Project title	Molecular methods for detection of stem nematode (*Ditylenchus dipsaci*) in soil and predicting risk of damage to onions and leeks
Project number:	FV 415
Project leader:	Dr Steve Ellis, ADAS, High Mowthorpe, Duggleby, Malton, North Yorkshire YO17 8BP
Report:	Annual report, March 2014
Previous report:	None
Key staff:	Steve Ellis, ADAS High Mowthorpe Shaun Buck, ADAS High Mowthorpe David Norman, Precision Agronomy Renske Landeweert, ClearDetections
Location of project:	ADAS High Mowthorpe and ClearDetections, Wageningen, The Netherlands
Industry Representative:	Robert Brown, E C Brown Farms
Date project commenced:	1 April 2013
Date project completed (or expected completion date):	31 March 2015

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.

Steve Ellis

Entomologist

ADAS

Signature..  .....Date 31.3.14.....

Report authorised by:

Peter Bassett

Entomolgy Segment Head

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GROWER SUMMARY

Headline

The PCR analysis developed by ClearDetections is effective at detecting stem nematode either on its own or in the presence of other free-living nematode species from a limited range of UK soils.

Background

Stem nematode (*Ditylenchus dipsaci*) is potentially a very destructive pest of bulb onions and leeks. Quantifying soil infestation prior to drilling is recommended as a tool to determine the suitability of land for growing onions or leeks. In general, if stem nematode is present at moderate or high levels the land is rejected as a site for a following onion or leek crop. At low levels the onion crop is sometimes grown but treated with a nematicide. However, a lack of confidence in the ability of some laboratories to identify stem nematode means that fields may be unnecessarily rejected or treated. HDC Project FV 327 identified the optimum sampling scheme and soil extraction method to give the best chance of detecting stem nematode in soil. However, identification of stem nematode by microscopy is very difficult and there are few nematologists in the UK who are confident of doing this. There are a number of *Ditylenchus* species in soil and it is important that these can be differentiated to prevent unnecessary use of nematicides or rejection of land wrongly identified as being unsuitable for onions or leeks.

As presence or absence of stem nematode is usually considered sufficient to predict the risk of pest attack it is ideally suited to PCR analysis. This has the advantage of being rapid and does not rely on a limited number of individuals with the necessary nematological expertise. A PCR assay for stem nematode has been developed by a Dutch based company (ClearDetections, a recent start up) and ADAS is already working with this group to determine whether the technique is capable of detecting a UK isolate of stem nematode either in isolation or, more practically, in extracts containing a range of nematode species. Preliminary studies with ClearDetections investigated if the stem nematode PCR was able to detect a single stem nematode, a single stem nematode amongst other nematode species and also if the other nematode species produced any false positive results in the absence of stem nematode. There were five replicates of each treatment. The results were very promising and showed that the test was able to detect a single stem nematode in 100% of cases. In view of the success of these preliminary tests there is potential for an

HDC project to validate the technique from a range of sites across the UK onion and leek growing areas.

In future, under the Sustainable Use Directive (SUD), protecting crops from free-living nematode damage will become increasingly reliant on integrated strategies that combine cultural and chemical control. Robust risk assessment in which growers can be confident will be fundamental to the success of such IPM programmes.

The overall aim of the project is to validate a PCR technique for detection of stem nematode (*Ditylenchus dipsaci*) in soil as a basis for predicting risk of damage to onions and leeks. Specific project objectives are as listed below:

1. To validate the effectiveness and specificity of qualitative PCR analysis in detecting stem nematode in extracts of free-living nematodes from UK soil samples.
2. To determine the effects of sample pre-treatment and DNA extraction on the PCR analysis for detecting stem nematode in a range of soil types from different locations throughout the UK.
3. To investigate the potential of PCR analysis to distinguish between UK populations of the oat-onion race and giant bean race of stem nematode.
4. To communicate project results to deadline via annual and final project reports, an article in HDC News and dissemination of the sampling protocol.

Summary

Year 1 of the project concentrated on Objective 1. Onion plants showing symptoms of stem nematode infestation were collected from the field and extracted by cutting them open and immersion in water for 24 hours. The identity of the nematodes was confirmed by microscopy by ADAS.

The PCR analysis was undertaken by ClearDetections in the Netherlands. The PCR tests have been developed for routine use on DNA extracts originating from nematode suspensions and utilise a SYBRGreen based detection system for ‘real time’ visualisation of PCR product.

