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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.

AUTHENTICATION

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

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CONTENTS

GROWER SUMMARY

Headline

- The UK horticulture industry now has an optimised and validated molecular qPCR test for the assessment of fields for club-root disease. This test can be carried out quickly (within days of laboratory receipt) with an accuracy of >90%. It is quantitative and has a detection sensitivity of 1000 spores per gram of soil tested.

- This molecular test provides capability to accurately detect disease at low level and at specific points in the field. Using this approach the application of tailored and cost effective treatments can be made for management of club-root.

- This approach provides growers with an integrated disease management system for control of club-root disease and meets the criteria of the European Sustainable Use Directive (SUD).

- A lateral flow test has been developed and validated with 67% accuracy* for the measurement of club-root in soils. The test has a detection sensitivity of >10,000 spores per gram of soil. It provides a club-root risk of either zero to low (≤10,000 spores g^{-1} soil) or medium to high risk (>10,000 spores g^{-1} soil).

- Further work is planned by the University of Worcester scientists to improve the efficiency of the lateral flow test.

* compared to grower field observation
Note: The lateral flow test is less accurate than the qPCR as a result of varied soil types and sample size.

Background

Brassica crops are of great economic importance in the United Kingdom. One of the most important diseases affecting Brassica crops is club-root, caused by the soil borne organism *Plasmodiophora brassicae*. Mild club-root 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. Club-root resting spores

are capable of inducing disease in vegetable Brassica crops years after initial infestation of the soil.

Once soil has been contaminated club-root spores can remain viable for up to 18 years. In the UK, growers of horticultural crops frequently rent land on an annual basis, often with limited knowledge of previous cropping histories. The capability to forecast club-root disease risk prior to contractual agreements being made, would be beneficial. The level of soil infestation by club-root resting spores has been shown to directly affect the amount of club-root 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). The soil, and prevailing environmental conditions during key cropping periods, will also affect the risk of disease development and the severity of crop symptoms.

As *Plasmodiophora brassicae* only grows within living tissues it is not possible to use standard dilution plating techniques to quantify numbers of pathogenic propagules within soil samples. The traditional technique used to assess potential risk of the disease in soils has been to infect bait plants grown under optimal disease conditions. It takes six to eight weeks for disease symptoms to be visible on bait plants, which makes the technique time consuming and expensive. Growers have reported that this type of test tends to 'over predict risk' compared to what actually happens in the field. With the development of new detection methods based at the molecular level (DNA, Antibody, Aptamers) these approaches may prove to be more accurate, quicker and less expensive. Whilst many molecular tests require a high degree of precision, knowledge, and are generally laboratory based, the lab on a stick (lateral flow / in-field test) offers an inexpensive alternative that could easily be used by a grower or agronomist.

The lateral flow device has the potential to be used by growers using field soils, as well as in water based systems such as reservoirs and irrigation lines used by vegetable brassica propagators. A quantitative measurement of club-root 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 of whether the crop is at risk can be made as well as the level of risk i.e. low, medium or high.

Determining the concentration of club-root resting spores in soils is an essential component in the development of an integrated disease management programme. Club-root spores are reported as having a half-life of approximately four years. If resting spore numbers can be maintained below the disease threshold (10,000 spores g^{-1} soil) then rotation and an

increased pH level ≥7.2 through liming should prove useful in reducing disease risk. Where an increased risk of disease is identified (>10,000 spores g^{-1} soil) the application of targeted treatments such as Limex, a by-product of the British sugar industry, could be applied. The use of club-root resistant varieties has also been shown to be useful in reducing the severity of disease expression when growing in infested soils.

The development and expression of the disease in the crop will be dependent on a number of factors: the resting spore concentration, the conducive or suppressive nature of the soil type, the environmental conditions over the growing season, and the brassica cultivar planted.

The deliverables from this project are:

- Validation of assay systems to test soils for club-root prior to planting the crop
- Adoption of integrated pest management systems: field mapping of club-root resting spore distribution and application of targeted treatments

Summary

Club-root testing services and co-ordination of soils for sampling: A laboratory molecular based test and a 10 minute 'in field' test, were assessed for their ability to measure and predict risk of club-root disease in UK horticulture grade soils. Over a two year period a total over 100 soils across the UK were sampled for the measurement of *Plasmodiophora brassicae* infestation (club-root resting spores). At each sampling site, two hectare blocks were identified and spatially assessed by the collection of 48 soil samples at points across a W grid format. Soil cores were taken to a depth of 6-8" (15-20 cm). At each point a core soil sample of approx. 50 g was collected. In Year 1 of the project, the collected soils were individually air dried, sieved and mixed ahead of the test processes. This was to ensure that any disease present would be evenly distributed throughout the sample. However this process was labour intensive, time consuming and with high sample volumes required considerable space. Depending on the moisture content of the soils the process could take upwards of three weeks.

Assessment of soils for club-root resting spore concentration

In each of the two years, the molecular (laboratory quantitative PCR) and immunological test (in field test) gave some disparity in the prediction of club-root risk for the soils tested. It is known however that soil type can influence DNA extraction and subsequently the outcome of PCR amplification, as a result of the presence or absence of inhibitory

substances. Humic and fulvic acids, soil clay content and the presence of heavy metals have all been reported to affect DNA extraction of a target organism from soil. One of the most common ways to identify soil inhibitors is to dilute the samples in molecular grade water. By doing this, the extraction of club-root DNA for the soils tested in 2013 (Year 1) either remained the same or was improved. These results provided an opportunity to also improve the soil preparation process. Bringing the soil water content up to 50% created a slurry type suspension which could be stirred by hand. This was a simple, quick and robust process used to mix the soil sample uniformly before testing. The addition of water to samples was also useful in reducing the effect of soil inhibitors.

Using this system in Year 2 of the project enabled soil samples to be processed quickly and the results reported within days of sample receipt. The risk of club-root was reported via an interactive web page which the co-ordinators had access to via a personal login. In Year 1, the soils processing had resulted in growers receiving results between three and five weeks after sample receipt. Modification of the molecular test in Year 2 not only resulted in faster sample analysis time but with an improved accuracy. Compared to grower observations, the PCR (molecular DNA) was 92% accurate in predicting club-root disease risk. This was further improved to 96% when compared to the industry standard (bait test data). The rationale for this is that high numbers of resting spores are required in soils (>10000 g^{-1} naturally infested soil) before above ground disease symptoms on infected plants are observed i.e. stunting, yield loss, wilting of plants during hot and prolonged dry weather. The industry bait test scores club-root risk based on below ground disease symptoms, i.e. root swellings / clubbing. For this reason the bait test will identify low level plant infection of *Plasmodiophora brassicae* (club-root) even though symptoms are not apparent to the plant above ground level. This is why the bait test is often seen as over predicting disease risk when compared to the grower outcome. In fact, both the molecular test and the bait test are able to measure risk well in advance of the crop outwardly exhibiting disease symptoms. Based on the data set provided, the bait test provided an accuracy of 70.5% compared to the grower outcome. When the bait test was compared to the results of the molecular and lateral flow test the accuracy of predicting club-root resting spores was improved to 88.5%.

Early detection of club-root infestation ahead of above ground symptom expression is critical if the disease is to be controlled successfully. This is amply demonstrated by a 'club-root negative' soil (S11). This soil was designated by the grower as showing no evidence of club-root. The PCR molecular test however designated 3040 spores g^{-1} soil. A club-root positive result was provided by the bait test but at a low level (8.3%). This spore concentration would be indicative of the healthy crop outcome observed. However,

successive plantings of brassicas would, over a short time, result in the occurrence of club-root disease symptoms, as the spore concentration was close to the disease threshold (>10000 g^{-1} naturally infested soil). This was shown to good effect in soil (S8) where the molecular test identified disease at 1080 spores g^{-1} (bait test negative). The first cropping showed no signs of club-root but the second cropping recorded yield loss to club-root. By adopting a four year rotation period the concentration of resting spores could, before the first cropping, have been reduced by up to 50%. (Club-root resting spores are proposed to have a half-life of four years). Alternatively, at this spore concentration the application of lime to raise the pH to 7.2 may have had beneficial effect in disease control. Where resting spore concentrations are identified above this level (≤10000 g^{-1}), HDC project FV 349 showed that an application rate of Limex at or above 10 tons ha^{-1} could reduce club-root symptoms. The capability to accurately detect disease at low level and at specific points in the field provides growers with the opportunity to map soils on a year by year basis. This will provide a cost effective and tailored approach towards treatment of specific field areas for club-root disease risk.

