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Project leader:	Dr Tim O'Neill, ADAS
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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headlines

- High levels of the root lesion nematode *Pratylenchus thornei* have limited impact on growth of strawberry.
- Novel molecular tests allow accurate identification and rapid quantification of nematodes in soil.

Background and expected deliverables

Nematodes are important pests of strawberries and raspberries and can cause crop losses through direct feeding damage on roots, transmission of viruses and possibly increasing susceptibility to Verticillium wilt caused by *Verticillium dahliae*. Nematode problems in strawberry and raspberry are of increasing concern to growers, especially with tighter rotations. However, the relative occurrence of different nematode species in UK soil-grown soft fruit crops is unknown. An improved understanding of these pests is likely to become increasingly important particularly as the availability and use of soil disinfestation treatments for nematode control is decreasing.

There are a number of gaps in our understanding of free-living nematodes. Firstly, it is unclear how many root lesion nematodes (*Pratylenchus* spp.) are needed to cause direct feeding damage. Threshold levels used for assessing risk of direct damage to strawberry are based on anecdotal not experimental evidence. Secondly, there may be an interaction between the presence of *Pratylenchus* species and the incidence of Verticillium wilt. It is known that some Verticillium wilts (e.g. in *Acer* and potato) can be exacerbated by plant pathogenic nematodes, particularly of the genus *Pratylenchus*. Furthermore, despite the risk of serious losses from nematodes in soft fruit, expertise in their extraction, identification and evaluation is limited.

A quantitative molecular (QPCR) test has recently been developed (SF 97) for determination of *Verticillium dahliae* in soil which is able to detect and quantify inoculum of the pathogen within 24 hours with a high level of specificity. DNA extraction from soil to quantify *V. dahliae* offers prospects for rapid determination of any other pests and pathogens present, including nematodes, as the method extracts all DNA present. Recent advances in DNA techniques offer the potential to identify nematode species accurately and quickly without the need for taxonomic expertise.

This project aims to reduce losses in strawberry and raspberry caused by root nematodes through determination of threshold levels that cause direct damage and an increased

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understanding of their interaction with *Verticillium dahliae* to cause Verticillium wilt in strawberry.

The specific project objectives were:

1. To determine the nematode species most commonly found associated with soil-grown strawberry and raspberry crops in the UK.
2. To confirm the soil threshold level for direct root damage to strawberry by a *Pratylenchus* species.
3. To determine whether nematode species present in a soil sample can be identified by testing the mass DNA extracted from soil samples when testing for *V. dahliae* by molecular quantification.
4. To determine whether increasing levels of a root lesion nematode species increases the risk of strawberry Verticillium wilt caused by *V. dahliae*.

Summary of the project and main conclusions

Objective 1: Occurrence of nematodes in UK soils used for soft fruit production

The relative frequency of different genera of nematodes found in 92 soil samples submitted by soft fruit growers to ADAS and Fera and processed by ADAS Pest Evaluation Services between 2001 and 2011 was examined. Strawberries were the most frequently sampled crop/prospective crop followed by raspberries; together they accounted for 91% of samples processed.

The most commonly recovered nematodes were stunt/spiral nematodes (e.g. *Tylenchorynchus* spp.) which were present in 98% of samples (Table 1); these are considered one of the least pathogenic groups. Root lesion nematodes (*Pratylenchus* spp.) which can potentially damage soft fruit were the next most common nematode group, being found in 86% of samples, followed by needle nematodes (*Longidorus* spp.) which were present in 58% of samples. Stubby root nematodes (*Trichodorus* spp. were found in 49% of samples and cyst juveniles (*Globodera/Heterodera* spp.) and dagger nematodes (*Xiphinema* spp) in 30% or less of samples.

The current threshold levels for individual nematode groups are shown in Table 2. The proportion of nematode counts above thresholds for individual groups gives an indication of the potential crop area likely to be treated with a nematicide. These data are presented in Table 3.

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Table 1. Free-living nematodes recovered from 92 soil samples from fruit farms examined by ADAS 2001-2011: Numbers detected and range

Nematode group	Number/Litre		Proportion of samples with nematode present	Numbers of nematodes/Litre of soil comprising 90% of max-min range
	Min	Max		
Cyst juveniles	0	525	30	72
Dagger nematodes	0	395	23	12
Needle nematodes	0	2,835	58	330
Root lesion nematodes	0	3,025	86	671
Stubby root nematodes	0	3,475	49	221
Stunt/spiral nematodes	0	10,400	98	5309

Table 2. Anecdotal threshold levels for direct feeding damage and virus transmission to soft fruit crops from different nematode groups

Nematode group	Main genera	Threshold level (Number/L) for:	
		Direct damage	Virus transmission
Dagger	*Xiphinema* spp.	50	Any
Needle	*Longidorus* spp.	50	Any
Root lesion	*Pratylenchus* spp.	700	NA
Stubby root	*Trichodorus* spp.	200	NA
	Paratrichodorus spp.		
Stunt/spiral	*Tylenchorynchus* spp.	10,000	NA
	Helicotylenchus spp.		

NA – not applicable; these genera are not known to transmit viruses.

Table 3. Proportion of sites above threshold for different nematode groups in soil samples extracted for fruit/prospective fruit crops, 2001-2011

	% sites over threshold for:	
Nematode group	Direct feeding damage	Virus transmission
Dagger nematodes	5	15
Needle nematodes	29	54
Root lesion nematodes	10	NA
Stubby root nematodes	12	NA
Stunt/spiral nematodes	1	NA

NA – not applicable; these genera are not known to transmit viruses.

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Above threshold counts of needle nematodes were more common than for all other nematode groups for both direct feeding damage and virus transmission. Almost 30% of samples had threshold counts for direct feeding damage. Needle nematodes were present in 54% of samples and are potentially the most important virus vector in soft fruit crops.

Soil samples were taken from four strawberry crops and four raspberry crops considered to be at high risk of nematode problems due to their cropping history. Although no symptoms in the growing crop attributable to nematode damage or nematode-transmitted virus were reported at the time of soil sampling, a total of eight and 17 plant parasitic species were identified from the strawberry and raspberry soils respectively. Numbers of nematodes in the strawberry soils were relatively low while those in the raspberry soils were slightly higher. There was a potential for direct feeding damage in one of the strawberry and all of the raspberry crops based on current threshold levels.

Objective 2: Soil threshold levels for direct damage

A range of populations of a root lesion nematode (*Pratylenchus thornei*) was created by soil dilution in both year 1 and year 2 of the project. This involved mixing soil infested with the nematode with the same soil which had been sterilised by oven drying at 60ºC for 45 minutes. A total of 50 target populations were created in 15 cm diameter plant pots. A single strawberry plant (cv. Elsanta) was planted in each pot and maintained in a polythene tunnel. After approximately four months the plants were harvested. Dry matter yield of the foliage, crown, roots and total plant dry weight was assessed. The population of root lesion nematodes in each pot at harvest was also determined.

No nematode species other than *Pratylenchus thornei* were detected in the soil. Soil dilution was effective at providing a range of populations. Despite actual populations being lower than the target population the nematode counts ranged from approximately zero to 1200 root lesion nematodes/L soil in year 1 and from zero to 775 root lesion nematodes/L soil in year 2. Nematode numbers in year 1 were both well below and above the anecdotal threshold of 700 root lesion nematodes/L soil and so provided a good range over which to assess their impact on strawberry growth. Although numbers were lower in year 2 the highest population was still greater than the anecdotal threshold.

Results suggested that populations of *P. thornei* as high as 1200/L soil have limited impact on strawberry growth. In Year 1 there was a slight negative relationship with root dry weight decreasing with increasing nematode numbers at harvest. This relationship was not apparent in year 2. Numbers of nematodes in the created populations were well in excess of the anecdotal threshold (700/L) at both the start and end of the experiment. This

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suggests that the current thresholds may be too conservative and below the number of root lesion nematodes which can be tolerated by the crop.

If strawberries are more tolerant of nematodes than previously thought it will have a significant impact on nematicide use and potentially increase the profitability of the crop. However, it should be borne in mind that there are a range of species of root lesion nematodes which may not all exhibit the same degree of pathogenicity towards strawberries.

Objective 3: Identification of nematodes by molecular methods

DNA barcoding techniques were carried out at Fera to determine how well nematode species present in soil samples can be identified by testing the mass DNA extracted from soil samples. Large, moderately variable coding regions of conserved genes are considered useful for providing suitable resolution between taxa. The use of at least two of these barcoding genes are a good basis for a robust and reliable means of identifying free-living nematodes.

In Year 1, total DNA was extracted from 36 single-isolate nematode samples in water. Representative samples were chosen to evaluate the suitability of five candidate barcoding primer sets. Five isolates were initially barcoded. The resulting sequences were aligned using a database to give a 'best match' identification. All 'best match' identifications matched with the visual identification at either genus or species level.

In Year 2, the sequencing data obtained from the barcoding was used to develop specific assays for the detection and quantification for three pathogenic nematode species. Real-time PCR assays were developed to *Pratylenchus penetrans, Pratylenchus thornei,* and *Meloidogyne hapla*. All three free living nematode species tested using the new PCR assays were detected in at least some of the 32 soil samples tested supplied by soft fruit growers. *Meloidogyne hapla* was the most frequently detected, in 18 of the 32 soil samples. *Pratylenchus penetrans* and *P. thornei* were detected in 8 and 10 samples, respectively.

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Table 4. Results from comparisons between visual identification and barcoding identification from DNA extracts using SSU sequence analysis.

DNA extract reference number	Visual identification	ADAS reference number	Barcoding closest similarity
2	*Rotylenchus buxophilus*	7965.002	*Rotylenchus* goodeyi
5	*Bitylenchus dubius*	8036.001	*Bitylenchus dubius*
14	*Paratylenchus* sp.	8013.001	*Paratylenchus dianthus*
16	*Paratylenchus* sp.	8013.001	*Paratylenchus dianthus*
36	*Pratylenchus thornei*	8013.001	*Pratylenchus thornei*

Objective 4: To determine whether increasing levels of a root lesion nematode species increases the risk of strawberry Verticillium wilt caused by V. dahliae

An experiment was designed in which pots of soil with four defined levels (nil, low, medium and high) of predominantly one *Pratylenchus* species and four levels of *Verticillium dahliae* (nil, low, medium and high) were prepared and planted with a strawberry variety susceptible to Verticillium wilt (cv. Elsanta). All combinations of the different *Pratylenchus* and *V. dahliae* levels were examined, resulting in 16 treatments. Nematode numbers at the start of the experiment were 0, 42, 517 and 875/L soil. This declined to 0, 42, 192 and 268/L soil after two months but numbers then remained relatively constant for the rest of the experiment. The nematode species was identified as *Pratylenchus thornei,* a species pathogenic to strawberry, raspberry, blackberry and other fruit crops.

Levels of *V. dahliae* at the start of the experiment as determined by qPCR and expressed as cfu/g equivalents were 0.3 (nil), 5.1 (low), 13.1 (medium) and 38.0 (high). Levels as measured by wet sieving followed by selective nutrient agar were 7, 33, 55 and 122 cfu/g. Levels showed little change between the start and end of the experiment and a clear distinction was maintained between the zero, low, medium and high infestations. It is difficult to directly compare the results from the qPCR test and the wet sieving method. However, the two methods did produce comparable relative values. *V. dahliae* was recorded in the zero infestation which was unexpected as a qPCR analysis of the original soil showed it to be negative for both *V. dahliae* and *V. albo-atrum*. In general, qPCR was less effective at detecting *V. dahliae* than wet sieving and culturing using selective nutrient agar.

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Plants were examined after 3 and 5 months for occurrence of Verticillium wilt, after 5 months for effect on plant growth (dry matter) and after 10 months for survival over winter. There was no indication that increasing nematode numbers influenced susceptibility to Verticillium wilt. Although treatments showed differences in leaf browning, dry weight and vigour, no plants showed a sudden collapse, typical symptoms of the disease. Leaf browning, reduced vigour and reduced dry weight were taken as possible symptoms of Verticillium wilt. There was a trend for increased leaf browning with increasing levels of *V. dahliae* at the 3 month assessment. There was no consistent increase in leaf browning or reduction in dry weight and crop vigour with increasing nematode levels. It should be borne in mind that in general, 2012 was not a good year for Verticillium wilt development in strawberries (or raspberries). This was probably due to the cool wet summer which failed to stress plants and made them less susceptible to the fungal infection. Under more stressful conditions it is possible that feeding by increasing numbers of nematodes could have an impact on susceptibility to Verticillium wilt.

It was interesting that crop vigour in August, leaf number and number of live plants in September and root dry weight and total dry weight at harvest were highest in the presence of nematodes. This suggests a possible compensatory response from the crop to nematode feeding. However it is also possible that the heat sterilisation procedure used to kill any free-living nematodes had a detrimental impact on the nutrient availability of the soil and consequently the growth of the strawberry plants.

