Final Report

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

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CONTENTS

1. INDUSTRY SUMMARY

- Six common DNA extraction protocols were compared to evaluate their efficiency to obtain quality DNA samples for four species of root-lesion nematodes (RLN). Among all methods tested, the method with glass beads proved to be efficient for *P. penetrans,* but also for *P. crenatus, P. neglectus* and *P. thornei.*
- A TaqMan hydrolysis probe method 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*. Specificity and sensitivity of the methods were confirmed by three experiments with different life stages, increasing numbers of species target and mixed samples. Such diagnostics provide invaluable tools for accurate identification and quantification of *Pratylenchus* spp., allowing better management decisions and greater research possibilities.
- 200 potato fields from fifteen counties in England with the highest area of potato production were sampled between September and November, in 2017 and 2019. RLN were detected in 88% of samples. Identification of RLN species from positive survey samples showed that: *P. neglectus* (31%) and *P. thornei* (19%) were the most distributed mainly in East of England and in South East and South West, respectively. *P. penetrans* (18%) was mainly found in the East and South East of England, and *P. crenatus* (9%) in the East and West of England.
- Eighteen samples from Scotland were also included and 94% fields showed the presence of RLN. *Pratylenchus neglectus* and *P. crenatus* were the most present and abundant species.
- A controlled environment 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). 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.
- Nematode density of *P. penetrans* had no effect on potato yield, although the yield was significantly affected by soil type with ST1 giving the lowest yield.
- Yield was not significantly affected by nematode densities even though RLN were recorded among all treatments, except controls with water, confirming invasion

occurred. Both species were detected within the roots of potatoes, confirming invasion occurred. In all three assays, reproduction factors (R_f) were below 1 in all treatments, suggesting that reproduction was limited on Maris Peer.

- 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.
- The current study confirmed the wide prevalence of root-lesion nematodes in potato fields of 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.
- Based on the results of soil tests, growers should consider measures such as rotation planning or the use of cover crops such as black oats or marigold (*Tagetes* spp.) as poor hosts for *Pratylenchus* spp.
- Lastly, further studies should be conducted to investigate resistance and/or tolerance of potato cultivars to root-lesion nematodes to reduce nematode populations and protect yields.

2. INTRODUCTION

Root-lesion nematodes (RLN) of the genus *Pratylenchus* are migratory endoparasites with worldwide economic impact on several important crops including potato (Sasser and Freckman, 1987; Castillo & Vovlas, 2007). 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). To date, species associated with potatoes worldwide are *P. alleni, P. andinus, P. brachyurus, P. coffeae, P. crenatus, P. flakkensis, P. neglectus, P. penetrans, P. scribneri*, and *P. thornei* (Oostenbrink, 1958, 1961; Brodie *et al*., 1993; Ingham *et al*., 2005; Scurrah *et al*., 2005; Castillo & Vovlas, 2007). Both adults and juveniles infect roots by entering behind the zone of elongation and feeding on parenchyma cells. 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, 1990a, 1990b). Such lesions typically lead to necrotic areas and cell death with a reduction in root growth (Castillo and Vovlas, 2007). Damaged roots impede uptake of water and nutrients, thus plants become stunted and present leaf chlorosis (Duncan and Moens, 2013). The lesions on the surface of tubers can be brown to black, turning purple over time. 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. Root-lesion nematodes are difficult to manage once introduced into agricultural land and damage can be related to pathogenicity and population densities. 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.

2.1. *Aims*

- Compare six common methods for DNA extraction from root-lesion nematodes *Pratylenchus* spp. (RLN) to evaluate the most efficient
- Develop TaqMan real time PCR for rapid confirmation and quantification of four common species of RLN: *P. penentrans*, *P. neglectus*, *P. crenatus* and *P. thornei*
- Undertake a survey to determine the distribution and prevalence of *Pratylenchus* spp. in potato growing land in England and Scotland
- Determine pathogenicity and potato damage thresholds for *Pratylenchus* species in different soil types with a range of cultivars from the UK, under controlled conditions

3. MATERIALS AND METHODS

3.1. Comparing the efficiency of six common methods for DNA extraction from root-lesion nematodes (*Pratylenchus* spp.)

3.1.1. Six common methods of DNA extraction

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); (B) Heating and freezing before lysis extraction based on a method adapted from Williams *et al*. (1992); (C) Utilisation of glass beads to cause mechanical disruption of nematodes, adapted from Jesus *et al*., (2016); (D) Lysis of nematodes using Worm Lysis Buffer (WLB) based on a method modified from Holterman *et al*., (2006); (E) DNA extraction using NaOH (0.05 M), adapted from the method reported by Janssen et al., (2016); (F) DNA extraction using a PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific).

3.1.2. Experiments for comparison of different 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 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).

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.
2. DNA of one juvenile, one female and one male of *P. penetrans* was extracted, in three replicates for each method.
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.
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.

3.2. Development and validation of four TaqMan qPCR methods for the identification and quantification of *Pratylenchus crenatus, P. neglectus*, *P. penetrans* and *P. thornei*

3.2.1. Primer design and optimization of real-time PCR conditions

A set of two primers and one TaqMan probe were designed for each species target. After optimization, based on the Ct values obtained, the optimal conditions of the qPCR reaction were: 10 µl SensiFast Probe Hi-Rox Mix (Bioline Reagents), 0.6 µM of each primer, 0.25 µM of probe and 2 µl of DNA template. The amplification conditions were: 95ºC for 3 min followed by 35 cycles at 95ºC for 10 sec with 68–69ºC for 60 sec.

3.2.2. Specifivity, sensitivity and selectivity of diagnostics

Four standard curves were made by plotting known gene copy number, obtained by a log serial dilution of purified plasmids, with the corresponding Ct values. Four tests were performed to test the specificity, sensitivity and selectivity of primers and probe to detect each species (*P. crenatus, P. neglectus*, *P. penetrans* and *P. thornei*) with their own specific primers/probe set, separately.

- Eight different *Pratylenchus* spp. were used *(P. crenatus, P. penetrans, P. thornei, P. neglectus, P. coffae, P. pseudocoffae, P. vulnus* and *P. fallax*), including also other genera as nematode non-target such as *Globodera rostochiensis, Meloidogyne hapla, Trichodorus primitivus* and *Bitylenchus hispaniensis*. Each sample was tested in three replicates. Reactions and amplification conditions were as described above.
- One female of *P. penetrans, P. neglectus, P. crenatus* and *P. thornei* were picked up with a needle and transferred in one Eppendorf tube. Same procedure was applied for preparing samples with 10 females of each species in one tube and negative controls without species target. Positive controls were also prepared with one and ten females of species target per tube. Five replicates for each sample were prepared for DNA extraction and qPCR.
- Ten replicates for each life stage were prepared and qPCR was performed following qPCR protocols for each species target. Gene copy number per life stage was then estimated multiplying gene copy number obtained for each reaction and the total volume of each samples. Mean and standard error of mean (S.E.M.) were calculated for adults and juveniles. Values of gene copy number per individual were estimated from the average of gene copy number among adults and juveniles.

3.3. Detection and distribution of root-lesion nematodes in potato fields in England and Scotland

3.3.1. Sampling

Potato fields from fifteen counties of England were selected for a survey on the basis of the highest area of production (data reported by AHDB). The number of field sites per county were determined with a stratified method, with the overall aim to sample 200 fields. In particular, 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:

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

The counties with related number of fields are reported in Table 1.

Soil samples were collected after potato harvest, from September to October, 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, taking 60 sub-cores for a total volume of 1 kg soil per 1 ha of field in the gate entrance. The soil samples were taken using a corer with a half-cylindrical blade with 2.5 cm diameter, at 0-20 cm depth for each core. All soil cores 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).

Table 1: Top 15 counties of potato production In England based on data from AHDB. Areas of production are expressed in hectares. The number of fields to be sampled were calculated using the ratio between total number of samples to collected (200) and the total growing areas (77081) divided by the growing area of each county.

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

3.3.2. Nematode extraction and TaqMan real-time PCR:

A subsample of 200 g^{-1} soil from each soil sample was taken for nematode extraction. Nematodes were extracted from soil according the Whitehead tray method (Hooper, 1986). 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 hours. 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. The freeze-dried samples were then used for DNA extraction using Purelink DNA (Invitrogen) extraction kit, following the manufacturer's instructions. The qPCR diagnostics for *P. penetrans, P. neglectus, P. crenatus* and *P. thornei,* following the methods reported in Paragraph 3.2, were then assessed for all samples. The total number of each species per sample was calculated by dividing the gene copy number per sample and the gene copy number per individual for each species. The total count for genus was determined by the sum of qPCR numbers for each species. When no qPCR amplification was detected despite the confirmation on microscope these samples were counted as "other species of *Pratylenchus*". 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.
Growers were contacted to obtain background information about their fields via a questionnaire. 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)

3.3.3. Mapping the distribution of root-lesion nematodes in England and Scotland and statistical analysis

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). 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. All statistical analyses were performed using Genstat (19th edition, VSN International).

