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Location of project:	University of Worcester
Industry Representative:	Andy Richardson
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The results and conclusions in this report are based on an investigation conducted over a four 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.

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AUTHENTICATION

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

[Name]
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GROWER SUMMARY

Headline

- Laboratory and field-based tests have been developed to investigate the level of risk from clubroot.
- Improved methodology has reduced processing times and sample accuracy.

Background and Summary

Brassica crops are of high economic importance in the United Kingdom. One of the main diseases affecting Brassica crops is Clubroot, caused by the soil borne organism *Plasmodiophora brassicae*. Mild clubroot infections lead to slowed growth and delayed harvesting. Severe infections result in total crop failure. Infection is easily recognisable by swelling of root tissue causing galls and club shaped structures. Clubroot resting spores are capable of inducing disease in vegetable Brassica crops years after initial infestation of the soil.

Once soil has been contaminated clubroot spores can remain viable for up to 18 years. In the UK, growers of horticultural crops frequently rent land on a yearly basis, often with limited knowledge of previous cropping histories. The capability to forecast clubroot disease risk would be beneficial prior to contractual agreements made. The concentration of infestation of the soil by clubroot resting spores has been shown to directly affect the degree of clubroot infection. Resting spore concentrations in excess of 100,000 spores per gram of soil have been reported for severe and uniform disease expression on bait plants (Buczacki & Ockendon, 1978). Additional factors such as the conducive or suppressive nature of the soil may also influence the concentration required (Rouxel *et al*., 1988). The prevailing environmental conditions during key periods of the cropping period will also prove important in the risk and severity of the crop to clubroot disease development.

As the pathogen only grows within living tissues it is not possible to use standard dilution plating techniques to quantify numbers of pathogenic propagules within soil samples. Traditionally the use of bait plants grown under optimal disease conditions is required to assess potential risk of the disease in soils. This process has proved expensive and is carried out over a six to eight week period for disease to be visible on exposed plants. With the development of new detection methods based on molecular approaches the presence or absence of clubroot can be determined quickly in most soil samples. These tests are

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laboratory based and require a high degree of precision by the operator. However a competitive lateral flow device (lab on a stick / in-field test) has been developed and evaluated for use in UK commercial soils for the detection of clubroot resting spores. The device has been used by growers to detect clubroot spores within 10 minutes at epidemiological significant levels in artificially infested soils.

The lateral flow device has the potential to be used in soil by field growers and in water based systems such as reservoirs and irrigation lines (vegetable brassica propagators). A quantitative measurement of clubroot resting spore infestation can be made using the lateral flow test device when used in conjunction with a lateral flow reader and standard curve data. This means that a prediction on whether the crop is at risk may be determined and, at what level i.e low, medium or high risk.

Determining the clubroot resting spore number in soils using either a molecular or lateral flow test is an essential component in the development of an integrated disease management programme. Currently only two chemicals (cyazofamid – Ranman and fluazinam – Shirlan) approved for control of disease in potato crops have been demonstrated to have any potential for controlling clubroot in the field. However both these chemicals do not hold approval for clubroot control in vegetable Brassicas as their efficacy against clubroot has not yet been demonstrated. However Limex, a by-product of the British sugar industry (www.limex.co.uk) was found in HDC FV 349 to reduce the effect of clubroot infestation in soils on Brassica crop production over three consecutive years of the project. An application rate of Limex at or above 10 tons Limex ha-1 was found to be optimal in reducing clubroot disease.

The deliverables from this project are:

- Field mapping of clubroot resting spore distribution.
- Quick and cheaper testing of soils prior to planting the crop.
- Increased soil volumes assessed
- A choice of test formats (laboratory or ‘do it yourself’) and / or combination of both.
- Evaluation of test formats UK wide in different soil types for commercial uptake in 2015

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Financial Benefits

- The usage of the detection tests for risk assessment of clubroot will improve the control of this pathogen.
- Knowledge of resting spore concentration in soils will provide cultivators with information on optimal crop rotation patterns, varietal selections and appropriate control measures to prevent yield loss.

Action Points

Specific action points for growers at this stage in the project include:

- HDC levy payers can as part of this project have their soils tested for clubroot disease inoculum concentration. Contact a.wakeham@worc.ac.uk

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SCIENCE SECTION

Introduction

In year 1 of the two year project, two diagnostic tests were evaluated for the measurement of clubroot in soils UK wide. A molecular test has been developed and provides the measure/yardstick to assess an antibody-based lateral flow test (lab on a stick / in field test) for clubroot disease risk. The quantitative molecular PCR (polymerase chain reaction) requires a specialised laboratory facility, a lengthy soil preparation process to include DNA extraction from the resting spores in soil and is expensive given the low volume of sample assayed. Alternatively the lateral flow test has evolved to provide an inexpensive field test (approx. £5 / test) which shows potential for the estimation of clubroot resting spores in UK commercial soils and, without the need of a laboratory or skilled operator. Results reported in HDC FV 349, identified that out of 53 commercial soils assessed by each test there was statistical agreement between 30 of the soils. Of those soils which fell outside the confidence limit of the statistical analysis the majority were as a result of the lateral flow device over estimating the disease risk when compared to the molecular (q PCR) test. Ranking both sets of results into low, medium and high disease risk categories improved the correlation.

Sample inhibitors such as humic and fulvic acids, pesticide residues, organic material are all reported to inhibit the DNA polymerase enzyme (Kong *et al*., 2003). Equally, colloidal matter (to include clay) has a high affinity for DNA and was reported to affect PCR based measurement within environmental samples (Wilson, 1997; Theron & Cloete, 2004). The presence of these in field samples has the potential to affect the amplification process and test sensitivity (Cai *et al*., 2006; Lombard *et al*., 2011; Stewart-Wade, 2011). Similarly, soil inhibitors have been reported to affect antibody based assay systems. With this in mind the study has looked to evaluate each of the two tests across commercial soil types found in the UK for issues associated with soil inhibitors.

With a range of soil compositions likely, composed of differently sized aggregates and, with microbial populations that are not evenly distributed, a small sample volume for test analysis also requires consideration when developing a sampling strategy to provide a suitable ‘field’ test coverage. For this purpose work in Years 1 and 2 of the project will look to examine the potential to isolate and concentrate resting spores directly from soils. This is achieved by a process called ‘immunomagnetic fishing’. Specific antibody probes which recognise the resting clubroot spore are bound to coated magnetic spheres and mixed with

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the soil sample. On recognition to the spores the probe and sphere binds and are removed from the soil by means of a magnetic force. This process removes the need to air dry and mill the soils for uniform spore delivery in a limited sample volume for test application. Using immunomagnetic fishing the resting spores are delivered to either assay system in a concentrated form and free of soil inhibitors. Instead of a soil sample size of 0.25g assessed for clubroot resting spore presence, the magnetic bead assay will look at increasing soil sample volumes upwards of 50g lots. This should improve the sensitivity, reproducibility and robustness for each test format.

Materials and Methods

Advertise clubroot testing services and co-ordination of soils for sampling through the Brassica Growers Association

A Clubroot soils testing service was offered to HDC participating growers in the spring of 2013 through the Brassica Growers Association. Sample collection was organised through two sector leads and the soils sent to Worcester for processing by molecular qPCR and antibody based lateral flow devices (lab on a stick).

Assessment of commercial soils for clubroot resting spore concentration by qPCR (10g) and lateral flow (0.25g)

Soil samples were provided by Growers from 52 sites and considered as two sets. One set was defined as the 'Lincolnshire set' (36 soils) and the other as the 'Scottish set' (16 soils). Upon receipt the soil samples were booked in and given unique identifiers. The soils were air dried at room temperature with sample weight recorded daily and were considered fully dry once a stable weight had been maintained for two or more days.

After drying the samples were ground in a rota barrel (Balco Engineering Ltd. UK), with a metal bar at full power for 10 minutes. Soils were then sieved through a 2mm sieve and large stones discarded.

Soil DNA extraction was performed using a MoBio Powersoil 10g DNA extraction kit (MoBio Laboratories, Inc. Carlsbad, CA) following the manufacturer's instructions with amendment of the final elution step to 660µL elution buffer run through the filter three times to concentrate the DNA. A Polyvinylpolypyrrolidone (PVPP) DNA clean-up was subsequently performed on all DNA extracts using a protocol modified from Klemsdal *et al*., (2008): The PVPP, following rehydration with 500ul molecular H_2O, was left at room temperature for 5 min. Columns

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were then centrifuged for three min. at 1500 x g. Flow through was discarded, and these steps repeated. A further centrifugation of 4500 x g for 1 min was performed and columns placed in a clean 1.5ml low-bind microfuge. A 100µl DNA sample was applied to the surface of each PVPP microfuge and incubated at room temperature for five min. A final centrifugation step of 3500 x g for three min. eluted the DNA.

