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
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Key staff:	Steve Ellis, ADAS High Mowthorpe Shaun Buck, ADAS High Mowthorpe Ben Maddison, ADAS Nottingham Helen Rees, ADAS Nottingham David Norman, Precision Agronomy Renske Landeweert, ClearDetections Carla Oplaat, ClearDetections Pawel Deka, ClearDetections Winfried Mulder, ClearDetections
Location of project:	ADAS High Mowthorpe, UK and ClearDetections, Wageningen, The Netherlands
Industry Representative:	Robert Brown, E C Brown Farms
Date project commenced:	01 April 2013
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The results and conclusions in this report are based on an investigation conducted over a two-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

Senior Research Consultant

ADAS

Signature  ..... Date 12.5.2015.....

Report authorised by:

Peter Bassett

Entomology Segment Head

ADAS

Signature  .....Date 12.5.2015.....

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GROWER SUMMARY

Headline

Real-time Polymerase Chain Reaction (PCR analysis) developed by ClearDetections is effective at detecting stem nematode either on its own or in the presence of other free-living nematodes and is effective in a wide range of UK soil types. There was good agreement between microscopy and PCR analysis for detection of stem nematode.

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. AHDB Horticulture 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.

With the declining availability of nematicides and with the imposition of the Sustainable Use Directive (SUD), protection of crops from free-living nematode damage in the future will become increasingly reliant on integrated pest management (IPM) 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. Detecting the presence of stem nematode is a crucial component of any risk assessment and is the main subject of this project.

As presence or absence of stem nematode is usually considered sufficient to predict the risk of pest attack it is ideally suited to Polymerase Chain Reaction (PCR) analysis. This has the advantage of being rapid and does not rely on morphological identification by a limited number of technicians 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 in this project ADAS has collaborated 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 whether the PCR analysis was able to detect 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. The results 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 the current project was commissioned to validate the PCR analysis from a range of sites across the UK onion and leek growing areas.

The overall aim of the project was 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 AHDB Grower 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 immersing 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 detection system for ‘real time’ visualisation of the 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) *Heterodera* 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 tube containing this pest 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 therefore needed to be removed before PCR testing. 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.

Work in year 2 concentrated on Objectives 2 and 3. Work on Objective 2 was specifically designed to investigate the potential for PCR analysis to be inhibited by substances found in nematode suspensions from different UK soil types and consisted of two experiments as below:

- Experiment 1. Validating PCR analysis for stem nematode extracted from different soil types.
- Experiment 2. Validating PCR analysis for stem nematode inoculated into representative soil types

Nematode suspensions isolated from different soil types 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). To test this in Experiment 1 nematode suspensions were extracted from a range of soil types using the Seinhorst two-

flask technique. These samples were examined by microscopy. Some of the samples contained stem nematode and some did not.

These samples were submitted to ClearDetections for PCR analysis. In total 170 samples were submitted in two batches for analysis (24 clay, 43 loam, 24 organic, 39 sand, 40 silt). Results showed a 99% agreement between the results of microscopy and PCR analysis.

To ensure that a full range of UK soil types was studied (alliums may not be grown in all UK soil types) it was also decided to inoculate known numbers of stem nematodes into nematode suspensions extracted from a range of different UK soil types before being submitted for PCR analysis (Experiment 2). The soil types selected were a sand, a silt, a clay, an organic soil and a loam. There were twenty five replicates of each soil type inoculated with stem nematodes (125 samples in total). Microscopy was used to confirm that none of the selected soils were infested with stem nematode.

The stem nematodes for inoculation were collected by extracting infested plant material. These plants were extracted by cutting open the infested material and immersing in water in a Baermann funnel. The extracted nematodes were collected after 24 hours and four were inoculated into each of the 25 replicate nematode suspension samples from each of the five soil types. The identity of the nematodes was confirmed by microscopy.

Across all soil types (clay, loam, organic, sand, silt) 122 out of 125 samples were positive for stem nematode (97.6%). There were three unexpected negative results; one from loam and two from sand soils. For these samples an additional Real-Time PCR was performed using a general nematode DNA assay. The results of this troubleshooting analysis confirmed the presence of nematode DNA and absence of *D.dipsaci*. The reason for this result is unclear but it is possible that the stem nematodes were lost during sample handling or transport; they may have been missed during the DNA extraction, they may have stuck to the lid of the tube or they might have been lost during the volume reduction before DNA extraction.

Objective 3 concentrated on determining whether the ClearDetections PCR analysis was capable of differentiating between *D. gigas* (giant bean race) and the more common oat-onion race. This was not possible primarily because *D. gigas* is very difficult to find in soil. In the UK ADAS has failed to detect this species in 10 years of data collected from soils from all over the UK and only recorded it in infested samples of field beans. This suggests that *D. gigas* poses a limited threat to onions and leeks in the UK. ADAS will continue to look for *D. gigas* and if it can be found, most probably following extraction of bean samples, material will be made available to ClearDetections with which to test their PCR analysis.

In conclusion the results of this project show that the PCR analysis developed by ClearDetections is effective at detecting stem nematode from a wide range of UK soil types either on its own or in the presence of other free-living nematode species. PCR analysis could become a vital component of an integrated pest management strategy for *D. dipsaci* to help growers assess the risk from the pest. Detection of stem nematode in soil in the UK is labour-intensive and dependent on a dwindling level of expertise able to identify the pest using traditional microscopic examination. Molecular assays provides an opportunity for a rapid, standardised and validated detection test for stem nematode for UK onion and leek growers.

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. The benefit of a molecular assay is that it can be run by any molecular technician, analysing 96 samples in one run. The main costs incurred are for the PCR machine, disposables and reagents. Microscopic examination of samples can only be performed by a skilled technician one sample at the time. Real cost benefits therefore depend mostly on the laboratory under consideration and its potential sample throughput. Industry representative Robert Brown (E. C. Brown Farms) commented that the project 'is taking the next step towards operating a more adaptable method of detection, which helps alleviate the requirements of trained nematode identifying expertise. Whilst still offering a high detection test that is reliable.'

Action Points

There is nothing that an individual grower can do immediately. The output of the research suggests that a commercial sampling and testing scheme could be established. The benefits are from gaining a more reliable and rapid test for stem nematode with less reliance on a dwindling group of nematologists. A reliable and cost effective predictive test is more likely to be used by growers to allow them to avoid nematode damage and subsequent crop loss. There would also be more confidence in growing the crops without nematicide treatment.

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. AHDB Horticulture 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.

With the declining availability of nematicides and with the imposition of the Sustainable Use Directive (SUD), protection of crops from free-living nematode damage in the future will become increasingly reliant on integrated pest management (IPM) 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. Detecting the presence of stem nematode is a crucial component of any risk assessment and is the main subject of this project.

As presence or absence of stem nematode is usually considered sufficient to predict the risk of pest attack it is ideally suited to Polymerase Chain Reaction (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 in this project ADAS has collaborated 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. Results showed that the 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. There were no false positive results in the absence of stem nematode. In view of the success of these preliminary tests the current project was commissioned to validate the PCR analysis from a range of sites across the UK onion and leek growing areas.

The overall aim of the project was 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 AHDB Grower and dissemination of the sampling protocol.

In year 1 of the project work concentrated on Objective 1. 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.

In the second year of the project further work was done to investigate 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 (Objective 2) and also to investigate the potential of PCR analysis to distinguish between UK populations of the oat-onion race and giant bean race of stem nematode (Objective 3).

Materials and methods

Objective 2. Validating the sensitivity of PCR analysis in detecting stem nematode from a range of soil types

This objective involved two separate experiments.

Experiment 1. Validating PCR analysis for stem nematode extracted from different soil types

Stem nematodes isolated from different soil types and locations across the UK may amplify differently due to possible soil matrix effects dictated by the local soil composition. Nematodes were isolated from soil using the Seinhorst two-flask technique which was shown to be most effective for stem nematode in FV 327 Onions: Improving risk assessment for stem nematode. The identity of the nematodes were confirmed by microscopy.