There was no evidence to suggest that *V. dahliae* affects the overwintering survival of strawberry plants. However, as even the lowest level fungal infection in this experiment would be expected to significantly affect the growth of strawberries (cv. Elsanta) it is possible that all levels of *V. dahliae* were equally damaging. Although overwintering survival of strawberries did differ between nematode infestation levels there was no clear trend to the data and this result should be treated with caution.

Financial benefits

No immediate financial benefits from this work have been identified. However, several potential financial benefits are possible given confirmation of our results and further test development:

a) Savings in nematicide use through adoption of a higher threshold level for *P. thornei*;

b) Increased accuracy in the identification of nematode species present in soft fruit soils through use of DNA barcoding techniques;

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c) Use of a rapid pre-plant soil test for *P. penetrans* and *P. thornei* in samples where DNA has been extracted for *V. dahliae* determination;

d) Improved risk assessment for nematodes to develop more effective pest and disease control strategies based on barcoding methods and a new, higher threshold for *P. thornei.*

Action points for growers

- Growers should continue to sample land for free-living nematodes to assess the risk for those groups potentially damaging to fruit.
- Growers should adopt a new threshold limit of 1200 root lesion nematodes/L soil to replace the anecdotal threshold of 700 root lesion nematodes/L soil. This could result in potential savings on nematicide use.
- Growers should be aware that needle nematodes (*Longidorus* spp.) appear to be potentially the most damaging to soft fruit in view of the frequency and numbers at which they are recorded in soil samples.
- Growers should continue to use wet sieving followed by selective nutrient agar to detect the presence of *V. dahliae* until the precision of the qPCR analysis can be improved.

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SCIENCE SECTION

Introduction

Nematodes are important pests of strawberries and raspberries and can cause crop losses through direct feeding damage on roots, transmission of viruses and possibly increasing susceptibility to Verticillium wilt caused by *Verticillium dahliae*. Nematode problems in strawberry and raspberry are of increasing concern to growers, especially with tighter rotations. However, the relative occurrence of different nematode species in UK soil-grown soft fruit crops is unknown. An improved understanding of these pests is likely to become increasingly important, particularly as the availability and use of soil disinfestation treatments for nematode control is decreasing. Telone (94% w/w1,3-dichlororoprpene) has been withdrawn in the UK and Basamid (97% w/w dazomet), which has activity against both nematodes and *Verticillium dahliae*, may lose efficacy with regular use on certain soil types due to enhanced degradation.

There are a number of gaps in our understanding of free-living nematodes that need to be urgently addressed. Firstly, it is unclear how many root lesion nematodes (*Pratylenchus* spp.) are needed to cause direct feeding damage. Threshold levels used for assessing risk of direct damage by *Pratylenchus* species to strawberry are based on anecdotal and not experimental evidence. Secondly, there may be an interaction between the presence of *Pratylenchus* species and the incidence of Verticillium wilt. It is known that Verticillium wilts (e.g. in *Acer* and potato) can be exacerbated by plant pathogenic nematodes, particularly of the genus *Pratylenchus*, although cyst nematodes may also have a synergistic effect in potato wilt (Daami-Remadi *et al*., 2009; Rowe *et al*., 1985; Saeed *et al*., 1998). Thirdly, despite the risk of serious losses from nematodes in soft fruit, expertise in their extraction, identification and evaluation is limited.

A quantitative molecular (QPCR) test has recently been developed (SF 97) for determination of *Verticillium dahliae* in soil which is able to detect and quantify inoculum of the pathogen within 24 hours with a high level of specificity. DNA extraction from soil to quantify *V. dahliae* offers prospects for rapid determination of any other pests and pathogens present, including nematodes, as the method extracts all DNA present. Recent advances in DNA barcoding techniques (methods used for species identification based on the DNA sequence of conserved genes) offers the potential to identify nematode species accurately and quickly without the need for taxonomic expertise.

The specific project objectives are listed below:

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1. To determine the nematode species most commonly found associated with soil-grown strawberry and raspberry crops in the UK.
2. To confirm the soil threshold level for direct root damage to strawberry by a *Pratylenchus* species as identified in objective 1.
3. To determine whether nematode species present in a soil sample can be identified by testing the mass DNA extracted from soil samples when testing for *V. dahliae* by molecular quantification.
4. To determine whether increasing levels of a nematode species increases the risk of strawberry Verticillium wilt caused by *V. dahliae*.

Objective 1 was completed in Year 1 of this project. Objectives 2-3 were started in year 1 of the project and objective 4 did not begin until year 2.

Materials and methods

Objective 2: Soil threshold levels for direct damage

In year 1 of the project results suggested that root lesion nematodes (*Pratylenchus* spp.) had little, if any, impact on strawberry growth, even at numbers well in excess of the anecdotal threshold of 700 root lesion nematodes/L. This suggested that the current thresholds may be too conservative and well below the number of nematodes which can be tolerated by the crop. This result was unexpected and as a result, repeat studies were undertaken in year two of the project to test the results from year one.

Approximately 75 kg of field soil was collected from a site known to be infested with root lesion nematodes (*Pratylenchus* spp.). The soil was collected using spades to sample to a depth of approximately 15 cm at a range of points across the field and then stored in plastic dustbins. The bins were returned to the laboratory and sampled using a 15 cm deep x 2 cm diameter cheese corer. A total of 20 cores was taken from each bin and each sample extracted twice, once using the Seinhorst two flask technique (Seinhorst, 1955) and once using the Flegg modified Cobb technique (Flegg, 1967).

A range of nematode populations was created by taking a known volume of nematode infested soil and diluting this with a known volume of sterile soil. Populations were created in 15 cm diameter x 15 cm deep pots. Half of the soil collected was sterilised by oven drying at 60ºC for 45 minutes. This was done in cotton bags in 5 kg batches. After oven drying the soil was allowed to cool for at least 24 hours before using it to dilute the nematode infested soil.

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As each pot contained approximately 1.5 L soil, the nematode populations were prepared in 2 L soil. This provided enough soil to fill the pot and sufficient spare to check the accuracy of the created population. As an example, a target nematode population of 1,000 stubby root nematodes/L soil can be prepared by mixing 1 L of soil containing 2,000 root lesion nematodes/L soil with 1 L of sterile soil. The exact quantities of soil required to create the populations depended on the number of nematodes in the infested soil. The sterile and infested soil was mixed on a sheet of polythene. This was folded carefully from one side to another to ensure thorough mixing of the soil without damaging the nematodes. The mixed soil was carefully tipped into the pot until approximately 2.5 cm from the rim. The spare soil was retained and stored in a labelled polythene bag in a cold store at approximately 5ºC and was later extracted using the Seinhorst two flask technique to check the nematode population. There were 29 target nematode populations in the experiment ranging from 0-2,375 root lesion nematodes/L soil. This range covered that likely to be recorded in the majority of UK soils.

A single strawberry plant (cv. Elsanta) was planted into the nematode infested soil in each pot. Prior to planting the fresh weight of each plant was measured to assess the degree of variability. This was determined so that it could be used to help interpret any differences in plant weights at harvest.

A sample of 20 spare strawberry plants from the batch to be planted was destructively assessed for occurrence of nematode infestation of roots and *V. dahliae* infection of the crown as a health check. Roots were tested for nematodes by staining (acid fuchsin) followed by microscopic examination of 1 g of macerated root tissue.

Crown tissue was tested for *V. dahliae* by examination for brown staining and by isolation (four pieces/crown) onto potato dextrose agar (PDA) + streptomycin. Any suspect colonies of *V. dahliae* were examined by microscopy. The soil infested with root lesion nematodes was also tested for the presence of *V. dahliae* by culturing on agar.

Pots were maintained in a polythene tunnel and watered as necessary. After approximately five months the plants were harvested. Fresh weight and dry matter yield of the foliage, crown, roots and total plant dry weight was assessed. Dry weight was determined by oven drying at 80^{o}C for 48 hours. Fresh weight was measured to take account of the variation in size of strawberry plants at planting, which potentially could mask any change in weight of plants brought about by nematode feeding. The % change in fresh weight of all strawberry plants was calculated. Regression analysis was used to assess the effect of the initial nematode population on all growth parameters of strawberries. The pot soil was also

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extracted using the Seinhorst two flask technique to compare the initial and final nematode population.

Objective 3: Identification of nematodes by molecular methods

Materials and methods

DNA extraction

Isolates of *Pratylenchus penetrans, P. thornei* and *Meloidogyne hapla* were obtained from single-isolate nematode samples. DNA was extracted from pure nematode material using a standard chelex extraction or DNeasy blood and tissue kit (Qiagen). DNA was quantified when used as standards for quantification by real-time PCR. This was done using the nanodrop spectrophotometer (Thermofisher Scientific, Loughborough, UK). Absorbance values at 230, 260 and 280 nm were taken to assess DNA quality and quantity.

Soil DNA extraction was based on the method of Woodhall *et al*. (2012). Soil samples were air dried for two days prior to DNA extraction. 50 g samples were added to 250 ml Nalgene bottle (HDPE), with eight steel ball bearings. Ball bearings were 25.4 mm in diameter. 100 CTAB soil buffer (120 mM sodium phosphate buffer pH8, 2% cetrimonium bromide, 1.5 M NaCl, 3% Antifoam B) was added. Nalgene bottles were then placed on an Automixer commercial paint shaker (Merris Dispensing & Mixing Equipment, Czech Republic) for four minutes. A 40 ml subsample was then transferred to a 50 ml tube and centrifuged at 5,000 g for five minutes. 20 ml of the resulting supernatant was added to a clean 50 ml tube with 2 ml 5M potassium acetate and placed on ice for 10 minutes, followed by a five minute centrifugation at 12,000 g. The supernatant was then added to a clean 50 ml tube containing 15 ml isopropanol plus 800 µl acid washed silicon dioxide and placed on a flat bed shaker for 15 minutes at 70 rpm followed by five minutes centrifugation at 12,000 g. The supernatant was discarded and the pellet re-suspended in 2 ml lysis buffer A (Wizard Magnetic DNA Purification System). The tubes were placed in a shaking incubator on their side for 10 minutes at 65ºC at 100 rpm. The silica particles were then separated by centrifugation for five minutes at 12,000 g. The remainder of the procedure was according to the manufacturer's instructions for the Wizard Magnetic DNA Purification System in conjunction with a Kingfisher ML magnetic particle processor (Thermo Electron Corporation). All DNA samples were eluted into 200 µl Tris-EDTA (TE) buffer and stored at -30°C until required.

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Primer design and TaqMan real-time PCR

Sequences from the rDNA internal transcribed spacer region (ITS) for *Pratylenchus* and *Meloidogyne* were obtained from GenBank and aligned using clustalW. *Pratylenchus* spp. were chosen as they are important pests of soft fruit, including strawberries, and *Meloidogyne hapla* (Northern root knot nematode) has also been recorded as damaging strawberries and is thought to interact with *V. dahliae* on strawberries (Meagher and Jenkins, 1970). Examination of these sequence alignments indicated that there was a region of variation within the ITS region and potential primer/probe sites could be located. Primer Express 2.0 software (Applied Biosystems, Warrington, UK), was used to design specific primers and a probe was designed with putative specificity to the target species. The forward primer for the *P. thornei* consists of the final twenty base pairs of the forward primer used in the SYBR Green assay of Yan *et al.* (2012).

TaqMan PCR was carried out with the ABI Prism7900HT Sequence Detector System (Applied Biosystems) in 96 well plates. Environmental Master Mix 2.0 (Applied Biosystems) was used with samples originating from pure cultures and soils and consisted of half the total reaction volume of 25 µl. Primers (MWG Biotech, Germany) were added to a final concentration in the reaction of 300 nM. FAM-TAMRA probes were obtained from MWG Biotech, Germany whilst the FAM-MGB probe was Applied Biosystems - both were added to a final concentration of 100 nM. The remaining volume was made up with molecular grade water. Cycling conditions consisted of 50°C for two minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for one minute. The Ct value for each reaction was assessed using the Sequence Detection Software's default threshold setting of 0.2 ΔRn (fluorescence) units. A 10-fold dilution series was created for each pathogen (as DNA standards) to determine the detection limits for each assay.

Quantification of nematode DNA in soil

Thirty-two soil samples were taken from strawberry fields prior to planting; these consisted of samples submitted by growers for *V. dahliae* testing at Fera. Total nucleic acids were extracted from each soil using the Fera soil extraction method. The extracts were tested by real-time PCR using the new PCR assays for the target pathogens, *Meloidogyne hapla, Pratylenchus penetrans* and *P. thornei*. Each sample was tested in two replicates and an average cycle threshold (Ct) was taken.

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Objective 4: To determine whether increasing levels of a root lesion nematode species increases the risk of strawberry Verticillium wilt caused by V. dahliae.

An experiment was designed in which pots of soil with four defined levels (nil, low, medium and high; numbers/Litre) of predominantly one *Pratylenchus* species each with four levels of *Verticillium dahliae* (nil, low, medium and high; fg DNA/g soil and cfu/g soil) were prepared and planted with a strawberry variety susceptible to Verticillium wilt (cv. Elsanta). A fully randomised block design was used with the 16 treatments replicated four times and a 4 x 4 factorial treatment design. Each plot consisted of three pots giving a total of 192 experimental pots (16 treatments x 4 replicates x 3 pots per plot). The experimental treatments are given in Table 5. A full crop diary is given in Appendix 1.

