Final Report

Validation of quantitative DNA detection systems for PCN

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1. Summary

The plant-parasitic nematodes *Globodera pallida* and *G. rostochiensis* are the species of potato cyst nematode (PCN) that cause large losses in potato crops. Both PCN species are widespread throughout Europe. At the start of this project it was known that a new EU Council Directive on control of PCN was to come into force. It would replace the previous Directive (69/465/EEC) issued in 1969 and was expected to have implications in terms of both the intensity of PCN sampling, the volume of soil tested and the extraction method used. In anticipation of these changes work was commissioned to determine if approaches could be developed to reduce the cost and increase the speed of testing soil samples for PCN.

Traditional methods to detect and identify *G. pallida* and *G. rostochiensis* in soil involve separating cysts on the basis of different sedimentation rates in a flotation device (eg Fenwick can or Wye washer), followed by identification of the cysts either using a microscope or by applying DNA-based methods to the eggs. Some of the earliest DNA-based methods were not quantitative and therefore could not estimate PCN egg density in a soil sample. More recently, real-time PCR -based detection methods have been developed. These are quantitative and the results can be directly related to the numbers of PCN eggs present in a sample. At the start of the project staff at Plant and Food Research Ltd (formerly Crop & Food Research, New Zealand) had developed real-time PCR assays for *G. pallida* and *G. rostochiensis*, and staff at Central Science Laboratory (CSL) had worked on direct extraction of DNA from soil and the development of real-time PCR assays for pathogens of potato. The two groups were commissioned to bring together the expertise and prior research from CSL and Plant & Food Research to produce improved diagnostic tests for PCN for use by the British potato grower.

Summary of work

The Central Science Laboratory have developed a new real-time PCR assay using TaqMan DNA-based technology for the detection, identification and quantification of both species of PCN in soil. This method was chosen for development during the project on the basis of the relative cost of the reagents required for this assay compared to the existing Plant and Food Research assay. The TaqMan assay detects the presence of PCN DNA. Dead PCN eggs will also contain DNA but in the harsh environment of the soil this DNA is unlikely to survive in a detectable form for very long. Therefore, except for soil recently treated with a nematicide, any PCN detected in the soil is likely to have come from a viable egg.

The TaqMan assay developed by CSL has been used in conjunction with cysts extracted using flotation devices (eg., Fenwick can, Wye washer). In addition, methods for the direct extraction of DNA from soil samples have been tested in conjunction with the CSL TaqMan assay on its own. Methods to extract DNA from volumes of soil up 1.5kg were developed and shown to work. Estimates of the costs of the different PCN extraction and identification methods (eg Fenwick Can + TaqMan assay; direct soil extraction + TaqMan assay) were calculated. Working with the direct extraction of DNA from large (1.5kg) volumes of soil was not found to be cost effective. Overall, the methods developed can be tailored to specific needs yielding cysts numbers, egg numbers and individual species from between 250g and 1.5kg soil depending on specific requirements. The costs vary according to the level of information required/volume of soil processed.

A review of the available information on the relationship between PCN egg densities and yield loss was carried out and is provided in Annex 1. The review suggests that more work is required to allow an effective relationship between egg counts and yield loss to be determined for GB conditions and cultivars.

2. Experimental Section

Introduction

The plant-parasitic nematodes *Globodera pallida* and *G. rostochiensis* are the species of potato cyst nematode (PCN) that cause large losses in potato crops. Both PCN species are widespread throughout Europe. In England and Wales, over 60% of all potato lands are infested with PCN and *G. pallida is* the dominant species (Minnis *et al*., 2002). Yield losses of up to 90% have been found, resulting in economic losses of around £43 million per annum for the UK (Haydock & Evans, 1998).

PCN is considered a quarantine pest in the EU and the EU Directive (69/465/EEC), introduced in 1969, was in place to prevent the spread of the pest. This Directive was enhanced by the EU Plant Health Directive (Council Directive 77/93/EEC) in 1977 which listed all those organisms present in the Community but which should be controlled. In 2004, a working group was set up to review the Directives relating to PCN and a new Directive (2007/33/EC) has now been passed which will come into force in July 2010. It will have implications for the intensity of sampling and weight of samples taken for testing. The particular requirements will decided by each country. In England and Wales sample sizes will range from of 200 ml to 1500 ml depending on the requirements for particular fields and crop types.

The currently available methods to detect and identify *G. pallida* and *G. rostochiensis* in soil in the UK involve sampling up to 0.5 kg of soil and separating cysts on the basis of different sedimentation rates in a flotation device, followed by morphological and/or molecular identification. The methods are laborious and the extraction equipment is only capable of processing approximately 250g (Fenwick Can) or up to 750g (Wye Washer) of soil, depending on type, at any one time. The process can take several days and must be carried out by experienced nematologists. Identifying species, and viability of cysts and determining the number of viable eggs, if necessary, is very time consuming, costly and requires highly skilled staff.

Identification using on DNA-based techniques can simultaneously identify the presence or absence of PCN and determine whether either or both species are present. The first DNA-based PCN detection method was developed by Marshall and Crawford in 1987. More recently PCR-based detection methods have been developed (Bulman and Marshall, 1997). One of the main advantages of real-time PCR is the quantitative nature of the technology. The results gained can be directly related to the numbers of individuals present in a sample. While these methods work well, at the start of the project they had only been applied to nematodes separated from the soil in the traditional fashion (eg., using a Fenwick Can or elutriator). However, direct soil DNA extraction methods had progressed and small-scale extractions were being successfully used for routine diagnostics for some plant pathogens at CSL. Plant and Food Research had developed large volume bead mills and specific DNA extraction buffers for direct DNA extraction methods using samples from infested soils between 100 and 500 g, although the mills were not available in the UK.

In summary, the combination of direct soil DNA extraction and real-time PCR offered the potential for rapid and accurate identification of *Globodera* species in soil samples as well as the ability to quantify the viable numbers of each species present. The objectives of the project were to:

- compare all methods for the quantification and identification of PCN from both organizations and select the most effective and appropriate methods for use by the British potato industry.
- validate the most effective methods under British conditions and;
- analyse the costs of the method and compare the costs with those of the existing analytical methods

The aim was to provide a framework for a new level of service to the GB potato industry that is reliable and cost-effective and can meet the requirements of the new EU PCN Directive 2007/33/EC.

Material and methods

CSL Methods

Primer optimisation

The sequences of the primers and probes used for detection and the location relative to ITSI sequence for *G. pallida* and *G. rostochiensis* are given in Figure 1. Probes and primers were designed to place polymorphic nucleotides between the two species in the middle of a short probe, such that universal primers could be used with two probes for discrimination. The probe and primer combinations were designed using Primer Express software (PE-Biosystems). For *G. pallida* the initial probe had the 5′ terminal reporter dye FAM (6-carboxyfluorescein) and the 3′ quencher TAMRA (tetra-methylcarboxyrhodamine); the modified LNA probe had the 5′ terminal reporter dye FAM and the 3′ quencher was non-fluorescent (BHQ). For *G. rostochiensis* the initial probe had the 5′ terminal reporter dye JOE and the 3′ quencher TAMRA (tetra-methylcarboxyrhodamine); the modified LNA probe had the 5′ terminal reporter dye TET and the 3′ quencher was non-fluorescent (BHQ). In each case the dyes used enable multiplex detection and discrimination of *G. pallida* and *G. rostochiensis*.

FIGURE 1: AN ALIGNMENT SHOWING THE TAQMAN® PRIMERS AND PROBES ALIGNED WITH ITS1 SEQUENCE FROM BOTH *G. PALLIDA* AND *G. ROSTOCHIENSIS*. THE NUCLEOTIDES WHICH HAVE BEEN BOXED IN ARE THOSE WHICH ARE MODIFIED IN THE LNA PROBES.

TaqMan® reactions were set up in 96-well reaction plates using PCR core reagent kits (Applied Biosystems), following the protocols supplied. Plates were then cycled at generic system conditions (48°C/30 min, 95°C/10 min and 40 cycles of 60°C/1 min, 95°C/15 sec.) within the 7900HT Sequence Detection System (Applied Biosystems), using real time data collection.
In order to optimise assay performance, a primer concentration matrix was carried out to calculate the concentration of primers to be used in the assays. The 3 x 3 matrix of 50 nM, 300 nM and 900 nM primer concentrations were set up; the difference between each of the concentrations account for a difference of 2°C in actual T_m of the primer. The concentration of primers for each set was chosen as the lowest concentration that gave the highest normalised reporter fluorescence (ΔR_n) and the lowest threshold cycle (C_T). Further optimisation of probe concentrations was carried out to remove cross-reaction between the two assays.
Environmental Master Mix (Applied Biosystems) was also used for TaqMan PCR using the sample primer and probe conditions described above and following the protocols supplied.

Soil DNA extraction method

Protocols were developed to extract DNA from a range of compost and soil samples as part of the Defra project HH3214TFV (Identifying risks of *Rhizoctonia solani* to field vegetables and develop sustainable methods for quality production). The method developed utilises the natural affinity of DNA to bind to silica in the presence of chaotrophic salts, as described by Boom (1990). This natural property is combined with a magnetic particle extraction stage, to produce high quality DNA extracts with minimal staff input.

The protocol was adapted to allow the extraction of PCN from larger volumes of soil. Soil samples (between 50-1500g) were homogenised in 1.5 to 3 volumes of soil extraction buffer (120mM phosphate buffer (pH8), 1.5 M NaCl, 2% CTAB, 1% antifoam) in the presence of variable numbers (see results section) of 1 inch diameter steel ball bearings. The following protocol was then followed:

1. Shake the container on a Merris Automixer for 2 minutes to mix, and transfer 40 ml ground sample extract into a 50 ml screw cap tube and centrifuge at 5000g for 5 minutes. This centrifugation step can be repeated if necessary for difficult samples.

2. Transfer 20 ml of cleared extract into a clean 50 ml tube and add 0.1V of 5M potassium acetate to the extract. Vortex and leave on ice for 10 minutes.

3. Centrifuge the samples at 12000g for 5 minutes and transfer the cleared extract to a clean 50 ml tube, taking care not to disturb the pellet.

4. Add 0.8V isopropanol and 0.04V acid washed silica particles to each sample and place on a shaker for 10–15 minutes.

5. Centrifuge the samples at 12000g for 5 minutes and discard the liquid. Resuspend the silica pellet in 2 ml lysis buffer A (Promega Food Kit FF3750) and place the samples in a shaker at 65oC for 15 minutes.

6. Centrifuge the samples at 12000g for 5 minutes and transfer 1 ml of recovered buffer into a 2 ml tube.

7. Add 250 ul buffer B and 750 ul precipitation buffer (Promega Food Kit) to each tube. Vortex and spin at 13000g for 10 minutes.

8. Transfer 750 ul of cleared sample into a clean 2 ml tube containing 600 ul isopropanol and 50 ul vortexed Magnesil beads. Vortex and incubate at room temperature for 5 minutes.

9. Prepare a Kingfisher rack; one set of wells for each sample:

Well A – Sample with Magnesil
Well B – 1 ml buffer B
Well C – 1 ml 70% ethanol
Well D – 1 ml 70% ethanol
Well E – 200 ul TE buffer

Run the samples on the Kingfisher ML (Thermo Electron Corporation) using program ‘gDNA’

Internal control

No organisms are known to be present at a standard concentration in every soil; therefore in order to confirm negative results, an internal control must be added to each sample. An artificial plasmid was constructed and added to soil as an internal control. Oligonucleotide sequences CCGCTGCATTGCGAAACCCGCGCGTGCACCATTAACAGCGAACGCACCA and GGTGCGTTCGCTGTTAATGGTGCAC GCGCGGGTTTCGCAATGCAGCGGA were heated to 95^{o}C for 5min and then cooled slowly from 70^{o}C to 20^{o}C over 50 minutes and maintained at 20^{o}C for 30 minutes to allow then to hybridise. This hybridisation produced a short completely artificial (confirmed by

BLAST) double stranded piece of DNA with overhangs allowing it to be cloned and transformed into *E.coli* using the pGemTeasy PCR cloning kit (Promega). Large quantities of the plasmid, pLH-7 was produced and quantified using a Nanodrop ND-100 (Thermo Scientific). 150ng of plasmid were added to each 250g soil sample. A TaqMan assay using a primer (CCAGTGAATTGTAATACGACTCACTATAGG) based on the artificial insert and a primer (GGTGCGTTCGCTGTTAATGG) and probe (FAM-CGACGTCGCATGCTCCCGG-TAMRA) based on the plasmid was designed for detection of the plasmid.