An in-field ten minute test (lateral flow device) has also been developed and used for the measurement of club-root resting spores in water and directly in artificially infested soil samples (FV 259 & FV 349). A clear relationship has been established between the molecular PCR test and the lateral flow test for measurement of resting spores in artificially infested soil, (Figure 1). In water, a reliable test detection sensitivity of 1000 spores ml^{-1} is achieved.

The lateral flow test has been used by growers to detect club-root spores within 10 minutes at epidemiologically significant levels in artificially infested soil standards (≥ 10,000 spores / g soil detection sensitivity). At three grower group meetings the lateral flow tests have been used successfully by agronomists and growers to accurately measure resting spores in the soil standards. A good correlation between the predicted risk (10 minute lateral flow in-field test) and resting spore concentration in a club-root artificially infested soil was recorded at one the HDC Technology Transfer meeting in Edinburgh. However, when the test was used in naturally infested soils poor correlation was observed with the molecular PCR test.

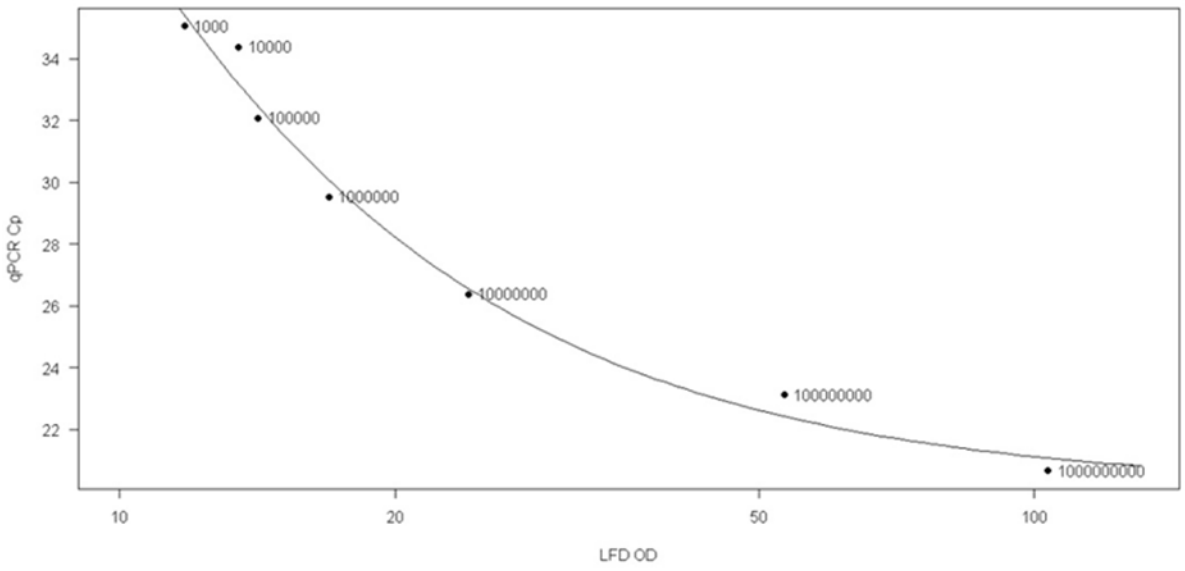


Figure 1. Relationship of the lateral flow test (LFD) and the molecular qPCR test for measurement of *Plasmodiophora brassicae* resting spores in artificially infested soil standards. Points on the graph relate to the number of club-root spores g^{-1} soil.

Compared with the grower observations for incidence of club-root disease, a 67% test accuracy rate was recorded when the commercial soils were tested with the lateral flow test (negative to low risk <10,000 spores g^{-1} soil; ≥10,000 medium to high risk club-root). However, like the molecular test, instances were recorded where the lateral flow test recorded positive for disease and this was subsequently confirmed by bait test. Examples of this are recorded for soils S10, S11 and Sb. Using the same analogy as described for the molecular test, an accuracy of 79% could therefore be assumed.

The S16 soil sample was recorded as a false negative result (0<1000 spores g^{-1} soil recorded) by lateral flow. The grower observed only low instances of disease in the crop. This result may reflect the sample process (0.25g tested by lateral flow) and patchy nature of the disease at this low level rather than the test accuracy. The molecular test recorded a resting spore concentration of 10,000 spores g^{-1} for this soil which is at or near the cusp of disease expression in the field. Also, only one of the two molecular tests identified disease presence.

When resting spore concentration was below the disease threshold for above ground symptom expression the bait test was able to detect disease presence at a low level in a number of the soils tested (S10, S11, Sb), S9 soil however remains as a bait false positive

(78.3% clubroot predicted risk). The molecular and lateral flow tests for this soil were clubroot negative. Grower observation recorded no sign of clubroot after the field was double cropped with brassicas. Nevertheless, the bait test when compared to the other diagnostic tests (lateral flow and molecular test) provides an accuracy of 88.5% for clubroot disease risk. For this reason, the bait test delivers a better measure of club-root disease potential than the lateral flow test but not as accurate as the molecular test. However the bait test requires large volumes of soil, a five to six week test turnaround and, is considerably more expensive than the lateral flow test.

Trouble shooting the on-site lateral flow test

In FV 349 the lateral flow test demonstrated different test sensitivities to different *P. brassicae* pathotypes, significantly race 5. (This pathotype was not considered to be a 'gene breaker' i.e. able to overcome Brassica club-root resistant varieties). Studies have shown that within a single club-root gall multiple pathotypes can be present. Differences shown by the lateral flow could in part result from the different *P. brassicae* pathotypes present in the soil. However it is more likely that the textural structure of the soil type and the small sample size will have influenced the accuracy of the test.

Studies reported in Year 1 showed that the interactions between silt, sand and clay were significant in reducing the capability of the qPCR molecular test for measurement of *P. brassicae* in soil. This effect on the lateral flow had not been considered. In variation to the PCR test, a complex extraction process is not followed or the removal of inhibitors made. Instead a simple flotation process is adopted where the larger soil particles are allowed to settle out of solution. This is needed to prevent the pores of the lateral flow (nitrocellulose membrane) from blocking, causing inhibition of the test. It is likely that this 'settling' process provides opportunity for resting spores to be taken out of solution. Also the positively charged resting spore has been reported to adhere to clay particles which are flat, plate-like and negatively charged. Too few results were available for a comprehensive study of soil textural significance on the efficiency of the lateral flow test. However sandy loam soils and those in association with clay might lend towards false positives or over prediction of disease. Gold complexes (to include colloids used in the lateral flow) have been shown to adsorb to organic matter, clays and iron. Each of these factors could affect the accuracy of the test. For this purpose, studies in year 2 continued to focus on isolation of the resting spores from the soil by immuno-magnetic extraction.

Immuno-magnetic fishing

Earlier studies at Warwick HRI, and later at NPARU, have shown that *P. brassicae* specific antibody coated magnetic beads can label and isolate resting club-root spores directly from a range of soil types. This process provided the capability for concentrating resting spores from 50g soil samples. In these studies the club-root resting spores were directly labelled with UW 249 monoclonal antiserum and indirectly isolated from soil with anti-mouse IgM super paramagnetic particles BE-M03/0.3 (Merck Chimie SAS). These particles were of suitable size for lateral flow test development (300nm). In Year 1 of the project, the super paramagnetic particles were withdrawn from the market place as a standard product, and work was conducted in Year 2 to produce a replacement. However, this area of work is likely to require significant investment of time to develop suitable alternative protocols.

Financial Benefits

- The use of the detection tests for risk assessment of club-root 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

- Consider annual sampling and precision application of targeted club-root control treatments using GPS mapping systems.