P. brassicae specific primers designed to amplify within 18S and ITS1 regions of *P. brassicae* rDNA were used in DNA amplification in a 20µl reaction mix comprising Lightcycler® 480 Sybr Green I Master (Roche Diagnostics, Burgess Hill, UK), primers, molecular grade H_2O and DNA. The soil DNA extractions were run against a series of standards and absolute quantification analysis, based on the second derivative maximum method, to quantify the number of *P. brassicae* spores g^{-1} soil (VanGuilder *et al.*, 2008).

The length of time taken to dry and process the samples ready for DNA extraction, along with the time taken to report the final result to Growers was recorded.

Based on the results further examination of the samples was then performed to assess the limitations of the 10g DNA extraction kit. Firstly, a comparison to a 0.25g soil DNA extraction kit was performed by extracting the DNA from the soils using the MOBIO UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Inc. Carlsbad, CA) following the manufacturers protocol. The vortex step was replaced with three runs on a FastPrep® Instrument (QBiogene, Irvine, CA) at speed 5.5 for 25s with samples resting on ice in between runs. Secondly, the DNA extracts obtained from both the 0.25g kit and the 10g were diluted ten-fold and one hundred fold into molecular grade H_2O and the results compared.

Quantification of the levels of *P. brassicae* was also determined by lateral flow device (LFD) following the protocol detailed in HDC Annual Report FV 349, May 2012.

Finally, to investigate the potential to speed up the processing and the overall time taken to report results to Growers, five fields were selected to undergo a comparison of soil processing methods. The method of drying and grinding the soil detailed above was taken to be the 'standard air drying' method. The other methods investigated were:

- 'Fresh processing' which extracted DNA from soil upon sample receipt regardless of the water content as detailed in the 0.25g MOBIO UltraClean Soil DNA Isolation Kit instructions.
- '25% soil water' in which the moisture content of the soil was measured using a SM200 Moisture Sensor and GP1 Data Logger (Delta T Devices, Cambridge, UK)

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and then brought up to 25% soil water content using distilled H_2O, followed by vigorous mixing of the soil using a spatula.

- '50% soil water' which was as for 25% soil water but the water content was brought up to 50%.
- 'Quick Dried' where aliquots were dried at 30^{o}C for 3 days in an oven.

Statistical analysis was performed on log transformed data.

Optimisation of the magnetic fishing process (IMF) for the isolation, collection and concentration of resting spores of P. brassicae from soils. To include studies to increase sample volume > 50g soil

Previous work had established an IMF protocol and detailed in HDC Annual Report FV 349 May 2012. This comprised 50g soil samples in 100mL sucrose solution, sieved through an 180µM sieve and then incubated with clubroot specific antibody (UW 249) for 1h. Thereafter anti-mouse IgM bound magnetic particles (MERK Chime, France) were added to the solution. Antibody labelled clubroot resting spores were later isolated by magnetic separation, processed by DNA extraction and their concentration measured by molecular qPCR. This work had been optimised using Merck anti-Mouse IgM magnetic particles which have since been discontinued. An alternative source of magnetic particles was identified in 2013 (BioMag® Goat anti-Mouse IgM, Bangs Labs Inc. USA). However this required re-optimisation of the assay due to differences in the antibody binding capacity of the magnetic beads and particle number of the two products.

Using the new bead set (Bangs Lab, USA), four 25g aliquots of artificially inoculated soil containing 1x10^{5} spores g^{-1} were suspended in 25mL 10% sucrose and vortexed (Genie 2, Scientific Industries, Inc. NY USA) at full speed for 10mins. Particulate material was then allowed to settle for 2 min. The aqueous phase of each tube was collected in to a 50mL centrifuge tube and a 100µL aliquot of clubroot specific antibody was added. After agitation for 1h, the magnetic anti-antibody conjugated particles were added at one of the following volumes; 50, 100, 200 or 400µL. The samples were agitated for a further 1h period. For each of the tubes, large soil particles were removed by passing the soil through muslin. Magnetic separation was performed over a 2 min. period and the retained material was collected in 1mL PBSTwC and stored at -20°C.

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This protocol was then applied to 20g of eight field soils which had tested negative for clubroot spores by qPCR and one soil which had tested positive as a positive control.

Later, six 50g aliquots of a soil with a moderately high clubroot spore level ($1x10^5$ spores g^{-1} soil) were processed using the protocol described. This time however the soils were mixed with 100mL 5% sucrose in 200mL Durans and rolled over a two hour period. After which soil particulate material was allowed to settle briefly, the liquid component decanted through an 180µM soil sieve and distributed equally between two 50mL centrifuge tubes, prior to storage at 4^oC overnight. To each tube, 200µL clubroot specific antibody was added and the tubes sealed and rolled over 1h. To each pair of tubes a treatment of 100, 250, 500, 1000, 2500, 5000µL BioMag® anti-Mouse IgM magnetic particles, were applied (equally divided between the two tubes). The tubes were rolled again for 30 mins. and placed in a magnetic field for 5 mins. The retained magnetic particles were re-suspended in 1mL PBSTwC prior to measurement of clubroot resting spore concentrations by q PCR.

As the new magnetic bead spheres provided a different binding capacity to the discontinued beads (i.e. those used in 2012), it was necessary to evaluate and optimise the magnetic sphere concentration for antibody binding to target analyte. This process was applied to a clubroot resting spore suspension free of soil.

A *P. brassicae* spore suspension containing $1.23x10^9$ spores/mL suspension was prepared by maceration of Brassica gall tissue in a blender. The solution was centrifuged at 2500 rpm for 10 min and the spore layer collected from the pellet. The spore suspension was diluted to $6.15x10^6$ spores/ml and divided into 50mL aliquots. The *P.* brassicae IgM mouse monoclonal antibody (UW249) was diluted to 0.1mg/mL in PBSTwC (PBS, Tween20, 0.1% casein). The antibody was applied to the 50mL spore suspension aliquots of 1, 5 or 10µL. The tubes were sealed with parafilm and mixed gently over 45 min to allow the antibody to seek and bind to homologous clubroot resting spore antigen. After which bead concentrations of 1, 2 or 3mL were added to the tubes and mixed for a further 30 min prior to magnetic separation. The supernatant of each tube was removed with care not to dislodge the magnetically bound spores from the side of the centrifuge tube. The retained material was re-suspended in 1mL PBSTwC and stored as previous prior to DNA extraction.

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Evaluate processes to improve the sensitivity of the lateral flow for quantification of IMF P. brassicae spores

One of the main limitations of the lateral flow device (lab on a stick) in quantifying IMF *P. brassicae* spore levels is that the antibody/antigen complex is a large structure when bound to the secondary conjugated anti-antibody magnetic particles. As a result of the high binding affinity and avidity of IgM antibodies (clubroot antibody) cross-linking to form clumping of spheres is likely. This limits the type of membrane and flow dynamics of the beads within the system. Enzymatic digestion of the IgM antibody, to isolate the smaller active analyte binding sites (i.e. the part which binds to the clubroot resting spore), could be conjugated directly to either magnetic or gold particles. This would provide a much smaller antibody labelled antigen carrier and provide greater flexibility in the lateral flow system used. This could improve significantly the sensitivity of the lateral flow system.

Three enzymes were investigated for their antibody digestion properties; pepsin, papain and trypsin. For pepsin and papain, the digestion was performed following pilot fragmentation protocols developed against IgG antibodies (Andrew and Titus, 2000). In brief, for pepsin, this comprises dialysing 2 mL of 3mg/mL purified IgM mouse monoclonal antibody (UW 249) against 200 mL acetate buffer (at both pH 4.0 and pH4.5) for 4 hours at 4^{o}C. The concentration of dialysed antibody was calculated using a Nanodrop 2000c UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc.) and adjusted to a concentration of 2.5 mg/mL. For both pH concentrations, 100µL antibody was transferred to each of 16, 1.5mL microfuge tubes. In eight of the tubes 100µL 0.1mg/mL pepsin was added to give an antibody/enzyme sample, while 100µL acetate buffer (pH4.0 or pH4.5) was added to the remaining tubes as controls. For papain, a 100µL 2mg/mL purified IgM (UW249) was aliquoted into 24, 1.5mL tubes. Two papain solutions at different concentrations (0.1mg/mL and 0.02mg/mL) were prepared in digestion buffer, and for each concentration 100µL aliquots were applied to eight of the 24 tubes that contained the purified IgM. To the remaining eight IgM antibody filled microfuge tubes, 100µL digestion buffer with no enzyme was added, and served as control samples.