Standards for real-time PCR

PCN DNA for use as standards was obtained from cysts cultured in sand. Fresh cysts were suspended in buffer A from the Promega food kit and disrupted using a micropestle. This sample was then DNA extracted as described in the soil extraction (step 7 onwards)

Field samples for parallel testing

10 samples of 150g of potato soil were tested using the standard Wye Washer method to isolate cysts and microscopic examination was used to determine total cysts, viable cysts and egg counts. The remaining 250g was extracted using the soil extraction method and TaqMan assayed alongside a series of standards.

DNA isolation from float material

Float / cysts material was suspended in 500ul buffer A from the Promega food kit in a 2ml tube and disrupted using a micropestle. 200ul of 0.5mm Zirconia / Silica beads (Thistle Scientific, UK) were then added and the tube placed in a bead beater (Biospec Limited) for 30 seconds at 50 thousand RPM. This sample was then DNA extracted as described in the soil extraction (step 7 onwards)

P&F Methods

Development of duplex primers for quantifying PCR

In work carried out before the start of the Potato Council Project the primers designed to discriminate between *G. rostochiensis* and *G. pallida* were found to produce some cross fluorescence signal when duplexed, which gave unexpected levels of eggs from both species when in a mixture. As a result the design of the probes and placement on the sequence was re-investigated.

Nematode cysts were ground in eppendorf tubes, using plastic micro-pestles with 500ul of solution containing 5M guanidine isothiocyanate, 10mM EDTA, 50mM Tris-HCl (pH7.5) and 8% mercaptoethanol. After room temperature incubation for up to 1 hour, the DNA-containing solution was extracted once with equal volumes of phenol and chloroform-isoamyl alcohol (24:1) and once with chloroform-isoamyl alcohol, then precipitated with 0.3M sodium acetate and two volumes of isopropanol. DNA was resuspended in 100ul of H2O.

Quantitative PCR was performed on the Perkin-Elmer ABI® 7700 Sequence Detector (TaqMan™).

The two primers used were:

PCNTQPr1f 5'-CACATGCCTCCGTTTGTTGT-3'
PCNTQPr1r 5'-CGCTCAACGACGCACAGA- 3'

The primers were designed to amplify the DNA from both *Globodera pallida* and *G. rostochiensis* equally well. The fluorescent probes were designed to be species specific and had the following sequences:

Pallida 5'Fam-ACAGCAATCGTCGAGTCACCCATTG-Tamra3'
Rostochiensis 5'Viv-CAGCAATCGTCGGCTCACCCATA-Tamra3'

All reaction components for quantitative PCR were purchased from Applied Biosystems. For the template 5ml of DNA was used in a final PCR reaction volume of 25ml. PCR reagents were 1x TaqMan™ universal PCR master mix, 300nM of each primer, 200nM of *pallida* probe and 100nM of *rostochiensis* probe. The thermal cycling conditions for the reactions were: a hold step for AmpErase activation at 50oC for 2 min; a second hold step for AmpliTaq Gold activation at 95oC for 10 min; then 40 cycles of a denaturation at 95oC for 15 seconds and an annealing phase at 60oC for 1 minute.

The effect of different initial PCN (*G. pallida*) population levels on potato yield and multiplication rates

To ensure a range of initial PCN population levels were included within the main potato plot trial, it was necessary to initially map the existing infestation. A 1.2-hectare research field known to contain PCN was intensively sampled using a sampling grid of 4 x 5 metres, where approximately 1 kg of soil was collected from each position. Soil samples were taken back to Plant & Food Research, Lincoln, New Zealand for assessment of cyst numbers using standard elutriation procedures. Assessment involved the soil samples being elutriated to recover cysts and then counted under a stereoscopic microscope. From this initial mapping of the infestation, it was possible to establish plots across the full range of initial PCN levels to assess the effect of differing initial population levels on potato yield and PCN multiplication rates (= Pf/Pi, where Pf is the final cyst population, determined at crop harvest, and Pi is the initial cyst population). Multiplication rates provide a measure of the PCN population dynamics during the growing season.

Thirty-two potato plots were established, where each plot consisted of 7 tubers by 5 rows. Soil samples were collected from each plot at the time of planting and analysed in the laboratory to determine the initial (Pi) populations at planting, which ranged from nil to 66 eggs/ml soil. This confirmed that the positioning of the plots for the main trial encompassed a range of initial PCN levels. The cultivar selected for this trial was Ilam Hardy as this particular cultivar is susceptible to PCN. To determine the number of eggs in a sample, the first step involves removing and counting cyst numbers, as described above. The eggs were then released from the cysts by staining with 0.1% w/v new blueR overnight and egg numbers determined in a Doncaster counting cell under a stereoscopic microscope at 30 x magnification.

The entire trial site was managed using standard commercial fertiliser, herbicide, and fungicide programmes. The plots were later harvested and soil samples collected. To exclude "edge effects", yield assessments for the main trial were based on the middle 5 plants by 3 rows; equivalent to an effective sampling area of 3.4 m2 per plot. Tubers were graded into table and seed, and the number and weights of each grade were recorded. Soil samples were collected from all 32 plots and assessed for cyst and egg counts to determine final (Pf) PCN populations. In addition, map coordinates of each of the 32 potato plots in the main trial were captured using Global Positioning System (GPS) technology to ensure the sites could be accurately found in future.

Results and Discussion

CSL

TaqMan Assay

Primer optimisation

Initial experiments with the primer/probe sets developed showed that the *G. pallida* probe was specific for *G pallida* and did not cross react with *G. rostochiensis* (figure 2A). The *G. rostochiensis* probe on the other hand gave significant cross-reaction with DNA extracted from *G. pallida* (Figure 3A).

Modifications were made to the probes in an attempt to make them more specific. LNA (locked nucleic acid) base pairs were incorporated into the sites of the polymorphisms. These synthetic nucleotides should give much greater binding to the complementary base pairs; in some cases this improved binding also allows the probe to be shortened such that it is more specific (see Figure 1).

Following amplification, the LNA modified *G pallida* assay surprisingly gave cross hybridisation with *G rostochiensis* (Figure 2B); the LNA modified *G rostochiensis* probe on the other hand was more specific and no longer gave cross hybridisation with DNA extracted from *G pallida* (Figure 3B).

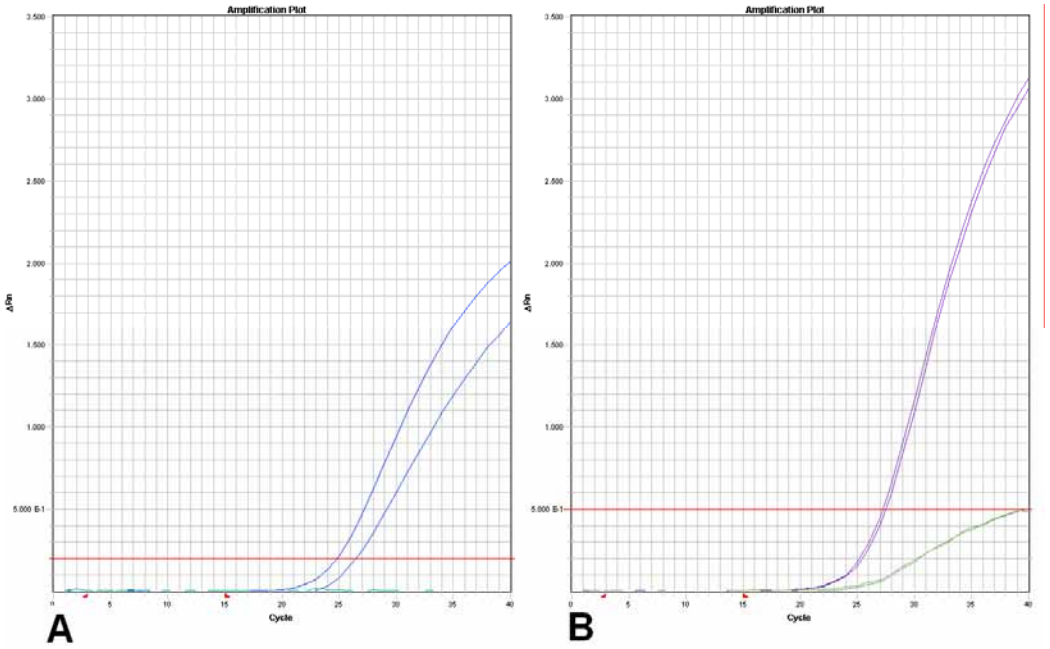


FIGURE 2: *G. PALLIDA* ASSAY OPTIMISATION. A: AMPLIFICATION PLOTS FOLLOWING AMPLIFICATION (IN DUPLICATE) USING THE UNMODIFIED FAM/TAMRA PROBE GIVING POSITIVE RESULTS FOR *G. PALLIDA* AND NEGATIVE RESULTS FOR *G. ROSTOCHIENSIS*. **B** SHOWS A FAM/BHQ PROBE WITH TWO MODIFIED LNA BASES TESTING THE SAME SAMPLES SHOWN IN A.

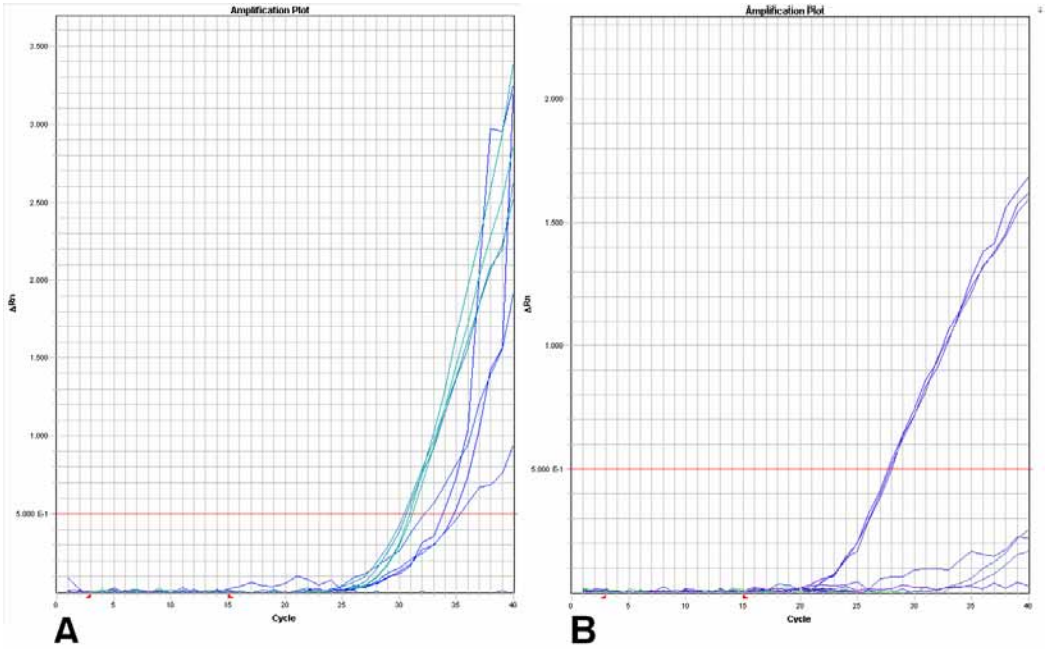


FIGURE 3: *G. ROSTOCHIENSIS* PROBE OPTIMISATION. A: AMPLIFICATION PLOTS FOLLOWING AMPLIFICATION (IN DUPLICATE) USING THE UNMODIFIED JOE/TAMRA PROBE GIVING POSITIVE RESULTS FOR BOTH *G. PALLIDA* AND *G. ROSTOCHIENSIS*. B: AMPLIFICATION PLOTS FOLLOWING AMPLIFICATION (IN DUPLICATE) USING THE LNA MODIFIED TET/BHQ PROBE TESTING THE SAME SAMPLES SHOWN IN A. IN THIS CASE POSITIVE RESULTS ARE ONLY ACHIEVED WITH THE HOMOLOGOUS DNA SAMPLE.