SCIENCE SECTION

Introduction

Test background

Two diagnostic tests were identified for their potential use as commercial tools to measure and predict risk of club-root in UK horticultural soils (FV 349). A molecular PCR test, using primers developed by Faggian and Parsons (2002) and an in-field monoclonal antibody based test (HDC FV 259; FV 349). The quantitative molecular PCR test (polymerase chain reaction) is reported to provide a test sensitivity of ≥ 1000 club-root resting spores / g soil but requires a specialised laboratory facility and a complex DNA extraction process. Conversely, the lateral flow test provides potential of an inexpensive field test (approx. £7 / test) with minimal soils preparation. However in soil, the sensitivity of the test for resting spore measurement is limited to ≥ 10,000 spores g^{-1} soil. 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.

Soil Test Inhibitors. 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.

Soil and Sample Size.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 examined the potential to isolate and concentrate resting spores directly from soils. This was achieved by a process called ‘immuno-magnetic fishing’. The principle of this approach

being that club-root specific antibodies are bound to coated magnetic spheres and mixed with the soil sample. On recognition to the spores antibody coated sphere binds and as a complex are removed from the soil by means of a magnetic force. Using this approach the resting spores are delivered to either assay system (molecular PCR or field lateral flow) in a concentrated from and free of soil inhibitors. Instead of a soil sample size of 0.25g assessed for club-root resting spore presence, the magnetic bead assay will enable increasing soil sample volumes upwards of 50g lots to be tested. This should improve the sensitivity, reproducibility and robustness for each test format. The separation of bacteria has been achieved by immuno-magnetic capture with isolation, concentration and detection reported from contaminated feedstuffs (Mansfield et al., 1993), faeces (Luk & Lindberg, 1991) aquatics (Bifulco & Schaefer, 1993) and soil (Mullins et al., 1995).

YEAR 1

Materials and Methods

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

A club-root 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 (on-site lab on a stick).

Assessment of commercial soils for club-root 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 which 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 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 (Van Guilder *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) 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 which is detailed in HDC Annual Report FV 349 May 2012. In short this comprised 50g soil samples in 100mL sucrose solution, sieved through an 180µM sieve and then incubated with club-root specific antibody (UW 249) for 1h. Thereafter anti-mouse IgM bound magnetic particles (MERK Chime, France) were added to the solution. Antibody labelled club-root 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 of club-root 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.

This protocol was then applied to 20g of eight field soils which had tested negative for club-root 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 club-root 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°C overnight. To each tube, 200µL club-root 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 then placed in a magnetic field for 5 mins. The retained magnetic particles were re-suspended in 1mL PBSTwC prior to measurement of club-root 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 the target analyte. This process was applied to a club-root 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 in volumes of volume 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 club-root 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.

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 (club-root 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 club-root 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 the accuracy of the test by reducing the potential for cross-linkage of the anti-mouse capture label during magnetism and then later during the competitive lateral flow assay process.

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°C using a micro-dialysis chamber (Andrew *et al.* 1999). Samples were stored at -20°C until use.

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°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°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 and 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.

Liaise with Growers to determine crop outcome based on club-root 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 club-root 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.

Assessment of commercial soils for club-root 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 2. Out of 52 soils tested, 18 tested positive for *P. brassicae* spores. The maximum level of *P. brassicae* spores in the Scottish soils was $2.49x10^4$ spores g^{-1} soil, and $1.42x10^5$ spores g^{-1} soil in the Lincolnshire soils. The mean spore level across both sets of soils was $8.15x10^3$ spores g^{-1} soil.

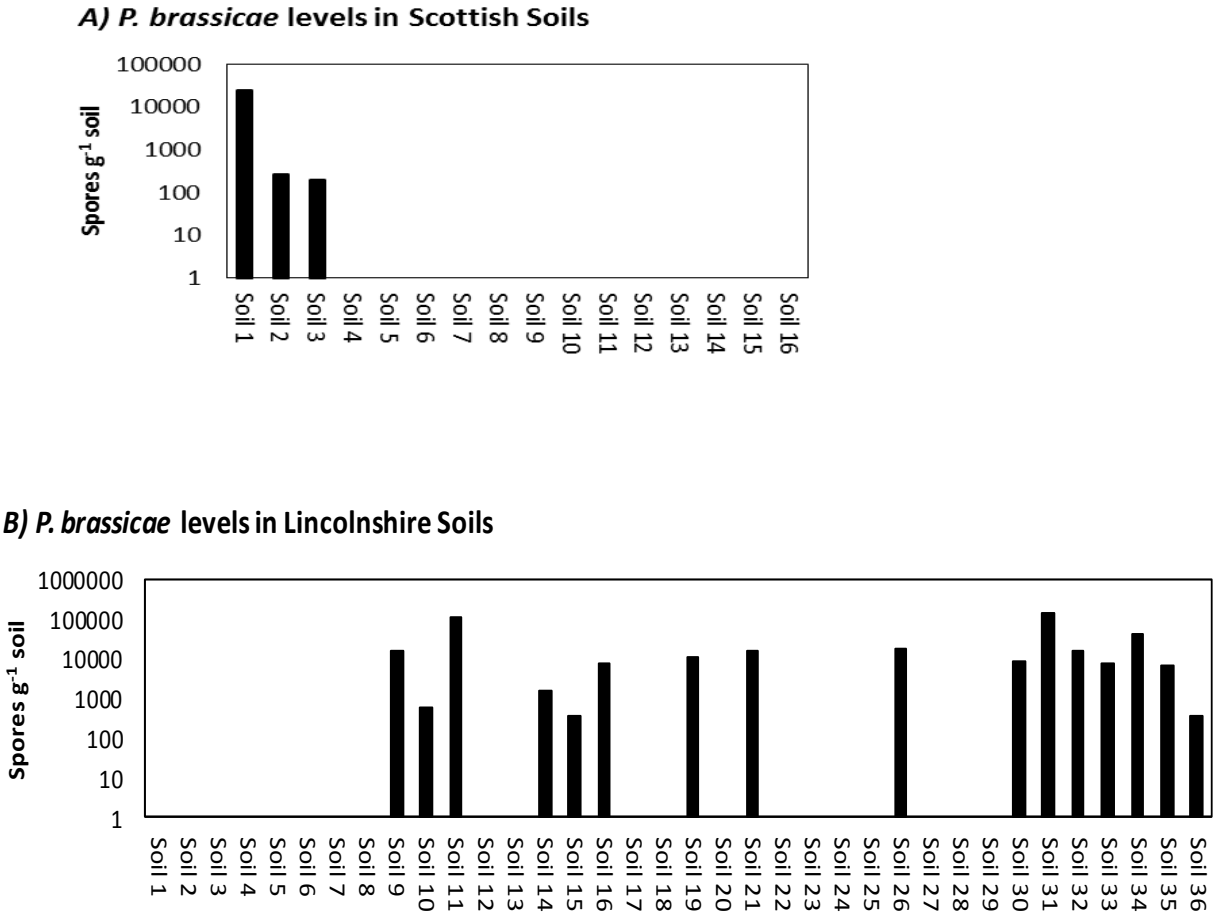


Figure 2. Initial levels of *P. brassicae* reported in Grower provided soils from Scotland (A) and Lincolnshire (B), following extraction with the Power Soil 10g DNA Extraction Kit.

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. This process was similar in time to that of the Industry standard (bait test).

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 3.

