Project title:	Strawberry and raspberry: using soil nematode threshold levels to reduce direct feeding damage on roots and interactions with Verticillium wilt
Project number:	SF 122
Project leader:	Tim O'Neill, ADAS
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Location of project:	ADAS, High Mowthorpe – poly tunnel, glasshouse and laboratory Fera – Laboratory work
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Date project commenced:	1 April 2011
Date project completed (or expected completion date):	31 March 2013

The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations

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

Headline

- 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, whilst other early results suggest that reductions can be made on nematicide use, even though current thresholds are at best anecdotal.

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*, although cyst nematodes may also have a synergistic effect in potato wilt. 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 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.

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

The specific project objectives are:

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 the predominant *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 the soil threshold level for the predominant nematode species which increases the risk of strawberry verticillium wilt caused by *V. dahliae*.

In year one of this project, Objectives 1-3 have been started. Objective 4 will not begin until year 2.

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 soils submitted by soft fruit growers to ADAS and Fera between 2001 and 2011 was examined. The site identification details were removed to retain the confidentiality of the grower submitting the sample. There were a total of 92 samples submitted to ADAS over this period.

In addition, four strawberry crops and four raspberry crops with a history of nematode problems, or sites considered at high risk (e.g. grown strawberries at least twice in last 10 years) were selected by ADAS Soft Fruit Consultants.

Of those soil samples from fruit crops submitted to ADAS Pest Evaluation Services, strawberries were the most frequently sampled crop/prospective crop followed by raspberries. Together these two crops 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.

Table 1. Free-living nematodes recovered from 92 soil samples from fruit farms submitted to 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	980	5,309

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.

Table 2. 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 for both direct feeding damage and virus transmission in soil samples extracted for fruit/prospective fruit crops, 2001-2011

	% sites over threshold	
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.

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 root lesion nematodes was created by soil dilution. This involved mixing soil infested with nematodes with the same soil which had been sterilised by oven drying at 60ºC for 45 minutes. For example, to achieve a target nematode population of 1,000 root lesion nematodes/L of soil, 1 L of soil containing 2,000 stubby root nematodes/L soil was mixed with 1 L of sterile soil. A total of 50 target populations was 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.

Soil dilution was effective at providing a range of populations of root lesion nematodes. In general the actual population was approximately one third that of the target population. Despite actual populations being lower than the target population the nematode counts ranged from approximately zero to 1,200 root lesion nematodes/L soil. This is 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.

Results suggested that populations of root lesion nematodes as high as 1,200/L soil had limited impact on strawberry growth; there was a slight negative relationship suggesting that root dry weight decreased with increasing nematode numbers at harvest. 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 populations were well in excess of this threshold at both the start and end of the experiment. This suggests that the current thresholds may be too conservative and below the number of 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. 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. Large, moderately variable coding regions are considered useful for providing suitable resolution between taxa. The use of at least two of those barcoding genes are a good basis for a robust and reliable means of identifying free-living nematodes.

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 using the SSU gene. 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. The sequencing data obtained from the barcoding will be used to develop specific assays for the detection and quantification for up to five pathogenic nematode species in Year 2 of the project.

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*

Financial benefits

It is not possible to estimate the potential financial benefits from this work until the project is completed. Benefits may arise from:

a) Reduced losses from nematodes and verticillium wilt;

b) Improved risk assessment for nematodes to develop more effective pest and disease control strategies;

c) Increased accuracy in the identification of nematode species present in soft fruit soils;

d) Development of a rapid pre-plant soil test for nematodes in samples where DNA has been extracted for *V. dahliae* determination;

e) Increased understanding of interaction between nematodes and *V. dahliae* in the development of verticillium wilt in soft fruit;

f) Availability of new information to help improve rational decision-making on planting decisions.

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 continue to use current thresholds (even though they are at best anecdotal) as there are still potential savings to be made 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.

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 has been withdrawn in the UK and Basamid, 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 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 (Dami-Remadi *et al*., 2009; Rowe *et al*., 2008; 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:

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 the predominant *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 the soil threshold level for the predominant nematode species which increases the risk of strawberry verticillium wilt caused by *V. dahliae*.

In year one of this project, Objectives 1-3 have been started. Objective 4 will not begin until year 2.