Further optimisation of the probe concentrations used in the duplex assay has removed any cross-reaction between the *pallida* and *rostochiensis* assays. Figure 4 shows the detection of *G. pallida* and *G. rostochiensis* in duplex assay

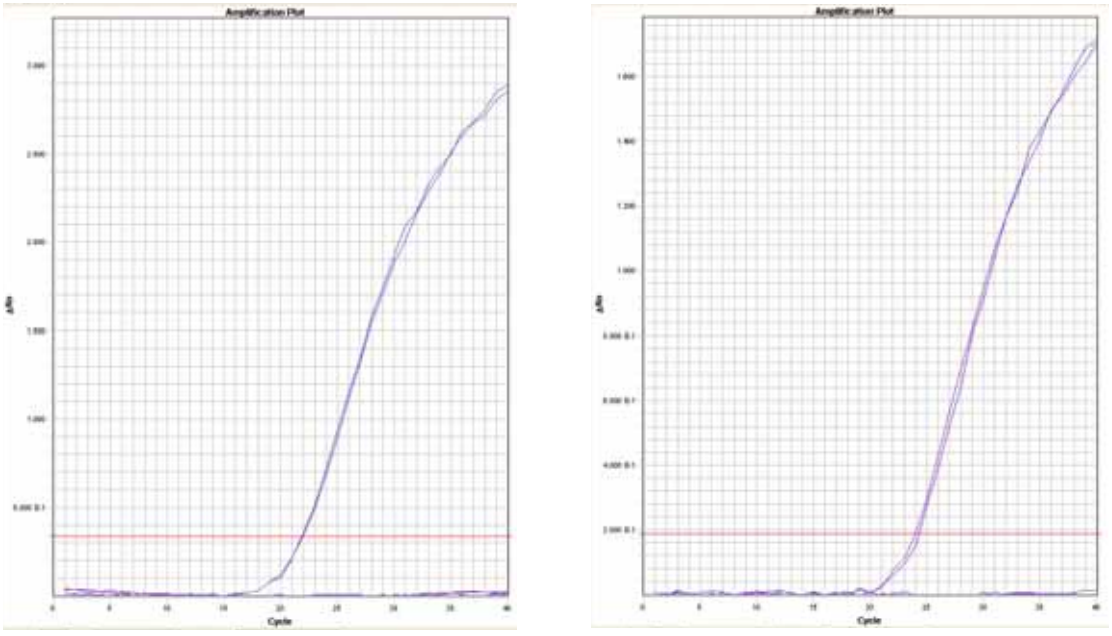


FIGURE 4. AMPLIFICATION PLOTS OF DUPLEX DETECTION OF *G. PALLIDA* (A) AND *G. ROSTOCHIENSIS* (B) SHOWING LACK OF CROSS-REACTION

Assay validation

Validation work has been completed using the *G. pallida* TaqMan assay compared to the standard conventional PCR (Bulman & Marshall, 1997) for speciation. The results (Table 1) using 109 sub samples of 13 confirmed *G. pallida* cysts show that the real-time PCR method is more sensitive than conventional PCR, and that taken as a whole (i.e. when the sub sample data is pooled), both assays correctly identified all 13 samples as *G. pallida.* Thus both sets of primers have the same specificity.

TABLE 1: COMPARISON OF THE CSL G. PALLIDA TAQMAN ASSAY WITH THE CONVENTIONAL PCR METHOD ILLUSTRATING DIAGNOSTIC SENSITIVITY ($\frac{A}{A+C}$) AND SPECIFICITY ($\frac{D}{D+B}$) IN EACH CASE. SAMPLES WHERE DNA EXTRACTS WERE SHOWN TO BE NON-AMPLIFIABLE (USING THE INTERNAL POSITIVE CONTROL) WERE EXCLUDED FROM THE ANALYSIS.

	Real-time PCR (TaqMan)			
		+	-	Total
Conventional PCR Method	+	88 (*A*)	0 (*B*)	88
	-	15 (*C*)	6 (*D*)	21
	Total	103	6	109

Notes: diagnostic sensitivity = 85.4% and diagnostic specificity = 100%

Both CSL assays have been tested against a series of PCN isolates, other *Globodera* species, nematodes and other soil borne potato pathogens (see Table 2 for list). The *G. rostochiensis* assay detected all the *G. rostochiensis* isolates and showed no cross reactions. The *G. pallida* assay detected all the *G. pallida* isolates, but was found to cross react with high concentrations of pure *G. tabacum* DNA (Figure 5) and give a moderate cross reaction with 5ug pure *G. tabacum* DNA. *G. tabacum* is currently not present in the UK. The shape of the amplification plot is also distinct with a very slow exponential phase (Figure 5). A weak cross reaction (Ct 38) between 20ng *Heterodera glycines* and the *G. pallida* assay was also found but this cross reaction was removed at 5ng DNA.

TABLE 2. LIST OF NEMATODE SPECIES USED TO VALIDATE THE ASSAYS

Species Tested	Phylum	Tissue	Isolate Code	origin	Host	Year of isolation
G. pallida	Nematoda	Cyst	Gp1	UK	Soil	2007
G. pallida	Nematoda	Cyst	Gp2	UK	Soil	2007
G. pallida	Nematoda	Cyst	Gp3	UK	Soil	2006
G. pallida	Nematoda	Cyst	Gp4	UK	Soil	2006
G. pallida	Nematoda	Cyst	Gp5	UK	Soil	2006
G. pallida	Nematoda	Cyst	Gp6	UK	Soil	2006
G. pallida	Nematoda	Cyst	Gp7	UK	Soil	2007
G. rostochiensis	Nematoda	Cyst	Gr1	UK	Soil	2007
G. rostochiensis	Nematoda	Cyst	Gr2	UK	Soil	2006
G. rostochiensis	Nematoda	Cyst	Gr3	UK	Soil	2007
G. rostochiensis	Nematoda	Cyst	Gr4	UK	Soil	2007
G. rostochiensis	Nematoda	Cyst	Gr5	UK	Soil	2007
G. rostochiensis	Nematoda	Cyst	Gr6	UK	Soil	2007
G. tabacum	Nematoda	Cyst	Gt1	UK	Soil	
G. achilleae/millefolii	Nematoda	Cyst	Ga1	UK	Soil	2007
Heterodera glycines	Nematoda	Cyst	N/a	USA	Soybean	pre-1990
Heterodera schactii	Nematoda	Cyst	113	Germany	Sugar beet	1987-1991
Rhizoctonia solani AG2-1	Basidiomycete	Hyphae	Y3	UK	Potato	2002
Rhizoctonia solani	Basidiomycete	Hyphae	R112	UK	Potato	2001

AG3-PT						
Spongospora subterranea	Plasmodiophoromycete	Spores from infected tuber	Ss1	UK	Potato	N/a
Polyscytalum pustulans	Ascomycete	Hyphae	Pp3	UK	Potato	unknown
Colletotrichum coccodes	Ascomycete	Hyphae/Conidia	Cc1499	UK	Potato	2002
Phytophthora infestans	Oomycete	Infected leaf tissue	N/a	UK	Potato	N/a
Helminthosporium solani	Ascomycete	Infected Tuber material	N/a	UK	Potato	N/a
Streptomyces species	Actinobacteria	Infected Tuber material	N/a	UK	Potato	N/a

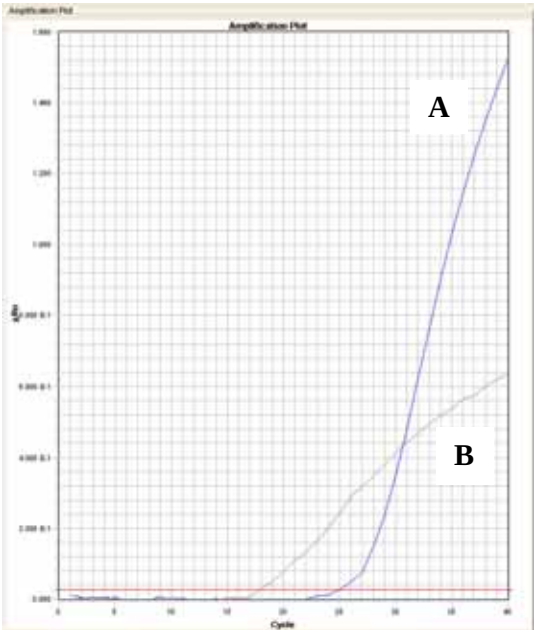


FIGURE 5 AMPLIFICATION PLOTS *FOR G. PALLIDA* (A) AND *G. TABACUM* (B) PRODUCED USING PURE DNA AND THE ASSAY

A sequence alignment (Figure 6) of the appropriate ITS1 region from various *Globodera* species shows that cross-reaction with the other non-PCN species *G. hypolysi* is unlikely.

A

	G pallida	*G rostochiensis*	*G tabacum*	*G millefolii*	*G hypolysi*	*G achillae*
***pallida* taqman set**	100	94	94	85	91	92
***rostochiensis* taqman set**	93	100	93	79	88	90

B

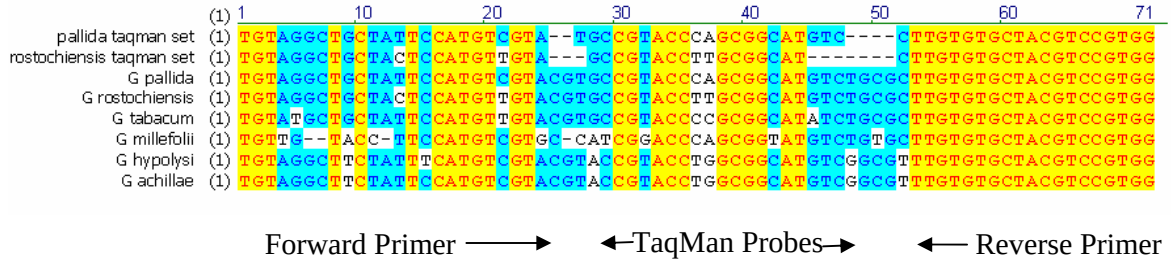


FIGURE 6 (A) DNA SEQUENCE IDENTITY (PERCENT) TABLE FOR CSL PCN TAQMAN ASSAYS AND APPROPRIATE ITS1 REGION FROM VARIOUS *GLOBODERA* SPECIES. (B) SEQUENCE ALIGNMENT OF SEQUENCES USED TO CONSTRUCT THE IDENTITY TABLE

We compared the CSL assay to the Plant & Food Research assay and found that the Plant & Food Research assays would only work with expensive Applied Biosystems master mix. The CSL assay works with this master mix but also with the standard CSL Core PCR reagents from less expensive suppliers. For this reason the CSL assay was selected for further development in the project.

Soil DNA extraction method

Direct soil extraction

Initial experiments were conducted (as part of Defra project HH3214TFV, but results are relevant here) to determine whether PCR inhibitors are co-extracted with DNA and also to determine the sensitivity of the DNA extraction method. A 1:5 serial dilution of *R. solani* sclerotia was prepared in extraction buffer. An equal proportion of sclerotial spike was added to samples containing soil and extraction buffer (soil dilution series) and to samples containing only extraction buffer (control dilution series).

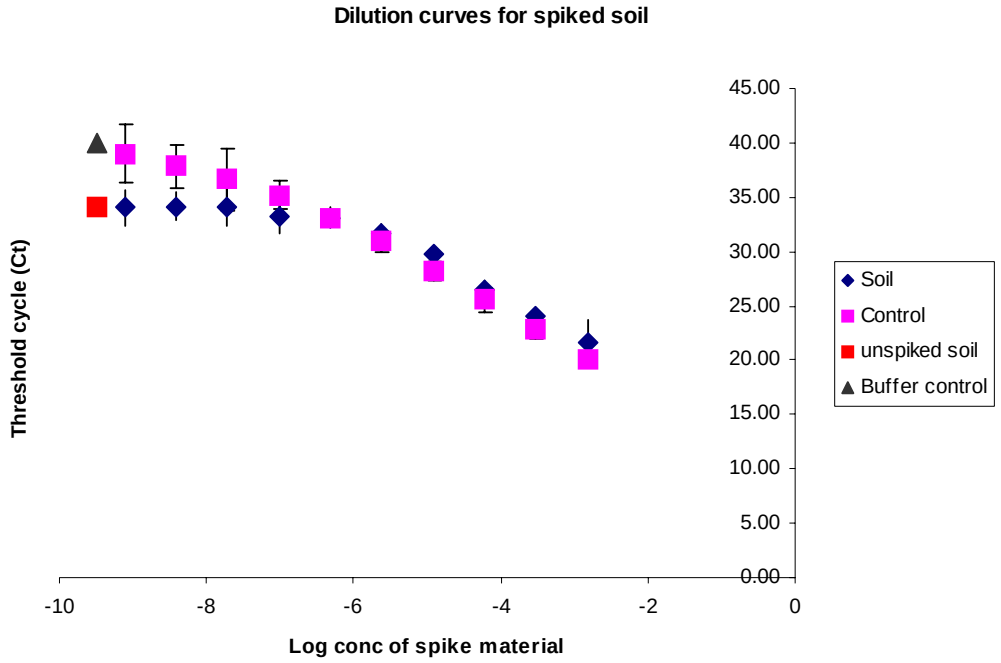


FIGURE 7. DETECTION OF *R. SOLANI* IN DILUTIONS OF EXTRACTION BUFFER AND SOIL (ERROR BARS ± STANDARD DEVIATION N=3).