All tubes were then incubated at either 37^{o}C (papain) or 4^{o}C (pepsin). For each, an antibody/enzyme and a control sample were removed at time points of 1, 2, 4, 6, 10, 24, 29 and 34h. At each time point the reaction was stopped. For pepsin this was by addition 40µL 2M Tris base, and for papain the addition of 20µL 0.3M iodoacetamide in PBS. The samples were vortexed and then dialysed against 1L PBS for 4 hours at 4^{o}C using a micro-dialysis chamber (Andrew *et al.* 1999). Samples were stored at -20^{o}C until use.

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For trypsin digestion the purified IgM (UW 249) was processed using an Immobilised TPCK Trypsin (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. In brief this comprises washing the immobilised TPCK Trypsin in digestion buffer before suspending in ~0.2mL digestion buffer. Purified IgM was diluted into the digestion buffer to a concentration of 1.7mg/mL and 500µL applied to a prepared immobilised TPCK Trypsin column. Eight samples were prepared in this manner, while in a further four samples the immobilised TPCK Trypsin was replaced by digestion buffer to serve as controls.

Samples were incubated over a 24h period and at 37^{o}C. The control samples were removed from incubation at time points of 0, 6, 16 and 24h. The antibody/trypsin samples were removed from incubation at 0, 2, 4, 6, 8, 16, 20 and 24h. For all samples the digestion process was stopped by centrifugation and the supernatants were retained and frozen at -20^{o}C until use.

Following the digestion process the samples were run on 8% non-reducing SDS PAGE gels using a discontinuous gel protocol detailed in Gallagher (2007) and 5X sample buffer. Gels were cast using the Mini Protein II Electrophoresis Cell (BioRad, USA) and run using a Power Pac 300 (BioRad, USA). Samples were run for between 35 min and 1 hour at either 150V or 200V, and a Multicolour High Range Protein Ladder (Thermo Fisher Scientific Inc.) was included for determination of antibody fragment size. Gels were stained in Coomassie Blue (Coomassie, Methanol, Acetic Acid) for at least 2h and de-stained overnight before imaging on a BioSpectrum MultiSpectral Imaging System, (UVP, USA).

The samples were then transferred to nitrocellulose membrane using a Mini Trans-blot® Electrophoretic Transfer Cell (BioRad, USA) at 30V for 1h. After which the membrane was blocked with 5% skimmed milk powder in TBST pH7.4 (Trizma base, NaCl, Tween20, distilled H_2O) for 1h, before washing three times with TBST and probing with Goat anti - mouse IgG+IgM (Heavy and Light chains, Sigma-Aldrich, USA) peroxidase conjugate (Thermo Fisher Scientific, Inc.). After a further wash step the membrane was developed with 3, 3', 5, 5' TMB liquid substrate systems for membranes (Sigma-Aldrich, USA). The nitrocellulose membranes were air dried and photographed. Immobilised and stained protein bands were identified and selected on both the cold pepsin and the trypsin digest membranes. These protein bands were estimated and excised in their native form from earlier gels in a minimal volume of buffer. To confirm successful 'protein band' isolation, the collected gel extracts were in part processed by gel electrophoresis. Protein band presence was then determined by staining with Coomassie blue as previously described.

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Liaise with Growers to determine crop outcome based on clubroot disease prediction

Feedback was obtained where possible from Growers to determine the crop outcome based on the disease prediction, generally this was in personal communication verbally or via email. It was difficult to determine a true comparison as not all the soils were to be planted with Brassicas and / or the history was unknown. Where a medium / high risk was provided this was thought to be accurate although there was a feeling that in general the tests were under predicting actual risk.

Results

Advertise clubroot testing services and co-ordination of soils for sampling through the Brassica Growers Association

After advertising and liaison with Growers the soil sampling was co-ordinated across two areas: Scotland and Lincolnshire. A total of 52 sites were initially received and considered as two sets depending on their source region. These were processed as detailed in the methods section and the results are included below. A further six soil samples were received several months after the main bulk of samples were completed. These were processed based on the findings of the two main sets and the results reported directly to the Grower (these six results will be detailed with the second year's data of the final report).

Assessment of commercial soils for clubroot resting spore concentration by qPCR (10g) and lateral flow (0.25g)

The first results reported to growers were based on the air dried, ground and sieved soil samples processed using the 10g soil DNA extraction kit, and are shown in Figure 1. Out of 52 soils tested, 18 tested positive for *P. brassicae* spores. The maximum level of *P. brassicae* spores in the Scottish soils was 2.49×10^4 spores g^{-1} soil, and 1.42×10^5 spores g^{-1} soil in the Lincolnshire soils. The mean spore level across both sets of soils was 8.15×10^3 spores g^{-1} soil.

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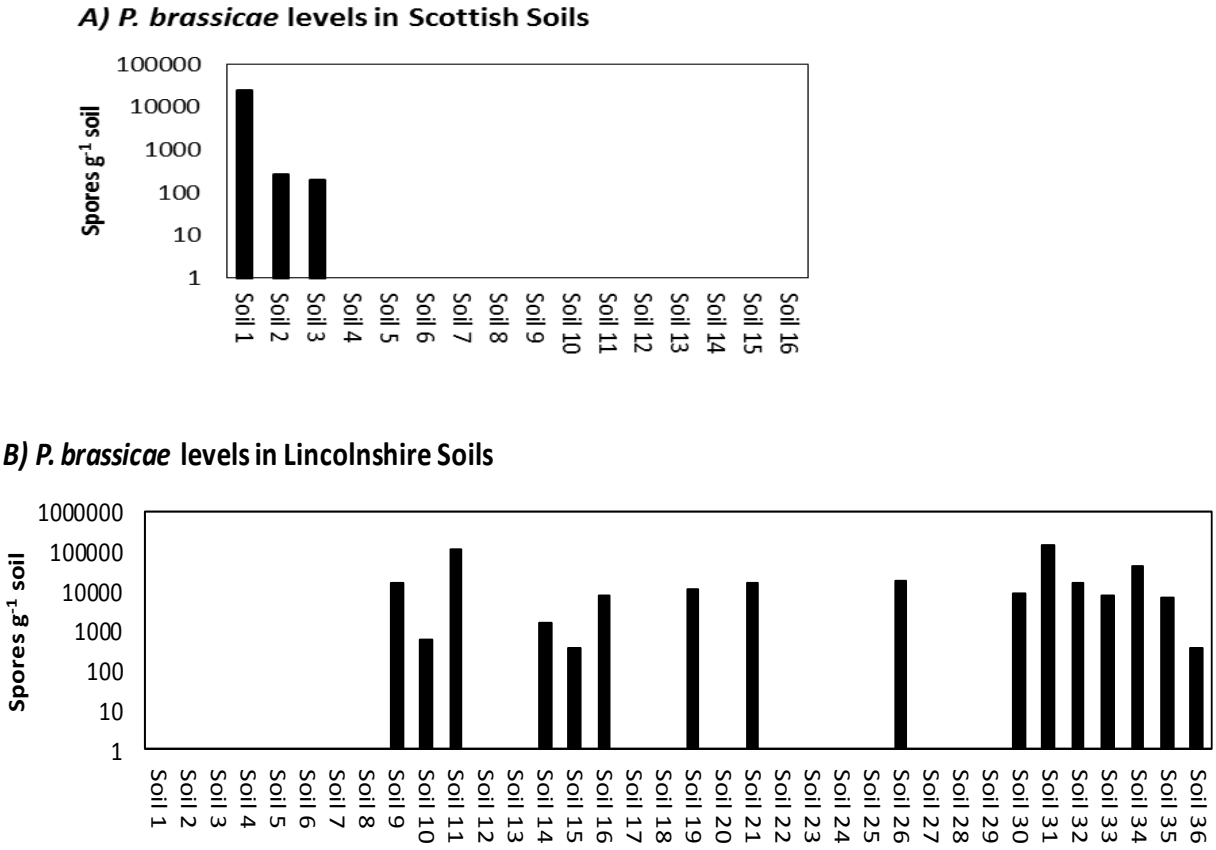

Figure 1. Initial levels of *P. brassicae* reported in Grower provided soils from Scotland (A) and Lincolnshire (B), following extraction with the Power Soil 10g DNA Extraction Kit.

The mean length of time needed to air dry the soil prior to DNA extraction was 19 days, with a maximum drying time of 26 days and the minimum 14 days. The maximum time to complete grinding, sieving and DNA extraction of the soils was 33 days, the minimum was 6 days and the mean was 26 days. From the time at which all DNA extractions were completed, the results were obtained by qPCR and sent to the growers within 1.5 days. Therefore the maximum time taken to turnaround results to growers was 60.5 days with a mean turnaround time of 40 days.