3.4. Development and yield of potato Maris Peer in soil infested with *Pratylenchus penetrans* and *P. thornei*

3.4.1. Nematode inoculum

Initial populations of *P. penetrans* and *P. thornei* were obtained from established carrot-disk cultures supplied by ILVO (Belgium). Monoxenic cultures of *Pratylenchus* ssp. 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). After nematode extraction using the Whitehead tray method (Hooper, 1986), different initial population densities (P_i) were used to infect each pot (see experiments for specific details). Water was used for untreated pots. Before using the inoculum, some 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.

3.4.2. Experiment 1

The experiment was carried out from July to August 2017 at Harper Adams University over six weeks. Densities were calculated on the basis of the total weight of 250 g soil per pot (Table 2). Three different type 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.

3.4.3. 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 eleven 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 (Table 2). Densities were calculated on the basis of the total weight of 500 g soil per

pot (Table 1). One type of soil, ST1 (10% JI N°2 and 90 % coarse sand) was used and six replicates for a total of 72 pots were arranged in a Latin square design.

3.4.4. 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 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 a versatile 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). In experiments 2 and 3, nutrients (Hoagland's No. 2 Basal Salt Mixture) were supplied weekly, after 5 weeks.

3.4.5. 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.

3.4.6. Estimation of nematode population density in soil and potato roots

Nematodes were extracted from the soil of each pot using the Whitehead tray method (Hooper, 1986) 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 40X magnification (Mazurek Optical Service, Meiji EMT) and expressed as nematodes g^{-1} root following the formula below:

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

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 P_i is the initial population density inoculated in each pot.

3.4.7. Statistical analysis

Data of numbers of tubers, weights of fresh shoots, roots, biomass (sum of weights of fresh shoots and roots) and tubers were subjected to two-way analysis of variance (ANOVA) to assess soil and population densities effects for experiment 1. Similarly, a one-way analysis of variance (ANOVA) for experiment 2 and 3 was performed to assess population densities effect. 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).

4. Results

4.1. Comparing the efficiency of six common methods for DNA extraction from root-lesion nematodes (*Pratylenchus* spp.)

4.1.1. DNA extraction and amplification of *P. penetrans*

Six methods of DNA extraction were tested with increasing numbers (one, five and ten females) (Figure 1) and different life stages (Figure 2) of *P. penetrans*. 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 1). There were no significant differences among life stages (P = 0.374, χ2 = 1.97, d.f. = 2) (Figure 2). Whereas significant differences (P < 0.001, χ2 = 24.92, d.f. = 5) were observed between different DNA extraction methods (Figure 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.

4.1.2. 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) and different life stages (Figure 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). 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 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 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.

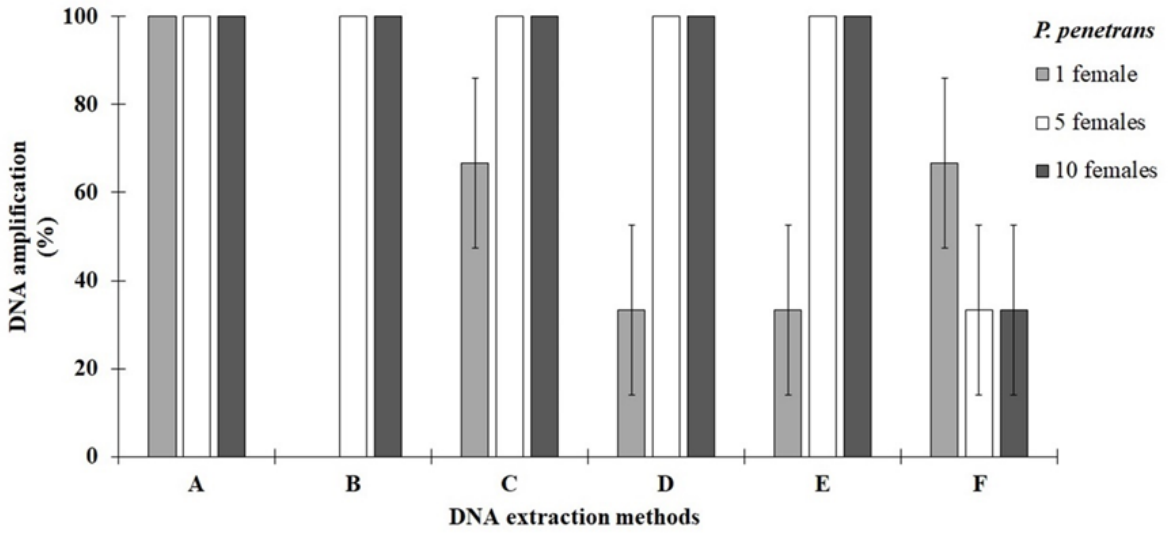


Figure 2: DNA amplification (%) of one male (n=3), one female (n=3) and one juvenile (n=3) of *P. penetrans* using six DNA extraction methods: (A) manual cut of nematode; (B) heating and freezing; (C) glass beads; (D) Worm lysis buffer; (E) NaOH; (F) PureLink DNA extraction kit. Error bars show the standard error of the mean.

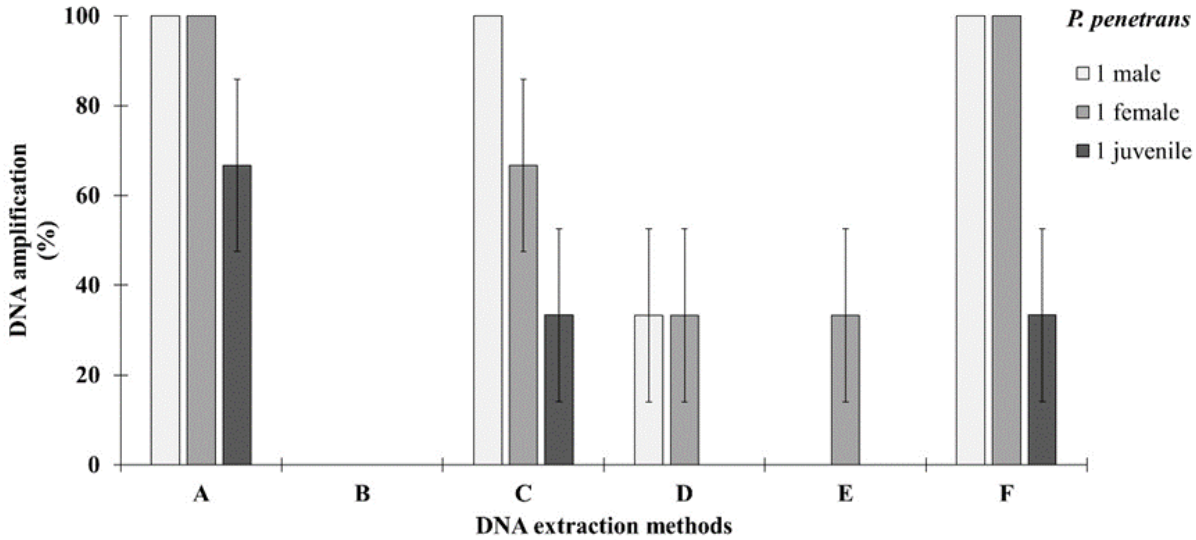


Figure 3: DNA amplification (%) of one female (n=3), five females (n=3) and ten females (n=3) of *P. crenatus, P. neglectus*, *P. thornei* and *P. penetrans* using a glass bead DNA extraction method (Method C). Error bars show the standard error of the mean.