The results (figure 7) show that *R. solani* AG2-1 was detected in the neat soil (red box figure 7), thus preventing the dilution reaching a lower quantity than that present in the soil sample. However, a straight-line was achieved, and although the error was greater for the soil sample, the results were comparable suggesting minimal inhibition of real-time PCR by extracts from soil.

This assay was then applied to soil spiked with PCN. Initially 100g of soil spiked with PCN cysts was extracted using the protocol described above. 100g of soil or 100ml water was mixed with 300ml soil extraction buffer, 12 balls and 2, 5 or 10 fresh *G. rostochiensis* cysts in a 500ml Nalgene bottle. These samples were then assayed using the CSL TaqMan assay. Figure

8 shows the results of this extraction. *G. rostochiensis* was detected in all the spiked samples with increasing amounts detected when more cysts were added. The results from the soil and water samples were similar suggesting that PCR inhibitors from the soil had not co-purified with the DNA.
Duplicate soil / cyst containing samples were disrupted and passed to a trained nematologist who was unable to identify any cysts or cysts debris in the homogenised soil using CSL traditional PCN extraction technique. This suggests that this method is able to completely disrupt PCN cysts.

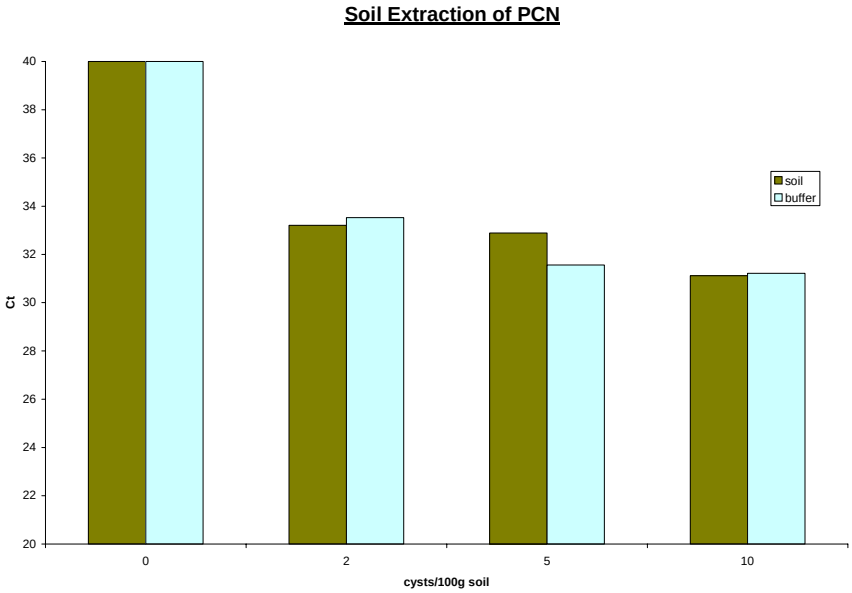


FIGURE 8. RESULTS OF *G. ROSTOCHIENSIS* TAQMAN PCR DNA EXTRACTED FROM 100G SOIL / BUFFER SAMPLES SPIKED WITH *G. ROSTOCHIENSIS* CYSTS.

Having shown that the technique is able to successfully disrupt cysts, experiments moved on to using eggs as the number of eggs in a cyst is variable. In order to show that the soil was being efficiently homogenised 250g of soil in 500ml buffer with 24 balls was spiked with 5 eggs /g *G. rostochiensis or G. pallida* eggs the soil extracted and 3 replicate samples of the homogenised soil extracted and assayed for PCN by TaqMan PCR. Figure 9 shows the results of the experiments

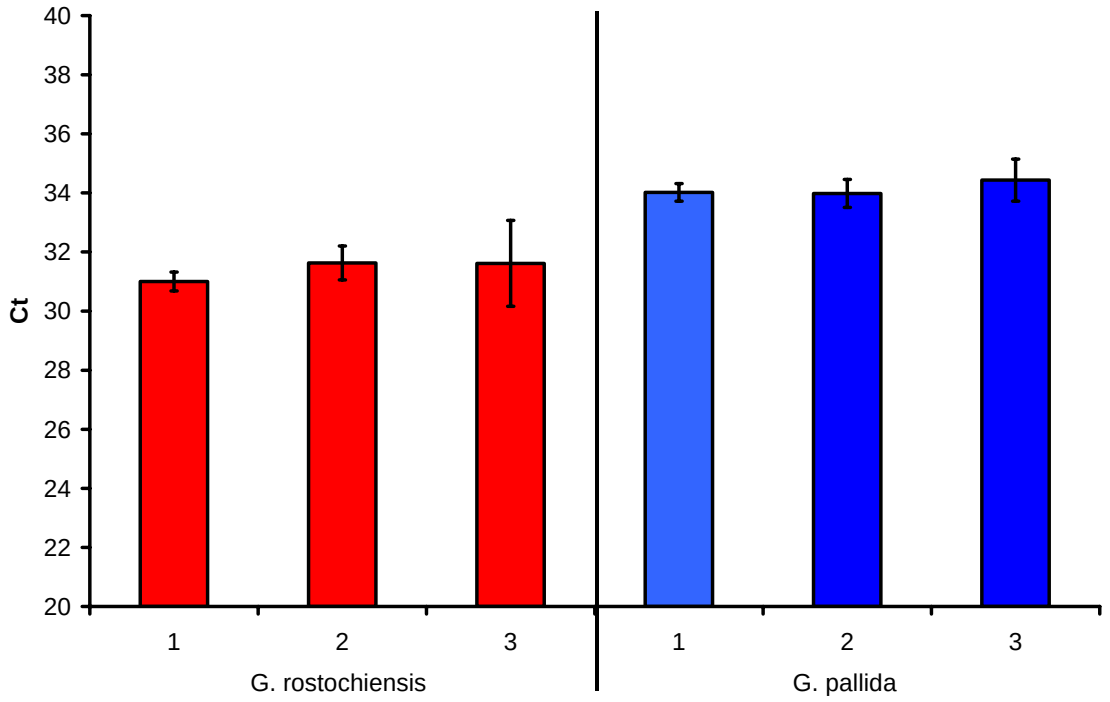


FIGURE 9 PCR DETECTION OF PCN DNA EXTRACTED FROM 250G OF SOIL. 3 REPLIACTE SAMPLES WERE TAKEN FROM EACH SAMPLE (ERROR BARS ± STANDARD DEVIATION N=3)

No significant differences were detected by ANOVA between the replicate samples showing that the soil was successfully homogenised.

During this project and other soil extraction projects we have successfully extracted DNA from numerous different soil types. One of the hardest is the clay soil found around CSL and this soil type has been used in all subsequent experiments.

To determine if the extraction was quantitative, known numbers of *G. rostochiensis and G. pallida* eggs were spiked into 250g samples of soil. The soil was extracted, assayed using PCR and a standard curve produced. Figure 10 shows the linear relationship between Ct value for *PCN and* eggs /g in the spiked soil. It also shows that this assay will detect PCN at levels below 0.5 eggs /g soil.

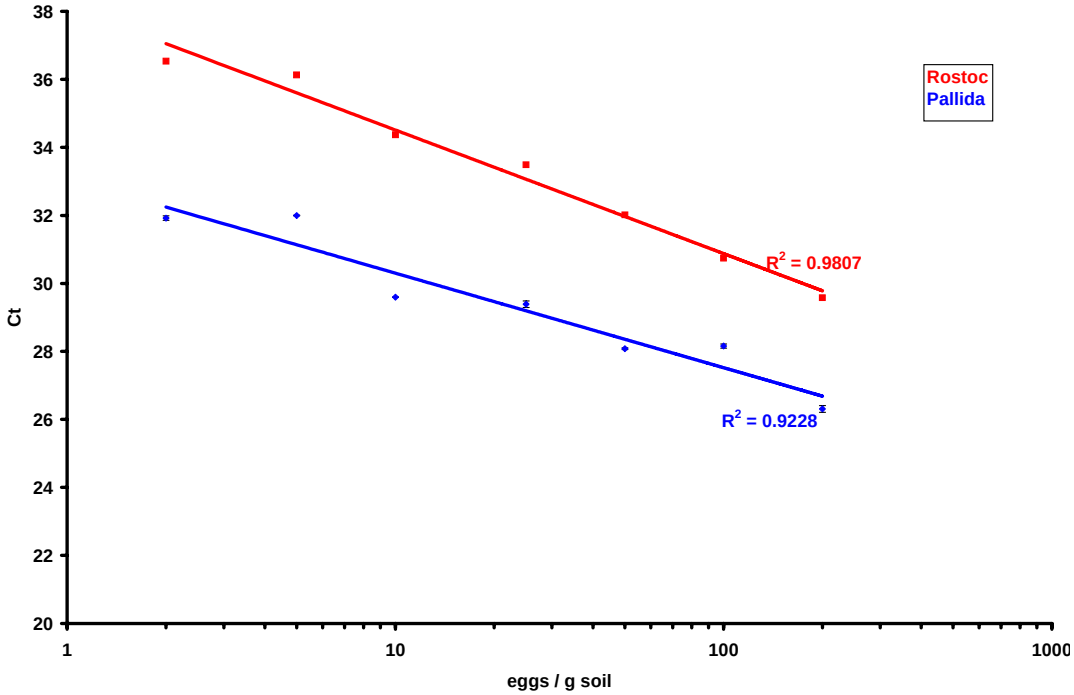


FIGURE 10. PCR OF KNOWN AMOUNTS OF *PCN* EGGS SPIKED INTO 250G OF SOIL AND EXTRACTED (ERROR BARS ± STANDARD DEVIATION N=3).

This 'eggs in soil' standard curve was then run against a dilution series of pure cysts DNA quantified using a Nanodrop spectrophotometer. Figure 11 shows the relationship between PCN DNA and TaqMan Ct. These assay standards will then run along side soil samples with unknown PCN content and the standards used to quantify the amount of PCN in the soil.

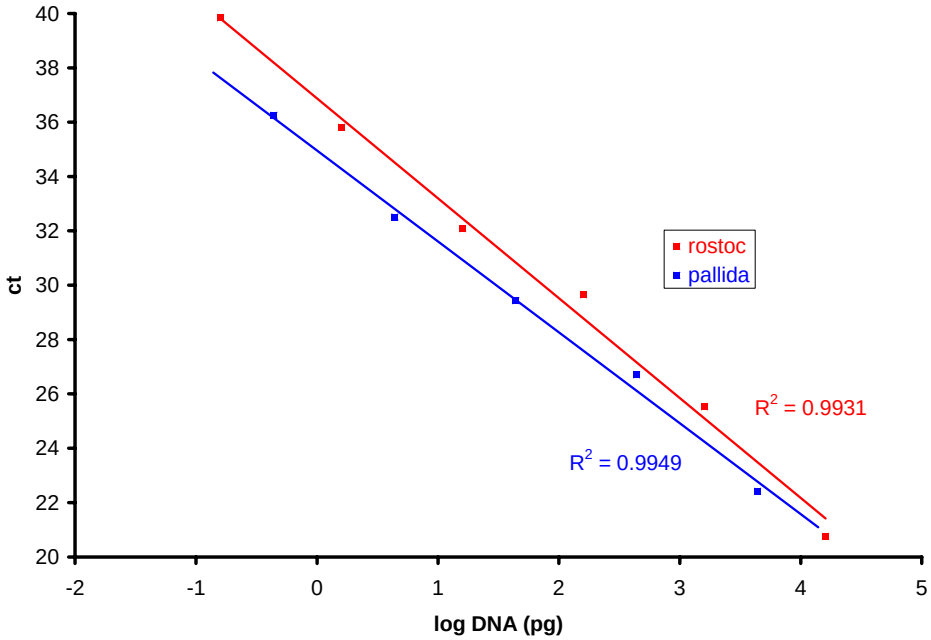


FIGURE 11 TAQMAN OF A DILUTION SERIES OF PURE PCN DNA

This work was carried out using 250g of soil. The new EU directive calls for the testing of up to 1.5kg of soil per hectare of field. To this end an assay was developed to extract soil from 1.5kg of soil. 1.5kg of soil, 3 eggs /g *G. pallida*, 2.5L of buffer and either 30 or 60 steel balls were placed in duplicate Nalgene 4.5L HDPE bottles, homogenised in the Automixer and 40ml

DNA extracted. Figure 12 shows the results of PCR to determine the presence of *G. pallida* DNA. The 60 ball extraction gave a good Ct and produced little variation between the two replicates. As will be discussed in the costings section although this assay works well it was found not to be cost effective and therefore further work was done using the 250g assay.