Materials and methods

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

The relative frequency of different genera of nematodes found in soils submitted by soft fruit growers to ADAS between 2001 and 2011 were examined. The site identification details were removed to retain the confidentiality of grower submitting the sample. There was a total of 92 samples submitted to ADAS over this period. In addition, four strawberry crops and four raspberry crops with a history of nematode problems, or sites considered at high risk (e.g. grown strawberries at least twice in last 10 years), were selected by ADAS Soft Fruit Consultants. A soil sample was taken from each of these sites according by zig-zagging across the area in an extended 'W' path and taking approximately 50 cheese-corer cores to a depth of 15 cm. This provided a bulked soil sample of approximately 2 kg.

Site details, including cropping history and use of soil disinfestation (e.g. methyl bromide, chloropicrin, Basamid, Caliente meal, Mustard), was collected for the preceeding 10 years. Any symptoms of suspect nematode damage (e.g. poor vigour, reduced yield, virus symptoms) were recorded.

The soil was extracted using both the Seinhorst two-flask method (Seinhorst, 1955) and the Flegg modified Cobb method (Flegg, 1967). The Seinhorst two-flask method provides the best estimate of small to medium sized species such as root lesion (*Pratylenchus* spp.) and stubby root (*Trichodorus* and *Paratrichodorus* spp.) nematodes and the Flegg modified Cobb method is best for larger species such as needle (*Longidorus* spp.) and dagger nematodes (*Xiphinema* spp.). Representative samples of nematodes were identified to species level by Fera. A total of 36 potentially pathogenic free-living nematodes were isolated in single culture and processed to obtain total DNA for barcoding.

Objective 2: Soil threshold levels for direct damage

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 contained 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 and once using Flegg modified Cobb technique.

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 for each nematode group 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.

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 50 target nematode populations in the experiment ranging from 0-3,000 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 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 per 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. A Tiny Talk data logger was located in one pot to monitor soil temperature throughout the experiment. After approximately four months the plants were harvested. Dry matter yield of the foliage, crown, roots and total plant dry weight was assessed by oven drying at 80^{o}C for 48 hours. The pot soil was also extracted using the Seinhorst two-flask technique to compare the initial and final nematode population.

Objective 3: Identification of nematodes by molecular methods

Ten free-living nematode species that are potentially pathogenic to soft fruit were obtained from 36 single-isolate nematode samples (Table 5). Total DNA were obtained from each suspension of nematodes in water. Representative samples (N1, N2, N19, N28, N33-36) were chosen to evaluate the suitability of five candidate barcoding primer sets.

Table 5. List of free-living nematode species obtained from soil extracts

DNA extract number	Visual identification	Adas ref number
1,2	*Rotylenchus buxophilus*	7965.002
3	*Longidorus macrosoma*	7965.002
4,5	*Bitylenchus dubius*	8036.001
6.7	*Merlinius brevidens*	8013.002
8	*Pratylenchus thornei*	8013.002
9	*Merlinius brevidens*	8013.001
10 -16	*Paratylenchus sp (*cf *nanus)*	8013.001
17-22, 34-36	*Pratylenchus thornei*	SF122
23	*Trophurus imperialis*	SF122
24-33	*Xiphinema diversicaudatum*	8036 001

DNA extraction was undertaken with the Qiagen blood and tissue kit using the manufacturer's instructions except that DNA was eluted in 100 µl of buffer. Conventional PCR was undertaken using standard conditions of Fermentas 2x master mix with 50 µl samples. Two µl of 10 µM primer stocks were used for forward and reverse primers with 4

µl of DNA template used. PCR consisted of a 95ºC incubation for 3s followed by 35 cycles of 95ºC for 30s, 53 for 30s and 72ºC for 60 s with a final extension of 72 for three minutes.

Primers sets chosen to perform the barcoding were: large ribosomal subunits (LSU) D1/D2 domain, LSU D2/D3 domain, the ribosomal DNA ITS region, CoxI region and the ribosomal small subunit (SSU) 18S region (Nadler *et al*, 2003; Holterman *et al*, 2006)

The resulting DNA sample was purified using a Qiagen PCR clean-up kit according to the manufacturer's instructions and a suitable aliquot was sent for sequencing by MWG Biotech Ltd. Both forward and reverse DNA strands were sequenced and a consensus sequence formed. This consensus sequence was then compared with sequences held on the NCBI Blast database.

Five isolates were initially barcoded using the SSU gene. The resulting sequences were aligned using the NCBI nucleotide Blast search to give a best match identification.