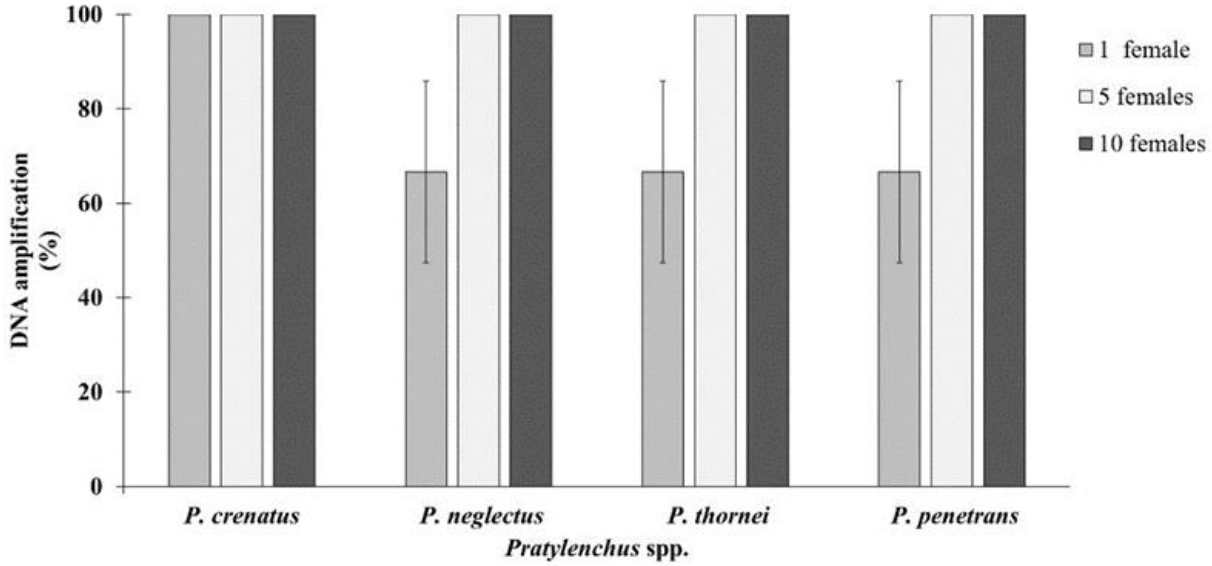
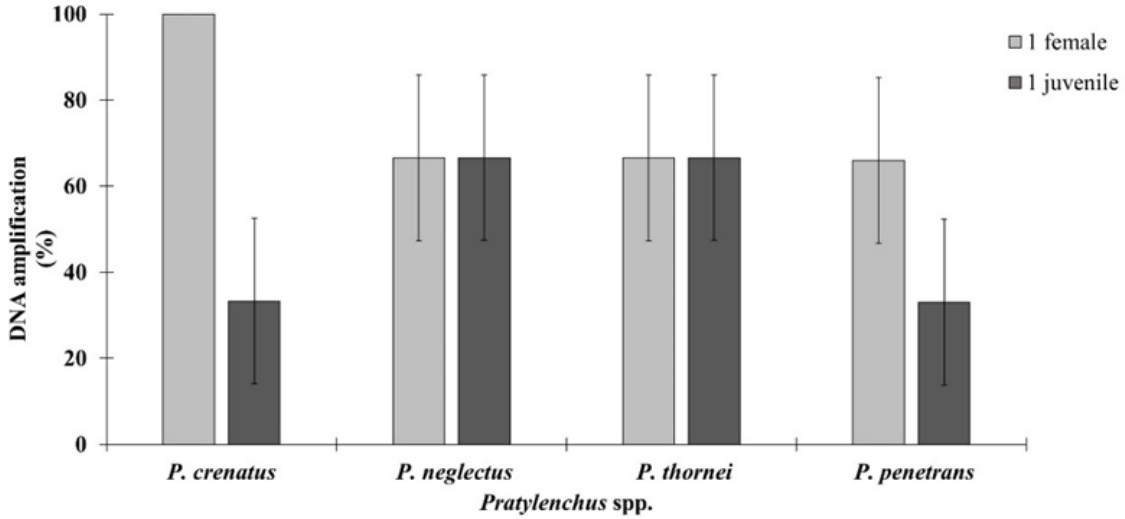


Figure 4: DNA amplification (%) of one female (n=3) and one juvenile (n=3) of *P. crenatus*, *P. neglectus*, *P. thornei* and *P. penetrans* using a glass bead extraction method (Method C). Error bars show the standard error of the mean.



4.2. Development and validation of four taqman qpcr methods for the identification and quantification of *Pratylenchus crenatus, P. neglectus, P. penetrans* and *P. thornei*

4.2.1. Standard curves

Standard curves 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% was recorded (Figure 5). No amplification signals were detected for negative controls samples with non-species targets and water (NTC), for all four diagnostics.

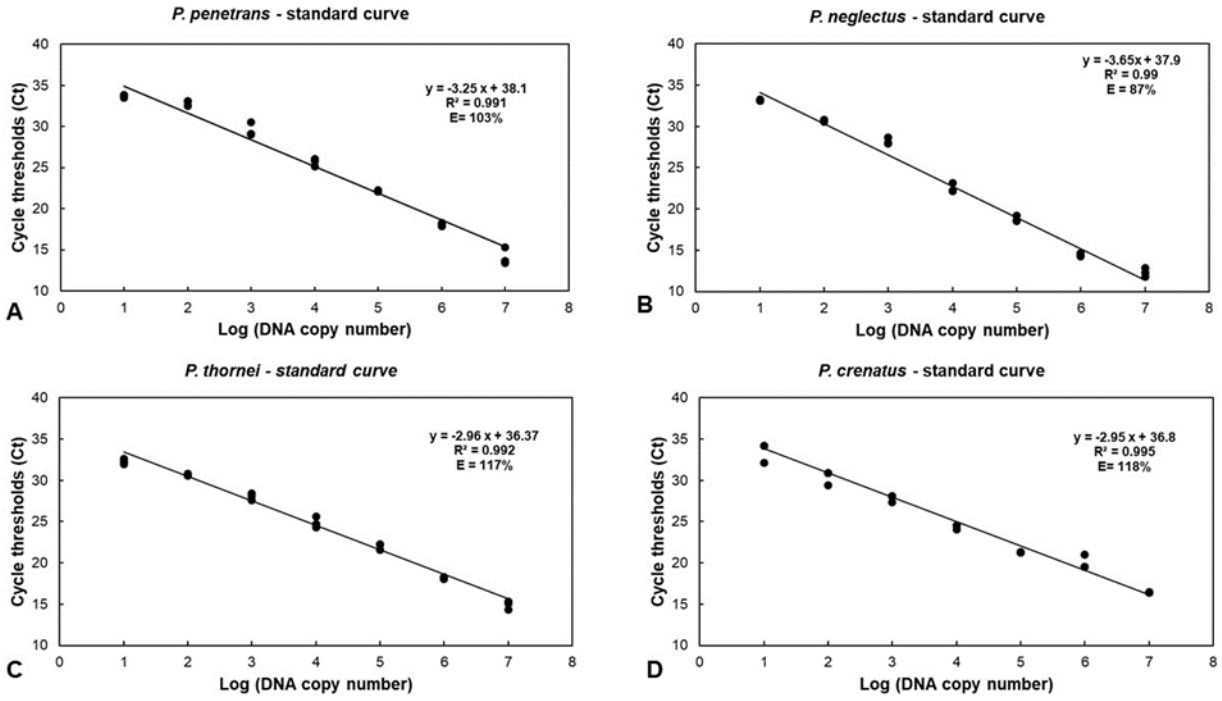


Figure 5: Standard linear curves of cycle threshold (Ct) values plotted against log-transformed gene copy number of A: *Pratylenchus penetrans*; B: *P. neglectus*; C: *P. thornei* and D*: P. crenatus*. The standard curves were run in triplicate for each *Pratylenchus* species. E is the efficiency of standard curve calculated by the equation: E % = [(10(−1/slope)) -1] X 100.

4.2.2. Test 1 - Specificity of primers and probe to detect each species target

Each diagnostic assay was tested for one adult of each species-target from different locations and/or sources, in three replicates (Table 4). The qPCR method was applied to three different populations of *P. penetrans*, with one individual in each tube, in three replicates. Consistent Ct values, in the range of 30.1 - 30.5, were obtained for one individual of *P. penetrans*, confirming the reproducibility of the method among different populations. 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 species-target. Three populations of *P. crenatus* from different locations were also tested. One sample from Scotland (code 452) showed Ct values of 28.8 ± 0.12 that was just 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 sample 452 and the other Scottish sample (Je2), neither 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*).

4.2.3. Test 2 - Selectivity of primers and probe in mixtures of *Pratylenchus* spp.

The assays were tested against samples prepared with a mixture of *Pratylenchus* species to identify the selectivity of each diagnostic assay (Table 5). One female of *P. penetrans* was detected (Ct =29.8 ± 0.09) in a mixture of 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). Similar results were obrained also for mixed samples of other species *P. thornei, P. crenatus* and *P. neglectus*. All the other samples with mixed species without the target species were not amplified, as well as negative controls with distilled water. 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 mixed closely related species.

4.2.4. Test 3 – Amplification of different life stages

Each diagnostic assay was tested for the amplification of different life stages (Table 6). Overall, the average DNA copy number per individual were: 7317 ± 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). Males 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 = 0.003, P = 0.02 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 = 0.009) lower than adults (9463 ± 400). Negative controls produced no signal for all tests.

Table 4: Codes, host/sources and origin of *Pratylenchus* spp. and other nematode genera used in this study, together with the mean 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)	-	-	-	-

Table 5: Specificity of each diagnostic protocol for detection and quantification of one (n=6) and ten (n=6) target species in mixture of *Pratylenchus* spp. Ct values obtained by qPCR are reported by 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 target species	29.3 ± 0.14 a	30.8 ± 0.6 a	30.3 ± 0.5 a	30.7 ± 0.7 a
1 female of target species + 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 target species + 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 target species	-	-	-	-
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 6: Sensitivity of each assay for detection and quantification of different life stages (n=10) for each *Pratylenchus* target species. Ct values were obtained by performing qPCR protocol of each target species separately. Quantification of DNA copy number (D2-D3 copy number) of each target species 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 ($P \leq 0.05$). Values of gene copy number per individual were estimated from the average of gene copy number among adults and juveniles.