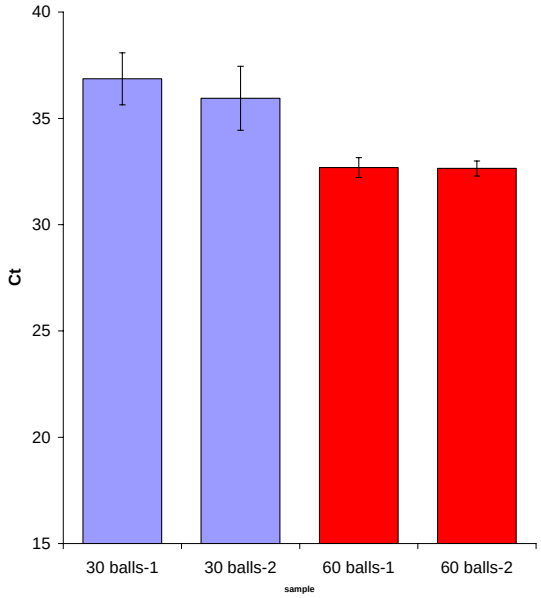


FIGURE 12. RESULTS OF TAQMAN ASSAY OF DUPLICATE EXTRACTIONS OF 1.5KG OS SOIL SPIKED WITH 3 EGGS / G *G. PALLIDA*. (ERROR BARS ± STANDARD DEVIATION N=3)

Parallel testing of field samples

Ten soil samples from potato fields were assayed for PCN with 150g of each sample being tested by Wye washer / microscopy method and 250g extracted using the direct soil extraction and TaqMan assay. Table 3 shows the cysts and egg counts obtained by each method.

TABLE 3. EGG AND CYSTS RESULTS OF PARALLEL TESTED FIELD SAMPLES. THE CYSTS COUNT WERE OBTAINED FROM THE WYE WASHER METHOD.

Sample	1	2	3	4	5	6	7	8	9	10
Total Cysts	75	52	89	26	26	18	3	84	41	16
Viable Cysts	51	16	62	14	12	6	2	62	37	7
Non-Viable Cysts	24	36	27	12	14	12	1	22	4	9
Species (TaqMan)	Pallida	Rostoc	Rostoc	Pallida	Pallida	Pallida	Pallida	Pallida	Pallida	Pallida
eggs/g (counts)	33	10	40	9	8	3	1	40	23	5
egg/g (Taqman)	346	275	161	46	27	0	0	39	30	25

Figure 13 shows a plot of egg counts obtained by the two methods. There was no correlation ($R_2 = 0.18$) between the values obtained by each method.

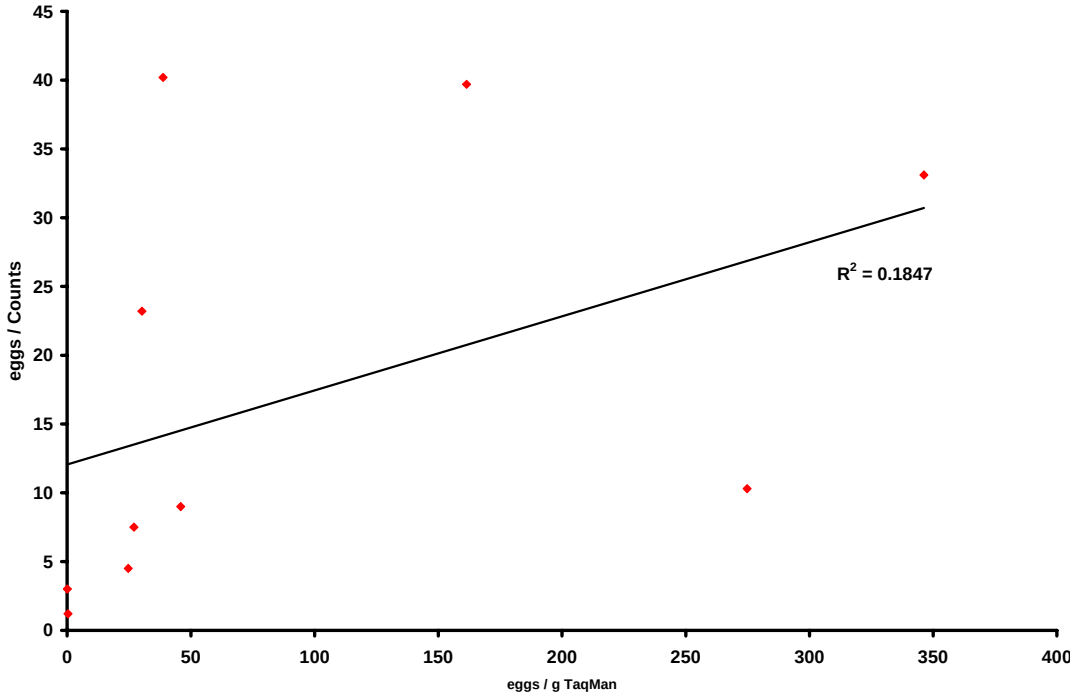


FIGURE 13 PLOT OF EGG COUNTS FROM FIELD SAMPLES TESTED BY TAQMAN AND WYE WASHER METHODS.

Internal controls (CSL)

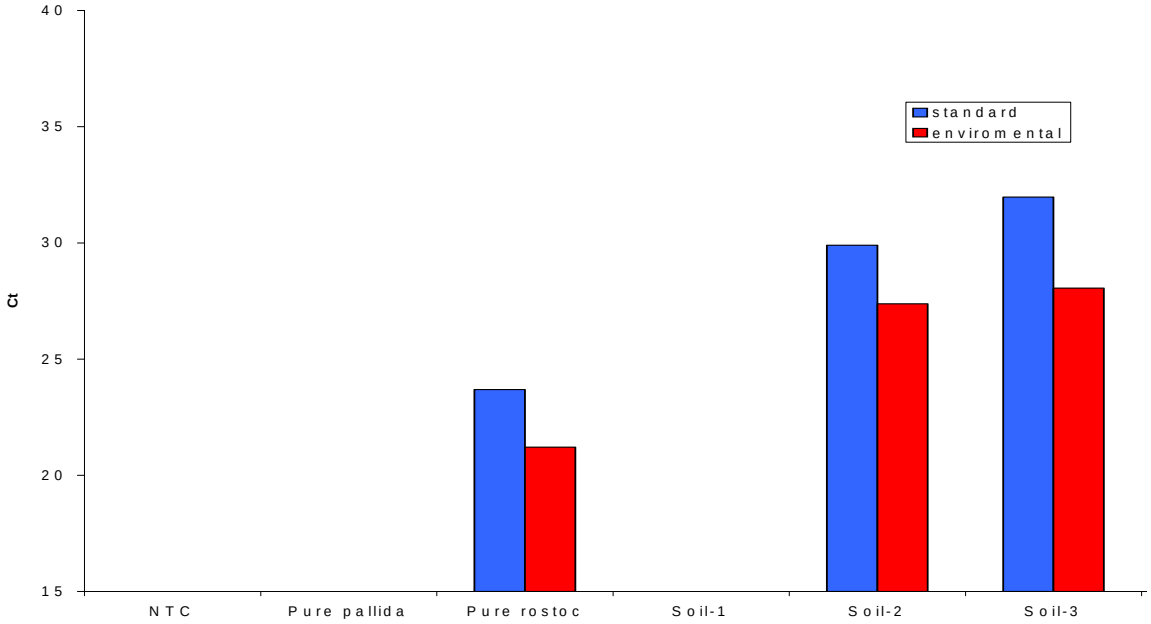
An artificial plasmid pLH-7 was constructed to act as an internal control for our extractions. A known amount (150ng) of plasmid is added to each soil sample prior to extraction and this was then detected using a specific TaqMan assay. If this assay fails or only gives a weak response then the extraction has failed. The plasmid construct was detected consistently in most, extractions. This method can be used for eliminating false negatives; where a sample contains the target organism, but due to a poor DNA extraction the target organism has not been detected.

Environmental TaqMan Master mix (CSL)

Occasionally samples fail to give positive results for the control plasmid or PCN even when a known amount of PCN had been added. Some samples also gave better Cts when the extracted DNA is diluted ten fold prior to amplification. This is indicative of PCR inhibitors and has always been a problem when performing PCR on samples extracted from soil.

Applied Biosystems have just released a new "Enviromental TaqMan master mix" designed specifically for soil samples. This was tested on a number of samples which had proved difficult to PCR in the past. Figure 14 shows the results of this comparison and that the environmental master mix was more sensitive even when testing the pure cysts DNA. In the case of soil sample 1 spiked with *G. pallida* the standard master mix failed to detect anything whereas the environmental master mix detected the spiked PCN DNA. We have now moved over to using this master mix for all our soil samples and will screen a collection of field samples with these reagents.

A



B

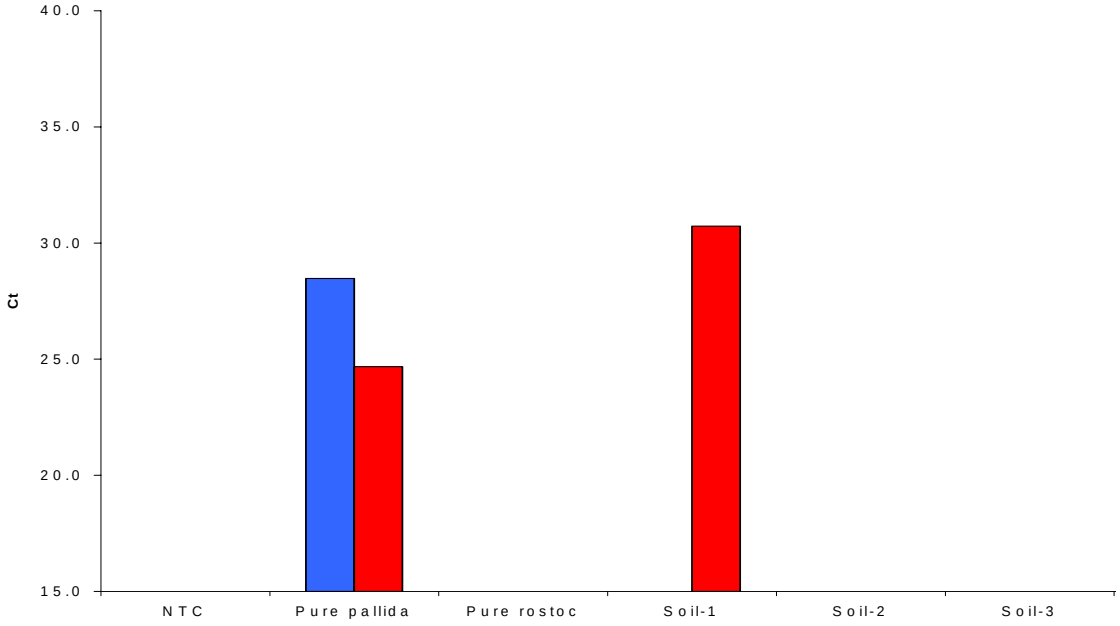


FIGURE 14. COMPARISON OF STANDARD PCR REAGENTS AND ENVIROMENTAL MASTER MIX USING TAQMAN ASSAYS FOR A: G. *ROSTOCHIENSIS* OR B: *G. PALLIDA* ON SAMPLES OF PURE CYSTS DNA OR SOIL SAMPLES SPIKED WITH EITHER *G ROSTOCHIENSIS* OR B: *G. PALLIDA*

Float testing

DNA has successfully been extracted from cyst material and a standard curve was produced using known quantities of eggs. Figure 15 shows the linear relationship between egg number and TaqMan results (in this case expressed as pg PCN DNA). This float standard curve was then run against a dilution series of pure cysts DNA (Figure 11). These assay standards will

then run along side float samples with unknown PCN content and the standards used to quantify the amount of PCN in the float.