Results

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

Over the period January 2001 - July 2011, a total of 92 soil samples were received by ADAS Pest Evaluation Services (PES) for extraction of free-living nematodes from fruit crops. All samples were subjected to two extraction methods, the Seinhorst two-flask technique (Seinhorst, 1955) for small to medium sized nematodes and the Flegg modified Cobb technique (Flegg, 1967) for large nematode species. The combination of these two methods is considered to give the best estimate of nematode numbers in a soil sample. In each sample eight main nematode groups were identified and counted, and numbers presented as numbers/litre soil. Of these, four main groups: dagger nematodes; needle nematodes; root lesion nematodes and stubby root nematodes, were considered to be those most likely to damage a soft fruit crop. All groups can potentially feed directly on the roots and dagger nematodes, needle nematodes and stubby root nematodes are potential virus vectors. There are no established thresholds for direct feeding damage on soft fruit and the presence of any virus vectors is potentially a risk as it is not possible to determine if they are carrying virus.

The range of crops/prospective crops from which samples were taken is given below (Table 6). Prospective fruit crops are those sites where the grower was considering planting a soft fruit/ cane fruit crop

Table 6. Fruit crops/prospective fruit crops from which soil samples were received by ADAS, Pest Evaluation Services for extraction of free-living nematodes, January 2001-July 2011.

Fruit crop/ prospective fruit crop	Number of samples received
Blackberries/strawberries	1
Blackcurrants	1
Fruit/Christmas trees	2
Raspberries	25
Soft fruit	1
Strawberries	34
Strawberries/cane fruit	3
Strawberries/raspberries	25
Total	92

Strawberries were the most frequently sampled crop or prospective crop, followed by raspberries. Together these two crops accounted for 91% of samples processed. The nematode counts from each soil extraction were used to advise farmers/growers and agronomists of the likelihood of the need for control (usually a nematicide) based on knowledge of the following crop and a best estimate (Table 7) of the numbers likely to cause damage.

Best estimates of the number of dagger, needle and stubby root nematodes that might cause direct feeding damage on the roots of a fruit crop are the same as those quoted for carrot, parsnip and sugar beet crops and so should be treated with some caution. The proposed threshold for root lesion nematodes is based on anecdotal evidence from ADAS Fruit Consultants although the figure may vary depending on the species present. For example, *Pratylenchus penetrans* is considered more pathogenic than *P. thornei*. Stunt/spiral nematodes are not considered major crop pests and so their guideline threshold is much higher than for other groups.

Table 7. Nematode groups identified and counted by PES, typical species and best estimates of numbers likely to cause damage (number/Litre soil)

Nematode group	Typical species	Guideline threshold
Dagger nematodes	*Xiphinema* spp.	50 or presence in case of virus transmission
Needle nematodes	*Longidorus* spp.	50 or presence in case of virus transmission
Root lesion nematodes	*Pratylenchus* spp.	700
Stubby root nematodes	*Trichodorus* spp. *Paratrichodorus* spp.	200
Stunt/spiral nematodes	*Tylenchorynchus* spp. *Helicotylenchus* spp.	10,000

All nematode counts from January 2001 to July 2011 were analysed to determine any trends or patterns in nematode incidence. This included the relative abundance of each nematode group, the range of numbers encountered, and proportion of samples in which the guideline threshold was exceeded.

Maximum and minimum counts of nematode groups and range

The minimum count for all nematode groups was zero, indicating that each group was absent from at least one sample between 2001 and 2011 (Table 8). The most commonly recovered nematodes were stunt/spiral nematodes, which were present in 98% of samples, but these are considered one of the least pathogenic groups, as previously discussed.

Root lesion nematodes, which can potentially damage soft fruit, were the next most common nematode group, being found in 86% of samples, followed by needle nematodes, which were present in 58% of samples.

Stubby root nematodes were found in 49% of samples and cyst juveniles and dagger nematodes in 30% or less of samples. The maximum nematode count was 10,400 stunt spiral nematodes/L soil. The next highest individual nematode counts were for stubby root nematodes (3,475/L soil) and root lesion nematodes (3,025/L soil).

The maximum and minimum counts, together with the numbers of nematodes within 90% of the maximum to minimum range, give a good indication of how many nematodes of each group are likely to be found in most soil samples. These data confirm the relative rarity of

dagger nematodes and cyst juveniles in soil samples processed for fruit crops. Dagger nematodes are a potential pest of fruit whereas there are no fruit cyst nematodes.