These samples were submitted to ClearDetections for PCR analysis. A number of samples from different locations were tested to validate PCR analysis and test matrix effects, under practical UK conditions. ADAS used industry contacts to select sites where stem nematode has caused crop damage and/or been detected in the past. Every effort was made to sample sites from a full range of UK soil types e.g. clay, silt, loam, sand, organic. The aim was to test 25 replicate samples from each soil type (125 samples in total). In practice this was not possible and the 125 nematode samples were collected from the range of soil types shown in Table 1. The number of samples from each soil type considered to be positive for stem nematode is also shown. The first batch of samples prepared (Batch 1) became

contaminated during transport with the result that only 45 out of 125 were suitable for PCR analysis. Consequently a second batch of samples was prepared (Batch 2) and these were all subjected to PCR analysis.

Table 1. Number of soil samples from a range of UK soil types submitted for PCR analysis and the number considered positive for stem nematode.

Soil type	**Number of samples submitted for PCR analysis**		**Number of samples +ve for stem nematode**	
	Batch 1	Batch 2	Batch 1	Batch 2
Clay	10	14	3	4
Loam	9	34	0	6
Organic	4	20	0	4
Sand	10	29	2	6
Silt	12	28	1	6
Total	45	125	6	26

Batches of nematode suspensions were judged to be infested with stem nematode following microscopic examination. These data were used to compare the relative precision of both the PCR analysis and microscopic examination.

In addition to samples collected specifically for the project, those submitted to ADAS Pest Evaluation Services were also used to increase the number examined from different soil types.

Preparation of samples for PCR analysis

The nematode suspensions were allowed to stand in the 50 ml sterilin tubes overnight. The next morning sufficient excess water was removed with a pipette to allow the remainder of the suspension to be transferred into a 15 ml tube. The suspension was then left undisturbed as long as possible at room temperature to allow the nematodes to sink to the bottom of the tube. The tubes were then centrifuged for 10 min at 500 g after which the supernatant was carefully removed with a pipette, to leave 0.5-2 ml in the bottom of the tube. This sample was then mixed by pipetting the liquid up and down three times and transferred to a siliconised 2 ml tube. A siliconised tube was used to prevent nematodes becoming stuck to the side of the tube. This tube was centrifuged for five minutes at 500 g and the supernatant removed leaving approximately 150 µl making sure not to disturb the nematodes at the bottom of the tube. The tubes were stored at 4°C before the next stage of the process.

Experiment 2. Validating PCR analysis for stem nematode inoculated into representative soil types

As alliums may not necessarily be grown in all soil types, examples of typical soils were collected and inoculated with stem nematode.

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). To ensure that a full range of UK soil types was studied (alliums may not be grown in all UK soil types) it was also decided to inoculate known numbers of stem nematodes into nematode suspensions extracted from a range of different UK soil types before being submitted for PCR analysis. The soil types selected were a sand, a silt, a clay, an organic soil and a loam. These soils were extracted and submitted for analysis to collect more data on the potential influence of the soil matrix on PCR test. There were twenty five replicates of each soil type inoculated with stem nematodes (125 samples in total). Microscopy was used to confirm that none of the selected soils were infested with stem nematode.

The stem nematodes used to inoculate the different soil types were collected by extracting infested plant material. These plants were extracted by cutting open the infested material and immersion in water in a Baermann funnel. The extracted nematodes were collected after 24 hours and four inoculated into each of the 25 replicate nematode suspension samples from each of the five soil types. The identity of the nematodes was confirmed by microscopy.

Preparation of samples for PCR analysis

The nematodes were collected in 50ml sterilin tubes. The suspension was left undisturbed overnight at room temperature to allow the nematodes sink to the bottom of the tube. The volume of the suspension was then reduced to approximately 1-1.5 ml using a pipette. During this stage it was important not to disturb the nematodes at the bottom of the tube. The reduced volume suspension was then mixed by pipetting it up and down three times and then transferred to a siliconised 2 ml tube. The suspension was then spiked by adding four stem nematodes to each tube and the tubes stored 4°C until the next stage of preparation.

DNA extraction and Real-Time PCR analysis

The Real-Time PCR analysis used was the same for both Experiment 1 and 2. Real-Time PCR analysis was undertaken by ClearDetections. The PCR tests have been developed for routine use on purified DNA extracts originating from nematode suspensions (containing

DNA of approximately 10,000 individual unknown nematodes). The real-time PCR tests use a fluorescent DNA binding dye based detection and enable the user to monitor the amplification of the nematode PCR product without the requirement for analysis on agarose gels. The specificity of these tests is demonstrated by routine analysis of both the cycle threshold (Cq) value and the melt temperature (Tm) of the PCR products detected. The standard operating procedure prescribes analysing the Cq value and Tm of any Real-Time PCR 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.

DNA extraction & purification

DNA extraction was performed using the DNA extraction and purification kit for nematode suspensions and multiple cysts (ClearDetections, The Netherlands) following manufacturers protocol. The nematode DNA was purified to remove any potential PCR inhibiting substances from soil. All samples were diluted before PCR analysis.

Real time PCR analysis

The presence of stem nematode DNA was analysed with the ClearDetections *D. dipsaci* Real-Time PCR detection and identification kit. Simultaneously, a DNA extraction control was performed using the external control available in the ClearDetections DNA extraction kit. For both PCR applications 5 µl of 80 or 160 fold diluted DNA was mixed with 2 µl of species-specific primers (end concentrations for both primers 250 nM), 3 µl PCR enhancer and 10 µl ClearDetections' PCR mix (all included in the ClearDetections kit) 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. In all Real-Time PCR assays a positive amplification control (PAC) and a negative amplification control (NAC) were included to monitor the PCR performance.

Objective 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

The 'giant race' of *Ditylenchus dipsaci* was first recorded in 1986 on broad beans (*Vicia faba*) in Algeria. In this population, adult nematodes were considerably larger than those commonly observed for *D. dipsaci*. This 'giant race' was then observed in other countries around the Mediterranean Basin. Symptoms caused by the 'giant race' on *V. faba* crops are generally more severe than those caused by other races of *D. dipsaci*, and more

infested seeds are produced. Several authors have suggested that *D. dipsaci* was a complex species (comprising at least seven species) and in particular, that the 'giant race' should be considered as a distinct taxon. On the basis of morphological, biochemical and molecular data obtained from several populations of *D. dipsaci* collected on *V. faba* from Southern Italy, Southern Spain and Lebanon, Vovlas *et al*. (2011) are now considering that the 'giant race' is a new and distinct nematode species and proposed to name it *Ditylenchus gigas*. The aim of Objective 3 was to try and determine if the ClearDetections primer set specific for *D. dipsaci* was able to distinguish *D. dipsaci* from *D. gigas.*

Results

Determination of DNA dilution factors

Nematodes extracted from soil can contain substances that can inhibit the PCR analysis. In order to minimise the effect of potential PCR inhibitors samples from each soil type were subjected to serial dilutions. Real time PCR analysis was then undertaken and the optimum dilution selected where inhibition was minimised whilst maintaining a good PCR signal. A two-fold decrease of the amount of target DNA should theoretically result in an approximately 1 Cq unit increase. Only slightly increasing (<0.5 Cq), constant, or even declining Cq values are an indication of high levels of PCR inhibiting compounds in the sample in which case a higher dilution factor should be selected. The first dilution factor at which the Cq values increases by approximately 1 Cq unit is the lowest dilution factor at which there is no PCR inhibition in all tested samples. This was the dilution factor selected for each particular batch of samples.

Determination of the DNA dilution factor for Experiment 1 for both batch 1 and batch 2 soil samples was based on a 20, 40, 80 and 160 fold dilution series of two samples per soil type. Results of the serial dilutions (Table 2, batch 2 samples only) suggested that for batch 1 soils a 40 fold dilution was suitable for clay, loam, sand and silt soils and a 160 fold dilution for organic soils. For batch 2 an 80 fold dilution was best for clay, loam, sand and silt soil and a 160 fold dilution for organic soil. The higher dilution for organic soil is indicative of the greater quantity of potential PCR inhibitors in nematode suspension extracted from this soil type.