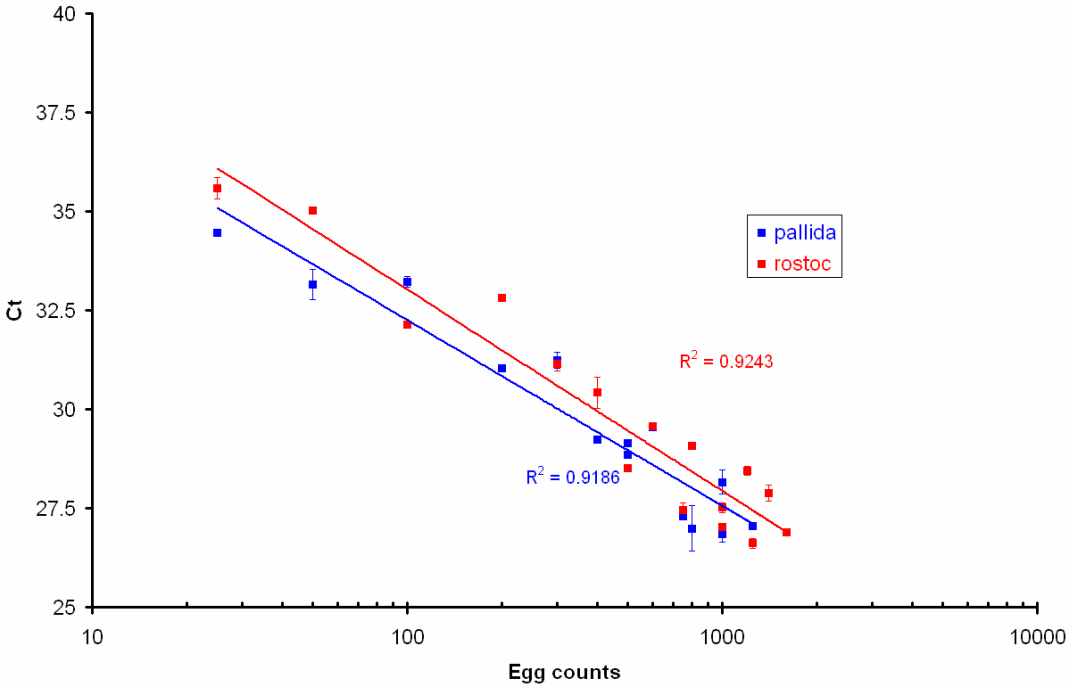


FIGURE 15. RELATIONSHIP BETWEEN EGG COUNTS AND PCN CT FROM SAMPLES OF EXTRACTED CYST MATERIAL.

Cost Analysis

The costs (in time and consumables) of different PCN extraction methods were calculated. Costs are included for 3 different soil sample sizes and 1 or 8 samples being run simultaneously. Costs shown in Table 4 are broken down into time (minutes) and consumables costs for the real-time PCR assays.

TABLE 4. COSTING FOR DIFFERENT PCN EXTRACTION METHODS

Fenwick Can

This method gives cyst counts but not egg counts or species determination. Consumable costs are zero as real-time PCR is not carried out on the sample(s).

		Fenwick Can					
		250g		500g		1500g	
		time (min)	cost (£)	time (min)	cost (£)	time (min)	cost (£)
1 sample	extraction from soil	40	0	70	0	190	0
	PCR						
	Column total	**40**	**£0**	**70**	**£0**	**190**	**£0**
8 samples	Extraction from soil	320	0	560	0	1520	0
	PCR						
	Column total	**320**	**£0**	**560**	**£0**	**1520**	**£0**

Fenwick Can (+Speciation 20 cysts)

This method gives cyst counts and is costed for speciation of 20 individual cysts but not egg counts.

		Fenwick Can (+Speciation 20 cysts)					
		250g		500g		1500g	
		time (min)	cost (£)	time (min)	cost (£)	time (min)	cost (£)
	extraction from soil	40	0	70	0	190	0
1 sample	PCR	180	48	180	48	180	48
	Column total	**220**	**£48**	**250**	**£48**	**370**	**£48**
8 samples	Extraction from soil	320	0	560	0	1520	0
	PCR	600	369	600	369	600	369
	Column total cost	**920**	**£369**	**1160**	**£369**	**2120**	**£369**

Wye Washer

This method gives cyst counts but not egg counts or species determination. Consumable costs are zero as real-time PCR is not carried out on the sample(s).

		Wye Washer					
		250g		500g		1500g	
		time (min)	cost (£)	time (min)	cost (£)	time (min)	cost (£)
	extraction from soil	20	0	20	0	40	0
1 sample	PCR						
	Column total	**20**	**£0**	**20**	**£0**	**40**	**£0**
8 samples	Extraction from soil	160	0	160	0	320	0
	PCR						
	Column total	**160**	**£0**	**160**	**£0**	**320**	**£0**

Wye Washer (+Speciation 20 cysts)

This method gives cyst counts and is costed for speciation of 20 individual cysts but not egg counts.

		Wye Washer (+Speciation 20 cysts)					
		250g		500g		1500g	
		time (min)	cost (£)	time (min)	cost (£)	time (min)	cost (£)
	extraction from soil	20	0	20	0	40	0
1 sample	PCR	180	48	180	48	180	48
	Column total	**200**	**£48**	**200**	**£48**	**220**	**£48**
8 samples	Extraction from soil	160	0	160	0	320	0
	PCR	600	369	600	369	600	369
	Column total	**760**	**£369**	**760**	**£369**	**920**	**£369**

Direct Soil Extraction

This method gives egg counts and speciation but not cyst counts

		Direct Soil Extraction					
		250g		500g		1500g	
		time (min)	cost (£)	time (min)	cost (£)	time (min)	cost (£)
	extraction from soil	180	12.5	180	20	240	50
1 sample	PCR	60	14	60	14	60	14
	Column total	**240**	**£27**	**240**	**£34**	**300**	**£64**
8 samples	Extraction from soil	180	100	180	160	360	400
	PCR	60	21	60	21	60	21
	Column total	**240**	**£121**	**240**	**£181**	**420**	**£421**

Hybrid Fenwick Can Extraction (egg counts)

This method gives egg counts and speciation using the newly developed float method but not cyst counts

		Hybrid Fenwick Can Extraction (egg counts)					
		250g		500g		1500g	
		time (min)	cost (£)	time (min)	cost (£)	time (min)	cost (£)
	extraction from soil	20		40		120	
1 sample	PCR	105	14	105	14	105	14
	Column total	**125**	**£14**	**145**	**£14**	**225**	**£14**
8 samples	Extraction from soil	160		320		960	
	PCR	150	21	150	21	150	21
	Column total	**310**	**£21**	**470**	**£21**	**1110**	**£21**

Hybrid Fenwick Can Extraction (egg + cyst counts)

This method gives egg and cyst counts and speciation using the newly developed float method

		Hybrid Fenwick Can Extraction (egg + cyst counts)					
		250g		500g		1500g	
		time (min)	cost (£)	time (min)	cost (£)	time (min)	cost (£)
	extraction from soil	40	0	70	0	190	0
1 sample	PCR	105	14	105	14	105	14
	Column total	**145**	**£14**	**175**	**£14**	**295**	**£14**
8 samples	Extraction from soil	320	0	560	0	1520	0
	PCR	150	21	150	21	150	21
	Column total	**470**	**£21**	**710**	**£21**	**1670**	**£21**

Hybrid Wye Washer Extraction (egg counts)

This method gives egg counts and speciation using the newly developed float method but not cyst counts.

		Hybrid Wye Washer Extraction (egg counts)					
		250g		500g		1500g	
		time (min)	cost (£)	time (min)	cost (£)	time (min)	cost (£)
	extraction from soil	15		15		35	
1 sample	PCR	105	14	105	14	105	14
	Column total	**120**	**£14**	**120**	**£14**	**140**	**£14**
8 samples	Extraction from soil	120		120		280	
	PCR	150	21	150	21	150	21
	Column total	**270**	**£21**	**270**	**£21**	**430**	**£21**

Hybrid Wye Washer Extraction (egg + cyst counts)

This method gives egg and cyst counts and speciation using the newly developed float method

		Hybrid Wye Washer Extraction (egg + cyst counts)					
		250g		500g		1500g	
		time (min)	cost (£)	time (min)	cost (£)	time (min)	cost (£)
	extraction from soil	20	0	20	0	40	0
1 sample	PCR	105	14	105	14	105	14
	Column total	**125**	**£14**	**125**	**£14**	**145**	**£14**
8 samples	Extraction from soil	160	0	160	0	320	0
	PCR	150	21	150	21	150	21
	Column total	**310**	**£21**	**310**	**£21**	**470**	**£21**

The best method to use depends on what information is required. The Wye Washer is cheaper than the Fenwick Can but both only give cyst numbers. For speciation, either morphological methods and/or PCR is required and this is most cheaply obtained using the float testing method developed as part of this project. If cyst number is not required then the Wye Washer can be combined with a direct float test which saves staff time as cysts don't need to be isolated and counted. If cyst number is required then these need to be isolated and counted before the cysts are extracted and PCR tested.

The direct soil method is more expensive than the Wye Washer hybrid method and this cost difference increases with the sizes of the extracted sample as the buffer used to extract the soil is quite expensive.

P&F Results

Development of duplex primers for quantifying PCR

New probes were designed and checked for sequence specificity by comparing for homology to all known sequences in the Genbank database using a BlastN search. This showed that the probes designed would possibly pick up some other species as well, namely *Punctodera punctata* and *Globodera tabacum*. The sequences for these two species of nematode were down-loaded from Genbank and compared with *G. rostochiensis* and *G. pallida* and an area of differences between all four investigated as the site to re-design the probes. Primers were redesigned as well to allow discrimination between all four species. Forward primers were designed to be species specific, reverse primer to amplify the DNA from both *pallida* and *rostochiensis* equally well. The fluorescent probes were designed to be species specific.

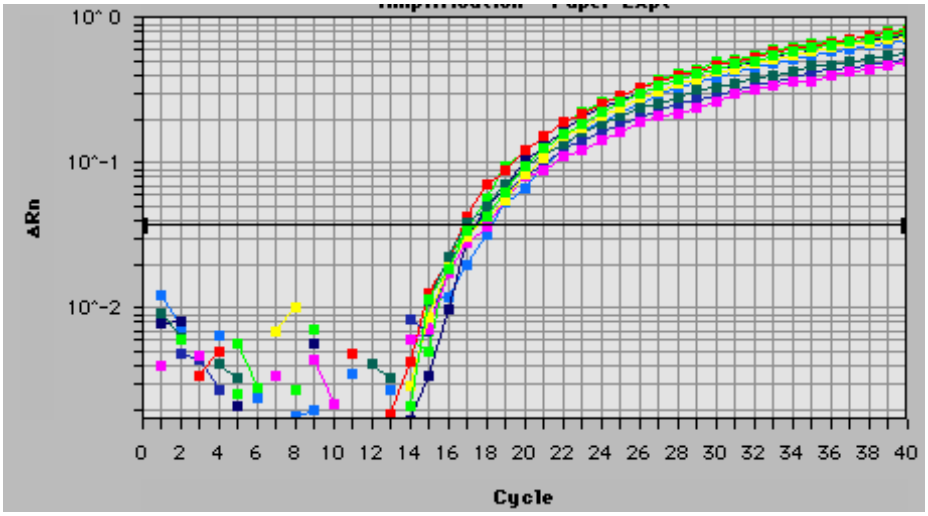


FIGURE 16: AMPLIFICATION PLOT FOR ROSTOCHIENSIS WITH / WITHOUT PALLIDA. THERE IS NO INTERFERENCE FROM PALLIDA.