Table 8. Free-living nematodes recovered from 92 soil samples from fruit farms 2001-2011: Numbers detected and range

Nematode group	Number/Litre		Proportion of samples with '0'	Numbers of nematodes comprising 90% of max-min range
	Min	Max		
Cyst juveniles	0	525	70	72
Dagger nematodes	0	395	77	12
Needle nematodes	0	2,835	42	330
Root lesion nematodes	0	3,025	14	671
Stubby root nematodes	0	3,475	51	221
Stunt/spiral nematodes	0	10,400	2	5,309

Proportion of nematode counts above threshold

The proportion of nematode counts above threshold for individual groups gives an indication of the potential crop area likely to be treated with a nematicide. These data are presented in Table 9. The proportion of sites over threshold for direct feeding damage and virus transmission are given. Thresholds for direct feeding damage should be treated with some caution, as already discussed, as their basis is at best anecdotal. Presence of a potential nematode vector is considered as sufficient to warrant nematicide treatment in the case of virus transmission.

Table 9. Proportion of sites above threshold for different nematode groups for both direct feeding damage and virus transmission in soil samples extracted for fruit/prospective fruit crops, 2001-2011

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

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, whereas for all other nematode groups the proportion over threshold was less than 12%. Stunt spiral nematodes only exceeded the guideline threshold in one sample, confirming the relatively low risk this group poses to fruit crops. Needle nematodes were present in 54% of samples and therefore posed a potential risk of virus transmission. Stubby root nematodes were present in 45% of samples and dagger nematodes in 15% of samples. Therefore needle nematodes are also potentially the most important virus vector in soft fruit crops.

Soil samples from strawberry/raspberry crops

A total of eight soil samples were taken from sites with a history of nematode problems, or sites considered at high risk (e.g. grown strawberries at least twice in last 10 years) by ADAS Soft Fruit Consultants. Four were strawberry crops and four from raspberry crops. The results of nematode extractions are given in Table 10. Site and crop details are given in Table 11. No symptoms suggestive of nematode damage were reported at any of the sites at the time of soil sampling.

Table 10. Number of nematodes/L soil recovered from strawberry and raspberry crops believed to be at risk from the pests, sampled in 2011

Crop	Nematode numbers/L soil				
	Dagger	Needle	Root lesion	Stubby root	Stunt spiral
Stawberrry 1	0	0	50	0	25
Strawberry 2	0	0	50	0	25
Strawberry 3	0	0	0	0	25
Strawberry 4	485	15	25	0	50
Raspberry 1	0	220	0	0	400
Raspberry 2	0	0	1,275	75	2,300
Raspberry 3	0	50	0	0	25
Raspberry 4	600	5	775	0	725

Table 11. Details of strawberry and raspberry sites considered at increased risk of soil nematode problems from which soil samples were taken – 2011

Site	County	Variety	No. years field in soft fruit	Date of last soil treatment	Method used
Strawberry					
1.	Cambs	Sweet Eve	6	2009	Chloropicrin
2.	Beds	Florence, Fenella, Symphony & Judi Bell	2	None	N/A
3.	Berks	Symphony	10	2009	Chloropicrin
4.	Cornwall	Symphony	11	None	N/A
Raspberry					
1.	Cambs	Octavia	17	1995	Metham sodium
2.	Oxon	Tulameen	4	2003-4	Methyl bromide
3.	Beds	Glen Ample	5	None	N/A
4.	Devon	Glen Ample	7	None	N/A

Numbers of nematodes in the strawberry samples were relatively low. Needle and dagger nematodes were present in Strawberry 4 and posed a risk of virus transmission. Numbers of dagger nematodes were also potentially high enough to cause direct feeding damage. In the raspberry samples the presence of dagger nematodes in Raspberry 4 and needle nematodes in Raspberry 1, 3 and 4 posed a risk of virus transmission. There was a potential of direct feeding damage in all raspberry samples due to a high count of needle

nematodes in Raspberry 1, a high count of root lesion nematodes in Raspberry 2, a relatively high count of needle nematodes in Raspberry 3 and a high count of both dagger and root lesion nematodes in Raspberry 4. Although there appears to be a high count of stunt/spiral nematodes in Raspberry 2 these are not considered an important pest species.

Nematodes were identified to species where possible and details for both strawberry and raspberry crops are shown in Table 12. The relative proportion of different species within each nematode group was calculated. In total eight plant parasitic nematodes were identified from strawberries and 17 in raspberries.

In strawberries and raspberries *Xiphinema diversicaudatum* was the only dagger nematode species recorded. *Pratylenchus thornei* was the most common root lesion nematode in strawberries, whereas in raspberries it was *P. penetrans* followed by *P. thornei*. The most common stunt/spiral nematodes in strawberries were *Merlinius brevidens* followed by *Bitylenchus dubius,* whereas in raspberries it was *B. dubius* followed by *Helicotylenchus vulgaris*. Stubby root nematodes were only found in raspberry samples. *Paratrichodorus anemones* was most common, followed by *P. pachydermus*.