After initial result reporting, the DNA extracts from the 10g soils were diluted and this increased the number of positive soils in the Scottish set from three to 15. There was no effect of diluting the DNA on the number of positive soils in the Lincolnshire set. The effect of the DNA dilutions in the Scottish samples can be seen in Figure 2.

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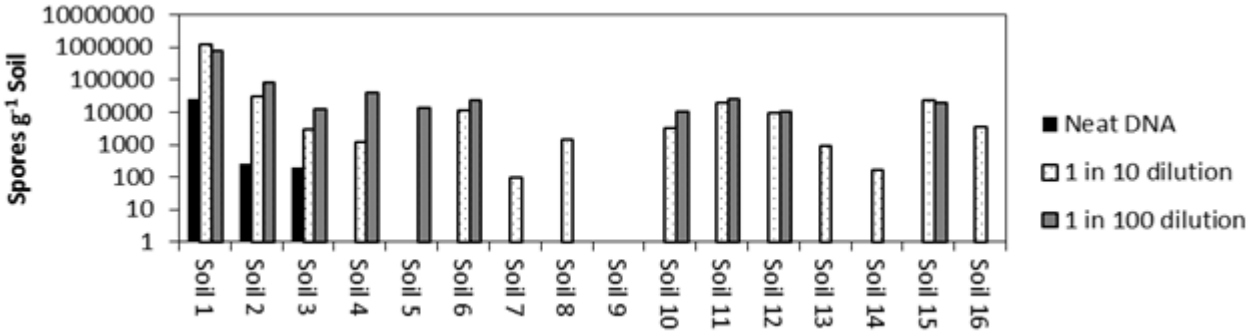
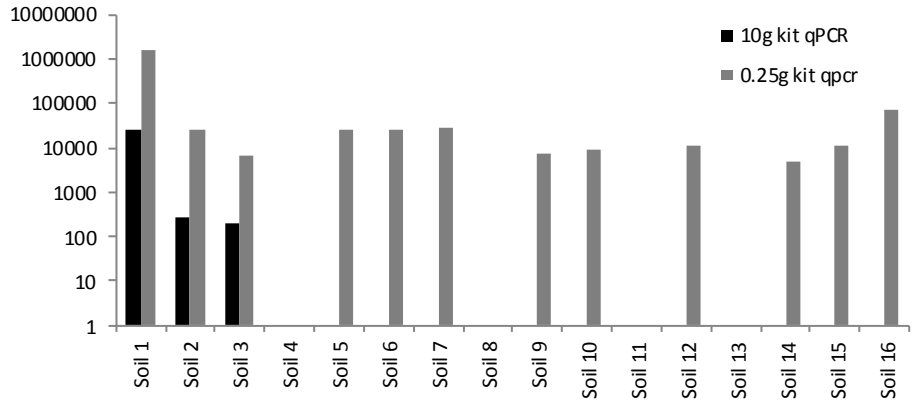


Figure 2. The increased number of *P. brassicae* positive soils following dilution of the DNA extracts in Scottish soils.

The soils were also extracted for DNA content using a 0.25g soil DNA kit. The neat DNA (undiluted) results were compared with the neat DNA 10g kit. An increase in the number of *P. brassicae* positive soils in both the Scottish and Lincolnshire soil sets (Figure 3) was observed with the 0.25g kit (neat DNA only). An additional eight soils were positive in the Scottish set and another 10 positive soils in the Lincolnshire set. Two soils which were positive with the 10g kit did not display any spores with the 0.25g kit in the Lincolnshire samples.

A) Scottish soils extracted with two soil DNA extraction kits



B) Lincolnshire soils extracted with two soil DNA extraction kits

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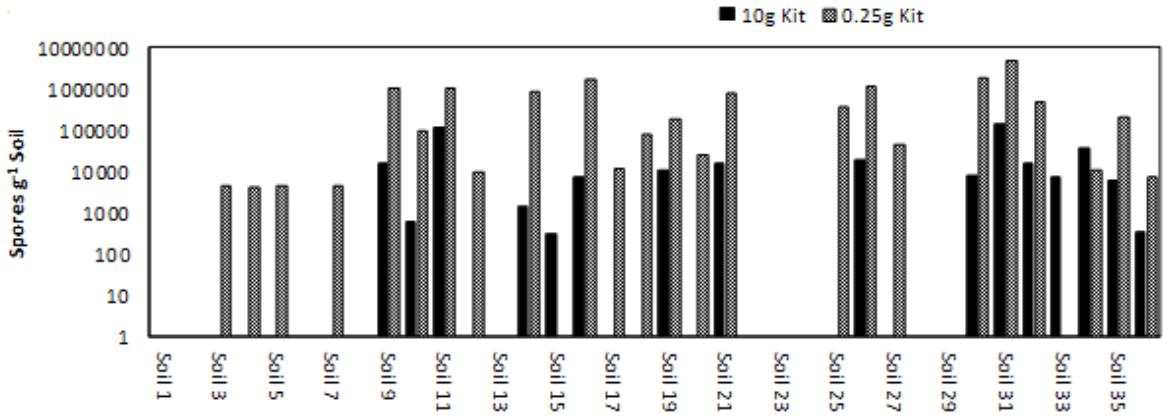
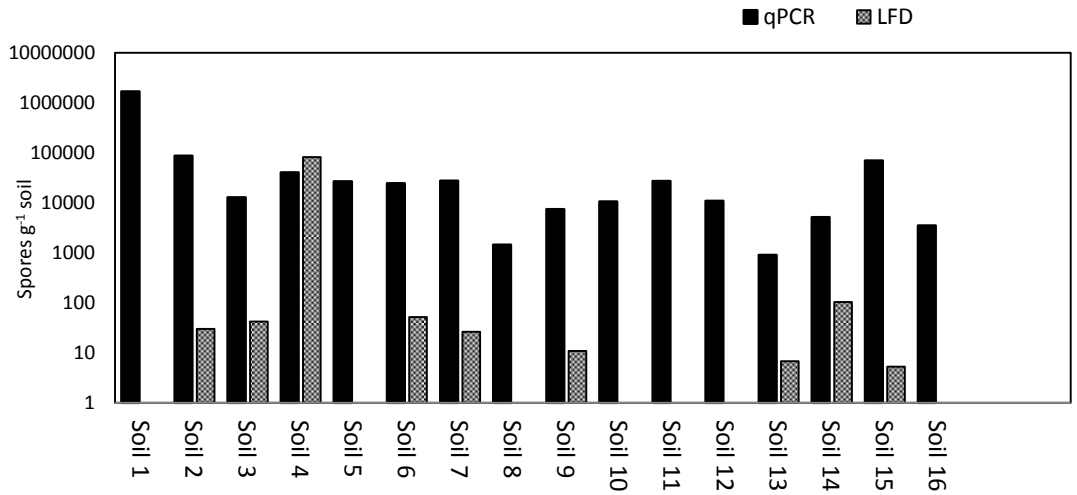


Figure 3. The Difference between *P. brassicae* levels in Scottish (A) and Lincolnshire (B) soils extracted with two soil DNA extraction kits (PowerSoil 10g DNA Extraction Kit and UltraClean 0.25g DNA Extraction Kit)

As a result of the DNA extract dilutions and extraction kit investigations, a final soil result was generated for each soil, based on the highest number of spores given from the DNA based methods (10g DNA extraction kit and dilutions or 0.25g DNA extraction kit). These results were compared with the 'on-site' LFD test (figure 4). In total, 41 out of the 52 soils tested were positive for *P. brassicae*. By LFD, fewer soils tested positive for *P. brassicae*. No dilutions were made for the LFD test which in turn could have been affected by soil inhibitors. Only 0.25g soil was assessed by LFD.

A) Final *P. brassicae* levels in Scottish Soils; qPCR and LFD



B) Final *P. brassicae* levels in Lincolnshire Soils; qPCR and LFD

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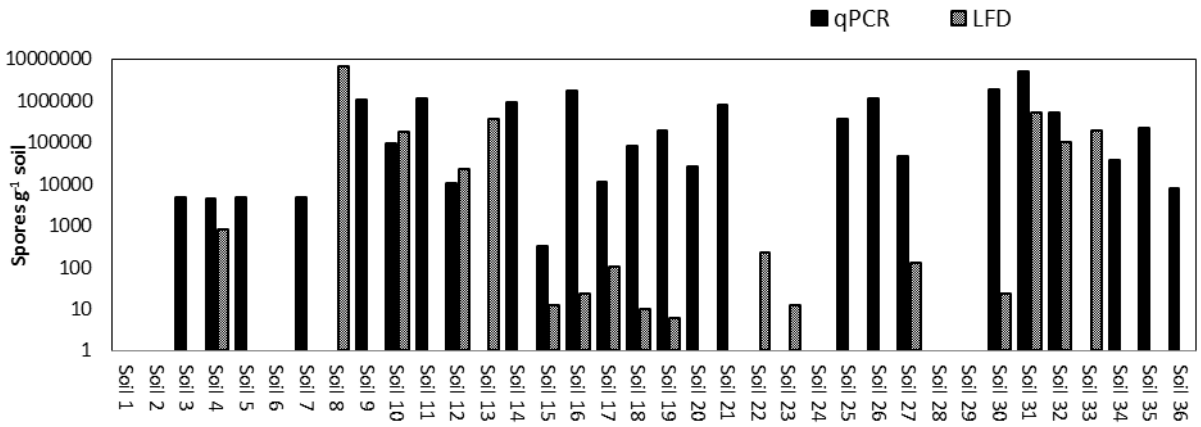

Figure 4. The final results of *P. brassicae* levels in Scottish (A) and Lincolnshire (B) soils determined from the highest levels given by one of the following methods; PowerSoil 10g DNA extraction kit and dilutions of the DNA extracts, and the UltraClean 0.25g DNA extractions, in combination with the results of the single LFD test (0.25g).

