and, consequently, also species-specific primers.

Moreover, particular attention is also given to genes that encodes *β*-1,4-endoglucanase, a cellulase within the glycosyl hydrolase family 5 (GHF5). These genes have been found in different bacteria and fungi, but also in plant parasitic nematodes and are concerned with the production of cell wall degrading enzymes (*β*-1,4-endoglucanase) during the penetration and

migration of nematodes into the roots (Kyndt *et al.*, 2008). Fanelli *et al.* (2014) sequenced four different endoglucanases in *P. vulnus* and in situ hybridization analysis revealed that transcripts were localized in the sub-ventral oesophageal glands and in the intestine. The same study showed that three endoglucanases were expressed in all life stages and they are produced at higher level in adult males and females of *P. vulnus*, suggesting that adults are also a parasitic stage in this species (Fanelli *et al.*, 2014). Uehara *et al.* (2001) were the first to confirm the presence of *β* -1,4-endoglucanases in *P. penetrans*. Studies on the *β*-1,4-endoglucanase genes of *Pratylenchus* (Mokrini *et al.*, 2013, 2014; Fanalli *et al.*, 2014; Peetz and Zasada, 2016) have identified that these genes could be used as a useful molecular marker to differentiate different species of this genus. Indeed, species-specific primers have been designed for *P. penetrans*, *P. thornei*, *P. crenatus, P. vulnus* and *P. neglectus* (Table 1.3). However, Peetz and Zasada (2016) have highlighted the possible heterogeneity of the *β*-1,4-endoglucanase gene within of *P. crenatus, P. neglectus, P. penetrans* and *P. thornei* from various locations in North America, but the authors highlight the possibility to design other species-specific primers to amplify the same gene from other countries.

1.4.4 Species-specific primers for Pratylenchus

Several species-specific primers for *Pratylenchus* have been designed focusing the attention mainly on rRNA genes (Table 1.3). Uahara *et al.* (1998) were the first to develop species-specific primers to identify *P. penetrans* based on ITS1 and ITS2 sequences. Two years later, an RFLP technique was developed to identify 18 different *Pratylenchus* species (Waeyenberge *et al.*, 2000). Large differences in length of ITS sequences were detected, and five restriction enzymes were used to digest the fragments. Although all species could be differentiated from each other by a combination of two enzymes, intraspecific variation for different populations of *P. coffeae* was found and the authors assumed that this was due to heterogeneity in ITS sequences within individuals (Waeyenberge *et al.*, 2000). Indeed, the same authors also tested a duplex PCR technique with ITS regions as target and showed high variability between and within populations of *P. penetrans*. However, three potentially useful species-specific primers were developed and tested on different *Pratylenchus* species and *P. penetrans* populations from different geographic origins. The test enabled *P. penetrans* to be distinguished in mixed nematode samples with different numbers of other *Pratylenchus* species (Waeyenberge *et al.*, 2009). Many other species-specific primers have been reported for qPCR assays, and these are discussed in the section 1.4.

Table 1.3: Published species-specific primers of ITS rDNA, 28S rDNA and *β*-1,4 endoglucanase for identification of common *Pratylenchus* spp. on potato. f – forward; r- reverse; * universal primer

Species	DNA region	Primer Sequence (5'-3')	PCR product size (bp)	MolecularAssay	Reference
P. brachyurus	ITS rDNA	f - TTGATTACGTCCCTGCCCTT r - GCWCCATCCAAACAAYGAG	267	Conventional PCR	Machado *et al.* (2007)
P. coffeae	ITS rDNA	f - ATGCGCACATTGCATTCA r - GAGCGAGAAACACCTCTCAC	632	Conventional PCR	Uehara *et al*. (1998a)
	ITS rDNA	f - ATGCCCACATTGCATTCAGC r - GAGAGAGAAACACCTCTCAC	638	Multiplex PCR	Saeki *et al.* (2003)
P. crenatus	28S rDNA	f - AAAGCCTGAATGCCCTGAG r - AAATTGAAAGAGGTCGGTCGT	610	Convetional PCR	Mekete *et al.* (2011)
	ITS rDNA	f - TTCTTGACAAGTTCATTGCTTC r – CACTCACGATGTGCTTCTG	116	TaqMan qPCR	Oliveira *et al.* (2017)
	β-1,4-endoglucanase	f -TCTCCTGGACGGACGTGCTC r - AGGCCGTCCAGGAAGGTGTAC	381	Convetional PCR	Peetz and Zasada (2016)
P. neglectus	28S rDNA	f - ATGAAAGTGAACATGTCCTC r - TCGGAAGGAACCAGCTACTA*	290	Convetional PCR	Al-Banna *et al.* (2004)
	28S rDNA	f - CGCAATGAAAGTGAACAATGTC r – AGTTCACCATCTTTCGGGTC	144	SYBR green qPCR	Yan *et al*. (2008)
	ITS rDNA	f – GGCACTGTGCGAAGTGTCCG r -TTAACACCTCAGGCGTCATGTAC	234	SYBR green qPCR	Yan *et al.* (2013)
	ITS rDNA	f - ACTGTGCGAAGTGTCCG r – GATCCACCGATAAGGCTAGA	121	TaqMan qPCR	Oliveira *et al.* (2017)
	β-1,4-endoglucanase	f - TGACCACAACGCGCAGAACCAC r – GCCACGTCCACGTCCTGGGA	293	Convetional PCR	Peetz and Zasada (2016)
P. penetrans	28S rDNA	f - TAAAGAATCCGCAAGGATAC r - TCGGAAGGAACCAGCTACTA*	278	Convetional PCR	Al-Banna *et al.* (2004)
	28S rDNA	f - ACATGGTCGACACGGTGATA r – TGTTGCGCAAATCCTGTTTA	520	Convetional PCR	Mekete *et al.* (2011)
	28S rDNA	f - GGTTTTCGGGCTCATATGGGTTC r - TTTACGCCGAGAGTGGGATTGTG	111	SYBR green qPCR	Baindoo *et al.* (2017)
	28S rDNA	f – GAGACTTTCGAGAAGGCGATATG r – AGGACCGAATTGGCAGAAG	176	TaqMan qPCR	Dauphinais *et al.* (2018)
	ITS rDNA	f - ATGATGGAAGTGTCCGCCT r – CCCAAACGACGGTCAAAAGG	462	Convetional PCR	Uehara *et al.* (1998b)
	ITS rDNA	f - ATTCCGTCCGTGGTTGCTATG r - GCCGAGTGATCCACCGATAAG	134	SYBR green qPCR	Sato *et al.* (2007)
	ITS rDNA	f - TGACTATATGACACATTTRAACTTG r - ATATGCTTAAGTTCAGCGGGT	660	Duplex PCR	Waeyenberge *et al*. (2009)
	ITS rDNA	f - AATGTGTCTCGCCCTGAGG r – GCAACCACGGACGGAATAC	80	TaqMan qPCR	Oliveira *et al.* (2017)
	β-1,4-endoglucanase	f - CCAACCTCTGCTACACTA r – CAGTGCCGTATTCAGTGA	-	TaqMan qPCR	Mokrini *et al.* (2013)
	β-1,4 endoglucanase	f - GGCATTTATGTG(A/C)TCGTGGATTGGC r - GTTGCCATCAGCGCTGACAGTG	528	Convetional PCR	Peetz and Zasada (2016)
P. scribneri	28S rDNA	f - AAAGTGAACGTTTCCATTTC r - TCGGAAGGAACCAGCTACTA*	286	Convetional PCR	Al-Banna *et al.* (2004)
	28S rDNA	f - ATGTGTTGCCATGCATCTG r – GTCCAGAACCCATTTGGACT	750	Convetional PCR	Mekete *et al.* (2011)
	ITS rDNA	f - AGTGTTGCTATAATTCATGTAAAGTTGC r - TGGCCAGATGCGATTCGAGAGGTGT	136	SYBR green qPCR	Huang andYan (2017)
P. thornei	28S rDNA	f - GAAAGTGAAGGTATCCCTCG r - TCGGAAGGAACCAGCTACTA*	288	Convetional PCR	Al-Banna *et al.* (2004)
	ITS rDNA	f - GTGTGTCGCTGAGCAGTTGTTGCC r- GTTGCTGGCGTCCCCAGTCAATG	131	SYBR green qPCR	Yan *et al.* (2012)
	β-1,4-endoglucanase	f - GGATGCGGTCATCAAGGC r - TTGGCTCTGGTGGTTCTG	88	TaqMan qPCR	Mokrini *et al.* (2014)
	β-1,4-endoglucanase	f - GGCTGGTCAGGAGTGAAGTCC r – GCCAGTTCCACCACTCGTTGG	364	Convetional PCR	Peetz and Zasada (2016)

1.4.5 Quantitative PCR (qPCR):

A highly useful development in molecular biology is the ability to perform quantitative PCR (qPCR), which allows not only the qualitative detection of target organisms but also the quantification. Generally, the qPCR, like the conventional PCR, consists of several changes on the temperature that are repeated 25 – 50 times and allow to measure the amplified product during the progress of the reaction. It is carried out in a thermal cycler with fluorescence detection programs to monitor the fluorescence during the amplification process. The fluorescent signal increases proportionally to the amount of PCR products and the threshold cycle (Ct) is an important value that correspond to the cycle number at which the fluorescent emission exceeds the fixed threshold (Bio-Rad, 2006). Lower Ct values (below 29 cycles) correspond to high amounts of target templates, while higher Ct values (above 35 cycles) indicate lower amounts of target nucleic acid.

Different types of qPCR are available according to the reporter used such as SYBR Green I, TaqMan, Eclipse probes, Amplifluor, Scorpions, LUX, and BD QZyme primers. The most used are SYBR Green I and TaqMan hydrolysis probes. In particular, SYBR Green I is a fluorescent chemical that binds the double strands of DNA increasing the fluorescence proportionally to the amount of acid nucleic present in the sample. It is a simple and cheap assay that require only two species specific primers without the need of probe design. Moreover, there is the possibility to construct melt curves that allow to test the specificity of the reaction and to distinguish the amplification products from primer dimers that melt at different temperatures. Instead, TaqMan probes is one of the most used probe-based chemistries. It requires a pair of species-specific primers, a probe with a fluorescent reporter at 5'-end and a quencher molecule at the 3'-end. The fluorescence is quenched when the specific target is not present; instead when the probe binds the specific DNA target, reporter and quencher are separated each other and the fluorescence signal increase proportionally to the number of products. This assay enables the detection of only the specific target sequence avoiding the amplification of non-specific products and can be adapted to perform multiplex reactions in a single test.

The advantage of qPCR is that it is much faster than traditional PCR because it allows a direct detection and quantification of the DNA targets without the need to make gel electrophoresis. If it is implemented directly on soil samples, it does not require nematode extraction from the soil which is also time consuming. Thus, this diagnostic test gives a faster alternative to the extraction, identification and counting of nematodes from soil samples which is time consuming. A limitation can be the possibility of false-positive reactions (fragments from unknown species not yet investigated), or false-negative reactions due to variation occurring between individuals. These limitations highlight that it is important to base diagnosis

on a combination of traditional and molecular taxonomic techniques, before developing a qPCR method. Another factor to consider that might cause problems on the calibration of qPCR assays, is the target gene selected. Indeed, some gene sequences are present in multiple copies in each cell of an individual and each individual has multiple cells; this might result in false-negative results. Moreover, gene copy numbers can change from one species to another or also among different developmental stages (Roberts *et al.*, 2016).

1.4.6 The qPCR method for identification and quantification of root-lesion nematodes

The first report on the application of qPCR for the quantification of nematodes was reported by Fleming and Powers (1998), which used ITS1 fragment as target for the detection and quantification of *Globodera rostochiensis* from cyst samples. A positive correlation was demonstrated between numbers of viable juveniles in cysts and the amount of DNA that could be extracted from them. Later, the qPCR method was adopted for the identification of many other plant parasitic nematodes such as *Meloidogyne javanica* (Berry *et al.,* 2008), *G. pallida* and *G. rostochiensis* (Bates *et al.,* 2002*)*, *Heterodera avenae* and *H. latipons* (Toumi *et al.*, 2015).

Although qPCR diagnostic assays have been developed for *P. zeae* (Berry *et al.* 2008), *P. penetrans* (Sato *et al.*, 2007, 2010; Goto *et al.*, 2011; Oliveira *et al.*, 2016), *P. thornei* (Yan *et al.*, 2012), *P. neglectus* (Yan *et al.*, 2013; Oliveira *et al.*, 2016) and *P. crenatus* (Oliveira *et al.*, 2016), these assays are based on ITS genes which, as discussed previously, have high levels of intra-specific and intra-individual variability. Using this DNA region significantly increases the risk of obtaining false-negative or false-positive reactions. Indeed, some of these authors reported underestimation or overestimation of nematodes using qPCR in comparison with traditional taxonomic methods. Sato *el at.* (2007) developed a qPCR for the detection and quantification of *P. penetrans* in a nematode community from 10 g of soil using the ITS1 as a target and SYBR green assay. The qPCR assay was able to detect one *P. penetrans* in a suspension with 800 other nematode taxa. Although high variation was detected in DNA extracts from different numbers of *P. penetrans*, the authors argued that the possible reasons were the variation in the DNA extraction and purification, or the presence of different developmental stages. To improve the efficiency of the detection of root-lesion nematodes, the soil was heavily compacted using a manually-operated compactor, doing so the nematodes were more destroyed and the Ct values of *P. penetrans* from compacted soils were lower than those from non-compacted soils (Sato *et al.*, 2010). However large differences of Ct values were found between the replicates. To counteract this issue, the authors improved the DNA extraction and purification reported by Sato *et al.* (2007) with a modified method adding salmon semen DNA, skimmed milk and other modifications on the protocol. The Ct

values obtained were lower than those using the original method suggesting that the method was improved. However, the authors continued to find a degree of variation within replicates in their results suggesting that the efficiency of this technique should be improved or to consider also possible problems due to soil types. Indeed, two types of soils, andosol and glay lowland soil, containing the same number of root lesion nematodes were tested and they gave different Ct values (Sato *et al.*, 2009, 2010). This suggested that the type of soil can also influence the efficiency of the DNA extraction or PCR reaction, so different calibration curves for different types of soil would be required to quantify nematodes (Sato *et al.*, 2010). Sato *et al.* (2010) compared the number of *P. penetrans* extracted from 20 g^{-1} soil using the Baermann method (nematode extraction using a tray and nematode counting under stereomicroscope) and the modified combination method reported by Sato *et al.* (2010) (soil compaction, DNA extraction and quantitative PCR). For 75% of the soil samples tested, *P. penetrans* was found in greater abundance when extracted by the combination method than when extracted by the Baermann method. The authors suggested that the combination method was able to detect nematode individuals that were not extracted by the Baermann method such as eggs or juveniles. However, all these studies require a specialized soil compactor that is not readily available. Goto *et al.* (2011) developed a multiplex qPCR assay to identify and quantify *M. incognita, P. penetrans, G. rostochiensis* and *H. glycines* in soil. The results showed an underestimation of *P. penetrans* in presence of other 1000 different nematodes, so an alternative multiplex PCR assay using SYBR Green for *P. penetrans* and *M. incognita*, using environmental DNA extracted from a soil infested with nematodes was developed. The Ct values in the SYBR Green assay were high when one single *P. penetrans* was mixed with 1000 other nematodes, suggesting that this assay can be applied when there are low numbers of target nematodes and high numbers of other taxa. However, this study also showed that *P. penetrans* densities were more than 10-fold higher than the estimate obtained with the traditional method, when qPCR was used. The same overestimation was found for the identification and quantification of other species, *P. thornei* (Yan *et al.*, 2012), *P. neglectus* (Yan *et al.*, 2013; Oliveira *et al.*, 2016) and *P. crenatus* (Oliveira *et al.*, 2016). In all these cases, species-specific primers from ITS1 rDNA were developed and they were highly specific and did not amplify DNA from other *Pratylenchus* spp. or other nematodes but, as in all the previous studies, the qPCR showed higher estimates compared to counts reported by the Baermann method. As previously discussed, ITS genes are known to have a high intraspecific and intra-individual variation in several animals including nematodes, so this can be an explanation of the variation present in all these studies which was not considered by the previous authors. Therefore, care must be taken because variation on ITS genes can seriously affect the specificity or sensitivity of any diagnostics and it is important to be sure that the

assay does not detect 'non-target' species, that have such ITS variation. Moreover, nematode counts, following different extraction methods, like for example the Seinhorst two flask technique or centrifugal flotation, should be compared to qPCR results. Different extraction methods may cause underestimation and variable results. Also, the target genes may cause overestimation due to possible false-negative results or higher estimation due to the presence of gravid female, sperm and eggs in bulk samples.

Another gene of interest for the development of qPCR methods for detection of different root-lesion nematode is the β-1,4-endogluconase gene. It has been investigated for *P. penetrans* (Mokrini *et al.*, 2013), *P. thornei* (Mokrini *et al.*, 2014) and *P. vulnus* (Fanelli *et al.*, 2014). Mokrini *et al.* (2013) was the first to develop a qPCR assay to detect and quantify *P. penetrans* focusing the attention on this gene target. The target gene from 21 different isolates from *P. penetrans* was amplified using designed species-specific primers. The DNA-extraction method was able to extract DNA from 80 individuals and the amount of DNA extracted increased according to the proportional increasing number of nematodes. The qPCR method allowed the detection of a single individual of *P. penetrans* in mixed populations with *P. thornei*. Comparing the Ct values at different annealing temperatures, 63°C was the optimal temperature to obtain a dissociation curve with high peaks for *P. penetrans* and very low or null for *P. thornei.* No amplicons were obtained when the primers were used in presence of other 18 *Pratylenchus* species and other plant-parasitic nematodes. Moreover, a high correlation was present between the number of nematodes in solution and the amount obtained by the qPCR method. Later, the same authors developed a similar qPCR assay based on *β*-1,4-endoglucanase gene for the detection and quantification of *P. thornei*, designing species-specific primers for this species (Mokrini *et al.*, 2014). In this case, the optimal annealing temperature was 69 °C that allow to detect a single *P. thornei* with high peaks at 85.5°C in dissociation curves and no peaks for *P. pentrans* and the negative control. Also, in this study, a single specimen of *P. thornei* was detected in mixed populations with 80 individuals of *P. penetrans,* and Ct values for negative controls were always undetermined. In addition, the authors included an experiment to test the robustness of the method comparing the number of nematodes detected visually under stereomicroscope with numbers calculated with qPCR method, and both results were very similar. In this case, there were no cases of underestimation or overestimation as previously reported for qPCR methods performed with ITS primers. The qPCR methods developed in these two studies (Mokrini *et al.*, 2013, 2014) suggest that the method can be used as tool for diagnosis of root lesion nematodes; however, both studies were conducted using nematode suspensions in water, whereas DNA extraction directly from soil would provide a much faster method and avoids potential errors during nematode extraction from the soil and/or nematode counting under stereomicroscope.

In the last two years, particular attention is given also to the D2-D3 expansion segments of 28S rDNA as potential target for qPCR assays. In 2008, species-specific primers on this fragment were designed to identify *P. neglectus* and *P. thornei*, developing a method of DNA extraction directly from the soil which allowed the identification of 1 juvenile in 1 g of soil (Yan *et al.*, 2008). Species-specific primers for *P. penetrans* were designed by Bandoo *et al.* (2017) for a SYBR green assay and their specificity was investigated using 8 populations of *P. penetrans* and 31 other nematodes. This qPCR method allowed the detection of a single juveniles (Cq = 31.38) of *P. penetrans* in 1 g of soil without evidence of cross-reaction with other root lesion nematode species or plant parasitic nematodes, showing then a high level of specificity and sensitivity. However, this assay included only local species, so this should be tested also with populations from other countries. Thus, another problem to consider is that some species-specific assays for *Pratylenchus* spp. have been developed on a limited number of populations. Moreover, as previously discussed for other studies (Yan *et al.* 2012), also this qPCR assay presented overestimation of nematode numbers compare to the conventional counting under microscope; this might be due to nematode extraction efficiency or amplification of different developmental stages that are not detected with visual counting (Bandoo *et al.*, 2017). A TaqMan qPCR method reported by Dauphinais *et al.* (2018) also showed high specificity and sensitivity improved by an exogenous internal positive control (EIPC) for the prevention of false negative results, but in this case the quantification estimated by the qPCR assay was lower than the visual counting under microscope.

Despite several studies that report the molecular diagnostics of different root-lesion nematodes species, there is still the need to find a consistent and unique tool that will allow a faster and reliable identification compare to the current methods reported in literature.

1.5 ROOT-LESION NEMATODES: BIOLOGY AND DISTRIBUTION

1.5.1 Biology and life cycle

Root-lesion nematodes are migratory endoparasitic nematodes, found in diverse environments and on a variety of different crops. They are considered to be the third most important species of plant parasitic nematode in terms of their economic impact on global crop production (Jones *et al.*, 2013). Root-lesion nematodes are polyphagous, feeding directly on roots, tubers and cortical tissues. Typically, they are found inside the roots where they feed and reproduce, but they can also be found in the soil surrounding the roots. However, when their feeding source is depleted, nematodes leave the roots and migrate through the soil to infect new host roots. During adverse conditions, they can also survive as eggs or through anhyrodrobiosis until favourable conditions return (Van Gundy, 1965).

Juvenile and adults are vermiform, and all stages can infect the roots. Males are common in some species (*P. penetrans, P. coffee, P. vulnus*) but in others are rare or absent (*P. neglectus, P. thornei, P. zeae*) and in this case they reproduce by parthenogenesis, females produce fertile eggs without copulation by males (Duncan and Moens, 2013). Instead, when males are present, they reproduce by amphimitic reproduction. Females lay eggs which can be found in clusters inside the roots or also in the surrounding soil. Little is known about the life cycle of root lesion nematodes under field conditions, with almost all the information available originating from studies conducted in controlled conditions for different species in different hosts (Castillo and Vovlas, 2007). The first moult is within the egg on which the first-stage juvenile (J1) becomes a second-stage juvenile (J2). The J2 stage remains inside the egg until the root diffusates stimulate its hatch, usually after one week from egg deposition. The J2 starts moving the stylet toward one pole of the egg, disrupting the eggshell and eventually emerging from the egg. Following hatching, the J2 moults into J3 and then J4, within 35 to 40 days, after that the nematodes moult into adults, becoming either female or male (Figure 1.4) (Castillo and Vovlas, 2007; Duncan and Moens, 2013).

In general, the life cycle is completed in 3-4 weeks in tropical regions, where the temperatures are higher, whereas 5-8 weeks are required in temperate regions (Duncan and Moens, 2013). The life cycle is typically completed between 3 to 8 weeks depending on the species, host plant, temperature and other abiotic factors such as moisture of soil (Loof, 1991). For example, *P. penetrans* has a life cycle of 54-65 days and each female produces 16–35 eggs at a rate of 1-2 eggs per day in red clover (Turner and Chapman, 1972), whereas *P. thornei* has a shorter life cycle of about 25-35 days on carrot discs (Castillo *et al.*, 1995). Temperature can be an important factor in determining the time of the life cycle. Increasing the temperature from 17 to 30 °C decreased the life cycle of *P. penetrans* from 46 to 22 days

in Ladino clover roots, with 25 °C being considered optimal for this species to complete its life cycle in 26 days (Mizukubo and Adachi, 1991). In contrast, Mokrini *et al.* (2019) reported an optimum temperature of 20 °C for the reproduction of *P. penetrans* and *P. thornei* on carrot discs.

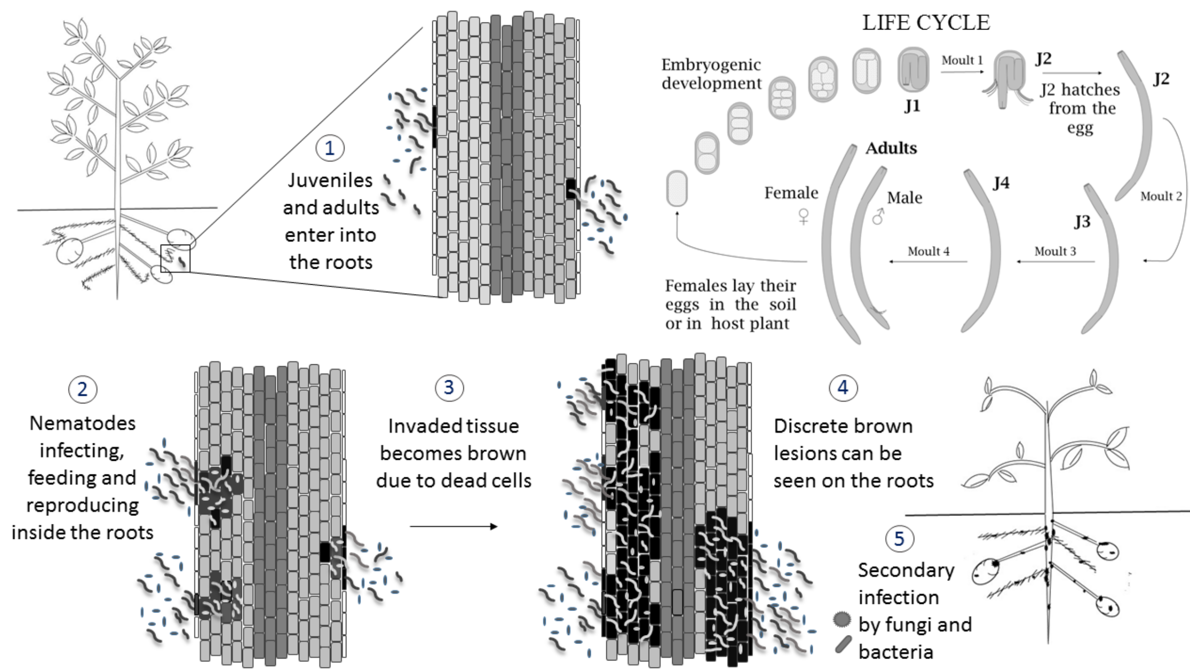

Figure 1.4: Representation of life cycle and pathogenisis by *Pratylenchus* spp. on potatoes. Drawings are the authors own.

1.5.2 Crop damage caused by root-lesion nematodes

Root-lesion nematodes are classified as migratory endoparasites as they can feed and reproduce within the root system, although they may also feed on the root surface without entering the root tissue (Duncan and Moens, 2013). *Pratylenchus* feed directly on roots, tubers, and cortical tissues. Both adults and juveniles infect roots by entering behind the zone of elongation and feeding on parenchyma cells (Castillo *et al*., 1996). The nematodes degrade cell walls with mechanical movements of the stylet, and by secreting enzymes that degrade the cytoplasm within cells leading to brown lesions at the points of entry and root migration (Zunke, 1990 a, b) (Figure 1.4). Such lesions typically lead to necrotic areas and cell death with a reduction in root growth (Castillo and Vovlas, 2007). The lesions on the surface of tubers can be brown to black, turning purple over time. Damaged roots impede uptake of water and nutrients, thus plants become stunted and present leaf chlorosis (Duncan and Moens, 2013). Root-lesion nematodes occur in patches (foci) in potato fields (Holgado and Magnusson, 2012). Fifteen species of root lesion nematodes are known to infect potatoes, but *P.*

penetrans, P. crenatus, P. neglectus, P. alleni, P. thornei and *P. scribneri* are the most common species associated with this crop. Depending on the species, different symptoms can also be present on potato tubers. For instance*, P. scribneri* causes tubers to have a scabby appearance whilst *P. penetrans* has been associated with wart-like protuberances (Brodie *et al.*, 1993). There are reports of seed potato tubers infected by *P. penetrans* (Olthof and Wolynetz, 1991; Khan and Hussain, 2004; Holgado *et al.*, 2009; Holgado and Magnusson, 2012), *P. neglectus* (Olthof and Wolynetz, 1991), *P. scribneri* (Olthof and Wolynetz, 1991), and *P. brachyurus* (Koen and Hogewind, 1967; Koen, 1969). Infected seed potato tubers may lead to the transfer and subsequent spread in potato fields that were previously uninfested.

1.5.3 Abiotic factors influencing reproduction of root-lesion nematodes

Abiotic factors, such as soil texture, soil moisture, temperature and pH, can have different effects on the reproduction and, consequently, pathogenicity of root lesion nematodes. Indeed, sometimes nematodes alone have no significative effects on the development and yield of the plant, but together with specific biotic factors, they can enhance damage on crops (Wallace, 1973).

Soil texture is one of the major factors influencing reproduction and distribution of root lesion nematodes; indeed, it can influence nematode movement, penetration into host roots, reproduction and survival, moreover it affects water and nutrient uptake for the plant. In potato crops, *Pratylenchus* species have been found to be related to different soil types. For example, *P. crenatus* has been found mostly in potatoes growing in loams and silt loams, whereas *P. penetrans* tends to be associated with sandy soil (Florini *et al.*, 1987). Soil type was also found to influence damage of corn roots (*Zea mays*) by *P. penetrans*, with greater damage occurring in sandy loam than in silty loam (Townshend, 1973). Soil texture was found to influence the reproduction and pathogenicity of *P. neglectus* on rangeland grasses where the nematodes' activities were greater in a sandy loam compared to a clay loam (Griffin, 1996). In wheat fields, *P. neglectus* and *P. thornei* have been detected in silt loams, clay loams, and irrigated sandy loams (Smiley, 2010). In general *P. penetrans* is often found in sandy soils, whereas *P. neglectus* prefers clay and loamy soil, and *P. crenatus* is usually found in loams and silt loams (Castillo and Volvas, 2007; Brodie *et al.*, 1993). In England and Wales, Corbett *et al.* (1970) found that *P. neglectus* occurs in all type of soils, *P. crenatus* and *P. fallax* in sandy soils, whereas *P. thornei* was found in clay soils.

Together with soil type, nematode penetration and migration are also affected by soil moisture. Nematode population dynamic between seasons might depend on variations in soil moisture due to rainfall or a dry period (Wallace, 1973). The maximum migration of nematodes

is at field capacity (amount of water content held in the soil after excess water has drained away) and then it is significantly reduced in very dry or wet soils. However, dry conditions favour the infection and subsequent damage to wheat crops by *P. neglectus* and *P. thornei* (Smiley, 2010). Additionally, the survival of *P. penetrans* and *P. neglectus* increased as soil moisture declined in corn fields (Townshend, 1973). In dry soils, nematodes tend to move less, therefore requiring less energy reserves which in turn might lead to longer survival. In wet soil, however, nematodes are totally immersed in water, so they need to maintain an osmotic balance that imposes a loss of energy (Kable and Mai, 1968). Moreover, moist soils can favour the proliferation of microbial species pathogenic to nematodes affecting their survival, thus reducing the infection to the host plant. However, Kimpiski *et al.* (1976) reported high numbers of *P. neglectus* in wheat at early spring when there was a high level of soil moisture due to rainfall and low nematode populations under dryer conditions during summer. Kable and Mai (1968) found that *P. penetrans* survival and infection of alfalfa roots was suppressed by low or very high soil moisture content, but this was dependent on soil type. For example, in clay loam the increase of soil moisture did not increase nematode populations and infection in the roots.

Soil temperature also influences the movement and reproduction of root-lesion nematodes, but differences are found among species. For example, *P. alleni, P. brachyurus, P. coffeae, P. neglectus, P. scribneri* and *P. zeae* have optimum temperature of 30°C on soybean, whereas the optimum temperature for *P. penetrans* and *P. vulnus* is 25°C, being able still to reproduce at 15°C (Acosta and Malek, 1979). The optimum temperature for *Pratylenchus penetrans* in alfalfa was at 18°C (Kimpinski and Willis, 1980), instead for *P. neglectus* in corn roots it was 30°C (Townshend, 1972). In barley, *P. neglectus* was also able to reproduce at a low temperature (7.7°C), whilst a high temperature of up to 25°C did not favour its reproduction (Umesh and Ferris, 1992). Also, *P. penetrans* is still able to reproduce at low temperatures (7°C) causing problems to onions in the Autumn of Indiana, US (Ferris, 1970). The reproduction of *P. penetrans,* in alfalfa and Timothy, was favoured by the increase of temperatures, whilst the increase of temperature from 10°C to 30°C decreased the movements of *P. crenatus* in vertical soil columns in alfalfa and high temperature resulted in a reduction in the numbers of nematodes (Kimpinski and Willis, 1980). Usually low temperatures (below 5°C) reduce population of root lesion nematodes. For instance, exposure of *P. penetrans* to −4°C for 4 weeks caused 90% mortality, whilst 4h of exposure to -12°C resulted in 92% of mortality (Kimpinski and Dunn, 1985). Low temperatures may be then useful to reduce populations of *P. penetrans* in tubers of potato after storage, indeed temperatures below 5°C decreased numbers of *P. penetrans* in potato tubers after 8 months of storage

(Olthof and Yu, 1999; Olthof and Wolynetz, 1991), the same reduction was reported for *P. brachyurus* after 15 weeks of potato stored at 5°C (Koen and Hogewind, 1967).

Soil pH is another factor that can affect the survival and reproduction of nematodes; however, the optimum pH range for root lesion nematode species varies among the species and host plants. For example, an increase in pH from 5 to 6.9 in alfalfa increased the numbers of *P. penetrans* but reduced *P. crenatus;* but for both species there was less root invasion of the grass Timothy by both species. The optimum pH to favour the movements of *P. penetrans* was recorded to be 6.0, whereas the locomotion of *P. crenatus* was not affected when pH ranged from 5 to 7 (Kimpinski and Willis, 1980). Previous studies in vetch (*Vicia sativa* L.) and alfalfa, showed that *P. penetrans* reproduce better at pH ranging from 5.2 to 6.4, whilst a population decrease was observed at pH 7 (Morgan and MacLean, 1968; Willis, 1972). Similarly, the reproduction of *P. thornei* on mint was greatest at pH 6.0 when compared to pH 3.0 or 9.0 (Shukla *et al*., 1998).

1.5.4 Dispersal and distribution of root-lesion nematodes on potato crops

Plant parasitic nematodes can be transferred through movement of infected plant materials or soil, meaning that they can spread within the same field, increasing the area of infection, or they can be dispersed to other fields. *Pratylenchus* spp. are found worldwide, with occurrence depending of their optimal temperature. For example, *P. thornei*, *P. fallax* and *P. crenatus* are typical in temperate regions, whereas *P. coffee, P. brachyurus,* and *P. loosi* prefer tropical and sub-tropical regions. Then, their distribution in each region can vary according to the species, host plants and soil type.

Pratylenchus penetrans was reported and described for the first time in Rhinebeck, New York (USA) and since then it has been found all over the world (Corbett, 1973). There are reports infecting potato plants in North America (Kimpiski, 1979; Olthof and Wolynetz, 1991; Brown *et al*., 1980) and Australia (Harding and Wicks, 2007), whilst *P. crenatus* has been reported mostly on cereals and grasses (Loof, 1991), but also in potato in the US (Florini *et al*., 1987; Brown *et al*., 1980) and Canada (Kimpiski, 1979; Olthof *et al*., 1982; Kimpinski and Smith, 1988). Other species like *P. neglectus* was first recorded in Germany and later in many other countries on different hosts such as cereals and wheats, and potatoes in North America (Brown *et al*., 1980; Olthof and Wolynetz, 1991), whereas *P. thornei* has mainly been found infecting cereals and fruit trees, and there are no reports on potato except one record in Ohio, North America (Brown *et al*., 1980).

In a survey conducted in seventy-one potato fields in Prince Edward Island, *P. penetrans* and *P. crenatus* were the most common species present, with the respective nematodes found

in 66% and 94% of fields; *P. penetrans* was always found in higher densities than *P. crenatus* (Kimpiski, 1979). Also, in Wisconsin, the most common species associated with potato damage was *P. penetrans*; whereas *P. crenatus* was less present and it was found mostly in sandy soil (Dickerson *et al.*, 1964). On the other hand, *Pratylenchus thornei, P. vulnus* and *P. neglectus* were found only in one field. Brown *et al.* (1979) found several species of root lesion nematodes in seventy-three Ohio fields cultivated with "Superior" potatoes; in particular *P. crenatus, P. penetrans, P. scribneri, P. alleni, P. thornei,* and *P. neglectus* were detected in 65% and 84% of the potato soil and root samples, respectively; *P. crenatus* was the most common species found mostly in silt loam soils, followed by *P. penetrans* that was found mostly in sandy soils. In 1982, 50 potato fields were sampled in Ontario and *P. penetrans* was identified in 32 fields with nematode dentisity of 1700 kg^{-1} soil, instead *P. crenatus* and *P. neglectus* only in 12 and 7 fields, with densities of 860 kg^{-1} soil and 320 kg^{-1} soil, respectively (Olthof *et al.*, 1982). In Australia, different species of *Pratylenchus* were found in 87% of and 92% of 77 potatoes fields with an average of 1 to 19 nematodes per gram of soil. In particular, *P. penetrans* was identified only in two potato fields, while *P. crenatus* and *P. neglectus* were identified from 60% and 27% of fields, respectively (Harding and Wicks, 2007).

In Europe, there are few studies and reports of *Pratylenchus* spp. in potato fields. Van Der Sommen *et al*. (2009) reported the presence of root lesion nematodes in 61 % fields surveyed in The Netherlands, with *P. neglectus* identified as the most abundant species and *P. penetrans* the most damaging. Furthermore, high densities of *P. penetrans* were detected in a patch of poor growth in a field in Norway (Holgado *et al*., 2009). Recently, a survey conducted in forty potato fields in Portugal (Esteves *et al*., 2015) revealed the presence of root lesion nematodes in 83% of soil and 78% of root samples; in this study, *P. penetrans* was the most abundant species, being found in 42 % of the samples. Additionally, *P. neglectus*, *P. crenatus* and *P. thornei* were recorded in 35%, 13% and 3% of the samples respectively.

1.5.5 Distribution of Pratylenchus spp. in the U.K.

Although the distribution of root-lesion nematodes in the UK is available for certain crops, there is a lack of information regarding the potato production areas affected and the associated crop losses. In the past, *P. pratensis* and *P. penetrans* were reported by Southey (1959) in several hosts in England. Later, Seinhorst (1968) described three new species of *Pratylenchus* and he reported the presence of *P. fallax* and *P. flakkensis* also in England, although the host range was not specified. Corbett (1969) found three species related to wheat in a field at Rothamsted: *P. thornei, P. neglectus* and a new species, that was described for the first time by Corbett (1969) and named *P. pinguicaudatus*. A survey conducted during June and July 1966 in England and Wales, in twenty-four barley and five wheat fields, revealed the high

presence of *Pratylenchus* spp. compared to other plant parasitic nematodes (Corbett, 1970). Indeed, root lesion nematode were up to 50% of total number of PPN in all samples, with only four exceptions. Five species were reported: *P. minyus* (syn. *P. neglectus*), *P. crenatus*, *P. fallax, P. thornei* and *P. pinguicaudatus*. Strangely, *P. penetrans,* a common species of root-lesion nematode, was not reported. *Pratylenchus neglectus* was the most common species of the survey; it occurred in all soil types, in some cases it was found with *P. thornei* in loam and clay soils or with *P. crenatus* and *P. fallax* in sandy soils. A survey based on eight English farms, consisting of a total of 33 raspberry cane revealed the presence of different root lesion nematodes (Cotten and Roberts, 1981). *Pratylenchus crenatus* was found in 20 out of the 33 (61%) raspberry beds, being followed by *P. thornei*, *P. neglectus* and *P. fallax*. *Pratylenchus penetrans* was only found only in one raspberry bed. A survey conducted on carrots in Scotland demonstrated that 63% of 59 farms surveyed presented *Pratylenchus* species (Boag, 1979). In 1980, *Pratylenchus* was found in a survey conducted on 664 soil samples collected from field used for growing peas and beans in Eastern Scotland. In this survey, *Pratylenchus* spp. were found in 46% pea fields and only 11% in beans (Boag, 1980). However, in both surveys there are no reports regarding the species detected. A further survey conducted in Scotland reported the *Pratylenchus* species recovered in 98 soil samples collected from fields in cereal production; 55% of fields had high population densities of *P. crenatus* (500 nematodes in 200 g^{-1} of soil) and only 2% having either *P. thornei* or *P. penetrans* (Boag, 1990). Earlier, Boag and Lopez-Llorca (1989) reported that *P. crenatus* was the main species recovered from 31 soil samples collected from fields with cereals, at a range of 30-90 nematodes per 200 g^{-1} of soil in 19% of the samples. In the same work, 26 fields of permanent pasture were sampled and although *P. crenatus* and *P thornei* were found in 8% of fields, they were both at low densities, 20 and 8 nematodes per 200 g^{-1} of soil, respectively.

1.5.6 Pathogenicity and damage thresholds

The term “pathogenicity” is referred to when a pathogen causes a disease in a host plant with visible effects. As mentioned before, infection by root lesion nematodes can present visible symptoms such as the presence of patches in fields with stunted and yellowish plants, usually with severe areas occurring in the centre of the patch. Symptoms can often be overlooked or mistaken for damage caused by other pests or biotic factors (Starr *et al.*, 2013; Palomares-Rius *et al.*, 2014 b). As such, it is important to conduct analysis of soil samples together with diagnostics to understand population densities of root-lesion nematode species, in order to adopt proper management practices. The impact of nematodes on the yield of a crop can be affected by different factors: the pathogenicity of the nematode species, the

population density at planting time, the tolerance or susceptibility of the host plant and environmental factors that are favourable to nematode activity (Schomaker and Been, 2013).

The damage threshold of a species is the level (population density) at which the pest starts to cause damage in the crop with economic losses, and it depends on the different species of nematodes and its host. By measuring the initial population density just prior to sowing (P_i), it is possible to calculate damage thresholds and to predict crop damage based on population densities on soil at the time of planting (Castillo and Volvas, 2007). Damage thresholds of each species can vary according the host plants, as shown in table 1.4.

Table 1.4: Examples of damage threshold densities reported for the host-*Pratylenchus* interaction (adapted from Castillo and Volvas, 2007)

Species	Host	Damage thresholds (nematode g^{-1} soil)	Reference
P. crenatus	Oat	0.33	Barker and Olthof, 1976
	Carrot	0.3-1.8	Potter and Olthof, 1993
P. neglectus	Alfalfa	2	Griffin and Gray, 1990
	Barley	1.5	Rivoal and Cook, 1993
P. penetrans	Alfalfa	1	Griffin, 1993
	Bean	0.5	Elliot and Bird, 1985
	Carrot	1	Vrain and Bélair, 1981
	Corn	0.25	Dickerson *et al*., 1964
	Onion	0.67	Olthof and Potter, 1973
	Sweet corn	0.67	Olthof and Potter, 1973
	Tobacco	2	Olthof *et al*., 1973
	Tomato	0.45	Miller, 1978
P. scribneri	Bean	0.5	Thomason *et al.,* 1976
P. thornei	Wheat	0.5-1	Rivoal and Cook, 1993
	Wheat	0.42	Nicol and Ortiz-Monasterio, 2004
	Wheat	2.5	Thompson, 1993

1.5.7 Damage thresholds of root lesion nematodes to potato crops

Root lesion nematode reproduce easily in sandy soils, which are also the optimal soil type for potato plants. In general, *P. crenatus*, *P. neglectus* and particularly *P. penetrans* are considered to be the most damaging species infecting potatoes (Palomares-Rius *et al.*, 2014 b), but there are also other pathogenic species such as *P. alleni*, *P. scribneri* and *P. thornei* (Brodie *et al.*, 1993). Studies on the thresholds of root lesion nematodes for potato damage have been previously published in Canada (Olthof, 1986, 1990; Kimpiski, 1988) and Scandinavia (Holdago *et al.*, 2009) (Table 1.5).

For *P. penetrans* and *P. scribneri* densities of 1-2 nematodes g^{-1} soil have been reported to cause damage to potatoes (Olthof and Potter, 1973; Olthof, 1986; Riedel *et al.*, 1985) whereas *P. neglectus* can induce damage at densities of around 0.6 nematodes g^{-1} soil (Olthof, 1990). In Norway, potato growth (cv. Saturna) was correlated negatively with different densities of *P. penetrans*, and a damage threshold of 100 specimens 250 g^{-1} of soil (0.4/ g^{-1} of soil) was estimated to cause a yield reduction of 50% (Holdago *et al.*, 2009). However, damage thresholds can vary according to cultivars and other environmental factors like soil texture, temperature and moisture. Indeed, Bernard and Laughlin (1976) studied four different potato cultivars (Katahdin, Kennebec, Superior and Russet Burbank) subjected to different population densities of *P. penetrans* in micro-plots with sandy clay loam and reported different effects on yield loss. The variety Superior presented a yield loss of 23 -30% caused by 0.38 nematodes nematodes g^{-1} of soil, whereas Kennebec was affected by 0.81 nematodes g^{-1} soil and Katahdin by 1.5 - 2 nematodes g^{-1} soil. In comparison, Russet Burbank was unaffected by *P. penetrans* at densities of 0.38 - 2 nematodes g^{-1} of soil. Similarly, Bird and Vitosh (1978) reported that Russet Burbank had tolerance towards *P. penetrans*. In contrast, Olthof (1983) found that Russet Burbank had a yield loss of 16% when exposed to 1.8 *P. penetrans* g^{-1} soil whilst the yield of the varieties Kennebec, Monona, Norchip, Superior and Yukon Gold were unaffected by 1.6 - 2 *P. penetrans* g^{-1} soil. Later, the same cultivars were tested in a micro-plot experiment with 10 nematodes g^{-1} soil and yield losses were observed; Superior was the most affected with 73% losses of marketable tubers, followed by Russet Burbank (61%), Kennebec (55%), Monona (46%), Norchip (43%) and Yukon Gold (25%) (Olthof, 1986).