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

4.3. Detection and distribution of root-lesion nematodes in potato fields in England and Scotland

4.3.1. 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 field samples contained species of *Pratylenchus* and only 12% did not (Figure 6 A). Consequently, root-lesion nematodes are prevalent in potato growing land in England. 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 (Figure 6 B) . 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 minor *Pratylenchus* species. A proportion of the samples were found to contain more than one species; *P. crenatus* was found with other species in 61% of fields, while *P. neglectus*, *P. penetrans* and *P. thornei* occurred in mixed populations in 52%, 62% and 42% of samples, respectively (Figure 7).

4.3.2. Distribution of root-lesion nematodes in England

Root-lesion nematodes were found in all counties with a high percentage of presence. Norfolk, the county with the highest potato production and consequently highest number of fields sampled, presented root-lesion nematodes in 95% of fields sampled. Root lesion nematodes were found also in Suffolk (100% fields sampled), Nottinghamshire (100%), Shropshire (94%), Herefordshire (92%), Cambridgeshire (100%), Lincolnshire (97%) and Yorkshire (83%). In Staffordshire, only 33% of fields presented root-lesion nematodes and only *P. crenatus* and *P. neglectus* were detected in the samples.

The map in Figure 8 shows the distribution of root-lesion nematodes in England. The genus was widely present in England with a significantly higher (X^2=23.35, d.f.=5, $P < 0.001$) distribution found in the East and North East of England. Each species presented a different distribution. Figure 9 shows four different distribution maps representing each species we detected. In particular, *Pratylenchus crenatus* is more dispersed in the East and West of England and it is less abundant than other species (Figure 9 A). *Pratylenchus neglectus* was found to have a significantly higher occurrence in the East of England (X^2=16.14, d.f.=5, $P < 0.001$) (Figure 9 B). *Pratylenchus penetrans* had a significantly higher occurrence (X^2=16.14, d.f.=5, $P = 0.006$) in East and South East of England (Figure 9 C) , whereas

Pratylenchus thornei is mainly found in the South East and South West (X^2=16.58, d.f.=5, *P* = 0.005) (Figure 9 D).

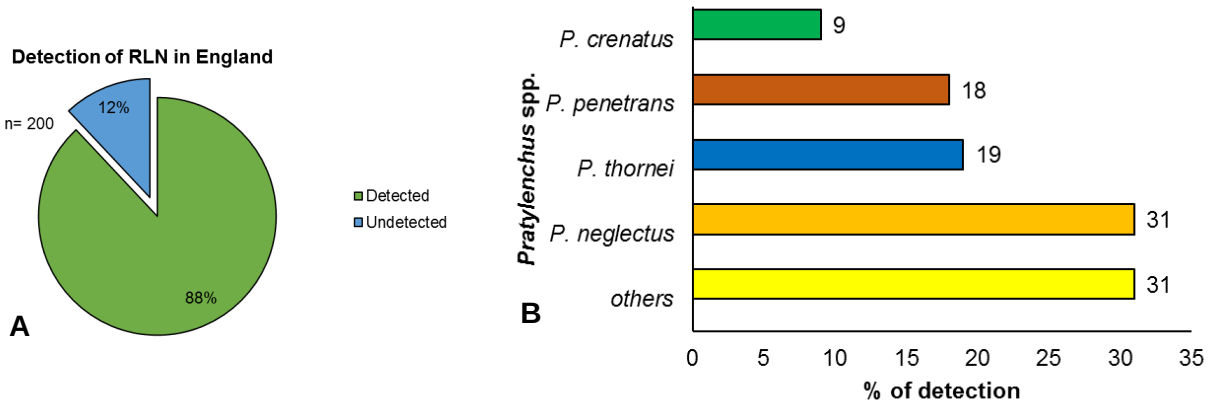


Figure 6: Percentage of root-lesion nematodes found in 200 potato fields from England (A); percentage of each *Pratylenchus* spp. detected in the survey (B).

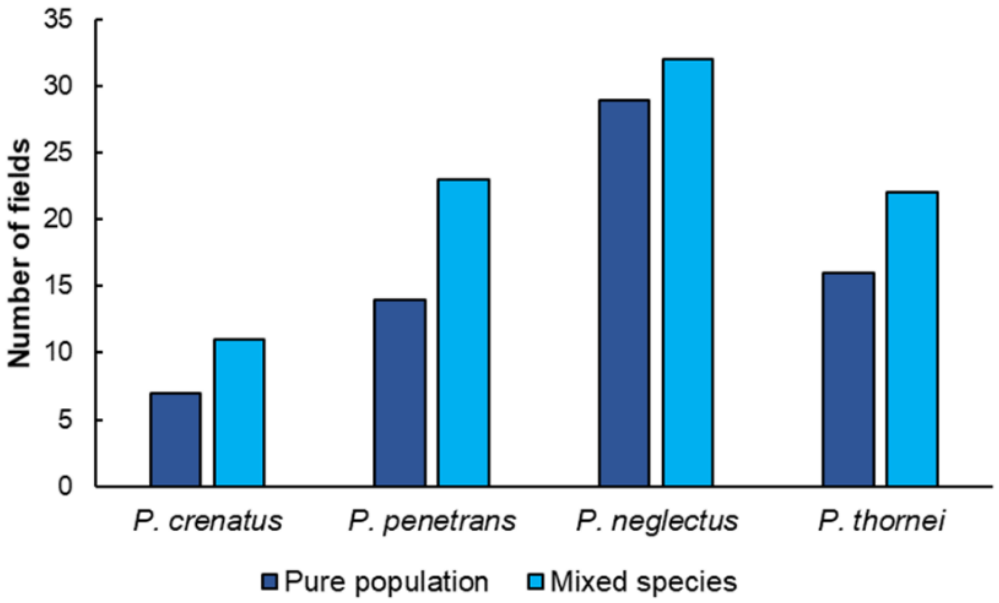


Figure 7: Number of positive fields from England where *P. crenatus*, *P. neglectus*, *P. penetrans*, *P. thornei* was found in isolation or in combination with other species.

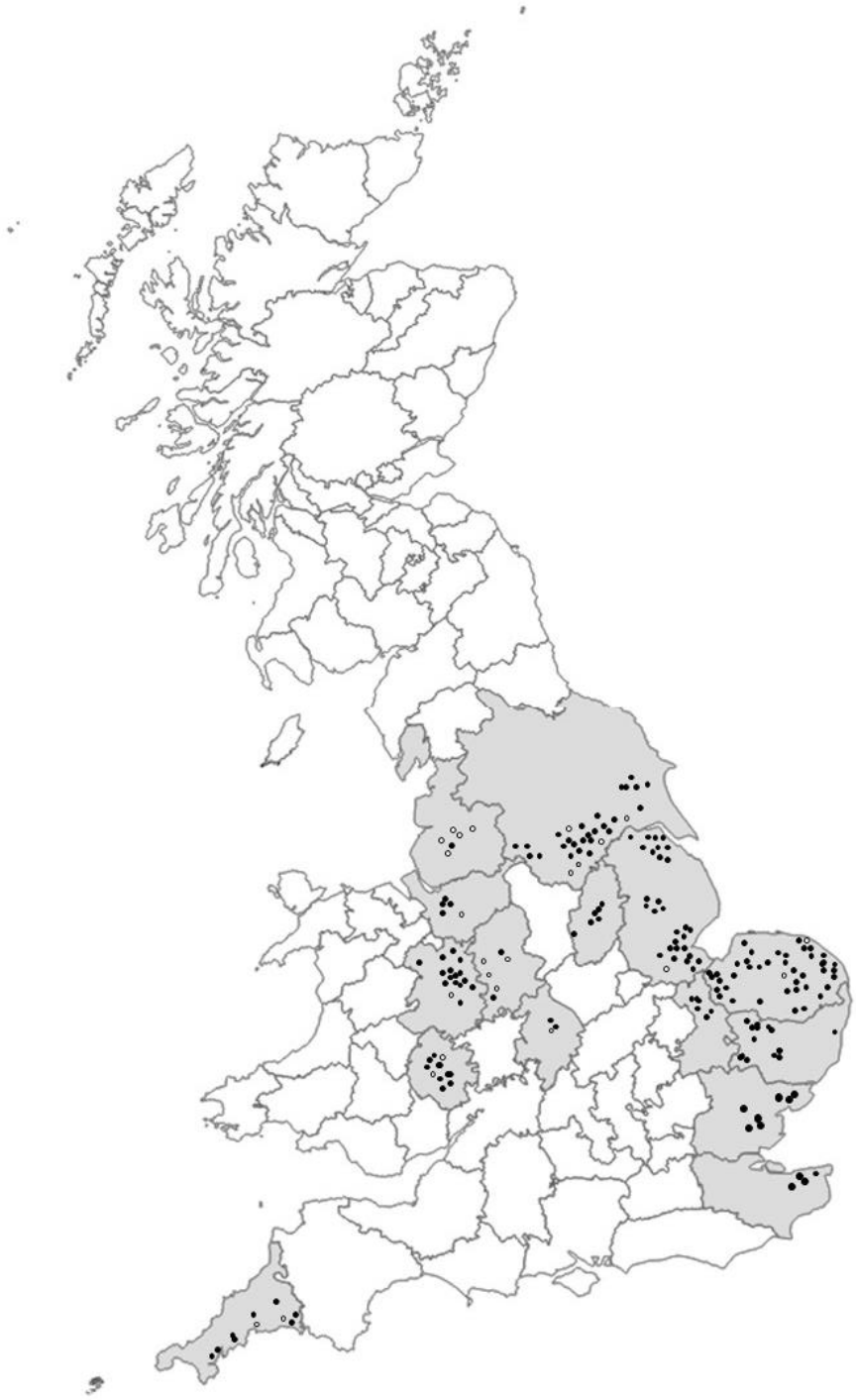


Figure 8: Distribution map of root-lesion nematodes in potato fields from England. Sampled counties are shaded grey colour, black dots indicate fields where root-lesion nematodes were positively detected, whereas white dots show fields without detection.