There were two Real-Time PCR controls included in the analysis these were as below:

- Positive Amplification Control (PAC) – This is an indicator that the Real-Time PCR is functioning adequately. The Cq value of the PAC should be less than 25 (Cq < 25).

A PAC threshold larger than 25 Cq > 25) indicates that the assay is not performing as expected which could result in false negative samples. If this was the case all results were considered unreliable and the assays were repeated.

- Negative Amplification Control (NAC) - For this control the sample is replaced by DNA dilution buffer from the kit. The NAC must be negative (Cq < 35). The NAC has to be included in every PCR run to confirm the absence of DNA contamination which can result in false positive results. If the NAC produced a positive result (Cq > 35) all data were ignored and the test was repeated. Ideally all sources of contamination are removed before repeating the test.

Results from both the PAC and the NAC indicated that the Real-Time PCR was functioning as expected and that the reagents and samples were not unintentionally contaminated with target DNA.

Table 2. Quantification cycle (Cq) values and melt temperatures (Tm) for *Ditylenchus dipsaci* and external control DNA in a dilution series of two soil samples per soil type (batch 2 soils). (PAC = positive amplification control, NAC = negative amplification control, N/A = no amplification)

Soil type	Dilution factor	External control		*D. dipsaci*		Result
		Cq	Tm	Cq	Tm	
Clay 1	20	26.08	78	29.49	85.5	Positive
Clay 1	40	26.97	78	30.72	85.5	Positive
Clay 1	80	27.85	78	31.27	85.5	Positive
Clay 1	160	28.71	78	33.83	85.5	Positive
Clay 2	20	25.93	78	29.07	85.5	Positive
Clay 2	40	26.45	78	30.29	85.5	Positive
Clay 2	80	27.38	78	31.51	85.5	Positive
Clay 2	160	28.14	78	33.31	85.5	Positive
Organic 1	20	29.52	78	N/A	None	Negative
Organic 1	40	27.05	78	N/A	None	Negative
Organic 1	80	27.41	78	34.22	85	Positive
Organic 1	160	28.56	78	32.48	85.5	Positive
Organic 2	20	26.7	78	N/A	None	Negative
Organic 2	40	27	78	35.1	85.5	Negative
Organic 2	80	27.7	78	32.7	85.5	Positive
Organic 2	160	28.86	78	33.44	85.5	Positive
Loam 1	20	25.21	78	31.13	85.5	Positive
Loam 1	40	26.19	78	31.25	85.5	Positive
Loam 1	80	26.95	78	31.56	85.5	Positive
Loam 1	160	27.86	78	31.92	85.5	Positive
Loam 2	20	25.54	78	31.15	85	Positive
Loam 2	40	26.25	78	30.91	84.5	Positive
Loam 2	80	27.22	78	32.42	84.5	Positive
Loam 2	160	28.33	78	33.14	84.5	Positive
Sand 1	20	25.71	78	29.96	85	Positive
Sand 1	40	26.29	78	30.56	85	Positive
Sand 1	80	27.47	78	32.11	85	Positive
Sand 1	160	28.34	78	32.41	85	Positive
Sand 2	20	25.67	78	29.4	85	Positive
Sand 2	40	26.44	78	31.01	85	Positive
Sand 2	80	27.52	78	31.95	85	Positive
Sand 2	160	28.57	78	32.79	85	Positive
Silt 1	20	25.51	78	29.99	85	Positive
Silt 1	40	26.45	78	29.99	85	Positive
Silt 1	80	27.24	78	31.12	85	Positive
Silt 1	160	28.09	78	31.64	85	Positive
Silt 2	20	25.47	78	30.4	85	Positive
Silt 2	40	26.26	78	30.65	85	Positive
Silt 2	80	27.11	78	31.36	85	Positive
Silt 2	160	27.98	78	32.85	85	Positive
PAC	0	-	-	24.12	85.5	Positive
PAC	20	24.98	78	-	-	Positive
PAC	40	25.72	78	-	-	Positive
PAC	80	26.8	78	-	-	Positive
PAC	160	27.77	78	-	-	Positive
NAC		N/A	None	N/A	None	Negative

Objective 2. Validating the sensitivity of PCR analysis in detecting stem nematode from a range of soil types

Experiment 1. Validating PCR analysis for stem nematode extracted from different soil types

Results of the PCR analyses on the nematode suspensions from the five different field collected soil types are shown in Tables 3 – 8. Table 3 is for batch 1 samples and tables 4-8 for batch 2 samples. The final two columns headed 'Result' show how well the detection of stem nematode by PCR analysis agreed with that done by microscopy. There was 100% agreement between PCR analysis and microscopy for batch 1 samples. For batch 2 samples there was 100% agreement between the two diagnostic methods for clay loam and sandy soils and 96.4% agreement for silt soils and 95% agreement for organic soils. With silt soils PCR analysis indicated that sample 10 was positive whereas it was negative by microscopy and in organic soils PCR indicated that sample 2 was negative whereas microscopy indicated that it was positive. These data were not subjected to statistical analysis due to the very high level of agreement between the two diagnostic methods. In summary over all field samples tested there was 99% agreement between microscopy and PCR analysis for detection of stem nematode.

Table 3. Quantification cycle values **(**Cq) and melt temperatures (Tm) for *Ditylenchus dipsaci*, external control and for general nematode primerset for batch 1 samples. Both the PAC and NAC controls meet the criteria but data are not shown in the table. (PAC = positive amplification control, NAC = negative amplification control, N/A = no amplification)

		D. dipsaci		**External control**		**General nematode**		**Result**	
Sample	**Dilution factor**	**Cq**	**Tm**	**Cq**	**Tm**	**Cq**	**Tm**	**PCR**	**Microscopy**
Silt 21	40	N/A	None	28.05	78	21.99	84.5	Negative	Negative
Silt 22	40	N/A	None	28.07	78	21.8	84	Negative	Negative
Silt 23	40	N/A	None	27.49	78	20.98	84	Negative	Negative
Silt 24	40	N/A	None	27.6	78	21.09	84.5	Negative	Negative
Silt 25	40	N/A	None	27.53	78	21.15	84.5	Negative	Negative
Silt 26	40	N/A	None	27.74	78	21.34	84.5	Negative	Negative
Silt 27	40	N/A	None	27.55	78	21.12	84.5	Negative	Negative
Silt 28	40	N/A	None	27.51	78	21.12	84	Negative	Negative
Silt 29	40	30.01	85.5	27.67	78	21.02	84	Positive	Positive
Silt 30	40	N/A	None	27.55	78	21.27	84	Negative	Negative
Silt 31	40	N/A	None	27.16	78	20.71	84	Negative	Negative
Silt 32	40	N/A	None	27.22	78	21.02	84	Negative	Negative
Organic 5	160	N/A	None	29.62	78	25.04	84.5	Negative	Negative
Organic 6	160	N/A	None	29.3	78	24.64	84.5	Negative	Negative
Organic 7	160	N/A	None	29.86	78	24.8	84.5	Negative	Negative