A total of 50 Eppendorf tubes, each containing a mix of free-living nematode (FLN) species (*Trichodorus* spp., *Tylenchorhynchus* spp., *Pratylenchus* spp. *Globodera* spp (juveniles) Heerodera spp (juveniles)) but no stem nematode, a single tube containing stem nematodes extracted from plant material, and six tubes with FLN from typical English onion soils were transported from ADAS to ClearDetections.

At ClearDetections single stem nematodes were manually extracted from the sample of stem nematodes using a mounted eye lash. A single stem nematode was added to 25 of the 50 tubes with a mix of FLN.

Nematode suspensions from certain soil types, especially those with a high organic matter content, may result in high levels of PCR inhibiting substances in the final nematode DNA extracts. These inhibitory substances need to be removed before PCR. To establish whether the ClearDetections nematode DNA extraction and purification kit is suitable for removing these substances from samples originating from English soil types, nematode suspensions from a typical English onion soil (sandy loam) were spiked with four stem nematodes (of Dutch origin) and nematode DNA was extracted and purified according to the standard protocol.

In total the following 81 nematode samples were analysed:

- 25 tubes with a single stem nematode
- 25 tubes with a single stem nematode among other FLN species
- 25 tubes with other FLN species and no stem nematode
- Six tubes with FLN from a typical English onion soil spiked with four stem nematodes

In 55 out of the 56 samples (98.2%) containing stem nematodes the pest was detected (positive result) either on its own or in combination with other free-living nematode species. All 25 free living nematode samples without a stem nematode were found to be negative.

Results to date suggest that the PCR analysis developed by ClearDetections is effective at detecting stem nematode either on its own or in the presence of other free-living nematode species from a limited range of UK soils.

Where no stem nematode was present the analysis always produced a negative result and did not result in any false positives.

Financial Benefits

A validated PCR assay for stem nematode will provide the industry with a rapid, standardised and validated method of assessing the risk of nematode damage to leeks and onions. In addition, a PCR assay has the potential to provide a more reliable and cost-effective risk assessment than current labour-intensive microscope examination which is heavily reliant on a restricted number of skilled nematologists who are able to identify the pest with confidence and consistency.

Action Points

As this is only the first year of the project there are no action points for growers to date. In Year 2 of the project, work will continue to validate the PCR analysis over a wider range of soil types and also to determine if it is capable of differentiating between the oat onion and giant bean race of stem nematode.

SCIENCE SECTION

Introduction

Stem nematode (*Ditylenchus dipsaci*) is potentially a very destructive pest of bulb onions and leeks. Quantifying soil infestation prior to drilling is recommended as a tool to determine the suitability of land for growing onions or leeks. In general, if stem nematode is present at moderate or high levels the land is rejected as a site for a following onion or leek crop. At low levels the onion crop is sometimes grown but treated with a nematicide. However, a lack of confidence in the ability of some laboratories to identify stem nematode means that fields may be unnecessarily rejected or treated. HDC Project FV 327 identified the optimum sampling scheme and soil extraction method to give the best chance of detecting stem nematode in soil. However, identification of stem nematode by microscopy is very difficult and there are few nematologists in the UK who are confident of doing this. There are a number of *Ditylenchus* species in soil and it is important that these can be differentiated to prevent unnecessary use of nematicides or rejection of land wrongly identified as being unsuitable for onions or leeks.

As presence or absence of stem nematode is usually considered sufficient to predict the risk of pest attack it is ideally suited to PCR analysis. This has the advantage of being rapid and does not rely on a limited number of individuals with the necessary nematological expertise. A PCR assay for the detection of stem nematode has been developed by a Dutch based company (ClearDetections) and ADAS is already working with this group to determine whether the technique is capable of detecting a UK isolate of stem nematode either in isolation or, more practically, in extracts containing a range of nematode species. Preliminary studies with ClearDetections investigated if the stem nematode PCR was able to detect a single stem nematode, a single stem nematode amongst other nematode species and also if the other nematode species produced any false positive results in the absence of stem nematode. There were five replicates of each treatment. Results showed that test was able to detect a single stem nematode in 100% of cases and a single stem nematode among other free-living species in 80% of cases. In the one test where no stem nematode was detected among other nematode species it is suspected that the stem nematode was not successfully transferred to the test equipment rather than any problem with the accuracy of the analysis. In view of the success of these preliminary tests there is potential for an HDC project to validate the technique from a range of sites across the UK onion and leek growing areas.