Table 12. Nematode groups and the % occurrence of species within each group for both strawberry and raspberry crops sampled in 2011

Nematode group	Species	% occurrence of species within group
Strawberries		
Dagger nematodes	*Xiphinema diversicaudatum*	100
Root lesion nematodes	*Pratylenchus thornei*	99
	Pratylenchus sp.	1
Stunt/spiral nematodes	*Bitylenchus dubius*	10
	Helicotylenchus sp.	35
	Merlinius brevidens	40
	Trophurus imperialis	10
	Tylenchorynchus sp.	5
Raspberries		
Dagger nematodes	*Xiphinema diversicaudatum*	100
Needle nematodes	*Longidorus macrosoma*	61
	Longidorus sp.	39
Root lesion nematodes	*Pratylenchus thornei*	30
	Pratylenchus sp.	8
	Pratylenchus penetrans	50
	Pratylenchus crenatus	12

Nematode group	Species	% occurrence of species within group
Stubby root nematodes	*Paratrichodorus anemones*	56
	Paratrichodorus pachydermus	31
	Trichodorus spp.	13
Stunt/spiral nematodes	*Bitylenchus dubius*	58
	Helicotylenchus sp.	1
	Merlinius brevidens	0
	Rotylenchus buxophilus	2
	Rotylenchus sp.	2
	Tylenchorynchus sp.	0
	Helicotylenchus vulgaris	36

Objective 2: Soil threshold levels for direct damage

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 13. If 100% of variation is accounted for this represents a perfect fit between target and actual nematode populations.

Table 13. 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)

Nematode group	Regression line equation		Probability		% variation accounted for	
	At start	At harvest	At start	At harvest	At start	At harvest
Root lesion	y = 0.3x + 25.9	y = 0.3x + 1	<0.001	<0.001	60.1	35.3

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 much lower at harvest than at the start of the experiment. In general the actual population was

approximately one third that of the target population. Despite actual populations being lower than the target population the nematode counts ranged from approximately zero to 1,200 root lesion nematodes/L soil. This range is from well below to well 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 86% of the initial population, suggesting that there was minimal change in numbers throughout the study.

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 infestation.

There was no clear significant relationship between the initial nematode population and foliar, crown, root and total dry weight. This result suggests that nematodes had limited, if any, impact on strawberry growth. Examination of the root systems before oven drying showed no clear differences in growth between pots. A high level of *Verticillium dahliae* (15.6 propagules/g soil) was recorded in soil containing the high populations of root lesion nematode soil but this did not appear to have any effect on crop growth over the period of the experiment.

However, a limited subset of the data in which plants had similar fresh weights at planting was re-analysed to determine if variability in plant fresh weight was masking any impact of nematode feeding on strawberry growth. This showed that there was a significant linear correlation between root dry weight and numbers of root lesion nematodes at harvest. The equation of the fitted line was root dry weight = 1.02 - 0.000523 x root lesion nematode number at harvest. The slope of the line was negative, suggesting that root dry weight decreased with increasing nematode number. The slope was significantly different from zero ($P=0.036$).