To examine whether it was possible to increase turnaround time and to provide a useful commercial service to growers, several different processing methods were tested on five soils, the results of which are shown in Figure 5. After data transformation analysis by two-way ANOVA with replication showed that the difference in processing methods was significant; $F(3,80)=7.43$, MSE = 3.66, $p=<0.001$, as were the different soils; $F(4,80)=28.32$, MSE = 13.95, $p=<0.001$, and the interaction between them; $F(12,80)=5.59$, MSE = 2.76, $p=<0.001$. Examination of the transformed means showed that the 25% Soil Water samples gave the highest levels (Mean = 4.14, SD = 1.47), followed by the 50% Soil Water samples (Mean = 4.04, SD = 1.18). The air dried samples gave the lowest levels (Mean = 3.12, SD = 1.17).

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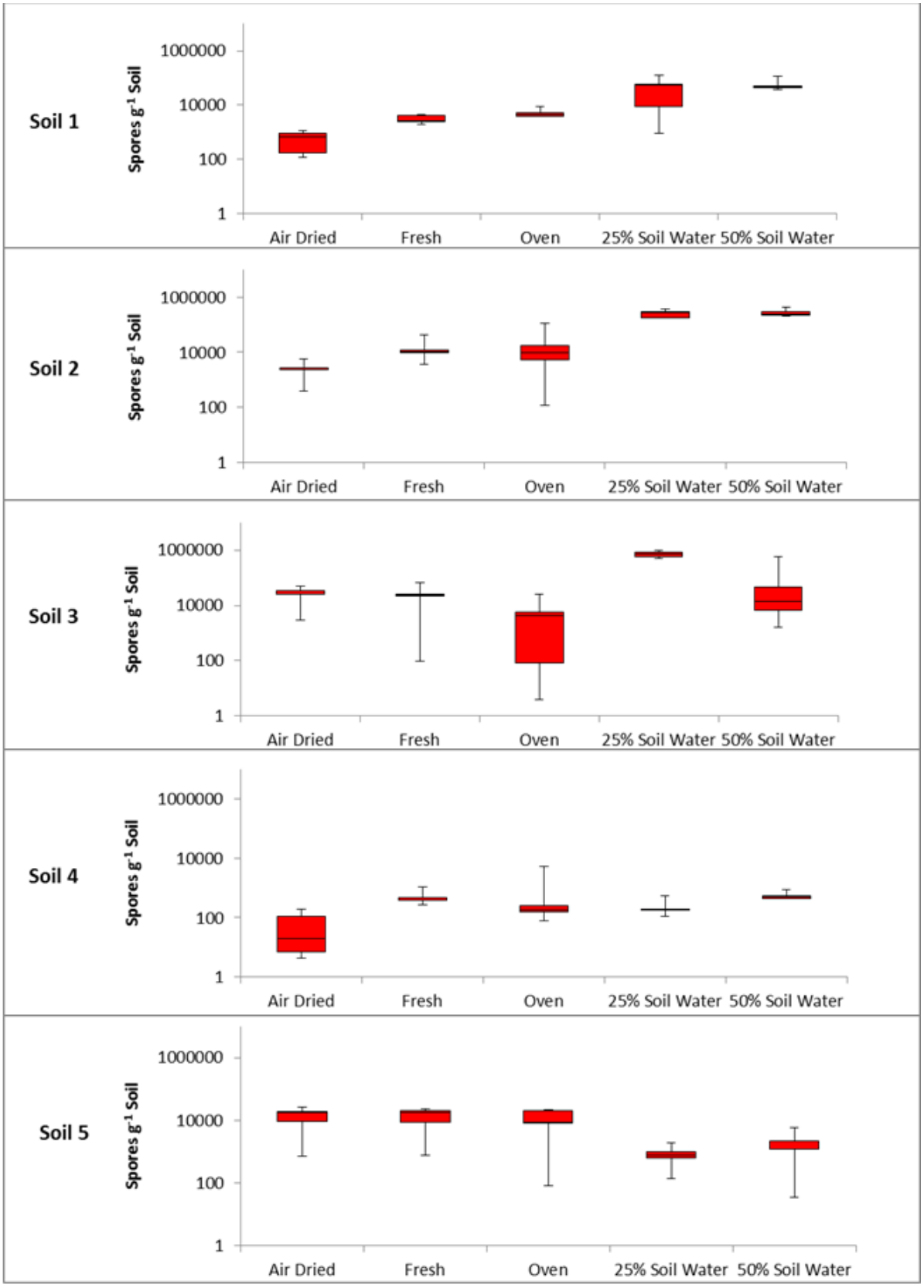

Figure 5. Graphs to show the different levels of *P. brassicae* reported based on different processing methods across five soils with varying degree of clubroot infestation.

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Optimisation of the magnetic fishing process (IMF) for the isolation, collection and concentration of resting spores of P. brassicae from soils. To include studies to increase sample volume > 50g soil

With the initial optimisation experiment it was found the maximum number of spores extracted was $5.85x10^5$ spores g^{-1} (Figure 6). The soil had been inoculated with $1x10^6$ spores g^{-1} soil and so contained $2.5x10^7$ spores in total. This demonstrates that the beads were not successful at concentrating the number of spores, although they were extracting a good proportion.

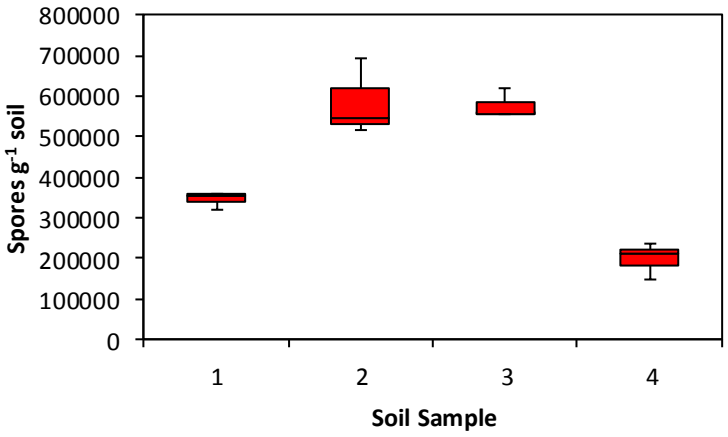

Figure 6. Immunomagnetic separation of *P. brassicae* spores from four soil samples for the optimisation of magnetic particles (beads) and antibody concentration. Samples 1, 2, 3, 4 contain 50, 100, 200 or 400µL beads respectively, all in combination with 100µL IgM (~3.5mg/mL).

When this protocol was applied to twenty soil samples, only one sample was positive for *P. brassicae* and this contained $5.4x10^4$ spores g^{-1} soil. This was comparable to the 0.25g DNA extractions which had been previously performed on these soils (data not shown) however it should have contained approximately $1x10^5$ spores in total.

Due to the lack of spore volume concentration by the magnetic particles, an optimisation experiment in spore suspension had been performed using a range of antibody and magnetic particle concentrations. The spore suspension used had a concentration of $6.15x10^6$ spores/mL, giving a total of $3.08x10^8$ spores in the 50mL suspension. The maximum number of spores extracted using the magnetic particles was $2.96x10^7$, thus falling short of the total number of spores present. For all three antibody concentrations the combination with the maximum bead concentration resulted in the highest levels of *P. brassicae* resting spores isolated from the suspension (samples 3, 6 and 9 on Figure 7).