In future, under the Sustainable Use Directive (SUD), protecting crops from free-living nematode damage will become increasingly reliant on integrated strategies that combine cultural and chemical control. Robust risk assessment in which growers can be confident will be fundamental to the success of such IPM programmes.

The overall aim of the project is to validate a PCR technique for detection of stem nematode (*Ditylenchus dipsaci*) in soil as a basis for predicting risk of damage to onions and leeks. Specific project objectives are as listed below:

1. To validate the effectiveness and specificity of qualitative PCR analysis in detecting stem nematode in extracts of free-living nematodes from UK soil samples.
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3. To investigate the potential of PCR analysis to distinguish between UK populations of the oat-onion race and giant bean race of stem nematode.
4. To communicate project results to deadline via annual and final project reports, an article in HDC News and dissemination of the sampling protocol.

Materials and methods

Preliminary studies

Before starting the current project some preliminary analyses were done with ClearDetections to get an indication of whether their PCR test for stem nematode was effective with nematodes extracted in the UK. Onion plants infested with stem nematode were cut open at ADAS High Mowthorpe and immersed in water to allow the nematodes to escape. The nematodes were confirmed as being stem nematode by microscopy. These nematodes were then used to create three experimental treatments as listed below:

1. A single stem nematode
2. A single stem nematode in a background of non-related free-living nematodes
3. Non-related free-living nematodes

There were five replicates of each treatment and each was created in water in a single snap top Eppendorf tube. The stem nematodes were fished from a sample of the pests using a mounted eyelash. The non-related free-living nematodes were taken from soil samples which had been extracted to determine their suitability for cropping with carrots and did not

contain stem nematode. The batch of fifteen tubes was dispatched to ClearDetections and subject to their PCR analysis.

Objective 1. Validating the effectiveness and specificity of PCR for stem nematode (Year 1)

Onion plants showing symptoms of stem nematode infestation were collected from the field by Precision Agronomy. These plants were processed at ADAS High Mowthorpe by cutting open the infested material and immersion in water in a Baermann funnel. The extracted nematodes were collected after 24 hours. The identity of the nematodes was confirmed by microscopy.

It was also intended to extract stem nematode samples from soil to complement those extracted from plants. Extraction from soil is necessary as it is possible that soil will contain substances that inhibit the PCR analysis. Unfortunately prior to the samples being transported, an audit of ClearDetections by the Netherlands Plant Health revealed that it was necessary to obtain documentation endorsed by both Netherlands and UK Plant Health to accompany the samples in transit. This caused an unavoidable delay so that at the time of writing this report the samples using nematodes from soil have not yet been dispatched to ClearDetections. However, the approval documentation has now been obtained and endorsed by both the Netherlands and UK Plant Health so the work will proceed as soon as possible.

The PCR analysis was undertaken by ClearDetections. The PCR tests have been developed for routine use on DNA extracts originating from nematode suspensions (containing DNA of approximately 10,000 individual unknown nematodes). The real-time PCR tests use a SYBRGreen based detection and enable the user to monitor the amplification of the nematode PCR product without the requirement for analysis on agarose gels, and can allow the further development of effective quantitative PCR based assays. The specificity of these tests is demonstrated by routine analysis of both the cycle threshold (Ct) value and the melting temperature (Tm) of the PCR products detected. The standard operating procedure of the SYBRGreen based test prescribes analysing the Ct value and Tm of any qPCR product formed and a test result can only be positive if these are found to be within the assay parameters. This confirmatory analysis of the amplified product is especially important when the test is performed on DNA extracts with unknown contents, which is often the case when testing soil samples.

Sample preparation

A total of 50 Eppendorf tubes, each containing a mixture of free-living nematode (FLN) species (*Trichodorus* spp., *Tylenchorhynchus* spp., *Pratylenchus* spp. *Globodera* spp (juveniles) Heerodera spp (juveniles) but no stem nematode, a single tube containing a sample of stem nematodes extracted from plant material, and six tubes with FLN from a typical English onion soil (sandy loam) were transported from ADAS to ClearDetections.

At ClearDetections single stem nematodes were manually extracted from the supply of stem nematodes using a mounted eye lash. A single stem nematode was added to each of 25 of the 50 tubes with a mixture of FLN.