These contrasting results may also be explained by the different ranges of initial population densities used in each study or the different environmental conditions under which experiments were conducted. Some authors (Kimpiski, 1982; Martin *et al.*, 1982) suggested that damage by root lesion nematodes may be greater when potatoes are exposed to drought conditions. Indeed, Martin *at al.* (1982) discussed the contrasting results seen in these different studies and suggested soil moisture as the main factor; in a dry condition (270 mm

rainfall), 75 nematodes per 100 g^{-1} soil (0.75 g^{-1} of soil) were hypothesised to cause a reduction in yield, whereas in wet conditions (560 mm rainfall) higher densities up to 260 per 100 g^{-1} soil (2.6 g^{-1} of soil) may not cause yield losses. Kimpiski *et al.* (1982) reported that moisture level of 35-55% field capacity reduced significantly the tuber weights of Superior potatoes growing in pots in fine sandy loam in greenhouse and also the interaction between soil moisture and *P. penetrans* inoculum (25 nematodes g^{-1} of soil) significantly reduced the yield. Possibly, in a stressed environment such as under drought conditions, root damage is more significant, and nematodes can infect the roots because they may disrupt the cell wall more easily with their stylet and secretions. Damage thresholds may decrease when plants are exposed to drought periods or poor soil nutrition, whereas they may increase in fields that are routinely irrigated regularly and soil nutrients are readily available, especially during the growing period (Smiley, 2010). On the other hand, irrigation can enhance nematode movement within a field and this may cause further damage to the crop. Moreover, other environmental factors, such as soil texture, may affect the establishment of damage thresholds.

Table 1.5: *Damage threshold densities of potato-*Pratylenchus *interaction*

Species	**Potato Damage thresholds (nematode g^{-1} soil)**	**Reference**
P. neglectus	0.6	Olthof, 1990
	1.5	Umesh and Ferris 1994
P. penetrans	1-2	Olthof and Potter, 1973
	0.4	Holdago *et al.*, 2009
P. scribneri	1-2	Riedel *et al.*, 1985

1.5.8 Interactions between root-lesion nematodes and pathogenic fungi

Interactions between plant-parasitic nematodes and pathogenic fungi, presenting severe problems in different crops, are well documented (Powell, 1971; Mai and Abawi, 1987; Evans and Haydock, 2000). When the interaction between nematode and fungi results in an increase of plant damage compared to the sum of damage caused by the single pest or pathogen, this is called "synergism" (1+1 > 2); when the association results in a plant damage that is less than the damage caused by the sum of the individual pathogens, this is called "antagonism" (1 + 1 < 2); "neutral interaction" is when the damage caused by nematodes and fungi together are equal to the sum of the individual pathogens (1 + 1 = 2). A synergistic interaction between two organisms cause what it is known as a "disease complex". Abiotic factors such as temperature, soil moisture and soil type may increase diseases caused by these interactions. The mechanisms of synergistic interactions between nematodes and fungi can be of different origins. Nematodes may play a role of wounding agents or induce physiological changes in plants together with the fungus or also influencing the chemical composition and quantity of root exudates that may influence the development of the fungus and consequently the disease (Back *et al.*, 2002).

Atkinson (1892) was the first to report a case of an interaction between *Fusarium oxysporum* f. sp. *vasinfectum* and root-knot nematodes on cotton. Later, the same disease complex was reported in other crops such as alfalfa, tomato, beans, chickpeas, coffee and banana (Back *et al.*, 2002). Other plant parasitic nematodes have been reported to be involved in complexes disease with fungi, like for example *Globodera* spp., *Heterodera* spp., *Rotylenchulus* spp., *Pratylenchus* spp. and ectoparasitic nematodes such as *Xiphinema* spp. and *Longidorus* spp. Endoparasitic nematodes are commonly in association with two type of fungi: wilt fungi (*Fusarium* and *Verticillium*) and the root-rot pathogens (*Pythium*, *Phytophthora* and *Rhizoctonia)* (Table 1.6).

Root-lesion nematodes enter the roots and migrate intracellularly in the cortical tissues causing lesions on roots that consequently provide access to other pathogens like bacteria or fungi. These secondary pathogens increase root decay and discoloration compared to that caused by the root lesion nematodes alone. Several studies have investigated the interactions between *Pratylenchus* spp. and the wilt fungi *Fusarium* and *Verticillium* in different host plants. In 1994, it has been reported that *P. neglectus* was associated with different fungi of wheat roots such as *Fusarium oxysporum, F. acuminatum, F. equiseti, Microdochium bolleyi, Gaeumannomyces graminis, Bipolaris sorokiniana, Pythium irregular, Pyrenochaeta terrestris* and *Rhizoctonia solani* (Taheri *et al.*, 1994). In addition, the same authors showed that nematodes reproduced significantly in wheat when they were co-infected with *R. solani, M. bolleyi, B. sorokiniana, P. irregulare*, or in combination with both *G. graminis* and *F. equiseti*;

instead, they reduced in numbers when co-infected with *G. graminis* and *R. solani*. This might suggest that some fungi favour the reproduction of root lesion nematodes within roots, instead others reduce them in numbers, so possibly these interactions may influence nematode reproduction. Also, in other studies, the reproduction of root lesion nematode was influenced by the presence of fungi. Indeed, populations of *P. penetrans* increased in alfalfa when co-infected with *V. dahliae* (Vrain, 1987) and the same for *P. thornei* on chickpea in presence of *F. oxysporum* f. sp. *ciceris* (Castillo *et al.*, 1998). However, the severity of a mint disease, caused by the interaction of *P. pentrans* with *V. dahliae,* depended on the fungal vegetative compatability group (VCG). Interactions of *P. penetrans* with *V. dahliae* VCG 2B increased the disease severity, whereas with VCG 4A there was no difference (Johnson and Santo, 2001). *Pratylenchus penetrans* has been reported to be associated with the fungus *Rhizoctonia fragariae* increasing the severity of a disease on strawberry called black root rot (La Mondia *et al.*, 2003). A split-root technique was developed to investigate this interaction, and plants were found to have a higher degree of disease in the roots where both pathogens were inoculated, demonstrating that *P. penetrans* increased the severity of the disease in a local rather than systemic manner. Possibly, death of cells on the cortical tissue, caused by the direct feeding of *P. penetrans*, resulted in increased susceptility to the infection of *R. fragariae* and this increases the disease.

Table 1.6: Examples of nematode-fungus disease complexes involving *Pratylenchus* species

***Pratylenchus* spp.**	**Fungus**	**Host**	**Reference**
P. crenatus	*Verticillium dahliae*	potato	Riedel *et al.*, 1985
P. penetrans	*Rhizoctonia fragariae*	strawberry	La Mondia *et al.*, 2003
P. penetrans	*Verticillium dahliae*	mint	Johnson and Santo, 2001
P. penetrans	*Verticillium dahliae*	alfalfa	Vrain, 1987
P. penetrans	*Verticillium dahliae*	potato	Riedel *et al.*, 1985
P. penetrans	*Rhizoctonia solani*	potato	Kocton *et al.*, 1985
P. neglectus	*Rhizoctonia solani*	potato	Riedel *et al.*, 1985
P. scribneri	*Verticillium dahliae*	potato	Wheeler *et al.*, 1994
P. thornei	*F. oxysporum f. sp. ciceris*	chickpea	Castillo *et al.*, 1998
P. thornei	*Rhizoctonia bataticola*	chickpea	Bhatt and Vadhera, 1997

1.5.9 Disease complexes between nematodes and fungi in potatoes

Several cases of interaction between nematode and soil-borne fungi have been also reported in potato. The most common and studied are the disease complexes represented by *Globodera*–*Verticillium dahliae* and *Pratylenchus*–*Verticillium dahliae.* In both cases, these interactions enhance a potato disease called "potato early dying" (PED). In general, this disease can be caused by either *Verticillium dahliae* or *Verticillium albo-atrum*, with both *Verticillium* species causing premature plant death and reduction of tuber yields. Potato early dying disease can be enhanced by the interaction of these fungi with *Pratylenchus* spp. and potato cyst nematodes, *G. rostochiensis* and *G. pallida* (Back *et al.*, 2002).

The interaction between potato cyst nematodes and *V. dahliae* has been reported in different studies showing an increase of the severity of disease in potatoes (Evans, 1987). Some potato cultivars have different degrees of resistance to *V. dahliae*, and this can change by the presence (or absence) of potato cyst nematodes (Evans, 1983). For example, the cultivar Pentland Javelin showed mild symptoms when both nematode and fungus were present whereas Maris Anchor produced severe symptoms in the presence of *V. dahliae* alone, and Maris Peer only presented increased disease symptoms when it was exposed to both nematode and fungus (Evans, 1983). These different responses might be related to the root system and variations in their endodermis, nematodes only being capable of disrupting the endodermis of certain cultivars to facilitate entry by *V. dahliae.* Evans (1987) demonstrated that Verticillium wilt symptoms appeared much earlier on early than in maincrop cultivars.

However, the most investigated disease complex in potato is the interaction between *Pratylenchus* spp. and *V. dahliae* (Riedel *et al.*, 1985; Rowe *et al.*, 1985; Wheeler *et al.*, 1994; Back *et al.*, 2002). The interaction between these organisms can change depending on the *Pratylenchus* species (Riedel *et al.*, 1985, Rowe *et al.*, 1985) and the fungal VCG's (Botseas and Rowe, 1994). *Pratylenchus crenatus*, *P. penetrans* and *P. scribneri* are the main species reported to interact synergistically with *V. dahliae* causing wilt disease (Riedel *et al.*, 1985; Rowe *et al.*, 1985; Wheeler *et al.*, 1994). However, in a two-year field experiment, Riedel *et al.* (1985) reported that the highest concentration of *P. penetrans* and *V. dahliae* caused a yield reduction of 20 and 39% and severe potato early disease symptoms each year of the field experiment, whereas *P. crenatus* and *P. scribneri* did not interact with the fungus and no PED symptoms were detected. *Pratylenchus penetrans* and *Verticillium dahliae* together reduced potato yields of Superior (Botseas and Rowe, 1994; Martin *et al.*, 1982; Rowe *et al.*, 1985) and Russet Burbank (MacGuidwin and Rouse, 1990; Saeed *et al.*, 1997, 1998) more than when each pathogen was present alone. Boatseas and Rowe (2004) have reported that the synergism between *V. dahliae* and *P. penetrans* occurs in potatoes only with one vegetative compatibility group of *V. dahliae,* called VCG 4A. However, as discussed

previously, other authors have reported that VCG 4A together with *P. penetrans* did not cause increase of wilt disease on peppermint, whereas synergism effects were detected with VCG 2B (Johnson and Santo, 2001). Disease thresholds of *V. dahliae* alone in potatoes have been reported to range between 5–30 colony-forming units (cfu) cm^{-3} of soil, but this decreases to 2–13 cfu cm^{-3} soil when *P. penetrans* is present (Powelson and Rowe, 1993). Martin *et al.* (1982) reported 36, 60 and 75% reductions in potato yield when 15, 50 and 150 *P. penetrans* per 100 cm^3 soil were inoculated respectively with *V. dahliae*, whereas no effects were reported when each pathogen was present alone, with the exception of the highest nematode densities, 150 *P. penetrans* per 100 cm ^{3} soil, that caused 12% of yield reduction. In a controlled experiment on the influence of *V. dahliae* and *P. penetrans* on gas exchange of Russet Burbank cultivar, the authors found lower values of stomatal conductance after co-infection by both pathogens compared to the control and the values after infection with either pathogen alone (Saeed *et al*., 1997a). Similar results were found in another experiment on which co-infection by both pathogens significantly reduced net photosynthesis, stomatal conductance and transpiration, at 45 days after planting, and gas exchange parameters were greater in old leaves than in young leaves (Saeed *et al*., 1997b). Also, Rotenberg *et al*. (2004) reported a significant reduction in transpiration in plants infected with both pathogens that contained a greater number of cfu in stem sap than those infected by *V. dahliae* alone. This was also confirmed in field experiments reported by Saeed *et al.* (2007) in which both pathogens caused a reduction on leaf light use efficiency, leaf stomatal conductance, leaf water use efficiency and an increase on intercellular CO_2 compared with plants infested by each pathogen alone and the untreated control. Overall, this research demonstrates that infection of potatoes by *P. penetrans* and *V. dahliae* affects the physiology of plants and ultimately increases disease symptoms.

Rhizoctonia solani is another important pathogen of potatoes causing stem cankers, stolon lesions, black scurf, deformations, elephant hide and dry core. *Rhizoctonia solani* has been reported to be involved to form disease complexes with some plant parasitic nematodes such as cyst (*Heterodera* and *Globodera*), root knot (*Meloidogyna* spp.) and root-lesion (*Pratylenchus* spp.) nematodes (Back *et al*., 2006, 2010). Although there are several studies about the interaction of plant parasitic nematodes and *R. solani*, less is known about the interaction with root lesion nematodes, especially on potatoes. Benedict and Mountain (1956) reported significant association between *R. solani* and *P. neglectus* in winter wheat fields in Canada causing root rot disease. In both greenhouse and field experiments, a synergistic interaction was found between the two pathogens and when soil was treated with both nematicide and fungicide the growth of wheat plants was more than twice of plants treated with each treatment separately. Clearly, this showed that treatments of both pathogens

enhanced the growth of wheat, although there is no evidence to support the hypothesis that *P. neglectus* influences *R. solani* infections of the roots. Taheri *et al.* (1994) reported different fungi (*Fusarium oxysporum, F. acuminatum*, *F. equiseti* and others) associated with *P. neglectus* infestations in Australian wheat fields, but in a glasshouse experiment, including *R. solani,* a strong positive interaction was only found with *R. solani*, which enhanced the number of nematodes per pot when they were inoculated together. However, root rotting of wheat did not increase unless each pathogen was inoculated separately. Kocton *et al.* (1985) investigated the interactions of *V. dahliae*, *Colletotricum coccodes* and *R. solani* with *P. penentrans* in the early dying syndrome on Russet Burbank potato in microplots and found that *R. solani* had no significant effects on the severity of disease symptoms or tuber yield, even when in combination with *P. penetrans*.

Five potato cultivars (Asterix, Romano, Sante, Nadine and Maris Piper) have been tested to study the interaction of *P. penetrans* and *R. solani* under controlled conditions (Kenyon and Smith, 2007). All cultivars showed a significant reduction of tuber yield in plants infected with *R. solani* alone, but a further significant reduction was detected when *R. solani* and *P. penetrans* (500 nematodes L^{-1} soil) were added together in the cultivars Asterix, Nadine and Maris Piper. Maris Piper was the only cultivar presenting a reduction in yield also with treatments at lower nematode densities (100 nematodes L^{-1} soil) and *R. solani*, indicating that this was the most susceptible to both pathogens. Björsell *et al.* (2017) reported a spatial distribution of root-lesion nematodes (*Pratylenchus* spp.), stubby-root nematodes (Trichodoridae) and potato cyst nematodes (*Globodera* spp.) together with the incidence of stem canker caused by *R. solani* in potato fields in Sweden. A spatial correlation between stem canker symptoms and stubby-root nematodes as well as potato cyst nematodes was detected, but this was not the case for root-lesion nematodes. This lack of interaction of *R. solani* with *Pratylenchus* spp. was confirmed also by Viketoft *et al.* (2017). In this study, a glasshouse experiment with six potato cultivars (Erika, Fontane, King Edward VII, Kuras, Perlo and Rosagold) was performed to study the interaction of *R. solani* and plant parasitic nematodes, dominated mainly by *P. crenatus* and *P. neglectus*. There were no differences between plants inoculated with nematodes and *R. solani* or either pathogen alone. The authors did not use pure populations of root-lesion nematodes, but a full nematode community where *P. crenatus* and *P. neglectus* were dominant. In this study, the nematode treatments reduced the yield of Kuras and Perlo tubers more than the untreated control, fungus treatment and the combination of nematode and fungus. On the other hand, a higher concentration of nematodes was recovered in the treatments with both pathogens, suggesting that nematode reproduction was positively influenced by the presence of the fungus.

In summary, few studies have been conducted to study the interaction between root-lesion nematodes and *R. solani* and it is still unclear if a synergistic interaction occurs or not. Therefore, further work is required to to understand how root lesion nematodes and *R. solani* enhance (or not) potato disease.

1.6 MANAGEMENT OF ROOT-LESION NEMATODES ON POTATO CROPS

The term “management” refers to all measures adopted to avoid the introduction of a pest in a field or its limitation under the damage thresholds. Nematodes can be spread due to human activities with movements of infected soils through the equipment, irrigation water or infected plant material, so it is important to prevent any of these activities adopting specific practices in the field (Viaene *et al.*, 2013). Like many other PPN, *Pratylenchus* spp. are difficult to control once introduced into previously uninfested land. The history of the crop, nematode diagnosis and soil types are important factors for determining the management practices in a specific field. A correct diagnosis of nematodes in the field is fundamental for planning the strategies to choose. Over the last 10 years, several nematicides have been revoked due to environmental concerns e.g. Vydate (oxamyl) revoked on the 24th December 2020. In response, alternative methods to control plant-parasitic nematodes have been investigated, developed and applied. Different measures can be adopted to minimise damage caused by *Pratylenchus* spp. that include different cultural practices, chemical and biological control. The most suitable strategy is focused on the reduction of the initial nematode population density and to avoid their reproduction during the growing season.

The history of the crop, species of root-lesion nematode, and soil texture are important factors to consider in the selection of management strategies for a specific field (Duncan and Moens, 2013). Physical and chemical management of soil, crop rotation, organic amendments, cover crops, biofumigation, elimination of weeds in the harvest, and off season are examples of important cultural practices for the control of root-lesion nematodes (Castillo and Vovlas, 2007). The following sections provide greater detail on each type of nematode management.

1.6.1 Resistance and Tolerance

The use of resistant crops has been widely applied for pest management for different crops in the place of chemical, biological and cultural practices. Resistance to nematodes has been investigated for many years on different nematode-host interactions, however the molecular mechanism of plant resistance is still not fully understood.

The first physical barrier of plants is the plant cell wall, which is a complex of polysaccharides and proteins. Nematodes, with their stylet and secretions of cell wall degrading enzymes, can overcome this barrier and migrate within the roots. Cell wall degrading enzymes include cellulase, pectase lyases, xylanases, polygalacturonases, arabinases, arabinogalactan galactosidases and expansins. After nematode invasion and migration, plants may respond with various defence responses like upregulation or downregulation of specific genes or pathways. The plant immune system is characterized basically by two layers of responses: pattern-triggered immunity (PTI), also known as basal defence, and effector-triggered immunity (ETI). Plants recognize pathogen molecules using cell surface pattern recognition receptors (PRRs) and PTI. However, nematodes can also produce effector proteins to suppress the basal defence of the plant, thus plants may activate a secondary defence, called ETI, that is often mediated by a hypersensitive response in the tissue where the nematodes are trying to feed (Gheysen and Jones, 2013).

A plant is considered “resistant” when it can suppress nematode feeding and reproduction thus causing a reduction in population density. In contrast, a plant is considered “susceptible” when nematode feeding and reproduction is unhindered. Whereas “tolerance” is the ability of a crop plant to compensate for pathogen damage and still produce a good yield (Starr *et al.*, 2013). Resistance can be a good strategy to use for improving the yield of field crops when nematodes exceed damage thresholds. The use of resistant cultivars and crop rotation play an important role in reducing nematode densities in the field. Some crops, however, may be only partially resistant. Plants can have different degrees of tolerance, with some varieties maintaining modest yields even in the presence of nematodes in high densities, while other varieties are sensitive to low densities of the nematode. Moreover, the tolerance limits may vary depending on host plant (or cultivar), nematode species and other edaphic factors. Resistant cultivars have the potential to improve the yield of the crop whilst limiting multiplication of nematode population densities (Starr *et al.*, 2013).

In the case of *Pratylenchus* species, there are some crops plants that are damaged by low population densities and others that present little, if any, symptoms in the presence of high popultation densities. Unfortunately, germplasm with resistance to root-lesion nematodes is not widely available, although resistant cultivars have been developed in crops like strawsberries, raspberries, potato, banana and different cereals such as wheat, oat and corn (Castillo and Volvas, 2007; Jones and Fosu-Nyarko, 2014). So far, there are no single dominant resistant genes found in any host plant against root-lesion nematodes. Only a few studies in wheat (Williams *et al.*, 2002) and barley (Sharma *et al.*, 2011) have identified loci linked to resistance to *Pratylenchus*. Williams *et al.* (2002) have identified a locus called Rlnn1, to be responsible for the resistance of Australian wheat cultivars against *P. neglectus*.

However, information about the mechanisms and genes involved in host resistance to root lesion nematodes are very limited, and there is still more to investigate.

In the case of potatoes, Brodie and Plaisted (1993) studied the resistance of different potato clones to *P. penetrans*, and most clones that were less susceptible to *P. penetrans*, were also resistant to *G. pallida* and *G. rostochiensis*. This suggests that resistance to *P. penetrans* might be controlled not by a single gene but multiple genes. The cultivar Butte was reported to be resistant to *P. penetrans* and *P. neglectus* (Davis *et al.*, 1992), and the cultivars Peconic and Hundson were considered resistant to *P. penetrans* (Brodie and Plaisted, 1993). However, it seems that the resistance or susceptibility of a cultivar depends also by populations. Indeed, France and Brodie (1995) found that cv. Butte was resistant to one *P. penetrans* population from Cornel but susceptible to a population from Long Island in the US. The same was found for cv. Hudson that was reported as resistant to *P. penetrans* by Dunn (1973) but then as susceptible by Kotcon *et al.* (1987). France and Brodie (1995) tested the same populations of previous studies, demonstrating that after 20 years the population from Cornel (used by Dunn, 1973) did not reproduce on cv. Hudson, whereas a *P. penetrans* population from Long Island reproduced well, confirming that different populations may have different responses to the cultivar being used. However, these differences in host suitability of the same potato cultivars to *P. penetrans* might depend also on the existence of different races of *P. penetrans* or a misidentification of one of the populations tested in the experiments. This variability reported in literature may highlight the importance of a correct diagnosis of *Pratylenchus* species before any further experiments in glasshouse or field.

1.6.2 Cultural Practices:

Crop rotation can be employed for nematode management, but in practice this is difficult to apply for root-lesion nematodes due to their wide host range. For example, oats (*Avena sativa*) and rye (*Secale cereale*), often used in rotation with potatoes, favour multiplication of root-lesion nematodes (Olthof, 1980; Bélair *et al.*, 2002). Bélair *et al.* (2002) reported that *P. penetrans* not only reproduced on rye but also on brown mustard (*Brassica juncea*), soybean (*Glycine max*), Japanese millet (*Echinochloa frumentacea*), oilseed rape (*Brassica napus*), buckwheat (*Fagopyrum esculentum*), white mustard (*Sinapis alba*), foxtail millet (*Setaria italica*), oats, maize (*Zea mays*), bromegrass (*Bromus inermis*), and perennial ryegrass (*Lolium perenne*) under greenhouse conditions. Only forage pearl millet (*Pennisetum glaucum*) was found to decrease the number of *P. penetrans*. In a one-year rotation, forage pearl millet significantly reduced *P. penetrans* populations, increasing the yield of potato by 10% compared to oats (Bélair *et al.*, 2005). Similarly, both forage pearl millet and marigold

(*Tagetes* spp.) reduced *P. penetrans* populations, increasing potato yields (Ball-Coelho *et al.*, 2003). Marigold may act as a non–host or a poor host for plant-parasitic nematodes, and they produce chemical compounds such as alpha-terthienyl that are toxic or inhibit the development of plant-parasitic nematodes.

Cover crops grown as intercrops, such as legumes, brassicas, and grasses, planted after harvest of the main crop, are usually grown through winter until spring when a new main crop is planted. Certain cover crop species, such as marigolds, alfalfa (*Medicago sativa*), redtop (*Agrostis alba*), and red fescue (*Festuca rubra*), can also help to reduce population densities of *Pratylenchus* spp. (Castillo and Vovlas, 2007). For example, marigolds are reported to be effective in the suppression of *P. penetrans* (Reynolds *et al.*, 2000; Alexander and Waldenmaier, 2002; Evenhuis *et al.*, 2004; Pudasaini *et al.*, 2006) and can be used in rotation with potato (Alexander and Waldenmaier, 2002; Pudasaini *et al.*, 2006). Kimpinski *et al.* (2000) reported the ability of different *Tagetes* species (*T. tenuifolia* 'Nemakill' and 'Nemanon', *T. patula* 'Nana', and *T. erecta* 'Crackerjack') to reduce *P. penetrans* reproduction and to enhance tuber yields by about 14% more than other cover crops such as annual ryegrass, red clover, soybean, and meadow fescue. Red clover and soybean cover crops resulted in the highest population densities of root-lesion nematodes and consequently lowest potato tuber yields in rotation with these two crops (Kimpinski *et al.*, 2000). Biofumigation is an alternative practice that consists of the use of brassica plants to limit the reproduction of the soilborne pests. Volatile chemicals, such as isothiocyanates, are released from decomposing *Brassica* tissues to suppress plant-parasitic nematodes. The impact of tissue amendments from several varieties of *Brassica napus, B. oxyrrhina, B. rapa, B. nigra, B. carinata*, and *B. juncea* were found to cause 56%–95% mortality of *P. neglectus* under laboratory conditions (Potter *et al.*, 1999). While total glucosinolate content within root amendments had no significant correlation with *P. neglectus* mortality, the level of 2-phenylethyl glucosinolate was significantly correlated with the suppression of *P. neglectus* (Potter *et al.*, 1999). In a greenhouse and field microplot study, *B. campestris* and *Raphanus sativus* green manures reduced populations of *P. neglectus* by up to 60% when grown prior to planting potato in the USA (Al-Rehiayani and Hafez, 1998). Significant reductions of *P. penetrans* ranging from 66% to 74% were also reported by Yu *et al.* (2007) using *B. juncea* seed meal and bran soil amendments as biofumigants prior to potato, strawberry, and maize planting in the greenhouse. Overall, the use of Brassicaceous crops appears to offer a good method for the management of root-lesion nematodes, but further studies are necessary to evaluate its effectiveness in commercial scale agriculture. For example, in a six-year study reported by Korthals *et al.* (2014), *B. juncea* 'Energy' was not effective in the management of *P. penetrans* and *V. dahliae*, and even increased nematode populations in some instances.

1.6.3 Chemical control

Chemical treatment is still, perhaps, the main crop protection method used by farmers to reduce damage caused by nematode feeding. Root-lesion nematodes can be managed with soil fumigants or non-fumigant nematicides (Table 1.7). Chloropicrin, methyl bromide, dazomet, 1,3-dichloropropene, metam-sodium are alternative fumigant nematicides historically used to treat root-lesion nematodes impacting potato production (Olthof, 1987, 1989; Whitehead, 1998). However, the use of fumigant nematicides globally is restricted due to human health safety, environmental concerns, and their negative effects on non-target organisms (Haydock *et al.*, 2013). Among all fumigants, only metam-sodium and dazomet are currently permitted for use to manage plant parasitic nematodes in Europe (Lainsbury, 2019). Non-fumigant nematicides like oxamyl, ethoprophos, aldicarb, carbofuran, fensulpathion, and disulphoton have been reported to supress *P. penetrans* in potato crops (Bernard and Laughlin, 1976; Kimpinski, 1982; Olthof *et al.*, 1985; Olthof, 1986; Kimpinski and McRae, 1988), but many have also been banned, and only fosthiazate is currently approved in Europe (Lainsbury, 2019).

Table 1.7: Nematicides used to manage root-lesion nematodes. Status under Reg. (EC) No 1107/2009 based on EU Pesticides database (http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public)

Active substance	Chemical Group	State of formulation	Status under Reg. (EC) No 1107/2009
FUMIGANTS			
1,3 – Dichloropropene	Halogenated hydrocarbon	Liquid	Not approved
Dazomet	Methyl isothiocianate liberator	Microgranulate	**Approved**
Metam (incl. -potassium and -sodium)	Methyl isothiocyanate liberator	Liquid Liquid	**Approved**
Methyl bromide	Halogenated hydrocarbon	Gas	Not approved
CARBAMATES			
Aldicarb	Oxime carbamate	Microgranulate	Not approved
Carbofuran	Carbamate	Microgranulate Liquid	Not approved
Oxamyl	Oxime carbamate	Microgranulate Liquid	Not approved
ORGANOPHOSPATES			
Cadusafos	Organophosphorus	Liquid Microgranulate	Not approved
Ethoprophos	Organophosphorus	Microgranulate	Not approved
Fosthiazate	Organophosphorus	Microgranulate	**Approved**
Thionazin	Organophosphorus	Microgranulate	Not approved

1.6.4 Biological control

Biological control is a strategy which involves the use of other living organisms to control a pest species. Although the use of biological control for nematodes on potatoes has excellent potential, it is still not well established or used in field crops (Palomares-Rius *et al.*, 2014; Stirling, 2014). Some organisms have been shown to be effective for the control of plant parasitic nematodes, like for example *Pochonia chlamydosporia* and *Verticillium suchlasporium* that have been shown to be effective against potato cyst nematodes (Palomares-Rius *et al.*, 2014).

Root-lesion nematodes are considered difficult targets for biological control because they mainly inhabit plant roots (Stirling, 2014). Certain "trapping" fungi such as *Arthrobotrys oligospora, Hirsutella rhossiliensis, Monacrosporium ellipsosporum, Verticillium balanoides, Drechmeria coniospora*, and *Nematoctonus* spp. that produce adhesive conidia have been investigated for potential biological control of *P. penetrans*, but only *H. rhossiliensis* has shown to be effective in potatoes (Timper and Brodie, 1993). In the same study, *A. dactyloides, A. aligospora,* and *M. ellipsosporum* were successful in causing mortality in adults and juveniles of *P. penetrans* under *in vitro* conditions. Only *H. rhossiliensis* and *M. ellipsosporum* were capable of causing a reduction of 24%–25% in field conditions, whilst *V. balanoides, D. coniospora*, and *Nematoctonus* spp. were weak or nonpathogenic to *P. penetrans* (Timper and Brodie, 1993). In another study, *H. rhossiliensis* caused a 25% reduction of *P. penetrans* entering potato roots with the suppression of nematode penetration (Timper and Brodie, 1994). There is scant information on the efficacy of bacteria to reduce *Pratylenchus* spp. in potatoes. Sturz and Kimpinski (2004) used an *in vitro* assay to study the effects of different endophytic bacteria isolated from African (*T. erecta*) and French (*T. patula*) marigold on *P. penetrans* mortality. Among 49 species of bacteria recovered from these plants, *Microbacterium esteraromaticum, Pseudomonas chlororaphis, Kocuria varians, K. kristinae,* and *Tsukamurella paurometabola* showed activity against *P. penetrans*, with *M. esteraromaticum* and *K. varians* causing the greatest level of *P. penetrans* mortality. Recently, in a study of five potato farms in Colorado, Castillo *et al.* (2017) reported a correlation between *P. neglectus, Meloidogyne chitwoodi* and rhizosphere bacteria, often present in potato soils. This study showed that farms with the fewest nematodes had greatest densities of *Bacillus* spp., *Arthrobacter* spp., and *Lysobacter* spp., whereas farms with greater abundances of *P. neglectus* and *M. chitwoodi* had a lower abundance of bacteria. This may suggest that some soil bacteria play an important role in suppressing *P. neglectus* in potato growing land.

1.7 CONCLUDING REMARKS

Root-lesion nematodes are frequently overlooked in potatoes, which may lead to an erosion of yield if they are not detected. Morphological and molecular diagnostics are often used to identify root-lesion nematodes, but the lack of expertise for morphological identification and issues with the available molecular methods highlight the need for alternative approaches that provide a quick and reliable diagnosis. Correct diagnosis and quantification are a key component of nematode management. Damage thresholds for *P. penetrans*, *P. neglectus*, and *P. scribneri* affecting potatoes have been reported (Olthof and Potter, 1973; Riedel *et al.*, 1985; Olthof, 1986, 1990), but they can vary according to cultivar and environmental factors, for example, soil texture, temperature, and moisture. Furthermore, species such as *P. brachyurus*, *P. coffeae*, *P. crenatus*, and *P. thornei* have been found in soil associated with potato but there is no information available about their pathogenicity and symptoms on potatoes. Consequently, further work is required on the recognition of clear symptoms caused by different *Pratylenchus* spp. and their impact on the yield of potatoes. Lesions on the roots caused by *Pratylenchus* spp. provide entry points for secondary pathogens such as fungi, resulting in interactions that enhance crop damage. Variability in experimental data suggests that interactions between *Pratylenchus* spp. and fungi are complex. Several factors play important roles in this interaction but there are still knowledge gaps on how multiple species of plant-parasitic nematodes combine to enhance diseases caused by potato pathogens.

Different measures can be adopted to minimize damage caused by *Pratylenchus* spp. including the application of nematicides, and cultural practices such as crop rotation, cover crops, biofumigation, and biological control. Each of these measures has limitations that make decisions on their use challenging. Further, the wide host range of some *Pratylenchus* spp. causes problems in the design of crop rotations, which is further confounded with a lack of available resistant and tolerant potato cultivars. Cultivar resistance offers a potential solution as it could unlock a sustainable solution for root-lesion nematode management in potatoes. However, this is a long-term process to breed and bring to market a resistant cultivar. As with other plant-parasitic nematodes, farmers still rely heavily on nematicides. However, only dazomet, metam-sodium, oxamyl, and fosthiazate are currently available for use against plant-parasitic nematodes, including *Pratylenchus* spp., in Europe.

Increasingly stringent pesticide legislation makes them unreliable in the long term, and nematologists are focusing their attention on the efficacy of alternative methods to reduce the use of nematicides in agricultural systems. Whilst crop rotation with forage pearl millet, cover crops such as *Tagetes* spp., and biofumigation with Brassica crops have been shown to be the most effective methods for reducing root-lesion nematodes on potato, further studies are necessary to evaluate their effectiveness in agriculture. A multifaceted approach combining

different methods may give good long-term field results on the limitation of root-lesion nematodes and this topic certainly needs further studies for a reliable application in agricultural systems.

1.8 AIMS OF PROJECT

Few UK agronomists and crop advisors are aware of the damage and symptoms caused by *Pratylenchus* spp. in England and Scotland, and in general there is little in the way of a management strategy used to combat these pest species. Although the distribution of root-lesion nematodes in the UK is available mainly related to cereals, there is a knowledge gap about the areas of potatoes affected or losses on the potato crops. Thus, it is important to conduct a survey within Great Britain in order to have a clear overview of the *Pratylenchus* species that they might be causing problems in GB arable crops, especially on potato.

The objectives of the present project were:

1. To undertake a survey to determine the distribution and prevalence of *Pratylenchus* spp. in potato growing land in England and Scotland

2. To identify *Pratylenchus* spp. present in England and Scotland and to develop molecular assays for rapid confirmation and quantification

3. To determine pathogenicity and potato damage thresholds for *Pratylenchus* species in different soil types, under controlled conditions

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Chapter 2

2. GENERAL MATERIALS AND METHODS

2.1 MONOXENIC CULTURES OF *PRATYLENCHUS* SPP. IN CARROT DISCS

Initial populations of *P. penetrans, P. crenatus* and *P. thornei* were obtained from established carrot-disc cultures supplied by East Malling Research (UK) and ILVO (Belgium) and then sub-cultured in other carrot discs following the method reported by Speijer and De Waele (1997). A population of *P. neglectus* was found in infested potato roots recovered from a crop in the UK (Shropshire, UK) and then cultured on carrot discs. Monoxenic cultures of *Pratylenchus* spp. were assessed using the method described by Speijer and De Waele (1997).

2.1.1 Solution of 4000 mg L^{-1} Streptomycin Sulfate for sterilizing nematodes

Streptomycin sulphate is a water-soluble antibiotic obtained from *Streptomyces griseus*. Streptomycin sulphate is ideal for creating a sterilized area because it is highly active against bacteria. The solution was prepared under a sterile laminar airflow cabinet, and 0.4 grams were weighted in a small Petri dish, using a balance. Sterile water was added with a syringe, let the salt to dissolve and then transferred into 100 mL bottle through a 0.2 µm filter. The bottle was stored at 4°C.

2.1.2 Preparation of carrot discs

Carrots with fresh leaves (Waitrose & partners) were used and washed with tap water and sterile water. After disinfection of laminar flow hood with industrial methylated spirit, carrots were topped and tailed before being sterilized under flame using a safety Bunsen burner (Fireboy plus, Integra Biosciences). Carrots were peeled from the top to the tip using a sterilised knife, cut into 3 mm thick discs and each disc transferred with sterilized forceps into a Petri dish (35 mm) (Figure 2.1). Petri dishes were sealed with parafilm and incubated at 23°C in the dark.

Figure 2.1: Example of carrot discs prepared for nematode infection

2.1.3 Extraction of nematode from carrot discs

After 2 months from infection, carrots showed signs of like becoming brown on the top of disc and the presence of white spots in the lid of Petri dishes (Figure 2.2 A). Nematodes were then extracted using a Baermann funnel. Nematodes could also be recovered from the lid of Petri dishes (Figure 2.2 B) and they were collected in a 50 mL Falcon tube with tap water. Each carrot disc was then placed in a Baermann funnel as described in Figure 2.3. Nematode were collected from the Baermann funnel after 12 h. In the case that larger quantities nematodes were required, nematodes were extracted from carrots using the Whitehead and Hemming method with trays (described in section 2.2) (Whitehead and Hemming, 1965). In this method, the carrots were cut into 1 cm pieces and left in the tray for 5 days or 1 week to let nematodes emerge from carrots.

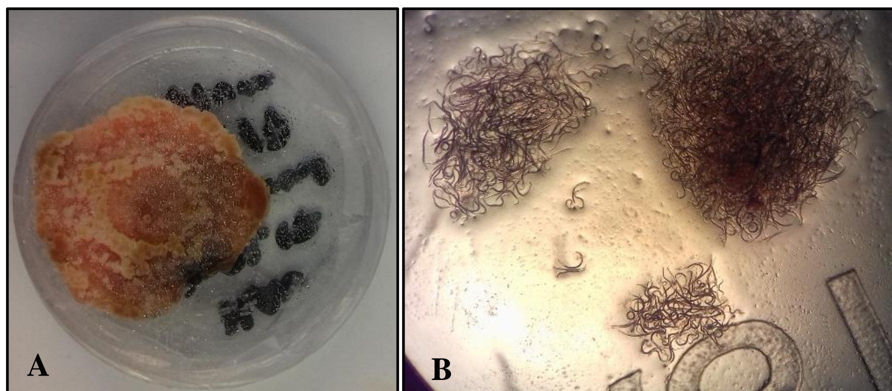

Figure 2.2: (A) Carrots with signs of *Pratylenchus* infection such as dark discolouration on thetop of the disc; (B) root-lesion nematodes in the lid of Petri dish.

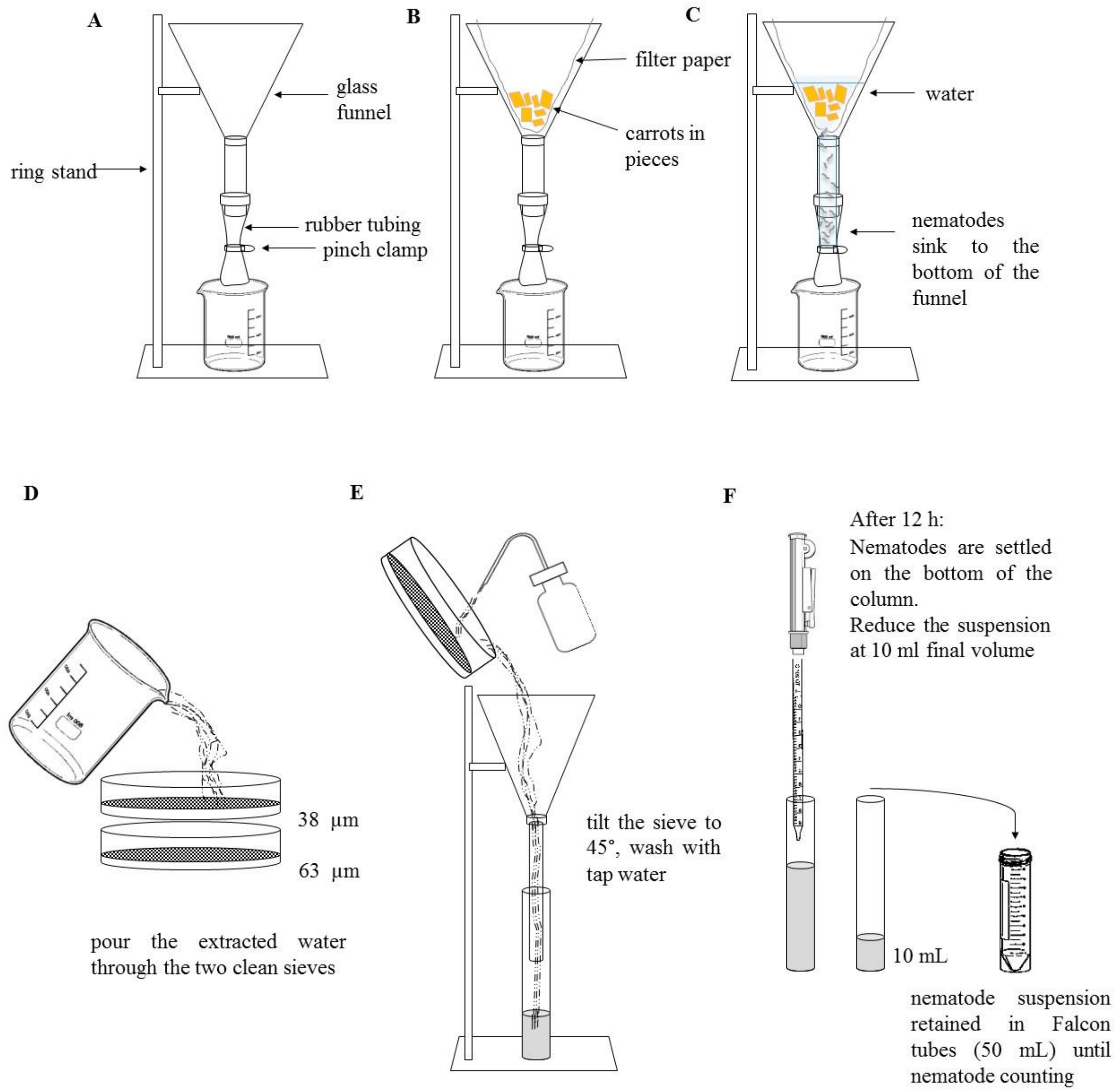

Figure 2.3: Diagram of Baermann method for nematode extractions from carrot discs.

2.1.4 Surface sterilizing of nematodes and infection of new carrot discs

Nematodes were transferred with a glass pipette from a counting dish to a staining block and the same volume of 4000 mg L^{-1} streptomycin sulphate was added to equal the volume of nematode suspension present in the staining block. The staining block was covered and stored overnight at 4°C. The following day, the supernatant above the settled nematodes was removed gently with a glass pipette and sterile water was added, nematodes were let to settle down for 1 h and the supernatant was removed again, and this process was repeated three times. Thirty gravid females were transferred into the cortex of a carrot disc, previously prepared for infection. The Petri dishes were sealed with Parafilm and labelled with the date, species and name of operator. All carrots were stored in plastic boxes, one for each species, and incubated at 23°C. After 2 months, carrots were ready for nematode extractions.

2.2 NEMATODE EXTRACTION FROM SOIL

There are several methods to extract nematode from the soil. It is important to choose the most appropriate method according to the aim of the extraction, time and equipment available, the nematode genus, life stage and features of the soil. The Baermann method (Figure 2.3-2.4) is the most common procedure used to extract active nematodes from soil and plant material. Here, the nematodes move through filter paper and sink to the bottom of the funnel. A method described by Whitehead and Hemming (1965) is widely used as a standard method for extracting free-living nematodes from 200 g of soil samples. The Whitehead and Hemming tray method (Figure 2.5) is a modified Baermann extraction method using a tray instead of a funnel. It is less efficient for non-active or large nematodes such as those belonging to the family Longidoridae, but it is quite a simple method, fast, not laborious, cheap, and good to recover active nematodes. The procedure to prepare the sample requires 5 to 15 min for each sample. Then, the nematode extraction will take 48 h during which nothing else is required from the operator. Collecting the extracts takes 10-15 min for each soil sample. Care must be taken to avoid contamination from the soil in the final suspension. Nematodes attach to any type of debris contamination, so the presence of the soil can cause problems for the identification and counting of nematodes.