Table 5. Treatment list showing the target initial levels of *P. thornei* and *V. dahliae*

Treatment	*Pratylenchus* sp. (No./L)	*V. dahliae* (cfu/g)
1.	Nil (0)	Nil (0)
2.	Nil	Low (1)
3.	Nil	Medium (5)
4.	Nil	High (20)
5.	Low (~30)	Nil
6.	Low	Low
7.	Low	Medium
8.	Low	High
9.	Medium (~900)	Nil
10.	Medium	Low
11.	Medium	Medium
12.	Medium	High
13.	High (~3,000)	Nil
14.	High	Low
15.	High	Medium
16.	High	High

Cfu/g = colony forming units per gram of soil

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Preparation of nematode populations

Nematode populations were created using the same technique described in Objective 2. This involved taking a known volume of nematode infested soil and diluting this with a known volume of sterile soil. Populations were created in 15 cm diameter x 15 cm deep pots. As each pot contained approximately 1 L soil, the nematode populations were prepared in 1.5 L soil. This provided enough soil to fill the pot and sufficient spare to check the accuracy of the created population. The exact quantities of soil required to create the populations depended on the number of nematodes in the infested soil. Also the calculations took into account the fact that additional *V. dahliae* infested soil was to be added to the pot which potentially could have diluted the nematode population. Once the amount of *V. dahliae* soil was known, a calculation was done to amend the nematode infestations. The spare soil was retained and stored in a labelled polythene bag in a cold store at approximately 5ºC. This soil was used to check the nematode population. A Seinhorst two-flask extraction was used to determine the number of root lesion nematodes.

Preparation of Verticillium dahliae *infestation densities*

Microsclerotia of *V. dahliae* were prepared for each of five isolates of the fungus, two from strawberry, two from raspberry and one from Acer. The isolates were mixed together to create the final population.

The isolates were grown in autoclaved sand and maize meal in conical flasks. Each flask contained 97 g of washed silver sand, 3 g of maize meal (Polenta) and 15 ml of distilled water. This made approximately 100 ml in volume of the culture media. The flasks were autoclaved for 20 minutes at 120^{o}C and then allowed to cool in a laminar flow cabinet. Sufficient medium was prepared for 12 small Sterilin bags (five isolates x two bags per isolate). For each isolate two 250 ml flasks of sterile sand/ maize meal were inoculated with around 10 plugs (8 mm) of one of the *V. dahliae* isolate using sterile technique. The contents of each flask were then mixed thoroughly to get uniform distribution of microsclerotia and incubated for 2-3 weeks at 20^{o}C. The flasks were tumble-mixed every two to three days to ensure growth throughout the medium

After 2-3 weeks, the inoculum was checked for *V. dahliae* microsclerotia and any flasks containing *Penicillium* sp. or other contaminants were discarded. A single clean flask of each isolate was mixed in a single bag and thoroughly mixed. This formed the high concentration source of *V. dahliae* inoculum.

The inoculum was diluted with John Innes compost, at a ratio of 1 part inoculum in 1,000 parts compost and thoroughly mixed. A total of three sub-samples were sent to Fera to determine the mean quantity of *V. dahliae* as fg/g and the result expressed as an equivalent

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number of microsclerotia per gram. The known inoculum was then diluted with sterile John Innes soil to achieve 200 cfu/g. This was mixed with nematode infested soil in a ratio of 1 part inoculum in 10 parts infested soil to achieve the target high concentration of 20 cfu/g. Similarly, the known inoculum was diluted to 50 and 10 cfu/g, then mixed with the nematode infested soils at 1 in 10 to achieve the medium target of 5 cfu/g and low target of 1 cfu/g.

When the final four populations of *V. dahliae* had been created these were characterised by both PCR (Fera) and culturing on agar (ADAS).

Planting the pots

Sufficient soil of each treatment was prepared to fill 192 pots of 1.0 L (main experiment). There were 28 pots of 1.0 L (4 *Pratylenchus* levels x 5 months + 8 *V. dahliae* levels, four at the start and four at the end of the experiment) to monitor any change in populations of nematodes and *V. dahliae* with time over the course of the experiment.

Pots were planted with cold-stored runners of certified A-grade cv. Elsanta. A sub-sample of the batch (10 plants) was checked for freedom from nematodes and *V. dahliae* by destructive assessment as described for Objective 2.

Potted plants were grown in a polythene tunnel for five months (July – December 2012), irrigated by overhead irrigation. It was decided not to apply any liquid feed as if plants are stressed they are more likely to show symptoms of wilt. The soil was kept moist initially to encourage infection by *Pratylenchus* and *V. dahliae*. After eight weeks, when plants had established, they were kept dry to encourage development of Verticillium wilt symptoms. This is likely to have caused a reduction in numbers of nematodes but by this stage they would have fed for sufficient time to allow any interaction with *V. dahliae.* Plants were regularly monitored for the presence of any fungal disease (other than Verticillium) or pest and a fungicide or insecticide was applied if necessary. The set of three pots in each plot was placed on an upturned tray with drainage holes to prevent inter-plot interference by movement of nematodes or *V. dahliae* in drainage water.

Monitoring levels of nematodes and of V. dahliae

At two and three months after the start of the experiment three spare pots were extracted to determine the level of infestation by root lesion nematodes (one from each level of infestation, except zero). This required 18 additional pots. Levels of nematodes at the start of the study were checked using spare soil from the created populations and 64 experimental pots were extracted for nematodes at the end of the study (one replicate pot from each nematode treatment per block) to see how numbers had changed. All extractions were done using the Seinhorst two-flask technique. Levels of *V. dahliae* were

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determined at the start and end of the experiment by both plating on selective nutrient agar and using QPCR. This did not require additional pots as spare soil used to create the experimental infestations was analysed to determine pathogen levels at the start of the study and soil from experimental pots was analysed to determine levels at the end of the study.

Disease assessments

The level of infection with Verticillium wilt was assessed at three months after planting (11 October 2012) and five months after planting at harvest (7 December 2012). There were two assessments, firstly the number of plants that were wilting or dying and secondly the number showing yellowing and/or browning and collapse of the outer whorl of leaves. Both assessments were scored on a scale from 1-3. Un-wilted plants were scored 1, wilted plants 2 and dead plants 3. Where there was no yellowing/browning plants were scored 1, where yellowing/browning was present they were scored 2 and dead plants were again scored 3.

After five months the incidence of crown staining due to Verticillium wilt was also assessed. This was a destructive assessment at harvest.

Harvest assessments

Foliar, root and crown fresh and dry weight was assessed at harvest. Samples were oven dried at 100 ^{o}C for 24 hours. In addition the total number of leaves, number of dead leaves, the number of crowns with staining and the number of dead plants was also assessed. The percentage of dead leaves per plant and proportion of plants that had stained crowns or were dead at harvest were calculated and subjected to analysis of variance.

Other assessments

In addition to the assessments described above, crop vigour was assessed on 2 August using a scale of 0-3 (0= dead plant, 3 = vigorous plant), leaf number per plant was assessed on 26 September 2012 and the number of live and dead plants was assessed on 2 October 2012. The main species of root lesion nematode was also determined.

Results

Objective 2: Soil threshold levels for direct damage

The nematode species extracted from soil and used for the experiment was identified as *Pratylenchus thornei.* The following crops have been recorded as being susceptible to this

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nematode – strawberry, raspberry, blackberry, pear, cherry, nectarine, apricot, plum (including some rootstocks) and boysenberry

Comparison of actual and target nematode numbers

Regression analysis was used to compare the target population of root lesion nematodes to the actual population achieved by soil dilution. The actual population was measured twice, once immediately after the population was created and secondly at the end of the experiment. The equation of the regression line and the percentage variation accounted for is given in Table 6. If 100% of variation is accounted for this represents a perfect fit between target and actual nematode populations.

Table 6. Results of regression analyses to compare target and actual nematode populations at both the start of the experiment and at harvest (y = actual population, x = target population) - 2012

Nematode group	Regression line equation		Probability		% variation accounted for	
	At start	At harvest	At start	At harvest	At start	At harvest
Root lesion	y = 0.2x + 57.7	Y=0.1x + 17.2	<0.001	<0.001	65.9	55.6

Regression analyses showed a very highly significant fit between the actual population at the start of the experiment and at harvest and the target nematode population (P<0.001, Figures 1 and 2) although the percentage variation accounted for was lower at harvest than at the start of the experiment. In general the actual population was approximately one fifth of the target population. Despite actual populations being lower than the target population the nematode counts ranged from approximately zero to 775 root lesion nematodes/L soil. This is both below and above the anecdotal threshold of 700 root lesion nematodes/L soil and so provided a good range over which to assess their impact on strawberry growth. A regression of the initial nematode population and that at harvest showed a highly significant fit (P<0.001, Figure 3). Nematode numbers at harvest were about 40% of the initial population suggesting that they declined throughout the study. However, nematodes would be expected to have the greatest impact on strawberry growth early in the life of the crop soon after it was planted. At this stage there was a wide range of nematode numbers both above and below the anecdotal threshold.

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Impact of nematodes on strawberry growth

Root staining (acid fuchsin) followed by microscopic examination of a sample of root tissue from the original strawberry plants just before planting showed no evidence of a significant infestation of nematodes. Also, examination of plants for infection by *V. dahliae* showed none to be present. Therefore it was concluded that the strawberry plants were in good health at the start of the experiment and not compromised by either nematode or Verticillium wilt infection.

From linear regression analyses, there was no clear significant relationship between the initial nematode population and foliar, crown, root and total fresh weight and dry weight of strawberry plants. There was also no significant relationship between the initial nematode population and % change in fresh weight of strawberry plants. These results are in agreement with those from year 1 of the project. These data are presented in Figures 4 – 9.

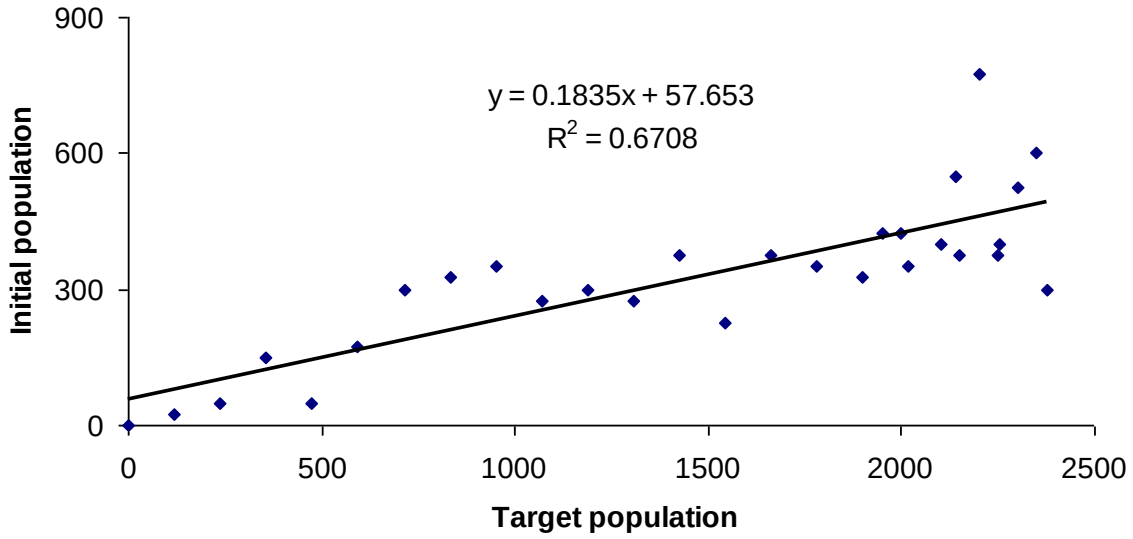

Figure 1. Initial created nematode populations against target populations (*P. thornei*) - 2012