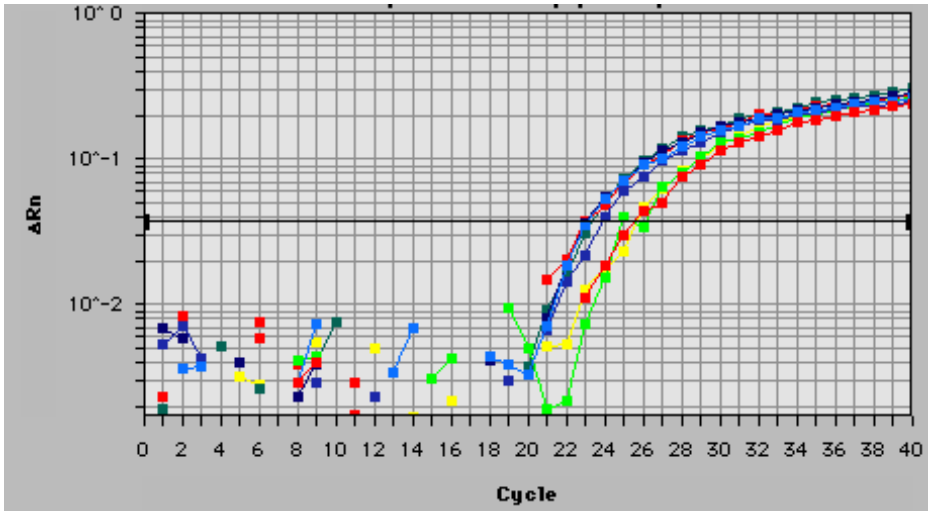


FIGURE 17: AMPLIFICATION PLOT FOR PALLIDA WITH / WITHOUT ROSTOCHIENSIS. ROSTOCHIENSIS IS INTERFERING AND CAUSING AN ADDITIVE EFFECT TO THE PALLIDA SIGNAL.

The *rostochiensis* primers and probe are specific for *G. rostochiensis* and are not picking up any *G. pallida* (Fig 16). The *pallida* primers and/or probe are not as specific and in mixtures are picking up *G. rostochiensis* as *G. pallida* (Fig 17). A high level of one species does interfere with detection of the other species if that is at low levels. When both species are at the same level, the detection is on target with expectations.

Field Trials

Nematode responses - Main trial

The multiplication rates (= Pf/Pi) were approximately x 8 for the range of Pi levels found within the potato plots (Figure 18). To assist with the interpretation of the data, Figure 18-1 also shows the theoretical x 1 and x 10 multiplication rates. At high Pi levels, a theoretical maximum final population is reached, beyond which a decrease in the population is known to occur, largely because of increasing competition between individuals and decreasing food supplies (Southey 1978; Been et al. 1995). This would be illustrated in Figure 18 as a drop in the multiplication rate below the x 1 line for high Pi levels. Clearly, such conditions did not

occur in this plot trial, and, instead, at all Pi levels there was a similar increase in the PCN population.

Potato yields were directly influenced by the PCN infestation. The infestation had a greater impact on the more marketable table grade, with a reduction in tuber numbers and weight being observed. Figure 19 illustrates the substantial decline in table grade yield with increasing Pi. This decline is apparent from Pi levels of 5-eggs/ml soil and greater. It is unlikely that an equilibrium density has been reached, as there appears to be no change in the rate of yield decline. The equilibrium density can be defined as the population at which no further plant response is observed. The highest level of Pi is 66 eggs/ml, at which point yields are approximately 30% of that obtained from the nematode-free plots. Clearly, this is a marked reduction in yield that is likely to affect the marginal profit for a grower.

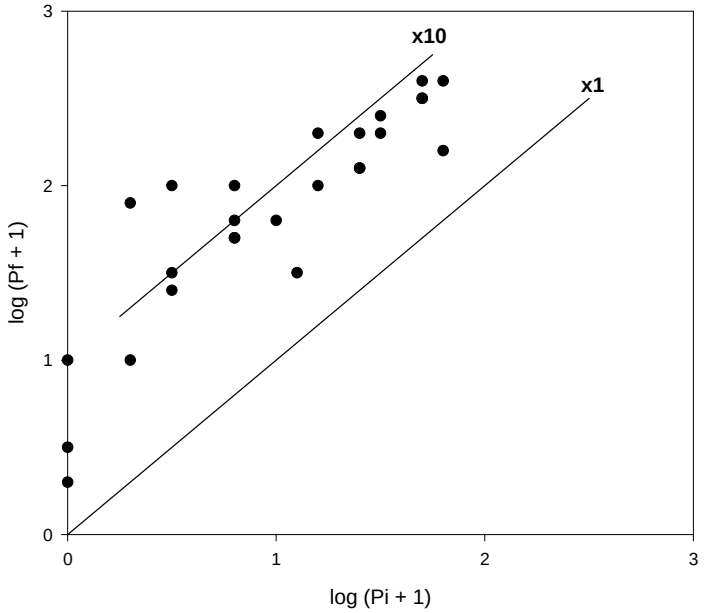


FIGURE 18. RELATIONSHIP BETWEEN THE INITIAL PCN POPULATION (PI) AND POST-HARVEST POPULATIONS (PF), WITH THEORETICAL X 1 AND X 10 MULTIPLICATION RATES ALSO SHOWN.

Results from the main trial clearly illustrate the effect of initial PCN populations on both the final populations and potato yields. To ensure PCN levels remain manageable, it is recommended that crop rotations and effective soil sampling techniques are employed as part of integrated control practices. By taking lightly infested fields with no groundkeepers out of potato production for at least 4 years, PCN populations will probably decline to undetectable levels. However, heavily infested fields, with groundkeepers present, even at low levels, will require a considerably longer period of time out of potato production before negligible levels of PCN are achieved. Consequently, if potato production is to continue on infested land, it will be necessary to grow more resistant cultivars, but there are few choices if *G. pallida* is present.

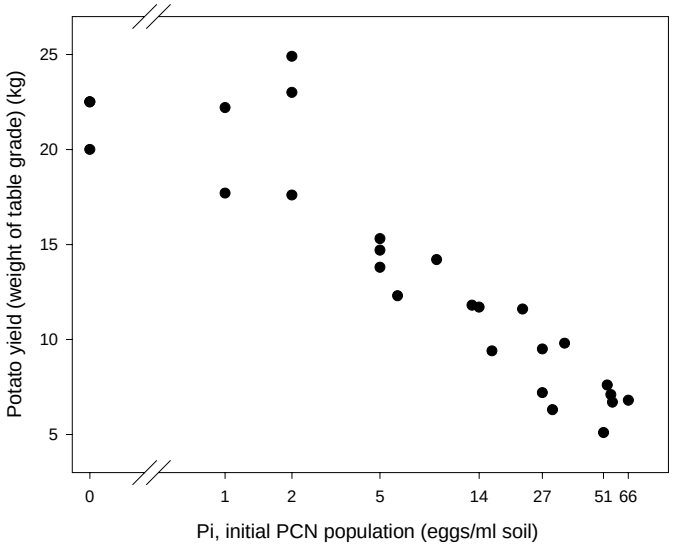


FIGURE 19. RELATIONSHIP BETWEEN YIELD OF ILAM HARDY (WEIGHT OF TABLE GRADE) AND POPULATION DENSITY OF PCN AT PLANTING TIME.

Soil sampling of fields should ideally be conducted at such intensity that the probability of detection is high. Recent Dutch research into soil sampling strategies for PCN detection show that a recommended sampling grid of 5 x 6 m provided a detection probability of 90% (Been & Schomaker 2000).

Discussion

During this research programme, work was carried out by P&FR staff in New Zealand on the existing assays, in addition a new duplex TaqMan assay was developed in GB. The latter allows the quantification and speciation of *G.pallida* and *G. rostochiensis* and only cross reacts with high concentrations of *G. tabacum* DNA. This is unlikely to be a problem in GB, as this species is not currently present here and is reportedly not a potato pest. If *G. tabacum* were to become common in the GB then a *G. tabacum* assay could be designed to specifically detect this organism.

The lack of correlation between the Wye washer and the direct soil extraction / TaqMan assay is disappointing but not unexpected. PCN cysts are known to cluster, so obtaining different results from two halves of a sample is to be expected. A more useful test of the assays would be to use samples of soil spiked with PCN. Spiked samples are regularly used at Fera with consistent accurate results for the training of new staff in the use of the Wye washer (data not shown). Figure 10 shows the soil extraction and TaqMan assay tested with spiked samples and the correlations between eggs spiked and Ct are very good (R^2>0.9). This suggests that both assays are accurate and quantitative. Some differences may be accounted for as the Wye washer only detects whole cysts whereas the TaqMan method will detect any PCN in the sample including any eggs that have already been released. The TaqMan assay also detects the presence of PCN DNA. Dead PCN eggs will contain DNA but in the harsh environment of the soil this DNA is unlikely to survive in a detectable form for very long. Therefore, except for soil recently treated with a nematicide, any PCN detected in the soil is likely to have come from a viable egg.

We have shown that the direct soil and float material assays are able to disrupt cysts and extract DNA in a quantitative manner and that the soil is completely homogenised in the assay. The direct soil assay allows the quantitative extraction of PCN DNA from soil sample sizes ranging from 100g to 1.5kg. However, the larger scale extraction was found not to be cost effective in this project it was proved to be effective and is available if required in future projects. The 250g assay was chosen as it was a good compromise between cost and scale allowing relatively high throughput in the lab.

The use of the Applied Biosystems Environmental Master mix seems to have removed any final traces of PCR inhibitors which were left behind by the direct soil extraction process.
A series of different options for PCN testing have been costed, ranging from the simple Fenwick Can and Wye Washer to direct soil detection. These options vary in cost and the information they provide. A solution can be tailored to the specific needs of the grower. CSL has recently gained UKAS accreditation for the use of the assay developed as part of this project for individual PCN cyst speciation and this has streamlined their service. Once the validation is complete the intention is to apply for UKAS accreditation for the hybrid Wye Washer / float test PCR method as this will greatly reduce the current costs. The method can be used to provide cyst as well as egg count data ha with only a small increase in costs. The direct soil method works well but is more expensive than the hybrid Wye Washer / float test. The extracted DNA can be tested for multiple pathogens and this assay opens the way for a cost-effective potato soil multi-pathogen screening service. The results of the new assays can be converted into eggs/g soil and should be given directly to growers, as data relating yield loss to egg counts is somewhat limited for UK conditions (see Annex 1).

Conclusions

This programme has developed a duplex TaqMan assay for the quantification and speciation of *G. pallida* and *G. rostochiensis*. Procedures which extract PCN DNA from between 100g and 1.5kg soil and PCN DNA from cysts/ float material have been developed. Protocols combining these methods, allowing tailoring of the methods in terms of cost and data required, have also been produced. This is particularly important as discussion with different end users has identified differing requirements.

Examination of the literature and consultation with other nematologists has shown that more work is required to allow an effective relationship between egg counts and yield loss to be determined for UK conditions. Information from field trials in New Zealand has been provided by Plant & Food Research to GB levy payers.

This study has shown that DNA-based methods are at the very least the equivalent of traditional monitoring techniques. Therefore the only impediments to adoption of the new technology are capacity and cost. Local laboratories must be able to demonstrate the capacity not only to conduct the tests but also the ability to process a large and sudden influx of samples during an incursion. DNA-based methods can be automated, and progress towards that end has been made in this study by the utilisation of real-time PCR and the trialling of robotic equipment. These measures will reduce labour inputs into testing and therefore reduce costs, which are expected to be considerably less than for traditional sample processing and testing.

The project has delivered newly developed test protocols that provide industry with tools to detect PCN in soil with a high degree of sensitivity and specificity

3. Glossary

Deoxyribonucleic acid (DNA): the chemical inside the nucleus of a cell that carries the genetic instructions for making living organisms

Polymerase chain reaction (PCR): a fast, inexpensive technique for making an unlimited number of copies of any piece of DNA. Real-time PCR is a sensitive, reproducible and accurate method to quantify DNA in samples. The amount of PCR product produced in every cycle step of the PCR reaction is monitored, either using fluorescent probes (e.g. TaqMan) or dyes (e.g. SYBR Green)

Primer: a short molecule of single stranded DNA used in a polymerase chain reaction

Probe: a piece of labelled DNA that is complimentary to a segment of DNA in the target organism (in this case *G. pallida* or *G. rostochiensis*).