Figure 9: Distribution of *P. crenatus* (A); *P. neglectus* (B); *P. penetrans* (C) and *P. thornei* (D) in potato fields in England.

4.3.3. Survey samples and analysis

Two hundred soils were sampled between September and November of 2017 and 2019. 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 10 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. Sugar beet and oats were previous crops in 6 and 3% of the samples, respectively. Other crops including carrots, cauliflower, mustard, onion, peas and pumpikins were the previous crop in 16% of fields (Figure 10 B). Both the crop at sampling and the previous crop did not significantly influence the distribution (P = 0.61 and P = 0.124), neither the abundance (P = 0.733 and P = 0.1) of root-lesion nematodes. 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) Soil type is another important factor to consider in the distribution of root-lesion nematodes. In our survey, 41% of fields sampled were categorised as a loamy soil, 24% were sandy clay loams, 8% were sandy loams and only 3% were peaty (Figure 11). 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 in the year of sampling (Figure 12). 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, and 22% did not receive irrigation (Figure 13). In our study the presence and abundance of root-lesion nematodes were not significantly influenced by soil type (P = 0.659), nematicide (P = 0.123) and irrigation (P = 0.372). Similarly, each factor did not significantly influence the distribution and abundance of each 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). The history of potato cyst nematodes (PCN) and root-lesion nematodes (RLN) is another aspect to take in consideration (Figure 14). The survey showed that 45% of fields had PCN present, while 40% did not have a record of PCN and 15% of the respondants did not provide an answer. Only 10% of growers indicated that RLN was present in their fields and the remainder (90%) did not know if RLN were present or not in their field.

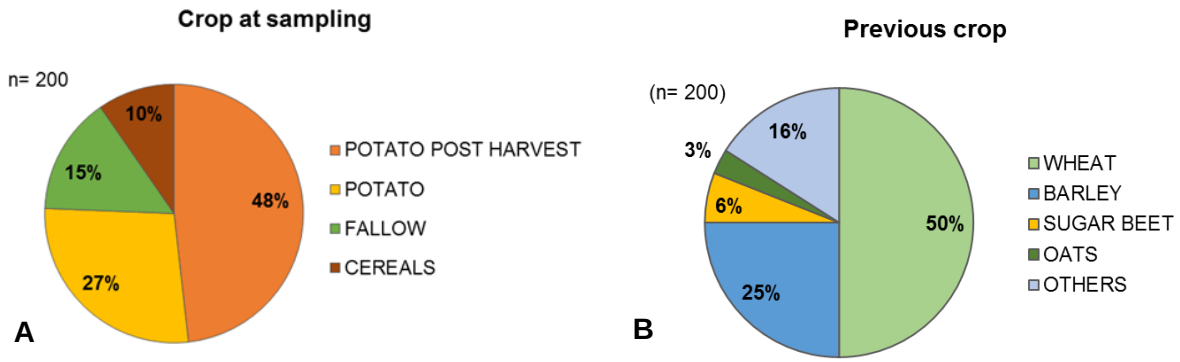


Figure 10: Percentage of each crop type at sampling (A) and as the previous crop (B).

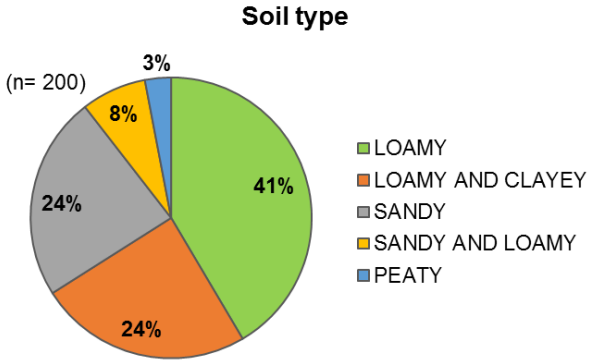


Figure 11: Percentage of each soil type from the potato fields sampled in England between 2017 and 2019 (n=200).

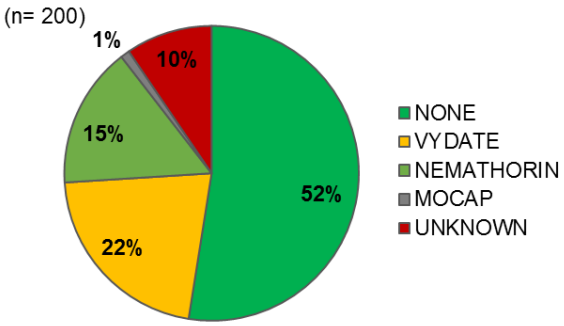


Figure 12: Percentage of nematicides applied in the fields sampled (n=200) in England between 2017 and 2019.

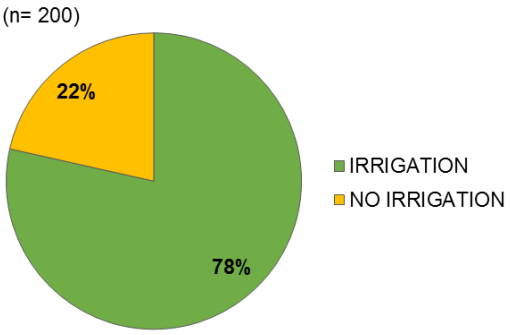


Figure 13: Percentage of irrigation in the fields (n=200) in England between 2017 and 2019.

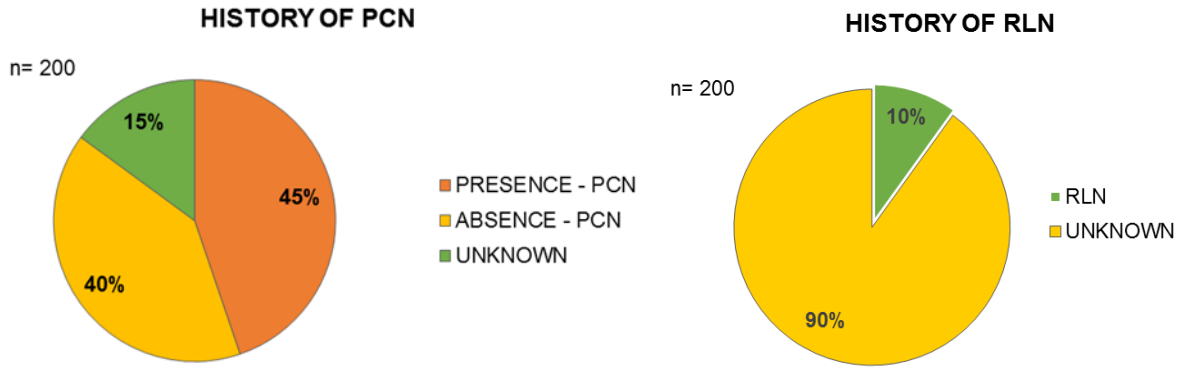


Figure 14: Percentage of farmers that confirmed the presence of potato cyst nematodes (right) and root-lesion nematodes (left) in their potato fields in a study of 200 fields in England

A wide range of potato cultivars were grown in the field sites sampled, with some fields containing more than one cultivar (Figure 15). The most commonly grown was Maris Piper (11% of fields sampled), followed by Melody (7% of fields sampled), Accord (5% of fields sampled), Maris Peer (5% of fields sampled), Russet Burbank (5% of fields sampled), Marfona (5% of fields sampled), Estima (4% of fields sampled), Nectar (4% of fields sampled) and Sagitta (4% of fields sampled). King Edward, Olympus, Desire, Premiere were grown in 3% of the fields sampled, whereas Royal and Taurus were each grown in 2% of the field sampled. The remainder of sites were represented by a collective of lesser grown cultivars (35%).