Organic 8	160	N/A	None	29.31	78	24.84	84.5	Negative	Negative
Sand 21	40	N/A	None	27.42	78	20.45	84	Negative	Negative
Sand 22	40	N/A	None	27.75	78	20.88	84	Negative	Negative
Sand 23	40	N/A	None	27.31	78	20.08	84	Negative	Negative
Sand 24	40	N/A	None	27.12	78	20.4	84	Negative	Negative
Sand 25	40	N/A	None	27.18	78	20.77	84	Negative	Negative
Sand 26	40	29.01	85.5	27.27	78	20.2	84	Positive	Positive
Sand 27	40	N/A	None	27.28	78	20.04	84	Negative	Negative
Sand 28	40	27.6	85.5	27.41	78	20.67	84	Positive	Positive
Sand 29	40	N/A	None	27.61	78	21.07	84	Negative	Negative
Sand 30	40	N/A	None	27.35	78	21.08	84	Negative	Negative
Clay 17	40	N/A	None	27.31	78	21.51	84	Negative	Negative
Clay 18	40	29.03	85.5	27.36	78	20.56	84	Positive	Positive
Clay 19	40	28.72	85.5	27.33	78	21.14	84	Positive	Positive
Clay 20	40	N/A	None	26.72	78	21.44	84	Negative	Negative
Clay 21	40	N/A	None	27.23	78	21.04	84	Negative	Negative
Clay 22	40	N/A	None	27.57	78	21.4	84	Negative	Negative
Clay 23	40	28.92	85.5	27.48	78	21.19	84	Positive	Positive
Clay 24	40	N/A	None	27.2	78	21.17	84	Negative	Negative
Clay 25	40	N/A	None	27.42	78	21.27	84	Negative	Negative
Clay 26	40	N/A	None	27.67	78	22.38	84	Negative	Negative
Loam 21	40	N/A	None	27.22	78	20.61	84	Negative	Negative
Loam 22	40	N/A	None	27.32	78	20.4	84	Negative	Negative
Loam 23	40	N/A	None	27.93	78	21.9	84	Negative	Negative
Loam 24	40	N/A	None	27.43	78	21.17	84	Negative	Negative
Loam 25	40	N/A	None	27.7	78	21.02	84	Negative	Negative
Loam 26	40	38.7	90.5	27.06	78	20.59	84.5	Negative	Negative
Loam 27	40	N/A	None	27.47	78	20.94	84	Negative	Negative
Loam 28	40	N/A	None	27.33	78	20.84	84	Negative	Negative
Loam 29	40	N/A	None	27.39	78	20.52	84	Negative	Negative

Table 4. Quantification cycle values (Cq) and melt temperatures (Tm) for *Ditylenchus dipsaci* and external control for the clay soil field samples. Both the PAC and NAC controls meet the criteria but data are not shown in the table. (Batch 2 samples) (PAC = positive amplification control, NAC = negative amplification control, N/A = no amplification)

Soil type	Dilution factor	External control		*D. dipsaci*		Result	
		Cq	Tm	Cq	Tm	PCR	Microscopy
Clay 1	80	27.74	78	N/A	None	Negative	Negative
Clay 2	80	27.41	78	25.48	85	Positive	Positive
Clay 3	80	27.37	78	N/A	None	Negative	Negative
Clay 4	80	27.41	78	25.48	85	Positive	Positive
Clay 5	80	27.2	78	N/A	None	Negative	Negative
Clay 6	80	26.88	78	N/A	None	Negative	Negative
Clay 7	80	27.07	78	N/A	None	Negative	Negative
Clay 8	80	27.42	78	26.61	85	Positive	Positive
Clay 9	80	27.36	78	N/A	None	Negative	Negative
Clay 10	80	27.34	78	N/A	None	Negative	Negative
Clay 11	80	27.14	78	26.12	85	Positive	Positive
Clay 12	80	27.39	78	N/A	None	Negative	Negative
Clay 13	80	27.09	78	N/A	None	Negative	Negative
Clay 14	80	27.12	78	N/A	None	Negative	Negative

Table 5. Quantification cycle values (Cq) and melt temperatures (Tm) for *Ditylenchus dipsaci* and external control for the loam soil field samples. Both the PAC and NAC controls meet the criteria but data are not shown in the table. (Batch 2 samples) (PAC = positive amplification control, NAC = negative amplification control, N/A = no amplification)

Soil type	Dilution factor	External control		*D. dipsaci*		Result	
		Cq	Tm	Cq	Tm	PCR	Microscopy
Loam 1	80	27.13	78	N/A	None	Negative	Negative
Loam 2	80	27.86	78	N/A	None	Negative	Negative
Loam 3	80	27.69	78	N/A	None	Negative	Negative
Loam 4	80	28.06	78	N/A	None	Negative	Negative
Loam 5	80	27.96	78	30.02	85.5	Positive	Positive
Loam 6	80	27.83	78	N/A	None	Negative	Negative
Loam 7	80	28.19	78	29.54	85.5	Positive	Positive
Loam 8	80	27.84	78	N/A	None	Negative	Negative
Loam 9	80	27.88	78	N/A	None	Negative	Negative
Loam 10	80	27.80	78	30.71	85.5	Positive	Positive
Loam 11	80	28.04	78	N/A	None	Negative	Negative
Loam 12	80	28.00	78	N/A	None	Negative	Negative
Loam 13	80	28.29	78	N/A	None	Negative	Negative
Loam 14	80	27.99	78	N/A	None	Negative	Negative
Loam 15	80	28.05	78	29.13	85	Positive	Positive
Loam 16	80	27.80	78	N/A	None	Negative	Negative
Loam 17	80	28.25	78	N/A	None	Negative	Negative
Loam 18	80	27.80	78	N/A	None	Negative	Negative
Loam 19	80	28.09	78	N/A	None	Negative	Negative
Loam 20	80	27.97	78	N/A	None	Negative	Negative
Loam 21	80	27.94	78	32.27	85.5	Positive	Positive
Loam 22	80	27.71	78	N/A	None	Negative	Negative
Loam 23	80	27.49	78	N/A	None	Negative	Negative
Loam 24	80	27.69	78	N/A	None	Negative	Negative
Loam 25	80	27.57	78	N/A	None	Negative	Negative
Loam 26	80	27.80	78	N/A	None	Negative	Negative
Loam 27	80	28.09	78	30.67	85.5	Positive	Positive
Loam 28	80	28.23	78	N/A	None	Negative	Negative
Loam 29	80	28.34	78	N/A	None	Negative	Negative
Loam 30	80	28.31	78	N/A	None	Negative	Negative
Loam 31	80	28.28	78	N/A	None	Negative	Negative
Loam 32	80	28.07	78	N/A	None	Negative	Negative
Loam 33	80	28.06	78	N/A	None	Negative	Negative
Loam 34	80	28.02	78	N/A	None	Negative	Negative

Table 6. Quantification cycle values (Cq) and melt temperatures (Tm) for *Ditylenchus dipsaci* and external control for the organic soil field samples. Both the PAC and NAC controls meet the criteria but data are not shown in the table. (PAC = positive amplification control, NAC = negative amplification control, N/A = no amplification) (Samples for which PCR and microscopy are in disagreement are shaded) (Batch 2 samples).

Soil type	Dilution factor	External control		*D. dipsaci*		Result	
		Cq	Tm	Cq	Tm	PCR	Microscopy
Organic 1	160	28.36	78	N/A	None	Negative	Negative
Organic 2	160	28.16	78	N/A	None	Negative	Positive
Organic 3	160	28.14	78	31.07	85	Positive	Positive
Organic 4	160	28.26	78	N/A	None	Negative	Negative
Organic 5	160	28.36	78	N/A	None	Negative	Positive
Organic 6	160	28.78	78	N/A	None	Negative	Negative
Organic 7	160	28.13	78	31.19	85	Positive	Positive
Organic 8	160	28.49	78	N/A	None	Negative	Negative
Organic 9	160	28.68	78	N/A	None	Negative	Negative
Organic 10	160	27.74	78	30.51	85	Positive	Positive
Organic 11	160	28.21	78	N/A	None	Negative	Negative
Organic 12	160	28.43	78	N/A	None	Negative	Negative
Organic 13	160	28.61	78	N/A	None	Negative	Negative
Organic 14	160	28.38	78	N/A	None	Negative	Negative
Organic 15	160	27.90	78	N/A	None	Negative	Positive
Organic 16	160	28.28	78	N/A	None	Negative	Negative
Organic 17	160	28.07	78	N/A	None	Negative	Negative
Organic 18	160	28.13	78	N/A	None	Negative	Negative
Organic 19	160	28.17	78	N/A	None	Negative	Negative
Organic 20	160	28.38	78	N/A	None	Negative	Negative

Table 7. Quantification cycle values (Cq) and melt temperatures (Tm) for *Ditylenchus dipsaci* and external control for the sand soil field samples. Both the PAC and NAC controls meet the criteria but data are not shown in the table. (Batch 2 samples) (PAC = positive amplification control, NAC = negative amplification control, N/A = no amplification)