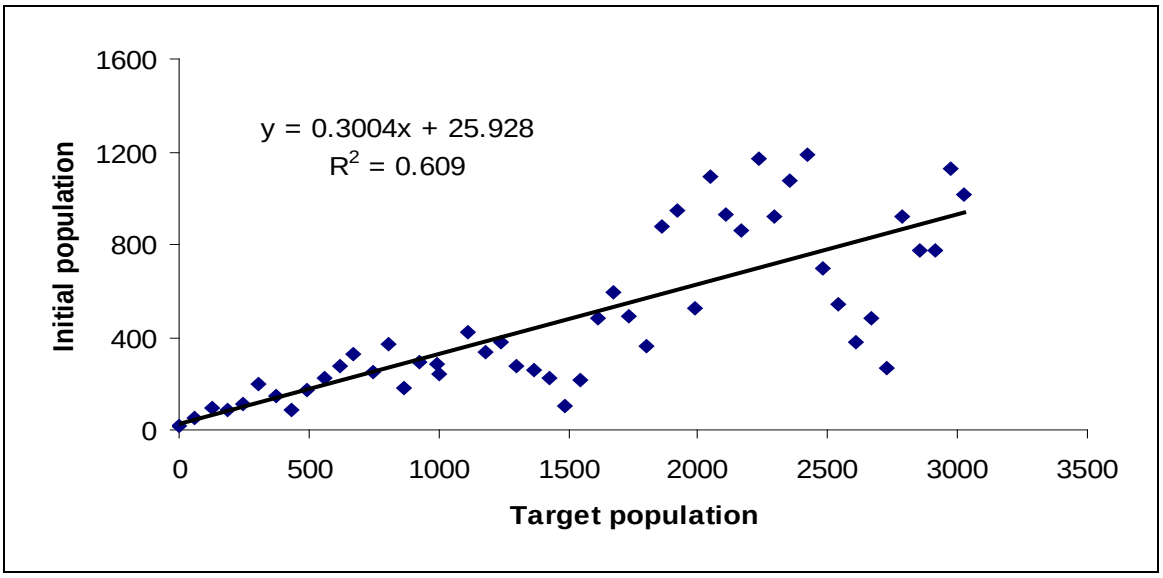

Figure 1. Initial created nematode populations against target populations

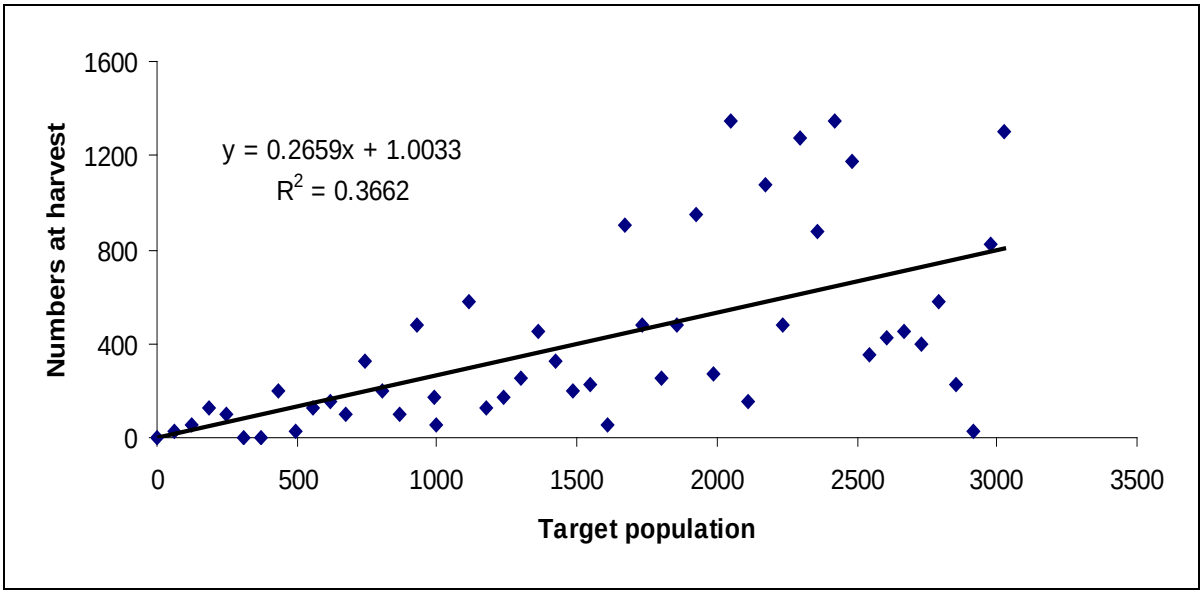

Figure 2. Nematode numbers at harvest against target populations

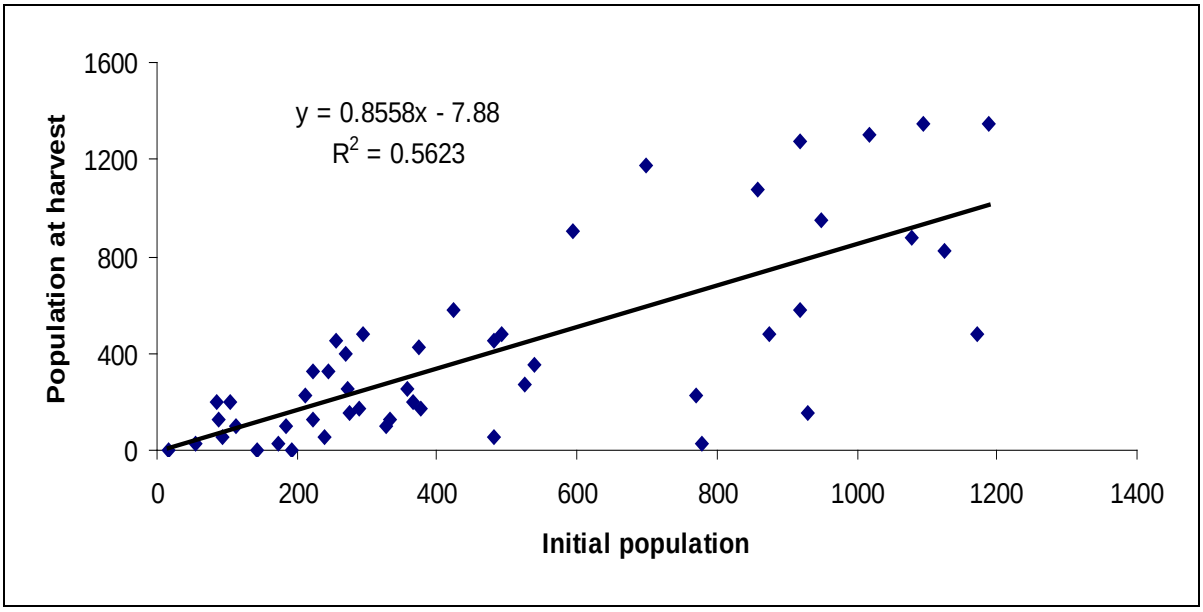

Figure 3. Graph of nematode numbers at harvest against initial created populations.

Objective 3: Identification of nematodes by molecular methods

The results from the comparison between the visual identification and SSU sequence identification (barcoding) are given in Table 14. Of the five isolates tested, there was complete agreement at the genus level when the results from the visual identification and identification obtained by barcoding were compared. Two out of three isolates, where classification to species level was possible by visual identification, agreed with the identification obtained by barcoding.

Table 14. Comparison between visual identification and barcoding identification from DNA extracts using SSU sequence analysis; results shown are based on closest similarity using the NCBI Blast database.

Visual identification	Closest barcoding identification to genus level	Top three barcoding identification to species level	Maximin Blast identification
Rotylenchus buxophilus	Rotylenchus	*R. goodeyi*	97%
		R. robustus	95%
		R. uniformis	95%
Bitylenchus dubius	*Tylenchorus*	*T. dubius*	*99%*
	(*Bitylenchus)*	*B. dubius*	*98%*
		T. maxiumus	*97%*
Paratylenchus sp.	*Paratylenchus.*	*Para. dianthus*	*99%*
		Para. microdorus	*96%*