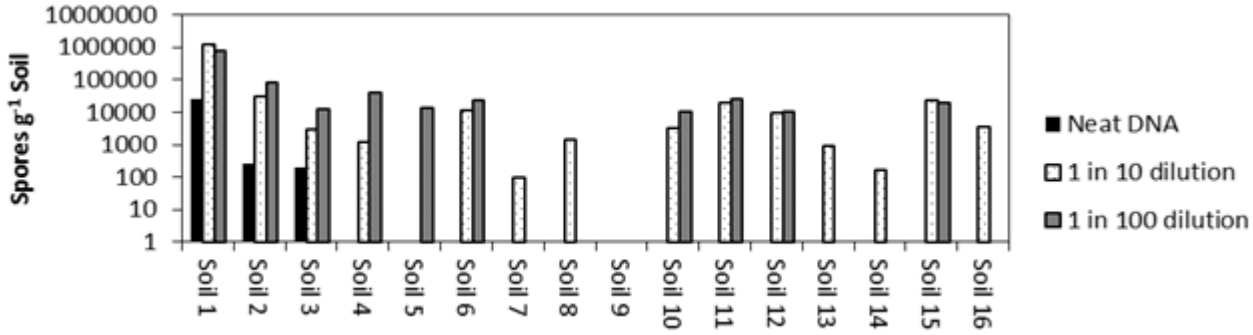
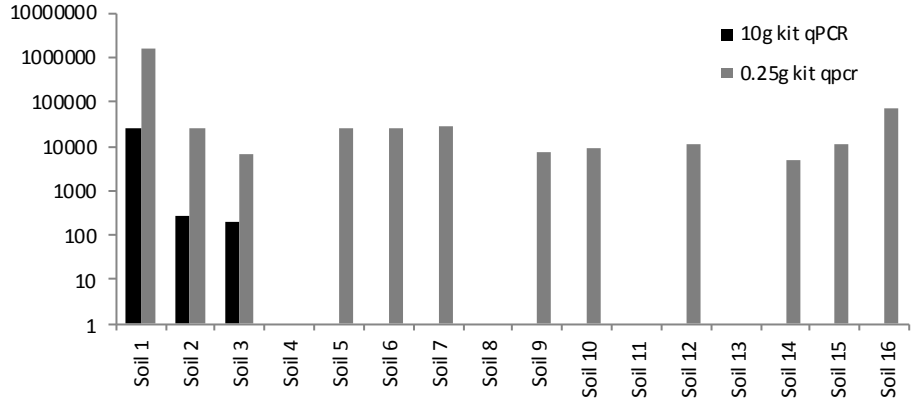


Figure 3. The increased number of *P. brassicae* positive soils following dilution of the DNA extracts in Scottish soils.

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 4a,b) 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

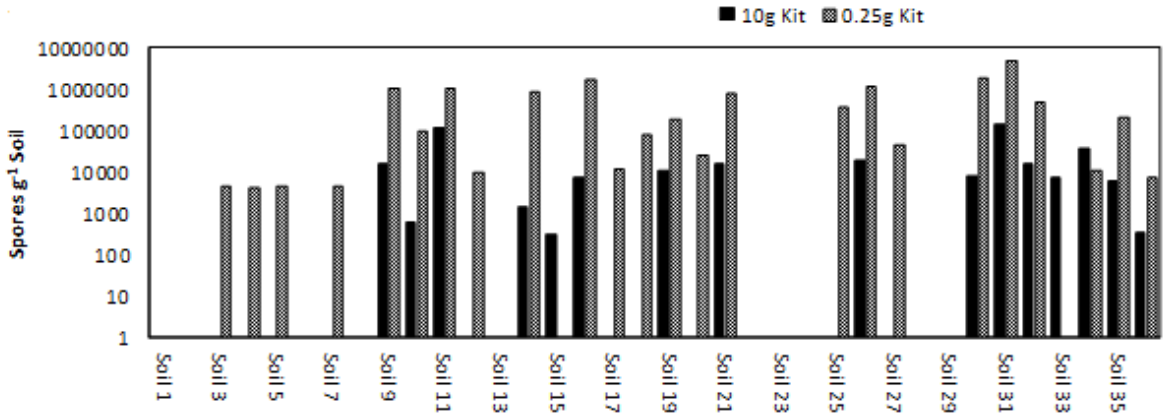
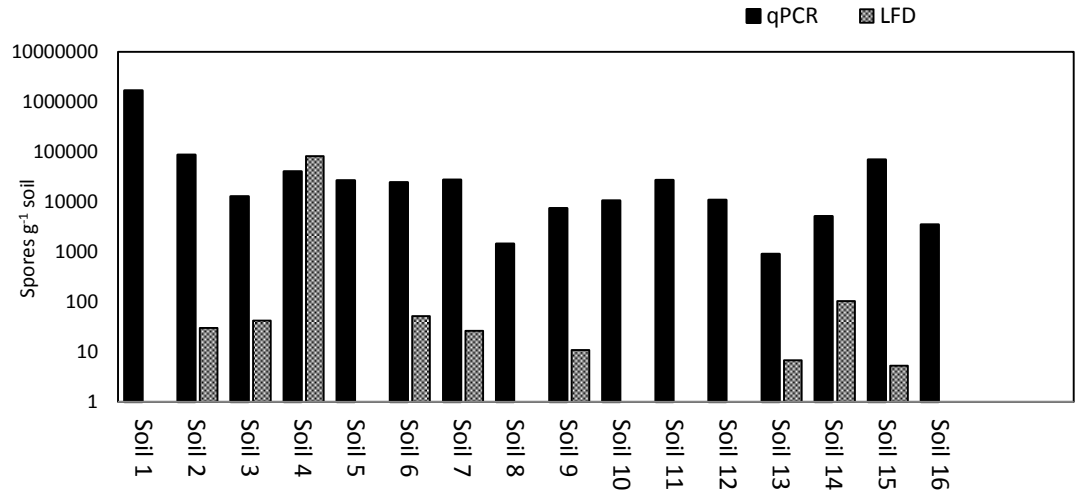


Figure 4. The Difference between *P. brassicae* levels in Scottish (A) and Lincolnshire (B) soils extracted with two soil DNA extraction kits (PowerSoil 10g DNA Extraction Kit and UltraClean 0.25g DNA Extraction Kit)

As a result of the DNA extract dilutions and extraction kit investigations, a final 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 5). 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

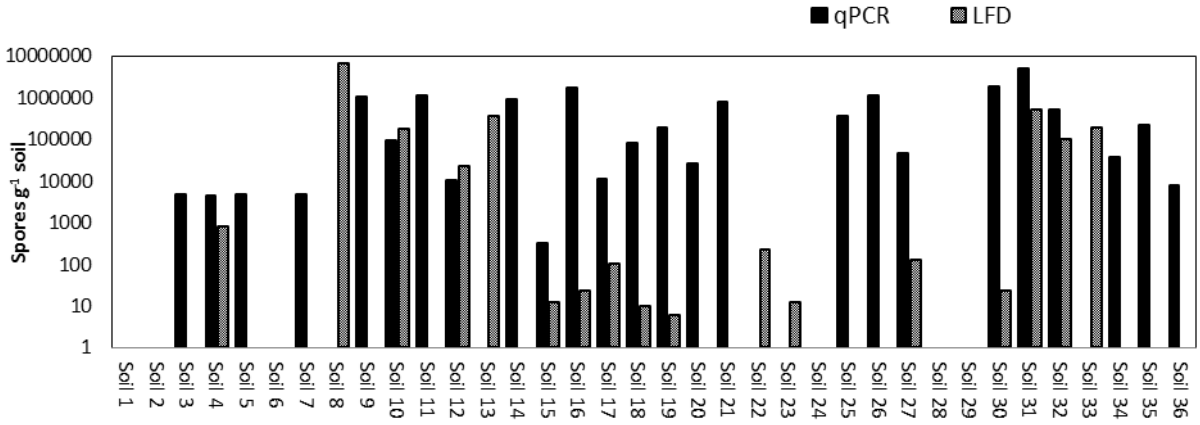


Figure 5. The final results of *P. brassicae* levels in Scottish (A) and Lincolnshire (B) soils determined from the highest levels given by one of the following methods; PowerSoil 10g DNA extraction kit and dilutions of the DNA extracts, and the UltraClean 0.25g DNA extractions, in combination with the results of the single LFD test (0.25g).

To examine whether it was possible to improve 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 6. 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).