Ct value: (Threshold cycle value) relates to how the amount of DNA present in a sample is measured. It is the PCR cycle at which a significant increase of the fluorescence signal is first detected. Ct values form the basis for quantitative comparison of individual PCR reactions. The smaller the Ct value is, the bigger the quantity of target DNA in a sample.

Duplex TaqMan assay: allows two target organisms to be detected/quantified in a single reaction- in this case it allows a sample to be tested for both *G. pallida* and *G. rostochiensis* at the same time.

Modified from glossary provided courtesy of National Human Genome Research Institute

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Annex 1: Relationship between PCN egg densities and yield loss: a review

A search of the literature and enquiries with relevant nematologists found relatively little recent published information for Great Britian relating numbers of eggs per g and/or cysts of PCN found in pre-planting soil samples with subsequent yields of those potato crops. Barker (2006) reported on trials investigating PCN management strategies in GB. He made an assumption that for every 5 eggs/g soil, one tonne of yield was lost from an average yield of 50 tonnes /ha, although he also stated that this was based on uniformly infested areas, which clearly does not happen in practice. However, amongst the report's findings were instances where Pf/Pi ratios did not seem related to yields for either treated or untreated areas. His investigation also compared Pi and Pf values with the yield of clones, but the comparative lifting date was only 42 days.

Further work was done by Keer (2006), who assessed Pf:Pi ratios and yields of different cultivars. He concluded that cultivars and irrigation greatly influenced PCN population dynamics, both in the presence and absence of a nematicide. None of the Pf:Pi ratios was less than 1 (Table A1).

Table A1. Pf:Pi values and yield data (after Keer, 2006)

Cultivar, with (T) or without chemical treatment	Pf:Pi values	Total Yield (t/ha)
Hermes	12.8	49.4
Hermes (T)	11.7	58.0
Lady Rosetta	6.0	47.5
Lady Rosetta (T)	3.4	64.2
Saturna	25.2	53.7
Saturna (T)	17.5	54.8
Maris Peer	26.6	26.0
Maris Peer (T)	14.1	55.8
Cara	50.3	80.0
Cara (T)	25.1	81.9
Sovereign	21.4	56.8
Cultivar, with (T) or without chemical treatment	Pf:Pi values	Total Yield (t/ha)
Sovereign (T)	11.4	73.3
Maris Piper	29.7	75.9
Maris Piper (T)	14.7	81.3
Estima	23.2	43.8
Estima (T)	13.3	58.1
Sante	5.8	55.7
Sante (T)	3.3	83.6
Desiree	37.6	45.6
Desiree (T)	27.2	55.3
Marfona	15.6	33.1
Marfona (T)	11.0	50.9
Pentland Dell	32.4	43.3
Pentland Dell (T)	26.7	54.5

Everest	3.0	76.0
Everest (T)	2.2	83.7
King Edward	19.9	48.9
King Edward (T)	12.7	62.2

First attempts at predictive modelling of the relationship of potato yields to population density of *G. pallida* highlighted the importance of cultivar tolerance and site effects (e.g. soil type and management) (Trudgill et al., 1996; Phillips et al., 1998). No modelling work specific to pure populations of *G. rostochiensis*, or for mixtures of the species, which occur in 33% of fields in England and Wales (Minnis et al., 2002), is available.

For growers with a significant infestation of *G. pallida* the Potato Council CD, 'Integrated control of potato cyst nematode (PCN) *Globodera pallida*, the white potato cyst nematode' (Anon, 2005) can be used. The CD provides a computer program that can be used to raise awareness of the changes in PCN populations that can occur under different cropping situations. It requires on-farm data to determine likely yield loss, such as soil type, cultivar tolerance or resistance, estimated length of rotation, etc. It requires eggs per g to be entered for an estimate of the effect on yield. There are no similar models for *G. rostochiensis.*

Recent work investigating the dynamics of a *G. pallida* infestation in The Netherlands (Van den Berg et al., 2006), done over three years on one site of reclaimed Polder soil, included the cultivars Agria, Bintje, Darwina, Santé, Turbo and Van Gogh. It compared the experimental results using an extended Ricker model with the model of Philips *et al.* (1991), which is similar to the one used in the Potato Council CD, and also concluded that both models required more research to improve accuracy. They found that each year of their experiment resulted in different estimates of model parameters. More research aimed at understanding the yield response of cultivars was recommended. In both cases it is believed more testing is required to enhance confidence in the models.

Been (personal communication) considers that the only model that is biologically and mathematically sound is the Seinhorst model (Seinhorst, 1998):

$$y = m + (1 - m)0.95^{P/T - 1}$$

where P is the density at planting of various nematode species
y is the plant weight (expressed as a proportion of plant weight at nematode densities $P<T$)
T is the tolerance limit, the largest density P that does not cause growth reduction
m is a constant smaller than 1

The model is believed to form the basis of the NemaDecide (www.nemadecide.com), an on-line decision support system in The Netherlands where an estimate of the numbers of eggs per 200ml is one of the parameters required for the production of an integrated control program. This has not been fully assessed by as part of this review as it is not known how relevant it is to UK conditions.

As might be expected, the damage threshold of PCN eggs/juveniles varies worldwide. In Venezuela, the Seinhorst model was used to estimate the tolerance limit of a new potato clone; this suggested a low threshold compared to European models (Anaya et al., 2005).

Trudgill *et al.*, (2003) reviewed the control of *G. pallida* and identified many factors responsible for the poor control of this species. He also highlighted the problems of different sampling regimes and for this reason often dismissed threshold values and simply advocated

action as soon as PCN species were detected. Further evidence of the difficulties in relating Pi or Pf values to yields if chemical treatments are used has been reported by Trudgill et al., (2003) and Barker (2006), who both failed to find consistent differences in yield between chemically treated and untreated potato cropping areas. Manipulation of crop management systems to involve not only the detection, speciation and level of PCN present, but also soil type, rotations, cultivars, irrigation and the prevailing weather are all factors that should be considered.

Only one unpublished report discussed the relationship of Ct values from TaqMan™ procedures with egg content in cysts, namely that produced by van der Weerden et al., (2001). This recommended more research was required to fully understand the relationship. This has been addressed in the Potato Council project reported here.

Summary
Any relationship between egg numbers prior to cropping and subsequent yield rely on crude estimates produced from a variety of sampling methods. However, it is clear that a given level of infestation is not the only factor involved in estimating yield loss. Attempts to produce models for *G. pallida* have met with some success but improvements are required, and more data is required to improve models developed in the UK. One option is a web-based version of a model, which would allow farmers to enter details including the actual final yield alongside the estimated final yield. This would allow further improvement of the model through comparison of the estimates of yield predicted by the model and actual yields in practice.

Annex 2: Background Intellectual Property research generated by Crop & Food Research prior to the Potato Council project and contributed to the project

TaqMan Assay

Development of duplex primers for quantifying PCN
Various levels of *G. pallida* cysts (100, 75, 50, 25, 10 5 and 1 cyst) were extracted from spiked soils to obtain the DNA. The resultant DNA was then analysed on the TaqMan™ in triplicate and an amplification plot of the DNA was generated (Figure A2.1; mean data for each level is presented).

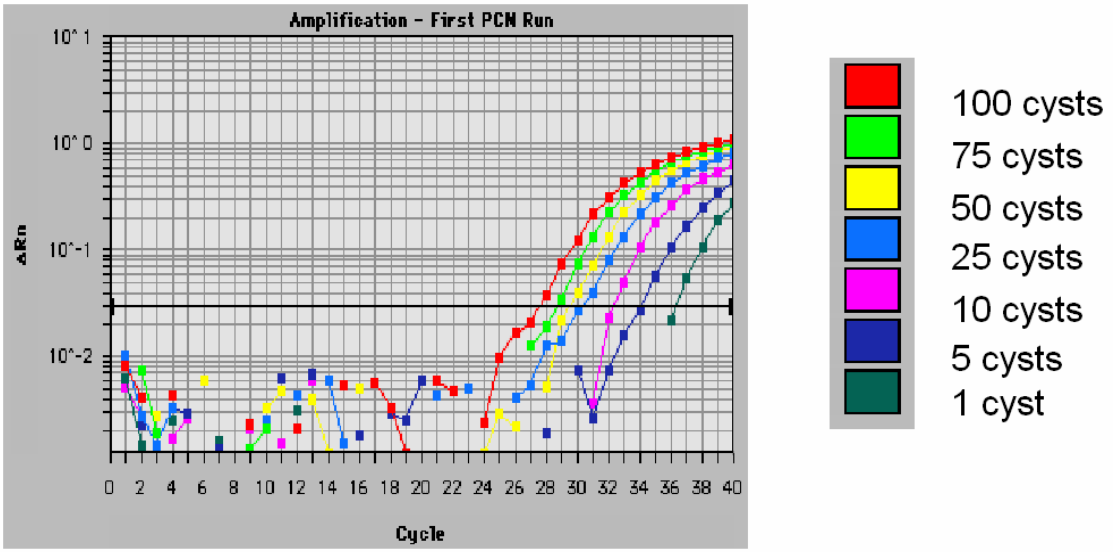

FIGURE A2.1: PLOT OF THE FLUORESCENCE SIGNAL DETECTED AS AN INDICATION OF THE AMPLIFICATION OF DNA. A LOW CYCLE NUMBER INDICATES HIGH CONCENTRATIONS OF DNA.

The TaqMan™ Ct value was then plotted against the log number of cysts extracted to allow a regression line to be fitted. The mean data of one run conducted in triplicate is presented in Figure A2.2. This produced an equation to estimate the relationship between the Ct value and the amount of cysts. This relationship can then be used to estimate the number of cysts contained in an unknown sample. The Ct values of egg content in cysts relating to field populations were determined. This involved counting all the cysts in a given soil sample, taking a portion of the cysts and counting the eggs contained, extracting DNA from remaining cysts and using the eggs contained in a portion of the cysts to obtain the number of eggs contained in extracted cysts. The DNA was put through the TaqMan as before and Ct values were subsequently related to the egg counts obtained. A weak relationship was identified between egg content, cyst numbers and Ct values.

The variability in the relationship between Ct values and egg count was caused by large uncontrolled variability in the number of eggs per cyst between samples.

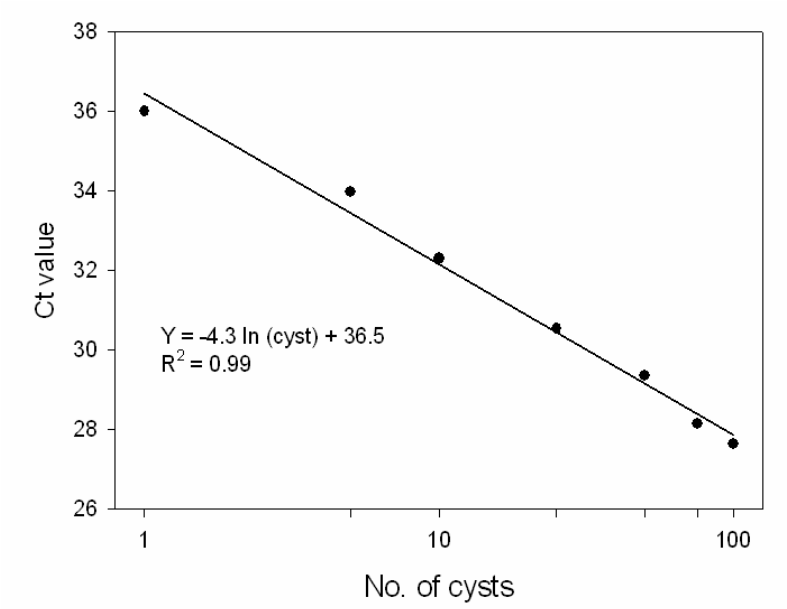

FIGURE A2.2. RELATIONSHIP BETWEEN CT VALUE FROM TAQMAN™ AND CYST NUMBERS (MEAN OF ONE RUN IN TRIPLICATE). FITTED LINE WITH REGRESSION EQUATION IS SHOWN.

This method of detection has been successful for the individual species of *G. pallida* and *G. rostochiensis*, with a corresponding minimum detection level of 1 cyst per 100g of soil sample.