		Para. staeleni	*96%*
Paratylenchus sp.	*Paratylenchus*	*Para. dianthus*	*99%*
		Para. microdorus	*97%*
		Para. staeleni	*96%*
Pratylenchus thornei	*Pratylenchus*	*P. thornei*	*99%*
		Zygotelenchus quevarae	*94%*
		T. maxiumus	*94%*

Sequencing data obtained from three common pathogenic nematodes (*Pratylenchus* sp., *Xiphinema* sp. and *Rotylenchus* sp.) were examined using five target barcoding regions (LSU 1/2, LSU 2/3, SSU, Cox1, ITS) (Table 15). The results indicate that the LSU and SSU regions are able to amplify the target nematode DNA.

Table 15. Success of barcoding primers pairs across three genera of nematodes

Target	*Pratylenchus*.	*Xiphinema*	*Rotylenchus*
SSU (18S) rDNA	yes	yes	yes
LSU rDNA (28S) D2/D3 region	yes	yes	yes
rDNA ITS region	yes	no	yes
LSU rDNA (28S) D1/D2 region	yes	yes	yes
Cox (I3-M11 partition)	no	yes	yes

Discussion

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

Between January 2001 and January 2011 a total of 92 soil samples were examined by ADAS PES to determine levels of free-living nematodes. This is approximately nine samples per year. This is significantly lower than for crops such as carrots and parsnips for which 1,500 to 2,000 samples are processed per year. Strawberries and raspberries were the most frequently sampled crops, accounting for 91% of samples processed. Of those nematode groups potentially damaging to soft fruit, root lesion nematodes were the most common, followed by needle nematodes, stubby root nematodes and dagger nematodes.