Nematode suspensions from certain soil types, especially those with a high organic matter content, may result in high levels of PCR inhibiting substances in the final nematode DNA extracts. These inhibitory substances need to be removed before PCR. To establish whether the ClearDetections nematode DNA extraction and purification kit is suitable for removing these substances from samples originating from English soil types, nematode suspensions from a typical English onion soil were spiked with four stem nematodes (of Dutch origin) and nematode DNA was extracted and purified according to the standard protocol.

In total the following 81 nematode samples were analysed:

- 25 tubes with a single stem nematode
- 25 tubes with a single stem nematode among other FLN species
- 25 tubes with other FLN species and no stem nematode
- Six tubes with FLN from a typical English onion soil spiked with four stem nematodes

DNA extraction & purification

From the 25 tubes with a single stem nematode DNA was extracted using a ClearDetections nematode DNA extraction kit for single nematodes and cysts. The DNA extract obtained from these single nematode samples was not purified. From all other nematode samples DNA was extracted with the ClearDetections nematode DNA extraction and purification kit. From these samples part of the extracted nematode DNA was purified over a glass filter plate to remove potential PCR inhibiting substances from soil.

Real-Time PCR analysis

The presence of stem nematode DNA was analyzed with the ClearDetections *D. dipsaci* Real-Time PCR detection and identification kit. For PCR application 5 µl of 5x diluted template was mixed with 2 µl species-specific primers (end concentrations for both primers 250 nM), 3 µl PCR enhancer (ClearDetections), and 10 µl qPCR SYBR Green mix (ClearDetections) in a total reaction volume of 20 µl. Thermal cycling was performed on a Bio-Rad CFX Connect thermal cycler (Bio-Rad, Hercules, CA) and consisted of 95^{o}C for 3 min; followed by 40 cycles of 95^{o}C for 10 sec, 63^{o}C for 1 min and 72^{o}C for 30 seconds.

Objective 2. Validating the sensitivity of PCR analysis in detecting stem nematode from a range of soil types (Years 1 & 2)

This objective addressed stem nematodes isolated from different soil types and locations across the UK. Soil samples from these locations may amplify differently due to possible soil matrix effects dictated by the local soil composition. Different nematode suspensions isolated from soil may have pronounced effects on the PCR efficiency as components of the soil samples co-purifying with the nematode DNA may be inhibitory to the PCR reaction (sample matrix effects). Every effort will be made to sample sites from a full range of UK soil types, e.g., clay, silt, loam, sand, organic. As alliums may not necessarily be grown in all soil types examples of typical soils will be collected and inoculated with stem nematode collected from infested plants. These soils will be extracted and submitted for analysis to collect more data on the potential influence of the soil matrix on PCR test. Variation in PCR Ct value against a standard curve would be indicative of sample-to-sample variation due to matrix effects of the soil extract and would need to be taken into consideration when interpreting any nematode PCR results.

The collection of these samples has also been delayed by the need for Plant Health documentation as described in Objective 1. Now this is available the work will proceed as soon as possible and it is not envisaged that the delivery of the final report for this work will be affected.

Objective 3. To investigate the potential of PCR analysis to distinguish between oat-onion and giant bean races of stem nematode (Years 1 & 2)

In the UK both the oat-onion and giant bean race of stem nematode are present in soil. The giant bean race is not a threat to onions and so it is important to be able to distinguish races so that land is not wrongly rejected as being unfit for onions and leeks. Both races of stem nematode will be extracted from soil or from plant material using the methods described at Objective 1, and the specificity of the PCR will be evaluated. To date it has not been

possible to locate a sample of the giant bean race of stem nematode but ADAS is liaising with the Pulse Growers Research Organisation to obtain bean samples infested with this pest. These can then be extracted and samples of both races of stem nematode sent to ClearDetections.

Results

Preliminary studies

The results of the PCR analyses on the preliminary samples are given in table 1.

Table 1. PCR threshold values (Cq) for nematode samples containing free- living nematodes (FLN) and/or stem nematodes (*D. dipsaci*). Blank is molecular grade water (H_2O). Positive control is plasmid DNA containing part of the stem nematode ribosomal (SSU) DNA.