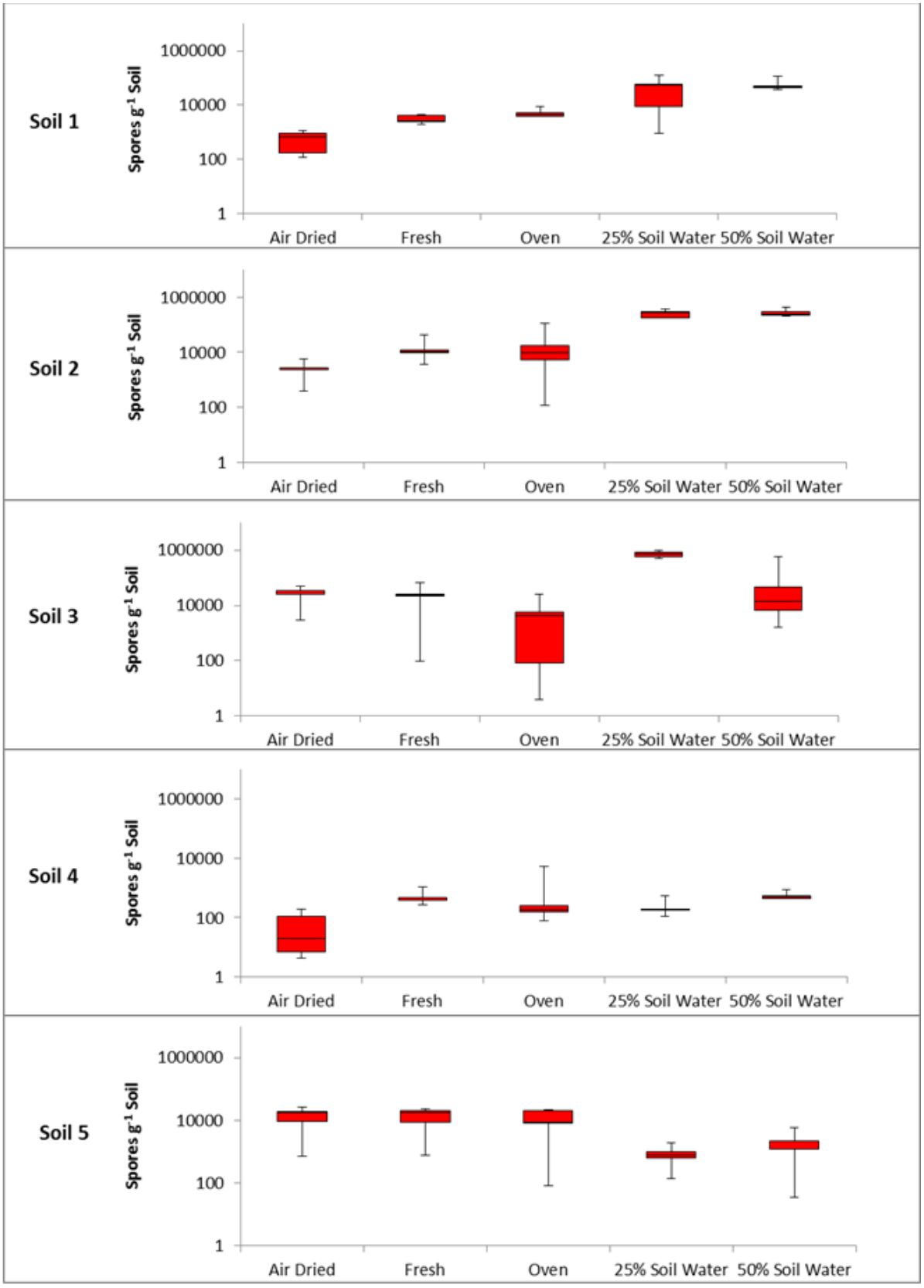


Figure 6. Graphs to show the different levels of *P. brassicae* reported based on different processing methods across five soils with varying degree of club-root infestation.

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 5.85 x 10^5 spores g^{-1} soil (Figure 7). The soil had been inoculated with 1x10^6 spores g^{-1} soil and so contained 2.5 x 10^7 spores in total. This demonstrates that the beads were not successful at concentrating the number of spores, although they were extracting a good proportion.

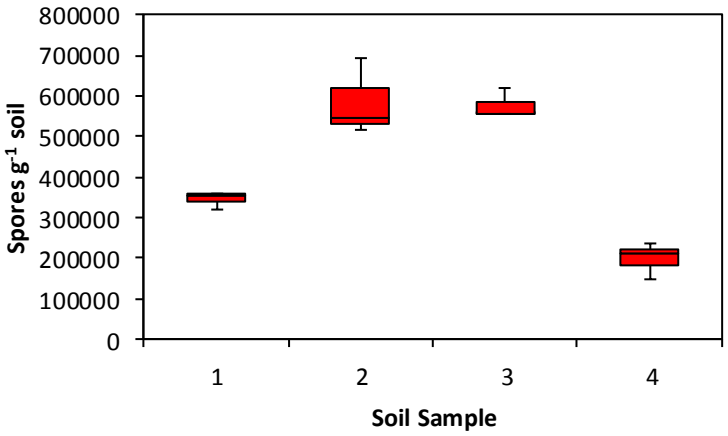


Figure 7. Immuno-magnetic separation of *P. brassicae* spores from four soil samples for the optimisation of magnetic particles (beads) and antibody concentration. Samples 1, 2, 3, 4 contain 50, 100, 200 or 400µL beads respectively, all in combination with 100µL IgM (~3.5mg/mL).

When this protocol was applied to twenty soil samples, only one sample was positive for *P. brassicae* and this contained 5.4 x 10^4 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 6.15 x 10^6 spores/mL, giving a total of 3.08 x 10^8 spores in the 50mL suspension. The maximum number of spores extracted using the magnetic particles was 2.96 x 10^7, 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 8).

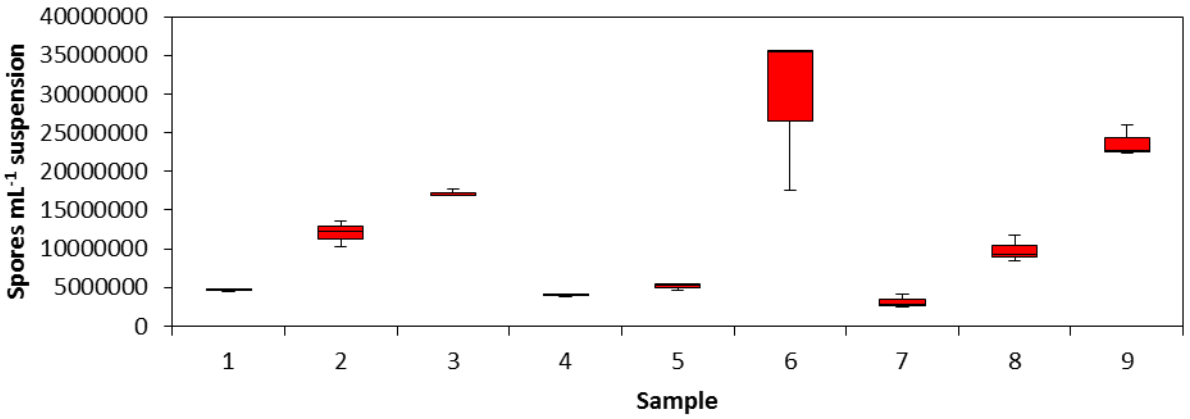


Figure 8. Immunomagnetic separation of *P. brassicae* spores from suspension for optimisation of magnetic particles (beads) and antibody levels. Samples are as follows; 1 = 1mL beads with1µL IgM, 2 =2mL beads with1µL IgM, 3= 3mL beads with1µL IgM, 4 = 1mL beads with 5µL IgM, 5 =2mL beads with5µL IgM, 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 9, 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.