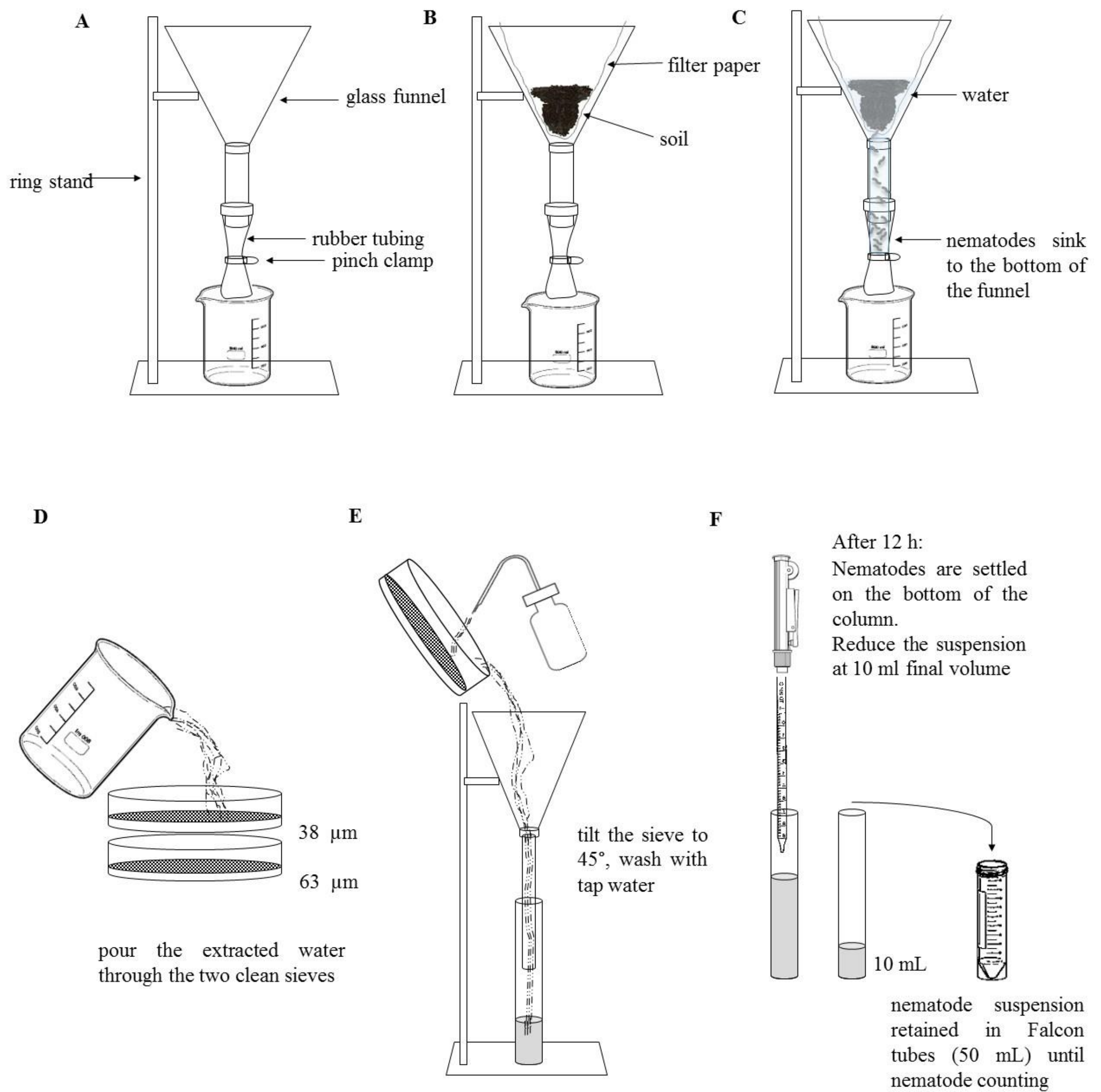

Figure 2.4: Diagram of Baermann method for nematode extractions from soil.

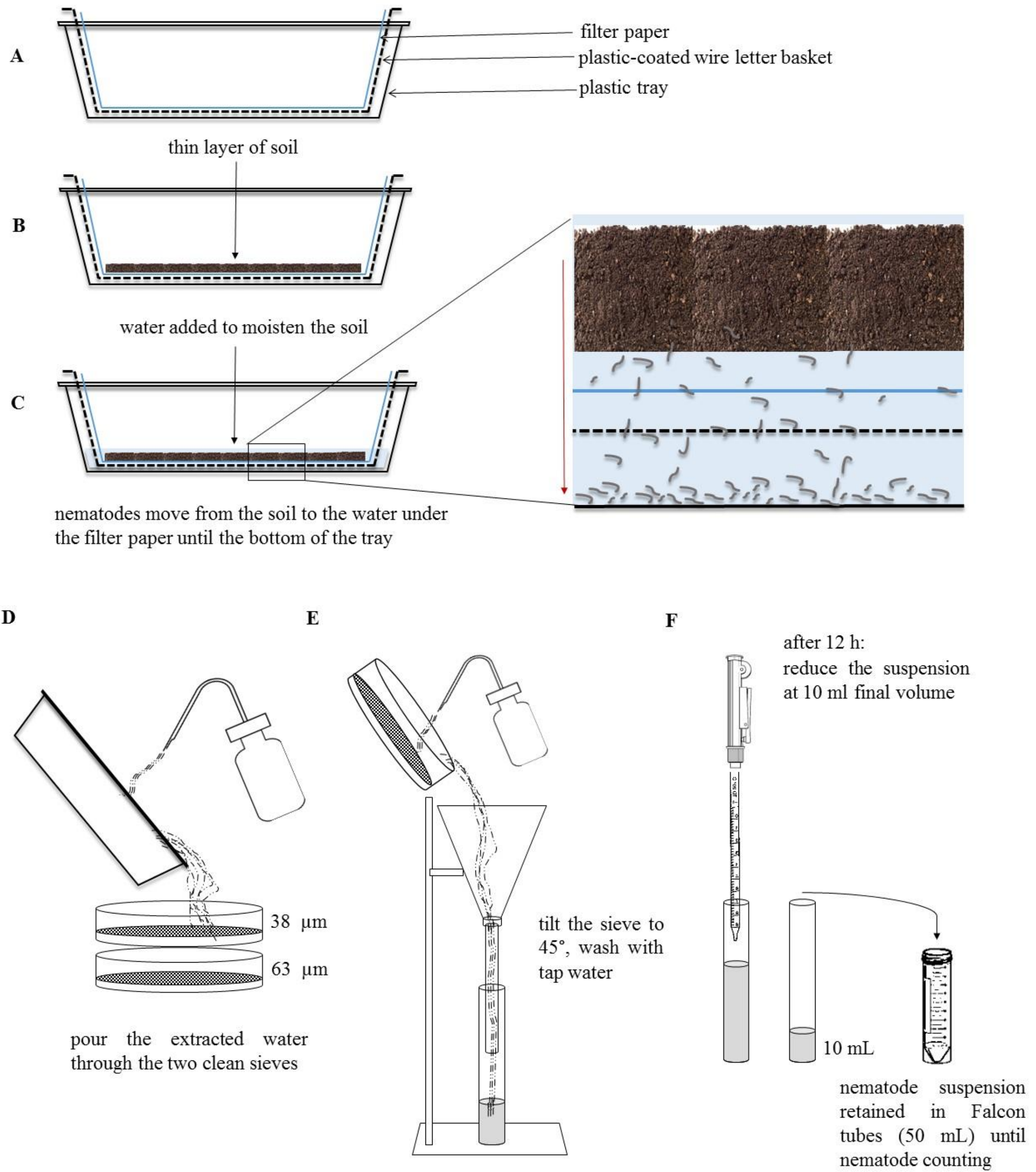

Figure 2.5: Summary of Whitehead and Hemming method for nematode extractions from soil

2.3 NEMATODE QUANTIFICATION AND IDENTIFICATION

Each nematode suspension (10 mL) was resuspended with a 10 mL pipette and a subsample of 1 mL was transferred into a counting dish for quantification. The number of nematodes belonging to the genus *Pratylenchus* was determined using a binocular microscope at 40 X magnification. Nematodes were counted in three subsamples (1 mL) and mean was then calculated. The total number of root-lesion nematodes extracted was then calculated using the following formula:

Total number per sample = Mean number per subsample x total volume of suspension (10 mL)

When *Pratylenchus* spp. were present, five to ten specimens were picked up with a needle and placed in 20 µL of water on a glass slide. A cover glass slip was gently placed in the glass slide and then sealed by nail polish. Each specimen in the slide was examined using a light microscope (Zeiss Axiolab, ZEISS, Germany) under 100 X magnification and identified at species level using taxonomic keys and monographs (Castillo and Vovlas, 2007; Geraert, 2013).

2.4 ROOT STAINING AND NEMATODE QUANTIFICATION

Root staining is an important technique that allows the quantification of nematodes after root invasion. Initially, harvested roots were carefully washed to remove all soil and debris. Samples were stored at -20°C until they were used. All roots were cut into 2 cm lengths to produce a total of 2 g per root. The samples were then placed in small bundles and wrapped in nylon mesh using copper wire before being transferred to 1000 mL thick glass (Pyrex) beaker with 600 mL of boiling acid-fuchsin stain (0.05% solution) for 3.5 min (Figure 2.6 A, B) (Hooper, 1986). Root bundles were then rinsed briefly with 600 mL of tap water and the contents transferred to a laboratory blender adding water sufficient to cover the sample and blended for 30 s (Figure 2.6 -C). The contents of the blending jug were then transferred into a 500 mL beaker and made up to 100 mL of solution with distilled water (Figure 2.6- D). After stirring, a volume aliquot of 2 mL was transferred with a 10 mL pipette into a clean De Grisse counting tray. Nematodes were counted under binocular microscope at 40 X magnification. The following formula was used to calculate the number of nematodes per gram of root:

$$Juveniles\ g^{-1}\ root = \frac{Total\ number\ of\ Juveniles\ in\ 2\ ml\ aliquot\ x\ 100}{Weight\ of\ root\ sample\ (normally\ 2g)}$$

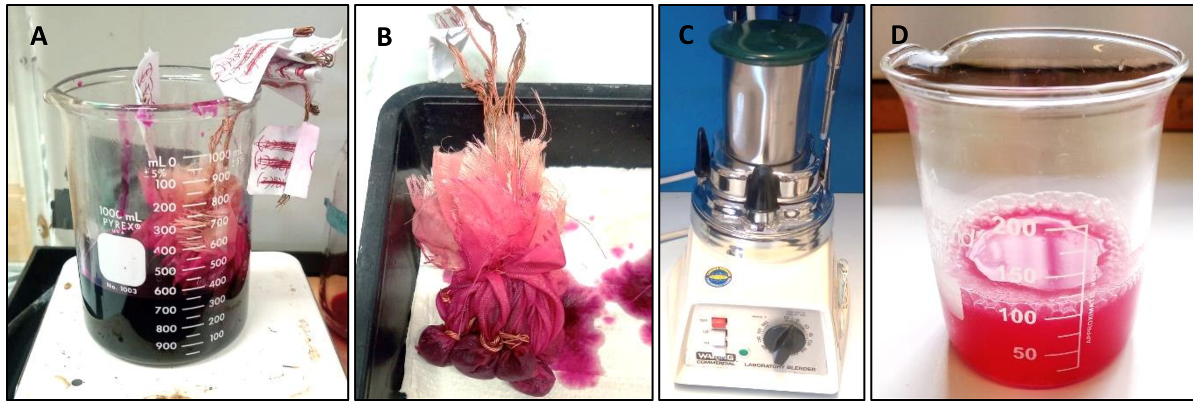

Figure 2.6: Acid-fuchsin stain method. (A) 1000 mL thick glass (Pyrex) beaker with 600 mL of acid-fuchsin stain (0.05% solution); (B) roots in pieces and placed into small bundles wrapped in nylon mesh using copper wire; (C) laborartory blender; (D) 500 mL beaker with blended roots and distilled water up to 100 ml.

2.5 NEMATODE DENSITIES

Different nematode densities (P_i) were prepared for the experiments described in Chapter 6. After nematode extraction from carrot discs, nematodes were counted following the method described in section 2.3. The total volume of suspension was made up to 50 mL on the basis of recovering a high number of nematodes following extraction from carrots. Subsamples of each suspension were taken and assessed under a stereomicroscope in order to reach the desired density, using the following calculation:

Final volume (mL) = Initial Volume / (Initial concentration/ Final concentration)

For example, if the total number of nematodes per suspension was 10,000 nematodes, and a concentration of 5,000 per sample was needed, 25 mL were transferred to a clean Falcon tube (50 mL). In order to have same final volume (10 mL) for all densities prepared before experiments, nematodes were left to settle in Falcon tubes overnight and the supernatant discarded until the final volume was 10 mL.

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Chapter 3

3. COMPARING THE EFFICIENCY OF SIX COMMON METHODS FOR DNA EXTRACTION FROM ROOT-LESION NEMATODES (*PRATYLENCHUS* SPP.)

Chapter modified from: Orlando, V., Edwards, S.G., Prior, T., Roberts, D., Neilson, R. and Back, M. 2020. Comparing the efficiency of six common methods for DNA extraction from root-lesion nematodes (*Pratylenchus* spp.). *Nematology* 23, 415-423.

3.1 ABSTRACT

Robust and accurate identification of root-lesion nematodes (*Pratylenchus* spp.) is an essential step for determining their potential threat to crop yields and, consequently, development of an efficient agronomic management strategy. It is recognised that DNA based techniques provide rapid identification of a range of plant-parasitic nematodes including *Pratylenchus* spp. Efficient and repeatable DNA extraction is central to molecular methodologies. Here, six common DNA extraction protocols were compared to evaluate their efficiency to obtain quality DNA samples for *Pratylenchus penetrans*. Samples with five and ten individuals of *P. penetrans* were successfully extracted and amplified by all extraction methods tested, whereas samples with a single nematode presented challenges for DNA amplification. Among all methods tested, the DNA extraction protocol with glass beads proved to be efficient for *P. penetrans* and all other species tested (*P. crenatus, P. neglectus* and *P. thornei*), generating high quality DNA at comparatively low cost and with a rapid sample throughput.

3.2 INTRODUCTION

Nematodes are the most abundant phyla on earth with plant-parasitic nematodes in a global context typically representing 25-30% of the total nematode community (Van den Hoogen, *et al.*, 2019). Root-lesion nematodes (*Pratylenchus* spp. Filipjev, 1936) are migratory endoparasitic nematodes of several crops with a worldwide distribution (Castillo and Vovlas, 2007; Jones *et al.*, 2013; Orlando *et al.*, 2020a). Correct species diagnosis is central to supporting agronomic management strategies to mitigate the impact of plant-parasitic nematodes on crop yield and quality. Many species of *Pratylenchus* share similarities for some important morphological characters that confound species identification (Castillo and Vovlas, 2007; Geraert, 2013). Further, identification of *Pratylenchus* spp. by microscopy is time consuming and requires well trained taxonomists that are diminishing in number (Coomans, 2000). Several molecular techniques have been developed to assist with identification and to study the intraspecific variability of root-lesion nematodes (Uehara *et al.*, 1998, 2001; Al-Banna *et al.*, 1997, 2004; Waeyenberge *et al.*, 2000, 2009; Subbotin *et al.*, 2008; Yan *et al.*, 2008, 2012, 2013; De Luca *et al.*, 2004, 2011; Oliveira *et al.*, 2017; Mokrini *et al.*, 2013, 2014; Fanelli *et al.*, 2014, 2018; Peetz and Zasada, 2016; Janssen *et al.*, 2017a, b). Many of these diagnostic methods have been summarised and discussed in a recent review by Orlando *et al.* (2020).

Effective molecular diagnostics depend upon efficient and robust extraction of DNA from one or more target individuals. Nematodes can be crushed in a drop of water and the DNA directly amplified by polymerase chain reaction (PCR) (Powers and Harris, 1993), or alternatively, homogenised or cut into several pieces using a small blade or needle. However, to enhance and ensure repeatability of DNA extraction, lysis via proteinase K (Tanha Maafi *et al.*, 2003; Subbotin *et al.*, 2008), or worm lysis buffer (Holterman *et al.*, 2006; Waeyenberge *et al.*, 2000, 2009; De Luca *et al.*, 2011; Peetz and Zasada, 2016) has been considered best practice. Lysis buffers can easily be prepared, and they usually release DNA in 2 to 3 h, providing sufficient and clean DNA without any further DNA purification step. Alkaline lysis with NaOH solution is another common protocol reported for nematode DNA extraction that does not require previous disruption of the nematodes and needs only 15 min at 95 °C for lysis (Stanton *et al.*, 1998; Floyd *et al.*, 2002; Janssen *et al.*, 2016). There are also several chemical treatments used for DNA purification and concentration such as phenol or phenol with chloroform. A simple alternative is the use of commercially available DNA extraction kits; however, they are typically more expensive if there are high numbers of samples to process. The choice of the extraction method depends on the purpose of the study, equipment available and the species targeted.

To our knowledge there are no studies testing the efficiency of DNA extraction methods for *Pratylenchus* species and only a few have reported such data for other genera (Harris *et al.*, 1990; Stanton *et al.*, 1998; Adam *et al.*, 2007). Thus, the choice of DNA extraction method for *Pratylenchus* spp. is not straightforward and may prove problematic for a new laboratory, particularly in optimising DNA extraction from a single individual. The aim of the present work is therefore to compare commonly used methods of DNA extraction to determine the most efficient for extracting DNA from different *Pratylenchus* species and life stages.

3.3 MATERIALS AND METHODS

3.3.1 Nematode population

Initial populations of *P. penetrans*, *P. crenatus* and *P. thornei* were obtained from established carrot-disc cultures supplied by East Malling Research (UK) and ILVO (Belgium) and sub-cultured on other carrot discs following the method reported by Speijer and De Waele (1997). A population of *P. neglectus* was recovered from infested potato roots (Shropshire, UK) and also cultured on carrot discs. Nematodes were extracted from infested carrot discs using a Baermann modified method (Whitehead and Hemming, 1965) with individual nematodes handpicked using a sterile needle under a stereomicroscope (Mazurek Optical Service, Meiji EMT) and transferred to sterile Eppendorf (500 µl) tubes for DNA extraction.

3.3.2 Tests for comparison of six DNA extraction methods

Four tests were performed to compare six different methods of DNA extraction. For each test, lysis was assessed by the success of ITS rRNA sequence amplification of *Pratylenchus* spp. DNA extracts.

Test 1. DNA of one, five and ten females of *P. penetrans* was extracted, in three replicates for each method. This comparison was used to determine the most suitable method for DNA extraction.

Test 2. DNA of one juvenile, one female and one male of *P. penetrans* was extracted, in three replicates for each method. This test aimed to identify any differences between DNA extraction methods among life stages.

Test 3. The most consistent lysis method showing the greatest DNA amplification success rate from Tests 1 and 2, was selected and used for DNA extraction and amplification of one, five and ten specimens of *P. crenatus, P. neglectus, P. penetrans* and *P. thornei* with 3 replications. This test aimed to detect differences of DNA extraction and amplification between species.

Test 4. The most consistent lysis method from Tests 1 and 2 was selected and used for DNA extraction and amplification from one juvenile and one female of *P. crenatus, P. neglectus, P. penetrans* and *P. thornei* with 3 replications. This test aimed to assess whether differences in DNA extraction existed among life stages and species.

3.3.3 DNA extraction methods

Six methods of DNA extraction were tested for their ability to lyse individuals of four target *Pratylenchus* species:

(A) Manual cutting of nematodes under a binocular microscope based on a modification of the method described by Tanha Maafi *et al.* (2003). One, five and ten specimens, depending upon the test, were handpicked under a stereomicroscope (Mazurek Optical Service, Meiji EMT) at 40X magnification using a sterile needle and then placed into 20 µl PCR water, previously pipetted onto a glass slide. Each nematode was cut into 4-5 pieces using a scalpel before the contents transferred with a pipette into an Eppendorf (500 µl) tube. Two µl of PCR 10X Buffer (GoTaq, Promega, UK), 3 µl proteinase K (600 µg ml^{-1}) and 5 µl PCR water were added to the tube and incubated at 65 °C for 1h and 95 °C for 15 min. Samples were allowed to cool before being centrifuged at 16,000 *g* (Heraeus Pico 17 Ventilated Microcentrifuge, Thermo Fisher Scientific).

(B) Heating and freezing before lysis extraction based on a method adapted from Williams *et al.* (1992). Whole nematodes (one, five or ten) were placed into an Eppendorf tube with 20 µl PCR water. Tubes were incubated at 95 °C for 15 min and stored at -80 °C overnight. Samples were thawed before 2 µl of PCR 10X Buffer (GoTaq, Promega, UK), 3 µl proteinase K (600 µg ml^{-1}) and 5 µl PCR water were added to each tube. Samples were incubated at 65 °C for 1h and 95 °C for 15 min and cooled before being centrifuged at 16,000 *g*.

(C) Utilisation of glass beads to cause mechanical disruption of nematodes, adapted from Jesus *et al.*, (2016). Each specimen was handpicked using a needle and placed into a tube with 20 µl of 10X PCR buffer (GoTaq, Promega, UK). Three 1 mm glass beads (Thermo Fisher Scientific) were added into each tube and homogenised using a Retsch M300 tissue disruptor (Retsch, Germany) for 30 s at 30 Hz. Thereafter, 4 µl of proteinase K (100 µg ml^{-1}) and 1 µl of 10X PCR buffer (GoTaq, Promega, UK) were added to each tube. Samples were incubated at 60 °C for 1h, 95 °C for 15 min and 10 °C for 10 min. After DNA extraction, tubes were centrifuged at 16,000 *g*.

(D) Lysis of nematodes using Worm Lysis Buffer (WLB) based on a method modified from Holterman *et al.*, (2006). Whole nematodes (one, five or ten) were placed into a tube with 10 µl WLB (0.2 M NaCl, 0.2 M Tris-HCl pH 8.0, 0.1 M dithiotreitol) and 2 µl proteinase K (800 µg ml^{-1}). The mixture was incubated at 65 °C for 1h and 95 °C for 15 min before being cooled and centrifuged at 16,000 *g*. Finally, 18 µl of PCR water was added to the tube.

(E) DNA extraction using NaOH (0.05 M), adapted from the method reported by Janssen *et al.*, (2016). Whole nematodes (one, five or ten) were handpicked with a needle and

transferred to Eppendorf tubes (500 µL) with 10 µL NaOH (0.05 M) before 1 µL Tween 20 (4.5 %) was added. Samples were incubated at 95 °C for 15 min, and then allowed to cool down. Tubes were centrifuged at 16,000 *g* and 19 µL of PCR water was added.

(F) DNA extraction using a PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific). As previously, individual nematodes were handpicked and transferred into an Eppendorf tube (2 mL) with 40 µl of PCR water. All steps were performed according to the instructions listed by the manufacturer, with DNA eluted in 40 µL genomic elution buffer (10 mM Tris-HCl, pH 9.0, 0.1 mM EDTA).

3.3.4 DNA Amplification and detection of PCR products

The molecular target for DNA amplification was ITS rRNA, using the universal primers VRAIN2F (CTT TGT ACA CAC CGC CCG TCG CT) and VRAIN2R (TTT CAC TCG CCG TTA CTA AGG GAA TC) (Vrain *et al.*, 1992). Each PCR reaction contained: 5X PCR MyTaq Red Reaction Buffer (Bioline, UK), 0.4 mM of each primer, 0.5 µL of MyTaq Red DNA Polymerase (Bioline, UK), 2 µl of DNA sample and double sterile water for a total volume of 15 µl for each PCR reaction. PCR conditions were: denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. A final extension was performed at 72 °C for 5 min. PCR products were separated and visualised on a 1 % agarose gel using 6X GelRed loading buffer (Biotium).

3.3.6 *Comparison of qPCR amplification from different life stages of* P. penetrans *obtained by different DNA extraction methods*

The DNA Samples extracted using six different protocols were used for q PCR method to detect differeces on the amplification of the molecular target. A Biorad CFX96 Touch Real-Time PCR Detection System (Biorad) was used. Each reaction contained: 10 µl SensiFast Probe Hi-Rox Mix (Bioline Reagents), 0.25 µM of probe, 0.6 µM of primers, distilled water calculated to arrive at 18 µL as final volume. Then, 2 µl of DNA template were added to each reaction. The amplification conditions were: 95˚C for 3 min followed by 35 cycles at 95°C for 10 sec with 69°C for 60 sec.

3.3.5 Statistical analysis

DNA amplification data were expressed as the percentage of successful PCR amplification. A two-way ANOVA with Bonferroni's test ($P < 0.05$) was used for Test 1, whereas a Pearson Chi-squared test was carried out for Tests 2 - 4. All statistical analyses were performed using Genstat (19th edition, VSN International Ltd, UK).

3.4 RESULTS

3.4.1 DNA extraction and amplification of P. penetrans

Six methods of DNA extraction were tested with increasing numbers (one, five and ten females) (Figure 3.1) and different life stages (Figure 3.2) of *P. penetrans.* It took ca.15 minutes of microscope work to prepare a set of five samples for DNA extraction Methods B-F. Method A, however, required ca.30 min for picking and cutting of nematode specimens. Overall, the estimated total time for each method was: 2h for Method A, 24 h for Method B, 1h and 40 min for Method C, 1h and 30 min for method D, 30 min for Method E and 3h for Method F.

Assessments of the DNA quality were made by PCR amplification of ITS rRNA sequences. DNA extractions of five and ten nematodes were successful for all methods with 100 % DNA amplification, apart from DNA extracted with the commercial kit (Method F), which had a lower efficiency compared to the other methods. Overall, DNA amplification for one nematode was significantly lower ($P < 0.001$, df = 34, %CV = 34.9) than amplifications for five and ten nematodes. Moreover, comparing each extraction method for increasing nematode abundance, only Method B had a significantly lower ($P = 0.012$) success rate for DNA extraction from a single nematode. Considering the data on individual nematodes, Method A was the most successful with 100% DNA amplification. Method C, using glass beads, was reasonably successful in amplifying the DNA from a single individual, and was faster than Method A.

Except for Method B, all extraction methods resulted in successful amplification of DNA for individual nematodes (Figure 3.1). There were no significant differences among life stages ($P = 0.374$, $\chi^2 = 1.97$, d.f. = 2) (Figure 3.2). Whereas significant differences ($P < 0.001$, $\chi^2 = 24.92$, d.f. = 5) were observed between different DNA extraction methods (Figure 3.2). Method B did not yield DNA amplification for any life stage, whereas Method D, with WLB, had lower DNA amplification efficiency for males and females, and no amplification for juveniles. Method E, with NaOH, yielded DNA amplification only for females but with low efficiency. Method A, C and F were the most successful for DNA extraction from all life stages.

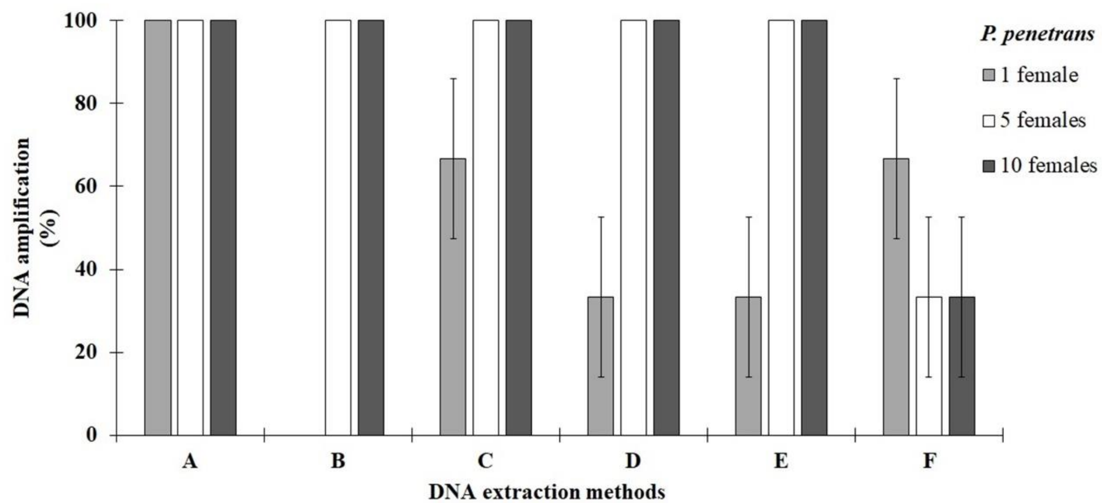

Figure 3.1: DNA amplification (%) of one (n=3), five (n=3) and ten (n=3) *P. penetrans* females using six DNA extraction methods: (A) manual cut of nematode; (B) heating and freezing; (C) glass beads; (D) Worm lysis buffer; (E) NaOH; (F) PureLink DNA extraction kit. Error bars show the standard error of the mean.

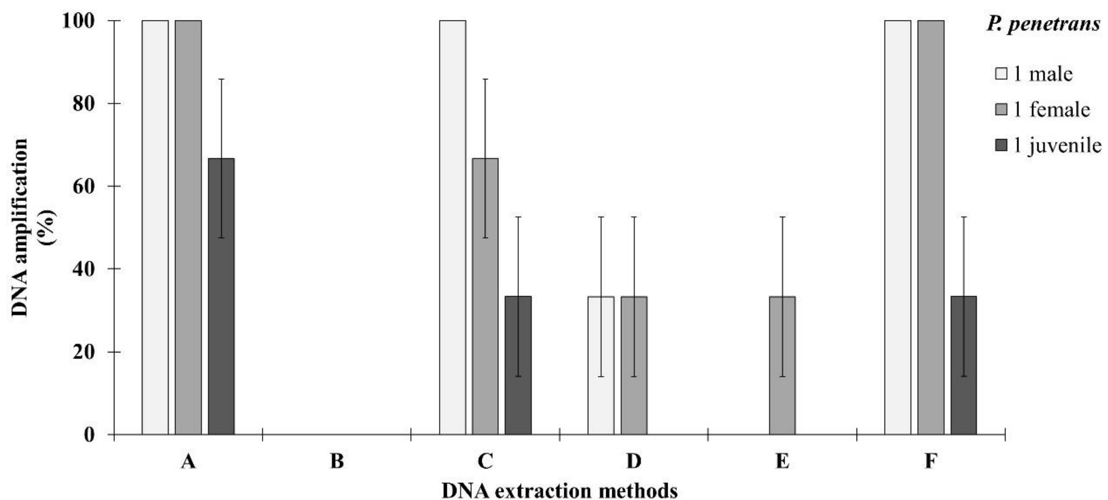

Figure 3.2: DNA amplification (%) of one male (n=3), one female (n=3) and one juvenile (n=3) of *P. penetrans* using six DNA extraction methods: (A) manual cut of nematode; (B) heating and freezing; (C) glass beads; (D) Worm lysis buffer; (E) NaOH; (F) PureLink DNA extraction kit. Error bars show the standard error of the mean.

3.4.2 DNA extraction and amplification of P. crenatus, P. neglectus, P. penetrans *and* P. thornei

DNA was extracted with Method C from four species of root-lesion nematodes (*P. crenatus, P. neglectus, P. penetrans* and *P. thornei*) with increasing number of individuals per sample (Figure 3.3) and different life stages (Figure 3.4). When DNA was extracted from one, five and ten individuals, there were no significant differences among different species (P = 0.942, χ^2 = 0.39, d.f. = 3) (Figure 3.3). Extraction using one female resulted in 100 % amplification of *P. crenatus* replicates and 66.6 % amplification of *P. neglectus, P. thornei* and *P. penetrans*. Similarly, considering single juveniles and females (Figure 3.4), Method C did not show significant differences between species (P = 0.528, χ^2 = 2.22, d.f. = 3), or among life stages (P = 0.178, χ^2 = 1.82, d.f. = 1).

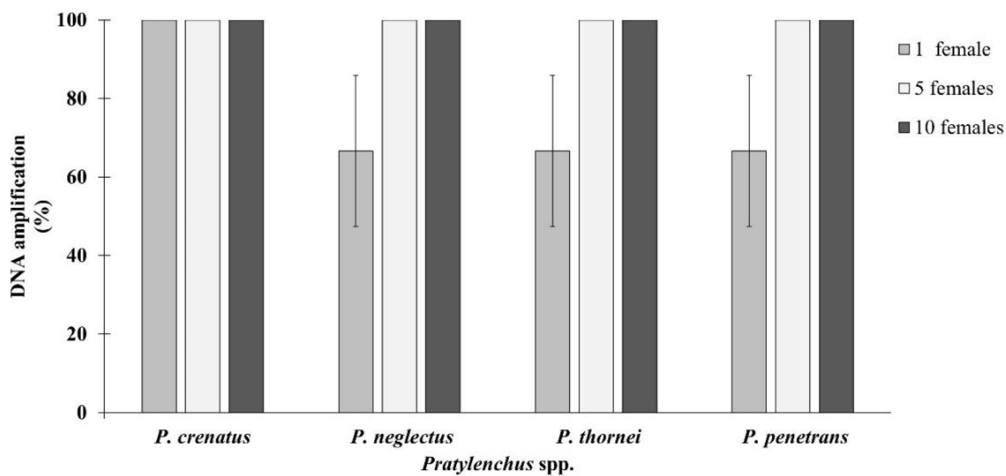

Figure 3.3: DNA amplification (%) of one female (n=3), five females (n=3) and ten females (n=3) of *P. crenatus, P. neglectus, P. thornei* and *P. penetrans* using a glass bead DNA extraction method (Method C). Error bars show the standard error of the mean.

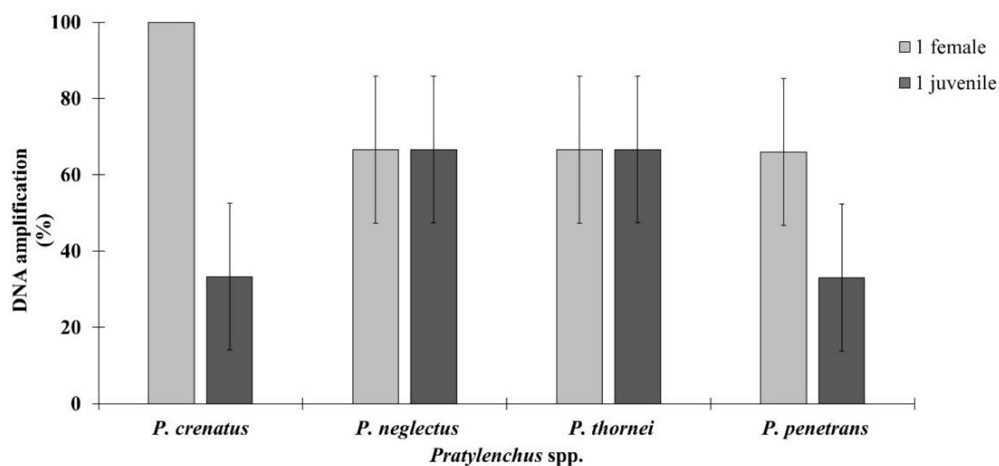

Figure 3.4: DNA amplification (%) of one female (n=3) and one juvenile (n=3) of *P. crenatus, P. neglectus, P. thornei* and *P. penetrans* using a glass bead extraction method (Method C). Error bars show the standard error of the mean.

3.5 DISCUSSION

DNA extraction is an important step for molecular identification of nematodes. Several protocols for nematode DNA extraction have been published. Depending on the purpose of the study, DNA extraction can be performed on a single specimen or from the whole suspension extracted from soil or roots. There are also protocols for direct DNA extraction from soil or infested roots. Each method can have an impact on yield and purity of DNA, influencing DNA amplification and further molecular analysis.

Six common protocols were tested for their efficiency of DNA extraction and amplification of *P. penetrans*. Methods that used manual cutting of the nematodes (Method A) and the use of glass beads (Method C) were the most efficient for extracting DNA from a single nematode. Thus, mechanical disruption of the cuticle and body of the nematode appears to be an important step to achieve successful and consistent DNA amplification. Method C was slightly faster (1h 40 min) than Method A (2h) and less laborious because it did not require the step of manual cutting, which can be time-consuming and impractical with either large numbers of nematodes per sample or many samples to process. The method involving heating and freezing before lysis (Method B) did not generate successful amplification for samples with one individual, possibly due to less disruption of the cuticle and cell membranes and subsequently less DNA released. Moreover, it required more time (24h) to complete the procedure respect to the other protocols. Method with WLB (Method D) required a total time of 1h and 30 min to complete the protocol but was less efficient for individual nematodes compared to Methods A, C and F. Despite being the quickest to perform, the protocol with NaOH (Method E) had low efficiency and only resulted in DNA amplification from females. Lastly, the commercial kit (Method F) was relatively quick to perform (ca.3h) but had lower efficiency with DNA extraction.

In this study, DNA extraction methods did not include a DNA purification step and crude DNA extracts were directly used for PCR amplification. Before performing any molecular assays, it is important to remember that many compounds used for DNA extraction can inhibit DNA amplification, in addition to the inhibitors present in soil (Schrader *et al.*, 2012). As a consequence of PCR inhibition, the sensitivity of any molecular assay will be decreased (Roberts *et al.*, 2016). The failure of PCR amplification for some methods tested, like extractions with NaOH or WLB buffers, could have been caused by PCR inhibitors within the buffers such as Tween 20, dithiothreitol or proteinase K. Some PCR inhibitors may degrade DNA samples or disrupt the annealing of the primers to DNA templates, whereas others can directly degrade the DNA polymerase or inhibit its activity. Chemicals such as Nonidet P-40, Tween 20, EDTA, dithiothreitol, dimethyl sulphoxide or mercaptoethanol may be necessary for efficient cell lysis but, at high concentrations, they can cause PCR inhibition (Schrader *et al.*, 2012).

There are several DNA extraction and purification methods and commercial kits available that have been tested for individual nematodes and nematode communities. However, the efficiency of DNA extraction may vary between commercial kits depending on the buffers and the matrix used (Schrader *et al.*, 2012). Donn *et al.* (2008) compared five different extraction methods including three commercial kits for nematode communities. DNA extraction with phenol chloroform purification and a Purelink PCR purification kit were the most efficient methods yielding consistently high-quality DNA templates (Donn *et al.*, 2008). While NaOH extractions gave the highest yields as measured by absorbance, they were not amplified by PCR. The authors suggested the possibility of protein contaminations leading to the high recorded values for absorbance. Also, Waeyenberge *et al.* (2019) showed the variation of DNA extraction efficiency on nematode species richness comparing fifteen extraction methods, including commercial kits from different companies. In their study, pre-treatment in liquid nitrogen followed by Qiagen method was the most successful with greatest DNA yield. Similarly, four DNA extraction protocols (chelex, worm lysis buffer Method, Holterman lysis buffer Method and FastDNA kit) were tested to compare the efficiency of DNA extraction and amplification of *Meloidogyne javanica* (Carvalho *et al.*, 2019). Extraction with the FastDNA provided low DNA concentration and failure on PCR amplification, whereas the WLB method was the most efficient for extracting DNA, confirming that efficiency varied among different methods (Carvalho *et al.*, 2019). In our results, DNA extracted with Purelink commercial kit (Method F) presented a low efficiency for five and ten individuals of *P. penetrans* and a relatively greater efficiency than the other methods for one individual.

Few studies have assessed DNA extraction methods for plant-parasitic nematodes, and those that have mostly focus on *Meloidogyne* spp. (Harris *et al.*, 1990; Stanton *et al.*, 1998; Adam *et al.*, 2007; Carvalho *et al.*, 2019). Adam *et al.* (2007) used a combination of worm lysis buffer and manual cutting of single second-stage juvenile of *Meloidogyne* spp. and PCR amplification products were obtained from 95 % of the extracts. Harris *et al.* (1990) reported a comparison of different lysis protocols on juveniles and eggs of *Meloidogyne incognita, M. hapla, M. javanica*, and *M. arenaria*. These authors included methods such as squashing the specimen with a micropipette tip, a proteinase K method and freezing and thawing protocol. However, only methods that included manual disruption of individuals provided consistent DNA amplification (50 %), whereas the other methods were less efficient. Furthermore, a lysis method using NaOH (24 h) showed consistent results with 81 % amplification for *Meloidogyne* juveniles, whilst squashing of the nematodes resulted in 50 % amplification and a proteinase K protocol, without nematode squashing gave 20% amplification efficiency (Stanton *et al.*, 1998). In our study, the NaOH protocol had low efficiency and only resulted in DNA amplification from *P. penetrans* females.

Our results showed that the six DNA extraction methods did not differ regarding the amplification of DNA extracted from five or ten *P. penetrans* adults. In contrast, successful DNA extraction from one individual was dependent upon the method used. *P. penetrans* DNA was successfully amplified

by PCR for all methods tested, with the exception of Method B where amplification for one single nematode was unsuccessful. Manually cutting nematodes (Method A) was the most successful method but it is laborious and time-consuming. In contrast, Method C, using glass beads, was easy to use and effective for successful PCR amplifications. The glass beads mechanically disrupt cells facilitating DNA extraction and provide a simple, rapid and relatively affordable extraction method that favours DNA extraction from single nematodes. This was the most consistent method among different life stages, increasing numbers of specimens, and species of *Pratylenchus* tested (*P. penetrans*, *P. crenatus, P. neglectus* and *P. thornei*).

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Chapter 4

4. DEVELOPMENT AND VALIDATION OF FOUR TAQMAN QPCR METHODS FOR THE IDENTIFICATION AND QUANTIFICATION OF *PRATYLENCHUS CRENATUS*, *P. NEGLECTUS*, *P. PENETRANS* AND *P. THORNEI*

4.1 ABSTRACT

Pratylenchus crenatus, *P. neglectus, P. penetrans* and *P. thornei* are globally the most common species of root-lesion nematodes (*Pratylenchus* spp.). Correct identification and quantification of these nematodes is important for strategic management such as rotation choice and nematicide use. A real-time quantitative PCR can provide a fast and reliable alternative to morphological identification which requires significant taxonomic experience. A TaqMan hydrolysis probe method based on the 28S rDNA D2-D3 expansion region was developed and validated for identification and quantification of these four species of root-lesion nematodes. A set of two primers and one TaqMan probe were designed for each target species. Four standard curves were made by plotting known gene copy number, obtained by a 10-fold serial dilution of purified plasmids, with the corresponding Ct values. The range was from 10^8 to 10 DNA copy number, with 10 copy number as lowest measurable standard. Each standard curve showed a strong linear correlation ($R^2 = 0.99$) between Ct value and DNA copy number. The PCR efficiencies of the standards were between 87-118%, confirming the sensitivity of each diagnostics. Consistent amplifications for samples with target species from different locations were detected, whereas a lack of amplification was found for non-target species such as *P. coffeae, P. pseudocoffeae, P. vulnu*s, *P. fallax*, *Globodera rostochiensis, Meloidogyne hapla, Trichodorus primitivus* and *Bitylenchus hispaniensis*. Specificity and sensitivity of the method were confirmed by consistent detection and amplification among different life stages and increasing numbers of target species. Methods allowed the detection of one and ten individuals from a target species when combined with up to 30 individuals of non-target species. Fifteen samples from potato fields were used to compare estimated abundance from qPCR with traditional counting by microscopy. A strong relationship ($R^2 = 0.78$) was found between the two approaches, thus confirming the robustness of the methods. In summary, the qPCR TaqMan methods developed in this study provides a highly specific, sensitive, fast and accurate quantification of *P. crenatus, P. neglectus, P. penetrans* and *P. thornei*.

4.2 INTRODUCTION

Root-lesion nematodes (*Pratylenchus* spp.) are migratory endoparasitic nematodes of several crops worldwide, including potato (Castillo and Volvas, 2007; Jones *et al.*, 2013; Orlando *et al.*, 2020). The identification and quantification of *Pratylenchus* is challenging and new methods would improve the diagnosis. Morphological identification using microscopy is time consuming and requires specific training for laboratory staff (Powers *et al.*, 2004; Palomares-Rius *et al.*, 2014). Moreover, morphological identification is not always possible due to similarities among different species and several differences between populations of the same species, and the presence of mixed species in the same soil sample making identification more difficult (Janssen *et al.*, 2017 a, b). An accurate molecular diagnostic can therefore help to overcome these issues and speed up the diagnostic process, especially when many samples need to be processed.

Detection and amplification of nucleic acid are important diagnostic methodologies widely used in nematology. Some analysis requires only qualitative detection but others also quantitative. The choice of methodology for diagnostics is an important step that requires a broad knowledge of the available techniques for the nematode target of interest. Quantitative real-time PCR (qPCR) is a molecular technique for detection and quantification of target organisms based on the increase of fluorescent signals obtained during amplification of target DNA (Subbotin *et al.*, 2021). There are two categories of fluorescent chemistries: DNA-binding dyes, such as SYBR Green I, and dye-labelled probes such as TaqMan, Molecular Beacons and Scorpions. The TaqMan hydrolysis probe is one of the most used chemistries for probe-based real-time qPCR. The TaqMan assay requires a pair of species-specific primers and a probe that contain a fluorescent reporter at 5'-end (e.g. FAM, 6-carboxyfluorescin) and a quencher at the 3'-end (e.g. TAMRA, 6-carboxytetramethylrhodamine). When the probe hybridizes to the specific target DNA between the two primer binding sites, 5' exonuclease activity of thermostable polymerase (Taq or Tth) cleaves off the reporter during the extension phase of the PCR. Then, reporter and quencher are separated from each other and the fluorescence signal is proportional to the quantity of amplified products (Schaad and Frederick, 2002; Roberts *et al.*, 2016; Subbotin *et al.*, 2021). This assay detects only the specific target sequence avoiding fluorescent signals from non-specific products. Although it can be highly specific, there are some limitations such as the possibility of false-positive reactions (fragments from unknown species not yet investigated), or false-negative reactions due to variation occurring between individuals (Schrader *et al.*, 2012). A full validation of each diagnostic is an important step to ensure the methodology is reliable, reproducible, and applicable to other laboratories (EPPO, 2018). Criteria for validation methods are fully described by EPPO (2018) and they mainly include: analytical sensitivity, diagnostic sensitivity, analytical specificity, diagnostic specificity, repeatability and reproducibility.