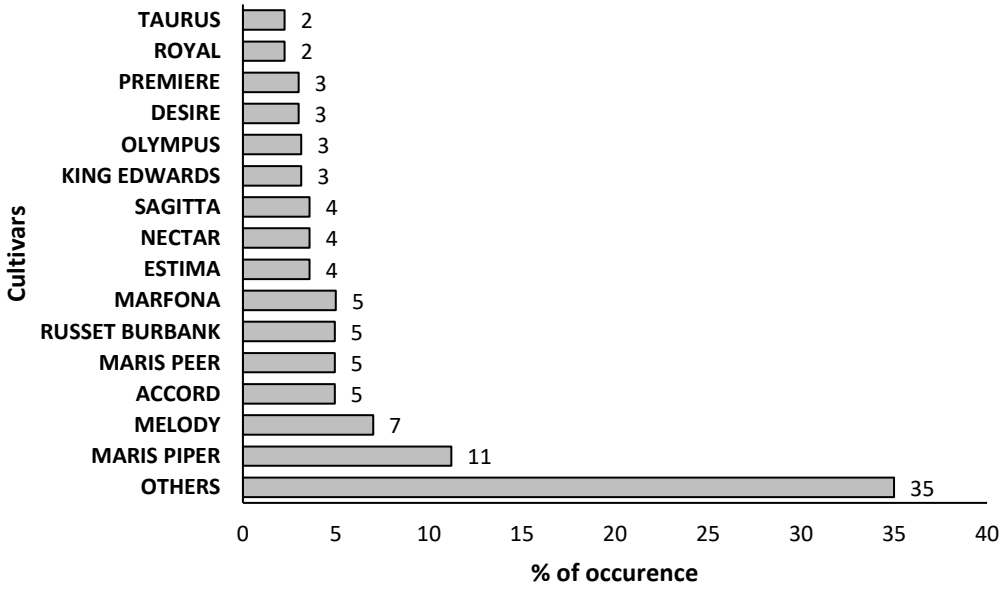


Figure 15: The choice of potato cultivar (n=223) grown in 200 fields sampled for a survey of *Pratylenchus* spp in England.

4.3.4. Detection of root-lesion nematodes in Scotland

Eighteen samples from potato fields of Scotland were included for the identification and quantification of root-lesion nematodes. The genus was detected in 94% of fields and only 6% did not present any *Pratylenchus* species (Figure 16 A-B). Species were often mixed (83%) and only three fields contained pure species. 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. *Pratylenchus penetrans* was found in ten fields. In terms of abundance, *P. crenatus* was found to be the most abundant in fields where it was detected, 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.

In terms of estimated abundance, *P. crenatus* was found to be the most abundant in fields where it was detected, 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 (data not shown).

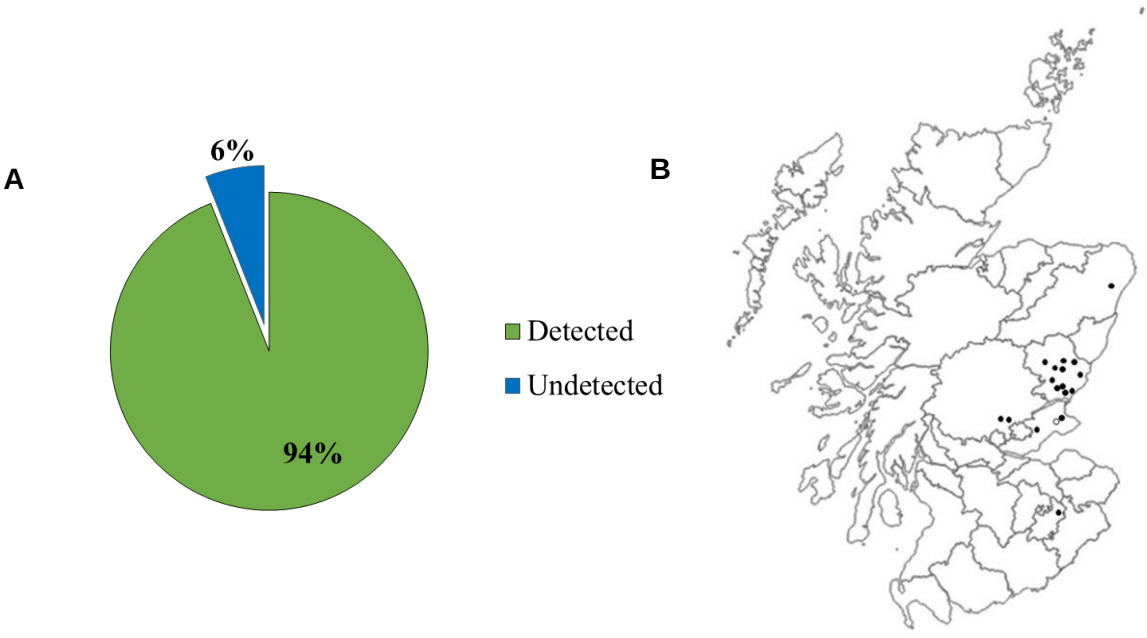


Figure 16: Percentage (A) and distribution map (B) of root-lesion nematodes in potato fields (n=18) from Scotland. Black dots indicate fields where root-lesion nematodes were positively detected, whereas white dots show fields without detection. Samples were collected by JHI between 2015 and 2017.

4.4. Development and yield of potato Maris Peer in soil infested with *Pratylenchus penetrans* and *P. thornei*

4.4.1. Experiment 1:

Almost all minitubers produced potato plants within three weeks with only three pots within soil type 2 (ST2) that failed to emerge. After six weeks, plant measurements were taken to predict the yield of potatoes. Plants grown in soil type 1 (ST1) and soil type 2 (ST2) were less developed than those grown in soil type 3 (ST3) (Figure 17). Eggs and adults were detected inside roots of almost all treatments except control (Figure 18 A-C) confirming that RLN invasion on roots occurred.

Height and fresh weight of haulms, fresh weight of roots and fresh weight of tubers were significantly affected by soil type. Plant height was significantly affected (P = 0.012) by the soil medium used, with those growing in soil type 3 having a greater height than those grown in soil type 1 (ST1) (Figure 19-A). Considering fresh weights of shoots (Figure 19-B), all three types of soil were all significantly different 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 19-C). Total weight of tubers (Figure 19-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.

Final population densities (P_f) were calculated for each treatment, and in each soil type group, densities increased according the increase of P_i, but in general, the P_f were lower than the P_i (Table 8). 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 presented highest value (P = 0.01) of 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 9).

Figure 17: 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.

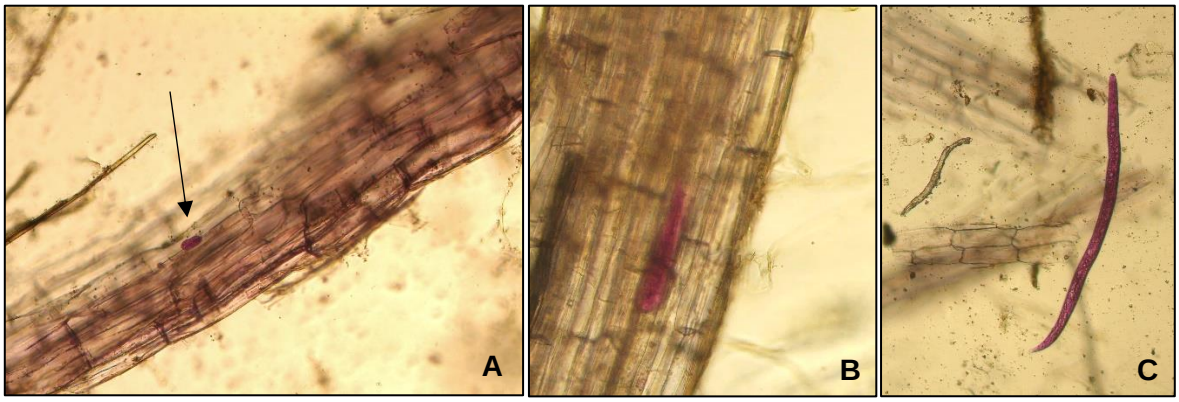


Figure 18: Egg (A) and adults (B-C) of *P. penetrans* inside potato root tissues after root staining with acid fuchsin method (Hooper, 1986). Arrow indicate the position of the egg in the root. Pictures were taken at 40X magnification.