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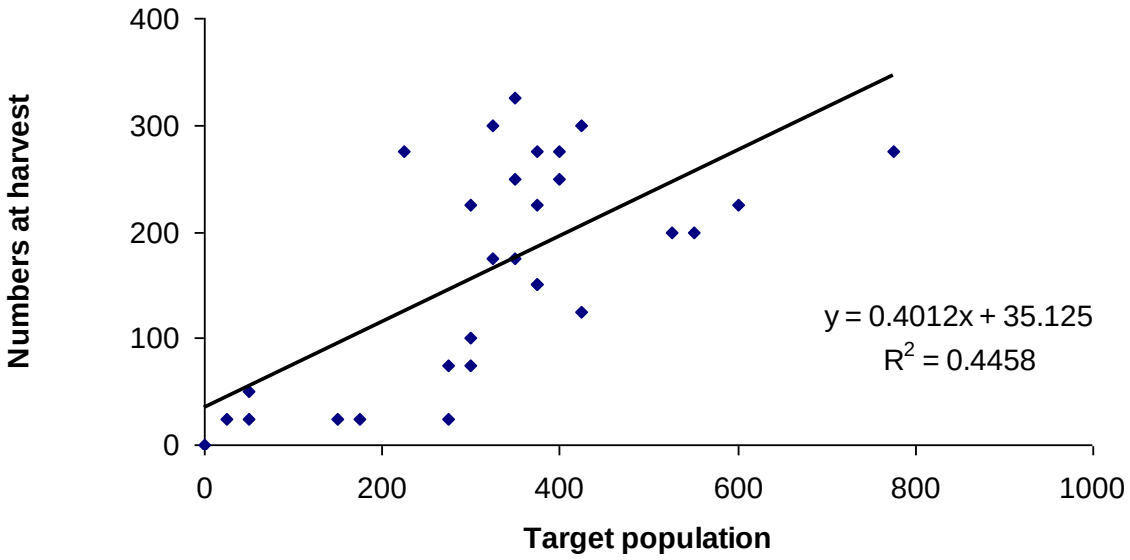

Figure 2. Nematode numbers at harvest against target populations (*P. thornei*) -2012

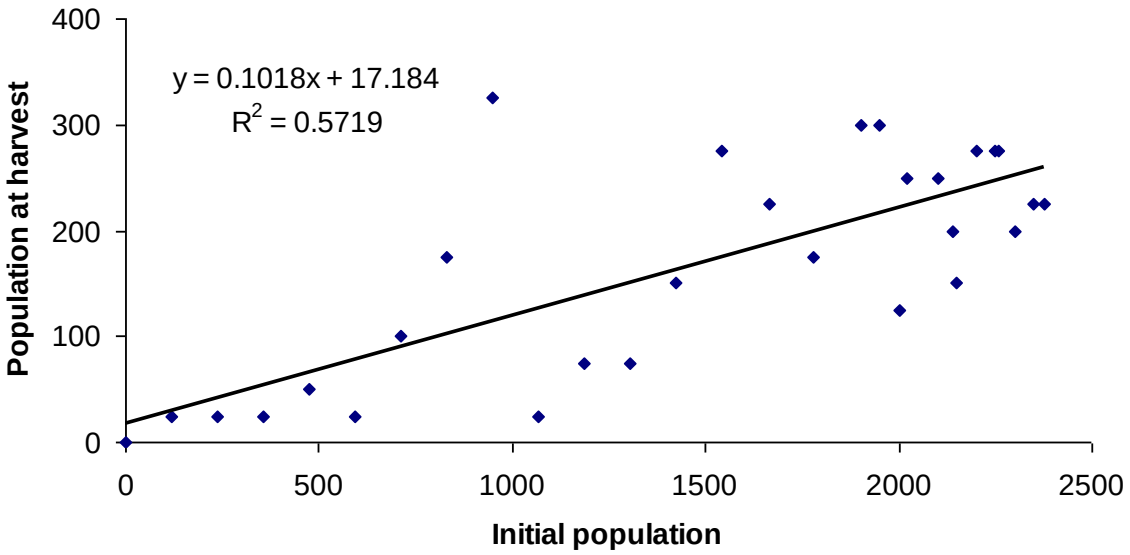

Figure 3. Nematode numbers at harvest against initial created populations (*P. thornei*)-2012

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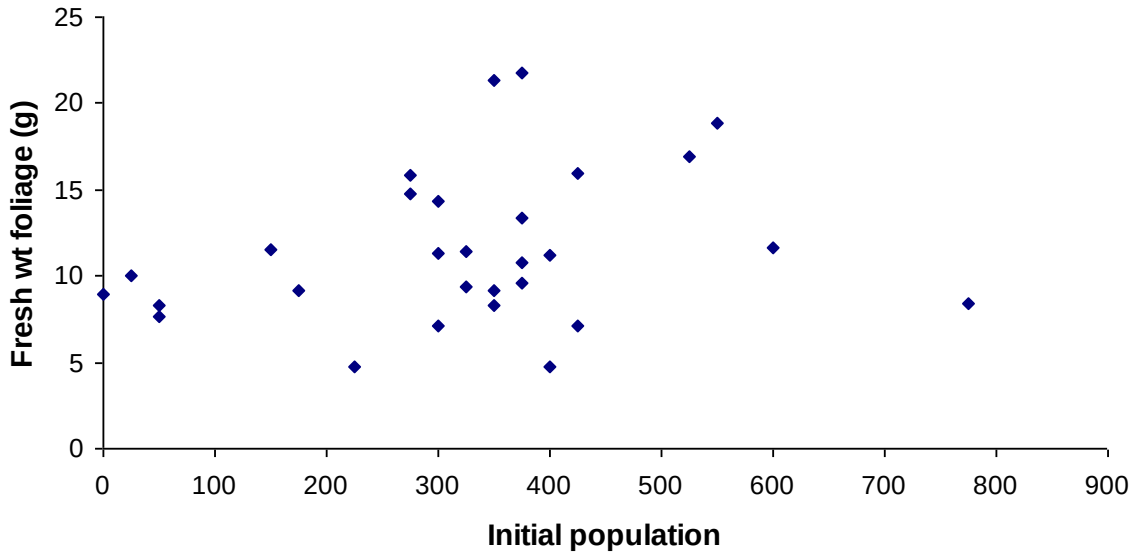

Figure 4. Fresh weight (g) of strawberry foliage against initial population of *P. thornei*

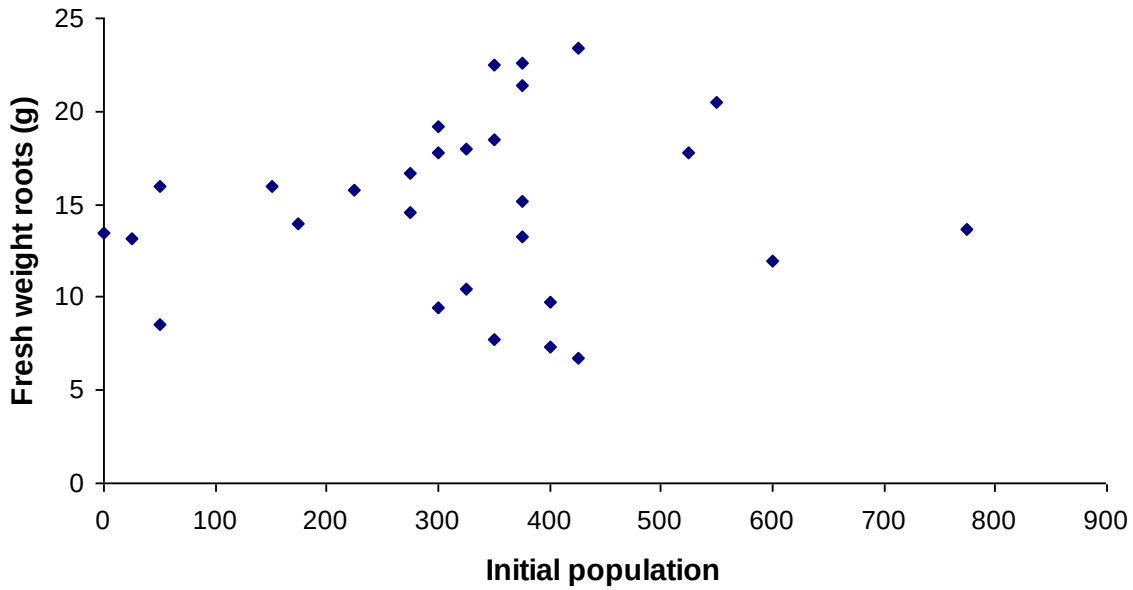

Figure 5. Fresh weight of strawberry roots (g) against initial population of *P. thornei*

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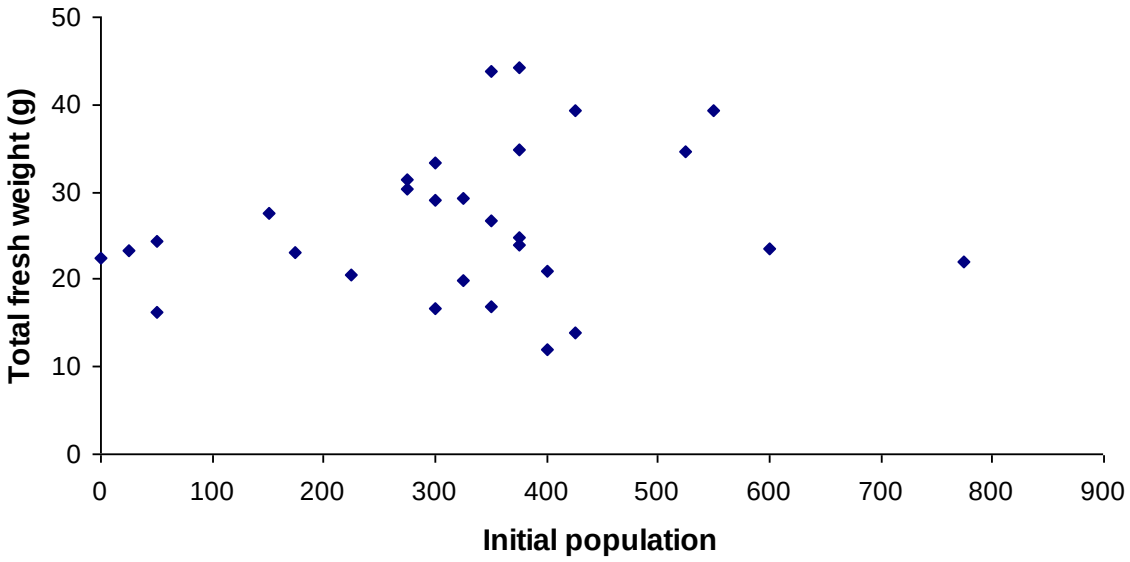

Figure 6. Total fresh weight of strawberry plants (g) against initial population of *P. thornei*

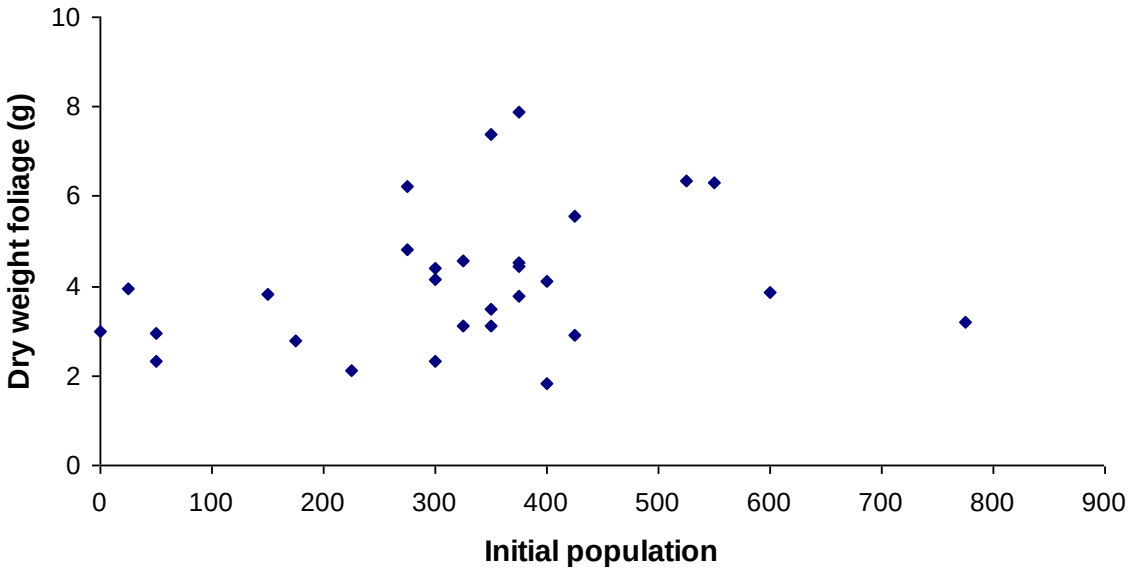

Figure 7. Dry weight of strawberry foliage (g) against initial population of *P. thornei.*