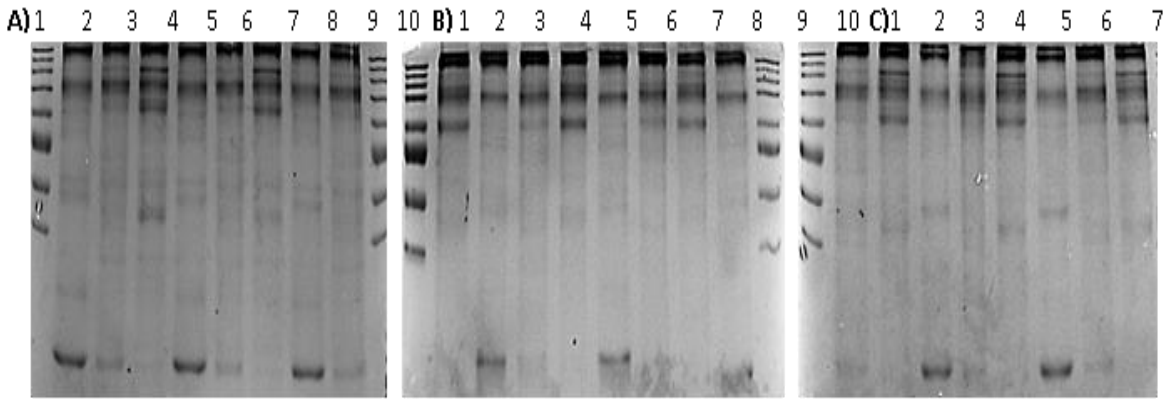


Figure 9. Papain digested *P. brassicae* IgM Antibody on non-reducing SDS PAGE gel. Lanes A1, A10, B1, B10 and C1 = Protein Ladder markers - from top bands represent 300, 250, 180 130, 100, 70, 50 and 40kDa, Lanes A2=1h 0.1mg/mL, A3=1h 0.02mg/mL, A4=1h control, A5=2h 0.1mg/mL, A6=2h 0.02mg/mL, A7=4h 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 (figure 10) reveal a different digestion pattern to the gel images 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.

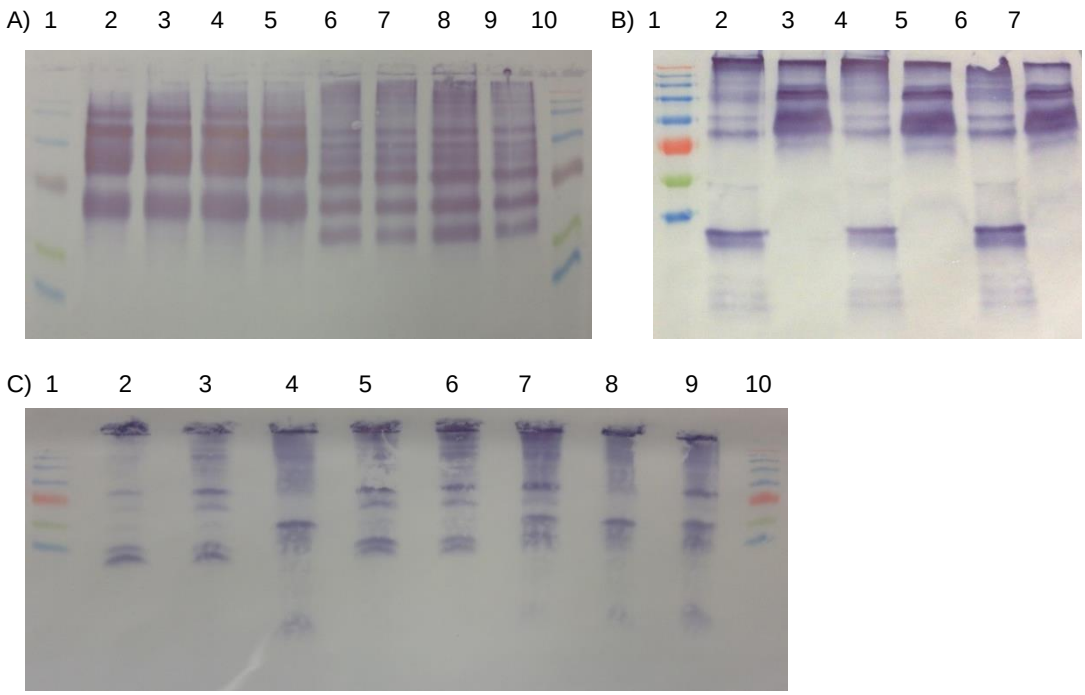


Figure 10. Pepsin digested *P. brassicae* IgM antibody on nitrocellulose membrane after western blotting with Goat anti mouse IgG+IgM (H+L chains) Peroxidase conjugate (Thermo Fisher Scientific). A) Papain digested samples, lanes 1 and 10 = protein ladder marker, A2=24h control, A3=10h control, A4=6h 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

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 immuno-staining by Western blot (figure 1
1).

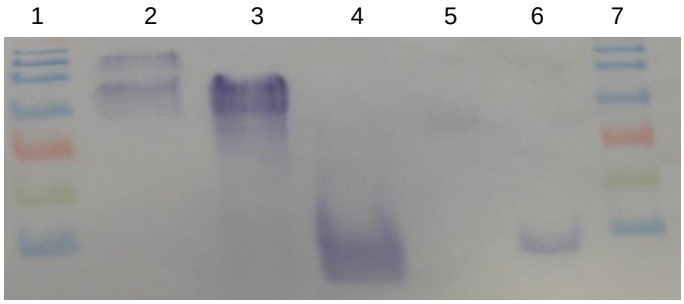


Figure 11. Trypsin and pepsin digested *P. brassicae* IgM antibody on nitrocellulose membrane after western blotting with Goat anti mouse IgG+IgM (H+L chains) Peroxidase conjugate (Thermo Fisher Scientific). Lanes 1 and 7 = protein ladder marker - from top, bands represent 300, 250, 180 130, 100, 70, 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 club-root 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 club-root 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.

Year 2

Materials and Methods

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

As in year one, a club-root soils testing service was offered to HDC participating growers and advertised through the Brassica Growers Association. Sample collection was organised through two sector leads and the soil samples sent to Worcester University for processing by real time molecular PCR (qPCR) and antibody based lateral flow devices.

*Assessment of commercial soils for club-root resting spore concentration by qPCR (10g) and lateral flow (0.25g).*Results to be provided back to respective growers within 4 weeks of receipt of soils*.

After advertising and liaison with sector co-ordinators, the soil sampling was across two areas: Scotland and Lincolnshire, as in year one. A total of 78 commercial horticultural soils were processed for estimation of club-root infestation by q PCR and the 'in-field' lateral flow test. Forty one soil samples were provided by Growers during the spring and eight sites during the autumn period. A further 23 samples were processed as part of a precision mapping exercise. This was to examine the distribution of spores within a grid referenced field. Six soils were processed in early 2015

On receipt of the samples at NPARU, unique identifiers were provided to each soil. Each soil was brought up to a 50% water content with distilled H_2O (measured using a SM200 soil water sensor and GP1 Data Logger, Delta-T Devices, UK). A DNA extraction was performed as described in Year 1 of the report using both the 0.25g and 10g soil DNA extraction kits (page 13). However, this time the manufacturer's instructions were followed and with no amendments made. Amplification and measurement of the soil DNA samples was also as described previous using the *P. brassicae* specific primers. (page 13).

Quantification of *P. brassicae* in soils was also determined by lateral flow device (LFD) following the protocol detailed in HDC Annual report FV 349, May 2012. Increasing the soil volume (w/v at an average of 15% soil moisture content) from 0.25g to 7.5g was assessed; soil at 7.5g was brought up to 20mL with buffer (B2 components), shaken vigorously and allowed to settle for five min. A 100µl aliquot from the liquid phase was applied to the lateral flow device as for the 0.25g lateral flow.

Textural analysis was also performed on the soils using a bouyoucos hydrometer (Bouyoucos, 1936, Bouyoucos, 1951) and the results were plotted on a soil textural triangle based on the soil classification system of the Soil Survey of England and Wales.