Data confirmed the value of thresholds as a means of deciding on the need for nematicide treatment as the soils differed greatly in the numbers and species of nematodes present. Thresholds for free-living nematodes are at best anecdotal but showed that less than a third of crops would have required chemical control to combat direct feeding damage. There is potential for virus transmission if any nematode vectors are present but again, soil sampling showed that only just over half of samples would have required nematicide treatment. Thresholds often tend to be conservative and even where there is a chance of virus transmission it is possible that very low counts of nematodes still pose minimal risk. Therefore it is possible that even less sites would have benefited from nematicides. Clearly soil sampling to determine nematode risk is an important component in developing integrated control strategies for these pests and worthy of further investigation.

Results of soil sampling from current strawberry/raspberry crops confirmed the importance of assessing nematode levels as a means of determining the likely risk of damage. Pest numbers in strawberry fields were generally lower than in raspberry fields. Examination of

the range of nematode species suggested that *Xiphinema diversicaudatum* was the most important dagger nematode, whereas a range of root lesion nematodes were recovered, together with three species of stubby root nematode and two of needle nematodes. These results should be treated with some caution in view of the low number of sites sampled.

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, which would have been very difficult to achieve using field sites. The actual population was approximately one third of the target population. 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 approximately zero to 1,200 root lesion nematodes/L soil. This range is from well below to well 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.

It is interesting that root lesion nematodes did not have any significant 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 irrigated and it is possible that this helped them to tolerate the impact of nematode feeding. Limiting the irrigation 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*. If the soil contained a relatively low proportion of *P. penetrans* compared with other *Pratylenchus* species, then one might expect less damage. The identity and relative proportions of different *Pratylenchus* species were not determined, as currently this is not normal practice 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. This suggests 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 if there is sufficient resource, repeat studies will be undertaken in year two of the project to confirm the results from year one and to test some higher populations.

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 change in fresh weight to be related to nematode numbers. This was not possible as dry weight was assessed. However, 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. However, re-analysis of a subset of data in which plants had a similar fresh weight at planting did show a significant correlation between root dry weight and nematode numbers at harvest. This was a slight negative relationship, suggesting that root dry weight did decrease with increasing numbers of root lesion nematodes and is worthy of further investigation. .

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

There was good agreement between visual identification and barcoding using the SSU region of nematode genome in the five isolates tested, at least to genus level. There was only one disagreement whereby *Rotylenchyus buxophilus*, identified visually, had been ascribed to *R. goodeyi* by SSU barcoding. More work needs to be carried out to test all single-isolate cultures to determine whether barcoding continues to provide good identification of all the pathogenic free-living nematodes extracted. Also, in addition to barcoding based on the SSU region, work will continue during the remaining time in the project to investigate how the addition of further barcoding regions improves the identification of target nematodes. Work to date suggests that barcoding the LSU and SSWU regions together might offer a robust identification tool.

Conclusions

- Examination of historical sampling data suggests that soil extraction and assessment of nematodes is a useful means of differentiating soils with regard to nematode risks.
- Results suggest that a reduction can be made on nematicide use, even though current thresholds are at best anecdotal.
- 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.
- Stubby root nematodes, root lesion nematodes and dagger nematodes, all of which can potentially damage fruit, were also recovered from soil samples.
- *Xiphinema diversicaudatum*, a dagger nematode, was the only species of this group recovered from strawberry/raspberry crops. A range of species were found for the other nematode groups potentially damaging to fruit.
- 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* species) 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.
- Molecular barcoding technology using the SSU region of nematode DNA was successful in identifying four genera of pathogenic free-living nematodes.

Knowledge and Technology Transfer

Article

O'Neill TM, Ellis S & Peters J (2012). Assessing nematode risk to soft fruit. *HDC News* (in press).

Glossary

Seinhorst two-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).

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).

Barcoding

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

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Appendix 1 – Crop diary

Date	Task	Staff responsible
14.7.2011	Soil collected from field from site with high populations of root lesion nematodes and stored in walk-in fridge	SA
21.7.2011	Strawberry plants delivered (cv Elsanta). Fifty plants potted up in 50 different nematode populations and maintained in a poly-tunnel.	DL
22.7.2011	Spare soil from each population extracted using the Seinhorst two-flask technique	DL & JN
26-27.7.2011	Extracted soil samples examined microscopically and numbers of nematodes counted	SB
21.7–21.11.2011	Plants maintained in a poly-tunnel and watered as necessary	JS
21.11.2011	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
6.12.2011	Soil extracted using Seinhorst two-flask technique	DL & JN
16-18.12.2011	Extracted soil samples examined microscopically and numbers of nematodes counted	HM
15.2.2012	Statistical analysis of experimental data	CD