Several studies have been conducted to develop qPCR protocols for quantitative identification of different species of root-lesion nematodes (Sato *et al.*, 2007, 2010; Mokrini *et al.*, 2013, 2014; Fanelli

et al., 2014; Berry *et al.* 2008; Yan *et al.*, 2008; Goto *et al.*, 2011; Yan *et al.*, 2012, 2013; Oliveira *et al.*, 2016; Bandoo *et al.*, 2017; Dauphinais *et al.*, 2017; Arora *et al.*, 2020; Lin *et al.*, 2020). Currently, there are protocols of real-time PCR for different species such as *P. zeae*, *P. penetrans*, *P. thornei*, *P. neglectus, P. scribneri* and *P. crenatus.* Some methods have been developed using ITS rDNA sequences as target (Sato *et al.*, 2007, 2010; Berry *et al.* 2008; Goto *et al.*, 2011; Yan *et al.*, 2012, 2013; Oliveira *et al.*, 2016; Arora *et al.*, 2019), but it has been demonstrated that this molecular target has a high frequency of intra-specific and intra-individual variability for *Pratylenchus* spp. (De Luca *et al.*, 2004, 2010; Subbotin *et al.*, 2008; Janssen *et al.*, 2017 a, b). This may significantly increase the risk of obtaining false results for some species, with underestimation or overestimation of nematodes compared to traditional approaches, such as microscope counting. Other molecular targets have been investigated for qPCR assays of *Pratylenchus,* such as β-1,4-endogluconase (Mokrini *et al.*, 2013, 2014; Fanelli *et al.*, 2014) and the D2-D3 expansion segments of the large subunit 28S rDNA (Yan *et al.*, 2008; Bandoo *et al.*, 2017; Dauphinais *et al.*, 2017; Lin *et al.*, 2020). The D2-D3 expansion region has been selected as molecular marker for phylogenetic relationships to distinguish closely related species of *Pratylenchus* because it possesses a higher degree of interspecific genetic variability and low intra-specific variation (Al-Banna *et al.*, 1997, 2004; De Luca *et al.*, 2004, 2010; Subbotin *et al.*, 2008; Janssen *et al.*, 2017 a, b). Recently, species-specific primers focused on D2-D3 expansion segment of 28S rDNA were designed by Baidoo *et al.* (2017) and Dauphinais *et al.* (2017) for qPCR assays for the identification and quantification of *P. penetrans.* Also, Lin *et al.* (2020) developed a duplex qPCR assay based on this region for the simultaneous identification and quantification of *P. neglectus* and *P. thornei.*

The aim of this work was to develop and validate a TaqMan qPCR method focusing on the D2-D3 expansion fragment for identification and quantification of *P. penetrans, P. crenatus, P. neglectus* and *P. thornei.*

4.3 MATERIALS AND METHODS

4.3.1 Nematode populations and cultures of Pratylenchus *spp.*

Initial populations of *P. penetrans* (PP), *P. thornei* (PT) and *P. crenatus* (PC) were obtained from established carrot-disk cultures supplied by ILVO (Belgium). *Pratylenchus neglectus* (PN) was extracted from potato soils (Norfolk, UK) and *P. thornei* (PTU) from soil samples in a field of bean at Harper Adams University (UK). Nematodes were extracted from infested carrot discs and soil samples using the modified Baermann method (Whitehead and Hemming, 1965) and then sub-cultured following the method described by Speijer and De Waele (1997) for monoxenic culture of *Pratylenchus* spp. in carrot discs. Nematodes were morphologically identified under a light microscope (Zeiss Axiolab, ZEISS, Germany) at 100 X magnification and using taxonomic keys and

monographs (Castillo and Vovlas, 2007; Geraert, 2013). These species were used for the development of four qPCR methods. Other DNA samples and specimens for the validation of the assays were obtained from other locations (Table 4.1).

Table 4.1: Origin and codes of *Pratylenchus* spp. and other nematodes used in this study

Code	Species	Host/sources	Origin
PC	*Pratylenchus crenatus*	Carrot disc	Belgium
452	*P. crenatus*	DNA extract	Scotland (UK)
Je2	*P. crenatus*	DNA extract	Scotland (UK)
PN	*P. neglectus*	Potato soil	England (UK)
Li03	*P. neglectus*	Potato soil	England (UK)
Li10	*P. neglectus*	Potato soil	England (UK)
491	*P. neglectus*	DNA extract	Scotland (UK)
PP	*P. penetrans*	Carrot disc	Belgium
NO03	*P. penetrans*	Potato soil	England (UK)
Li25	*P. penetrans*	Potato soil	England (UK)
PT	*P. thornei*	Carrot disc	Turkey
PT-U	*P. thornei*	Beans	England (UK)
PCof	*P. coffae*	Carrot disc	Ghana
286	*P. fallax*	DNA extract	The Netherlands
PsCof	*P. pseudocoffae*	Carrot disc	Iran
189	*P. vulnus*	DNA extract	The Netherlands
PV-It	*P. vulnus*	Carrot disc	Italy
90236	*Globodera rostochiensis*	Potato soil	England (UK)
MeH	*Meloidogyne hapla*	Tomato soil	England (UK)
368	*Bitylenchus hispaniensis*	DNA extracts	Scotland (UK)
Trich	*Trichodorus primitivus*	Potato soil	England (UK)

4.3.2 DNA extraction

Single individuals of each *Pratylenchus* species were used for DNA extraction. Each specimen was picked-up with a needle under a stereomicroscope and then transfered into a 200 µl Eppendorf, containing 18 µl of 1X PCR buffer (GoTaq, Promega, UK). Four to five 1 mm glass beads were added into each tube and incubated in a Retsch M300 tissue disruptor for 30 s at 30 Hz. Then, 4 µl of proteinase K (100 µg ml^{-1}) and 1 µl of 1X PCR buffer (GoTaq, Promega, UK) were added to each tube. Tubes were incubated at 60°C for 60 min, 95°C for 15 min and 10°C for 10 min. After DNA

extraction, tubes were centrifuged at 16,000 *g* (Heraeus Pico 17 Ventilated Microcentrifuge, Thermo Fisher Scientific) and stored at −20°C. For samples with more than 10 individuals, DNA was extracted using PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific).

4.3.3 Conventional PCR and direct sequencing

To confirm the microscope-identification of the target nematodes, the D2-D3 expansion fragment of 28S rDNA was amplified using the universal primers D2A (5'-ACA AGT ACC GTG AGG GAA AGT TG-'3) and D3B (5'-TCG GAA GGA ACC AGC TAC TA'3) primers (De Ley *et al.*, 1999) and sequenced. For direct sequencing, each PCR reaction contained: 5X PCR GoTaq Buffer (Promega, UK), 12.5 mM of each dNTP (Promega, UK), 0.4 mM of each primer, 1 unit of GoTaq Polimerase (Promega, UK), 2 µl of DNA and double sterile water for a total volume of 15 µl for each PCR reaction. PCR conditions were: denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. A final extension was performed at 72°C for 5 min. PCR products were separated and visualised with Sybr Safe (Thermofisher) on a 1% agarose gel. ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific) was used for enzymatic clean-up of amplified PCR products according to the manufacturer's instructions and they were incubated at 37°C for 30 min followed by 10 min at 80°C and 5 min at 10°C. PCR products were then sequenced by the James Hutton Institute in-house sequencing service, in one direction using D2A forward primer. A total of 49 sequences (Appendix 8.1) were generated and used for confirmation of species identification.

4.3.4 Conventional PCR and cloning

To generate products for cloning, PCR reactions were prepared with 1X PCR High Fidelity buffer (Invitrogen, UK), 2 mM $MgSO_4$ (Invitrogen, UK), 0.2 mM of each dNTP (Promega, UK), 0.4 mM of each primer, 1 unit of High-Fidelity Platinum Taq polymerase (Invitrogen, UK), 2 µl of DNA and double sterile water for a total volume of 15 µl for each PCR reaction. PCR conditions were: denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 68°C for 1 min. A final extension was performed at 68°C for 5 min. PCR products were purified with gel purification using the QIAquick Gel extraction Kit (Qiagen) according to the protocol given by the manufacturer. Before ligation, additional ATP was added to each purified PCR product to enhance the ligation into the vector: 2 µl GoTaq Buffer (5X), 2 µl dATP (0.2 mM), 1 µl GoTaq Flexi Polymerase, 4 µl purified PCR products and nuclease-free water to a final volume of 10 µl; samples were incubated at 72°C for 15 min. Two µl of purified PCR products were ligated into pGEM-T Easy® vector (Promega, UK) following the manufacturer's protocol. The resulting plasmids were transformed into *Escherichia coli* DH5α competent cells (Invitrogen, UK).

White colonies with recombinant plasmids were selected and DNA purified using QIAprep Spin Miniprep kit (Qiagen, UK) according to the manufacturer's instructions. Purified plasmids were sequenced using universal M13F and M13 primers, in forward and reverse direction by the James Hutton Institute in-house sequencing service. Contigs were assembled using DNA baser sequencing assembler (http://www.dnabaser.com). A total of 72 sequences (Appendix 8.2) were generated and used for confirmation of species identification, for primer/probe design and development of standard curves.

4.3.5 Primers and probe design

The above-generated sequences, along with all sequences of D2-D3 expansion fragment of 28S rDNA for all *Pratylenchus* spp. available in GenBank database (accessed "01-02-2018") were used to generate an alignment of 743 sequences using Clustalw (http://www.genome.jp/tools-bin/clustalw) for the detection of potential conserved regions useful for primers and probe design. Jalview Version 2 was used to visualize variable regions among species (Waterhouse *et al.*, 2009). Four primers/probe sets for each target species were designed using the on-line tool for real time qPCR TaqMan primer design of genscript (https://www.genscript.com/tools/real-time-pcr-tagman-primer-design-tool) and then synthesised by PrimerDesign, Southampton, UK. Probes were labelled at the 5'-end with FAM reporter dye and at the 3'-terminal with TAMRA quencher.

4.3.6 PCR optimisation

A gradient of annealing temperatures from 58°C to 70°C was tested using Biorad CFX96 Touch Real-Time PCR Detection System (Biorad). Combinations of different concentrations of primers (0.3 µM, 0.6 µM and 0.9 µM) and probe (0.25 µM and 0.5 µM) were also tested.

4.3.7 Standard curves

The DNA concentration of each plasmid was measured by spectrophotometer (Beckman Coulter DU 640 spectrophotometer) and used to calculate the corresponding number of copies for each target using the online tool DNA copy number and dilution calculator (http://scienceprimer.com/copy-number-calculator-for-realtime-pcr) based on the sum of length of the p-GEMT vector (3015 bp) and our DNA inserts (~ 780 bp). The DNA copy number for each plasmid was calculated with the following formula reported by Daniell *et al.* (2012):

$$\text{Number of copies} = (\text{amount (ng)} * 6.022\text{x}10^{23}) / (\text{length (bp)} * 1\text{x}10^{9} * 650)$$

A pre-dilution in 10mM Tris (pH 8.0) of each plasmid was carried out to obtain a concentration stock of 1 x 10^{10} copies μl^{-1}. Two microlitres of this pre-dilution stock was linearized by digestion with 2 µl of NotI enzyme (Promega, UK) in 2 µl of buffer and 16 µl sterile UV-treated water and incubated at 37°C for 90 min and at 65°C for 15 min (Daniell *et al.*, 2012), to obtain a final concentration of 1 x 10^9 copies μl^{-1}, for each plasmid. A serial dilution ranging from 10^8 to 10^1 copies μl^{-1} in 10mM Tris (pH 8.0) was prepared in triplicate and used to generate standard curves for real time PCR. These standards were included in all real-time qPCR reactions to allow quantification of the target nematodes in samples.

Efficiency of each standard curve was calculated following the equation:

$$E\ \% = [(10^{(-1/slope)}) - 1] \times 100$$

4.3.7 Real-time PCR

Real-time PCR amplifications were performed initially using a StepOnePlus Real-Time PCR System (Applied Biosystems) for development of methods, then a Biorad CFX96 Touch Real-Time PCR Detection System (Biorad) for the optimization and validation of methods. Each reaction contained: 10 µl SensiFast Probe Hi-Rox Mix (Bioline Reagents), 0.25 µM of probe, 0.6 µM of primers, UV-treated sterile distilled water to give a volume of 18 µL. Where additional primers were required, the same concentration 0.6 µM for each primer was used. Then, 2 µL of template DNA were added to each reaction. The amplification conditions were: 95˚C for 3 min followed by 35 cycles at 95°C for 10 s with 68-69°C for 60 s. Positive controls with plasmids and negative controls with sterilized water were included for each test performed.

4.3.8 Specificity of primers and probe to detect each species target

After optimization of annealing temperature, primer and probe sets were tested for their specificity on the detection of each target species. Eight different *Pratylenchus* spp. were used (*P. crenatus*, *P. penetrans, P. thornei, P. neglectus, P. coffae, P. pseudocoffae, P. vulnus* and *P. fallax*), as well as other non-target nematode genera (*Globodera rostochiensis, Meloidogyne hapla, Trichodorus primitivus* and *Bitylenchus hispaniensis)*. Each sample was tested in three replicates. Reactions and amplification conditions were as described above.

4.3.9 Selectivity and sensitivity of each diagnostic PCR

Three different tests were performed to test the specificity of primers and probe to detect each species (*P. crenatus, P. neglectus*, *P. penetrans* and *P. thornei*) with their own specific primer/probe set, separately.

Test 1. Selectivity of each target species in samples with mixed species was tested. One female of *P. penetrans, P. neglectus, P. crenatus* and *P. thornei* were picked up with a needle and transferred to a 500 µL Eppendorf tube. The same procedure was applied for preparing samples with 10 females of each species in one tube and negative controls without target species. Positive controls were also prepared with one and ten females of pure target species per tube. Five replicates for each sample were prepared for DNA extraction and qPCR.

Test 2. Sensitivity of each diagnostic PCR was tested with different life stages of each target species. Each specimen was picked up with a sterilised needle and transferred individually in 500 µL Eppendorf tube containing 20 µL of 10X PCR buffer for DNA extraction. Ten replicates for each life stage were prepared and qPCR was performed following qPCR protocols for each target species. DNA copy number per life stage was then estimated multiplying DNA copy number obtained for each reaction and the total volume of each sample. Mean and standard error of mean (S.E.M.) were calculated for adults and juveniles. Values of DNA copy number per individual were estimated from the average of DNA copy number among adults and juveniles.

Test 3. Sensitivity of each diagnostic PCR was tested with increasing numbers of *P. penetrans, P. thornei* and *P. crenatus* in separate Eppendorf tubes, in three replicates. For *P. penetrans* one, ten, 100 and 1000 females were prepared in separate tubes, whereas for *P. thornei* one, ten and 1000 and one, ten and 50 nematodes for *P. crenatus*. Due to limited numbers of individuals per culture, only 50 individuals of *P. crenatus* were possible to use for this test. For the same reason, data for *P. neglectus* were not included because only sample with 10 individuals in triplicate was available. Negative controls were included in three replicates using 2 µL distilled water in place of DNA.

4.3.10 Quantification of Pratylenchus *spp. with qPCR from nematode extractions derived from soil samples and comparison with visual counting on microscope*

Soil samples from the survey described in Chapter 5 were used: CO03, CO06, YO05, YO20, Li01, Li11, Li10, Li17, Li20, Li25, HE01, HE08, NO02, NO03, NO05. These samples were ramdomly selected among the positive samples with RLN from microscopy from across the full range of counts. Briefly, the nematode suspensions from 15 samples were first counted using a binocular microscope (Mazurek Optical Service, Meiji EMT) before the contents were transferred to a 2 mL Eppendorf tubes and freeze dried. The DNA was extracted using Purelink DNA (Invitrogen) extraction kit. All four TaqMan PCR assays were performed on each DNA sample. TaqMan PCR estimates were calculated based on the DNA copy number per individual for each species and the total number was the sum of each species present in the sample. Microscope counts were compared with data from qPCR methods developed in the present study.

4.3.11 Statistical analysis

Data of Ct values and DNA copy number for each test were subjected to a two-way ANOVA with Tukey's test (P < 0.05). Data obtained by microscopy and by qPCR were analysed with a linear regression model. All statistical analyses were performed using Genstat (19th edition, VSN International).

4.4 RESULTS

4.4.1 Alignment and primer/probe design for P. penetrans, P. thornei. P. neglectus *and* P. crenatus *diagnostic PCR*

A total of 121 new sequences (Appendix 8.1 and 8.2) were generated and used for an alignment with 638 sequences that were available in GenBank at the date of analysis (February 2018), for a total of 759 sequences. An alignment was performed with all sequences present in GenBank including all new sequences obtained in this study (Appendix 8.3). Based on this alignment, a set of primers and probe was designed for *P. crenatus*, *P. neglectus*, *P. penetrans* and *P. thornei* (Table 4.2).

Fourteen clones and sequences were obtained from four *P. penetrans* individuals. Based on position 1026-1097 of the D2-D3 rDNA sequence, one forward (Pen-F2) covered most sequences of *P. penetrans*, but a further 2 forward primers (Pen-AltF2b and Pen-AltF2c) were included to cover all sequences available for *P. penetrans*. None of the non-target *Pratylenchus* species were covered by the forward primers. The reverse primer covered 42 out of 49 *P. penetrans* sequences while excluding closely related nematode species. However, the probe covered all *P. penetrans* sequences, but it included also sequences of *P. fallax* and *P. dunensis*. Overall, the non-target species did not align with one or two of the three important components (primers and probe), so their sequences were highly unlikely to be amplified.

Thirty-two clones and sequences from six individuals of *P. thornei* were obtained. Six sequences were used to perform alignments with all sequences of *Pratylenchus* from GenBank. The forward primer covered most sequences except eight *P. thornei* sequences, but none of the non-target species. The reverse primer covered all the *P. thornei* sequences, except six sequences. The probe covered all *P. thornei* sequences, except for five that included a population of *P. thornei* from Australia reported by Subbotin *et al.* (2008). The primer/probe set was located at position 814-900 of D2-D3 expansion fragment of *P. thornei* (Appendix 8.4).

Eleven clones were obtained from 4 adults of *P. neglectus*, but only one sequence was successful for alignment. Primers and probe were designed. The additional primer Neg-AltF2 covered all sequences of *P. neglectus* in the alignment, except one. The reverse primer covered all sequences.

The probe covered all sequences, except six. All three set of sequences were tested *in silico* and no match with other species were observed (Appendix 8.5).

Twelve clones and sequences were obtained from 4 females of *P. crenatus*. After alignment, primers and probe were designed. The primer Cren-AltF and probe covered all D2D3 sequences of *P. crenatus* in the alignment (Appendix 8.6), except five. The reverse primer covered all sequences, except four. Primers/probe sets were at 754-922 and 851-978 for *P. neglectus* and *P. crenatus,* respectively.

The primers and probe used in this study are reported in Table 4.2. An *in-silico* analysis was carried out for each primer and probe using BLAST to detect the specificity of each diagnostic against sequences reported in GenBank, revealing designed primers were highly specific and suitable for species-specific diagnostics of four species studied.

Table 4.2: Primer/probe sets for diagnostic PCR of *Pratylenchus crenatus*, *P. neglectus, P. penetrans* and *P. thornei*.

Species	**Primer/Probes**	**Sequence (5' → 3')**
P. crenatus	Cren-AltF2	CCAAGTGGTGCATTTGCAGGT
	Cren-R	GAACATCACTCCTCCAGTCC
	Cren-Probe	ATGAAGCCGCCCCAGGAGCC
P. penetrans	Pen-F2	ATGGGTTCGAATTGGTGTGG
	Pen-AltF2b	ATGAGTTCGAGTTGGTGTGG
	Pen-AltF2c	ATGGGTTCGCGTTGGTGTGG
	Pen-R2	AGGACCGAATTGGCAGAAGG
	Pen-Probe2	CACATGTTGCATGCAACTGCCACC
P. neglectus	Neg-AltF2	AGCGTATCGGGCCAGCATTC
	Neg-R	CAAAAGCAGGTTCACACCG
	Neg-Probe	ACAACCCCCACTCCGTCCCAATCT
P. thornei	Th-AltF3	AGATTGGGACGGAGTTGGG
	Th-AltR3	CAACACCTCGAACAGCTCAG
	Th-AltProbe3	ACCGCCCGTGGTGCATTTGCA

4.4.2 PCR optimisation

Based on the Ct values, 68°C was kept as the optimal annealing temperature for *P. crenatus* and *P. penetrans*, without any amplification signals due to primer-dimers or non-specific targets. By contrast, 69°C was the optimal annealing temperature for *P. neglectus* and *P. thornei.* Five

combination of primers and probe concentrations were also tested for PCR optimisation, obtaining consistent results using a concentration of 0.6 μM for primers and 0.25 μM for probes.

4.4.3 Standard curves

Standard curves produced for *P. penetrans*, *P. neglectus*, *P. thornei* and *P. crenatus* had a strong linear correlation ($R^2 > 0.99$) between cycle threshold and DNA copy number with a 10^6 dynamic range. A limit of quantification of 10 DNA copies μL^{-1} and PCR efficiencies between 87-118% were found (Figure 4.1 A-D). No amplification signals were detected for negative controls samples with non-target species and water (NTC), for all four diagnostics.

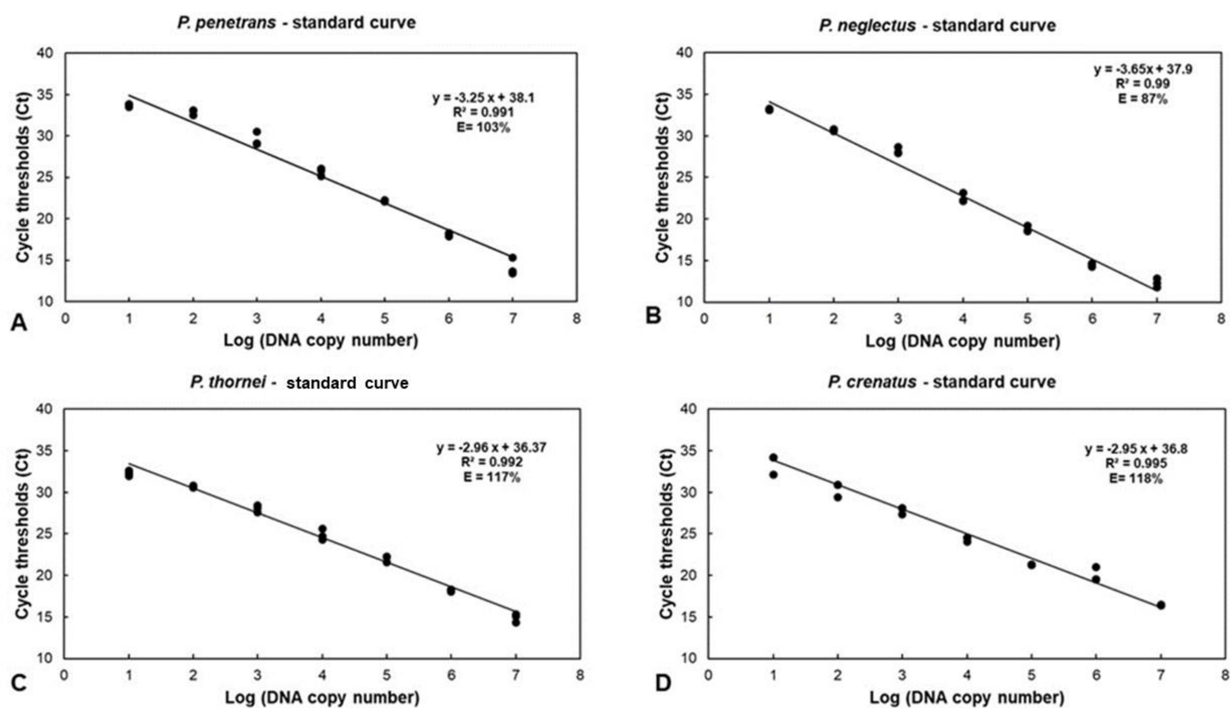

Figure 4.1: Standard linear curves of cycle threshold (Ct) values plotted against log-transformed DNA copy number of A: *Pratylenchus penetrans*; B: *P. neglectus*; C: *P. thornei* and D: *P. crenatus*. The standard curves were run in triplicate for each *Pratylenchus* species. E is the PCR efficiency calculated by the equation: E % = $[(10^{(-1/slope)}) -1] \times 100$

4.4.4 Specificity of primers and probe to detect each target species

Each diagnostic assay was tested for one adult of each target species from different location and/or sources, in three replicates (Table 4.3). The qPCR method was applied to three different populations of *P. penetrans*, with one individual in each tube. Ct values, in the range of 30.1 - 30.5, were obtained for one individual of each *P. penetrans* population, confirming the reproducibility of the method among different populations from two different countries (UK and Belgium). Four populations of *P. neglectus* also showed stable Ct values, in the range of 29.8 and 30.3. Similarly,

two populations of *P. thornei* were tested with the specific diagnostics and resulted with amplification with similar Ct values in the range of 29.5 and 30.7. All three diagnostic assays for these species showed no statistical differences ($P > 0.05$) among Ct values of different populations of same target species. Three populations of *P. crenatus* from different locations were also tested. One sample from Scotland (code 452) had Ct values of 28.8 ± 0.12 that was significantly lower ($P = 0.037$) than a population from England (code PC) with Ct values of 30.1 ± 0.3. No significant differences were observed between 452 and another Scottish sample (Je2), nor between Je2 and PC. The specificity of primers and probe for each diagnostic assay was confirmed by the lack of amplification for any other non-target *Pratylenchus* spp. (*P. penetrans, P. thornei, P. neglectus, P. coffae, P. pseudocoffae, P. vulnus* and *P. fallax*) and other nematode species (*Bitylenchus hispaniensis, Globodera rostochiensis, Meloidogyne hapla, Trichodorus primitivus*).

Table 4.3: Codes, host/sources and origin of *Pratylenchus* spp. and other nematode species used in this study, together with the Ct values (mean ± SEM). Each sample was tested in three replicates.

Code	Species	Host/sources	Origin	Ct values (mean ± SEM)			
				P. crenatus	*P. neglectus*	*P. penetrans*	*P. thornei*
PC	*Pratylenchus crenatus*	Carrot disc	Belgium	30.1 ± 0.3	-	-	-
452	*P. crenatus*	DNA extract	Scotland (UK)	28.8 ± 0.12	-	-	-
Je2	*P. crenatus*	DNA extract	Scotland (UK)	29.3 ± 0.04	-	-	-
PN	*P. neglectus*	Potato soil	England (UK)	-	29.8 ± 0.3	-	-
Li03	*P. neglectus*	Potato soil	England (UK)	-	30.1 ± 0.8	-	-
Li10	*P. neglectus*	Potato soil-	England (UK)	-	29.5 ± 0.22	-	-
491	*P. neglectus*	DNA extract	Scotland (UK)	-	30.3 ± 0.5	-	-
PP	*P. penetrans*	Carrot disc	Belgium	-	-	30.5 ± 0.4	-
NO03	*P. penetrans*	Potato soil	England (UK)	-	-	30.1 ± 0.15	-
Li25	*P. penetrans*	Potato soil	England (UK)	-	-	30.3 ± 0.3	-
PT	*P. thornei*	Carrot disc	Turkey	-	-	-	29.5 ± 0.19
PT-U	*P. thornei*	Beans	England (UK)	-	-	-	30.7 ± 0.3
PCof	*P. coffae*	Carrot disc	Ghana	-	-	-	-
286	*P. fallax*	DNA extract	The Netherlands	-	-	-	-
PsCof	*P. pseudocoffae*	Carrot disc	Iran	-	-	-	-
189	*P. vulnus*	DNA extract	The Netherlands	-	-	-	-
PV-It	*P. vulnus*	Carrot disc	Italy	-	-	-	-
90236	*Globodera rostochiensis*	Potato soil	England (UK)	-	-	-	-
MeH	*Meloidogyne hapla*	Tomato soil	England (UK)	-	-	-	-
368	*Bitylenchus hispaniensis*	DNA extracts	Scotland (UK)	-	-	-	-
Trich	*Trichodorus primitivus*	Potato soil	England (UK)	-	-	-	-

4.4.5 Selectivity and sensitivity of diagnostic assays

4.4.5.1 Test 1 – Selectivity of primers and probe in mixtures of Pratylenchus *spp.*

Assays were tested against samples prepared with a mixture of *Pratylenchus* species to identify the specificity of each diagnostic (Table 4.4).

One female of *P. penetrans* was detected (Ct =29.8 ± 0.09) in a mixture of the other three species (*P. crenatus*, *P. neglectus* and *P. thornei*), with no significant differences ($P > 0.05$) compared to samples of *P. penetrans* with one individual (Ct =30.8 ± 0.6). Equally, 10 specimens of *P. penetrans* were detected (Ct =27.3 ± 0.08) in a mixture with 30 individuals of the other three species. The Ct values were statistically similar ($P > 0.05$) with values obtained from samples with ten individuals (Ct =27.7 ± 0.5).

Similarly, one female of *P. thornei* was detected (Ct =29.49 ± 0.2) in a mixture of *P. crenatus*, *P. neglectus* and *P. penetrans*. Also, ten females of *P. thornei* were detected (Ct =25.6 ± 0.09) in a mixture with 30 females of *P. crenatus*, *P. neglectus* and *P. penetrans* in the same sample. Each Ct value in mixed samples were like samples of one (30.7 ± 0.7) and ten pure females (25.7 ± 04). Similarly, the diagnostic for *P. neglectus* detected one female (Ct =29.86 ± 0.19) in a mixture of *P. crenatus*, *P. thornei* and *P. penetrans,* showing a similar result to pure samples with one female (30.3 ± 0.5). Ten females of *P. neglectus* were detected (Ct =27.9 ± 0.17) in a mixture with 30 females of *P. crenatus*, *P. thornei* and *P. penetrans* in the same sample with Ct values similar to pure samples with ten females of *P. neglectus* (28.14 ± 0.08).

The sensitivity of the diagnostic was tested for one female of *P. crenatus* (Ct =33.7 ± 0.15) in a mixture of *P. neglectus*, *P. thornei* and *P. penetrans*. In this case, values were significantly higher ($P < 0.05$) than Ct values for one nematode (29.3 ± 0.14). In addition, ten females of *P. crenatus* were detected (Ct =27.9 ± 0.06) in a mixture with 30 females of *P. neglectus*, *P. thornei* and *P. penetrans* in the same sample, with comparable values with ten *P. crenatus* samples (27.3 ± 0.4).

All the samples with mixed non-target species as well as negative controls did not show amplifications. This test confirmed the specificity and sensitivity of each protocol on the detection and quantification of *P. penetrans, P. crenatus*, *P. thornei* and *P. neglectus* in the presence of closely related species.

Table 4.4: Specificity of each diagnostic protocol for detection and quantification of one (n=6) and ten (n=6) species target in mixture of *Pratylenchus* spp. Ct values obtained by qPCR are reported as mean ± SEM. Data with different letters are significantly different according to Tukey's test ($P \leq 0.05$).

Mixture of *Pratylenchus* spp.	Ct values (Mean ± SEM)			
	P. crenatus	*P. penetrans*	*P. neglectus*	*P. thornei*
1 female of species target	29.3 ± 0.14 a	30.8 ± 0.6 a	30.3 ± 0.5 a	30.7 ± 0.7 a
1 female of species target + 3 other *Pratylenchus* spp.	33.7 ± 0.15 b	29.8 ± 0.09 a	29.86 ± 0.19 a	29.49 ± 0.2 a
10 females of species target + 30 other *Pratylenchus* spp.	27.9 ± 0.06 c	27.3 ± 0.08 b	27.9 ± 0.17 b	25.6 ± 0.09 b
Mixture of 30 *Pratylenchus* spp. females without species target	-	-	-	-
10 females of *P. crenatus*	27.3 ± 0.4 c	-	-	-
10 female of *P. penetrans*	-	27.7 ± 0.5 b	-	-
10 females of *P. neglectus*	-	-	28.14 ± 0.08 b	-
10 females of *P. thornei*	-	-	-	25.7 ± 04 b

Table 4.5: Sensitivity of each assay for detection and quantification of different life stages for *P. penetrans* (n=10), *P. neglectus* (n=10), *P. thornei* (n=10) and *P. crenatus* (n=6 for juvenile and n=10 for adults). Ct values were obtained by performing qPCR protocol of each species target separately. Quantification of DNA copy number (D2-D3 copy number) of each species target was obtained by fluorescence comparison with standard plasmid curves. Data of Ct values and DNA copy number are reported by mean ± SEM. Data with different letters in the same column are significantly different according to Tukey's test ($P \leq 0.05$). Data for copy number per Individual is the average of DNA copy number of the life stages analysed.

Life stage	***P. crenatus***		***P. penetrans***		***P. neglectus***		***P. thornei***	
	Ct	Copy number	Ct	Copy number	Ct	Copy number	Ct	Copy number
Juvenile	31.3 ± 0.1 a	4961 ± 191 a	31.8 ± 0.11 a	3807 ± 405 a	31.1 ± 0.2 a	1777 ± 59 a	30.45 ± 0.3 a	2398 ± 168 a
Female	29.3 ± 0.14 b	9463 ± 400 b	30.8 ± 0.19 ab	9572 ± 1153 ab	29.8 ± 0.4 b	8806 ± 637 b	29.5 ± 0.2 b	4845 ± 164 b
Male	-	-	29.7 ± 0.03 b	15285 ± 1071 b	-	-	-	-
Individual	-	7775 ± 199	-	9555 ± 297	-	5292 ± 266	-	3624 ± 109

4.4.5.2 Test 2 – Amplification of different life stages

Each diagnostic assay was tested for different life stages (Table 4.5). Different life stages (juvenile and female) of *P. crenatus, P. neglectus, P. penetrans* and *P. thornei* were prepared in separate Eppendorf tubes and DNA was extracted and amplified to quantify the DNA copy number. Male of *P. penetrans* and *P. thornei* were also included. Samples were prepared in six or ten replicates for each life stage. DNA copy number per microlitre was calculated from the qPCR and the total DNA copy number per nematode was then calculated based on the whole volume of each DNA extract.

Overall, the average DNA copy number per individual were: 7775 ± 199 for *P. crenatus*, 9555 ± 297 for *P. penetrans*, 5292 ± 266 for *P. neglectus* and 3624 ± 109 for *P. thornei*.

Juveniles (n=10) of *P. penetrans* had lower DNA copy number (3807 ± 405) compared to females (n=10, 9572 ± 1153) and males (n=10, 15285 ± 1071). Male DNA copy number was significantly higher than juveniles ($P < 0.05$) with females copy number intermediate between males and juveniles.

Also, individual juveniles (n=10) and females (n=10) of *P. thornei* and *P. neglectus* DNA were extracted and amplified by qPCR. In both cases, DNA copy numbers were statistically ($P < 0.05$ respectively) higher for females compared to juveniles. Four samples for *P. crenatus* juveniles failed to amplify. The DNA copy number of juveniles (4961 ± 191) were statistically ($P < 0.05$) lower than adults (9463 ± 400). Negative controls produced no signal for all tests.

4.4.5.3 Test 3 – Assessing the response of the quantitative assay over increasing target abundance

The sensitivity and linearity of the assay was tested with increasing numbers of nematode individuals for *P. penetrans* (1, 10, 100, 1000 individuals), *P. thornei* (1, 10, 1000 individuals) and *P. crenatus* (1, 10, 50 individuals) in three replicates (Figure 4.2-4.4). Results showed strong linear correlations (R^2 = 0.91-0.99) between number of nematodes and cycle thresholds. The negative controls with water (NTC) were always not amplified.

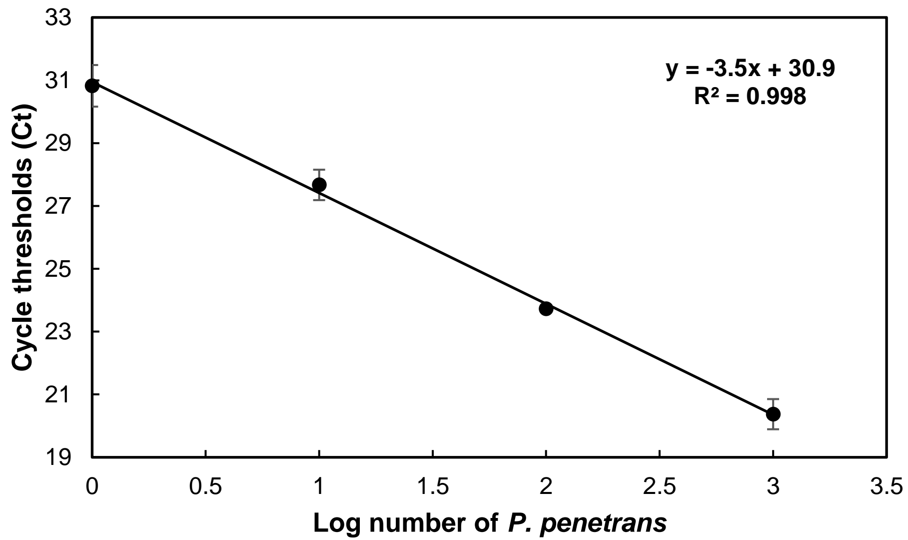

Figure 4.2: Linear regression of the qPCR cycle threshold (Ct) for *Pratylenchus penetrans* against the log number of *P. penetrans* individuals (1, 10, 100, 1000) (n = 3).

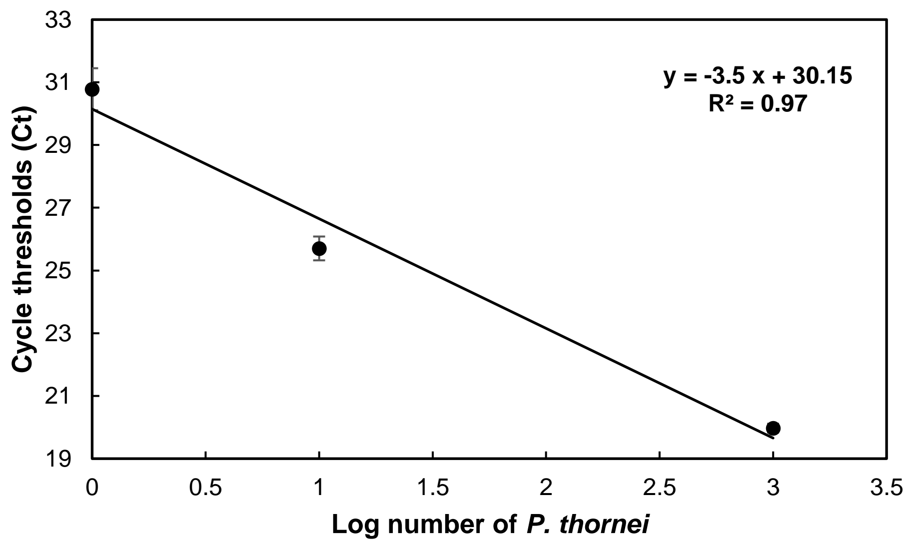

Figure 4.3: Linear regression of the qPCR cycle threshold (Ct) for *Pratylenchus thornei* against the log number of *P. thornei* individuals (1, 10, 1000) (n = 3).

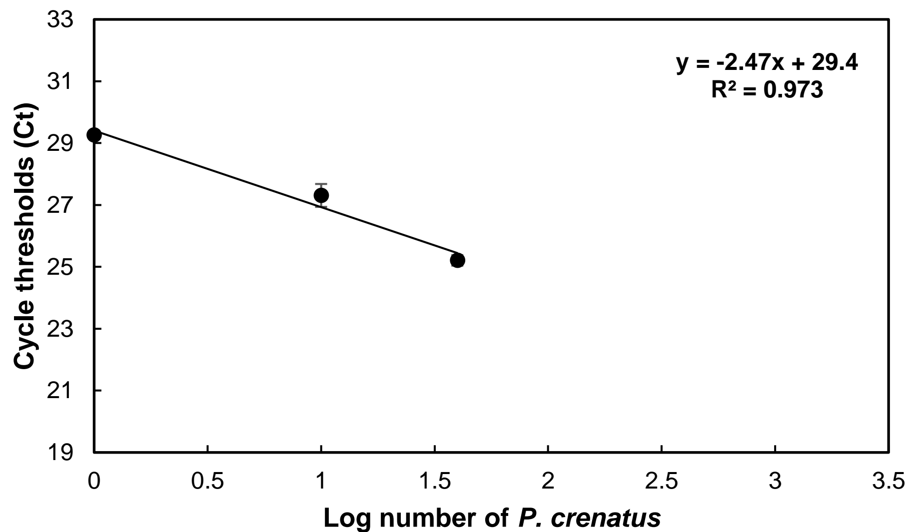

Figure 4.4: Linear regression of the qPCR cycle threshold (Ct) for *Pratylenchus crenatus* against the log number of *P. crenatus* individuals (1, 10, 50) (n = 3).

4.4.6 Comparison of nematode counts obtained from microscopy with qPCR

The qPCR protocol was also tested by comparing numbers of *Pratylenchus* spp. counted by microscopy and the numbers estimated by qPCR. The numbers of root-lesion nematodes in fifteen soil samples from the survey of ware potato growing land in England (Chapter 5) were determined using traditional counts. In addition, whole nematode suspensions (from the same samples) were used for DNA extraction. The total number of *Pratylenchus* determined by qPCR were calculated by the sum of results from each of four diagnostics. Soil samples contained between 4 and 343 *Pratylenchus* nematodes. All the samples tested positive for presence of *Pratylenchus* through qPCR and microscopy. A highly significant ($P < 0.001$) and strong relationship ($R^2 = 0.79$) between qPCR estimates and the microscopy counts was found (Figure 4.5) confirming the robustness of the qPCR estimates. At low densities, estimates by microscopy were slighly higher compared to qPCR estimates, whereas at high densities (above 100 nematodes) molecular diagnostics appeared to overestimate compared to microscopy.

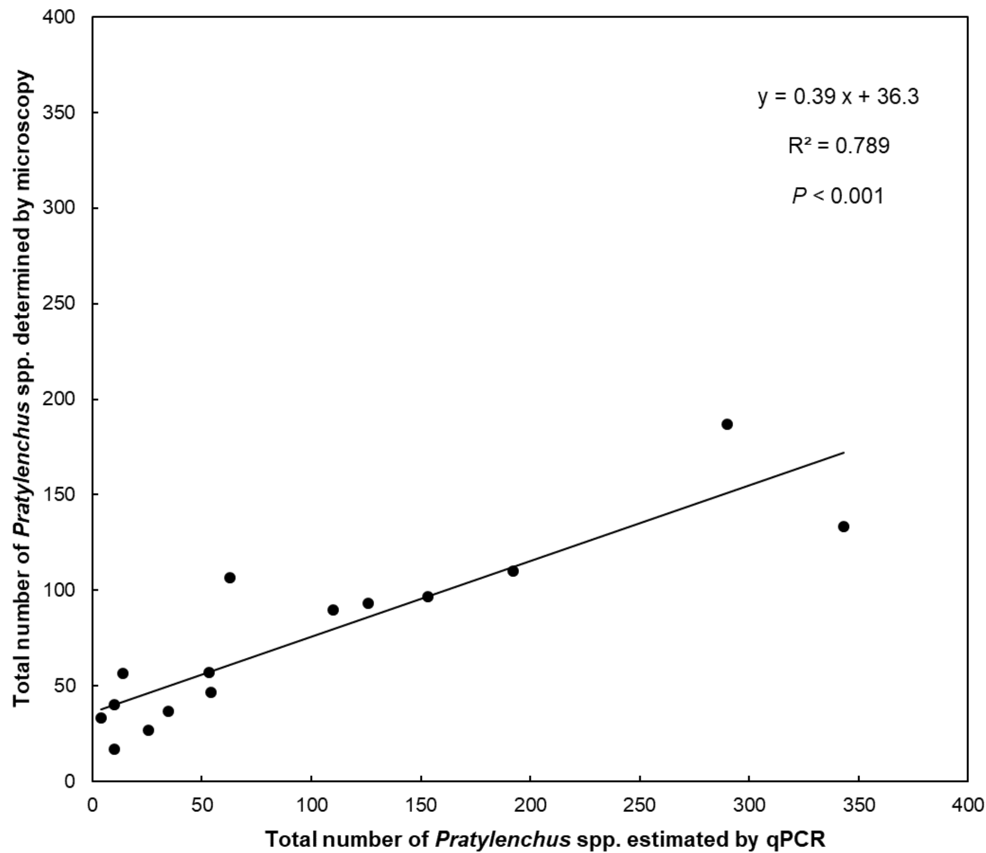

Figure 4.5: The relationship between total number of *Pratylenchus* spp. determined by the estimated total numbers by microscopy (morphological characteristics) and qPCR for 15 soil samples collected from arable fields in England.

4.5 DISCUSSION

Root-lesion nematodes (*Pratylenchus* spp.), although one of the most common plant-parasitic nematodes worldwide, are often difficult to identify at species level. Molecular diagnostic methods can help the rapid identification and quantification of nematodes including different species of *Pratylenchus* and enhance their management where infested fields occur. The qPCR method is an accurate method for the identification and quantification for specific targets based on correlation between the Ct value and DNA concentration or known DNA copy number. In recent years, different qPCR protocols have been published for *P. crenatus* (Oliveira *et al.*, 2016), *P. penetrans* (Sato *et al.*, 2007, 2010; Goto *et al.*, 2011; Oliveira *et al.*, 2016), *P. thornei* (Yan *et al.*, 2012), *P. neglectus* (Yan *et al.*, 2013; Oliveira *et al.*, 2016) and *P. zeae* (Berry *et al.* 2008), mainly using either fluorescent reporter dye SYBR Green or hydrolysis probes. Orlando *et al.* (2020a) summarized all species-specific primers and methods reported for several *Pratylenchus* spp. until 2017. Many protocols have been developed using the ITS region, although its inherent intra-specific and intra-individual variability has caused concerns in several studies (De Luca *et al.*, 2004, 2010; Subbotin *et al.*, 2008; Janssen *et al.*, 2017 a, b), leading to questions on the accuracy of quantification when this target is

used in qPCR. In contrast, the D2–D3 expansion segment of 28S rRNA seems to have a high degree of interspecific variation and low intra-specific variation among different species of *Pratylenchus* spp., suggesting this region is a suitable target for root-lesion diagnostics (Al-Banna *et al.*, 2004; De Luca *et al.*, 2004; Subbotin *et al.*, 2008; Yan *et al.*, 2008; Baidoo *et al.*, 2017; Dauphinais *et al.*, 2018). For this reason, in this study, four TaqMan qPCR protocols for the identification and quantification of *P. crenatus, P. penetrans, P. neglectus* and *P. thornei* based on the D2-D3 region of the large subunit rDNA were developed.