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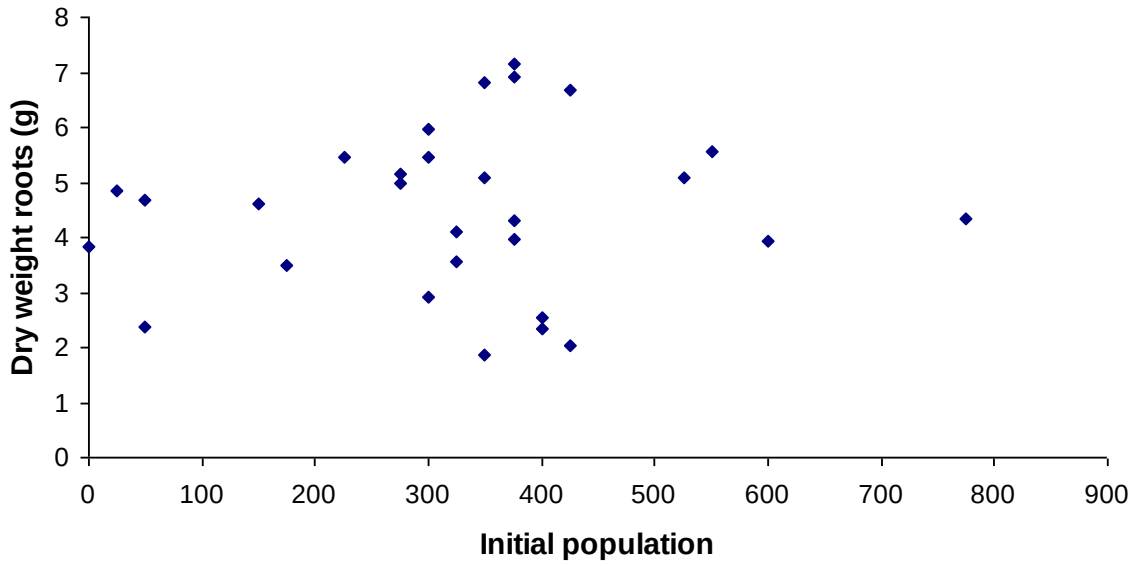

Figure 8. Dry weight of strawberry roots (g) against initial population of *P. thornei*

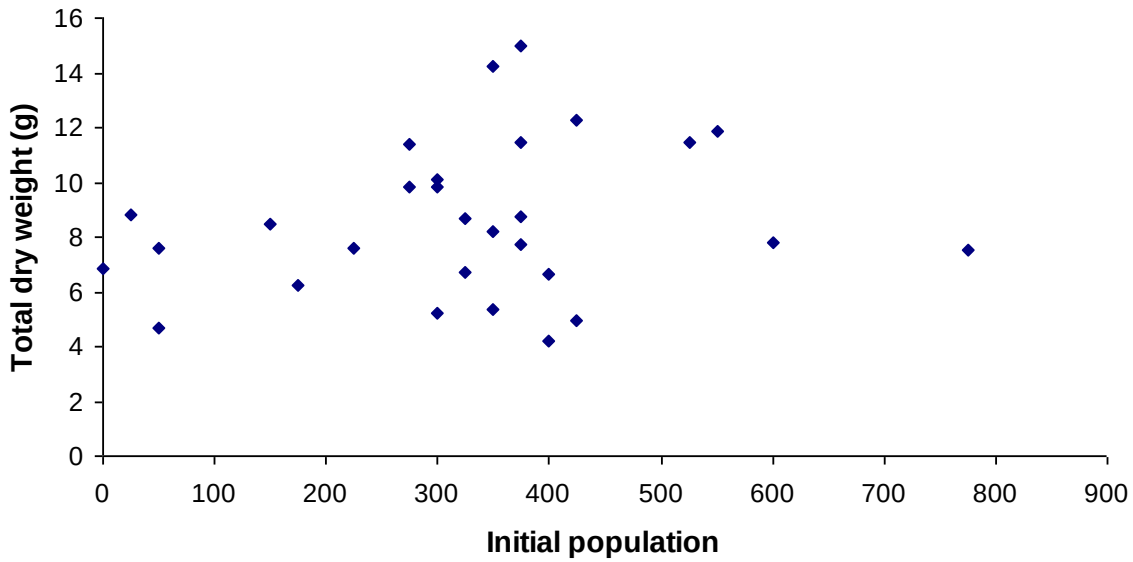

Figure 9. Total strawberry dry weight (g) against initial population of *P. thornei*

Objective 3: Identification of nematodes by molecular methods

Assay design

Three putative assays (Table 7) were designed for three species: *Meloidogyne hapla, Pratylenchus penetrans* and *Pratylenchus thornei*. All were designed from the ribosomal DNA internal transcribed spacer region. The assays only detected the target species for

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which they were designed. The non-target species listed in Table 8 were not detected using the three PCR assays. However, it should be noted that further testing of cross-reactivity is required as only a limited number of nematode target species have been tested so far.

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Table 7. Details of sequences for all forward (_F) and reverse (_R) primers and probes (_P) used in this study.

Target Group	Primer/Probe	Modification	Sequence	Target region
Pratylenchus penetrans	Ppen_F	-	GGG CGA GAC ACA TTT TGC AT	ITS1
	Ppen_R	-	TGC TGC TGG ATC ATT ACT TTG T	ITS1
	Ppen_P	MGB	CAAGTTTAAATGTG TCATATAGT	ITS1
Pratylenchus thornei	Pthorn_F	-	GTC GCT GAG CAG TTG TTG CC	ITS1
	Pthorn2_R	-	ATA GGG AAA GCA GCT CTA CCG TG	ITS1
	Pthorn2_P	TAMRA	CTC GTC CGT GGC TGT GAT GAG GC	ITS1
Meloidogyne hapla	Mhap_F	-	TGG TTC AGG GTC ATT TTT CTA TAA AGT A	ITS2
	Mhap _R	-	GAC AGC GAA AAG AGT TAT TCA TTT ACA	ITS2
	Mhap _P	TAMRA	CCA TTG GCA CTA TAA CTT TTA ATG TTG GTA CGC AG	ITS2

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Table 8. Species, code, host and origin of the nematode isolates used in the study to validate the assays

Species	Isolate code	Original host	Origin
Hirschmanniella gracilis	Nembar 489	Not known	UK
Hirschmanniella spp.	Nembar 286	Cyptocoryne	Indonesia (UK Interception)
Meloidogyne chitwoodii	1A	Not known	
	4.1	Not known	
Meloidogyne hapla	1.1	Not known	
	1.2	Not known	
	2.1	Not known	
Paratylenchus spp.	N10	Soil	UK
	N12	Soil	UK
	N14	Soil	UK
	N16	Soil	UK
Pratylenchus crenatus	NB643	Turfgrass	UK
	NB644	Turfgrass	UK
Pratylenchus neglectus	NB645	Soil	UK
Pratylenchus penetrans	N54	Soil	UK
	N56	Soil	UK
Pratylenchus thornei	N19	Soil	UK
	N36	Soil	UK
	N58	Soil	UK
	N59	Soil	UK

The theoretical limit of detection for all assays was below 1pg μl^{-1} (Figure 10). A good relationship between DNA level and Cycle threshold was observed for all assays (R^2 > 0.99). Reaction efficiency for all the assays was moderate to excellent. 85% for the *P. penetrans* assay, 93% for the *P. thornei* assay and 109% for the *M. halpa* assay.

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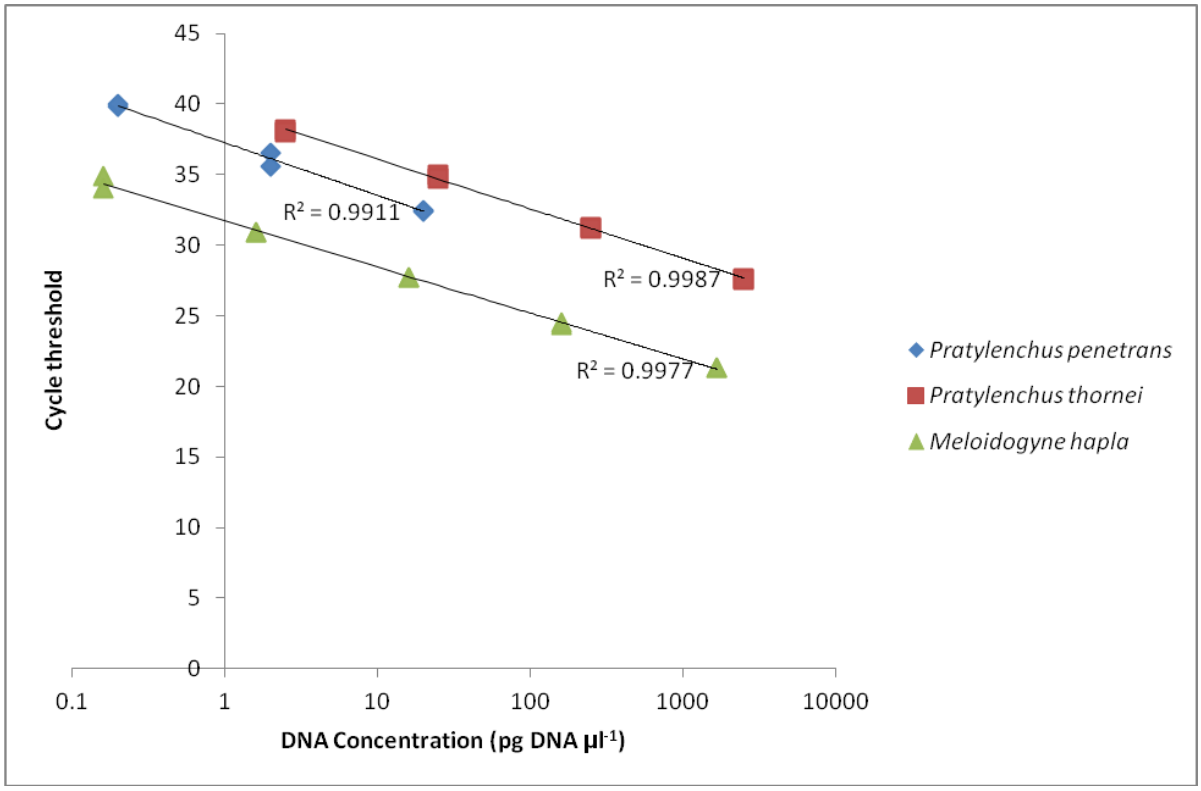

Figure 10. Relationship between PCR cycle threshold and amount of DNA extracted from pure cultures of *Pratylenchus penetrans*, *P. thornei* and *Meloidogyne hapla*.

All three free living nematode species tested using the new PCR assays, *Pratylenchus penetrans*, *P. thornei* and *Meloidogyne hapla*, were detected in at least some of the 32 soil samples tested (Table 9). *Meloidogyne hapla* was the most frequently detected, in 18 of the 32 soil samples. *Pratylenchus penetrans* and *P. thornei* were detected in eight and 10 samples, respectively.

Table 9. Detection of three nematodes by real-time PCR in 32 naturally infested strawberry field soil samples. Data presented as incidence (%), average and range of nematode quantity presented as pg DNA

Target nematode species	Soil samples positive out of 32 (and %)	Mean DNA quantity (pg DNA per g soil) detected in positive samples	Range (pg DNA per g soil)
Pratylenchus penetrans	8 (28%)	834	41 to 3,682
Pratylenchus thornei	10 (31%)	12,845	96 to 87,452
Meloidogyne hapla	18 (56%)	242	0.1 to 2,331

Seven soils from different fields, each with a known level of *Pratylenchus* species as determined by conventional soil sieving and microscope counts, were supplied by ADAS to Fera for testing by the new qPCR assays for nematodes. The samples were coded 1 to 7.

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Two sub-samples (labelled a and b) were taken from each soil. Three of the seven soils tested positive for *P. thornei* (Table 10). One (from two) sub-samples tested positive for *P. penetrans.* No *M. hapla* DNA was detected. Soil samples number 1 to 4 and 6 to 7, had *V. dahliae* extracts ranging from 721 to 601,185 fg DNA/g soil. *Verticillium dahliae* was not detected in samples from soil number 5.

Table 10. Levels of *Pratylenchus penetrans*, *P. thornei, Meloidogyne hapla* and *Verticillium dahliae*, tested using real-time PCR, in seven soils containing differing levels of *Pratylenchus* species as determined by conventional tests. Data presented as fg DNA/g soil.

Sample	Number.root lesion nematodes/litre (ADAS count)	Nematode species (fg DNA/g soil)			Fungal species (fg DNA/g)
		P. thornei	*P. penetrans*	*M. hapla*	*V. dahliae*
1a	0	<50	<50	<50	**601,185**
1b		<50	<50	<50	**240,611**
2a	175	<50	<50	<50	**29,980**
2b		<50	<50	<50	**22,576**
3a	475	<50	<50	<50	**145,584**
3b		<50	61	<50	**105,278**
4a	1,025	10,847	<50	<50	**721**
4b		30,053	<50	<50	**687**
5a	1,400	<50	<50	<50	**<220**
5b		<50	<50	<50	**<220**
6a	2,175	1,007	<50	<50	**6,915**
6b		755	<50	<50	**919**
7a	3,250	966	<50	<50	**912**
7b		502	<50	<50	**2,051**

Objective 4: To determine whether increasing levels of a root lesion nematode species increases the risk of strawberry Verticillium wilt caused by V. dahliae

Monitoring levels of nematodes and of V. dahliae

Nematode numbers declined most noticeably between July and September (Figure 11). Pots were kept moist in July and August to allow the strawberry plants to establish but subsequently were allowed to dry out to encourage the development of Verticillium wilt and

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this would not have favoured nematodes. After the initial reduction in nematode numbers they did not change significantly throughout the experiment and a clear difference was maintained between low, medium and high levels.