Sample number	Sample reference	PCR threshold cycle (cq)
1A	Single stem nematode	28.61
1B	Single stem nematode	29.09
1C	Single stem nematode	28.38
1D	Single stem nematode	29.24
1E	Single stem nematode	28.68
2A	Single stem nematode + non-related FLN	29.43
2B	Single stem nematode + non-related FLN	29.32
2C	Single stem nematode + non-related FLN	28.88
2D	Single stem nematode + non-related FLN	29.26
2E	Single stem nematode + non-related FLN	N/A
3A	Non-related FLN	N/A
3B	Non-related FLN	N/A
3C	Non-related FLN	N/A
3D	Non-related FLN	N/A
3E	Non-related FLN	N/A
Positive control	*D. dipsaci* DNA	23.19
NTC	No template control (H_2O)	N/A

In 11 out of 12 cases where stem nematode was present (91.6%) it was detected by the PCR analysis. No stem nematode were detected in those tubes that only contained the non-related free-living nematodes.

Objective 1. Validating the effectiveness and specificity of PCR for stem nematode (Year 1)

In 55 out of the 56 samples (98.2%) containing stem nematodes the pest was detected (positive result) either on its own or in combination with other free-living nematode species. In all cases the Cq values were far below the cut off value of Cq > 35 (Table 2). No stem nematode was detected in sample no. 7 (FLN + single stem nematode). All 25 free living nematode samples without a stem nematode were found to be negative (Table 2).

The almost parallel amplification curve slopes (grey slopes, Figure 1A), and the identical Ct values (± 1 Ct) of the stem nematode PCR indicates there is no substantial PCR inhibition in the DNA extracts from English onion soil nematode suspensions.

Table 2. Cq values and melting temperatures (Tm) for nematode samples containing free-living nematodes (FLN) and/or stem nematodes (*D. dipsaci*). Blank is 10 mM TE pH 8.0. Positive control is plasmid DNA containing part of the stem nematode ribosomal (SSU) DNA.

	Single stem nematode		FLN only		FLN + single stem nematode		FLN from onion soil + 4 stem nematodes	
Sample no	Cq	Tm	Cq	Tm	Cq	Tm	Cq	Tm
1	25.53	85.5	N/D	None	29.95	85.5	26.93	85.5
2	26.16	85.5	N/D	None	31.58	85.5	26.77	85.5
3	25.61	85.5	N/D	None	29.03	85.5	26.62	85.5
4	25.09	85.5	N/D	None	28.55	85.5	26.30	85.5
5	25.01	85.5	N/D	None	29.06	85.5	26.90	85.5
6	24.50	85.5	N/D	None	29.51	85.5	26.75	85.5
7	25.13	85.5	N/D	None	N/D	None		
8	25.04	85.5	N/D	None	28.83	85.5		
9	25.48	85.5	N/D	None	29.08	85.5		
10	24.83	85.5	N/D	None	29.73	85.5		
11	25.14	85.5	N/D	None	29.63	85.5		
12	25.01	85.5	N/D	None	29.06	85.5		
13	25.09	85.5	N/D	None	29.41	85.5		
14	25.79	85.5	N/D	None	29.07	85.5		
15	25.01	85.5	N/D	None	29.60	85.5		
16	25.23	85.5	N/D	None	30.28	85.5		
17	25.84	85.5	N/D	None	29.22	85.5		
18	27.02	85.5	N/D	None	29.31	85.5		
19	25.34	85.5	N/D	None	28.56	85.5		
20	25.66	85.5	N/D	None	28.74	85.5		
21	24.26	85.5	N/D	None	29.32	85.5		
22	25.44	85.5	N/D	None	28.79	85.5		
23	25.24	85.5	N/D	None	29.83	85.5		
24	25.78	85.5	N/D	None	30.01	85.5		
25	25.03	85.5	N/D	None	30.24	85.5		
blank							N/D	None
Pos. ctrl.							21.92	85.5