The primer/probe sets used in this study were located at positions 1026-1097, 814-900, 754-922, and 851-978, on the D2-D3 rDNA sequence of *P. penetrans, P. thornei, P. neglectus* and *P. crenatus,* respectively. After optimization of primer/probe concentration and annealing temperature, four standard curves were constructed for identification and quantification of each species target. Standard curves were established by plotting cycle thresholds (Ct values) against a serial dilution of cloned D2-D3 fragments from the target species, ranging from 10^7 to 10^1 copies μl^{-1}, showing very strong linearity ($R^2 = 0.99$) for all four diagnostics. The qPCR methods successfully discriminated each isolate of *P. penetrans, P. crenatus, P. neglectus* and *P. thornei* from seven other *Pratylenchus* species and four other non-target genera tested. Overall, the specificity of primers and probe for each diagnostic was confirmed by the absence of amplifications for non-target species and genera.

Adults of each target species from different locations and/or sources were tested against each diagnostic. Although *P. penetrans, P. neglectus* and *P. thornei* showed no statistical differences ($P > 0.05$) among Ct values from different populations, one *P. crenatus* isolate from Scotland (code 452) showed significantly ($P < 0.05$) lower Ct values than the other isolates. This might be due to the presence of more than one individual/juvenile accidentally picked up during preparation of sample or gravid female with eggs or full spermatheca that may have decreased Ct values compared to the other samples, as suggested by other authors (Roberts *et al.*, 2016; Oliveira *et al.*, 2017; Baidoo *et al.*, 2017; Arora *et al.*, 2020). It is also possible that *P. crenatus* has a slight intra-specific variation for either the D2-D3 fragment or copy-number among different populations. No fluorescent signals were obtained from any of the non-target species included in this study, confirming the specificity of the diagnostics developed in this study. Although *P. fallax* is a species closely related to *P. penetrans* (Ibrahim *et al.*, 1995; Waeyenberge *et al.*, 2000; Carta *et al.*, 2001; Handoo *et al.*, 2001; Janssen *et al.*, 2017b), and can be easily misidentified using morphology, this study showed that the qPCR method developed for *P. penetrans* was specific and did not show amplification for *P. fallax*.

All four diagnostics were sensitive, allowing the detection and correct quantification of as few as one *Pratylenchus* in samples with a mixture of other non-target *Pratylenchus*, even ten times as many non-targets as target nematodes. However, a further test including samples with 2000-3000 nematodes might be performed to reproduce the field representative populations. All four diagnostics did not produce signals with any of the non-target species and negative controls. Ct values were comparable with pure samples with one or ten females for each species, demonstrating the

consistency of the method in mixed samples. The only exception was represented by the diagnostic for *P. crenatus* that showed Ct values for mixed species with one female of *P. crenatus* in a mixture of species (Ct =33.7 ± 0.15) significantly ($P < 0.05$) higher than Ct values for one pure nematode (29.3 ± 0.14). A lack of amplification of four replicates of juveniles in test 2 was also found. Despite this issue, the other diagnostic methods detected small juveniles of each corresponding species, showing methods to identify and quantify juveniles and adults.

DNA copy number per individual was estimated for each species: 7775 ± 199 for *P. crenatus*, 9555 ± 297 for *P. penetrans*, 5292 ± 266 for *P. neglectus* and 3624 ± 109 for *P. thornei*. Overall, each species presented a different DNA copy number, with *P. thornei* having a significantly ($P < 0.05$) lower copy number than *P. penetrans*. This may be due to differences in life stages; for example, the presence of males may increase the estimate of DNA copy number of *P. penetrans* per individual due to the presence of sperm. Additionally, the degree of inter-specific variation within the D2-D3 segment may also affect the DNA copy number. Recently, Lin *et al.* (2020) confirmed that the intraspecific variations of the D2-D3 expansion fragment for *P. neglectus* and *P. thornei* ranged from 0.1–2% and 0.0–1.7% respectively, while interspecific variations between these two species ranged from 14.7% to 20.3%.

It is also important to consider that the test included limited numbers of populations for each species and from a limited number of geographical locations. It would be recommended that preliminary tests with populations of target-species and non-target species from each country before relying on qPCR methods to quantify populations in soil samples.

The qPCR assays were consistent in amplifying increasing numbers of *P. penetrans* (1, 10, 100, 1000 individuals), *P. thornei* (1, 10 and 1000 individuals) and *P. crenatus* (1, 10 and 50 individuals) with highly significant linear relationships found between Ct values and number of nematodes. This test suggests that the diagnostic is accurate over a wide range of target-abundance.

A strong relationship ($R^2 = 0.78$) was observed comparing numbers of *Pratylenchus* spp. counted by microscopy and the numbers estimated by qPCR, suggesting the robustness of the assay. At high densities (300-350 nematodes 200 g^{-1} soil), the estimate by qPCR was higher than to the microscopy count; this may be due to the increase of the chances to have samples containing gravid females with eggs in each species or also males with sperm for *P. penetrans* increasing the overall DNA copy number per sample. Baidoo *et al.* (2017) found similar results from artificial infested soil comparing qPCR estimates of *P. penetrans* and number obtained by microscopy, with a slightly higher variation between the two methods at high densities. The authors considered this might be due to a reduction on DNA extraction efficiency and variation in DNA among different life stages. Regarding this aspect, Sato *et al.* (2007) reported that Ct values of female (23 ± 0.3) and male (22 ± 0.7) of *P. penetrans* were significantly lower than juveniles (25.1 ± 0.4), which agrees with this study. Indeed, juveniles of *P. penetrans* had higher Ct values and lower DNA copies per individual compared to females and males. Despite this difference in DNA copy number, only males were

statistically different ($P < 0.05$) from juveniles. Whereas juveniles of *P. thornei, P. neglectus* and *P. crenatus* were statistically different in terms of DNA copy number compared to females. Some explanations might be also that gravid females containing eggs inside their body and males with sperm will generate a lower signal than non-gravid females or males without sperm (Oliveira *et al.*, 2017). Another potential explanation is the increased risk of juveniles (J2) or eggs being missed by the diagnostician during counting by microscopy.

The D2-D3 expansion region expansion segments of the large subunit (28S) has been selected as molecular markers for investigating phylogenetic relationships, and in particular, distinguishing between closely related species of *Pratylenchus* (Al-Banna *et al.*, 1997, 2004; De Luca *et al.*, 2004, 2010; Subbotin *et al.*, 2008; Janssen *et al.*, 2017 a, b). Species-specific primers on D2-D3 expansion fragment were designed to identify *P. neglectus* and *P. thornei*, developing a method of DNA extraction directly from the soil which allowed the identification of one juvenile in 1 g of soil (Yan *et al.*, 2008). Baidoo *et al.* (2017) reported a qPCR method for detection of a single *P. penetrans* juvenile in 1 g of soil, but the qPCR assay overestimated nematode numbers when compared to counts undertaken under a microscope. This might be due to DNA extraction efficiency or amplification of different developmental stages that are not detected with visual counting as discussed by the author (Baidoo *et al.*, 2017). In contrast, a TaqMan qPCR method reported by Dauphinais *et al.* (2017) for identification and quantification of *P. alleni* and *P. penetrans* showed high specificity and sensitivity, although the qPCR underestimated when compared to visual counting under microscope. This assay was improved by an Exogenous Internal Positive Control (EIPC) for the prevention of false negative results. Another consideration with qPCR methods is the absence of information on other *Pratylenchus* species that are not detected by the assays like for example *P. fallax, P. convallariae, P. pratensis* which would be identified by traditional microscopy. Moreover, often mixed population occur in the same sample, quantification of each species is not possible by microscopy that usually is performed at low magnification giving a total count of the genus but not of each species. Whereas if different qPCR diagnostics to each sample are applied, accurate quantification for each target species can be obtained when mixed species occur in a sample. Recently, Lin *et al.* (2020) developed a duplex qPCR assay based on 28 rDNA D2D3 expansion region for the simultaneous identification and quantification of *P. neglectus* and *P. thornei*. For first time, two different species of *Pratylenchus* were detected and quantified at the same time with qPCR, and in samples with up to 100 individuals of non-target nematodes. The authors were able to construct two linear equations with strong correlations between the Ct values and numbers of *P. neglectus* or *P. thornei*, quantifying both species simultaneously.

Some limitations of qPCR methods should be also considered. Indeed, the diagnostics developed in this study need to be performed separately, meaning that DNA samples would need to be processed in four replicates, one for each target species. Further work may be focused on the performance of a multiplex qPCR using the primer/probe sets developed in this study with different

fluorophores to obtain results in a single analysis rather than running four monoplex assays. This would reduce the amount of template and reagent required that would be used only once instead of four times. Multiplex reactions would also minimize the possibility of laboratory contamination.

As discussed above, the developed diagnostics only permits the quantification of four common *Pratylenchus* species per sample, meaning that other minor species would not be counted. A preliminary check using microscopy for the genus in nematode suspensions, may be needed before DNA extraction and TaqMan qPCR, to determine if “other species” of *Pratylenchus* are present in the samples. Indeed, if the samples are positive to the genus, but negative to each TaqMan qPCR, then we can assume another species, or multiple species, is present. In this case, morphological identification of specimens together with DNA sequencing would be required for identification.

DNA templates were obtained from nematode suspensions, after nematode extraction from soil, which involves several stages and therefore more time. Another limitation to consider is that also DNA extraction efficiency of nematode suspensions can vary according to the method selected (Orlando *et al*., 2020b; Chapter 3), consequently reducing the sensitivity of molecular assay for nematode identification and quantification (Roberts *et al*., 2016). Five different DNA extraction methods including three commercial kits for nematode communities were compared showing significant differences among the results (Donn *et al*., 2008). The method with phenol chloroform purification and a Purelink PCR purification kit were the most efficient methods (Donn *et al*., 2008). An implementation of DNA extraction directly from the soil would reduce time and variability due to different efficiency on different nematode extraction methods. However, current protocols reported in literature for nematodes are limited to 1-10 g of soil for direct extraction of DNA from soil, and this may not be comparable with the standard volume of 200-300 g^{-1} soil used for nematode extraction. Also, residuals of soil particles may inhibit the amplification of DNA template reducing the qPCR efficiency (Schrader *et al*., 2012). Sato *et al*. (2007) tested two types of soils, andosol and clay lowland soil, containing the same number of root-lesion nematodes and they found significant differences in Ct values, revealing that type of soil may also influence the efficiency of the DNA extraction and quality of PCR products. In addition, several studies have reported an overestimation of qPCR Ct values compared to numbers estimated by visual counting after Whitehead tray extraction method (Sato *et al*., 2010; Min *et al*., 2012; Yan *et al*., 2012, 2013; Baidoo *et al*., 2017). Additionally, different approaches to nematode extraction may result in variations in extraction efficiency and therefore discrepancies in the final counts (EPPO, 2013; Sato *et al*., 2007; Donn *et al*., 2008; Bellvert *et al*., 2008; Yan *et al*., 2013; Baidoo *et al*., 2017; Cesarz *et al*., 2019). For that, results between laboratories can vary greatly, possibly due to differences in the equipment used, adaptations of the methodology within each laboratory or different experience of the operators (EPPO, 2013). Ideally, a standardised protocol for nematode extraction should be used in all nematology laboratories around the world (Cesarz *et al*., 2019).

To conclude, the four methods developed in this study will be useful for discrimination and quantification of *P. penetrans, P. crenatus, P. neglectus* and *P. thornei* within mixed nematode extracts from soil. This methodology can replace the step of morphological identification that is frequently time-consuming when performed under the microscope, overcoming the requirement for taxonomic experience with nematode identification and allows for the correct quantification of each of the four species within a mixed population. In summary, TaqMan qPCR methods have the potential to be useful tools for accurate diagnosis and therefore the implementation of management strategies for these common *Pratylenchus* species.

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Chapter 5

5. DETECTION AND DISTRIBUTION OF ROOT-LESION NEMATODES IN POTATO FIELDS IN ENGLAND AND SCOTLAND

5.1 ABSTRACT

Potato, *Solanum tuberosum*, is a herbaceous plant and host to various plant-parasitic nematodes, including root-lesion nematodes (*Pratylenchus* spp.). *Pratylenchus neglectus* and particularly *P. penetrans* are the most damaging species for potato crops. In the UK, potato cyst nematodes are well documented to cause severe damage on different potato cultivars; however, less is known about the significance of *Pratylenchus* spp. The aim of this study was to assess the presence, distribution, and abundance of root-lesion nematodes in potato growing fields from England and Scotland. Two hundred sites from the top fifteen counties with largest area of potato production in England were sampled. The number of field sites per county was determined using a stratified method and soil samples were collected following potato harvest, between September and November, in 2017 and 2019. Samples from eighteen potato fields provided by The James Hutton Institute (Scotland) were also included. Root-lesion nematodes were detected in 88% of soil samples from potato fields in England and in 94% of samples from Scotland. This survey confirms that root-lesion nematodes are widely distributed within potato fields of England and Scotland. Two or three species of *Pratylenchus* were sometimes found co-occurring in a single sample. In England, the genus was mainly present in the East and North East, with *P. neglectus* and *P. thornei* being the most frequently detected species, whereas *P. crenatus* and *P. penetrans* were less common. *Pratylenchus thornei* was mainly found in South East and South West England, *P. neglectus* was mainly in the East and *P. penetrans* in North East and South East of England. Biotic factors that may influence the presence and prevalence of each species, such as soil type, previous crop, crop at sampling, irrigation and nematicide application, did not have significant effects on the presence and abundance of the genus or of each species. *Pratylenchus* was also highly prevalent in Scotland, with *P. neglectus* and *P. crenatus* the most widespread and dominant species. This study represents the first time that the distribution of root-lesion nematodes has been documented for potato growing land in England and Scotland.

5.2 INTRODUCTION

Potatoes represent one of the most important crops in the UK with 117,466 ha of total planted area registered in 2020 (AHDB, 2020). In 2020, East of England (Bedfordshire, Cambridgeshire, Essex, Hertfordshire, Norfolk and Suffolk) was the area with the highest potato production with 33,390 ha of planted area (AHDB, 2020). However, potatoes can be subject to a range of pests and diseases, including plant-parasitic nematodes (PPN), which are known to affect the yield and the quality of the tubers and, consequently, their production. Root-lesion nematodes (RLN), *Pratylenchus* spp., are migratory endoparasitic nematodes that feed directly on roots, tubers and cortical tissues. The most common species associated with infected potatoes are *P. penetrans, P. crenatus, P. neglectus, P. alleni, P. thornei* and *P. scribneri*. Symptoms can be overlooked or mistaken for damage caused by other pests or biotic factors (Palomares-Rius *et al*., 2014). Once infested, plant symptoms include poor growth with yellow foliage, discrete reddish-brown- lesions on the roots (nematode feeding sites) and small lesions on the stolons and tubers (Duncan and Moens, 2013). The lesions on the surface of tubers can be brown to black, turning purple over time (Holgado *et al*., 2009; Holgado and Magnusson, 2012; Figueiredo *et al*., 2021). In particular, *P. penetrans* (Olthof and Wolynetz, 1991; Khan and Hussain, 2004; Holgado *et al*., 2009; Holgado and Magnusson, 2012; Figueiredo *et al*., 2021), *P. neglectus* (Olthof and Wolynetz, 1991), *P. scribneri* (Olthof and Wolynetz, 1991), and *P. brachyurus* (Koen and Hogewind, 1967; Koen, 1969) are reported to directly infect seed potato tubers, thus a potential distribution mechanism to non infested fields.

Pratylenchus spp. are found worldwide, but their species distribution varies and is driven by availability of host plants, temperature, and soil type. For example, *P. thornei*, *P. fallax* and *P. crenatus* are typical of temperate regions, whereas *P. coffee, P. brachyurus,* and *P. loosi* prefer tropical and sub-tropical regions (Castillo and Volvas, 2007). In general, *P. penetrans* are more commonly found in sandy soils, whereas *P. neglectus* prefers clay and loamy soil (Castillo and Volvas, 2007; Brodie *et al*., 1993). In England and Wales, Corbett *et al*. (1970) found that *P. neglectus* occurs in all type of soils, *P. crenatus* and *P. fallax* occur in sandy soils, whereas *P. thornei* is found in loamy and clay soils. Although the distribution of root-lesion nematodes in the UK is available for some crops such as wheat, barley, carrot, pea and bean (Southey, 1959; Seinhorst, 1968; Corbett, 1969, 1970, 1972, 1973, 1974, 1976, 1983; Boag, 1979, 1980; Boag and Lopez-Llorca, 1989; Boag *et al*., 1990; Dale and Neilson, 2006), there is a lack of information regarding the potato production areas affected and the associated crop losses. *Pratylenchus pratensis* and *P. penetrans* were the main species reported by Southey (1959) in several hosts in England, and *P. fallax* and *P. flakkensis* were later reported by Seinhorst (1968) but the host range was not specified. Corbett *et al*. (1969) reported the presence of *P. thornei, P. neglectus* and *P. pinguicaudatus* in wheat. Later, twenty-four barley and five wheat fields were sampled and *P. neglectus*, *P. crenatus*,

P. fallax, P. thornei and *P. pinguicaudatus* were reported, with *P. neglectus* being the most common species found (Corbett, 1970). Similarly, the most common species on a survey conducted in raspberry cane beds by Cotten and Roberts (1981) was *P. crenatus* that was found in 20 cane beds, followed by *P. thornei*, *P. neglectus* and *P. fallax*. *Pratylenchus penetrans* was found only in one cane bed. In Scotland, *P. penetrans, P. neglectus, P. crenatus* and *P. thornei* were the species found in carrot fields (Boag, 1979), in pea and bean fields (Boag, 1980), cereals (Boag and Lopez-Llorca, 1989; Boag, 1990) and permanent pasture (Boag and Lopez-Llorca, 1989). The presence of *P. penetrans*, *P. crenatus* and *P. neglectus* was confirmed recently also by Oliveira *et al.* (2017) in samples from potato field in Scotland.

The aim of this chapter was to conduct a survey in England and Scotland to have a clear overview of the distribution of root-lesion nematodes on potato growing lands.

5.3 MATERIALS AND METHODS

5.3.1 Selection of fields for sampling

Potato fields from fifteen counties of England were selected for the survey based on the highest area of production (data reported by AHDB - Potato Data Centre - data obtained on the 9th of March 2017). The number of field sites per county were determined with a stratified method, with the overall aim to sample 200 fields.To determine the number of fields from each county, the top 15 counties with the highest potato production were selected and then the number of samples from each county was calculated with the following formula:

$$N° \ of \ samples = \frac{Total \ number \ of \ samples \ for \ survey}{Total \ growing \ areas \ of \ 15 \ counties} * growing \ area \ of \ each \ county$$

The counties with related number of fields are reported in Table 5.1.

Table 5.1: Top 15 counties of potato production in England based on data from AHDB (data obtained on the 9th of March 2017) with area of production (ha) and number of fields to be sampled based on a stratified selection.

TOP 15 COUNTIES OF POTATO PRODUCTION			
	County	Area (ha)	N°of fields
1	Norfolk	15084	42
2	Lincolnshire	12787	30
3	Yorkshire	11629	30
4	Suffolk	6133	17
5	Shropshire	5556	16
6	Herefordshire	4591	12
7	Cornwall	3721	10
8	Lancashire	3135	7
9	Essex	2611	7
10	Nottinghamshire	2539	6
11	Cambridgeshire	2157	6
12	Staffordshire	2150	6
13	Cheshire	2089	4
14	Kent	1463	4
15	Warwickshire	1436	3
	Total area (ha)	77081	200

5.3.2 Soil sampling and storage

Soil samples were collected after potato harvest, from September to November, for a total of 100 samples per year in autumn 2017 and 2019. Before sampling, each field was identified using Google Earth Pro® in order to allocate the gate entrance and the point of sampling. Each field site was then mapped using a handheld GPS coordinates using a mobile phone app called Survey-it. The application was used to plot the area (1 ha) in the gate entrance to each field and the sampling pattern in each field. Soil was collected following a W pattern (Figure 5.1) (Evans and Haydock, 2000; Schomaker and Been, 2013), taking 60 sub-cores for a total volume of 1 kg soil per 1 ha of field in the gate entrance. Soil samples were taken using a corer with a half-cylindrical blade with 2.5 cm diameter (Figure 5.2), at 0 - 20 cm depth (Schomaker and Been, 2013). All 60 subcores were combined and retained in labelled plastic bags. Soil samples were kept in plastic boxes in a cold store at 4°C located in the Field Technology Centre, CERC (Harper Adams University).

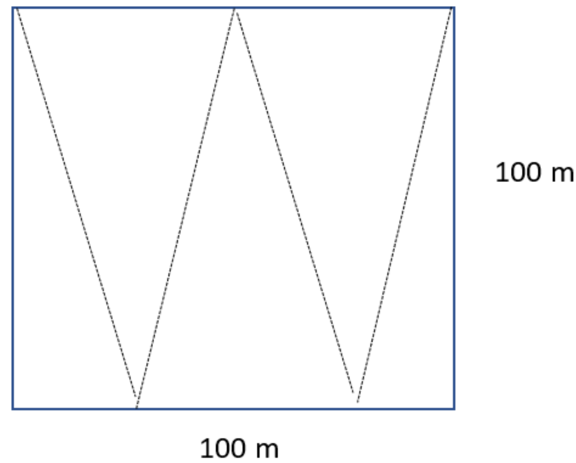


Figure 5.1: W pattern selected for root-lesion nematode soil sampling (Evans and Haydock, 2000; Schomaker and Been, 2013)

Figure 5.2: Potato beds (left) before harvest and corer with half-cylindrical blade (right) used during sampling.

5.3.3 Survey questionnaire

Growers were contacted to obtain background information about their fields via a questionnaire (Appendix 8.7). Information collected included details on: -

- Potato cultivars grown
- Total area of potatoes grown
- The crop rotation
- Previous history of plant-parasitic nematodes in the field
- Soil type
- Irrigation
- Nematicide (use and type of product)

5.3.4 Nematode extraction, quantification, and identification

A subsample of 200 g soil from each soil sample was taken for nematode extraction. Nematodes were extracted from soil according to the Whitehead and Hemming tray method detailed in section 2.2.2. Nematodes were identified and quantified following the procedure described in section 2.3. Each nematode suspension was assessed for *Pratylenchus* spp. using a binocular microscope (Mazurek Optical Service, Meiji EMT) at 40X magnification and then the same suspension was transferred to a 10 mL Falcon tube for molecular analysis and allowed to settle for 24 h. The volume of the sample was reduced to approx. 1.5 mL, resuspended, and transferred to a 2 mL Eppendorf tube. The Eppendorf was kept at -20°C. All nematode suspensions (200 samples) were processed for freeze drying procedure using a LyoDry Freeze Dryers (Mechatech systems, UK), leaving samples in the machine for one week. Freeze-dried samples were used for DNA extraction using a Purelink DNA (Invitrogen) extraction kit, following the manufacturer's instructions. qPCR quantification for *P. penetrans*, *P. neglectus*, *P. crenatus* and *P. thornei* was conducted following the methods reported in Chapter 4 for all 200 samples. The estimated total number of each species per sample was calculated by dividing the DNA copy number per sample and the average DNA copy number per individual for each species (calculated in Chapter 4). The total count for the genus was determined by the sum of qPCR numbers for each species. When no qPCR amplification was detected despite the confirmation by microscopy samples were counted as "other species" of *Pratylenchus*.

5.3.5 Samples from Scotland

DNA samples of eighteen potato fields from Scotland were provided by The James Hutton Institute. The sampling was conducted by JHI between 2015 and 2017. Each field was sketched out (before arrival) and a 'buffer strip' of ca. 5 m was made around the outer edge. The field was split into six approximately equal areas. Each area was numbered from 1 to 6 starting from the 'area' closest the field gate. Within each area, a random-location was selected for the sample-spot (within the grid) and a square of a size 10 m square was placed around that 'spot' and selected for the sampling. A W pattern grid was followed using a grass sampler to 10 cm depth for each point of sampling, collecting a total of 60 sub-cores per square for a total of around 800 g^{-1} soil. Soil samples were kept in plastic bags until nematode extraction. Nematodes were extracted from 200 g^{-1} soil using a modified Baermann funnel for 48 h (Brown and Boag, 1988). Samples were preserved by freeze-drying using Christ Alpha 1-2 LO plus Freeze dryer (SciQuip, Newtown, Shropshire, UK) and then stored at -20°C until DNA extraction was performed. The freeze-dried samples were then used for DNA extraction using Purelink DNA (Invitrogen) extraction kit, following the manufacturer's instructions.

5.3.6 Mapping the distribution of root-lesion nematodes in England and Scotland

GPS coordinates were recorded for each potato field sampled and used for constructing distribution maps with RStudio (Affero General Public License version 3) using the packages ggplot2 and ggmap (Kanle and Wickham, 2013). Counties of England were grouped in six different regions: East (Norfolk, Suffolk, Notthinghamshire, Essex and Cambridgeshire), West (Staffordshire, Shropshire, Herefordshire and Warwickshire), North East (Yorkshire and Lincolnshire), North West (Cheshire and Lancashire), South East (Kent) and South West (Cornwall).

5.2.7 Statistical analysis

Data for the distribution of *Pratylenchus* spp. were subjected to Pearsons-Chi square tests. Data on the presence and absence of *Pratylenchus* spp. for distribution and interactions with other factors (regions, counties, soil type, cultivars, previous crops, crop at sampling) were subjected to a stepwise analysis of deviance using a Bernoulli distribution followed by linear regression analysis. Based on the linear regression model, predictions of mean and S.E. were obtained for presence of root-lesion nematodes in each county. A stepwise analysis of deviance using a Bernoulli distribution followed by linear regression analysis was also applied for each species. For data of root-lesion nematodes with more than 70% presence, a stepwise analysis of variance was performed for count data (Log 10 transformed) followed by unbalanced ANOVA with Bonferroni test ($P < 0.05$) as post hoc test for multiple comparison. All statistical analyses were performed using Genstat (19th edition, VSN International).

5.4 RESULTS

5.4.1 Detection of root-lesion nematodes in England

Nematodes were extracted from all soil samples (n=200). Where root-lesion nematodes were found, they were identified and quantified. Overall, 88% of potato growing field samples contained species of *Pratylenchus* (Figure 5.3). Regarding the type of species present, 9% of samples contained *P. crenatus*, 18% had *P. penetrans*, 19% had *P. thornei*, while the most abundant species found was *P. neglectus*, present in 31% of the fields sampled. Apart from these known species, 31% of the fields assessed contained other *Pratylenchus* species, that were not identified. Such species may have been *P. fallax, P. convallariae, P. pratensis* or other *Pratylenchus* species, that are species recorded in England (Southey, 1959; Seinhorst, 1968; Corbett, 1969, 1970, 1972, 1973, 1974, 1976, 1983). A proportion of the samples were found to contain more than one species (Figure 5. 4). *P. crenatus* was found with other species in 11 fields, while *P. neglectus*, *P. penetrans* and *P. thornei* occurred in mixed populations in 32, 23 and 22 of positive samples, respectively.

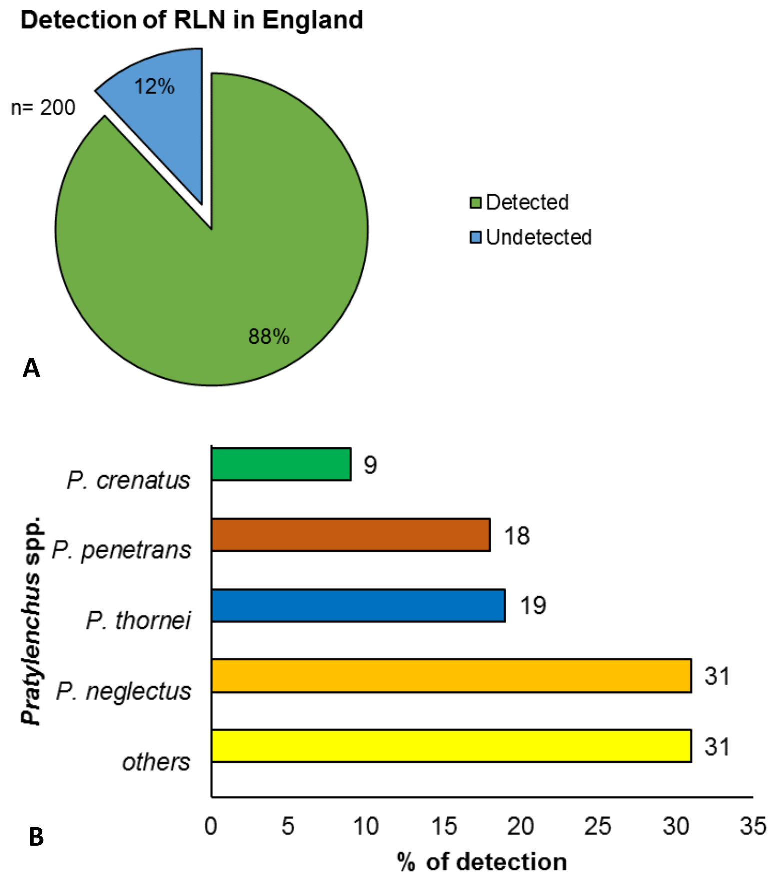


Figure 5.3: Percentage of root-lesion nematodes found in 200 potato fields from England (A); percentage of each *Pratylenchus* spp. detected in the survey (B).

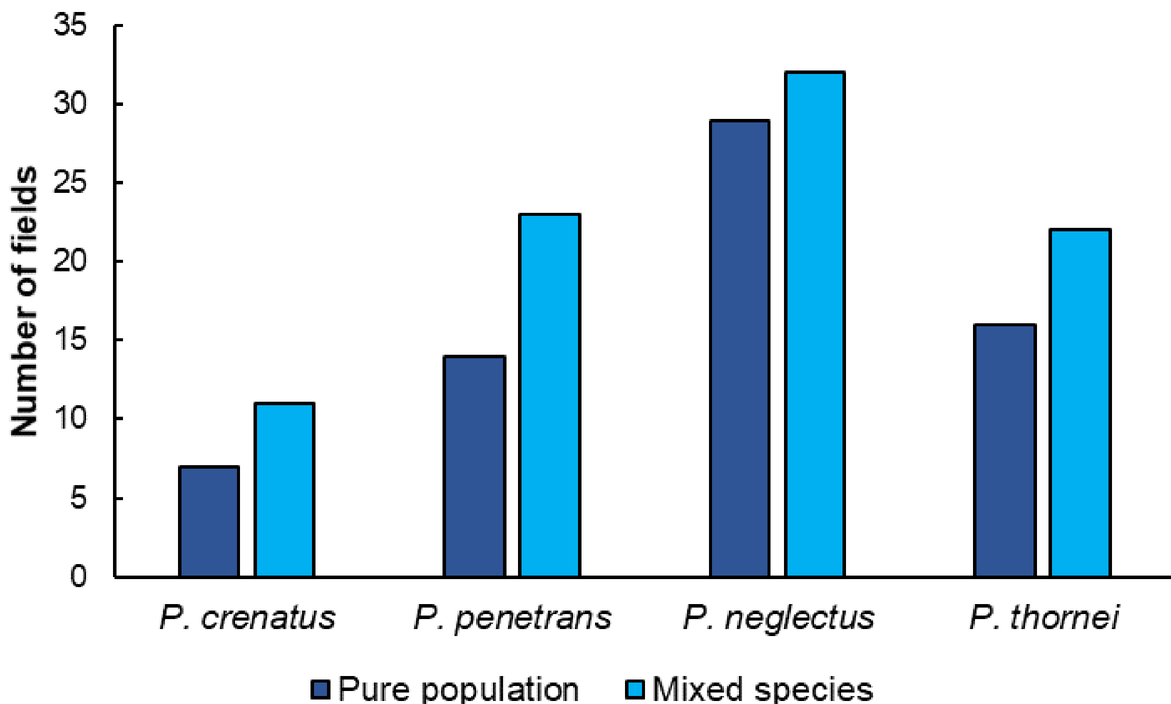


Figure 5.4: Number of positive fields from England where *P. crenatus*, *P. neglectus*, *P. penetrans* and *P. thornei* was found in isolation or in combination with other species.

5.4.2 Distribution of root-lesion nematodes in England

Root-lesion nematodes were found with a high incidence in all counties (Table 5.2). Norfolk, the county with the highest potato production and consequently highest number of fields sampled, presented 95% of fields positive to root-lesion nematodes, with all four species recorded in the county. Root-lesion nematodes were also found in Lincolnshire (97%), Suffolk (100%), Shropshire (94%), Herefordshire (92%) and Cambridgeshire (100%). In all these counties, all four common *Pratylenchus* species and others not identified were detected. *Pratylenchus penetrans, P. thornei* and *P. neglectus* were recorded also in Yorkshire, with 83% of fields positive to the genus. In Cornwall, 80% of fields presented root-lesion nematodes, with *P. penetrans* and *P. thornei* being the main species detected, whereas samples from Staffordshire (33%) only presented *P. crenatus* and *P. neglectus*. Samples from Lancashire had the lowest percentage of RLN detection (7%), and did not contain *P. crenatus, P. neglectus, P, penetrans, P. thornei*. Similarly, samples from Cheshire (80%) and Warwickshire (67%) did not contain common species, whereas samples from Kent (100%) contained *P. neglectus, P. penetrans* and *P. thornei.*

Table 5.2: Percentage of fields where root-lesion nematodes (RLN) were detected in each county in England. Presence (+) and absence (-) of *Pratylenchus* species in each county. n = the number of fields sampled in each county.

County	n	% fields	Species				
			P. crenatus	*P. neglectus*	*P. penetrans*	*P. thornei*	*other species*
Norfolk	42	95	+	+	+	+	+
Licolnshire	30	97	+	+	+	+	+
Yorkshire	30	83	-	+	+	+	+
Suffolk	17	100	+	+	+	+	+
Shropshire	16	94	+	+	+	+	+
Herefordshire	12	92	+	+	+	+	+
Cornwall	10	80	-	-	+	+	+
Lancashire	8	17	-	-	-	-	+
Essex	7	100	-	+	+	+	-
Nottinghamshire	7	100	+	+	+	+	-
Cambridgeshire	6	100	+	+	+	+	+
Staffordshire	6	33	+	+	-	-	-
Cheshire	5	80	-	-	-	-	+
Kent	4	100	-	+	+	+	-
Warwickshire	3	67	-	-	-	-	+

The map in Figure 5.5 shows the distribution of root-lesion nematodes in potato growing lands in England. The genus was widely present with a significant (X^2 = 23.35, d.f. = 5, $P < 0.001$) distribution found in the East and North East of England. Analysing the proportion of positive (black dots) and negative (white dots) fields in the map, West and North West presented higher numbers of negative fields compare to East and North East of England.

Each species presented a different distribution. Indeed, maps in Figure 5.6 shows four different distributions representing each species detected. In particular, *P. crenatus* is more dispersed in the centre of England (counties in the East and West of England) but, in the East, it is less prevalent than other species (Figure 5.6 A). *Pratylenchus penetrans* had a significantly high presence (X^2 = 16.14, d.f. = 5, $P = 0.006$) in East and South East of England (Figure 5.6 B), whereas *P. neglectus* was found to have a significantly occurrence in the East of England (X^2 = 16.14, d.f. = 5, $P < 0.001$) (Figure 5. 6 C). *Pratylenchus thornei* is mainly found in the South East and South West (X^2 = 16.58, d.f. = 5, $P = 0.005$) (Figure 5.6 D).

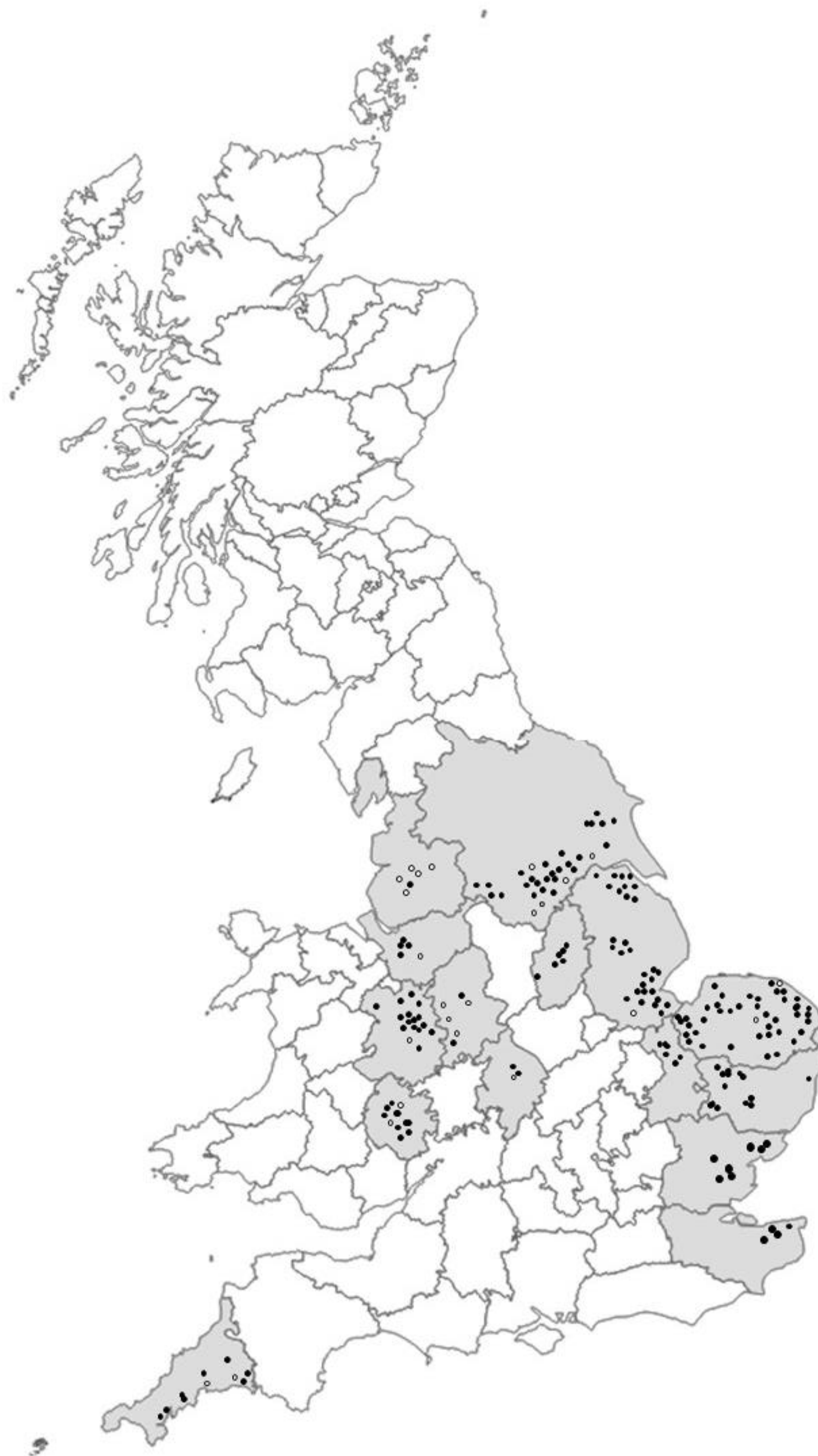


Figure 5.5: Distribution map of root-lesion nematodes in potato fields from England. Sampled counties are shaded grey colour, black dots indicate fields where root-lesion nematodes were positively detected, whereas white dots show fields without detection.

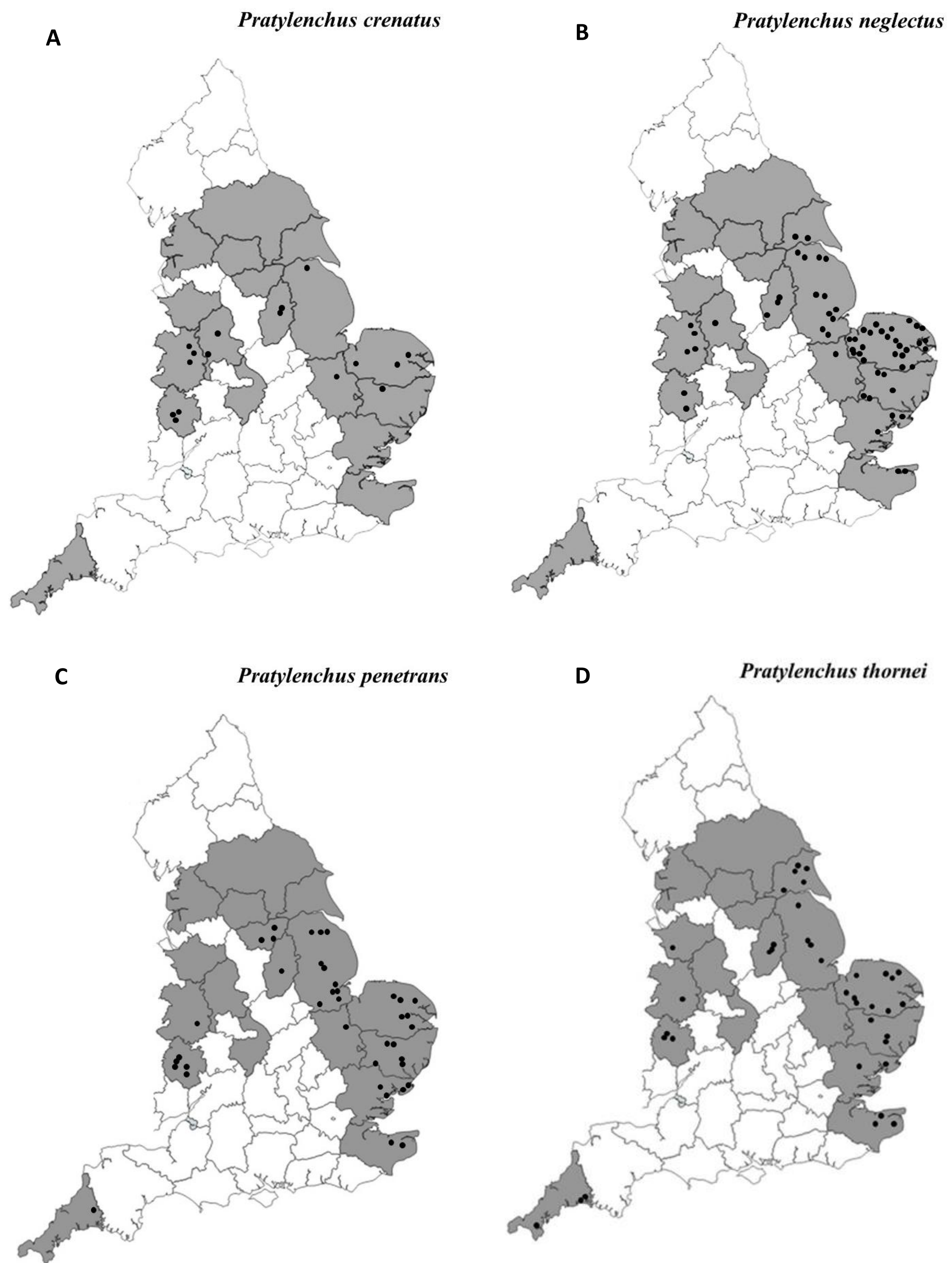
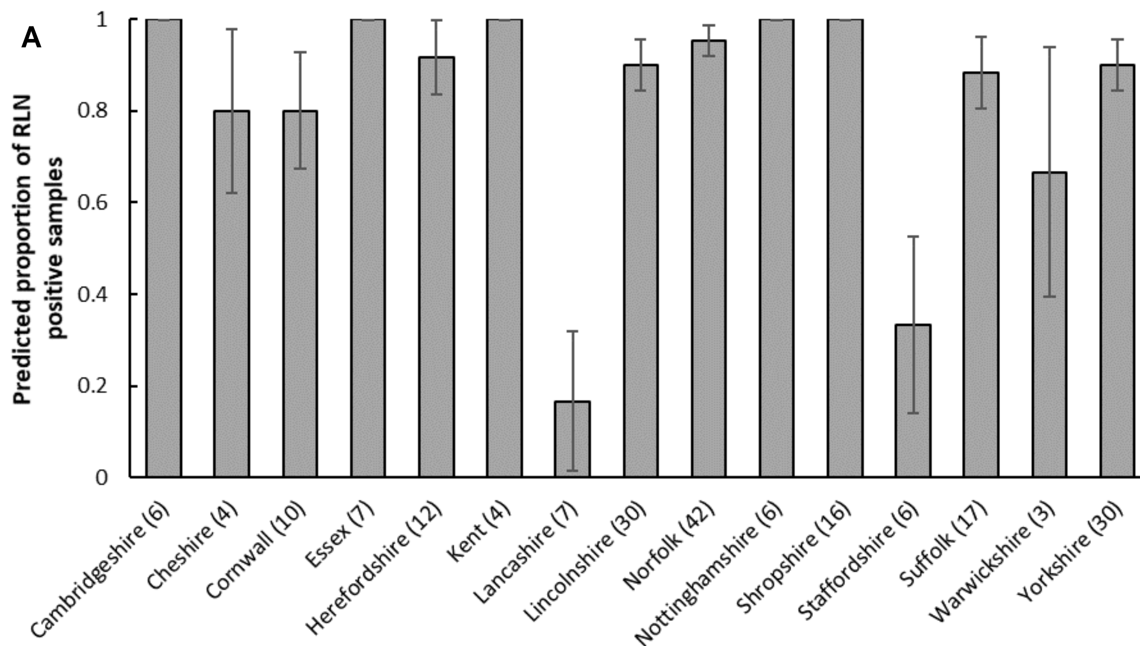


Figure 5.6: Distribution of *P. crenatus* (A); *P. neglectus* (B); *P. penetrans* (C) and *P. thornei* (D) in potato fields in England.