Levels of *V. dahliae* showed little change between the start and end of the experiment and a clear distinction was maintained between the zero, low, medium and high infestations (Figure 12). *Verticillium dahliae* was recorded in the zero infestation which was unexpected. A qPCR analysis of the original soil showed it to be negative for both *V. dahliae* and *V. albo-atrum* but wet sieving followed by culturing on selective nutrient agar showed *V dahliae* to be present at a level of 7.3 cfu/g.

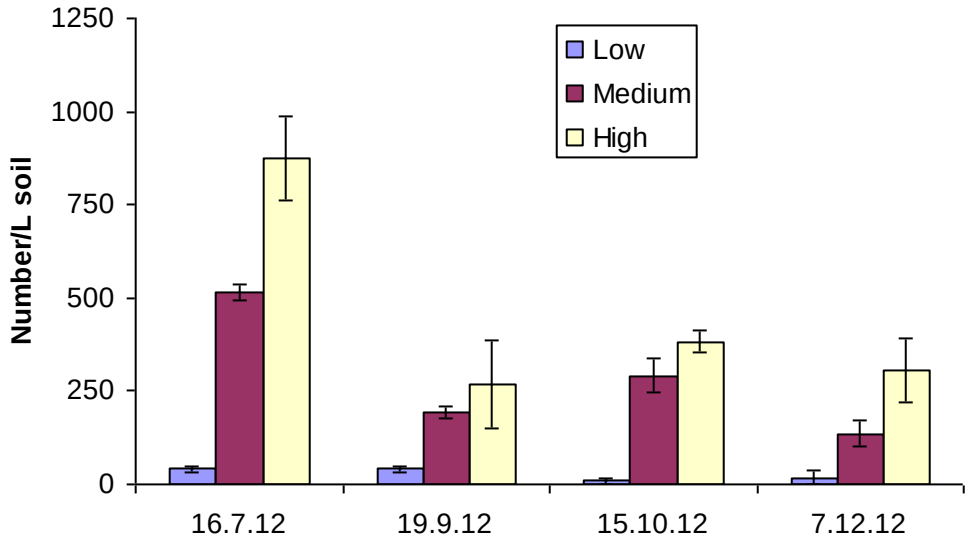

Figure 11. Variation in nematode numbers throughout the experiment. (Numbers were assessed at the start, after two and three months and at the end of the experiment)

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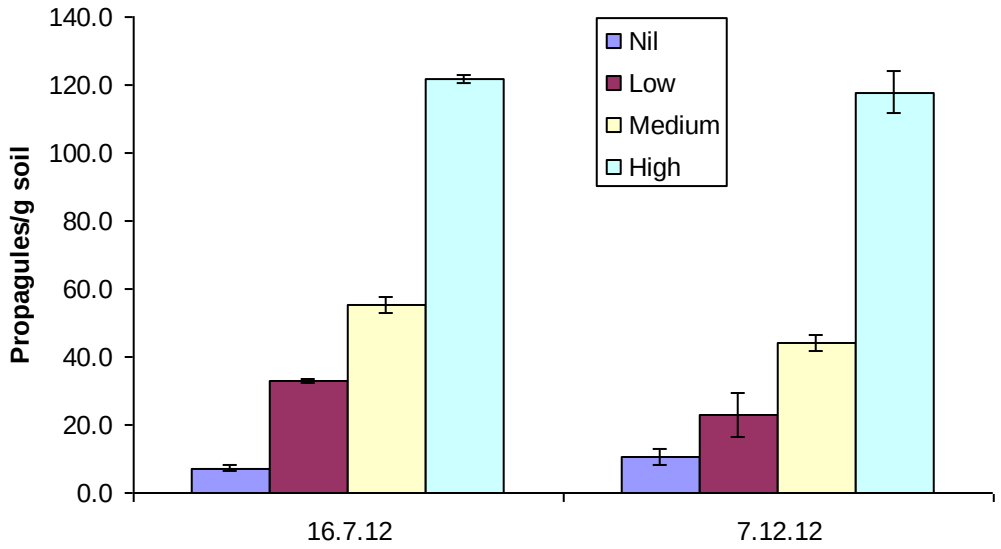

Figure 12. Levels of *V. dahliae* infestation at the start and end of the experiment, as determined by wet sieving followed by plating on nutrient selective agar

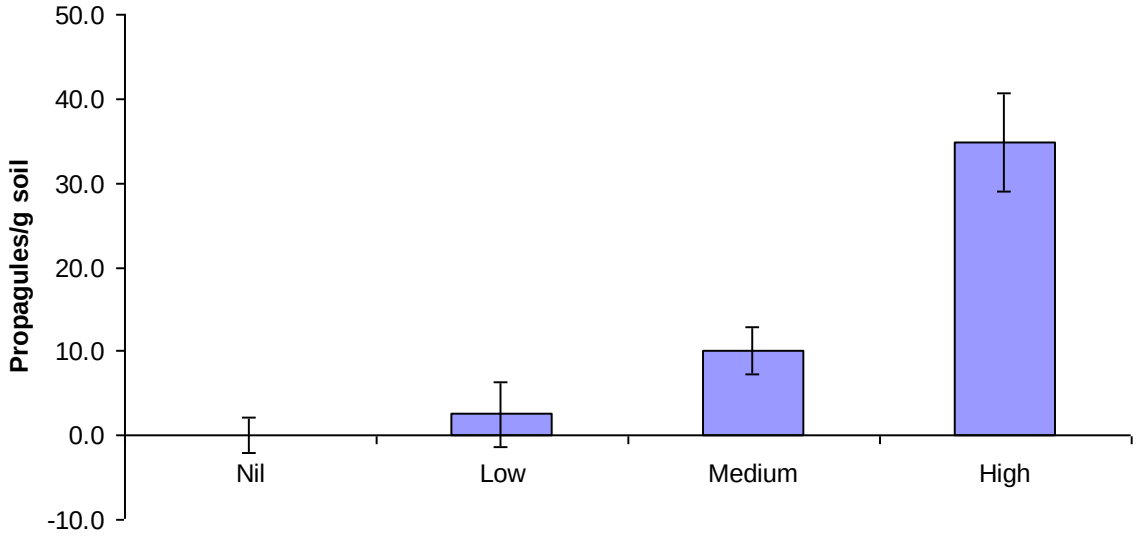

Figure 13. Levels of *V. dahliae* infestation at the start of the experiment as determined by qPCR

Levels of *V. dahliae* infestation were also determined using qPCR and these data are shown in Figure 13 and Table 11. In general, qPCR was markedly less effective at detecting *V. dahliae* than wet sieving and culturing using selective nutrient agar. Levels of *V. dahliae* as determined by wet sieving and nutrient agar were approximately 13, 6 and four times higher than the equivalent value determined by qPCR for the low, medium and

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high infestation soils respectively. There were four clear levels of infestation in the qPCR data but mean values at each infestation level were more variable than with wet sieving and selective agar.

Table 11. Levels of *V. dahliae* in four prepared soils at the start of the experiment as determined by a conventional test (wet sieving followed by selective nutrient agar) and qPCR

Soil sample relative level of *V. dahliae*	Levels of *V. dahliae*			Sensitivity of conventional compared with qPCR test*
	Conventional test (cfu/g)	qPCR (fg/g)	qPCR results (expressed as cfu/g)	
Nil	7.3	225	0.3	-
Low	33.0	513	5.1	6.5
Medium	55.2	1690	13.1	4.2
High	121.8	55154	38.0	3.2

* cfu/g conventional test/cfu/g from qPCR

Disease assessments

In general, 2012 was not a good year for Verticillium wilt infestation in strawberries (or raspberries). This was possibly due to the cool wet summer which failed to stress plants and made them less susceptible to the fungal infection. In an attempt to maximise the chances of the expression of symptoms of Verticillium wilt it was decided to split the final destructive assessment of the *V. dahliae/Pratylenchus* interaction study. As Verticillium wilt is thought to reduce the overwintering survival of strawberry plants, half the pots were assessed in December 2012 (blocks 1 and 3) and the remaining half (blocks 2 and 4) left until May 2013 once the first signs of growth were visible. The overwintered plants were maintained in the polytunnel and covered with horticultural fleece until 8 April to protect them from frosts.

At both the three and five month disease assessments there was very little wilting suggestive of Verticillium wilt and as a result these data were not subjected to statistical analysis. After three months the degree of yellowing/browning of the foliage differed significantly between levels of nematode inoculation ($P<0.001$, Table 12). There was no obvious relationship between yellowing/browning and increasing level of nematode infestation. Surprisingly, the highest level of yellowing/browning was recorded at the lowest nematode infestation and the lowest level of yellowing/browning was at the medium level of nematode infestation. There was a trend for increasing yellowing/browning with increasing level of *V. dahliae* ($P=0.052$).

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At five months there was no significant difference in the level of yellowing/browning between nematode and *V. dahliae* infestation levels (Table 12) and no statistically significant interaction.

Table 12. Mean scores for foliar yellowing/browning of strawberry plants (1 = no yellowing/browning, 2 = yellowing/browning, 3 = dead) at three and five months after planting (11/10/2012)

Levels of *Pratylenchus thornei*	Levels of *Verticillium dahliae*				
3 months	Nil	Low	Medium	High	Mean
Nil	1.3	1.3	1.9	2.0	1.6
Low	1.4	2.0	1.9	1.8	1.8
Medium	1.1	1.0	1.2	1.4	1.2
High	1.2	1.7	1.4	1.6	1.5
Mean	1.3	1.5	1.6	1.7	
SED (15DF) for comparison of *P. thornei* means = 0.16, P < 0.01					
SED (15DF) for comparison of *V. dahliae* means = 0.16, P = 0.052					
SED (15 DF) for comparison of means within the body of the table = 0.32, P = 0.560					
5 months	Nil	Low	Medium	High	Mean
Nil	1.9	2.0	2.0	2.1	2.0
Low	1.9	2.3	2.3	1.7	2.0
Medium	1.6	1.9	1.9	1.7	1.8
High	2.0	2.3	1.9	1.9	2.0
Mean	1.9	2.1	2.0	1.8	
SED (15DF) for comparison of *P thornei* means = 0.14, P = 0.203					
SED (15DF) for comparison of *V dahliae* means = 0.14, P = 0.153					
SED (15 DF) for comparison of means within the body of the table = 0.27, P = 0.763					

Harvest assessments

Root dry weight differed significantly between nematode infestation levels (P<0.05, Table 13). In general root dry weights were higher in the presence of nematodes than where they were absent. The highest root dry weight was at the lowest level of nematode infestation and the lowest dry weight was in pots with no root lesion nematodes.

There was a significant interaction between nematode and *V. dahliae* infestation levels for crown dry weight. This was probably because there was no consistent ranking in root dry

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weights between nematode infestation levels at each level of *V. dahliae* infestation and vice versa.

Dry weight of leaves did not differ significantly between nematode or *V. dahliae* infestation level (Table 13) but total dry weight differed significantly between nematode infestation levels ($P<0.05$, Table 13). Total dry weight was always greatest in the presence of nematodes. The highest total dry weight was in pots with a medium infestation of nematodes and the lowest dry weight where there were no nematodes.

There was no significant difference in the proportion of dead leaves per plant or the proportion of crowns with staining between either nematode or *V. dahliae* levels at harvest (Table 14).

The percentage of dead leaves per plant, proportion of crowns with staining and the proportion of dead plants at harvest did not differ significantly between nematode or *V. dahliae* infestation levels (Table 14).

Table 13. Mean dry weights for roots, crown, leaves and total dry weight at harvest (11.12.2012)

Levels of *Pratylenchus thornei*	Levels of *Verticillium dahliae*				
Dry weight roots	Nil	Low	Medium	High	Mean
Nil	2.23	2.88	2.40	2.10	2.40
Low	3.13	2.92	2.98	4.18	3.30
Medium	2.49	4.46	3.32	3.94	3.55
High	3.46	3.60	2.78	2.72	3.14
Mean	2.83	3.46	2.87	3.24	
SED (15DF) for comparison of *P. thornei* means = 0.353, P < 0.05					
SED (15DF) for comparison of *V. dahliae* means = 0.353, P = 0.262					
SED (15 DF) for comparison of means within the body of the table = 0.705, P = 0.287					
Dry weight crown	Nil	Low	Medium	High	Mean
Nil	1.86	2.27	2.36	1.98	2.12
Low	2.86	2.22	2.26	3.13	2.62
Medium	1.88	3.71	2.84	2.80	2.80
High	3.04	2.86	2.49	2.56	2.74
Mean	1.9	2.1	2.0	1.8	

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Table 13. Cont.