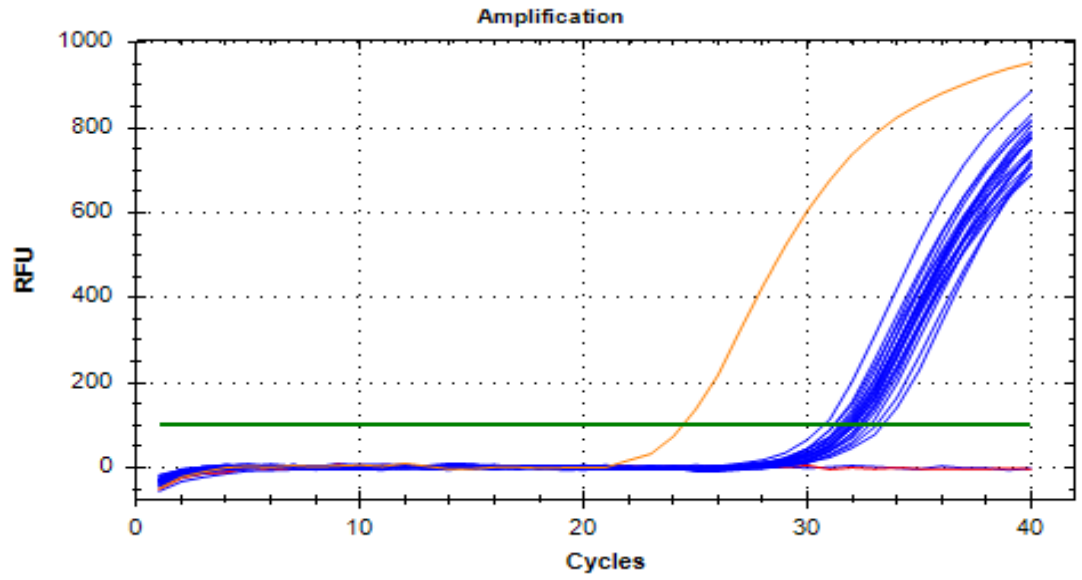
Soil type	Dilution factor	External control		*D. dipsaci*		Result	
		Cq	Tm	Cq	Tm	PCR	Microscopy
Sand 1	80	27.75	78	N/A	None	Negative	Negative
Sand 2	80	27.20	78	N/A	None	Negative	Negative
Sand 3	80	27.49	78	N/A	None	Negative	Negative
Sand 4	80	27.18	78	33.26	84.50	Positive	Positive
Sand 5	80	27.70	78	N/A	None	Negative	Negative
Sand 6	80	27.12	78	28.97	85.00	Positive	Positive
Sand 7	80	26.87	78	N/A	None	Negative	Negative
Sand 8	80	26.73	78	N/A	None	Negative	Negative
Sand 9	80	26.97	78	N/A	None	Negative	Negative
Sand 10	80	27.20	78	N/A	None	Negative	Negative
Sand 11	80	27.53	78	N/A	None	Negative	Negative
Sand 12	80	27.03	78	N/A	None	Negative	Negative
Sand 13	80	27.08	78	28.75	85.00	Positive	Positive
Sand 14	80	27.03	78	N/A	None	Negative	Negative
Sand 15	80	26.88	78	32.81	85.00	Positive	Positive
Sand 16	80	27.03	78	N/A	None	Negative	Negative
Sand 17	80	27.15	78	N/A	None	Negative	Negative
Sand 18	80	27.46	78	N/A	None	Negative	Negative
Sand 19	80	27.42	78	N/A	None	Negative	Negative
Sand 20	80	27.06	78	N/A	None	negative	negative
Sand 21	80	26.90	78	N/A	None	Negative	Negative
Sand 22	80	26.94	78	N/A	None	Negative	Negative
Sand 23	80	26.82	78	28.49	85.00	Positive	Positive
Sand 24	80	27.05	78	N/A	None	Negative	Negative
Sand 25	80	26.92	78	N/A	None	Negative	Negative
Sand 26	80	27.48	78	29.50	85.00	Positive	Positive
Sand 27	80	27.67	78	N/A	None	Negative	Negative
Sand 28	80	28.03	78	N/A	None	Negative	Negative
Sand 29	80	27.70	78	N/A	None	Negative	Negative

Table 8. Quantification cycle values (Cq) and melting temperatures (Tm) for *Ditylenchus dipsaci* and external control for the silt soil field samples. Both the PAC and NAC controls meet the criteria but data are not shown in the table. (Samples for which PCR and microscopy are in disagreement are shaded) (Batch 2 samples) (PAC = positive amplification control, NAC = negative amplification control, N/A = no amplification)

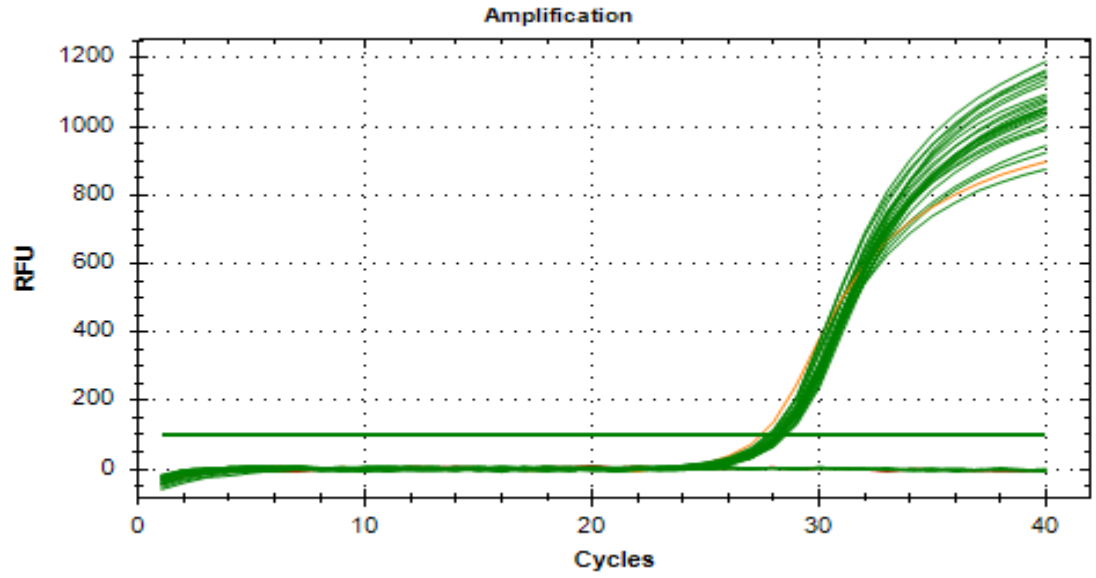
Soil type	Dilution factor	External control		*D. dipsaci*		Result	
		Cq	Tm	Cq	Tm	PCR	Microscopy
Silt 1	80	28.11	78	N/A	None	Negative	Negative
Silt 2	80	27.91	78	N/A	None	Negative	Negative
Silt 3	80	27.46	78	25.89	85.50	Positive	Positive
Silt 4	80	27.70	78	N/A	None	Negative	Negative
Silt 5	80	27.40	78	N/A	None	Negative	Negative
Silt 6	80	28.02	78	N/A	None	Negative	Negative
Silt 7	80	27.57	78	N/A	None	Negative	Negative
Silt 8	80	27.74	78	N/A	None	Negative	Negative
Silt 9	80	27.71	78	25.13	85.50	Positive	Positive
Silt 10	80	27.74	78	27.06	85.50	Positive	Negative
Silt 11	80	27.62	78	N/A	None	Negative	Negative
Silt 12	80	27.52	78	N/A	None	Negative	Negative
Silt 13	80	27.41	78	N/A	None	Negative	Positive
Silt 14	80	27.62	78	24.83	85.00	Positive	Positive
Silt 15	80	27.62	78	N/A	None	Negative	Positive
Silt 16	80	27.87	78	N/A	None	Negative	Negative
Silt 17	80	28.10	78	N/A	None	Negative	Negative
Silt 18	80	27.69	78	N/A	None	Negative	Negative
Silt 19	80	27.69	78	N/A	None	Negative	Negative
Silt 20	80	27.66	78	N/A	None	negative	negative
Silt 21	80	27.72	78	N/A	None	Negative	Negative
Silt 22	80	27.38	78	25.74	85.50	Positive	Positive
Silt 23	80	28.07	78	25.58	85.50	Positive	Positive
Silt 24	80	27.91	78	N/A	None	Negative	Negative
Silt 25	80	28.03	78	N/A	None	Negative	Negative
Silt 26	80	27.99	78	N/A	None	Negative	Positive
Silt 27	80	27.62	78	N/A	None	Negative	Negative
Silt 28	80	28.05	78	26.05	85.50	Positive	Positive