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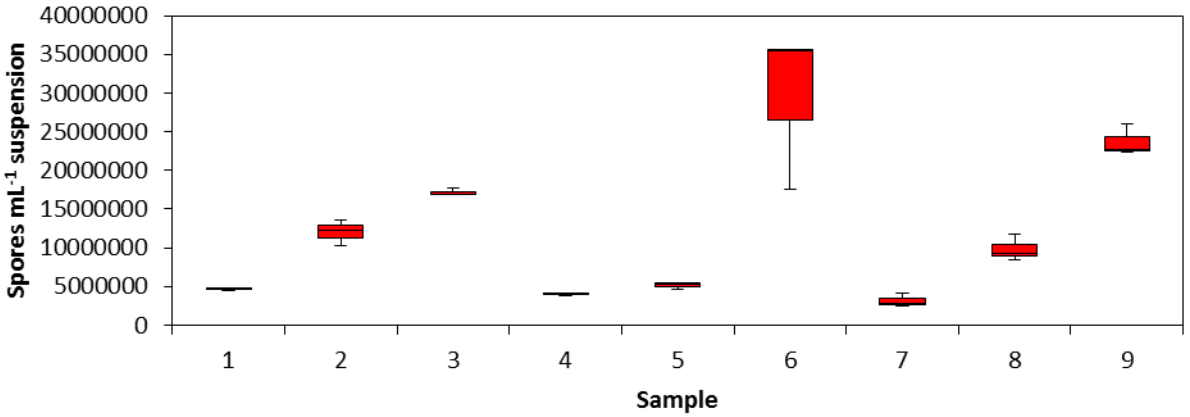

Figure 7. Immunomagnetic separation of *P. brassicae* spores from suspension for optimisation of magnetic particles (beads) and antibody levels. Samples are as follows; 1 = 1mL beads with1µL IgM, 2 =2mL beads with1µL IgM, 3= 3mL beads with1µL IgM, 4 = 1mL beads with 5µL IgM, 5 =2mL beads with5µL IgM, 6= 3mL beads with 5µL IgM, 7 = 1mL beads with 10µL IgM, 8 =2mL beads with10µL IgM, 9= 3mL beads with 10µL IgM. The starting IgM concentration is 0.1mg/mL and the starting bead concentration is 1mg/mL.

Evaluate processes to improve the sensitivity of the lateral flow for quantification of IMF P. brassicae spores

Initial gel images for the pepsin digest of UW 249 IgM antibody showed the non-fragmented IgM (whole antibody) present in the control wells as an intense band at >300kDa. There were no fragments or whole antibody present in the digested samples. This was the same for both sets of samples (pH4.0 and pH4.5). The papain gels displayed a degree of fragmentation as shown in figure 8, with different banding patterns occurring between the 0.1mg/mL, 0.02mg/mL papain digests and the control samples. Trypsin digested samples showed some differences between the treatments and the controls, but the gels were not as clear as for the papain digested samples.

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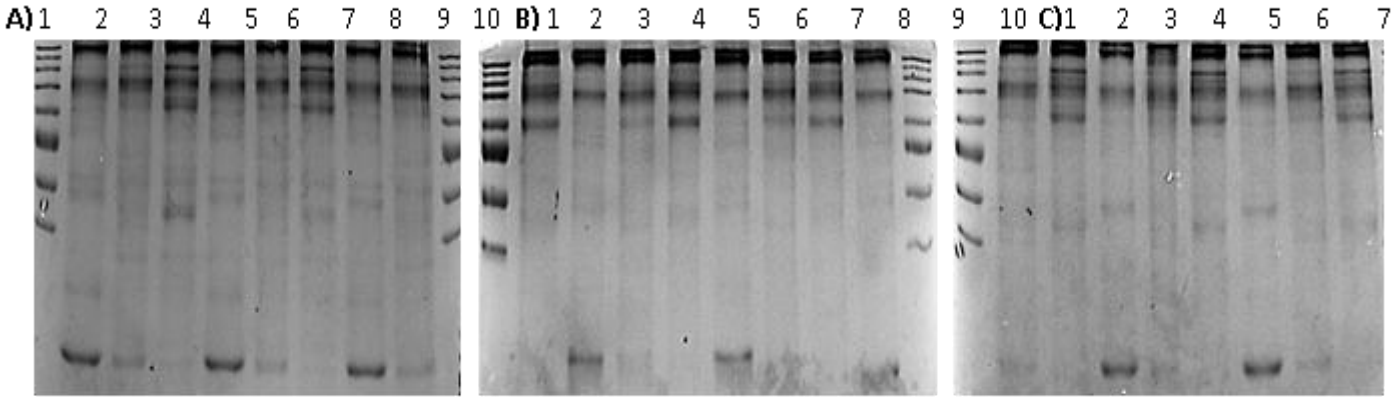

Figure 8. Papain digested *P. brassicae* IgM Antibody on non-reducing SDS PAGE gel. Lanes A1, A10, B1, B10 and C1 = Protein Ladder markers - from top bands represent 300, 250, 180 130, 100, 70, 50 and 40kDa, Lanes A2=1h 0.1mg/mL, A3=1h 0.02mg/mL, A4=1h control, A5=2h 0.1mg/mL, A6=2h 0.02mg/mL, A7=4h 0.1mg/mL, A8=4h 0.02mg/mL, A9=4h control. B2=4h control, B3=6h 0.1mg/mL, B4=0.02mg/mL, B5=6h control, B6=10h 0.1mg/mL, B7=10h 0.02mg/mL, B8=10h control, B9=24h 0.1mg/mL, C2=24h 0.02mg/mL, C3=24h control, C4=29h0.1mg/mL, C5=29h 0.02mg/mL, C6=29h control, C7=34h 0.1mg/mL, C8=34h 0.02mg/mL, C9=34h control

The Western blots reveal a different digestion pattern to the gel images (figure 9) and demonstrate an increased sensitivity for detection of the antibody fragmentation process. From this, it was possible to see bands across all enzyme digests where the Goat anti-mouse IgG+IgM (Heavy and Light chains, Sigma-Aldrich) peroxidase conjugate (Thermo Fisher Scientific) bound and identified the heavy or light chains of the digested *P. brassicae* IgM.

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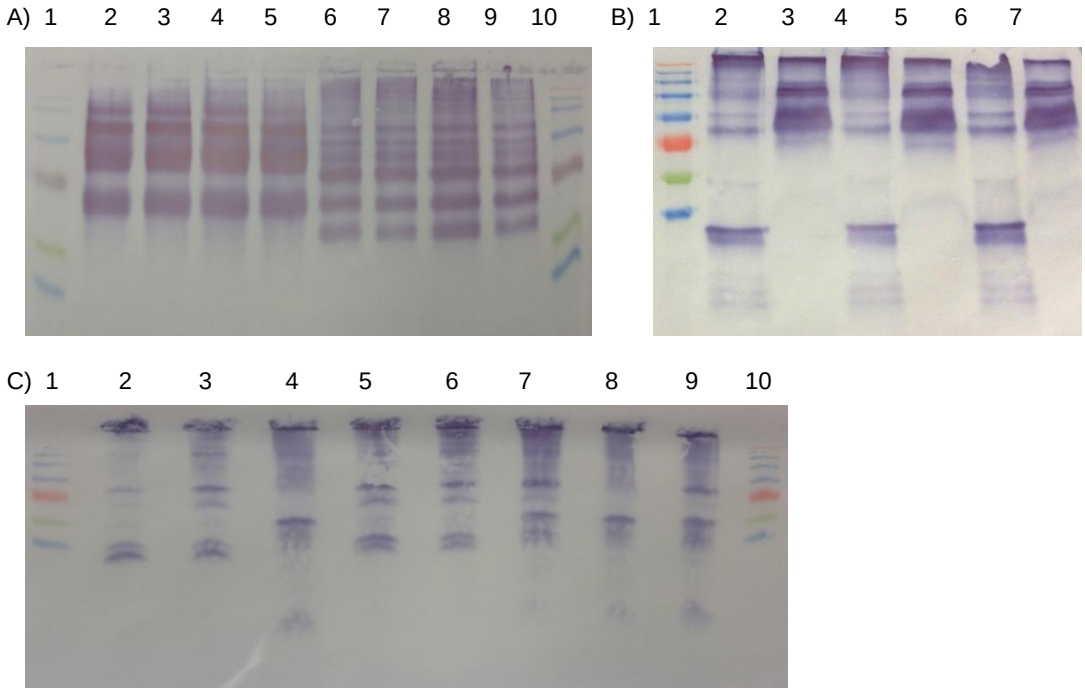

Figure 9. Pepsin digested *P. brassicae* IgM antibody on nitrocellulose membrane after western blotting with Goat anti mouse IgG+IgM (H+L chains) Peroxidase conjugate (Thermo Fisher Scientific). A) Papain digested samples, lanes 1 and 10 = protein ladder marker, A2=24h control, A3=10h control, A4=6h control, A5=4h control, A6=24h 0.1mg/mL, A7=10h 0.1mg/mL, A8=6h 0.1mg/mL, A9=4h 0.1mg/mL B) Pepsin digested samples (pH4.5), lane 1 = protein ladder marker, B2=24h control, B3=24h digest, B4=28h control, B5=28h digest, B6=33h control, B7=33h digest C) Trypsin digested samples, lanes 1 and 10 = protein ladder marker, C2=16h digest, C3=8h digest, C4=6h control, C5=6h digest, C6=4h digest, C7=2h digest, C8=0h control, C9=0h digest. For all images Protein Ladder markers - from top bands represent 300, 250, 180 130, 100, 70, 50 and 40kDa

With the papain digest there are three (or possibly four) bands in the digests between 70k and 130kDa which may not be present in the controls. However it is difficult to interpret due to the large volume of product around the same size in the control samples. There is a visible low band around 50kDa which is not present on the control samples. The protein ladder does not display the 70 or 100kDa marker (they appear to have merged) which could complicate sizing products in this region.