SED (15DF) for comparison of *P. thornei* means = 0.219, P < 0.05

SED (15DF) for comparison of *V. dahliae* means = 0.219, P = 0.422

SED (15 DF) for comparison of means within the body of the table = 0.437, P < 0.05

Levels of *Pratylenchus thornei*	Levels of *Verticillium dahliae*				
Dry weight leaves	Nil	Low	Medium	High	Mean
Nil	1.46	2.57	2.22	1.67	1.98
Low	2.58	2.24	2.27	3.14	2.56
Medium	2.44	4.15	3.16	2.83	3.15
High	3.45	3.15	2.60	2.32	2.88
Mean	2.48	3.03	2.56	2.49	

SED (15DF) for comparison of *P. thornei* means = 0.469, P = 0.120

SED (15DF) for comparison of *V. dahliae* means = 0.469, P = 0.617

SED (15 DF) for comparison of means within the body of the table = 0.939, P = 0.684

Total dry weight	Nil	Low	Medium	High	Mean
Nil	5.55	7.72	6.98	5.75	6.50
Low	8.58	7.37	7.52	10.45	8.48
Medium	6.81	12.31	9.32	9.57	9.50
High	9.95	9.61	7.88	7.60	8.76
Mean	1.9	2.1	2.0	1.8	

SED (15DF) for comparison of *P. thornei* means = 0.957, P < 0.05

SED (15DF) for comparison of *V. dahliae* means = 0.957, P = 0.417

SED (15 DF) for comparison of means within the body of the table = 1.915, P = 0.272

The overwintered plants were assessed on 3 May 2013 to determine the proportion of dead plants and proportion of stained crowns. The proportion of surviving plants was generally lower than was recorded in December and differed significantly between nematode infestation levels (P<0.05, Table 15). The lowest proportion of dead plants was at the medium nematode infestation level and the highest at the low infestation level. There was no significant effect of level of *V. dahliae* on the proportion of surviving plants and no significant interaction between nematodes and *V. dahliae.*

There was no significant difference between treatments (P=0.272) in the proportion of plants with stained crowns (Table 15). The majority of live plants (82%) had no staining in

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the crown, whereas the majority of dead plants (96%) had staining in the crown. There was no trend between either the level of nematodes or the level of *V. dahliae* on crown staining.

Table 14. Proportion of dead leaves, stained crowns and dead plants at harvest (11.12.2012)

Levels of *Pratylenchus thornei*	Levels of *Verticillium dahliae*				
Proportion of dead leaves	Nil	Low	Medium	High	Mean
Nil	0.35	0.55	0.38	40.3	0.43
Low	0.20	0.46	0.58	0.21	0.36
Medium	0.10	0.04	0.40	0.18	0.18
High	0.38	0.44	0.33	0.19	0.34
Mean	0.26	0.37	0.42	0.25	
SED (15DF) for comparison of *P. thornei* means = 0.227, P = 0.172					
SED (15DF) for comparison of *V. dahliae* means = 0.227, P = 0.336					
SED (15 DF) for comparison of means within the body of the table = 45.40, P = 0.682					
Proportion of stained crowns	Nil	Low	Medium	High	Mean
Nil	0.50	0.33	0.67	0.50	0.50
Low	0.67	0.83	0.67	0.33	0.63
Medium	0.50	0	0.50	0.17	0.29
High	0.17	0.33	0.33	0.17	0.25
Mean	0.46	0.38	0.54	0.29	
SED (15DF) for comparison of *P. thornei* means = 0.161, P = 0.107					
SED (15DF) for comparison of *V. dahliae* means = 0.161, P = 0.468					
SED (15 DF) for comparison of means within the body of the table = 0.322, P = 0.805					
Proportion of dead plants	Nil	Low	Medium	High	Mean
Nil	0.33	0.17	0.17	0.33	0.25
Low	0.17	0.33	0.33	0.17	0.25
Medium	0	0	0.17	0.17	0.08
High	0	0.17	0	0	0.04
Mean	0.13	0.17	0.17	0.17	
SED (15DF) for comparison of *P. thornei* means = 0.116, P = 0.191					
SED (15DF) for comparison of *V. dahliae* means = 0.116, P = 0.978					
SED (15 DF) for comparison of means within the body of the table = 0.231, P = 0.910					

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Table 15. Proportion of stained crowns and dead plants at harvest (3.5.2013)

Levels of *Pratylenchus thornei*	Levels of *Verticillium dahliae*				
Proportion of stained crowns	Nil	Low	Medium	High	Mean
Nil	0.50	0.33	0.67	0.83	0.58
Low	0.67	0.83	0.83	0.83	0.79
Medium	0.67	0.50	0.50	0.67	0.50
High	0.50	0.50	0.50	0.33	0.50
Mean	0.58	0.50	0.63	0.67	
SED (15DF) for comparison of *P. thornei* means = 0.143, P=0.146					
SED (15DF) for comparison of *V. dahliae* means = 0.143, P=0.688					
SED (15 DF) for comparison of means within the body of the table = 0.571, P = 0.567					
Proportion of dead plants	Nil	Low	Medium	High	Mean
Nil	0.50	0.33	0.67	0.83	0.58
Low	0.67	0.83	0.83	1.0	0.83
Medium	0.33	0.17	0.17	0.33	0.25
High	0.67	0.67	0.33	0.17	0.46
Mean	0.54	0.50	0.50	0.58	
SED (15DF) for comparison of *P. thornei* means = 0.154, P < 0.05					
SED (15DF) for comparison of *V. dahliae* means = 0.154, P = 0.938					
SED (15 DF) for comparison of means within the body of the table = 0.307, P = 0.533					

Other assessments

The nematode species was identified as *Pratylenchus thornei.*

Crop vigour was assessed on 2 August and differed significantly between nematode infestation levels (P<0.001, Table 16). There was a trend for crop vigour to increase with increasing level of nematode infestation. The most vigorous plants were in pots with the highest nematode infestation and least vigorous in pots with no nematodes.

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Table 16. Mean crop vigour score on 2 8.2012. (1 = least vigorous, 3 = most vigorous)

Levels of *Pratylenchus thornei*	Levels of *Verticillium dahliae*				
Crop vigour	Nil	Low	Medium	High	Mean
Nil	1.7	2.2	1.4	1.6	1.7
Low	2.2	1.3	1.8	1.6	1.7
Medium	2.1	2.7	2.8	2.2	2.4
High	2.7	2.2	2.8	2.4	2.5
Mean	2.1	2.1	2.2	1.9	
SED (15DF) for comparison of *P. thornei* means = 0.40, P < 0.001					
SED (15DF) for comparison of *V. dahliae* means = 0.40, P = 0.501					
SED (15 DF) for comparison of means within the body of the table = 0.80, P = 0.116					

An assessment on 26 September 2012 showed that both leaf number and the proportion of live plants differed significantly between nematode infestation levels (P<0.001 in each case, Table 17). Leaf number and proportion of live plants was always highest in the presence of nematodes. The medium level of nematode infestation had the highest number of leaves and proportion of live plants. The lowest number of leaves and proportion of live plants was lowest in the pots with no nematodes.

In the absence of nematodes, there was a trend for the proportion of live plants to decrease with increasing infestation levels of *V. dahliae.* In the presence of nematodes, there was no consistent pattern with regard to the effect of *V. dahliae* infestation level on the proportion of live plants.

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Table 17. Mean number of leaves per plant and proportion of live plants on 26 September 2012.

Levels of *Pratylenchus thornei*	Levels of *Verticillium dahliae*				
Number of live leaves	Nil	Low	Medium	High	Mean
Nil	3.5	4.2	3.4	2.5	3.4
Low	4.9	2.9	3.8	4.4	4.0
Medium	4.6	7.3	6.3	4.8	5.7
High	6.2	4.8	5.6	4.5	5.3
Mean	4.8	4.8	4.8	4.1	
SED (15DF) for comparison of *P. thornei* means = 22.70, P < 0.001					
SED (15DF) for comparison of *V. dahliae* means = 22.70, P = 0.530					
SED (15 DF) for comparison of means within the body of the table = 45.40, P = 0.243					
Proportion of live plants	Nil	Low	Medium	High	Mean
Nil	0.83	0.83	0.67	0.58	0.73
Low	0.83	0.58	0.75	0.83	0.75
Medium	1.00	1.00	0.92	0.92	0.96
High	1.00	0.75	1.00	1.00	0.94
Mean	0.92	0.79	0.83	0.83	
SED (15DF) for comparison of *P. thornei* means = 0.069, P < 0.001					
SED (15DF) for comparison of *V. dahliae* means = 0.069, P = 0.338					
SED (15 DF) for comparison of means within the body of the table = 0.138, P = 0.277					

Discussion

See Year 1 report for discussion on Objective 1.

Objective 2: Soil threshold levels for direct damage

Soil dilution proved to be a very effective way of creating a range of populations of root lesion nematodes in both years 1 and 2 of the project, which would have been very difficult to achieve using field sites. The actual starting populations were approximately one third of the target population in year 1 and one fifth of the target population in year 2. It is possible that some nematodes were killed when preparing the original soil dilutions even though every attempt was made to handle the soil as carefully as possible. Despite actual populations being lower than the target population the nematode counts ranged from

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approximately zero to 1,200 root lesion nematodes/L soil in year 1 and zero to 775 root lesion nematodes/L soil in year 2. Nematode numbers in year 1 were both well below and above the anecdotal threshold of 700 root lesion nematodes/L soil and so provided a good range over which to assess their impact on strawberry growth. Although numbers were lower in year 2 the highest population was still greater than the anecdotal threshold. Only one species of root lesion nematode was recovered from the field soil. This was *Pratylenchus thornei*.

It is interesting that root lesion nematodes did not have any significant deleterious effect on strawberry growth. It would be expected that plants would be most susceptible to the effect of nematode feeding at early stages of growth but there was no indication that this was the case, even at levels approximately double the anecdotal threshold. Plants were regularly watered and it is possible that this helped them to tolerate the impact of nematode feeding. Limiting the watering of pots would provide a more robust test of the plants' tolerance of nematode attack but also runs the risk of reducing pest activity. Nematodes will become less active at low levels of soil moisture.

Another possible factor that might have contributed to the lack of effect on strawberry growth is the identity and relative proportions of the root lesion nematode species in the soils used. As noted earlier, *Pratylenchus penetrans* is considered more pathogenic than *P. thornei*. As the soil contained only *P. thornei*, then one might expect less damage than if *P. penetrans* were present. The identity and relative proportions of different *Pratylenchus* species are not normally determined when testing soils pre-planting; identification is done to nematode genus level (*Pratylenchus* spp.). Development of barcoding for nematode species should improve the practicality of testing soils for individual nematode species.

There is no recognised threshold for root lesion nematodes in strawberries although anecdotal evidence suggests that 700/L soil may be damaging. Numbers of nematodes in the created population were well in excess of this threshold at both the start and end of the experiment in year 1. In year 2 numbers above the anecdotal threshold were recorded at the start of the experiment but did not exceed 300 root lesion nematodes/L soil at the end of the experiment. Despite this decline in numbers it is likely that at the start of the experiment levels were high enough to potentially affect strawberry growth at a time when the root system had not established and plants were most susceptible to attack. In general, results of two years work suggest that the current threshold may be too conservative and well below the number of this nematode species which can be tolerated by the crop.

In year 1 strawberry plants varied in fresh weight at planting from approximately 5-15 g. In retrospect an assessment of fresh weight at harvest would have allowed the proportional

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change in fresh weight to be related to nematode numbers. In year 2 the fresh weight of strawberry plants at planting and harvest were assessed. However, this did not show any impact of nematodes on strawberry growth. If nematodes had a significant effect on strawberry growth as has been suggested, it might have been expected that plant dry weight would have been reduced to such an extent in pots with high nematode numbers that there would be clear differences from those with low nematode numbers. This was not the case in either years 1 or 2. Re-analysis of a subset of data in year 1, in which plants had a similar fresh weight at planting, did show a significant linear correlation between root dry weight and nematode numbers at harvest. However, this was a relatively weak, negative relationship, suggesting that although root dry weight did decrease with increasing numbers of root lesion nematodes the impact on strawberry growth was slight.

If strawberries are more tolerant of nematodes than previously thought it will have a significant impact on nematicide use and potentially increase the profitability of the crop. However, it should be borne in mind that there is a range of species of root lesion nematodes, which may not all exhibit the same degree of pathogenicity towards strawberries.

Objective 3: Identification of nematodes by molecular methods

Molecular barcoding, using ITS1 and ITS2 regions of nematode DNA, was successful in developing real-time PCR assays to *Pratylenchus penetrans* (ITS1), *P. thornei* (ITS1) and *Meloidogyne hapla* (ITS2). These pathogens were chosen because *Pratylenchus* spp. and *Meloidogyne hapla* are known pathogens of strawberry. Thirty-two soil samples from strawberry fields were tested for levels of nematodes using the three real-time PCR assays. The assays suggest that *Meloidogyne hapla* was present in 56% of soil samples and was more common in the soils tested than *P. penetrans* (25%) and *P. thornei* (31%). From the 32 samples tested, just over 71% of samples were positive for one or more of the three nematodes tested by PCR.