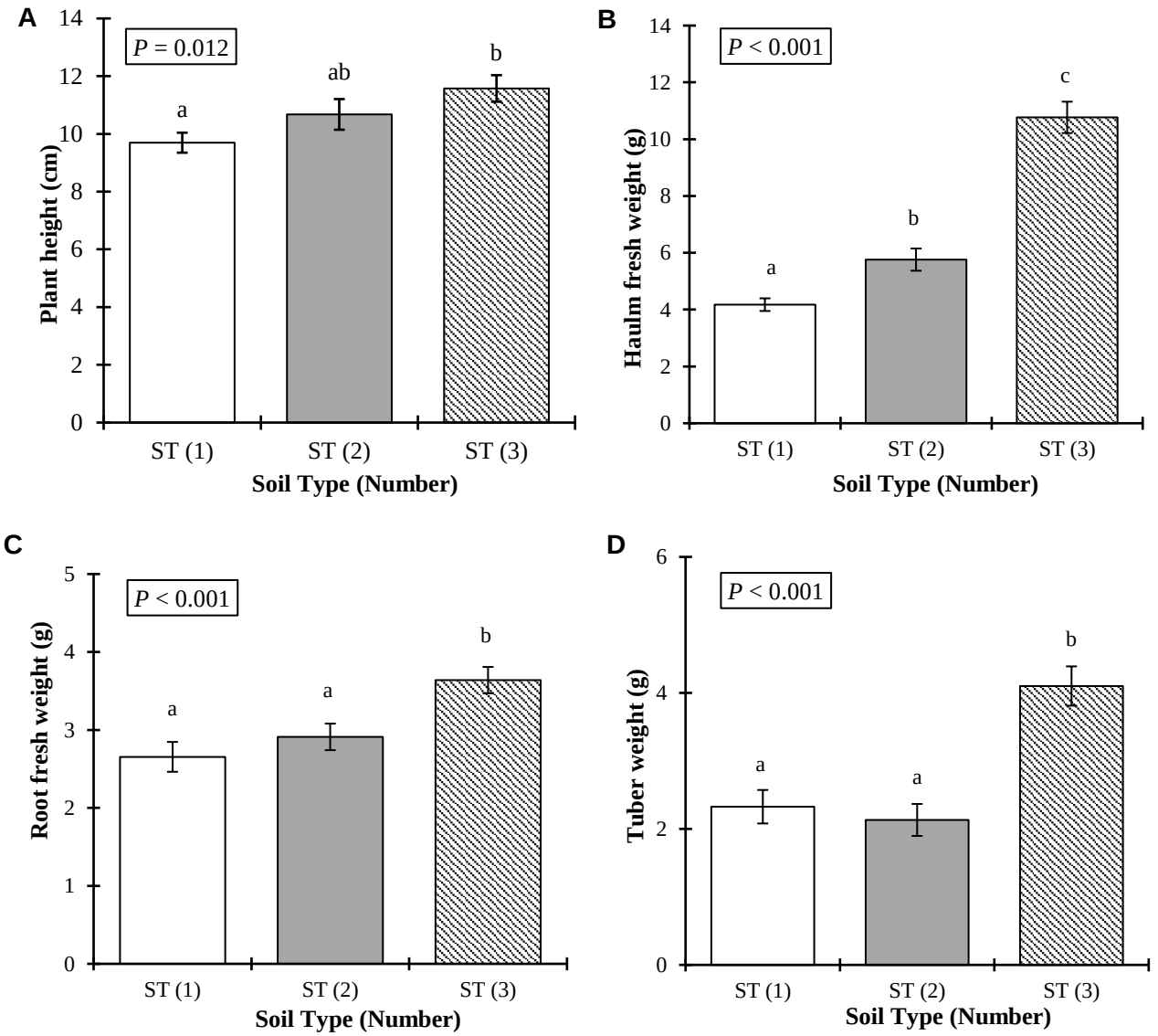


Figure 19: Plant height (cm) (A), Haulm fresh weight (g) (B), Root fresh weight (g) (C), and tuber weight (g) (D), of potatoes cv. Maris Peer growing in three different soil types, ST1 (10% JI N°2 and 90 % coarse sand), ST2 (20% JI N°2 and 80% coarse sand) and ST3 (40 % JI N°2 and 60% coarse sand) with different population densities (0, 0.125, 0.25, 0.5, 1, 2, 4 g^{-1} soil) of P. penetrans. Data are means (n = 28, collected at six weeks after planting and averaged) ± standard error of the mean (SEM). Columns with different letters are significantly different according to Bonferroni's test ($P < 0.05$).

Table 8: 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 (SEM). 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).

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 9: 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).

EXPERIMENT 1 – *P. penetrans*				
Initial population density (Pi)	Population density per pot (P)	Rf (Pf / P)		
		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

4.4.2. Experiment 2:

Potato plants emerged in all pots between 10 and 17 days. Potato plants attained final heights from 3.75 cm (±1.86) to 6.08 cm (±1.9) (Figure 20-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 nematodes g^{-1} soil, which was similar to the untreated control (9.38 g ± 1.64) (Figure 20-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 20-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. Initial population densities (P_i) did not significantly affect the yield of potatoes. Potato roots presented brown lesion (Figure 21), and root-lesion severity, associated with RLN nematodes, significantly increased with increasing P_i according to Kruskal-Wallis One Way ANOVA (P = 0.004, d.f.= 5, Chi-Square= 17.42) with a pairwise comparison (data not shown). Final population densities (P_f) increased significantly with increasing initial population densities, in both soil (P < 0.001) and roots (P = 0.022) (Table 9). 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 10).

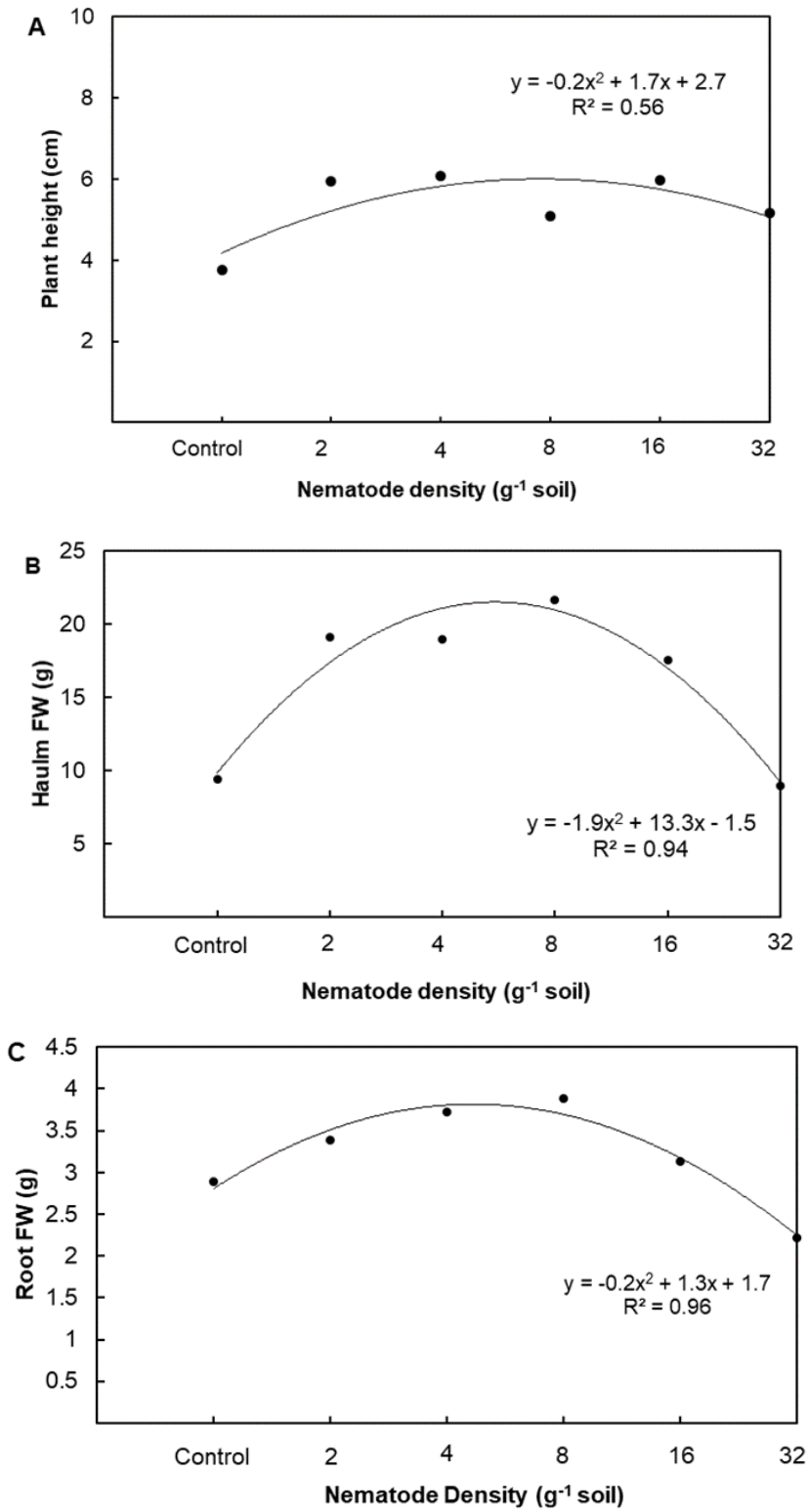


Figure 20: Plant height (A), haulm (B) and root fresh weight (g) (C) following exposure to different population densities (0, 2, 4, 8, 16, 32 g^{-1} soil) of *P. penetrans*. Data are means (n = 6) ± standard error of the mean (SEM). Differences between treatments are represented by a polynomial regression model.