Experiment 2. Validating PCR analysis for stem nematode inoculated into representative soil types

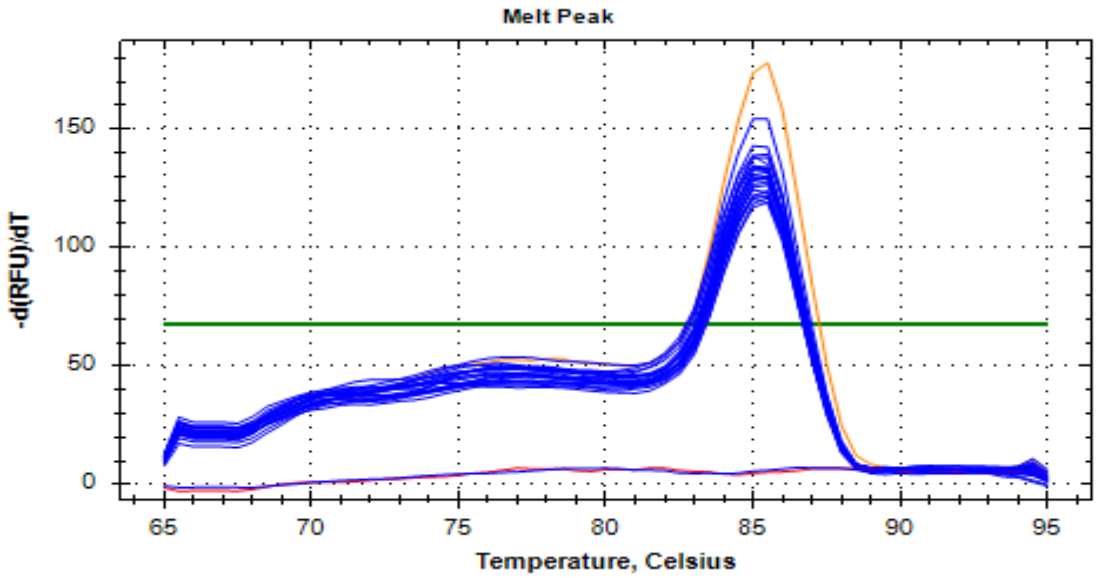
An example of the amplification curves for the inoculated loam soils are shown in Figure 1. For a positive identification it is essential that the sample Tm equals the Tm of the PAC (+/- 1^{o}C. Results of the PCR analyses on the nematode suspensions from the five different field collected soil types are shown in Tables 9 – 13.



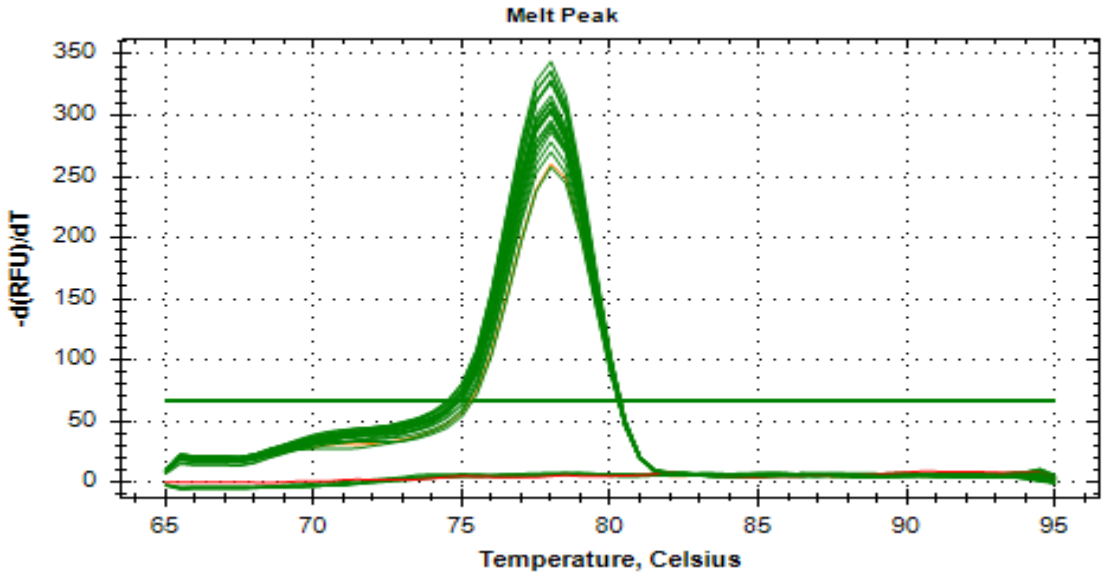
A



B



C



D

Figure 1. Amplification curves (A, B) and melting peaks (C, D) of the Real-Time PCR results for the inoculated loam soils. The positive amplification curve is shown in orange, the negative amplification control in red, the curve for *D. dipsaci* in blue and the curve for the external control in green. The graphs are adapted from the Bio-Rad CFX software.

Table 9. Quantification cycle values (Cq) and melt temperatures (Tm) for *Ditylenchus dipsaci* and external control for the inoculated clay soil samples. Both the PAC and NAC controls meet the criteria but data are not shown in the table (PAC = positive amplification control, NAC = negative amplification control, N/A = no amplification).

Soil type	Dilution factor	External control		*D. dipsaci*		Result
		Cq	Tm	Cq	Tm	PCR
Clay 1	80	27.85	78	31.27	85.5	Positive
Clay 2	80	27.38	78	31.51	85.5	Positive
Clay 3	80	28.33	78	33.10	85.5	Positive
Clay 4	80	28.14	78	33.10	85.5	Positive
Clay 5	80	28.45	78	32.19	85.5	Positive
Clay 6	80	28.22	78	34.57	85.5	Positive
Clay 7	80	28.21	78	33.32	85.5	Positive
Clay 8	80	28.40	78	32.93	85.5	Positive
Clay 9	80	28.65	78	33.85	85.5	Positive
Clay 10	80	27.80	78	32.26	85.0	Positive
Clay 11	80	27.21	78	32.23	85.0	Positive
Clay 12	80	28.36	78	32.93	85.5	Positive
Clay 13	80	28.23	78	33.04	85.0	Positive
Clay 14	80	28.26	78	33.49	85.0	Positive
Clay 15	80	28.79	78	31.32	85	Positive
Clay 16	80	29.08	78	34.32	85.5	Positive
Clay 17	80	28.73	78	34.30	85.5	Positive
Clay 18	80	28.47	78	32.39	85.5	Positive
Clay 19	80	28.28	78	32.93	85.0	Positive
Clay 20	80	28.23	78	33.15	85.0	Positive
Clay 21	80	27.90	78	33.11	85.0	Positive
Clay 22	80	28.20	78	32.34	85.5	Positive
Clay 23	80	27.66	78	34.11	85.0	Positive
Clay 24	80	28.40	78	33.15	85.5	Positive
Clay 25	80	28.0	78	31.96	85.5	Positive

Table 10. Quantification cycle values (Cq) and melt temperatures (Tm) for *Ditylenchus dipsaci* and external control for the inoculated loam soil samples. Both the PAC and NAC controls meet the criteria but data are not shown in the table (PAC = positive amplification control, NAC = negative amplification control, N/A = no amplification).