With the pepsin digest (pH 4.5) at 70kDa, a protein band is recorded in the digested samples and not in the control samples. An additional protein band at approx. 80-110kDa is visible after 1 hour. This increases in concentration to 33 hours. A band at 140kDa is seen after an hour of digestion and this becomes more prominent between 10 and 33 hours

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digestion. A smaller band is apparent after 10h and appears to remain at a constant concentration at approx. 70kDa.

With the Trypsin digest there are bands visible at 50kDa in both the control and digest samples. These are present at 0-2h but not in the digested sample from 4h onwards. After 4 hrs., two bands in the digested samples are visible at 35-45 kDa. There may be fragmentation at 4, 6 and 8hrs of digestion at or near 200kDa. Visible products are present only in the digested lanes near 60 and 70kDa.

Confirmation of the selected protein band isolations from the excised gels was obtained by running each on a gel and immunostaining by Western blot (figure 10).

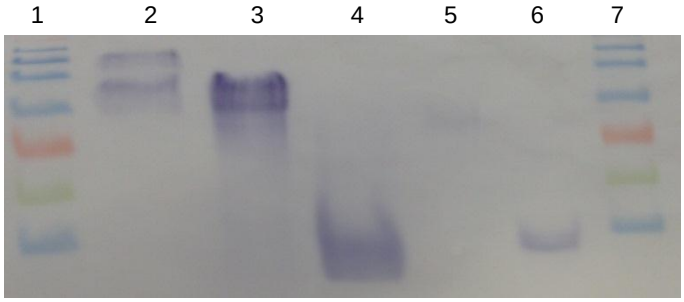

Figure 10. Trypsin and pepsin digested *P. brassicae* IgM antibody on nitrocellulose membrane after western blotting with Goat anti mouse IgG+IgM (H+L chains) Peroxidase conjugate (Thermo Fisher Scientific). Lanes 1 and 7 = protein ladder marker - from top, bands represent 300, 250, 180 130, 100, 70, 50 and 40kDa; 2= Pepsin Band ~150kDa; 3= Pepsin Band ~90-110kDa; 4= Pepsin Band ~70kDa; 5= Trypsin Band ~70kDa; 6= Trypsin Band ~35kDa.

Liaise with Growers to determine crop outcome based on clubroot disease prediction

It was difficult to determine a disease risk outcome as not all the soils were planted with Brassicas and the fields' history unknown. Where a medium / high risk was provided this was in general thought to be accurate. For a number of soils (particularly the Scottish set) an overview was that the tests considerably under predicted clubroot risk. This however was prior to the soils being retested by a different sample process and using dilution factors to mitigate factors associated with soil inhibitors.

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Conclusions

Clubroot soil testing service

All soils provided by growers participating in the 2013 HDC trials were processed within a two month period by molecular (DNA test) and lateral flow (on site 'lab on a stick' test). The results were supplied back to the co-ordinating grower representatives following completion. However the turnaround time and the occurrence of false negatives raised concern. Firstly, the turnaround time was not sufficient to enable growers to assess a field prior to planting and secondly, the number of soils testing positive for *P. brassicae* was less than would have been expected based on prior experience and knowledge of their land. As a result efforts were made to address these concerns by looking at factors which could affect the two test measurement assays for clubroot resting spores, and the length of time of the different processing methods.

Factors which can affect soil DNA extracts include humic and fulvic acids, soil clay content and the presence of heavy metals (Wilson, 1997; Menking *et al.,* 1999.). The inclusion of the PVPP clean-up step is designed to assist with DNA purification; however it is possible that there may be inhibitors that are not being sufficiently removed by this alone. One of the most common ways to look at the effects of inhibitors on successful DNA amplification by qPCR is to dilute the samples in PCR grade water (Kemp *et al.,* 2006) and this has been shown to have a beneficial effect in sediment samples such as in the work of Erdner (2010). When used here, the results showed that diluting the DNA extracts reduced the number of false negatives for the Scottish soils. There was no effect seen for the Lincolnshire co-ordinated soils. This may suggest a difference in soils between the two regions; however without further examination of the soil constituents this effect cannot be determined.

In previous studies problems associated with soil inhibitors had not been observed either during routine or experimental clubroot soils sampling (HDC FV 349). However the use of the 10g kit was in its infancy within this programme of work and the manufacturer different to the system used previously. This could lead to different extraction characteristics between the Powersoil DNA extraction kit (10g kit) and the characterised 0.25g Ultraclean Soil DNA extraction kit. In addition, it was determined after the 2013 HDC soils extraction process, that the manufacturer's instructions had not been followed in full. The final DNA elution step had been altered to increase sample concentration. This may have increased the concentration of inhibitors and / or affected the elution efficiency of the final step. For this reason the samples were diluted and a substantial number of soils previously scored as

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clubroot free / low risk were found to have epidemiologically significant number of resting spores present.

The inclusion of the 0.25g molecular test did not identify all of the soil samples that tested positive for clubroot using 'diluted' DNA from the 10g PowerSoil DNA extraction kit. It is possible that this may be due to the smaller volume of soil processed. Clubroot spores can have patchy distribution within soils. Equally it may prove beneficial to make dilutions of the DNA extract for the 0.25g soils. The area of DNA inhibition is complex and there are various techniques to improve reliability (Kemp *et al*., 2006, Erdner *et al*., 2010, Kemp *et al*., 2014). This is an area on which further work will be performed during the second year of this project, when soil samples are again provided by growers during the spring of 2014. This should determine the most reliable method of quantifying *P. brassicae* spores from soil.

The second area which had been raised as of concern was the length of time for results to be provided. Examining the different sections of the overall process, highlighted two areas that required significant time, space and high labour input. The commercial soils provided in 2013 were in many instances saturated. The air drying process at room temperature was upwards of three to four weeks in some cases. To ensure uniformity of the clubroot resting spores in the soils, an extensive grinding and sieving process was administered prior to the DNA extraction process. The DNA extraction and quantification by qPCR being short (1.5 days for all samples). In comparison to the length of time taken here, a Canadian commercial testing laboratory offers clubroot soils testing by PCR, with turnaround time of 7-10 days BUT based on the soil samples sent in being air dried by the grower (20/20 Seed Labs, Inc. 2014). In the UK, a laboratory molecular test is combined with a bait test, offering a 10-14 day turnaround time but requiring 14 days advance notice of samples being received for growth of bait plants. The results of this test provide only a presence/absence marker; they do not offer a quantitative service (Fera, 2014a; 2014b).

The air drying of the soil samples is largely dependent on the moisture content of the sample upon receipt and the temperature and humidity of the room used to dry the soils. To increase the speed of this process oven drying the soils at 30^{o}C for 3 days was proposed. The manufacturer's protocols for the DNA extraction kit also allow for wet processing of soil samples. This eliminates the need for drying and so protocols adapting the soil water to three different water contents were assessed in 2013. A benefit of using wet soil is that there is no requirement for grinding and sieving which was the other limiting factor in the processing of the soil samples.

It was found that by increasing to a 25% soil water content yielded the highest levels of *P. brassicae* resting spores in the soils tested by the molecular method. A much more rapid

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turnaround time of two days was observed with soils processed in this way. However the technicians' noted that getting the soil water content uniform at 25% was difficult despite thorough mixing of the soil. The second highest level of P. brassicae incidence was recorded by the 50% soil water content process. This methodology was found to be more consistent to attain the soil water content and provided uniform distribution of the soil matrix by stirring. Due to the patchy nature of *P. brassicae* spore distribution within the soil, mixing the samples by grinding and sieving during the 'traditional' air drying method was performed to ensure even distribution. Bringing the soil water content up to 50% creates a slurry type suspension which can be stirred by hand and it was hypothesised that this would be comparable to the mixing by grinding and sieving. The 50% soil water and air drying had very similar standard deviations, but the 25% soil water displayed a greater deviation from the mean over the range of samples tested. From the preliminary findings the results would confer that it is feasible to use a wet processing method for the extraction of *P. brassicae* resting spores from soil. The method is comparable to air drying in terms of ensuring an even spore distribution in the samples tested. These methods will continue to be investigated in year two of this project.