Pos. ctrl = Positive control

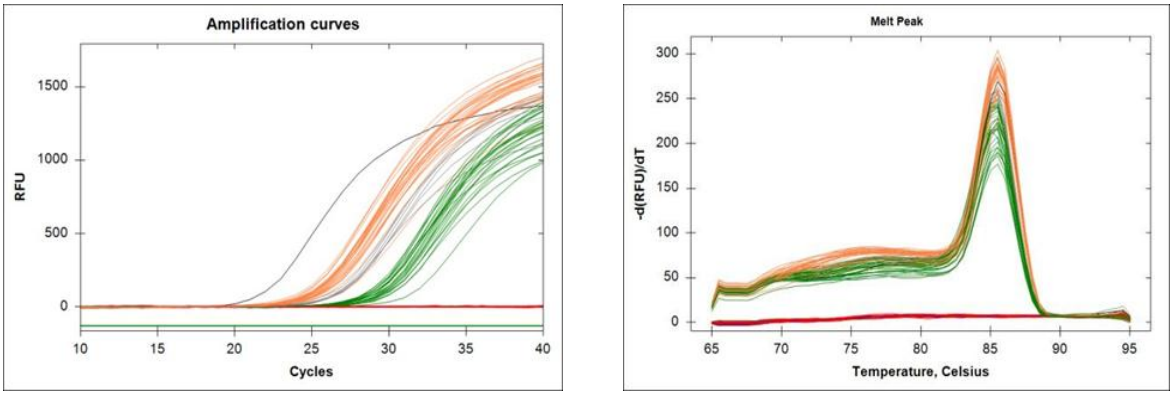

Figure 1. Amplification curves (left) and melting peaks (right) of *D. dipsaci* Real-Time PCR. Single stem nematode (Orange), FLN only (Red), FLN + single stem nematode (green), FLN from onion soil + 4 stem nematodes (grey), Positive control (black)

Discussion

The results of analyses of samples (preliminary samples and those for Objective 1) with and without stem nematode extracted from infested onion plants confirm that the ClearDetections *D. dipsaci* Real-Time PCR assay is able to detect single stem nematodes originating from England, even in suspensions of multiple other free living nematode species. Equally important, where stem nematode were not present the PCR analysis always produced a negative result suggesting that fields would not be mistakenly rejected by false positive results indicating the presence of stem nematode.

In only one sample in the preliminary studies and in those for Objective 1 was the presence of stem nematode not detected. It seems most likely that the nematode was lost during its transfer to the Eppendorf tube prior to analysis, which would not have been obvious to the human eye. Such a mistake is always possible with such a small organism and is very difficult to prevent. Under routine testing protocols, PCR analysis would not normally involve transfer of nematodes between sample tubes; and that this was only done in this project to create replicates of each nematode treatment for the experimental analysis. In practice a nematode sample will be treated to extract its DNA and any transfer of material between tubes will be minimised.

No substantial PCR inhibition was found in the DNA extracts from an English onion soil (clay loam) nematode suspensions. Therefore it can be concluded that the nematode suspensions as produced by ADAS from this soil type are suitable for DNA extraction and molecular detection of stem nematodes, using the protocols of ClearDetections. Future work will need to test a range of soil types that can be found in the UK to ensure that none of these inhibit the PCR analysis. This will be part of the work programme for year 2 of this project.

Conclusions

- Results to date suggest that the PCR analysis developed by ClearDetections is effective at detecting stem nematode either on its own or in the presence of other free-living nematode species from a limited range of UK soils.
- Where no stem nematodes were present the analysis always produced a negative result and did not record any false positives.

Knowledge and Technology Transfer

The project was discussed at the EMRA/HDC Soft fruit day at East Malling Research, Kent 21 November 2013 as a potential method for future proofing nematode diagnostics.

Glossary

Amplification curve – graphic depiction of the accumulation of PCR product during PCR cycling.

Baermann funnel – An extraction method designed to extract nematodes from soil or plants.

Cq value – Quantification cycle, PCR threshold value, point during PCR amplification where DNA product of PCR is being detected by PCR machine.

Eppendorf tube – Small plastic snap top tube (approx. 2.5cm long) used to transport nematode samples.

IPM – Integrated pest management, a control strategy which use non-chemical as well as chemical control options and designed to reduce reliance on chemicals.

PCR – Polymerase chain reaction, technology used to amplify a single or a few copies of a piece of DNA across several orders of magnitude, allows sensitive and specific detection of DNA

PCR enhancer – Reagents that increase the yield of the desired PCR product or alternatively neutralise soil derived PCR inhibiting substances.

Primers – Short single stranded DNA fragments which flank the target DNA sequence to be amplified and serve as a starting point for DNA synthesis during PCR

qPCR – Quantitative version of PCR designed to determine the numbers of particular organism present.

SYBRGreen - A fluorescent DNA dye that only binds with double stranded DNA, and whose fluorescence signal only increases on binding with DNA. Used in the PCR reaction as a method of detecting newly synthesised DNA.