A stepwise analysis of deviance with Bernoulli distribution showed that root-lesion nematodes were differently distributed (*P* < 0.001) among different counties. Prediction from linear regression model showed Lancashire as having the lowest values (for presence) compared to the other counties (Figure 5.7 A). A stepwise analysis of variance was performed also for count data of root-lesion nematodes (Log 10 transformed) followed by unbalanced ANOVA with Bonferroni test (*P* < 0.05) as post hoc test (Figure 5.7 B). Predictions from regression model showed that Lancashire had the lowest counts of root-lesion nematodes. Applying a stepwise analysis of deviance with Bernoulli distribution for each species, considering the positive records, the predictions from regression model showed that the distribution of *P. crenatus* was not significantly different (*P* = 0.319) between counties (Figure 5.8 A), whereas *P. neglectus* was detected the least in Yorkshire, Shropshire and Cambridgeshire and the distribution between counties was significantly different (*P* < 0.001) (Figure 5.8 B). *Pratylenchus thornei* was detected the least in Shropshire, Suffolk and Yorkshire and the most in Kent; a significant difference was found between the occurrence of *P. thornei* in the counties sampled (*P* = 0.008) (Figure 5.8 C). On the other hand, *P. penetrans* was most frequently recovered in Essex and least frequently in Norfolk and Cambridgeshire (*P* = 0.009) (Figure 5.8 D).



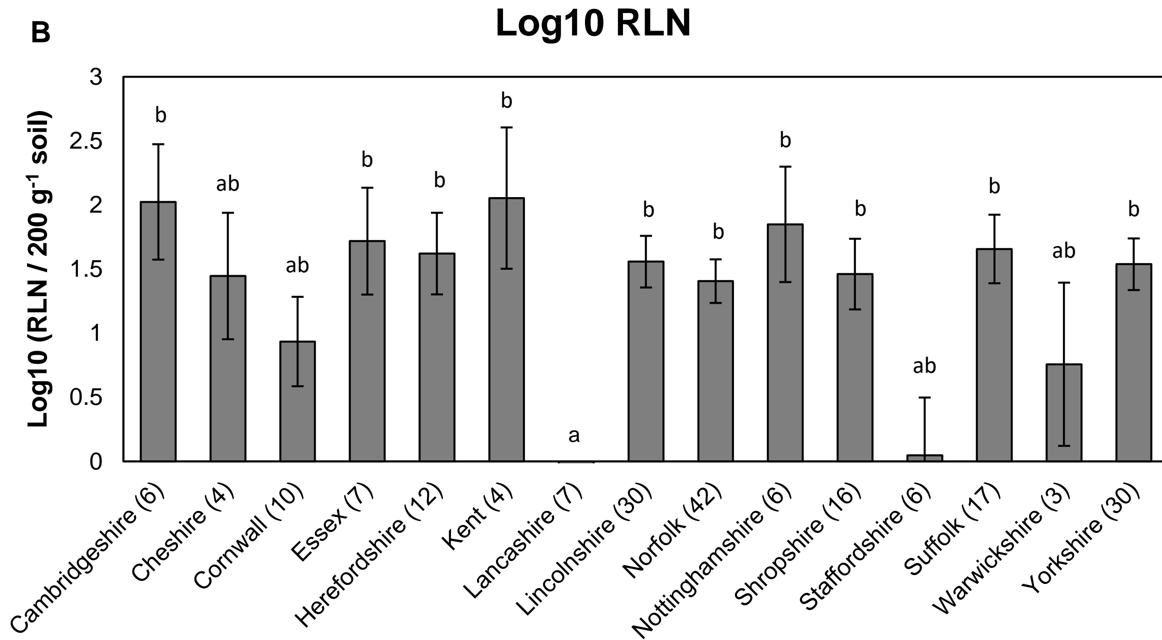
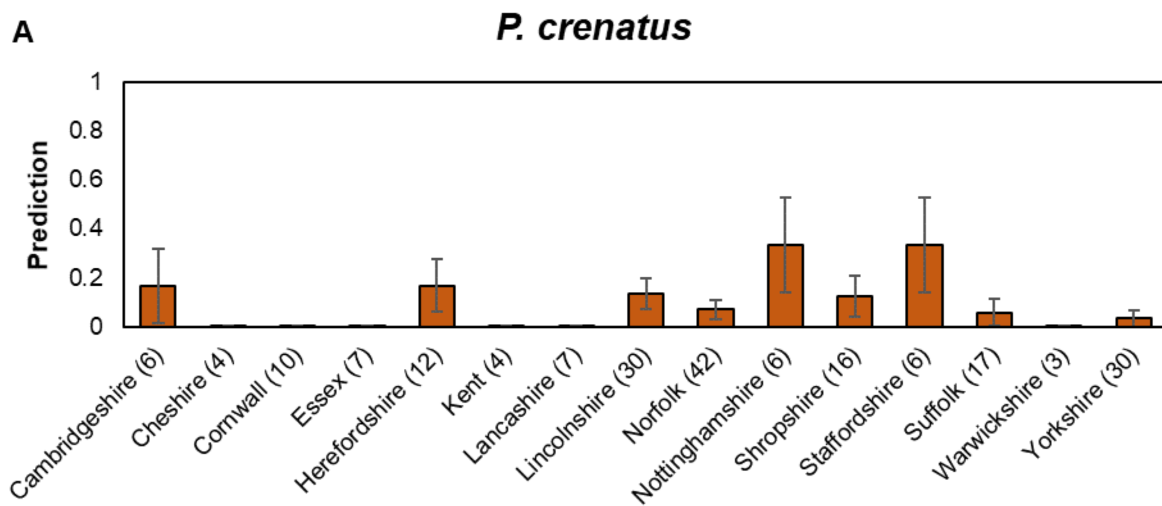


Figure 5.7: Prediction for the presence (A) and abundance (B) of root-lesion nematodes (RLN) in each county of England using a stepwise analysis of deviance with Bernoulli distribution for presence/absence and log10 transformation for abundance. Numbers between brackets are the number of fields in each county. Error bars represent the standard error of the mean. Letters represent significant differences according unbalanced ANOVA with Bonferroni test ($P < 0.05$).



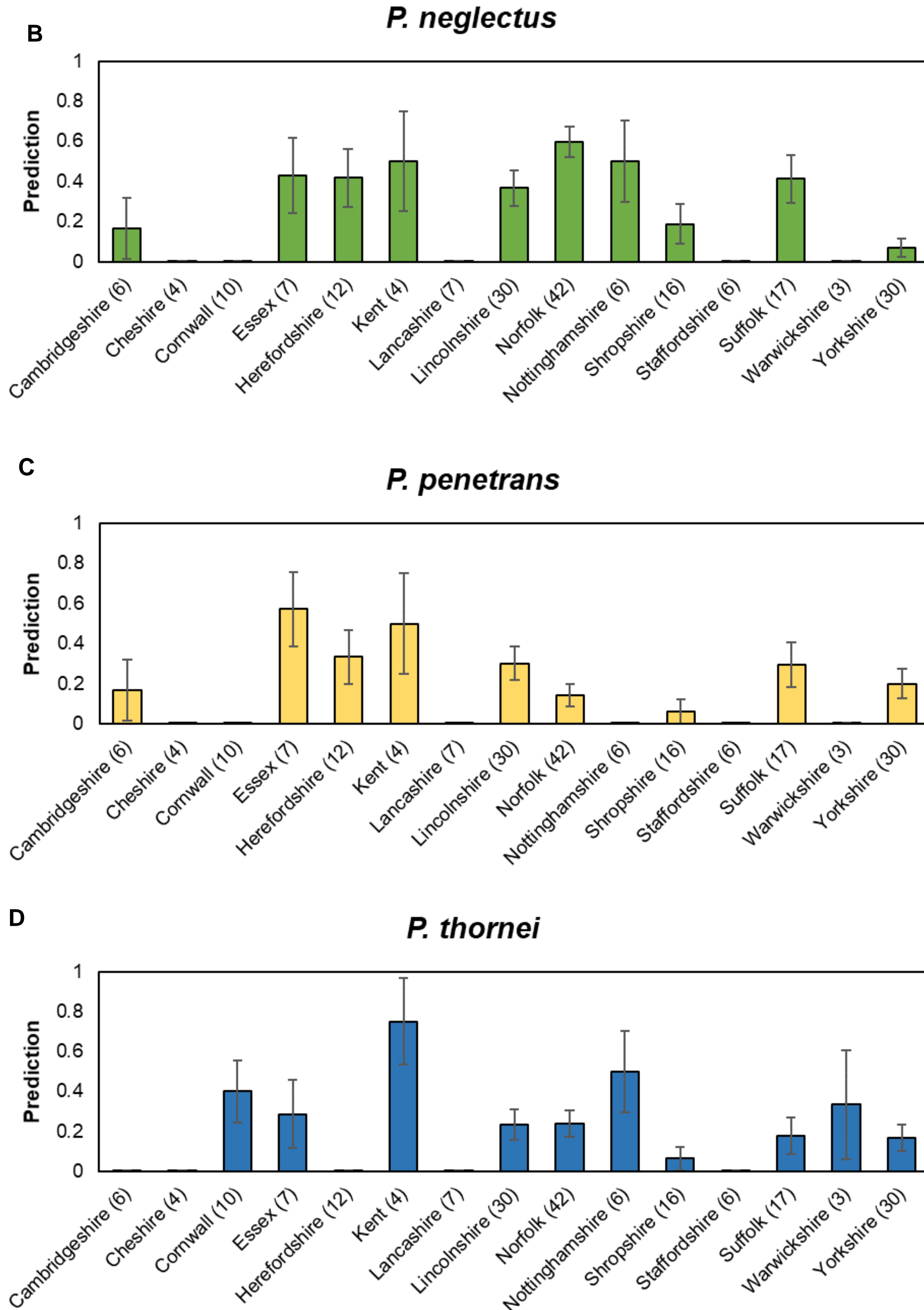


Figure 5.8: Prediction for the presence of *Pratylenchus neglectus* (A), *P. crenatus* (B), *P. penetrans* (C) *P. neglectus* and *P. thornei* (D) in each county of England. Numbers between brackets are the number of fields in each county. Error bars represent the standard error of the mean.

5.4.3 Survey samples and analysis

Two hundred soils were sampled between September and November of 2017 and 2019. Fifteen counties with the highest potato production were sampled using a stratified method. Overall, 48% of fields were sampled post-harvest of potatoes, 27% still had a potato crop in the field, 15% of fields were fallow and 10% had a cereal crop planted (Figure 5.9 A). Fallow included all fields with weeds, whereas post-harvest potatoes were fields presenting only soil at the time of sampling. Considering the previous crop, 50% of fields had wheat before potatoes and 25% had barley as the previous crop. Both these cereal crops can act as a good host for a variety of *Pratylenchus* species (Corbett, 1970; Mokrini *et al.*, 2018). Sugar beet and oats were previous crops in 6 and 3% of the samples, respectively. Other crops including carrots, cauliflower, mustard, onion, peas and pumpkins were the previous crop in 16% of fields (Figure 5.9 B). Both the crop at sampling and the previous crop did not significantly influence the distribution ($P = 0.61$ and $P = 0.124$), or abundance ($P = 0.733$ and $P = 0.1$) of root-lesion nematodes. Also, the presence of *P. crenatus* ($P = 0.881$), *P. penentrans* ($P = 0.145$), *P. thornei* ($P = 0.987$) and *P. neglectus* ($P = 0.77$) was not significantly influenced by the crop at sampling. Previous crop also did not significantly influence the presence of each species, *P. crenatus* ($P = 0.640$), *P. penentrans* ($P = 0.255$), *P. thornei* ($P = 0.758$) and *P. neglectus* ($P = 0.758$).

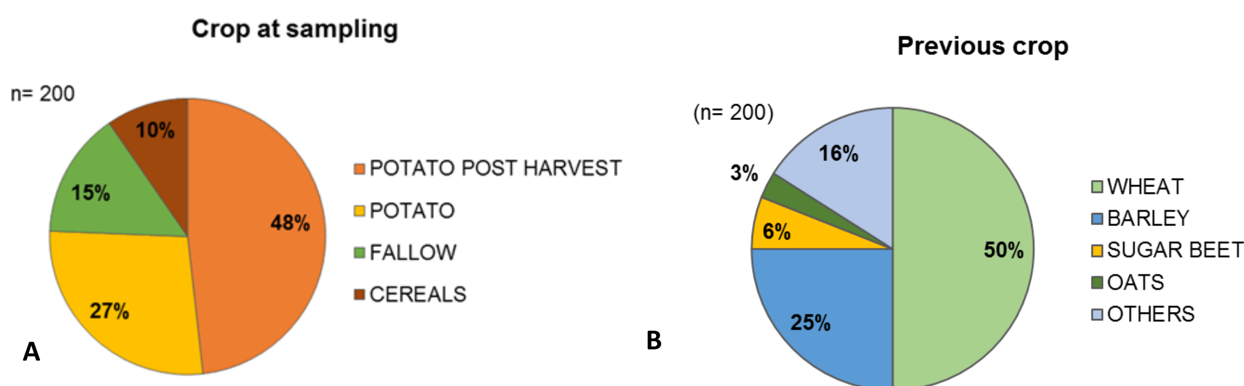


Figure 5.9: Percentage of each crop type at sampling (A) and as the previous crop (B).

Soil type is another important factor to consider for the distribution of root-lesion nematodes. In our survey, 41% of fields sampled were categorised based on farmer's information as a loamy soil, 24% sandy soil, 24% were sandy clay loams, 8% were sandy loams and only 3% were peaty (Figure 5.10). Nematicides were used in 48% of fields, with Vydate (oxamyl) used in 22% of the sites, Nemathorin (fosthiazate) used in 15% of sites and Mocap (ethoprophos) only used in 1% of the sites. The remainder of the fields sampled (52%) did not have a previous nematicide application within the last year (Figure 5.11). Irrigation can enhance nematode dispersion within fields and can be another

factor influencing root-lesion nematode distribution. In this study, 78% of fields were irrigated (Figure 5.12). In this study the presence and abundance of root-lesion nematodes were not significantly influenced by soil type (P = 0.659), nematicide (P = 0.123) or irrigation (P = 0.372). Similarly, each factor did not significantly influence the distribution and abundance of each single species, *P. crenatus* (P = 0.566; P = 0.436; P = 0.665), *P. thornei* (P = 0.659; P = 0.07; P = 0.359), *P. penetrans* (P = 0.823; P = 0.788; P = 0.256) and *P. neglectus* (P = 0.657; P = 0.630; P = 0.300).

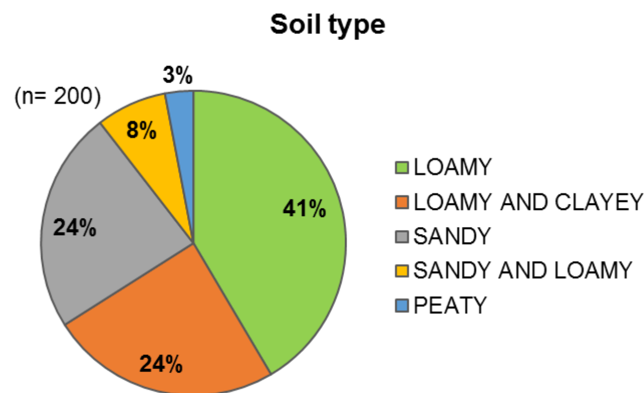


Figure 5.10: Percentage of each soil type from the potato fields sampled in England between 2017 and 2019 (n=200).

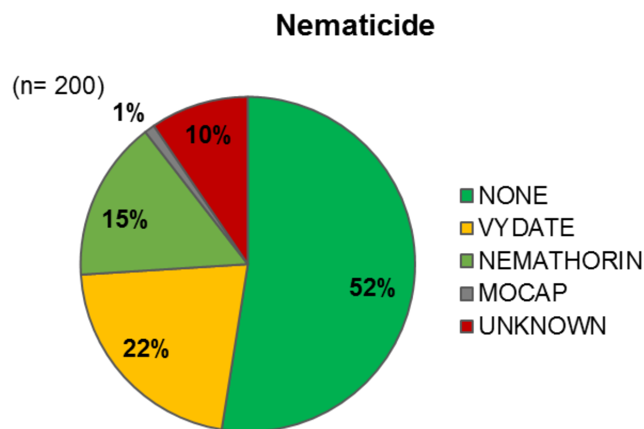


Figure 5.11: Percentage of nematicides applied in the fields sampled (n=200) in England between 2017 and 2019.

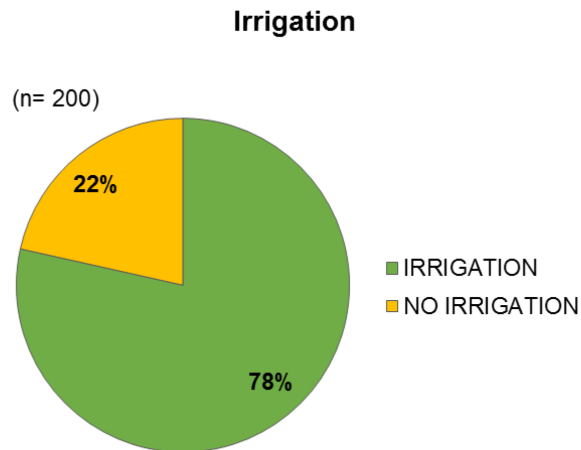


Figure 5.12: Percentage of irrigation in the fields (n=200) in England between 2017 and 2019.

The history of potato cyst nematodes (PCN) and root-lesion nematodes (RLN) were also recorded (Figure 5.13). Indeed, 45% of farmers indicated that PCN was present in their fields, 40% stated that PCN was not present and 15% did not provide an answer. Only 10% of the farmers indicated that RLN was present in their fields and the remainder (90%) did not know if RLN were present or not in their field.

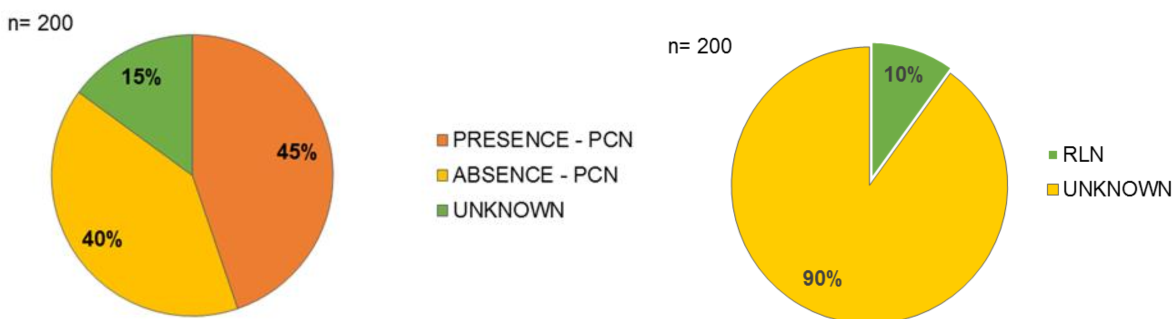


Figure 5.13: Percentage of farmers that confirmed the presence of potato cyst nematodes (left) and root-lesion nematodes (right) in the fields sampled (n=200) in England between 2017 and 2019.

A range of potato cultivars were grown in the field sites sampled, with some fields containing more than one cultivar (Figure 5.14). The most commonly grown was Maris Piper (11% of fields sampled), followed by Melody (7%), Accord (5%), Maris Peer (5%), Russet Burbank (5%), Marfona (5%), Estima (4%), Nectar (4%) and Sagitta (4%). King Edward, Olympus, Desiree, Premiere were grown in 3% of fields, whereas Royal and Taurus were each grown in 2% of fields. The remainder of sites were represented by a collection of lesser grown cultivars (35%).

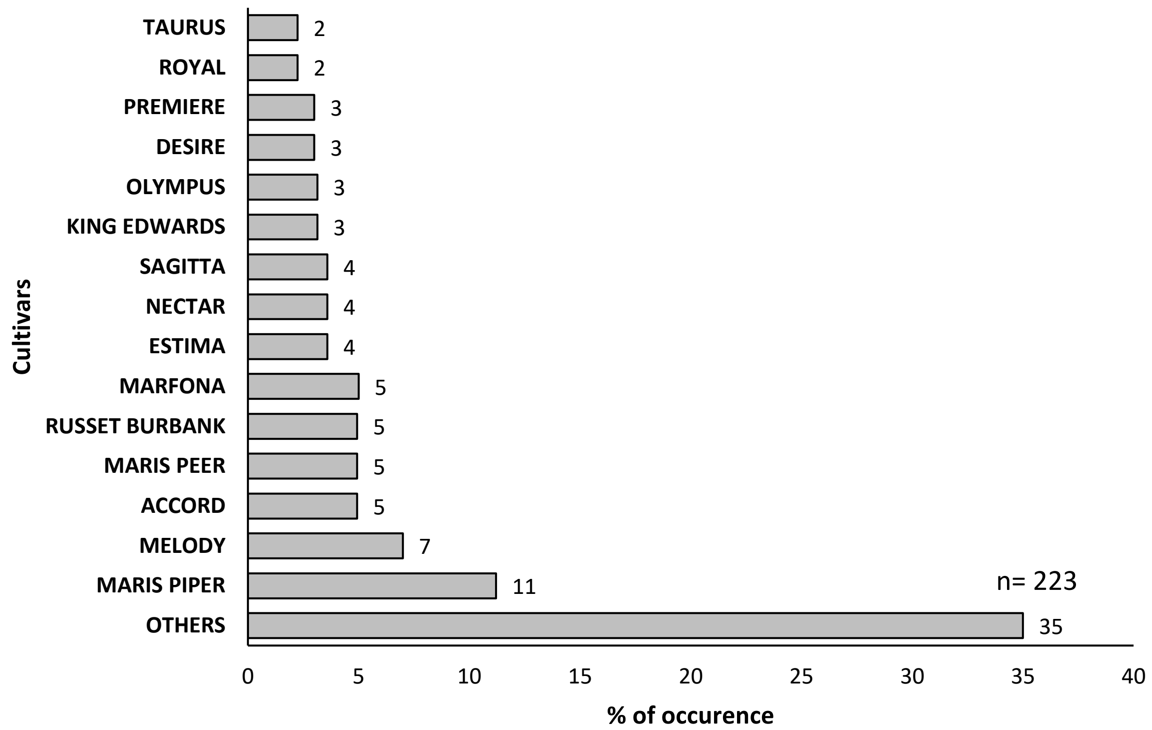


Figure 5.14: The choice of potato cultivar (n=223) grown in 200 fields sampled for a survey of *Pratylenchus* spp. in England.

5.4.4 Detection of root-lesion nematodes in Scotland

Eighteen samples from 18 potato fields of Scotland were included for the identification and quantification of root-lesion nematodes. *Pratylenchus* was detected in 94% of fields (Figure 5.15). Species were often mixed (83%) and only three fields contained single species (Table 5.3). The most common species was *P. neglectus*, being present in sixteen fields, followed by *P. crenatus* and *P. thornei* in fifteen and twelve fields, respectively (Table 5.3). *Pratylenchus penetrans* was found in ten fields.

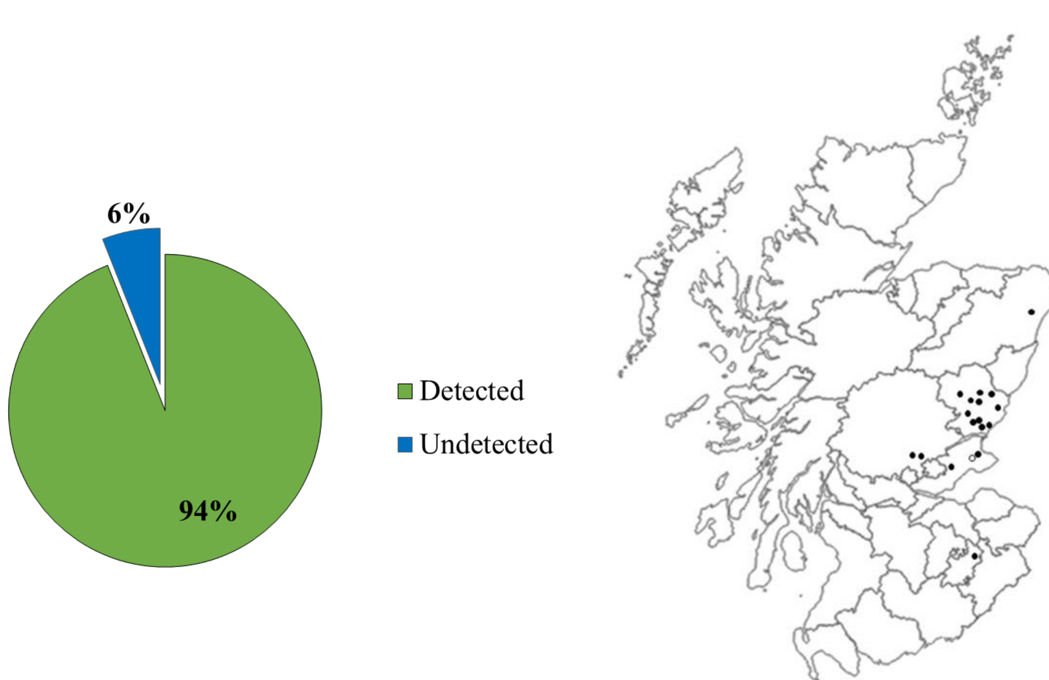


Figure 5.15: Percentage (A) and distribution map (B) of root-lesion nematodes in potato fields (n=18) from Scotland. Black dots indicate fields where root-lesion nematodes were positively detected, whereas white dots show fields without detection. Samples were collected by JHI between 2015 and 2017.

Table 5.3: Presence (+) and absence (-) of root-lesion nematodes in eighteen potato fields from Scotland, including the percentage of samples with mixed species.

ROOT-LESION NEMATODES IN SCOTLAND				
Fields	*P. crenatus*	*P. neglectus*	*P. penetrans*	*P. thornei*
3XM	+	+	-	+
2YS	+	+	+	-
2YT	+	+	-	+
2ZC	+	+	+	+
4XC	+	+	+	+
2ZNTL	+	+	+	+
L0	+	+	+	-
2LN	+	+	-	+
4XA	+	-	-	+
Q0	-	+	-	-
BO	+	+	+	-
2ZW	+	+	+	+
2YA	-	+	-	-
2ZEH	+	+	+	+
3ZW	+	+	+	+
MO	-	-	-	-
2ZS	+	+	+	+
3ZY	+	+	-	+
Percentage of samples with mixed species				83 %

In terms of estimated abundance, *P. crenatus* was found to be the most abundant in fields where it was detected (Table 5.4), whereas *P. neglectus* was generally found occurring at lower population densities. Whilst *Pratylenchus thornei* was less dispersed compared to other species, it was found at high densities in individual fields where it was detected, whereas *P. penetrans* showed the lowest dispersion and abundance at field sites in comparison with the other species.

Table 5.4: Densities (200 g^{-1} soil) of *Pratylenchus* spp. in eighteen samples from Scotland. Data are expressed as mean ± S.E.M.

ROOT-LESION NEMATODES IN SCOTLAND				
Fields	*P. crenatus*	*P. neglectus*	*P. penetrans*	*P. thornei*
3XM	136 ± 43	49	-	4 ± 0.73
2YS	334 ± 65	10 ± 4	52	-
2YT	337 ± 57	6 ± 0.8	-	10 ± 2
2ZC	311 ± 51	2864 ± 845	156	50 ± 30
4XC	663 ± 152	13 ± 2	31	649 ± 216
2ZNTL	78 ± 10	51 ± 3.1	698 ± 487	23 ± 9
LO	533 ± 50	10 ± 1.21	7 ± 1	-
2LN	96 ± 20	6	-	41
4XA	65 ± 21	-	-	-
QO	-	12 ± 1.4	-	-
BO	43 ± 6.4	12 ± 1.6	19	-
2ZW	89 ± 28	19 ± 2.2	2	4 ± 0.24
2YA	-	14 ± 2.5	-	-
2ZEH	377 ± 112	5 ± 0.52	34 ± 12	375 ± 170
3ZW	490 ± 101	3 ± 0.5	3 ± 0.3	423
MO	-	-	-	-
2ZS	379 ± 71	31	15 ± 8	497 ± 247
3ZY	957 ± 218	6	-	-

5.5 DISCUSSION

Root-lesion nematodes are often overlooked in potato fields, but they can be an important potato pests as reported in a range of studies (Olthof and Potter, 1973; Olthof, 1986; Riedel *et al.*, 1985; Holdago *et al.*, 2009; Bernard and Laughlin, 1976; Kimpiski, 1982; Martin *et al.*, 1982). Detection of *Pratylenchus* spp. in potatoes has been reported by authors mainly in North America (Kimpiski, 1979; Olthof and Wolynetz, 1991; Brown *et al.*, 1980; MacGuidwin, 1989; MacGuidwin and Stanger, 1991; Morgan *et al.*, 2002; Saeed *et al.*, 1998; MacGuidwin *et al.*, 2012), Australia (Harding and Wicks, 2007), The Netherlands (Van Der Sommen *et al.* 2009), Sweden (Björsell *et al.*, 2017; Viketoft *et al.*, 2017, 2020; Edin *et al.*, 2019), Norway (Holgado *et al.*, 2009; Holgado and Magnusson, 2012), Portugal (Esteves *et al.*, 2015; Gil *et al.*, 2021; Figueiredo *et al.*, 2021) and in Italy (Fantini *et al.*, 2019). However, there are no published data for the distribution and impact of these nematodes in potato growing land in a number of countries, including the United Kingdom. Indeed, there are few reports of root-lesion nematodes affecting crops in UK, although reports are available for lily, peas, bean, carrots and cereals (Corbett, 1969, 1970 a, b, 1972, 1973, 1974, 1976, 1983; Boag, 1979, 1980; Boag and Lopez-Llorca, 1989; Boag *et al.*, 1990; Dale and Neilson, 2006).

In this study, two hundred potato fields were sampled in England between September and November 2017 and 2019, focusing on the end of the potato growing season. Overall, 88% of fields in England were positive to *Pratylenchus* spp., confirming their wide presence across potato fields. In particular, *P. neglectus* (31%) and *P. thornei* (19%) were the most distributed mainly in East of England and in South East and South West, respectively, followed by *P. penetrans* (18%) in the East and South East of England, and *P. crenatus* (9%) in the East and West of England. Moreover, 31% of fields presented "other species" not identified but widespread in England. They could include *P. fallax, P. convallariae, P. pratensis, P. flakkensis, P. vulnus* and *P. pinguicaudatus* as each of these species has been reported to be present in the UK (Corbett, 1970a). Other than *P. vulnus*, the other species listed are not presently reported associated with potatoes. The limitation of having qPCR diagnostics available for four species, prevented the identification and quantification of these "other species" in this study. Although morphological identification, utilising the main characteristics for species diagnosis, was conducted prior to DNA extraction and qPCR, data were for limited individuals per samples and did not include morphometrics. The number of samples combined with the limited time available did not permit more detailed investigation of the other species found. The species were sometimes mixed together with two or three species in one sample, and similarly Corbett (1970 a) found *P. crenatus, P. fallax, P. neglectus, P. pinguicaudatus* and *P. thornei* in cereals of England and Wales. The same author reported *P. vulnus* in roses and *P. convallariae* in lily. Additionally, *P. pratensis, P. penetrans, P. fallax* and *P. flakkensis* were also reported in England (Southey, 1959; Seinhorst, 1968). These studies also confirm the co-occurrence of mixed species in their samples. Corbett (1970 b) provided a detailed description of British *Pratylenchus* spp. with a

corresponding key for identification. A survey was conducted during June and July 1966 to detect the species affecting cereals in England (Corbett, 1970 a), reporting the densities found in soil samples. The proportion of *Pratylenchus* in samples was often more than 50% of the total parasitic nematodes, except for four fields where they accounted for around 25-40%, confirming their abundance in barley and wheat. *Pratylenchus neglectus* was the most prevalent and abundant species found (between 1,000 and 11,000 per litre of soil), mainly present in barley fields in Nottinghamshire, Northamptonshire, Bedfordshire, Herefordshire, Lincolnshire, Norfolk, Suffolk and Wiltshire. It was found also in wheat fields of Yorkshire, Suffolk, Bedfordshire and Hertfordshire. In our results, *P. neglectus* was also the most prevalent RLN found in England, particularly in the East of England. Other species such as *P. crenatus, P. thornei* and *P. fallax* were also highly prevalent in the study of Corbett (1970 b) and often mixed with two or three RLN species in a single sample. *Pratylenchus crenatus* was present mainly in barley fields in Derbyshire, Northamptonshire, Herefordshire and Wiltshire, and in one wheat field in Bedfordshire mixed with *P. neglectus*. *Pratylenchus fallax* was recorded only in barley in Nottinghamshire, Derby and Leicestershire, whereas *P. thornei* was detected in barley fields in Lincolnshire, Northamptonshire, Norfolk, Suffolk, Bedfordshire, Hertfordshire and Wiltshire, and in wheat in Suffolk and Hertfordshire. *P. pinguicaudatus* was found only in one wheat field in Herefordshire, mixed with *P. neglectus* and *P. thornei.* In our results, *P. crenatus* was mainly distributed in the centre of England (East and West of England), while *Pratylenchus thornei* was mainly distributed in the South of England. Corbett (1970 a) did not report the presence of *P. penetrans*, while we found this species mainly in the North East and South East.

Cereals are often in rotation with potato, and in the current study, they represented 75% of previous crops. This agronomic information is certainly important for understanding the dispersion and prevalence of RLNs in potatoes. As in the studies by Corbett (1970 a,b), we also mainly found *P. neglectus, P. crenatus* and *P. thornei,* with species frequently occurring together at the same site; all of these species use cereals as a host plant. In this study, previous crop did not significantly influence the prevalence and abundance of each species tested, this may indicate that as these species have a wide host range, then they are unaffected by differences in rotation.

Generally, Corbett (1970 a, b) reported that *P. neglectus* occurred in all soil types, often in association with *P. thornei* in loamy and clay soils, whereas *P. crenatus* and *P. fallax* occurred in sandy soil. In contrast, in the current study, soil type did not significantly affect distribution and population densities of root-lesion nematodes and each individual species. However, *P. crenatus* was mainly found in peaty and sandy soil, *P. neglectus* in all soil types, although more abundant in sandy and loamy soils. Based on the analysis of other factors of the survey such as crop at sampling, use of nematicides and potato cultivar choice, no statistical differences were found to be related to the distribution and abundance of the genus and the species tested.

Eighteen samples from Scotland were also included and almost all fields (94%) showed the presence of root-lesion nematodes. Species were often mixed (70% of fields) and only four fields presented pure species. As in previous studies (Boag, 1979, 1980; Boag and Lopez-Llorca, 1989; Boag *et al.*, 1990; Dale and Neilson, 2006; Oliveira *et al.*, 2017), *P. neglectus* was the most common species found and was present in 89 % of fields, followed by *P. crenatus* (83 % of fields), *P. thornei* and *P. penetrans* (55% of fields, respectively). The most abundant species was represented by *P. neglectus* (2864 ± 845 nematodes 200 g^{-1} soil), followed by *P. crenatus* (957 ± 218 nematodes 200 g^{-1} soil), *P. thornei* (649 ± 216 nematodes 200 g^{-1} soil) and *P. penetrans* (698 ± 487 nematodes 200 g^{-1} soil). Similarly, a previous survey conducted in fifty-nine fields of Scotland on plant-parasitic nematodes associated with carrots revealed that *Pratylenchus* spp. was present in 63% of fields with a mean population of 31 nematodes 200 g^{-1} soil (Boag, 1979). In another survey on nematodes associated with cereals in Scotland, *P. crenatus* was found in 55% of fields, whereas *P. penetrans* and *P. thornei* was only found in 2% of fields, and *P. neglectus* was present in very few samples (number not specified). The abundance of *P. crenatus* was up to 550 neamtodes 200 g^{-1} soil in the heavy infested fields, and data suggested that *P. crenatus* preferred acidic soils because they were found mostly in soil with low pH and numbers significantly increased when pH decreased. Boag and Lopez-Llorca (1989) reported the presence of *P. crenatus* in 19% of thirty-one fields of cereal production, with an average of 37 nematodes 200 g^{-1} soil, occuring mainly in loamy soil. Also, twenty-six fields of permanent pasture were tested, showing that *P. crenatus* and *P. thornei* were both present in 8% of fields, showing a preference for sandy loam soils. However, the RLNs were found at low densities, with 20 *P. crenatus* 200 g^{-1} soil and 8 *P. thornei* 200 g^{-1} soil. A recent study conducted by Oliveira *et al.* (2017) included 235 potato fields across Scotland and they confirmed the high occurrence of *P. crenatus, P. neglectus* and *P. penetrans* in Scottish potato fields. Species were often mixed in the same samples as those in the present study. *P. crenatus* was the most prevalent and abundant (59 ± 40; 6-176 nematodes 200 g^{-1} soil), followed by *P. penetrans* (16 ± 22; 0-96 nematodes 200 g^{-1} soil) and *P. neglectus* (9 ± 13; 0-57 nematodes 200 g^{-1} soil). The most frequently occuring RLN species were *P. neglectus* and *P. crenatus* but the most abundant species was *P. neglectus*, confirming these two species are very common and abundant in potato fields in Scotland.

Few studies have been reported on root-lesion nematodes in potatoes in Europe (Holgado *et al.*, 2009; Van Der Sommen *et al.*, 2009; Esteves *et al.*, 2015; Fantini *et al.*, 2019). In the Netherlands, root-lesion nematodes were detected in 61% of fields surveyed with *P. neglectus* being the most abundant species, with *P. penetrans* cited as being the most damaging (Van Der Sommen *et al.*, 2009). In Portugal, Esteves *et al.* (2015) conducted a survey in forty potato fields with root-lesion nematodes detected in 83% of soil samples and 78% of root samples, with an average of 104 nematodes 200 g^{-1} soil. Contrary to this study, *P. penetrans* was the most abundant species, being found in 42% of the samples. Additionally, *P. neglectus*, *P. crenatus* and *P. thornei* were recorded

in 35%, 13% and 3% of the samples, respectively. In a recent study conducted in 2019 in potato fields of Salerno (Italy), morphological and molecular identification revealed the presence of two mixed species, *P. neglectus* and *P. thornei*, both common in Italy but mainly in cereals (Fantini *et al.*, 2019).

Crop damage is related to population densities of RLN (Schomaker and Been, 2013), and for this reason it is important to use diagnostics to determine the range of species present and adopt a strategic management plan. In the current study, positive fields for each species had a range of 1-6558 nematodes 200 g^{-1} soil for *P. neglectus*, 1-540 nematodes 200 g^{-1} soil for *P. thornei*, 1-1037 nematodes 200 g^{-1} soil for *P. penetrans* and 10-2406 nematodes 200 g^{-1} soil for *P. crenatus* were found in England. In Scotland, positive fields had a range of 92-957 nematodes 200 g^{-1} soil for *P. crenatus*, 3-2864 nematodes 200 g^{-1} soil for *P. neglectus*, 4-650 nematodes 200 g^{-1} soil for *P. thornei*, and 3-156 nematodes 200 g^{-1} soil for *P. penetrans*. Potato damage thresholds for root-lesion nematodes have been reported to be 200-400 nematodes 200 g^{-1} soil for *P. penetrans* (Olthof and Potter, 1973; Holdago *et al.*, 2009) and 120-300 nematodes 200 g^{-1} soil for *P. neglectus* (Olthof, 1990; Umesh and Ferris, 1994). Comparing the data of this study on the abundance of each species with published damage thresholds, we found that fifteen fields (7.5 % of total field sampled) exceeded damage thresholds for *P. neglectus* and thirteen (6.5% of total field sampled) for *P. penetrans* in England. In Scotland, only one field exceeded the damage threshold for each of the two species. So far, there are no damage thresholds reported for *P. crenatus* and *P. thornei* on potato to compare with current data of this study. Results highlight that root-lesion nematodes have the potential to cause damage to potato crops in Great Britain. Damage thresholds vary among species, cultivars, soil type and other biotic factors, so further studies on this topic should be conducted before recommendations are made for managing root-lesion nematodes in potatoes. Generally, potato soil sampling is best done prior to planting usually between October to March. Once the presence and identity of plant-parasitic nematodes has been established, decisions of strategic management can be taken before planting.

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Chapter 6

6. DEVELOPMENT AND YIELD OF POTATO MARIS PEER IN SOIL INFESTED WITH *PRATYLENCHUS PENETRANS* AND *P. THORNEI*

6.1 ABSTRACT

The potato, *Solanum tuberosum*, represents one of the most important crops in Great Britain with Maris Peer being in the top ten most popular cultivars in the country. Fifteen species of root-lesion nematodes (RLN) are known to infect potatoes, although *Pratylenchus penetrans* is generally considered to be the most damaging species. Three controlled environment experiments investigated the impact of *P. penetrans* and *P. thornei* on yield and quality loss of Maris Peer potatoes. The first assay assessed the impact of mixed juveniles and adult populations of *P. penetrans*, ranging from 0.125 to 4 nematodes g^{-1} soil, on growth and yield of Maris Peer, in three different sandy based soils. Different proportions of coarse sand and compost (John Innes No. 2) were mixed to form three different sandy based soils: ST1 (10% compost and 90% coarse sand), ST2 (20% compost and 80% coarse sand) and ST3 (40% compost and 60% coarse sand). Nematode density had no effect on potato yield at six weeks after inoculation and planting, although the yield was significantly affected by soil type with ST1 giving the lowest yield. However, root-lesion nematodes were detected within the roots of potatoes grown in each soil type, highlighting that *P. penetrans* was able to infect this cultivar without inducing yield loss at these population densities. Two further controlled experiments using a broader range of nematode densities, from 2 to 32 nematode g^{-1} soil were conducted using ST1 with *P. penetrans* and *P. thornei*. As with the first experiment, yield was not significantly affected by nematode densities even though root lesions were recorded among all treatments, confirming invasion occurred. In all three assays, final population density (P_f) was less than the initial population density (P_i) in all treatments, suggesting that reproduction was limited on Maris Peer indicating partial resistance by this cultivar. The length of experiment and other biotic factor such as temperature, moisture or soil matrix under pot trials may have influenced the reproduction of nematodes. Further studies using other GB potato cultivars, other common root-lesion nematode species like *P. neglectus* or *P. crenatus*, considering also stress factors such as drought conditions, may help provide a better understanding of the nematode's pathogenicity and yield losses in potato GB cultivars.

6.2 INTRODUCTION

Solanum tuberosum Maris Peer is one of the most popular potato cultivars in Great Britain being ranked at 7th position in 2020 (AHDB, 2020). Maris Peer is a second early potato crops that usually is planted between March and May, and then harvest by September, approximately 13 weeks from planting when the foliage begins to turn yellow. Tubers of Maris Peer have a firm, creamy yellow flesh and have all-round shape, being ideal for boiling, mashing, roasting and frying. This potato cultivar shows high yields and a good resistance to diseases like powdery scab, gangrene, damage, bruising and skin spot, but it is susceptible to potato cyst nematodes *Globodera rostochiensis* and *G. pallida* (AHDB, 2021). Indeed, plant-parasitic nematodes are one of the major pests of potatoes, affecting the yield and the quality of the tubers, which then impact upon their marketability (Palomares-Rius *et al.*, 2014). There are plant parasitic nematodes infesting potato plants that are polyphagous such as *Meloidogyne, Nacobbus* and *Pratylenchus*, while other species are highly host specific such as potato cyst nematodes, *Globodera* spp. and the tuber rot nematode, *Ditylenchus destructor* (Nicol *et al.*, 2011). Moreover, nematodes such as *Trichodorus* spp. and *Paratrichodorus* spp., cause damage due to feeding on the roots and by transmitting Tobacco Rattle Virus (TRV) to the plant (Taylor and Brown, 1997). *Pratylenchus neglectus* and particularly *P. penetrans* are the most damaging root-lesion nematode species infecting potatoes (Morgan *et al.*, 2002; Palomares-Rius *et al.*, 2014), but there are also reports for other pathogenic species such as *P. crenatus*, *P. alleni, P. scribneri* and *P. thornei* (Brodie *et al.*, 1993). Symptoms of root-lesion infestation in potato plants include patches in the field of poor growth plants with yellow foliage, brown-reddish spots on the areas of the roots where the nematode feed and small lesions occurring on the stolons and tubers (Brodie *et al.*, 1993; Holgado *et al.*, 2012; MacGuidwin and Rouse, 1990). The lesions on the surface of tubers can be brown to black, turning purple over time (Palomares-Rius *et al.*, 2014; Figueiredo *et al.*, 2021). Depending on the species, different symptoms can also be present on the tubers. For instance, *P. scribneri* causes tubers to have a scabby appearance, *P. penetrans* has been associated with wart-like protuberances (Brodie *et al.*, 1993), whilst *P. crenatus* produces grey-brown sunken cracked lesions (Olthof *et al.*, 1982). Recently, Figueiredo *et al.* (2021) found lesions induced by *P. penetrans* appeared as subtle dark spots or minor cross lesions on the potato skin.