Immunomagnetic separation of clubroot spores from soil and improving LFD sensitivity

From the results, the magnetic particles (Bangs Labs, USA) bound selectively to the *P. brassicae* IgM antibody and provided extraction of the *P. brassicae* spore material from both soil and spore suspensions. However the beads failed to concentrate the collected P*. brassicae* resting spores to the levels previously observed using a bead source from Merck Chime (HDC FV 349). These antibody conjugated beads are no longer available. The preliminary results from this study suggest that additional optimisation of the 'new' anti-antibody conjugated beads (Bangs), the specific clubroot antibody and the target analyte (clubroot resting spore) process is still required. Other sources of beads will be investigated in Year 2 of the project, as the current beads although useful in isolation and concentration for PCR processes, are too large for inclusion within lateral flow tests. Studies remain ongoing and the capability to directly conjugate clubroot specific antibody fragments directly to the magnetic beads is under investigation.

For this purpose the *P. brassicae* IgM antibody (UW249) has been purified and digested by pepsin. Some differences between the digested samples and the control (undigested samples) provide evidence that antibody fragmentation has occurred. Pascual and Clem (1992) observed a high molecular peptic fragment at approx. 130-140 kDa under non-denaturing SDS PAGE conditions (as was used above) and another fragment at approx. 67

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kDa for an IgM antibody fragmentation process. They speculated that under non-denaturing conditions, these two fragments appeared to be associated non-covalently, and that collectively these represented the $F(ab')_2$ fragments. This would concur with results obtained with the clubroot specific antiserum (UW249). The lower of these two bands (35 -40KDa) could speculatively be Fab. Fab' fragments are considered 5kDa bigger. There does not appear to be any $F(ab)_2$ as this would be expected to be at approximately 110 -150kDa.

When digested with trypsin, low molecular weight protein bands were observed. The smallest of which (35-40kDa) was proposed as Fab'. Plaut and Tomasi (1970) reported Fab fragments of 41kDa and $F(ab)_2$ fragments of 95kDa when 19S human IgM was digested with trypsin. However they recorded the molecular weight by the meniscus depletion method using absorption optics (Plaut and Tomasi, 1970). They also found intact IgM was 845kDa, and the $(Fc)_5$ region was 342kDa. There are protein bands in the gels at the speculated size for Fab and $F(ab)_2$ based on this. However the manufacturers' technical data (Thermo Scientific, 2014) suggests $F(ab)_2$ would be expected to be at approx. 150KDa while the size expected for Fab would be approx. 45kDa. This is close to that found by Plaut and Tomasi (1970). The trypsin digest provides some "IgG type-M" molecule at 4hrs, 6hrs and 8hrs into digestion (around 200kDa). The latter, if isolated and determined active towards *P. brassicae* spore antigen, could also prove useful in conjugation to the magnetic spheres.

Papain digestion is most frequently documented for IgG fragmentation, rather than IgM. Digestion of IgG can produce "rIgG" (around 75kDa) also known as reduced IgG or half IgG. These are usually produced in the presence of reducing agents though (which were not included in this study). From digestion of IgM these types of molecule may be larger (110kDa). Another possibility is $F(ab')_2$, which is usually around 150kDa from IgM but when obtained from digesting IgG is around 110kDa (Andrew and Titus, 2000). The bottom band at ~50kDa corresponds with the size of Fab' or Fab obtained when IgG is digested with papain (Andrew and Titus, 2000) and so is a strong candidate for isolation.

The protocols employed for digestion of the *P. brassicae* IgM antibody have produced banding patterns comparable with the production of functional antibody fragments. Further work in Year 2 will involve confirmation of this by running denaturing SDS-PAGE gels, size exclusion chromatography and enzyme-linked immunosorbent assays (ELISA's). Once antibody fragments have been identified and isolated they can be conjugated to magnetic particles or gold beads for incorporation into the lateral flow devices. Liu *et al.,* (2011) reported the use of magnetic Fe_3O_4 particles as the coloured agent in an LFD system. The

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existing clubroot lateral flow uses anti-species antibodies conjugated to gold spheres as the visual identifier. Magnetic particles could provide a dual system in the isolation and concentration of resting spores from increased soil volumes (>50g), as based on the IMF process. Transfer of the resting spore bound magnetic beads would require no additional labelling for the LFD process. Magnetic labels have shown to provide increased levels of sensitivity in this assay format and could significantly improve the detection limit of the assay to 100 spores per gram soil. Direct conjugation of antibody fragments, without the requirement of secondary antibodies, is likely to reduce cross-linking between sample components and, with smaller overall molecule size, improve travel within the LFD format.

Overview

Quantitative measurement of plant pathogens by molecular (DNA based) and immunological (antibody based) methods have in the past decade become an established procedure in the quantification of disease in many horticultural systems. In this project and using these methods, advances have been made to develop tests which can estimate disease potential of the clubroot pathogen in UK field soils. The ability to measure disease potential in field soils has useful applications in not only forecasting the risk of clubroot disease ahead of planting Brassica crops but in the management of the disease throughout the growing season.

Variation in the level of clubroot infestation in soils between the qPCR test and the lateral flow may result from the storage process of the soils ahead of testing. Dr Robert Faggian (University of Melbourne, Victoria: personal communication) reported that storage conditions effect clubroot resting spore DNA extraction level. Within one month at 4ºC storage, the DNA test signals of clubroot infested soils were seen to decline and the resultant qPCR value generated was significantly below that originally observed. In this study soils were air dried for up to a four week period to attain a consistent dry weight. Processes examined during this reporting year provide for the 2014 sampling series the adoption of a 50% soil moisture spore extraction approach which should reduce the sample storage and extraction time to less than two days. Soil type is also important in the context of test disease estimation. It is known that the soil type can influence DNA extraction and subsequently the outcome of PCR amplification due to the presence or absence of inhibitory substances (Lloyd-Jones and Hunter, 2001). This has also been noted for immunological tests (antibody tests i.e lateral flow device) and with particular reference to soils high in humic and fulvic acid.

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Soil textural parameters could also be linked to differences in test results. Soils collected in 2007, were assessed for soil textural type and approximate proportion of sand, silt and clay composition. Following analysis by qPCR it was determined that the silt content was significant in reducing test sensitivity of *P. brassicae* infestation in naturally infested soils. This study was extended in to newly collected soils which were identified as clubroot disease free. These soils were then artificially inoculated with clubroot disease over a concentration range of high to low. The study revealed that although silt was significant in reducing the qPCR signal in naturally infested soils the effect of sand and clay and, their interaction was significant. Further analysis of the soils in 2014, where inhibition was observed, could provide greater information on the significance of these factors for the accurate estimation of clubroot resting spores in soil. Equally, the peat based soils found in the low lying sea areas of West Lancashire may prove important in the performance of the lateral flow test and should not be limited to the effect of soil textural type but include analysis of humic and fulvic acid. Each of which could have a significant effect on the lateral flow assay and may explain the low levels of clubroot spores predicted in 2014 using this system. The current project (in Year 2) will look to address whether a simple diluent factor would, as observed with the molecular test, alleviate any inhibitory issues involved.

The development of the two test systems (laboratory test: *molecular qPCR* and field based grower test: *lateral flow device*) provides the UK horticultural industry with the ability to assess fields for clubroot disease and measure disease potential i.e. the number of disease propagules (resting spores) in the soil. Ultimately however the development and expression of the disease will be dependent on a number of factors: the resting spore concentration, the sampling procedure adopted, the conducive or suppressive nature of the soil type, the environmental conditions over the growing season and the Brassica cultivar planted.

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Knowledge and Technology Transfer

Publications

Alison Wakeham May 2013. Final Report HDC Project FV 349 entitled “Further development of an in field tests for resting spores of clubroot and the development of clubroot control based on detection

Seminars

23rd January, 2013. Roy Kennedy and Mary Lewis attended a HGCA meeting on Stoneleigh

HDC/BGA Brassica Technical Seminar workshop Demonstration of the Clubroot lateral flow device	Lincolnshire, 14 November 2013
HDC/Duchy College Brassica Variety Trial Event Clubroot Test Kit Demonstration	St Ives, 16th January 2014
HDC/BGA Brassica Technical Seminar Clubroot update and LFD test demonstration	Edinburgh, 28th January 2014

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