Seven soils were selected because they had different levels of root lesion nematodes (*Pratylenchus* spp.), from 0 to 3,250 nematodes/litre, as determined by conventional methods. Soils were tested by real-time PCR. QPCR was positive in samples between 1,085 to 3,250 nematodes/litre (except the sample with 1,400 nematodes/litre where no DNA was detected). There was broad agreement between *Pratylenchus* spp. nematode numbers above 475 nematodes/litre and QPCR results in three out of four positive root lesion samples. This provides a small snapshot of soil samples available that had different levels of *P. thornei* and *V. dahliae*. This showed that there was a small but negative significant relationship between *P. thornei* and *V. dahliae* where *P. thornei increased as V.*

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dahliae decreased (Figure 14, *P*=0.013). This relationship is based on a limited set of samples and would need to be corroborated using more samples. The new nematode real-time PCR assays will be a useful tool for investigating the inter-relationships between nematodes and fungal pathogens.

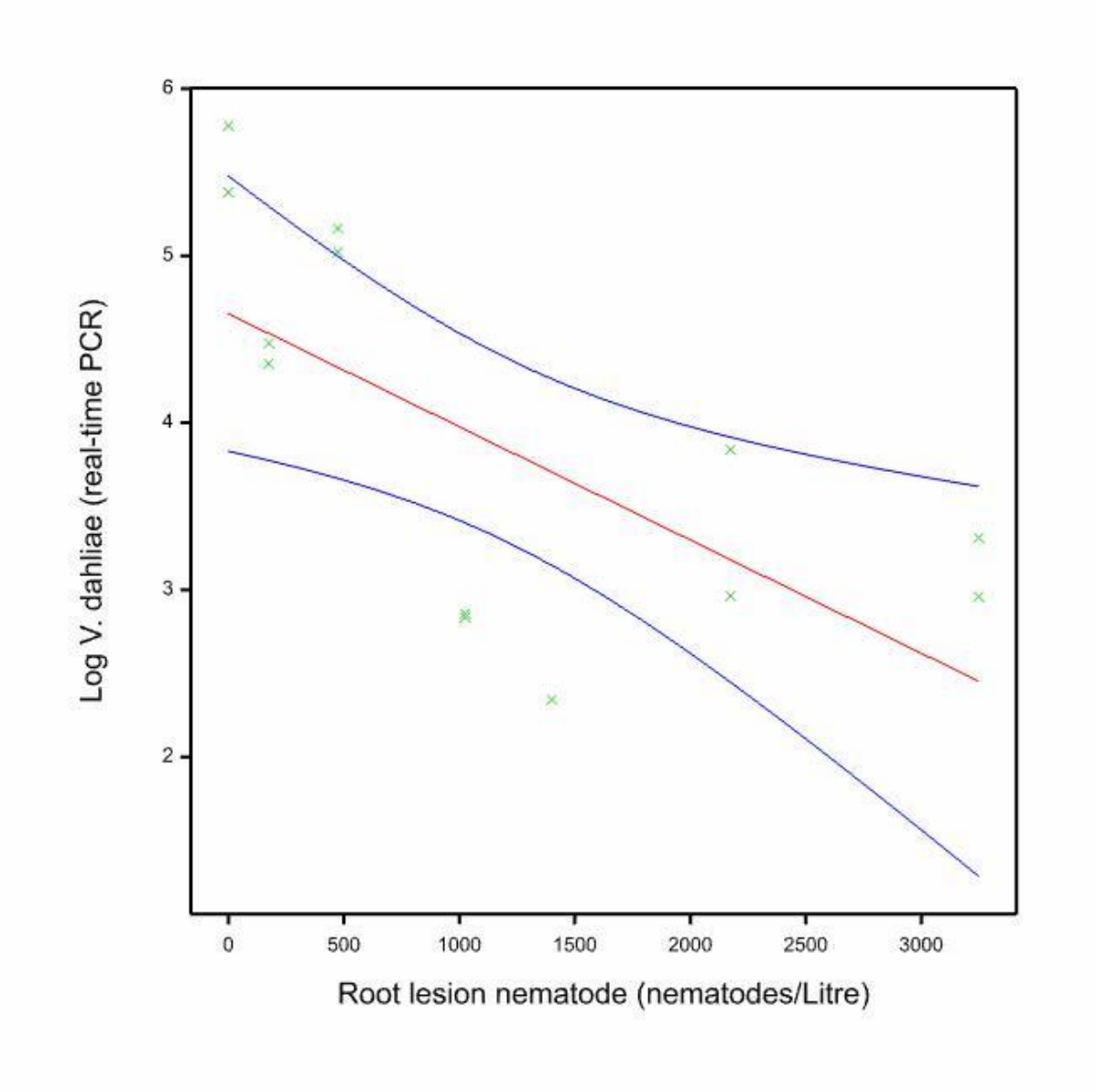

Figure 14. Relationship between log *Verticillium dahliae* real-time PCR levels and root lesion nematode/Litre.

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Objective 4: To determine whether increasing levels of a root lesion nematode species increases the risk of strawberry Verticillium wilt caused by V. dahliae

There was no obvious evidence of any interaction between numbers of root lesion nematodes and levels of *V. dahliae* in strawberry plants and nothing to suggest that increasing numbers of root lesion nematodes increases the susceptibility to Verticillium wilt.

Also there was no evidence to suggest that *V. dahliae* or nematodes reduced the overwintering survival of strawberry plants. The proportion of surviving plants decreased between December and January, which would have been expected due to the long cold winter, but this was not affected by the level of *V. dahliae* infestation. However, as the lowest level of *V. dahliae* infestation recorded by wet sieving and culturing on selective nutrient agar was well above that in which strawberries would normally be grown, it is possible that all levels of fungal infestation were equally damaging. Although overwintering survival did vary between nematode infestation levels there was no clear trend in the data, with most plants surviving at the low level of nematode infestation and least at the medium level of infestation. This result is difficult to explain and should be treated with a degree of caution.

It was interesting that crop vigour in August, leaf number and number of live plants in September and root dry weight and total dry weight at harvest were highest in the presence of nematodes. This suggests a possible compensatory response from the crop to nematode feeding. However, there is a possible alternative explanation for the apparent reduced vigour and dry weight of plants in the absence of nematodes. This soil had been heat sterilised to kill any free-living nematodes. This procedure may have had a detrimental impact on the nutrient availability of the soil and consequently the growth of the strawberry plants. Irrespective of whether crop compensation or reduced nutrient availability can explain the growth of strawberries in the presence or absence of nematodes, there was no indication that increasing nematode numbers influenced susceptibility to Verticillium wilt. However, it should be borne in mind that in general, 2012 was not a good year for Verticillium wilt infection in strawberries (or raspberries). This was possibly due to the cool wet summer, which failed to stress plants and made them less susceptible to the fungal infection. Under more stressful conditions it is possible that feeding by increasing numbers of nematodes could have an impact on susceptibility to Verticillium wilt.

There was a clear difference in the precision of qPCR and wet sieving followed by selective nutrient agar for quantification of *V. dahliae* in soil. In general, qPCR was markedly less effective at detecting *V. dahliae* than wet sieving and culturing using selective nutrient agar.

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Levels of *V. dahliae* as determined by wet sieving and nutrient agar for low, medium and high infestation levels were approximately 13, 6 and four times higher than the equivalent value determined by qPCR respectively. Also, a preliminary qPCR analysis of the soil collected for the experiment suggested that it was free of *V. dahlia,* whereas subsequent testing using wet sieving and selective nutrient agar showed there to be a mean of 7.3 propagules/g soil. This explains why it was not possible to achieve a nil level of *V. dahliae* in the experimental treatments as had been intended. Analysis using qPCR did indicate that inoculation achieved four clear levels of infestation of *V. dahliae* but the mean values at each infestation level were more variable than with wet sieving and selective agar. This difference in the precision of qPCR and wet sieving followed by culturing in selective nutrient agar has been recognised and is being addressed in HDC project SF 97a. A major objective of this work will be to increase the precision of qPCR analysis such that it is comparable with wet sieving and selective nutrient agar.

Conclusions

- Results suggest that a reduction can be made on nematicide use as there was no deleterious effect on strawberry plants by numbers in excess of the anecdotal threshold for a *Pratylenchus* species.
- Soil dilution was an effective method of creating a range of populations of root lesion nematodes which were assessed for their impact on strawberry growth.
- Populations of root lesion nematodes (*Pratylenchus thornei*) as high as 1,200/L soil did not appear to have a major impact on strawberry growth, although there was a slight negative relationship between root dry weight and nematode numbers at harvest. It is possible that nematodes could have an effect at lower levels of soil moisture which did not inhibit pest activity.
- Molecular barcoding technology using the SSU region of nematode DNA was successful in identifying four genera of pathogenic free-living nematodes.
- QPCR analysis for *Verticillium dahliae* was less sensitive than the wet sieving method but the molecular method was able to identify samples in the same order detected by the wet sieving method.
- There was broad agreement between *Pratylenchus* spp. nematode numbers above 475 nematodes/litre and QPCR results in three out of four positive root lesion samples

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- No evidence was found to support the hypothesis that *P. thornei*, one of several root lesion nematodes that can infect strawberries, increases the risk of Verticillium wilt.
- There was no evidence to suggest that *V. dahliae* affects the overwintering survival of strawberry plants. However, even the lowest level fungal infection in this experiment would be expected to have a significant effect on the growth of strawberries (cv. Elsanta), so it is possible that all levels of fungal infestation were equally damaging.
- Although overwintering survival of strawberries did differ between nematode infestation levels, there was no clear trend to the data and this result should be treated with caution.
- 2012 was a poor year for Verticillium wilt, probably because cool wet weather did not stress plants. Under more stressful conditions it is possible that feeding by root lesion nematodes would increase susceptibility to the disease.

Knowledge and Technology Transfer

Assessing nematode risk to soft fruit – Article for HDC News 2012

Linking nematodes to Verticillium wilt – Article for Soft Fruit Review Magazine December 2012.

Nematode thresholds revisted – Article for HDC News 2013

Glossary

Barcoding – Using sequences from specific DNA regions to identify organisms based on a nearest identification matches held on the NCBI database.

Flegg modified Cobb method (Flegg, 1967) – A method of extracting free-living nematodes from soil which is specifically recommended for large species (eg, dagger and needle nematodes).

Seinhorst 2-flask method (Seinhorst, 1955) – A method of extracting free-living nematodes from soil, which is specifically recommended for small to medium sized species (e.g. stubby root nematode, root lesion nematodes, stunt/spiral nematodes).

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Appendix 1 – Crop diary

Experiment 1. Interaction of nematodes and Verticillium wilt

Date	**Task**	**Staff responsible**
4.6.2012	Culture of *V. dahliae* prepared with which to inoculate pots	SB
21.6.2012	Soil collected from field from site with high populations of root lesion nematodes and stored in walk-in fridge	SA
2.7.2012	Strawberry plants delivered, 300 Elsanta 15-18 mm certified stock	
4.7.2012	Nematode populations made up by diluting infested soil with sterile soil. Strawberries planted and pots inoculated with four populations of *V. dahliae*. Additional strawberry plants potted up to provide four three of each nematode population to be extracted at regular intervals throughout the experiment to monitor population development. All pots arranged in poly tunnel. Soil samples infected with each *V. dahliae* population assessed for level of fungal infection by qPCR and plating out on selective nutrient agar	JS, DL, GM, SB
4.7.2012-May 2013	Plants maintained in a poly-tunnel and watered as necessary	JS
2.8.2012	Crop vigour assessed	SAE
19.9.2012	Twelve spare pots extracted using Seinhorst two-flask technique to determine nematode numbers	DL, LG, SB
10.10.2012	Twelve spare pots extracted using Seinhorst two-flask technique to determine nematode numbers	DL, LG, SB
11.10.2012	Visual assessment of disease levels on all pots.	SB
7.12.2012	Visual assessment of vert wilt levels in each pot	SB
20.12.2012	Pots from blocks 1 and 3 harvested. Plants assessed for disease levels. Plant fresh and dry weight determined. Soil analysed for levels of vert wilt and	JS, SB

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	nematodes. Remaining plants from blocks 2 and 4 covered with horticultural fleece for frost protection	
8.4.2013	Fleece removed from blocks 2 and 4	JS
3.5.2013	Blocks 2 and 4 assessed for levels of dead plants and crown staining	JS

Experiment 2. Impact of a range of population of stubby root nematodes (*P. thornei*) on strawberry growth

Date	**Task**	**Staff responsible**
21.6.2012	Soil collected from field from site with high populations of root lesion nematodes and stored in walk-in fridge	SA
2.7.2012	Strawberry plants delivered, 300 Elsanta 15-18 mm certified stock	
4.7.2012	Nematode populations (29) made up by diluting infested soil with sterile soil. Strawberries planted. All pots arranged in poly tunnel. Spare soil from each pot retained to determine nematode numbers	JS, DL, GM,
20-22.7.2012	Extracted soil samples examined microscopically and numbers of nematodes counted	DL, LG, SB
4.7-7.12.2012	Plants maintained in a poly-tunnel and watered as necessary	JS
7.12.2012	Pots harvested. Roots examined for any differences between nematode populations. Foliar, crown and root dry weight determined by oven drying. Soil retained for nematode extraction	JS
10.12.2012	Soil extracted using Seinhorst two-flask technique	DL & JN
18.12.2012	Extracted soil samples examined microscopically and numbers of nematodes counted	HM

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