Studies on the thresholds of root-lesion nematodes for potato damage have been previously published in Canada (Olthof,1986, 1990; Kimpinski, 1988); and Scandinavia (Holgado *et al.*, 2009). For *P. penetrans* and *P. scribneri*, densities of 1 - 2 nematodes g^{-1} soil have been reported to cause damage to potatoes (Olthof and Potter, 1973; Olthof, 1986; Riedel *et al.*, 1985) whereas *P. neglectus* can induce damage at densities of around 0.6 nematodes g^{-1} soil (Olthof, 1990). In Norway, potato growth (cv. Saturna) was correlated negatively with densities of *P. penetrans*, and a damage threshold of 0.4 nematodes per g^{-1} soil was estimated to cause a yield reduction of 50% (Holgado *et al.*, 2009). However, damage threshold values can change according to the susceptibility/tolerance

of each cultivar and other environmental factors that may affect the establishment of damage thresholds such as soil texture and temperature (Castillo and Vovlas, 2007).

To date, there are few or no reports about damage thresholds of *Pratylenchus* spp. on GB potato cultivars. In this study, the yield and quality loss of potatoes caused by *P. penetrans* and *P. thornei* and damage thresholds were investigated under controlled conditions using the cultivar Maris Peer.

6.3 MATERIALS AND METHODS

6.3.1 Monoxenic culture of P. penetrans and P. thornei

Initial populations of *P. penetrans* and *P. thornei* were obtained from established carrot-disk cultures supplied by ILVO (Belgium) (Figure 6.1). Monoxenic cultures of *Pratylenchus* spp. were assessed using the method described by Speijer and De Waele (1997). Petri dishes were sealed with parafilm and incubated at 23°C in the dark within an incubator (Panasonic, MIR-154-PE).

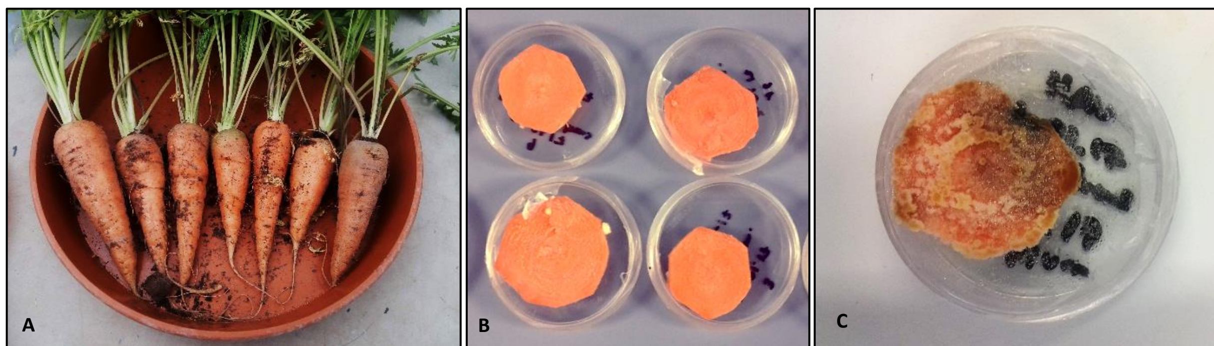

Figure 6.1: Carrots of the variety Nantes 2 grown in pots in the glasshouse (A); monoxenic culture of *Pratylenchus* spp. on carrot discs before infection (B) and after 3 months of infection (C).

6.3.2 Nematode inoculum

After nematode extraction using the modified Baermann method (Whitehead and Hemming, 1965), different initial population densities (P_i) were used to infect each pot (see experiments for specific details) (Table 6.1). Water was used for untreated pots. Before using the inoculum, suspensions were checked visually under a stereomicroscope at 40X magnification (Mazurek Optical Service, Meiji EMT) to confirm the viability of nematodes. Nematodes were inoculated at planting time using a glass pipette to equally distribute 10 mL of suspension into five holes of approximately 1 cm depth around the tuber.

Table 6.1: Log series of root-lesion soil population densities used for each treatment and experiment.

Experiment 1							
Treatments	Control	1	2	3	4	5	6
Log series	0	2^{-3}	2^{-2}	2^{-1}	2^{0}	2^{1}	2^{2}
g^{-1} soil	0	0.125	0.25	0.5	1	2	4
Pot (250 g^{-1} soil)	0	31	62	125	250	500	1000
Experiments 2 - 3							
Treatments	Control	1	2	3	4	5	
Log series	0	2^{1}	2^{2}	2^{3}	2^{4}	2^{5}	
g^{-1} soil	0	2	4	8	16	32	
Pot (500 g^{-1} soil)	0	1000	2000	4000	8000	16000	

6.3.3 Experiment 1

The experiment was carried out from July to August 2017 at Harper Adams University over 6 weeks. A dilution series of *P. penetrans* was made according to a log series of 2^x, with x ranging from −3 to 2, giving 7 population densities (0, 0.125, 0.25, 0.5, 1, 2, 4 nematodes g^{-1} soil of *P. penetrans*). Densities were calculated based on the total weight of 250 g soil per pot (Table 6.1). Three different types of soils were created using mixtures of John Innes N°2 (loam, peat, coarse sand and base fertiliser, John Innes Manufacturers Association, Reading, UK) and coarse sand to produce a total weight of 250 g^{-1} soil per pot. Soil type 1 (ST1) was prepared mixing 10% JI N°2 and 90% coarse sand, soil type 2 (ST2) with 20% JI N°2 and 80% coarse sand, and soil type 3 (ST3) contained 40% JI N°2 and 60% coarse sand. Four replicates for each combination of nematode density and soil type were used for a total of 84 pots in a complete randomized block design.

6.3.4 Experiment 2 and 3

The second and third experiments were carried out from May to August 2018 at Harper Adams University each over a period of 11 weeks. Six population densities (0, 2, 4, 8, 16, 32 nematodes g^{-1} soil) of *P. penetrans* (experiment 2) and *P. thornei* (experiment 3) were used. Densities were calculated based on the total weight of 500 g^{-1} soil per pot (Table 6.1). Schomaker and Been (2013) also reported that, in small pots, plants had a similar growth whether they were grown in soil with or without the presence of plant parasitic nematodes due to limited root development (plants becoming pot bound). For these reasons, experiments 2 and 3 were modified with 500 g soil pot volumes and a greater range of nematode densities than the previous experiment. Considering the results of experiment one, ST1 (10% JI N°2 and 90 % coarse sand) was used for the other two experiments and six replicates for a total of 36 pots were arranged in a Latin square design for each experiment.

6.3.5 Pot preparation and watering

For all experiments, mini-tubers of the cultivar Maris Peer were chitted for 2 weeks at room temperature before planting in order to produce apical sprouts of ca. 3 mm in length. A single mini-tuber was placed at the centre of each pot at 3 cm depth. The pots were kept within an environmental test chamber (Sanyo, MLR-350), which had a daytime temperature of 20°C and a night-time temperature of 10°C and a 16h photoperiod. Water was applied uniformly to each pot, every 2 days, by monitoring the % moisture content using a moisture probe (ProCheck Sensor, Decagon devices) at the rate equivalent to that typically applied through irrigation under field conditions. In experiments 2 and 3, nutrients (Hoagland's No. 2 Basal Salt Mixture) were supplied weekly, after 5 weeks.

6.3.6 Assessment of plant development and root lesion severity

After planting, each plant was monitored to determine the day of emergence and numbers of shoots. Length of shoots were measured throughout the growing period, once per week, using a standard ruler. At harvest, numbers of tubers, weights of fresh shoots, roots, biomass (sum of weights of fresh shoots and roots) and tubers were determined for each pot. For experiments 2 and 3, the severity of root lesion symptoms was assessed using the disease assessment key described in Table 6.2.

Table 6.2: Root-lesion nematode score index based on the numbers of lesions found in fresh root samples.

Score	Number of lesions on roots
1	No lesions
2	1-5 lesions
3	>5 lesion

6.3.7 Estimation of nematode population density in soil and potato roots

Nematodes were extracted from the soil of each pot using the modified Baermann method (Whitehead and Hemming, 1965) and counted in DeGrisse slides under a stereomicroscope at x40 magnification (Mazurek Optical Service, Meiji EMT). Final nematode population densities (P_f) were then calculated following the following formula: -

$$Pf(soil) = Total\ numbers\ of\ nematodes\ in\ subsample\ x\ \frac{total\ volume\ of\ suspension\ (20\ ml)}{volume\ of\ subsample\ (1\ ml)}$$

Two grams of roots were washed with water to remove soil and cut in pieces of about 1 cm in length. Roots were stained following the acid fuchsin method described by Hooper (1986). Extracted nematodes were counted in a DeGrisse slide under a stereomicroscope at x40 magnification (Mazurek Optical Service, Meiji EMT) and expressed as nematodes g^{-1} root following the formula below:

$$Nematodes\ \ g^{-1}\ root\ = \frac{Total\ nematodes\ in\ 2\ ml\ subsample\ x\ 100}{Weight\ of\ root\ sample\ in\ g}$$

Reproduction factor (R_f) was calculated for each experiment with the following formula:

$$R_f = P_f / Pi$$

P_f is the final population density and Pi is the initial population density inoculated in each pot.

6.3.8 Statistical analysis

Weight of fresh shoots, roots, biomass (sum of weights of fresh shoots and roots) and tuber data were subjected to two-way analysis of variance (ANOVA) to assess the effect of soil type and nematode densities on potato (experiment 1). Similarly, a one-way analysis of variance (ANOVA) for experiment 2 and 3 was performed to assess the effect of nematode densities on potato. Treatment means were compared by a Bonferroni test ($P < 0.05$). Kruskal-Wallis one-way ANOVA with pairwise comparison post hoc test was performed for root-lesion index data analysis. Statistical analyses were performed by using Genstat (19th edition, VSN International Ltd, UK).

6.4 RESULTS

6.4.1 Experiment 1

Almost all mini tubers produced potato plants within 3 weeks with only three pots within soil type 2 (ST2) that failed to emerge. For day of emergence with two way-ANOVA, there were no significant differences among the different nematode densities and soil types. During the five-week observation period, potato plants attained final heights of 8.9 ± 1.02, 9.6 ± 1.05 and 9.9 ± 1.24 cm, for each type of soil (ST1, ST2 and ST3), respectively. Although, potatoes grown in ST1 showed less growth compared to the other plants during the period of development (Figure 6.2), no statistical differences were found between the different soil types. Additionally, there were no significant differences in plant height between different nematode densities or an interaction between densities and soil type.

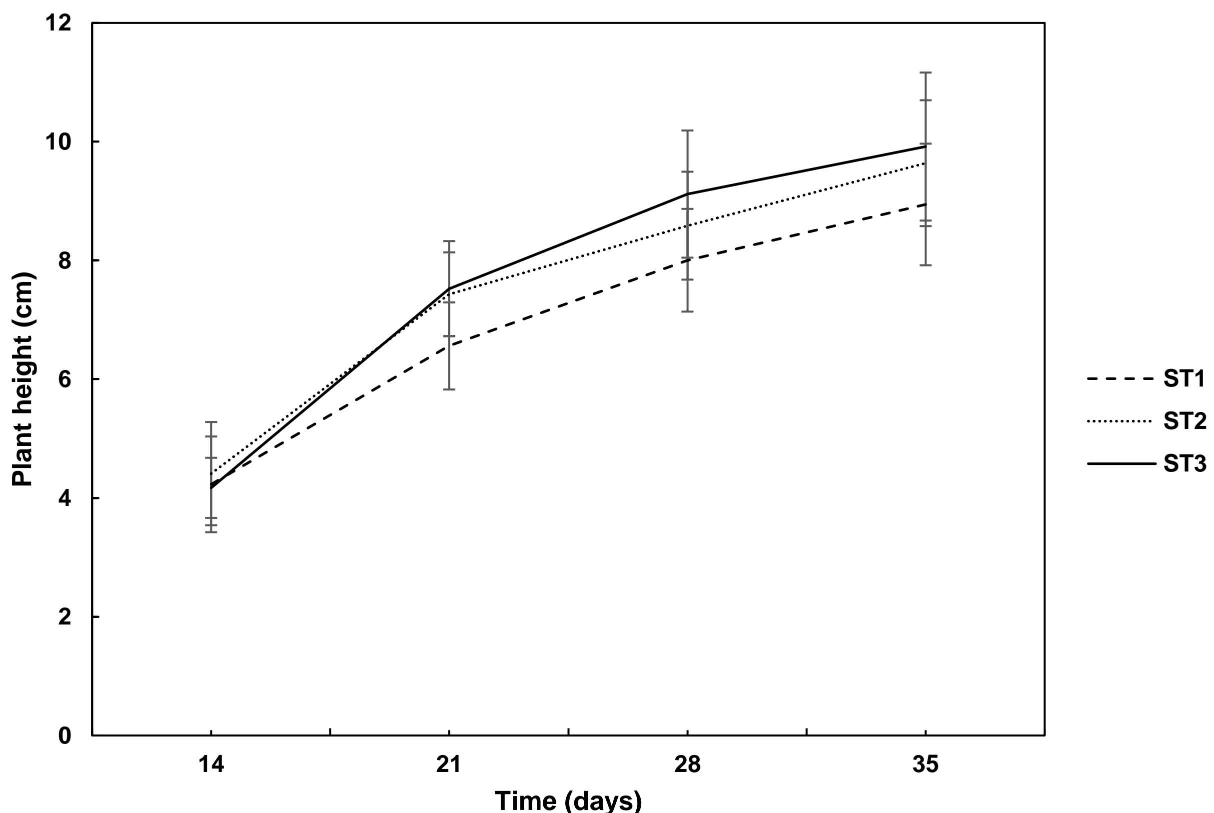

Figure 6.2: Plant height (cm) of potatoes growing in three different soil types, ST1 (10% JI N°2 and 90 % coarse sand), ST2 (20% JI N°2 and 80% coarse sand) and ST3 (40 % JI N°2 and 60% coarse sand) for 5 weeks under controlled conditions. Data are means (n = 28, collected at 14, 21, 28 and 35 days after planting). Bars represent the standard error of the mean.

After six weeks, plants grown in soil type 1 (ST1) and soil type 2 (ST2) were less developed than those grown in soil type 3 (ST3) (Figure 6.3). Height and fresh weight of haulms, fresh weight of roots and fresh weight of tubers were significantly affected by soil type (Figure 6.4). Plant height was significantly affected ($P = 0.012$) by the soil type used, with those growing in soil type 3 having a greater height than those grown in soil type 1 (ST1) (Figure 6.4-A). Considering fresh weights of shoots (Figure 6.4-B), all three types of soil were all significantly different from each other ($P < 0.001$), with ST3 plants weighing 11 g, compared with ST1 and ST2 plants weighing 4.2 ± 0.22 g and 5.8 ± 0.4 g, respectively. Also, the roots of the plants grown in ST3 were significantly heavier ($P < 0.001$) than those grown in ST1 and ST2 (Figure 6.4-C). Total weight of tubers (Figure 6.4-D) was affected by soil type ($P < 0.001$) with ST3 producing a higher yield of potatoes (c. 4 g) than the other two soil types (c. 2 g). Initial population densities (P_i) of *P. penetrans* did not significantly affect the development of plants and tubers in each soil type. However, eggs and adults were detected inside roots of almost all treatments (Figure 6.5 A-C) confirming that RLN invasion on roots occurred.

Figure 6.3: Examples of potato plants growing in three different soil types; ST1 (10% JI N°2 and 90 % coarse sand), ST2 (20% JI N°2 and 80% coarse sand) and ST3 (40 % JI N°2 and 60% coarse sand), harvested after 6 weeks.

Final population densities (P_f) were calculated for each treatment, and in each soil type group, densities increased with increasing P_i, but in general, the P_f were lower than the P_i (Table 6.3). At the highest density, there were higher number of nematodes recovered from soil with more nematodes occurring in ST1 (139 ± 24) compared to ST3 (61 ± 12.6). However, there were no significant differences ($P = 0.217$) between the P_f for soil types. The highest density of nematodes found in the roots was in ST1 (313 ± 96.5). The initial population densities of 125 g^{-1} soil in ST1 had the highest ($P = 0.01$) reproduction factor ($R_f = 1.12$) compared to the other densities. However, soil types did not significantly ($P = 0.06$) affect the reproduction of nematodes (Table 6.4).

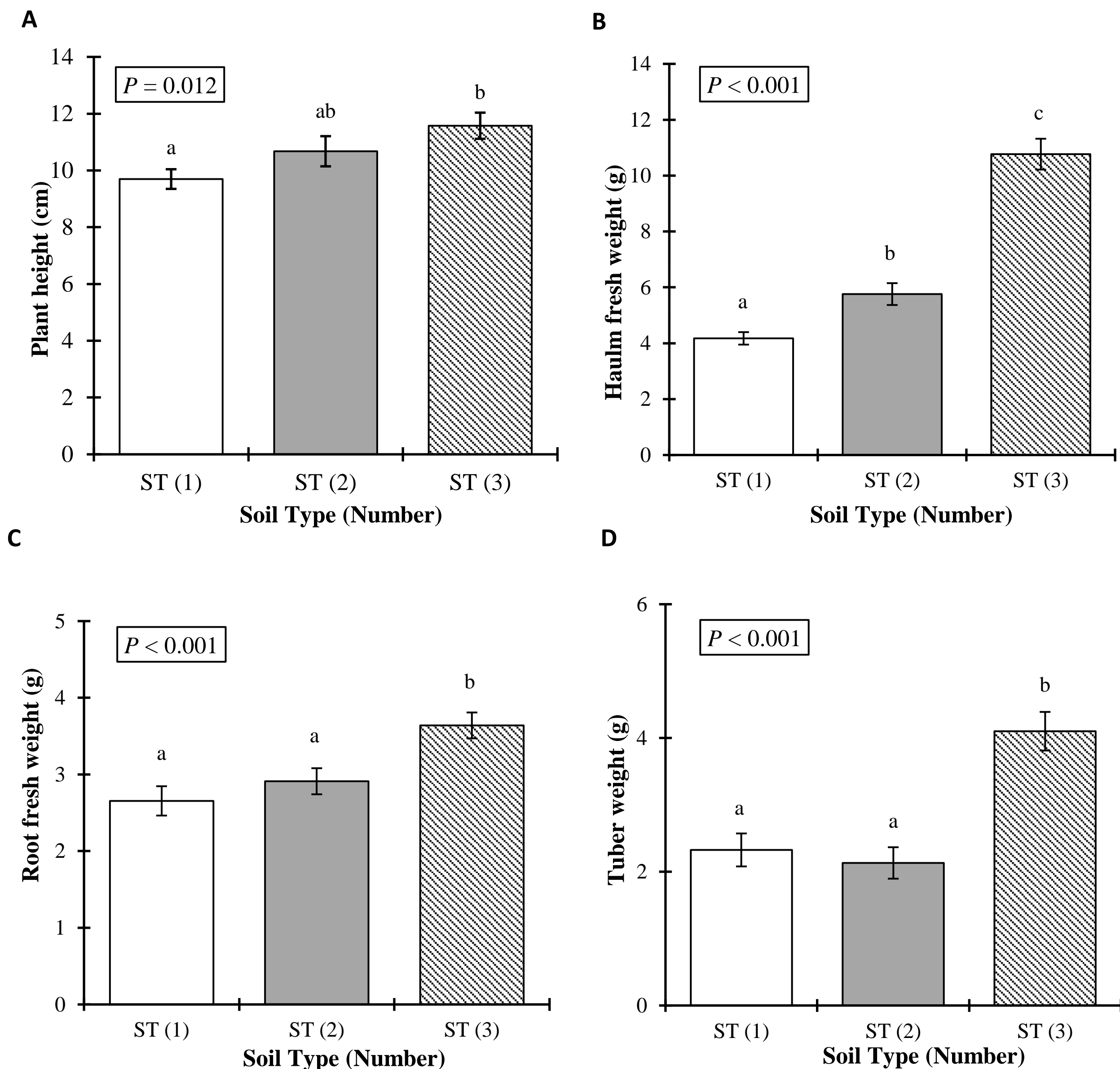

Figure 6.4: Plant height (cm) (A), haulm fresh weight (g) (B), root fresh weight (g) (C), and tuber weight (g) (D) of potatoes cv. Maris Peer growing in three different soil type, ST1 (10% JI N°2 and 90 % coarse sand), ST2 (20% JI N°2 and 80% coarse sand) and ST3 (40 % JI N°2 and 60% coarse sand) at six weeks after planting. Data are means (n = 28) ± standard error of the mean. Columns with different letters are significantly different according to the Bonferroni's test ($P < 0.05$).

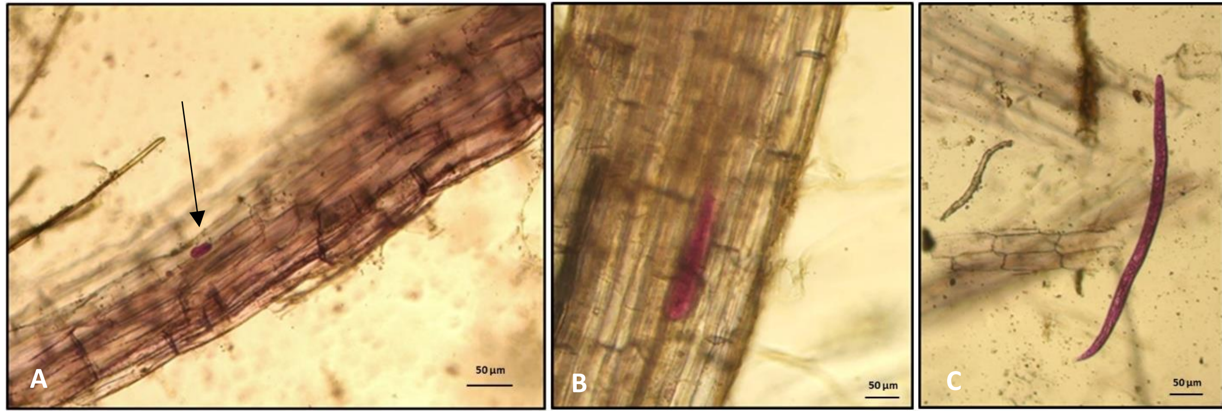

Figure 6.5: Egg (A) and adults (B-C) of *Pratylenchus penetrans* inside potato root tissues after root staining with acid fuchsin method (Hooper, 1986). Arrow indicate the position of the egg in the root. Pictures were taken at 40X magnification.

Table 6.3: Final population densities (P_f) of *P. penetrans* in soil (250 g^{-1} soil) and roots (g^{-1} root). Data are means (n = 4) ± standard error of the mean. ST= soil type. ST1 (10% JI N°2 and 90 % coarse sand), ST2 (20% JI N°2 and 80% coarse sand) and ST3 (40 % JI N°2 and 60% coarse sand). Data are means ± standard error of the mean.

EXPERIMENT 1 – *P. penetrans*							
Initial population density (P_i)	Population density per pot (P)	Final Population (P_f) Soil (250 g^{-1} soil)			Root invasion (Nematode g^{-1} root)		
		ST1	ST2	ST3	ST1	ST2	ST3
0	0	0	0	0	0	0	0
0.125	31	3 ± 1.4	2 ± 1.5	4 ± 2.6	0	0	0
0.25	62	7 ± 1.8	4 ± 2.3	2 ± 1.6	13 ± 13	13 ± 12.5	0
0.5	125	22 ± 11.4	20 ± 10.7	19 ± 8.7	91 ± 36.6	50 ± 50	0
1	250	34 ± 16.5	33 ± 7.6	32 ± 9.7	0	123 ± 78	25 ± 25
2	500	57 ± 19.8	53 ± 9.3	53 ± 20.5	192 ± 83	50 ± 50	71 ± 29
4	1000	139 ± 24	127 ± 44	61 ± 12.6	313 ± 96	263 ± 82	222 ± 28
		P-value ST		0.217	*P-value* ST		0.166
		P-value P		<0.001	*P-value* P		<0.001
		P-value STxP		0.291	*P-value* STxP		0.357
		S.E.D. ST		11.5	S.E.D. ST		21.7
		S.E.D. P		7.53	S.E.D. P		33.15
		S.E.D. STxP		19.92	S.E.D. STxP		57.4
		% CV		26.2	% CV		37.4

Table 6.4: Reproduction factor (R_f) of *P. penetrans* based on recovery from soil and root samples**.** ST= soil type. ST1 (10% JI N°2 and 90 % coarse sand), ST2 (20% JI N°2 and 80% coarse sand) and ST3 (40 % JI N°2 and 60% coarse sand). Reproduction factor (R_f) of *P. penetrans* calculated dividing Pf by P, based on recovery from soil and root samples.

EXPERIMENT 1 – *P. penetrans*				
Initial population density (Pi)	Population density per pot (P)	Rf (Pf / Pi)		
		ST1	ST2	ST3
0	0	0	0	0
0.125	31	0.07	0.05	0.12
0.25	62	0.32	0.3	0.04
0.5	125	1.12	0.7	0.2
1	250	0.14	0.6	0.2
2	500	0.5	0.2	0.2
4	1000	0.5	0.4	0.3
		P-value ST		0.06
		P-value P		0.01
		P-value STxP		0.21
		S.E.D. ST		0.1
		S.E.D. P		0.15
		S.E.D. STxP		0.26
		% CV		41.4

6.4.2 Experiment 2

Potato plants emerged in all pots between 10 and 17 days. After eleven weeks, potato plants attained final heights from 3.75 cm (±1.86) to 6.08 cm (±1.9) (Figure 6.6 A). The greatest haulm fresh weight (21.6 g ±15.1) was recorded for the treatment with 8 nematodes g^{-1} soil, while the lowest fresh weight (8.9 g ±1.7) was found in the treatment with 32 nematode g^{-1} soil, which was similar to the untreated control (9.38 g ± 1.64) (Figure 6.6 B). Then, at the highest densities (16 and 32 g^{-1} soil), haulm fresh weight declined. The effect of *P. penetrans* population density on the weight of potato roots is presented in Figure 6.6 C. As in the case of haulm fresh weight, between 0 and 8 nematodes g^{-1} soil, root fresh weight was found to increase with increasing P_i, but at the highest densities (16 and 32 g^{-1} soil), root weight declined. Although roots were invaded by nematodes (Figure 6.7), initial population densities (P_i) did not significantly affect the yield of potatoes (Figure 6.8). Nevertheless, root-lesion severity, associated with RLN nematodes, showed that treatment at 32 nematode g^{-1} soil was significantly different ($P = 0.004$, d.f.= 5, Chi-Square= 17.42) from the control, according to Kruskal-Wallis One Way ANOVA with a pairwise comparison (Figure 6.9).

Final population densities (P_f) increased significantly with increasing initial population densities, in both soil ($P < 0.001$) and roots ($P = 0.022$) (Table 6.5). At the highest P_i (32 g^{-1} soil), the highest P_f values (1913 ± 507 and 1050 ± 592) were recorded. However, nematodes losses occurred also in this experiment, indeed all R_f were below 1 among all treatments (Table 6.6).

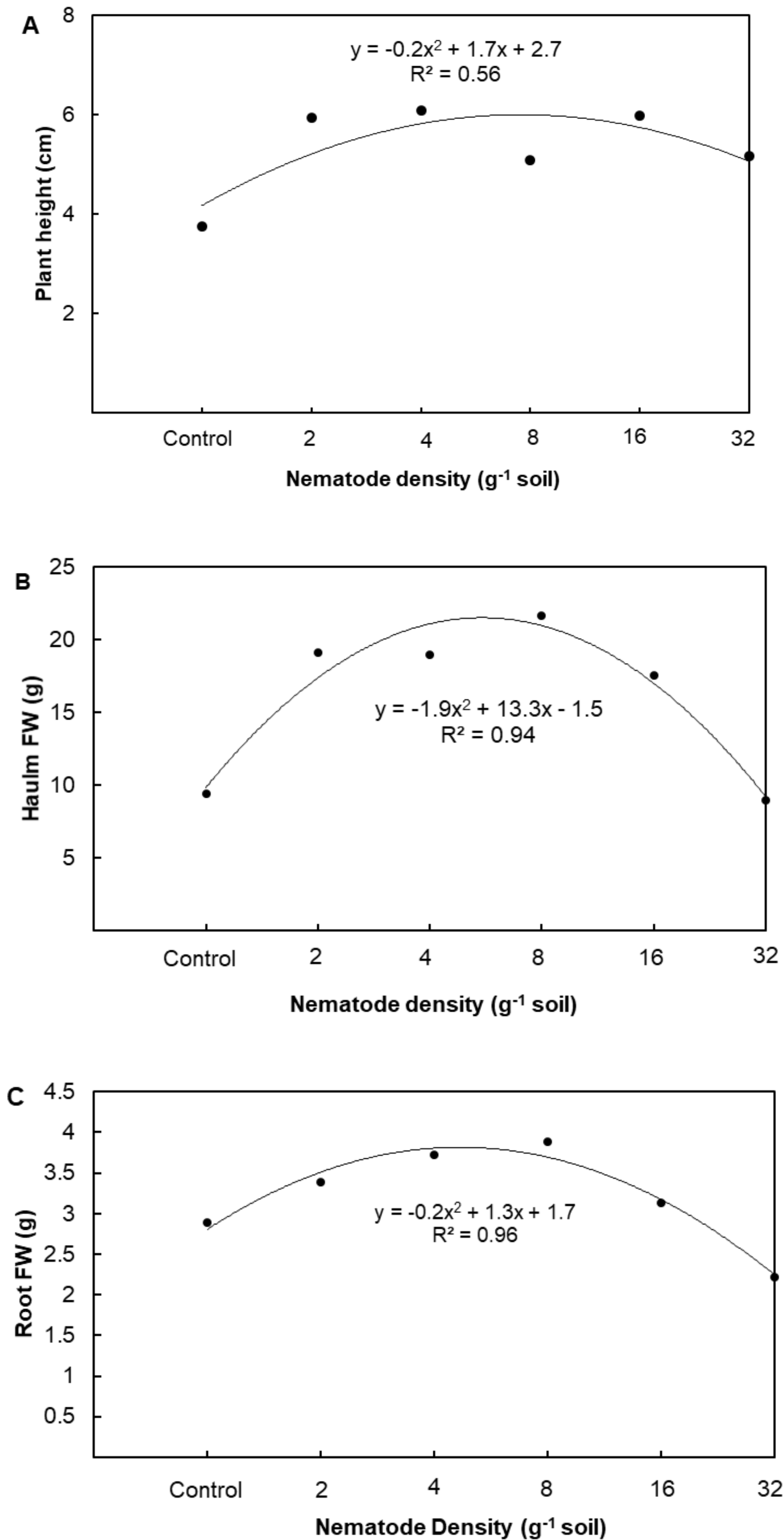

Figure 6.6: Plant height (A), haulm (B) and root fresh weight (g) (C) following exposure to different population densities (0, 2, 4, 8, 16, 32 g^{-1} soil) of *P. penetrans*. Data are means (n = 6) ± standard error of the mean (SEM). Differences between treatments are represented by a polynomial regression model.

Figure 6.7: Root-lesions on the roots of potato associated with invasion and colonisation by *P. penetrans*.

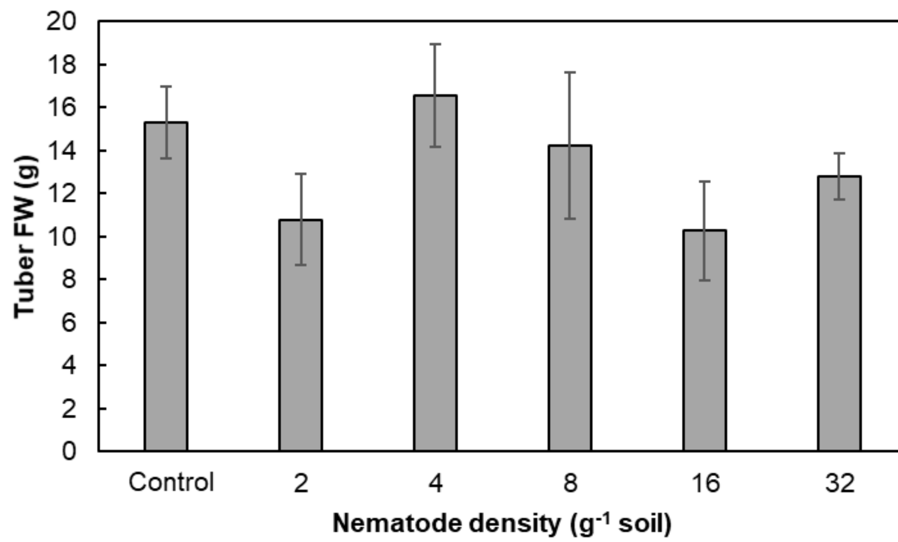

Figure 6.8: Tuber fresh weight (g) following exposure to different population densities (0, 2, 4, 8, 16, 32 g^{-1} soil) of *P. penetrans*. Data are means (n = 6) ± SEM.

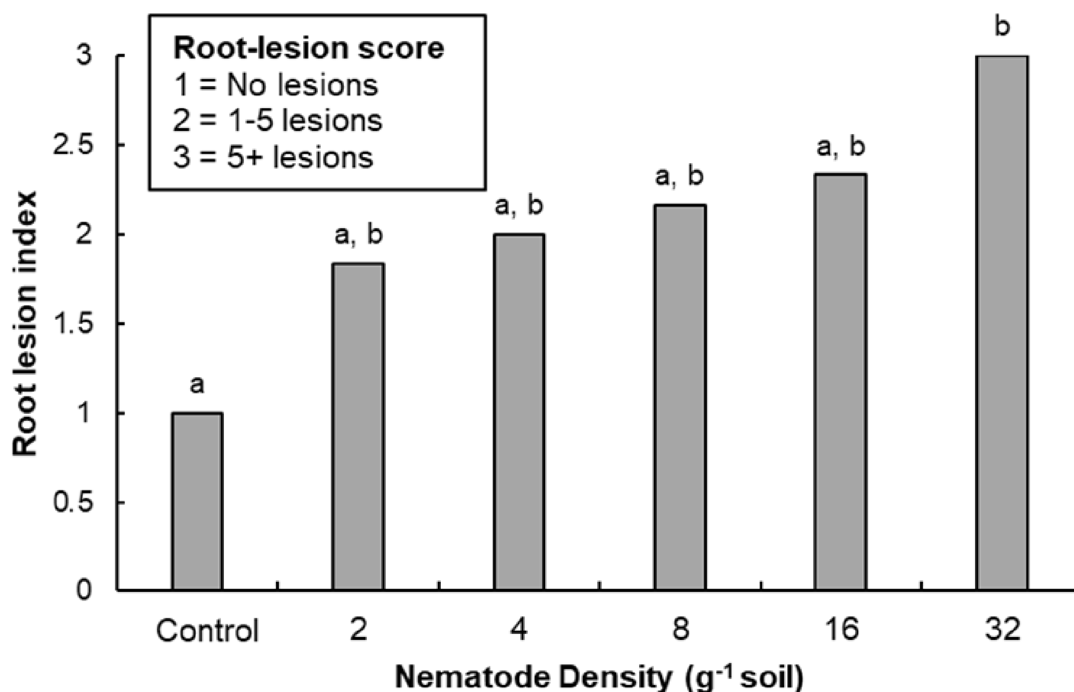

Figure 6.9: Mean root lesion disease index for potato plants grown in soil with different population densities of *P. penetrans.* Columns with different letters are significantly different ($P < 0.05$) according to Kruskal-Wallis one-way ANOVA with pairwise comparisons.

Table 6. 5: Final population densities (P_f) of *P. penetrans* in soil (500 g^{-1} soil) and roots (g^{-1} root). Data from soil are means (n=6) ± standard error of the mean (SEM). Data from roots are mean of the square-root (x+1) transformed data and in brackets are means (n=6) ± standard error of the mean (SEM). Reproduction factor (R_f) of *P. penetrans* calculated by (P_f/P) based on recovery from soil and root samples.

EXPERIMENT 2 – *P. penetrans*			
Initial population density (Pi)	Final Population (Pf) (500 g^{-1} soil)	Root invasion Square-root (x+1) (Nematode g^{-1} root)	Rf (Pf / Pi)
0	0	0	0
2	232±76	13.61(491±248)	0.69
4	300±84	19.14 (216±76)	0.26
8	332±123	25.8 (1266±577)	0.43
16	827±244	27.64(1458±1153)	0.29
32	1913±507	30.27 (1050±592)	0.19
P-value	0.002	0.022	0.231
S.E.D.	397.3	8.89	0.23
% CV	93.6	79.4	80.1

6.4.3 Experiment 3

The third experiment was conducted with *P. thornei*. Emergence occurred earlier than previous experiments, with plants emerging between 3 and 14 days, with no significant differences between pots with different P_i. After eleven weeks, potato plants attained final heights from 8.8 (± 0.27) cm to 10.9 (± 0.8) cm, with a negative correlation with P_i ($R^2 = 0.79$) (Figure 6.10 A). Haulm of plants decreased in weight with a negative correlation with P_i ($R^2 = 0.508$) (Figure 6.10 B). The lowest mean haulm weight of 5.8 (±0.8) g was recorded at the highest P_i (32 g^{-1} soil). A similar and moderate negative correlation was seen between P_i and root weight ($R^2 = 0.52$) (Figure 6.10 C). Whilst roots presented brown lesions (Figure 6.11), *P. thornei* did not significantly affect the yield of potatoes. As in Experiment 2, root-lesion severity was significantly higher ($P < 0.001$, d.f.= 5, Chi-Square= 21.5) at 32 nematode g^{-1} soil compare to the control (Figure 6.12). As with *P. penetrans* (Experiment 2), after nematode extraction from soil and potato roots, *P. thornei* final population increased with increasing P_i. The P_f values were lower than the P_i with low reproduction factors found for all treatments (Table 6.6), suggesting that multiplication did not occur during the experiment or some nematode losses occurred during the experiment or extraction.

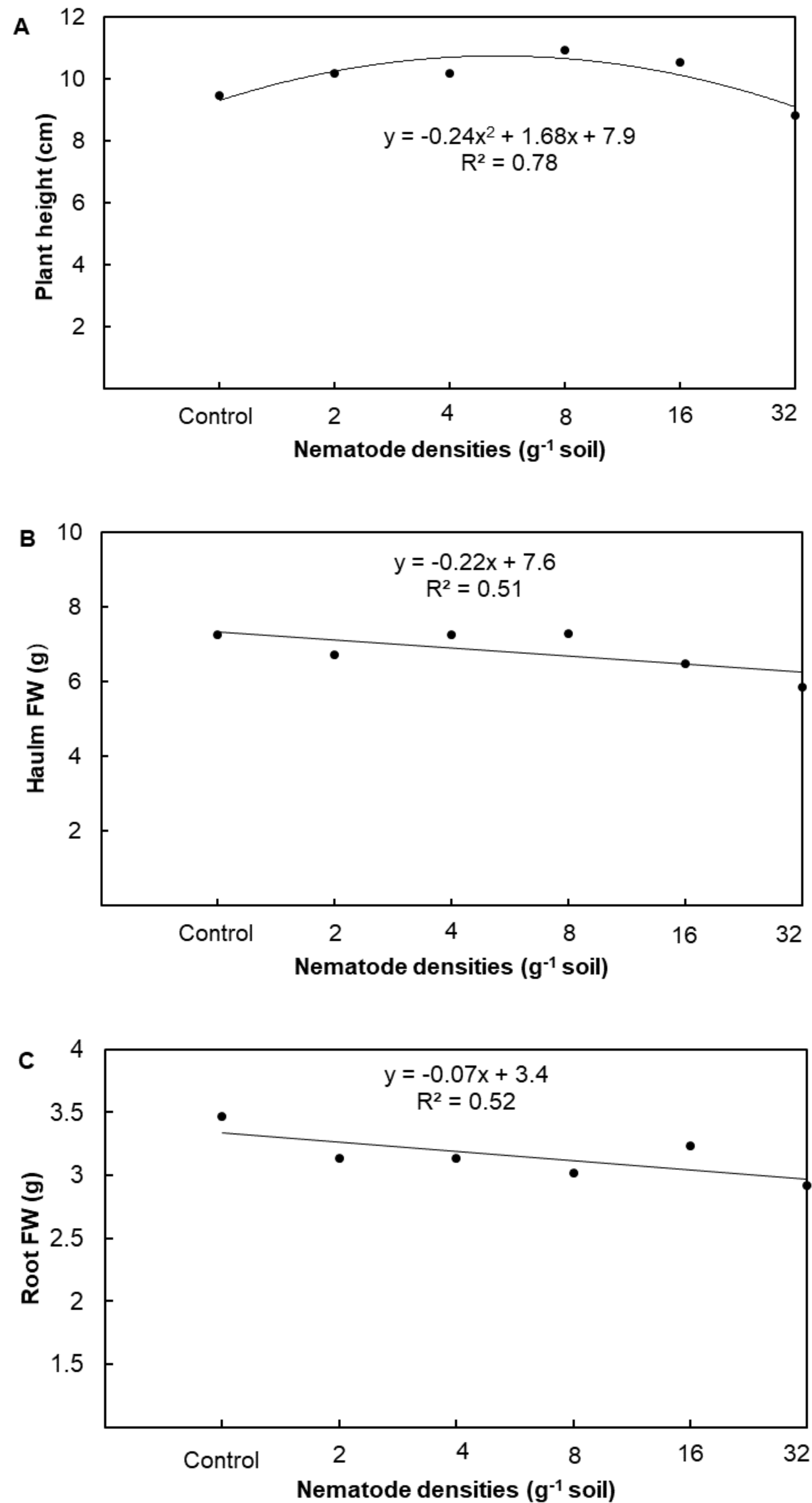

Figure 6.10: Plant height (cm) (A), haulm fresh weight (g) (B) and root fresh weight (g) (C) following exposure to different population densities (0, 2, 4, 8, 16, 32 g^{-1} soil) of *P. thornei*. Data are means (n = 6) ± standard error of the mean (SEM). Differences between treatments are represented by a polynomial regression model for plant height and linear model for haulm fresh weight and root fresh weight.

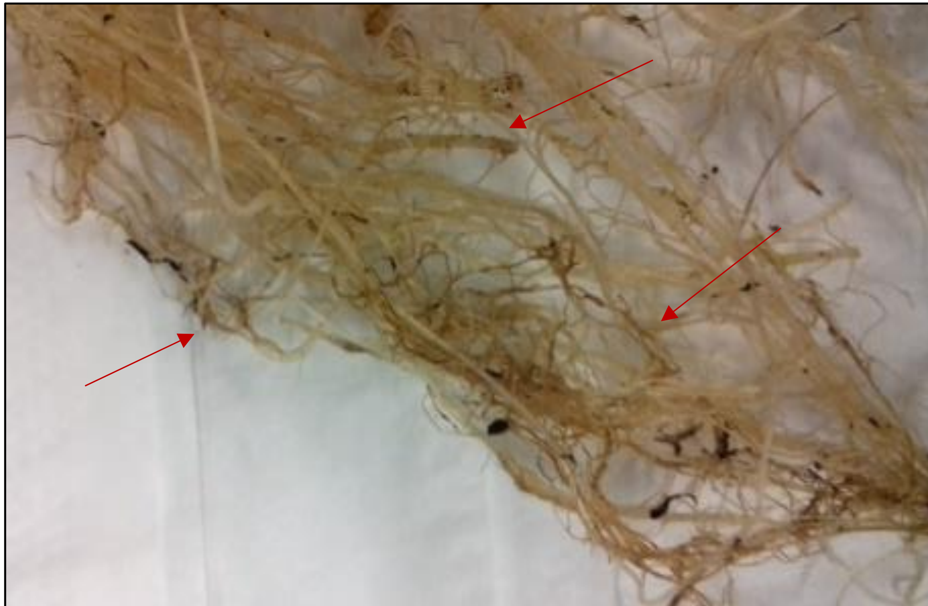

Figure 6.11: Root-lesions on the roots of potato associated with invasion and colonisation by *P. thornei*. Arrows point the brown lesion on the roots at harvest.

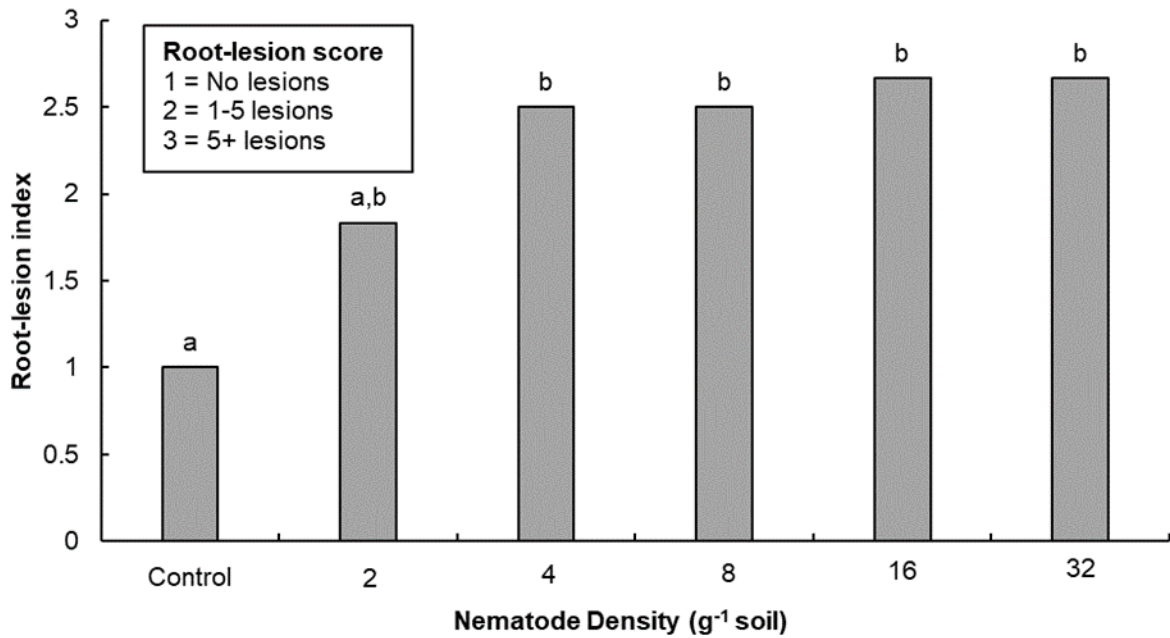

Figure 6.12: Mean root-lesion disease index for potato plants grown in soil with different population densities of *P. thornei.* Columns with different letters are significantly different ($P < 0.05$) according to Kruskal-Wallis one-way ANOVA with pairwise comparisons.