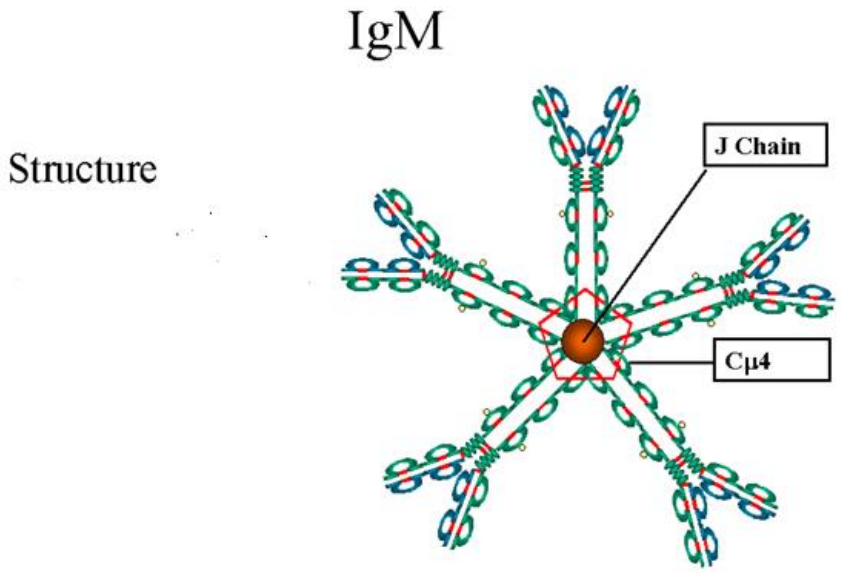
Liaise with Growers to determine crop outcome based on club-root disease prediction with each of the systems tested.

In order to communicate results emails were initially used to inform the co-ordinators. Subsequently a database for soil records was developed by the IT department at the University of Worcester. The two co-ordinators were provided with log-in details so they could view the results of the sampling and input their own details about the cropping history/outcome of the fields. Data fields completed by the University of Worcester included 'soil code', 'molecular test', '10 minute test', and 'risk', while data fields to be completed by the co-ordinators were 'cropping history', 'club-root disease history' and 'outcome'.

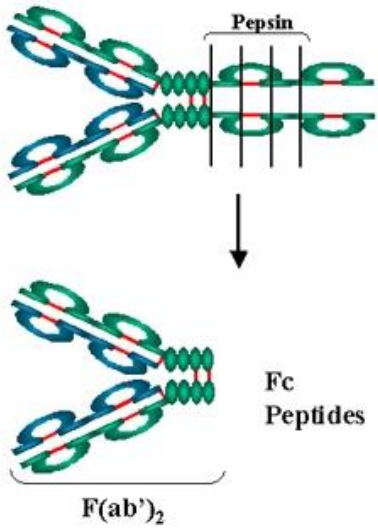
Optimisation of the magnetic fishing process (IMF) for the isolation, collection and concentration of resting spores of P. brassicae *from soils.*

Following on from the antibody fragmentation reported in Year 1 (page 16-17), further work was performed to optimise the binding of the *P. brassicae* antibody (IgM isotype) fragments to magnetic particles. Firstly, digestion of the IgM molecule using pepsin was studied, followed by separation of antibody fragments using gel filtration chromatography, confirmation of antibody fragments by western blotting and finally the coupling of the antibody fragment to magnetic particles.

IgM Digestion using Pepsin. IgM is produced by plasma cells as approx. 950kDa pentameric protein with ten antigen binding sites.



When used in LFD this large size and valency can cause issues with clumping, resulting in poor flow of antibody-antigen complex. To avoid this issue the antibody can be digested using pepsin to result in smaller molecules of IgM monomer (approx. 150kDa) and Fab 2 (approx. 100kDa), both with a valency of two.



A monoclonal antibody raised to *P. brassicae* and isotyped as IgM (coded UW 249) was purified using a Sepharose HiTrap IgM Purification HP column as per the manufacturer's instructions (GE Healthcare) to remove any bovine proteins present in the cell culture media. On average four to five passes through the purification column was required to collect all of the IgM in the culture media. The purified IgM was concentrated using Vivaspin

100kDa MWCO spin tubes (GE Healthcare), from around 40 mL to around 3mL. The concentrated IgM was then dialysed into digestion buffer (0.2M sodium acetate, 27.2g sodium acetate, pH to 4.5 using acetic acid, final volume 1L) overnight at 4ºC using a Slide-A-Lyzer dialysis cassette, MWCO 50kDa, (Thermo Scientific). After dialysis protein concentration was adjusted to 2mg/mL using Vivaspin 50kDa MWCO centrifuge concentrators or dilution with digestion buffer, depending on the concentration. A 0.16g/mL pepsin (Sigma Aldrich) solution was prepared in sodium acetate buffer, and an equal volume of pepsin solution added to 2mg/mL IgM solution i.e. 5mL of 0.16g/mL pepsin to 5ml of 2mg/mL antibody. Digestion was performed overnight (16 hours) and the reaction was stopped by adding 2M Tris base at 1:5 (200µL per mL). Samples were then concentrated to around 3mL (approx. 2mg/mL) using Vivaspin 50 KDa MWCO tubes and dialysed into PBS using the Slide-A-Lyzer dialysis cassette. Once digested the antibody fragments were separated using gel filtration.

Separation of IgM fragments. Sephacryl gel media S-300HR was selected for separation of IgM fragments as it has a molecular weight range of 10kDa–1500kDa, making it appropriate for separation of intact IgM (900kDa) and IgM monomer/Fab2 fragments (150kDa and 100kDa) respectively. Molecules of different molecular weights pass through at different speeds, larger molecules pass through quickly, smaller molecules more slowly. The S-300HR media was shaken by hand in the bottle to resuspend. Once suspended, 150mL of the media was pipetted into an EZSafe Chromatography Column (Sigma-Aldrich, UK) column, with the bottom tap closed, and allow to settle for 30 min. A further 150mL of separation buffer (Phosphate Buffered Saline, Sigma Aldrich) was then applied to the bulb at the top of the column before opening the tap at the bottom and allowing two column volumes to pass through. This process should remove all traces of the storage buffer in which the S-300HR media is supplied. For subsequent uses, the column was flushed with two column bed volumes of fresh separation buffer. A peristaltic pump at a speed of 2.5 units was then used to pack the column by forcing another column volume through the bed, compressing the gel matrix by around 10%. The running buffer was then allowed to flow out of the bottom of the bed until the level of buffer is level with the top of the bed. The IgM fragment samples were applied directly to the top of the bed using a 1ml pipette. The sample was allowed to flow into the bed, until the top of the sample is level with the top of the bed, then the bulb was topped up with separation buffer. One bed volume was loaded into the bulb in this manner, and then forced through the column using the peristaltic pump at a speed of 1.5 to 2 units. After 80mL had flowed through, the remaining outflow was collected in 5mL fractions up to 150mL. Protein concentration in each 5mL fraction was

determined using a Nanodrop 2000 (Thermo Scientific) to determine which fraction(s) contained protein. Aliquots from respective fractions were then 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 45min at 200V, and a Spectra Multicolour High Range Protein Ladder (Thermo Fisher Scientific Inc.) was included for determination of antibody fragment size. The samples were then transferred to nitrocellulose membrane using a Mini Trans-blot® Electrophoretic Transfer Cell (BioRad, USA) After which the membrane was blocked with 5% skimmed milk powder in TBST pH7.4 (Trizma base, NaCl, Tween20, distilled H2O) 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.

Coupling of IgM fragments to super paramagnetic particles. Fractions containing predominantly IgM monomer or Fab2 fragments were pooled and concentrated to 2mg/mL, then transferred to coupling buffer (50mM MES, pH 6.0, 0.025% (w/v) SDS) using 30kDa MWCO centrifuge concentrators before coupling to small Carboxyl-Modified Paramagnetic Estapor® Microspheres (Merck Millipore). The outline coupling protocol was provided by the manufacturer and was an adaptation of the two-step EDC/Sulfo-NHS coupling protocol (Hermanson, 2008). In brief 100µL 10% super paramagnetic microspheres were washed twice with 1.5mL coupling buffer before re-suspension in 1.9mL fresh activation buffer (2mL coupling buffer, 40mg Sulfo-NHS, 40mg N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and 0.025% (w/v) SDS). The microspheres were incubated in activation buffer for 15min at room temperature then washed twice in coupling buffer. After the final wash microspheres were re-suspended in 1.15mL coupling buffer. To achieve a microsphere coating concentration of 70mg antibody/g microspheres, 0.35mL of the IgM monomer/Fab2 fraction at a concentration of 2mg/mL was added to the microspheres in coupling buffer (total volume 1.4mL) and the solution thoroughly mixed and sonicated for 10s. The suspension was gently agitated on a roller for 2.5h, washed in 1.5mL coupling buffer then re-suspended in 1.8mL quenching buffer (50mM MES, pH 6.1, 0.025% (w/v) SDS, 100mM Tris base). The microspheres were then washed (with 15min incubation at room temperature) in storage buffer (PBS, 1% fish skin gelatin, 0.5% Tween-20) before final re-suspension in 1mL storage buffer. All re-suspension steps were performed in a cup horn sonicator (QSonica, LLC. USA) at 40amp for 20s. The coupled superparamagnetic microspheres were stored at 4ºC prior to use. The supernatant removed after microsphere

coating was retained and the quantity of protein measured (Nanodrop 2000, ThermoScientific) to confirm the binding of antibody to the microspheres.