Soil type	Dilution factor	External control		*D. dipsaci*		Result
		Cq	Tm	Cq	Tm	PCR
Loam 1	80	27.13	78	31.56	85.5	Positive
Loam 2	80	27.86	78	32.42	84.5	Positive
Loam 3	80	27.69	78	32.08	85.0	Positive
Loam 4	80	28.06	78	32.76	85.5	Positive
Loam 5	80	27.96	78	32.06	85.5	Positive
Loam 6	80	27.83	78	31.91	85.0	Positive
Loam 7	80	28.19	78	31.65	85.5	Positive
Loam 8	80	27.84	78	31.74	85.5	Positive
Loam 9	80	27.88	78	31.17	85.5	Positive
Loam 10	80	27.80	78	31.59	85.0	Positive
Loam 11	80	28.04	78	31.93	85.5	Positive
Loam 12	80	28.00	78	31.42	85.0	Positive
Loam 13	80	28.29	78	31.81	85.5	Positive
Loam 14	80	27.99	78	32.27	85.5	Positive
Loam 15	80	28.05	78	N/A	None	Negative
Loam 16	80	27.80	78	33.07	85.0	Positive
Loam 17	80	28.25	78	32.08	85.0	Positive
Loam 18	80	27.80	78	33.35	85.0	Positive
Loam 19	80	28.09	78	32.01	85.0	Positive
Loam 20	80	27.97	78	30.71	85.5	Positive
Loam 21	80	27.94	78	32.29	85.5	Positive
Loam 22	80	27.71	78	32.39	85.5	Positive
Loam 23	80	27.49	78	31.19	85.5	Positive
Loam 24	80	27.69	78	31.51	85.5	Positive
Loam 25	80	27.57	78	32.50	85.5	Positive

Table 11. Quantification cycle values (Cq) and melt temperatures (Tm) for *Ditylenchus dipsaci* and external control for the inoculated organic soil samples. Both the PAC and NAC controls meet the criteria but data are not shown in the table (PAC = positive amplification control, NAC = negative amplification control, N/A = no amplification).

Soil type	Dilution factor	External control		*D. dipsaci*		Result
		Cq	Tm	Cq	Tm	PCR
Organic 1	160	28.56	78	32.48	85.5	Positive
Organic 2	160	28.86	78	33.44	85.5	Positive
Organic 3	160	29.44	78	33.57	85.5	Positive
Organic 4	160	29.24	78	34.04	85.0	Positive
Organic 5	160	29.47	78	32.92	85.5	Positive
Organic 6	160	28.86	78	32.86	85.0	Positive
Organic 7	160	30.16	78	32.76	85.0	Positive
Organic 8	160	29.63	78	32.15	85.5	Positive
Organic 9	160	29.72	78	34.72	85.5	Positive
Organic 10	160	29.02	78	33.50	85.5	Positive
Organic 11	160	29.14	78	33.61	85.5	Positive
Organic 12	160	29.52	78	34.42	85.5	Positive
Organic 13	160	29.50	78	33.31	85.5	Positive
Organic 14	160	29.61	78	34.63	85.0	Positive
Organic 15	160	29.57	78	34.80	85.0	Positive
Organic 16	160	29.43	78	33.34	85.5	Positive
Organic 17	160	29.75	78	32.26	85.5	Positive
Organic 18	160	29.70	78	33.87	85.5	Positive
Organic 19	160	29.34	78	33.67	85.0	Positive
Organic 20	160	29.16	78	32.43	85.5	Positive
Organic 21	160	29.68	78	33.25	85.5	Positive
Organic 22	160	29.03	78	33.12	85.5	Positive
Organic 23	160	29.09	78	32.52	85.0	Positive
Organic 24	160	29.20	78	32.96	85.5	Positive
Organic 25	160	29.37	78	34.43	85.5	Positive

Table 12. Quantification cycle values (Cq) and melt temperatures (Tm) for *Ditylenchus dipsaci* and external control for the inoculated sand soil samples. Both the PAC and NAC controls meet the criteria but data are not shown in the table (PAC = positive amplification control, NAC = negative amplification control, N/A = no amplification)

Soil type	Dilution factor	External control		*D. dipsaci*		Result
		Cq	Tm	Cq	Tm	PCR
Sand 1	80	27.13	78	32.11	85.0	Positive
Sand 2	80	27.86	78	31.95	85.0	Positive
Sand 3	80	27.69	78	34.23	85.5	Positive
Sand 4	80	28.06	78	34.83	85.5	Positive
Sand 5	80	27.96	78	33.95	85.5	Positive
Sand 6	80	27.83	78	33.21	85.0	Positive
Sand 7	80	28.19	78	31.43	85.5	Positive
Sand 8	80	27.84	78	33.64	85.5	Positive
Sand 9	80	27.88	78	33.01	85.5	Positive
Sand 10	80	27.80	78	34.07	85.5	Positive
Sand 11	80	28.04	78	33.90	85.5	Positive
Sand 12	80	28.00	78	N/A	None	Negative
Sand 13	80	28.29	78	N/A	None	Negative
Sand 14	80	27.99	78	32.24	85.5	Positive
Sand 15	80	28.05	78	33.27	85.5	Positive
Sand 16	80	27.80	78	31.20	85.0	Positive
Sand 17	80	28.25	78	34.01	85.5	Positive
Sand 18	80	27.80	78	31.41	85.5	Positive
Sand 19	80	28.09	78	32.43	85.5	Positive
Sand 20	80	27.97	78	31.66	85.5	Positive
Sand 21	80	27.94	78	32.28	85.0	Positive
Sand 22	80	27.71	78	32.08	85.0	Positive
Sand 23	80	27.49	78	32.90	85.0	Positive
Sand 24	80	27.69	78	32.27	85.0	Positive
Sand 25	80	27.57	78	32.51	85.0	Positive

Table 13. Quantification cycle values (Cq) and melt temperatures (Tm) for *Ditylenchus dipsaci* and external control for the inoculated silt soil samples. Both the PAC and NAC controls meet the criteria but data are not shown in the table (PAC = positive amplification control, NAC = negative amplification control, N/A = no amplification)

Soil type	Dilution factor	External control		*D. dipsaci*		Result
		Cq	Tm	Cq	Tm	PCR
Silt 1	80	27.24	78	31.12	85.0	Positive
Silt 2	80	27.11	78	31.36	85.0	Positive
Silt 3	80	28.25	78	31.21	85.0	Positive
Silt 4	80	28.45	78	34.30	85.0	Positive
Silt 5	80	28.57	78	32.39	85.5	Positive
Silt 6	80	28.30	78	32.18	85.5	Positive
Silt 7	80	28.15	78	31.08	85.0	Positive
Silt 8	80	28.36	78	32.73	85.0	Positive
Silt 9	80	28.02	78	31.20	85.0	Positive
Silt 10	80	28.61	78	31.31	85.5	Positive
Silt 11	80	28.19	78	32.45	85.0	Positive
Silt 12	80	28.22	78	32.10	85.5	Positive
Silt 13	80	28.52	78	32.85	85.5	Positive
Silt 14	80	28.18	78	32.47	85.5	Positive
Silt 15	80	28.10	78	33.09	85.5	Positive
Silt 16	80	28.28	78	34.07	85.5	Positive
Silt 17	80	28.05	78	31.92	85.5	Positive
Silt 18	80	28.02	78	32.26	85.5	Positive
Silt 19	80	28.53	78	33.75	85.5	Positive
Silt 20	80	28.14	78	32.66	85.5	Positive
Silt 21	80	28.38	78	32.46	85.5	Positive
Silt 22	80	28.29	78	32.47	85.5	Positive
Silt 23	80	28.14	78	32.31	85.5	Positive
Silt 24	80	28.25	78	31.83	85.5	Positive
Silt 25	80	27.89	78	31.73	85.5	Positive

Across all soil types (clay, loam, organic, sand, silt) 122 out of 125 were positive for stem nematode (97.6%). There were three unexpected negative results; loam 15 (Table 10), sand 12 and sand 13 (Table 12). For these samples an additional Real-Time PCR was performed using a 'general nematode DNA' assay. A positive result from this primer set (Cq < 35) indicates that sufficient nematode DNA is present in the sample. A negative result indicates that no, or too little, nematode DNA is present in the tested DNA extract. In such a case, the DNA extraction procedure should be repeated with a new nematode sample. The results of this troubleshooting analysis are presented in Table 14, and the presence of nematode DNA and absence of *D.dipsaci* DNA was confirmed.