Table 6.6: Final population densities (P_f) of *P. penetrans* in soil (500 g^{-1} soil) and roots (g^{-1} root). Data from soil are means (n=6) ± standard error of the mean (SEM). Data from roots are mean of the square-root (x+1) transformed data (n = 6) and in brackets are means (n=6) ± standard error of the mean (SEM). Reproduction factor (R_f) of *P. thornei* calculated dividing P_f by P, based on recovery from soil and root samples.

EXPERIMENT 3 – *P. thornei*			
Initial population density (P_i)	Final Population (P_f) Soil (500 g^{-1} soil)	Root invasion (g^{-1} root)	Rf (P_f / Pi)
0	0	0	0
2	227±100	25±17	0.25
4	133±37	183±73	0.16
8	357±117	125±28	0.12
16	443±86	100±63	0.07
32	1570±477	258±114	0.11
P-value	<0.001	0.051	0.147
S.E.D.	289	85	0.07
% CV	110	127.7	86.7

6.5 DISCUSSION

Plant growth and potato yield of Maris Peer were significantly affected by the sand content of soil with the highest potato yield in soil with the lowest proportion of sand (ST3). Plants growing in ST3 were found to produce higher foliar and root biomass and were not found to be infected with nematodes at lower P_i, with no nematodes detected on roots under a density of 1 g^{-1} soil. Plants were subjected to the same conditions of temperature and moisture content. It is possible that the higher sand component had reduced nutrients and poorer moisture retention, meaning that the plants performed less well than others growing in other soil media. Nematode population densities did not appear to significantly affect plant growth or the tuber weight of potato significantly which meant that it was not possible to predict damage thresholds for *P. penetrans* on the cultivar Maris Peer at these nematode densities. In experiment 2, higher nematode densities than the previous experiment (from 2 to 32 g^{-1} soil) were tested. After eleven weeks, the greatest haulm fresh weight (21.6 g ±15.1) was recorded for the treatment with 8 nematodes g^{-1} soil, at the highest densities (16 and 32 g^{-1} soil), haulm fresh weight declined. At 32 nematodes g^{-1} soil, the potato roots reached their lowest weight (2 g) but the yield of potatoes was not affected when compared with the other treatments. At higher densities, an impact on yield may be observed. However, this may not reflect field conditions where population densities are not often recorded exceeding 32 nematode g^{-1} soil (Prior T. 2019, Fera Science Ltd, personal communication). This was confirmed also in the survey reported in Chapter 5, where maximum desities of 5 *P. penetrans* g^{-1} soil were reported. The initial increase in root weight might be due to proliferation of lateral roots in response to nematode feeding as observed for stubby root nematodes (Agrios, 2005; Palomares-Rius *et al.*, 2017). This might be a response of Maris Peer cultivar against *P. penetrans* as a hypersensitive response of plant to the pathogen to overcome its invasion. It is also possible that Maris Peer can withstand *P. penetrans* infection and being a tolerant host against this species. The presence of root-lesions in roots of all treatments, except controls, and the highest P_f values at the highest density (32 g^{-1} soil), confirmed nematode invasion in roots occurred. However, all reproduction factors were lower than 1, indicating that there was limited nematode multiplication, or some nematode mortality occurred after introduction into the soil. Another explanation may be a low efficiency of nematode extraction from soil using Baermann modified method have caused some nematode losses. Indeed, although Baermann modified method is an easy and common extraction method used in many laboratories, it presents some limitations such as less efficiency for the recovery of less active nematodes, reduction of nematode movements due to lack of aeration in the water and poor recovery from large samples (Viglierchio and Schmitt, 1983 a, b; EPPO, 2013; Neher *et al.*, 1995; Cesarz *et al.*, 2019; van den Hoogen *et al.*, 2020). Viglierchio and Schmitt (1983a) showed that

extraction efficiency depended by method, soil type and nematode species. Also tissue paper used to hold soil in the tray can inhibit the passage of nematodes, indeed retention of *Meloidogyne incognita* may vary between 5 up to 80% depending the tissue used, and for *D. dipsaci* this variation may vary from 5 to 35% (Viglierchio and Schmitt, 1983b). This was confirmed also by a recent study showing differences on nematode extractions and community composition when using Baermann-funnel method with different soil type, soil height, sieving, and filter type (Cesarz *et al.*, 2019). Indeed, the highest numbers of exctracted free-living nematodes were observed when using milk filters and the lowest soil height (1 cm height) of soil and sieving. These studies highlight that variations in the densities and community composition can vary between laboratories also depending the equipment used in the extraction protocol. A standardized extraction protocol would help to obtain similar results between laboratories.

In the third experiment conducted with *P. thornei*, fresh haulm ($R^2 = 0.508$) and root weight ($R^2 = 0.52$) moderately decreased in response to increasing P_i. Also, in this experiment, root lesion scores and final population densities increased significantly according to the increase of P_i, but a low percentage of nematode recovery was recorded. This suggested that nematode reproduction was minimal or that efficiency of extraction method was not optimal as discussed above for *P. penetrans* assays. Although roots were invaded by nematodes, *P. thornei* did not significantly affected potatoes yield, confirming the results obtained in the previous two experiments with *P. penetrans*.

Resistance occurs when plants can inhibit development and reproduction of infective nematodes (Trudgill, 1991), and it can be measured by the reproduction factor, that is multiplication rate of parasites, expressed as ratio Pf/Pi (Wallace, 1987). Tolerance is referred to a plant able to resist or overcome damage caused by nematode injuries and produce an acceptable yield (Trudgill, 1991). Considering these concepts and our results, Maris Peer may be partially resistant to both *P. penetrans* and *P. thornei*. Indeed, although invasion on roots occurred with both species, plants still had a similar yield to untreated plants, and they presented low nematode densities on roots suggesting that plants were able also to inhibit nematode reproduction. Different cultivars can have different responses to nematode attacks and consequently perform differently in terms of yield. Indeed, Bernard and Laughlin (1976) studied four different potato cultivars (Katahdin, Kennebec, Superior and Russet Burbank) subjected to different population densities of *P. penetrans* in micro-plots with sandy clay loam and reported different effects on yield loss. The variety Superior presented a yield loss of 23 - 30% caused by 0.38 nematodes g^{-1} soil, whereas Kennebec was affected by 0.81 g^{-1} soil and Katahdin by 1.5 - 2 g^{-1} soil. In comparison, Russet Burbank was unaffected by *P. penetrans* at densities of 0.38 - 2 g^{-1} soil. Similarly, Bird and Vitosh (1978) reported that Russet Burbank had tolerance towards *P. penetrans*. In contrast, Olthof (1983) found that Russet Burbank had

a yield loss of 16% when exposed to 1.8 *P. penetrans* g^{-1} soil whilst the yield of the varieties Kennebec, Monona, Norchip, Superior and Yukon Gold were unaffected by 1.6 - 2 *P. penetrans* g^{-1} soil. Later, the same cultivars were tested in a micro-plot experiment with 10 nematodes g^{-1} soil and yield losses were observed; Superior was the most affected with 73% losses of marketable tubers, followed by Russet Burbank (61%), Kennebec (55%), Monona (46%), Norchip (43%) and Yukon Gold (25%) (Olthof, 1986). Further studies with different UK cultivars may help to better understand the pathogenicity of these species and consequently resistance and tolerance of UK cultivars against root-lesion nematodes. Moreover, pot experiments may have some limitation and plants were possibly not stressed sufficiently to be impacted by root-lesion nematodes. Experiments with different moisture contents to simulate drought conditions may enhance the infection of the nematodes and help to clarify the pathogenicity of these nematodes. Another limitation to consider is the time of each experiment. The experiments were conducted in six (experiment 1) and eleven weeks (experiment 2 and 3), and possibly the time was not optimal for nematode reproduction. Indeed, Bernard and Laughlin (1976) conducting experiment with four different cultivars, they harversted Superior, Kennebec, Katahdin, and Russet Burbank at 116, 122, 133, and 147 days after planting, respectively. Olthof (1986) reported that plants of Superior, Yukon Gold and Monona were harvest after 118 days, whereas Norchip, Kennebec and Russet Burbank after 139 days. These times are longer than the time applied in the current study. The extension of the time may result with high multiplication of nematodes and significant impact on potatoes. Further experiments may investigate also the time of infection on RLN on potatoes.

Pathogenicity of root-lesion nematodes in UK potato cultivars is still unexplored, and this study represents the first investigation of *P. penetrans* and *P. thornei* infestation on Maris Peer cultivar, under controlled environment. Due to limited time, no further cultivars or species were included in this study, but further investigations on this topic would be recommended.

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Chapter 7

7. GENERAL DISCUSSION AND CONCLUSION

7.1 GENERAL DISCUSSION

The genus *Pratylenchus* represents one of the most common plant-parasitic nematodes known to have negative impacts on the production of a diverse range of crops, worldwide (Jones *et al.*, 2013). Generally, root-lesion nematodes are underestimated in agriculture and their symptoms are confused and attributed to other pests or biotic factors. On the other hand, symptoms and impacts of root-lesion nematodes on potatoes are reported in a number of countries (Olthof,1986; 1990; Kimpinski and MacRae, 1988; Holgado *et al.,* 2009; Olthof and Potter, 1973; Riedel *et al.*, 1985). Once detected in a field, eradication of root-lesion nematodes is not possible, but targeted control measures would limit the increase of population densities and consequently the damage on the crop. The correct diagnosis of root-lesion nematodes is difficult because the species are morphologically similar to each other and few laboratories have expertise on taxonomy to assist with identification, while symptoms on potato are challenging to assess (Palomares-Rius *et al.*, 2014). The first chapter of this thesis examined the current status of the diagnosis, pathogenicity, distribution and management of the main species of root-lesion nematodes reported to infect potatoes worldwide. Based on the literature, highlighting the main gaps, it was clear that a comprehensive study was required to understand the presence and impact of root-lesion nematodes in GB potato fields. This study was organized in three different sections: (1) development of molecular methods for a rapid identification and quantification of the four common *Pratylenchus* spp. (*P. crenatus, P. penetrans, P. neglectus* and *P. thornei*); (2) detection and distribution of *Pratylenchus* spp. on potatoes from England and Scotland, applying the molecular diagnostic methods developed in chapter 4; (3) assessing the pathogenicity of *P. penetrans* and *P. thornei* on the development of Maris Peer cultivar under controlled conditions.

Chapters 3 and 4 focused on the molecular diagnostics for four common *Pratylenchus* species to develop tools for DNA extraction and molecular identification using the real time qPCR method. Diagnosis of *Pratylenchus* spp. using microscopy is time consuming and difficult, while molecular diagnostics can provide useful methods for identification and quantification, which require less technical expertise. The first important step for a molecular diagnostic is the extraction of DNA. Existing DNA extraction methods for *Pratylenchus* spp. are not straightforward and no studies compare the efficiency of the available DNA extraction methods for *Pratylenchus* species (Harris *et al.*, 1990; Stanton *et al.*, 1998; Adam *et al.*, 2007;

Carvalho *et al.*, 2019). For these reasons, six common methods of DNA extraction were compared to determine the most efficient method for extracting DNA from *P. penentrans* and this method was then used to compare potential differences on DNA release and PCR amplification among four species such as *P. penetrans, P. crenatus, P. neglectus* and *P. thornei.* All methods did not differ in the amplification of DNA extracted from five or ten *P. penetrans* adults. However, methods involving cutting nematodes and using glass beads were the most efficient on extracting DNA from single *P. penetrans*, showing that mechanical disruption of the cuticle and body of the nematode appears to be an important step to achieve successful and consistent DNA amplification. Methods like extractions with NaOH or WLB buffers may have failed for DNA realise and PCR amplification because of the presence of PCR inhibitors within the buffers, such as Tween 20 or dithiothreitol. Indeed, Schrader *et al.* (2012) explained how some PCR inhibitors may degrade DNA samples or disrupt the annealing of the primers to DNA templates, whereas others can directly degrade the DNA polymerase or inhibit its activity. Between the two most successful methods tested for DNA extraction of *P. penetrans*, the use of glass beads seemed less laborious compared with the method of cutting the nematodes for this reason this technique was used for the comparison of DNA extraction among four species. The glass beads method was the most consistent method among different life stages, increasing numbers of specimens, and species such as *P. penetrans, P. crenatus, P. neglectus* and *P. thornei*. Consequently, this protocol has been applied for the development and validation of qPCR protocols in Chapter 4.

Chapter 4 focused on the development and validation of four qPCR protocols on the identification and quantification of *P. crenatus, P. penetrans, P. neglectus* and *P. thornei* based on the D2-D3 sequence of the rDNA gene. Molecular diagnostics in nematology has received more attention in recent years and a number of studies have been published on the rapid identification and quantification of root-lesion nematodes (Uehara *et al.*, 1998, 2001; Al-Banna *et al.*, 1997, 2004; Waeyenberge *et al.*, 2000, 2009; Subbotin *et al.*, 2008; Yan *et al.*, 2008, 2012, 2013; De Luca *et al.*, 2004, 2011; Oliveira *et al.*, 2017; Mokrini *et al.*, 2013, 2014; Fanelli *et al.*, 2014, 2018; Peetz and Zasada, 2016; Janssen *et al.*, 2017a, b). The D2-D3 expansion fragment of 28S rDNA is widely used in several studies on the phylogeny and taxonomy of root-lesion nematodes (de la Peña *et al.*, 2007; Subbotin *et al.*, 2008; Palomares-Rius *et al.*, 2010; De Luca *et al.*, 2012; Palomares-Rius *et al.*, 2014; Troccoli *et al.*, 2016; Zamora-Araya *et al.*, 2016; Janssen *et al.*, 2017a, b). This molecular gene target possesses high interspecific genetic variability and low intra-specific variation within the genus *Pratylenchus* as demonstrated by several authors (Al-Banna *et al.*, 2004; De Luca *et al.*, 2004; Subbotin *et al.*, 2008; Lin *et al.*, 2020). Subbotin *et al.* (2008) produced a phylogenetic analysis using several populations of *Pratylenchus* species from geographically distinct sites, showing that the D2-D3 expansion fragment recognised a higher degree of interspecific variation than the partial

18S rRNA (Subbotin *et al*., 2008). This was also confirmed by Janssen *et al.* (2017a, b), who demonstrated that 28S rDNA helped to resolve the separation of closely related species within the 'Penetrans group'. Although, the 28S rDNA has a higher mutation rate compared to the 18S rRNA gene, high intra-genomic variability associated with multiple copies of ribosomal genes needs to be considered (Janssen *et al.* 2017 a, b). Recently, the low intraspecific variations of the D2-D3 expansion fragment for *P. neglectus* and *P. thornei* was confirmed to be in the range of 0.1–2.0% and 0–1.7%, while interspecific variations ranged from 14.7% to 20.3%, respectively (Lin *et al*., 2020). For all these reasons, this gene target was selected for the development of qPCR protocols described in Chapter 4 of this thesis. Four TaqMan qPCR protocols for the identification and quantification of *P. crenatus, P. penetrans, P. neglectus* and *P. thornei* were developed. Each protocol is independent from the others and needs to be applied in separate reactions. Four standard curves were established with highly significant linearity (R^2 = 0.99). Considering the specificity of primers/probe set, validation of methods was assessed by four tests. *Pratylenchus penetrans, P. crenatus, P. neglectus* and *P. thornei* were successfully discriminated from other genera and *Pratylenchus* species, including *P. fallax* that is often confused with *P. penetrans*. The absence of amplifications for non-target species confirmed the high specificity of primers and probe designed in this study. Given that mixed RLN species are often found in individual samples, a second test was performed using samples prepared with a mixture of *Pratylenchus* species to distinguish target species from close related species mixed in the same sample. This test confirmed the detection and correct quantification of one and ten *Pratylenchus* spp. in samples with a mixture of the other non-species target. The Ct values were comparable with pure samples with one or ten females for each species, demonstrating the sensitivity of each method. Only *P. crenatus* showed Ct values for mixed species with one female significantly higher ($P < 0.001$) than Ct values for one pure nematode. However, ten females of *P. crenatus* mixed with thirty females of another *Pratylenchus* species showed the same Ct values of pure samples of ten *P. crenatus* females, demonstrating the consistency of the method for mixed samples. Test 3 involved the amplification of juveniles and adults of each target species. Each diagnostic protocol detected juveniles for each target species, confirming the accuracy of these methods to identify and quantify all life stages. Gene copy number was calculated for each life stage and estimates were made for individuals of each species. Overall, each species presented different gene copy numbers, and in particular *P. thornei* presented a lower copy number than the other species. This may be due to a level of inter-specific variation of D2-D3 segment that might be reflected in the different gene copy number. Alternatively, the DNA extraction efficiency may have differed among the four species of *Pratylenchus*. However, in Chapter 3, although juveniles presented lower percentage of DNA amplifications than adults and some differences were observed among species, these differences were not statistically significant. Consistent

amplifications for test 4, where *P. penetrans* (1, 10, 100, 1000 individuals), *P. thornei* (1, 10 and 100 individuals) and *P. crenatus* (1, 10 and 50 individuals) showed highly significant linear relationships between Ct values and increasing numbers of nematodes. These results demonstrated the high sensitivity of each method in the quantification of each target species. This was also confirmed by test 5 that compared counting obtained by q PCR with counting by microscopy. Here, a strong correlation ($R^2 = 0.78$) was observed, confirming the robustness of the methods developed within this study.

Methods developed in Chapter 4 were used for identification and quantification of *Pratylenchus* spp. in a survey of potato growing land in England and Scotland (Chapter 5). As discussed in previous chapters, root-lesion nematodes are often overlooked in potato fields, especially in the UK. Although there were some older publications on the distribution of root-lesion nematodes in lily, peas, bean, carrots and cereals in the UK (Corbett, 1969, 1970, 1972, 1973, 1974, 1976, 1983; Boag, 1979, 1980; Boag and Lopez-Llorca, 1989; Boag *et al.*, 1990; Dale and Neilson, 2006), there were no studies on their distribution in potato fields. The objective of Chapter 5 was to detect the presence and distribution of *Pratylenchus* spp. in potato growing lands in England and Scotland. Two hundred potato fields were sampled in England after the harvest of potatoes. Soil analysis revealed a wide distribution and abundance of *P. neglectus, P. thornei*, *P. penetrans* and *P. crenatus* were also found but not as extensively as *P. neglectus*. Other species not identified to a species level were also found and could include *P. fallax, P. convallariae, P. pratensis, P. flakkensis, P. vulnus* and *P. pinguicaudatus* as species reported previously in the UK (Southey, 1959; Seinhorst, 1968; Corbett, 1969, 1970, 1972, 1973, 1974, 1976, 1983). The species were sometimes mixed together as two or three species in single samples, while qPCR methods helped to quantify each species separately. The survey included eighteen samples from Scotland and almost all fields showed the presence of *Pratylenchus*, with *P. neglectus* and *P. crenatus* being the most common and abundant. These results were in line with previous reports in Scotland (Boag, 1979, 1980; Boag and Lopez-Llorca, 1989; Boag et al., 1990; Dale and Neilson, 2006; Oliveira *et al.*, 2017). Indeed, in a recent study of molecular diagnostics, Oliveira *et al.* (2017) included samples from potato land, reporting *P. crenatus* as the most common species followed by *P. neglectus* and *P. penetrans*. In the past, the presence of these species was also reported in other crops in Scotland such as cereals and carrots.

Crop damage is commonly related to the population densities of plant parasitic nematodes. Damage thresholds between 1 and 2 nematodes g^{-1} soil have been reported for *P. neglectus* and *P. penetrans* in potatoes in other countries (Olthof and Potter, 1973; Olthof, 1986, 1990; Riedel *et al.*, 1985), but, to date, there is no information about damage thresholds of root-lesion nematodes in the UK. For this reason, three controlled environment experiments were set up for the investigation of root-lesion nematodes pathogenicity against Maris Peer, one of

the most commonly grown GB cultivars (Chapter 6). The first experiment investigated the impact of *P. penetrans,* with densities ranging from 0.125 to 4 nematodes g^{-1} soil, on growth and yield of Maris Peer in three different sandy based soils. Potato yield was significantly affected by soil type, with ST1 (10% JI N°2 and 90 % coarse sand) giving the lowest yield, although the population density of *P. penetrans* had no effect. *Pratylenchus penetrans* was detected in the potato roots of all treatments, confirming that the nematode was able to infect the cultivar without inducing yield loss at the population densities tested. Similar results were obtained for two further controlled experiments with *P. penetrans* and *P. thornei*, respectively. Although a broader range of nematode densities, from 2 to 32 nematode g^{-1} soil were used, as in the first experiment, yield was not significantly affected by nematode densities. As in the previous experiment, both species of root lesion nematodes were recorded in the roots of all treatments, confirming invasion occurred. Moreover, reproduction factors were low for all three experiments, showing these species had a limited reproduction on Maris Peer. This result may indicate a partial resistance by this cultivar. However, more investigation must be undertaken to confirm this result and also at field conditions where the situation may change due to other factors like soil type, soil moisture, pH of soil and temperature. Soil moisture is an important factor to consider for nematode population dynamic. Dry conditions favour the infection and subsequent damage of *P. neglectus* and *P. thornei* (Smiley, 2010). Kable and Mai (1968) found that *P. penetrans* survival and infection of alfalfa roots was suppressed by low or very high soil moisture content. Moreover, moist soils can favour the proliferation of microbial species pathogenic to nematodes affecting their survival, thus reducing the infection to the host plant. No further experiments could be arranged due to COVID-19 restrictions, but it would be interesting to conduct more research exploring the pathogenicity of root-lesion nematode under stress condition such as drought. Furthermore, it would be interesting to test also other species common in GB potato fields like for example *P. crenatus* and *P. neglectus*. The pathogenicity of root-lesion nematodes to potatoes may vary between different cultivars as highlighted by Bernard and Laughlin (1976), who showed that *P. penetrans* caused different degrees of yield loss depending on the potato cultivar grown. For instance, 0.38 *P. penetrans* g^{-1} soil resulted in a yield loss of 23 -30% of the potato cultivar Superior, whereas cv. Kennebec was negatively affected by 0.81 *P. penetrans* g^{-1} soil and cv. Katahdin by 1.5 – 2 *P. penetrans* g^{-1} soil. In contrast, Russet Burbank was not affected by *P. penetrans* (Bernard and Laughlin,1976; Bird and Vitosh,1978). Further studies with different UK cultivars may help to better understand the pathogenicity of these species and consequently resistance and tolerance of UK cultivars against root-lesion nematodes. This was the first UK study to focus on root-lesion nematodes on potato for some time. Many aspects still need to be explored such as screening for tolerance and resistance of varieties against root-lesion nematodes, defining damage thresholds under field conditions and investigating potential complex

diseases with other pathogens such as *Rhizoctonia solani*, a common soil-borne pathogen, reported to interact with root-lesion nematodes in other countries (Kotcon *et al.* 1985, 1987; Kenyon and Smith, 2007; Viketoft *et al.,* 2017).

7.1.2 CONCLUSIONS AND RECOMMENDATIONS

Findings from the present study have improved our knowledge of the diagnosis of *Pratylenchus* spp. and it has been confirmed the distribution and species of RLN in potato growing lands for the first time in the England and Scotland. Such diagnostics provide invaluable tools for accurate identification and quantification of *Pratylenchus* spp., allowing better management decisions and greater research possibilities.

Molecular identification is an important and improving topic in nematology. After an extensive validation, the qPCR methods developed here were confirmed to be specific, accurate, sensitive and reliable. They can be used for diagnosis of *P. crenatus, P. penetrans, P. neglectus* and *P. thornei*, in place of morphological identification, but primary tests with individuals from specific regions (countries), and calibration of standards are recommended before performing the protocols in soil samples with mixed nematodes. Further research could look at the development of multiplex qPCR for the identification of different species within a single test, like for example available for both species of PCN (Bulman and Marshall, 1997; EPPO, 2017). Moreover, other RLN species should be studied in order to design species-specific primers and probes for detection and quantification. For example, *P. fallax* or *P. convallariae* are reported in the UK but are not associated with potatoes so far. *Pratylenchus fallax* has been mainly reported in barley and wheat (Corbett, 1970a) and raspberry (Cotten and Roberts, 1981), whereas *P. convallariae* is associated with lily (Corbett, 1970b). Since there are no studies on quantitative molecular diagnostics for these species, further research would improve the diagnosis of this genus.

The distribution of root-lesion nematodes in potato in the UK highlight the need to perform soil analysis in potato fields to exclude potential losses caused by these nematodes. The current study confirmed their wide prevalence in England and Scotland. Obtaining information about nematode densities for each field is important in order to get advice for nematode management from agronomists or accredited laboratories, focusing on the limitation of population densities during the crop season.

Pathogenicity of root-lesion nematodes in UK potato cultivars is still unexplored, and the present study reported the first investigation of *P. penetrans* and *P. thornei* infestation on Maris Peer cultivar, under controlled environment. Due to limited time, no further cultivars or species were included in this study, but further investigations on this topic would be really useful and interesting. Moreover, now that presence is widely confirmed, it would be recommended to

conduct field experiments to know the impact of these nematode on UK potato yields, considering drought condition. Although experiments conducted under field conditions may prove more challenging to establish, due to patchy RLN population densities, they might provide a more accurate assessment of damage thresholds in view of natural abiotic stresses. The presence of root-lesion nematodes themselves may not cause yield loss of some cultivar but they may cause to others not investigated so far. Moreover, root-lesion nematodes may interact with other pathogens like *Rhizoctonia solani* or *Verticillum dalhiae* resulting in complex-disease with severe losses in the potato yield. A lighter soil, such as sandy soil, may represent a risk factor to consider as potential spread of root-lesion nematodes and the presence of *V. dalhiae* in the field. A previous history of *Verticillium* wilt should be taken also into account when soil is tested for RLN. It would be advisable to conduct soil sampling once per year before growing potatoes, in the Autumn or Spring when the soil is typically moist. Plant Health Clinics should, where possible, provide advice on root-lesion nematodes. Based on the results of soil tests, growers can take measures such as rotation planning or use of cover crops such as black oats or marigold (*Tagetes spp.*) as poor host for *Pratylenchus* spp. Lastly, further studies on root-lesion management on UK potato cultivars would be very useful for advice to farmers to contain the nematode spread from infested lands.

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Appendices

Appendix 8.1: List of clones obtained for development of qPCR protocols. Values of %coverage, identity score and total score are obtained from GenBank.

Individual	Clones	Identification	%coverage	identity score	total score
PN1	PN1_1	*P. neglectus*	88	99	1358
	PN1_2	*P. neglectus*	89	98	1358
PN6	PN6_3	*P. neglectus*	89	99	1393
	PN6_5	*P. neglectus*	88	99	1399
	PN6_6	*P. neglectus*	91	99	1399
	PN6_7	*P. neglectus*	91	99	1399
PN7	PN7_1	*P. neglectus*	87	99	1387
	PN7_2	*P. neglectus*	88	99	1391
	PN7_3	*P. neglectus*	89	99	1426
	PN7_4	*P. neglectus*	90	98	1343
	PN7_5	*P. neglectus*	90	99	1404
PP1	pp1_3	*P. penetrans*	89	98	1362
	PP1_3_REP	*P. penetrans*	92	97	857
	PP1_4	*P. penetrans*	89	99	1380
	PP1_5	*P. penetrans*	89	99	1413
PP2	PP2_1	*P. penetrans*	93	98	1339
	PP6_6	*P. penetrans*	90	99	1406
	PP6_7	*P. penetrans*	90	99	1400
	PP6_8	*P. penetrans*	92	99	1417
	PP6_10	*P. penetrans*	90	99	1395
PP7	PP7_1	*P. penetrans*	89	99	1378
	PP7_2	*P. penetrans*	90	99	1369
	PP7_3	*P. penetrans*	87	99	1411
PC1	PC1_1	*P. crenatus*	82	97	1225
	PC1_2	*P. crenatus*	83	97	1225
	PC1_4	*P. crenatus*	85	98	1260
	PC1_5	*P. crenatus*	85	98	1245
PC2	PC2_1	*P. crenatus*	84	97	1214
	pc2_2	*P. crenatus*	86	98	1277
	PC2_2_rep	*P. crenatus*	93	99	889
	PC2_3	*P. crenatus*	84	98	1293
PC7	PC7_1	*P. crenatus*	84	98	1290
	PC7_3	*P. crenatus*	86	98	1284
	PC7_5	*P. crenatus*	85	99	1301
	pc7_7	*P. crenatus*	86	98	1271
PT1	PT1_1	*P. thornei*	89	99	1404
	PT1_2	*P. thornei*	90	98	1367
	PT1_3	*P. thornei*	91	99	1382

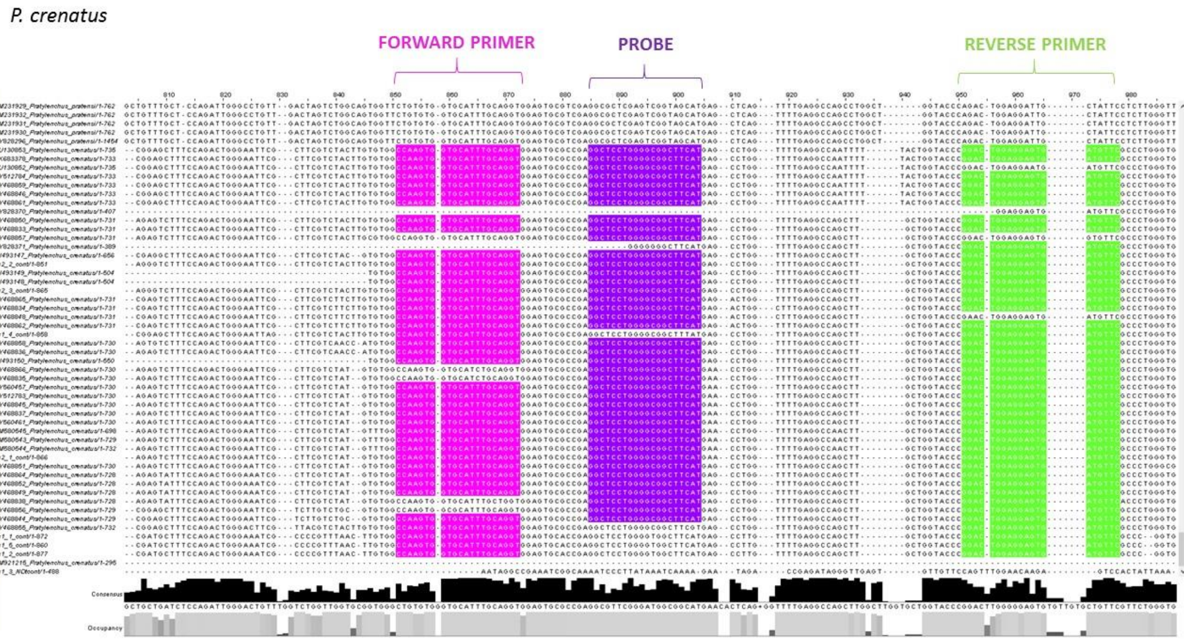
	PT1_4	*P. thornei*	91	99	1382
	PT1_5	*P. thornei*	91	99	1382
PT2	PT2_1	*P. thornei*	89	99	1423
	PT2_2	*P. thornei*	91	99	1406
	PT2_3	*P. thornei*	91	99	1406
	PT2_4	*P. thornei*	93	99	1421
	PT2_5	*P. thornei*	93	99	1426
	PT2_6	*P. thornei*	93	99	1426
	PT2_7	*P. thornei*	89	99	1386
	Pt2_8	*P. thornei*	91	98	1378
	PT2_8_rep	*P. thornei*	94	99	889
PT6	PT6_1	*P. thornei*	90	99	1421
	PT6_3	*P. thornei*	90	99	1205
	PT6_4	*P. thornei*	91	99	1432
	PT6_4 REP	*P. thornei*	91	99	1432
	PT6_5	*P. thornei*	90	99	1415
	PT6_6	*P. thornei*	90	99	1432
PT7	PT7_1	*P. thornei*	91	99	1382
	PT7_2	*P. thornei*	90	99	1426
	PT7_3	*P. thornei*	90	99	1432
	PT7_4	*P. thornei*	91	99	1421
	PT7_5	*P. thornei*	92	99	1421
PTU1	PTU1_1	*P. thornei*	92	99	1421
	PTU1_2	*P. thornei*	89	99	1424
	PTU1_3	*P. thornei*	91	99	1393
	PTU1_4	*P. thornei*	95	99	1426
	PTU1_5	*P. thornei*	87	99	1391
PTU2	PTU2_1	*P. thornei*	90	99	1293
	PTU2_2	*P. thornei*	91	99	1426
	PTU2_6	*P. thornei*	92	99	857
	Ptu2_6REP	*P. thornei*	89	99	1415
	PTU2_8	*P. thornei*	91	99	1424

Appendix 8.2: List of sequences used for alignment. Values of % coverage, identity score and total score were obtained from GenBank.

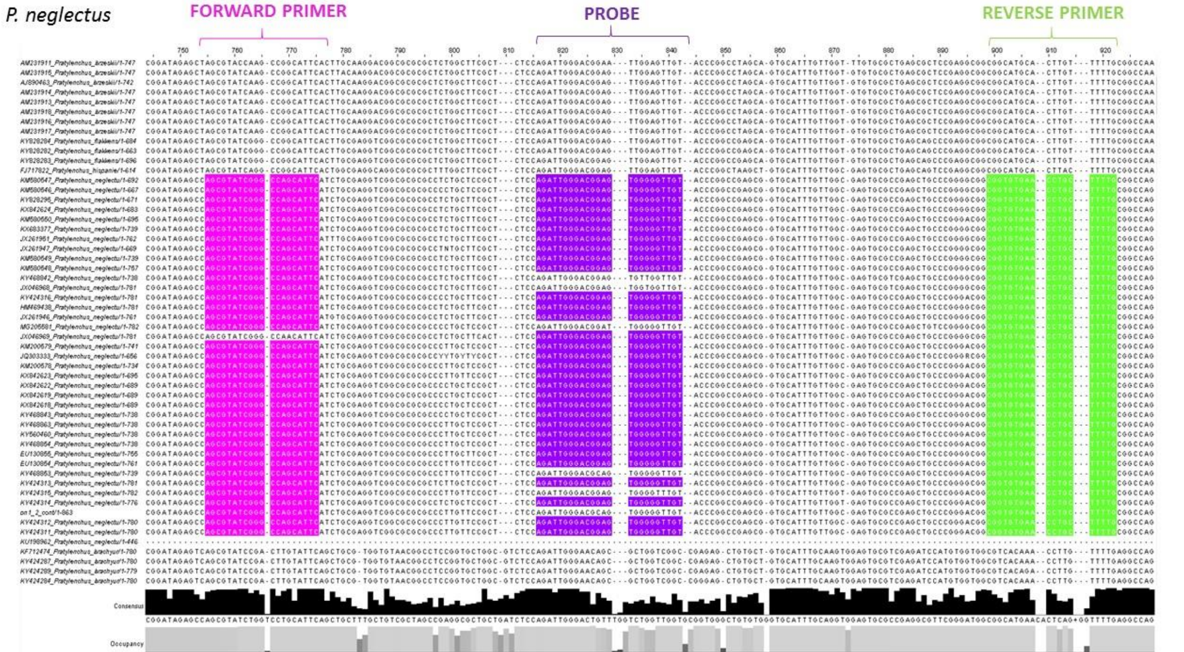
Sample number	Identification	% coverage	Identity score	Total score
PP1	*P. penetrans*	100	95	586
PP1_rep	*P. penetrans*	100	96	385
PP4	*P. penetrans*	98	98	784
PP4_rep	*P. penetrans*	97	97	527
PC3	*P. crenatus*	100	96	172
PC3rep	*P. crenatus*	100	97	243
489_1	*P. crenatus*	100	97	161
489_2	*P. neglectus*	100	98	732
489_2rep	*P. neglectus*	100	98	780
489_2Frep	*P. neglectus*	97	98	826
491_1	*P. neglectus*	100	98	736
491_1rep	*P. neglectus*	99	99	782
491_2	*P. neglectus*	99	99	824
491_2_rep2	*P. neglectus*	99	99	872
491_2rep	*P. neglectus*	99	99	808
491_3	*P. neglectus*	100	99	791
491_3_rep	*P. neglectus*	100	99	802
491_3_rep2	*P. neglectus*	100	99	800
492_1	*P. crenatus*	100	99	381
492_1rep	*P. crenatus*	100	99	381
492_2	*P. crenatus*	100	98	381
492_3	*P. crenatus*	100	98	381
st08	*P. crenatus*	97	97	122
st06	*P. crenatus*	98	96	130
su01_2	*P. neglectus*	99	100	750
je3_4	*P. crenatus*	100	99	196
je2	*P. crenatus*	100	99	169
223_1	*P. neglectus*	100	100	809
224_2	*P. crenatus*	98	100	167
224_3	*P. crenatus*	98	100	178
368_2	*P. thornei*	100	100	440
369_2	*P. crenatus*	98	100	163
370	*P. thornei*	96	100	91
420	*P. fallax*	96	100	416
446_1	*P. crenatus*	96	98	283
466_2	*P. thornei*	100	98	350
452	*P. crenatus*	98	96	283
459_3	*P. neglectus*	100	99	850
592	*P. thornei*	100	96	335
pc8	*P. crenatus*	100	99	124
pc9	*P. crenatus*	100	98	137

pt8	*P. thornei*	100	99	375
pt9	*P. thornei*	100	100	390
pn9	*P. neglectus*	100	100	734
pp9	*P. penetrans*	100	98	377
ptu3	*P. thornei*	100	99	257
ptu4	*P. thornei*	100	99	375

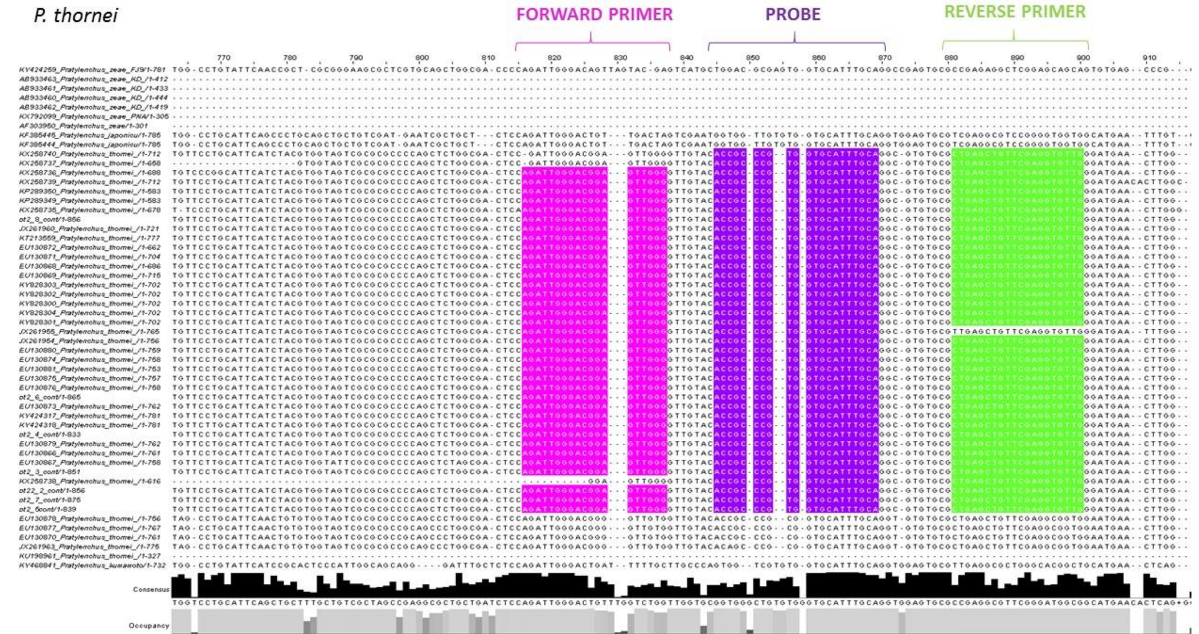
Appendix 8.3: Alignment and primers/probe design for *Pratylenchus crenatus*



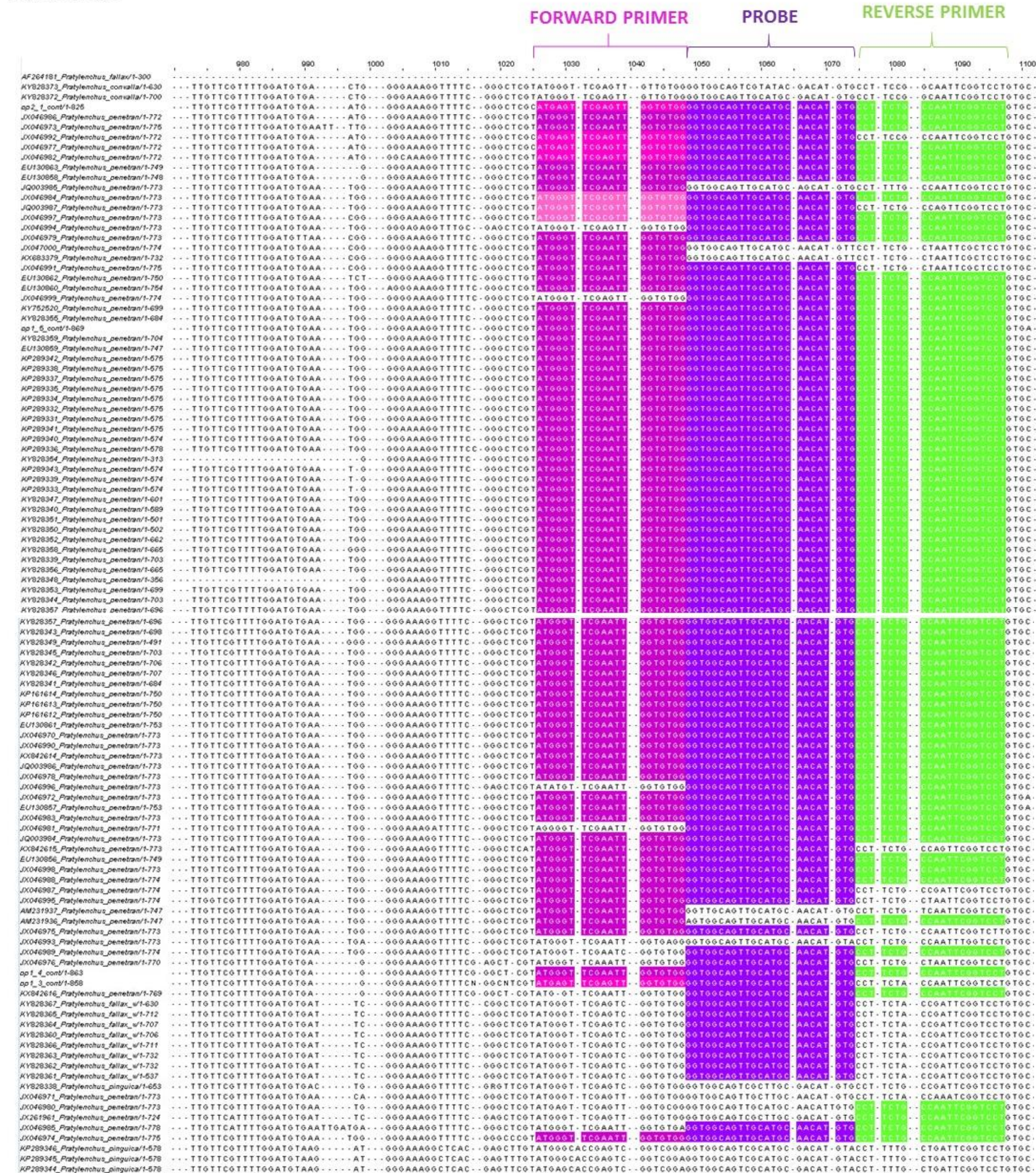
Appendix 8.4: Alignment and primers/probe design for *Pratylenchus neglectus*



Appendix 8.5: Alignment and primers/probe design for *Pratylenchus thornei*



Appendix 8.6: Alignment and primers/probe design for *Pratylenchus penetrans*



Appendix 8.7: Survey questionnaire

Harper Adams University

QUESTIONNAIRE- SURVEY OF ROOT LESION NEMATODES IN ENGLISH POTATO FIELDS

Ref. (for HAU use only): ____

Sampling date (to be completed by HAU):____

Name:

____

Address/Contact details:

____

____

__

County/Postcode:

____

Total area of potatoes grown in 2017: ______________Ha Ac (please delete as appropriate)

Varieties: _____________ ______________ ____________ ______________

Field name/code: ______________________________Field size (Ha or Ac)_________

Variety: ___________________________ Soil type: _______________________

Previous crop: _________________________ Potatoes last grown: ___________(Year)

Potato Cyst Nematodes (Yes/No): __________ Species (?): _______________________

Free living Nematodes (Yes/no): __________ Species/ Genus (?): _________________

Nematicides used: Yes/No _______________ Product:

____

Rate if different from standard: ____

Irrigation (Yes/No) __________ Applications or SMD: _______________

Crop present at the sampling date (to be completed by HAU):

____

Thanks!