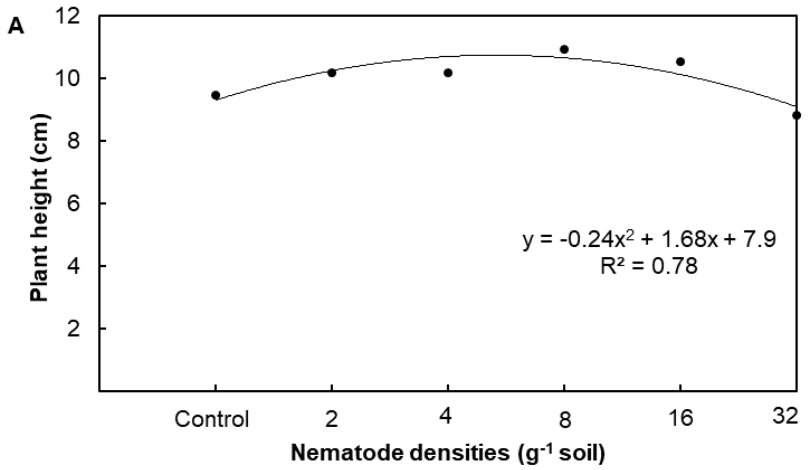
Figure 21: Root-lesions on the roots of potato associated with invasion and colonisation by *P. penetrans*. Arrows point the brown lesion on the roots (12 weeks).

Table 10: 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. penetrans* calculated by (P_f/P) based on recovery from soil and root samples.

EXPERIMENT 2 – *P. penetrans*				
Initial population density (P_i)	Population density per pot (P)	Final Population (P_f) Soil (500 g^{-1} soil)	Root invasion Square-root (x+1) (Nematode g^{-1} root)	Rf (P_f / P)
0	0	0	0	0
2	1000	232±76	13.61(491±248)	0.69
4	2000	300±84	19.14 (216±76)	0.26
8	4000	332±123	25.8 (1266±577)	0.43
16	8000	827±244	27.64(1458±1153)	0.29
32	16000	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

4.4.3. 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 22-A). Haulm of plants decreased in weight with a moderate negative correlation with P_i ($R^2 = 0.508$) (Figure 22-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 22-C). Whilst roots presented brown lesions (Figure 23), *P. thornei* did not significantly affect the yield of potatoes. Also in this case, root-lesion severity, associated with *P. thornei*, significantly increased ($P < 0.001$, d.f.= 5, Chi-Square= 21.5) with increasing P_i (data not shown). After nematode extraction from soil and potato roots, *P. thornei* final population increased in accordance to increases in P_i. As with *P. penetrans* (Experiment 2), P_f values were lower than the P_i with a low reproduction factor found for all treatments (Table 11), suggesting that multiplication did not occur during the the experiment.



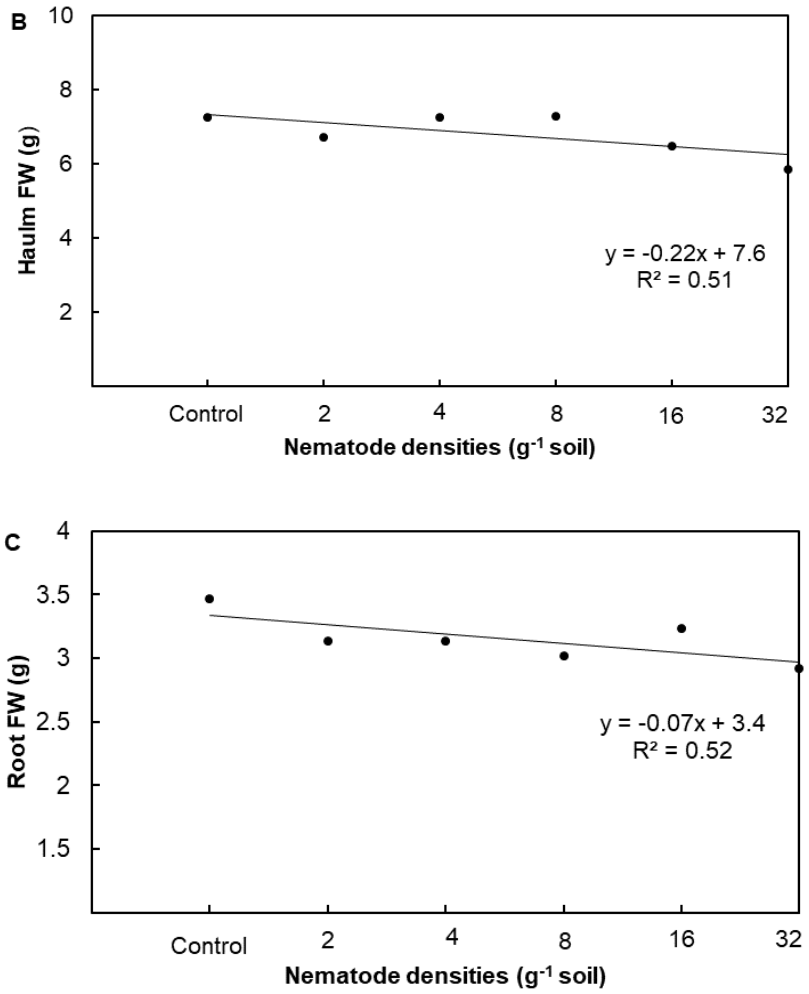


Figure 22: Plant height (cm) (A), haulm fresh weight (g) (B) and root fresh weight (g) (C) following exposure to different population densities (0, 2, 4, 8, 16, 32 g^{-1} soil) of *P. thornei*. Data are means (n = 6) ± standard error of the mean (SEM). Differences between treatments are represented by a polynomial regresion model for plant height and linear model for haulm fresh weight and root fresh weight.

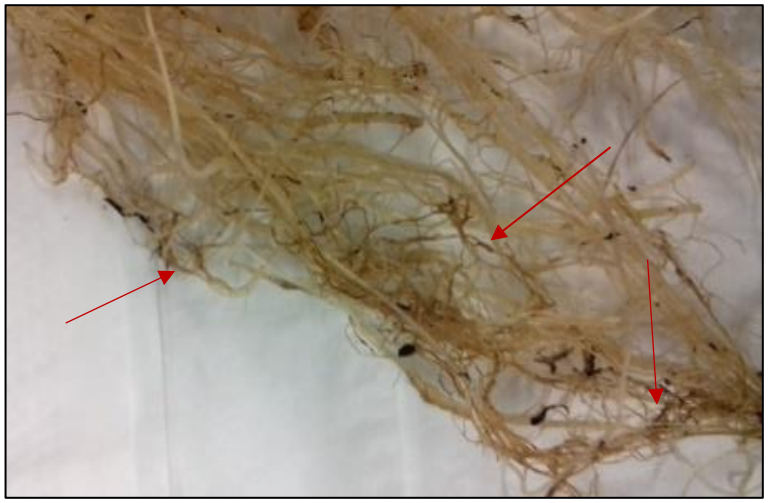


Figure 23: Root-lesions on the roots of potato associated with invasion and colonisation by *P. thornei*. Arrows point the brown lesion on the roots (12 weeks).

Table 11 **:** 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. penetrans* calculated dividing P_f by P, based on recovery from soil and root samples.

EXPERIMENT 3 – *P. thotnei*				
Initial population density (P_i)	Population density per pot (P)	Final Population (P_f) Soil (500 g^{-1} soil)	Root invasion (g^{-1} root)	Rf (P_f / P)
0	0	0	0	0
2	1000	227±100	25±17	0.25
4	2000	133±37	183±73	0.16
8	4000	357±117	125±28	0.12
16	8000	443±86	100±63	0.07
32	16000	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

4.5. 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). 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.

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*. 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.

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). Then, this molecular target was selected for the development of four TaqMan qPCR protocols for the identification and quantification of *P. crenatus, P. penetrans, P. neglectus* and *P. thornei*. 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 three tests. *Pratylenchus penetrans, P. crenatus, P. neglectus* and *P. thornei* were successfully discriminated from other genera and *Pratylenchus* species. 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 species target 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-target species. 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 species target. Each diagnostic protocol detected juveniles for each species target, 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*. These results demonstrated the high sensitivity of each method in the quantification of each species target.

Methods developed in this study were used for identification and quantification of *Pratylenchus* ssp. in a survey of potato growing land in England and Scotland. 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. 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). In the past, the presence of these species was also reported in other crops in Scotland such as cereals and carrots.

Crop damage are commonly related to the population densities of plant parasitic nematodes. Damage thresholds between 100 and 200 nematodes/100 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. 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. *P. penetrans* were 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* per 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* per g^{-1} soil and cv. Katahdin by 1.5 – 2 *P. penetrans* per 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 so far. 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).

5.1 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 the discipline of Plant 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* in 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 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. With climate change, the impact of these nematodes on potato production may increase in future. 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 *Verticillum dalhiae* or *Rhizoctonia solani* 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 in the damage caused by root-lesion nematodes, as well as 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. 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 in infested land.

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