Table 14. Results of the 'general nematode DNA' assay for the inoculated samples which were found to be negative. Both the PAC and NAC controls meet the criteria but data are not shown in the table (PAC = positive amplification control, NAC = negative amplification control, N/A = no amplification).

Soil type	Dilution factor	External control		*General nematode*		*D. dipsaci*		Result
		Cq	Tm	Cq	Tm	Cq	Tm	
Loam 15	80	28.02	78.0	22.52	84.0	N/A	None	Negative
Sand 12	80	27.91	78.0	21.53	84.0	N/A	None	Negative
Sand 13	80	28.15	78.0	21.13	84.5	N/A	None	Negative

Objective 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

The ClearDetections primer set specific for *D. dipsaci* has been designed to exclude *D. gigas* and should not give a signal with DNA from this nematode. However, all stem nematode races that occur within the *D. dipsaci* species are being detected with the *D. dipsaci* specific primer set. ClearDetections and ADAS have tried to find DNA of D*. gigas* in order to test it with the ClearDetections *D. dipsaci* primer sets. Material was finally received from Geves in France, which was tested but the results were inconsistent.

A total of 12 DNA samples of individual *D. gigas* specimens received from France were tested in 2014. Of these, seven gave no signal with the ClearDetections *D. dipsaci* Real-time PCR primer sets. These specimens originated from two sources in France. However, the remaining five samples gave a very low signal with a slightly lower melt temperature than expected for *D. dipsaci.* These five samples were said to originate from the UK.

In view of the limited information about the source and identification of the samples received from France it is difficult to draw any firm conclusions about the ability to differentiate *D.dipsaci* from *D. gigas* and further work is required. ADAS will continue to look for *D. gigas* and if found will send specimens to ClearDetections so that they can sequence each individual to identify the species and test the specificity of the *D. dipsaci* assay.

Discussion

The results indicate that DNA extraction was successful for all samples, both in the inoculated and natural field samples, as it was possible to detect external control (EC) DNA in all tested samples. In addition, all Real-Time PCR assays were successful as both the

negative and positive amplification controls always performed according to the criteria described in the ClearDetections manual.

In the inoculated samples *D. dipsaci* was detected in all clay, silt and organic soil samples. Unexpectedly, in two sand and a single loam sample tested negative for *D. dipsaci.* The reason for this remains unclear. It is possible that the stem nematodes were lost during sample handling or transport, they might have been missed during the DNA extraction, they may have stuck to the lid of the tube or they might have been lost during the volume reduction before DNA extraction.

In the natural field samples a total of six samples tested positive for *D. dipsaci* in the batch 1 and there was 100% agreement between the PCR analysis and microscopy. In batch 2 samples 26 tested positive for *D. dipsaci*; four in clay soil, six in sandy soil, seven in silt soil, three in organic soil and six in loam soil. There was 100% agreement between the two diagnostic methods for clay loam and sandy soils and 96.4% agreement for silt soils and 95% agreement for organic soils. With silt soils PCR analysis indicated that sample 10 was positive whereas it was negative by microscopy and in organic soils PCR indicated that sample 2 was negative whereas microscopy indicated that it was positive. In general over the two years of the project results showed that PCR analysis using the ClearDetections Real-Time PCR nematode identification kit was 99% as effective as microscopy for detecting *D. dipsaci* in soil samples. This result is reassuring as the number of UK nematologists is declining and an alternative method for assessing the suitability of soil for cropping with onions or leeks is urgently required should anything happen to those nematologists who can confidently identify the pest by microscopy.

It is clear that neither method is 100% reliable although results suggest that in the majority of cases both are valuable methods of assessing risk from *D. dipsaci*. Technician error can never be eliminated (identification of *D. dipsaci* is difficult and requires considerable experience and skill) and it is possible that a single stem nematode could be missed by microscopy. Also nematodes could be lost during volume reduction when preparing samples for PCR analysis. In both cases this is more likely when the pest population is very small and so, on the rare occasions when a false negative is recorded, it should have minimal impact on the crop. There is also the potential for false positive results. In this case there is the possibility of unnecessary pesticide use or rejection of land for cropping with onions or leeks. To try and minimise the risk of either false positive or negative results it may best to advise the use of multiple samples from the same field.

Results from analyses done on samples inoculated with stem nematode show that the PCR test is suitable for use in most UK soils although the dilution factor for organic soils is likely

to be higher than for other soil types. Results from year 1 show that the PCR analysis is capable of detecting a single *D. dipsaci* either in isolation or when it is among other free-living nematode species. Over both years of the project there was 97% agreement between microscopy and PCR analysis for detection of stem nematode in nematode suspensions from a range of soil types that had been inoculated with the pest. This result shows a slightly lower level of agreement between the two diagnostic techniques than for field collected samples (99%) and probably reflects the difficulties of trying to manually inoculate such low numbers of such a small nematode.

It was not possible to determine whether the ClearDetections PCR analysis was capable of differentiating between *D. gigas* (giant bean race) and the more common oat-onion race. This was primarily because *D. gigas* is very difficult to find in soil. In the UK ADAS has failed to detect this species in 10 years of data collected from soils from all over the UK. The only time *D. gigas* has been found was in infested field bean seed samples. This suggests that *D. gigas* poses a limited threat to onions and leeks in the UK. *D. dipsaci* is a much more significant pest and can be detected by either microscopy or PCR analysis. ADAS will continue to look for *D. gigas* and if it can be found, most probably following extraction of bean samples, material will be made available to ClearDetections with which to test their PCR analysis.

In summary, PCR analysis could become a vital component of an integrated pest management strategy for *D. dipsaci* to help growers assess the risk from the pest and make rational decisions on the need for control measures or whether or not grow a susceptible crop.

Conclusions

- This project confirms that a robust testing scheme based on this Real-Time PCR method can provide a reliable alternative to the current scheme which relies on a dwindling pool of nematological expertise.
- Results suggest that the Real-Time 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.
- Real-Time PCR was effective in a wide range of UK soil types using different DNA dilution factors.
- There was a high degree of agreement between microscopy and PCR analysis for detection of stem nematode. Over all samples tested there was 99% agreement

between the two diagnostic techniques for field collected samples and 97% agreement for nematode suspensions inoculated with stem nematode.

- PCR analysis could become a vital component of an integrated pest management strategy for *D. dipsaci* to help growers assess the risk from the pest.

Knowledge and Technology Transfer

The project was discussed at the EMRA/AHDB Horticulture (formerly HDC) Soft fruit day at East Malling Research, Kent 21 November 2013 as a potential method for future proofing nematode diagnostics. Results will also be presented at two bulb/narcissus grower events in Cornwall and Lincolnshire in May 2015 (13th and 20th). The project has also been the subject of articles in AHDB Horticulture publications as listed below.

AHDB Grower August 2014. First pass for Dutch test on English land.

AHDB Grower October 2014 Field Vegetables Review (Supplement to AHDB Grower) - Genetic test gives clear nematode warning.

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 the 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.

Negative Amplification Control (NAC) - For this control the sample is replaced by DNA dilution buffer from the kit. The NAC must be negative (Cq < 35). The NAC has to be included in every PCR run to confirm the absence of DNA contamination which can result in false positive results.

Positive Amplification Control (PAC) – This is an indicator that the Real-Time PCR is functioning adequately. The Cq value of the PAC should be less than 25 (Cq < 25).

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

Real-Time PCR – A laboratory technique based on the polymerase chain reaction which is used to amplify and simultaneously detect or quantify a target DNA fragment using an intercalating dye.

References

Vovlas N., Troccoli A., Palomares-Rius J.E., Luca F., de Liébanas G., Landa B.B., Subbotin S.A., Castillo P. (2011). *Ditylenchus gigas* n. sp. parasitizing broad bean: a new stem nematode singled out from the *Ditylenchus dipsaci* species complex using a polyphasic approach with molecular phylogeny. *Plant Pathology* **60**(4), 762-775.