To confirm activity of the *P. brassicae* antibody once conjugated to the super paramagnetic microspheres a series of tests were made. Firstly, the microspheres without conjugation to *P. brassicae* antibody, secondly super paramagnetic particles which were coated in fragmented antibody (IgM monomer and Fab 2 fragments (UW249)) and thirdly superparamagnetic particles coated with intact IgM (UW 249). Each of the magnetic particle sets were prepared using the previously detailed adaptation of the two-step EDC/Sulfo-NHS coupling protocol (Hermanson, 2008). For the negative control (no conjugation of the beads with *P. brassicae* specific Ab) the beads were processed according to the protocol but in the absence of the specific club-root antibody. Following conjugation the magnetic particles were applied to a series of club-root spore suspensions at $1x10^9$, $1x10^7$, $1x10^5$, $1x10^3$ and 0 club-root resting spores ml^{-1} storage buffer (PBS, 1% fish skin gelatin, 0.5% Tween-20). The super paramagnetic particles were added at a concentration of 0.05mg mL^{-1} to a final volume of 20ml. The super paramagnetic spore suspensions were rolled for 1h and then magnetically separated (Sepmag) for a five min.duration. For each sample the unbound liquid phase was removed using tubing attached to a 50mL syringe. On removal of the sample from the magnetic field the retained super paramagnetic particles were resuspended in 1mL storage buffer. Using a haemocytometer each of the solutions was examined for *P. brassicae* resting spores and a count made.

Thereafter each of the super paramagnetic samples was placed in a state of magnetism for a five min period. During this time a 300µL supernatant (free of super paramagnetic spheres and bound material) was removed from each sample and run on triplicate (100µl / lateral flow) club-root LFDs (conjugate pad UW 249 at 1 in 1600).

Note: Earlier studies (unpublished) had shown the super paramagnetic beads (300nm for lateral flow application) blocking non-specifically at the test line and at entry of the immunochromatographic test strip (Millipore 240 or 180 HF membrane). A range of running buffers had been assessed to overcome this. However, it was not possible to run the beads directly on the lateral flows following antibody conjugation. Other workers have reported similar problems and required dissociation of the antigen from the microsphere. For the purpose of this study the lateral flows were used to assess 'free' resting spores and soluble *P. brassicae* resting spore antigen in the absence of the superparamagnetic capture beads. In effect a competition stage was provided by the magnetic beads for binding to the resting spore or soluble antigen before testing this reaction by lateral flow.

Results

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

Results of the 78 soils (Spring and Autumn sampling) were disseminated to the sector co-ordinators either by email contact or later through the club-root web soils database page. Spring samples were processed within 5 - 21 days of sample receipt. In the autumn a turnaround time of 12 days was recorded.

Assessment of commercial soils for club-root resting spore concentration by qPCR (10g) and lateral flow (0.25g).

The soil samples received were processed as detailed in the methods section (Year 2). Out of 41 soils tested in the spring 21 were positive for *P. brassicae* resting spore infestation using the 10g DNA extraction kit. Only 10 were positive using the 0.25g DNA extraction kit. An indication of spore load was provided if a value between 1 and 1000 was extrapolated from the standard curve. Below 1000 spores g^{-1} soil it is not possible to accurately quantify the number of spores, however previous studies have shown a correlation between detection of very low levels of DNA and the presence of spores (data not shown), thus this information was provided to growers with an explanation. By the 0.25g DNA extraction kit 11 soils were reported in this manner and 13 with the 10g DNA extraction kit.

Below ≤10000 spores g^{-1} soil it is not possible to accurately quantify the number of spores when tested by lateral flow. For this reason the lateral flow provides results of a negative to low risk or high risk. Of the soils tested 13 were reported as <10,000 (low to zero risk of club-root).

Previous work in Year 1 had shown that dilution of the 10g DNA extracts increased the number of positive samples due to a hypothesised reduction in qPCR inhibition. Therefore the highest value achieved from the samples across neat, 1:10 and 1:100 dilutions was taken to be the value of *P. brassicae* spores present in the soil, and was the result reported to growers. A two tailed paired t-test showed there was significant difference between the two molecular DNA methods ($t=4.847$, $df=40$, $P<0.0001$), with the mean number of spores detected following 10g DNA extraction and q PCR being higher than the 0.25g DNA q PCR process. When the same comparison was done between the 10g and 0.25g LFD a two tailed paired t-test showed there was significant difference between the two methods

(t=2.235, df=37, P <0.05), with the mean number of spores detected following 0.25g LFD being higher than with the 10g LFD.

Textural soils analysis. In Year 1, results from soil textural analysis determined that silt and the effect of sand and clay and, their interaction was significant in reducing the q PCR signal in club-root infested soils. This effect on lateral flow was not considered. A textural analysis was performed on the soils in 2014 using a bouyoucos hydrometer as previously described (Bouyoucos, 1936, Bouyoucos, 1951). The results of the analysis are shown below (Table 1a, b) and for each soils group (Scotland and Lincolnshire) plotted on a soil textural triangle based on the soil classification system of the Soil Survey of England and Wales (Figures 12a, b). Soil texture and the interaction of this with the resting spore measurement by q PCR (DNA molecular test) and lateral flow is considered (Figure 13).

Table 1a. Scottish soils group

Soil Code (Scotland Group)		Soil Texture	% Sand	% Clay	% Silt
1		Sandy Loam	68	8	24
2		Sandy Loam	72	6	22
3		Sandy Loam	67.8	5	27.2
4		Sandy Loam	66	6	28
5		Sandy Loam	78	9	13
6		Sandy Loam	62.2	8.4	29.4
7		Sandy Loam	74	8	18
8		Sandy Loam	78	13	9
9		Sand	90	4.8	5.2
10		Sandy Loam	76	8	16
11		Sandy Loam	72	16	12
12		Loamy Sand	80	8	12
13		Sandy Loam	78	8	14
14		Sandy Loam	74.4	6	19.6
15		Sand	88	4.8	7.2
16		Sandy Loam	67.8	6	26.2
17		Sandy Loam	71	10	19
18		Sandy Loam	70.2	4	25.8
19		Sand	88	2	10
20		Loamy Sand	80	4	16
21		Loamy Sand	82.2	6	11.8
22		Loamy Sand	84	6	10
23		Sandy Loam	72	6	22
24		Loamy Sand	78.2	6	15.8
25		Sandy Loam	68	6	26
26		Loamy Sand	80	6	14
27		Sandy Loamy	66	10	24
28*		Loamy sand	82	2	16

Table 1b. Lincolnshire soils group

Soil Code (Lincolnshire Group)	Soil Texture		% Sand	% Clay	% Silt
1	Clay Loam		45	20	35
2	Clay Loam		50	20	30
3	Sandy Silt Loam		46	14	40
4	Sandy Loam		56	16	28
5	Sandy Clay Loam		55	20	25
6	Clay		30	42	28
7	Sandy Loam		68	13	19
8	Sandy Loam		77	10	13
9	Sandy Silt Loam		40	15.6	44.4
10	Sandy Loam		66	4	30
11	Sand		92	5	3
12	Sand		90	2	8
13	Sand		90.2	2	7.8
14	Sandy silt loam		32.4	18	49.6
15	Sand		92	4	4
16	Loamy sand		84	6	10
17	Loamy sand		78.4	4	17.6
18*	Loamy sand		82	2	16

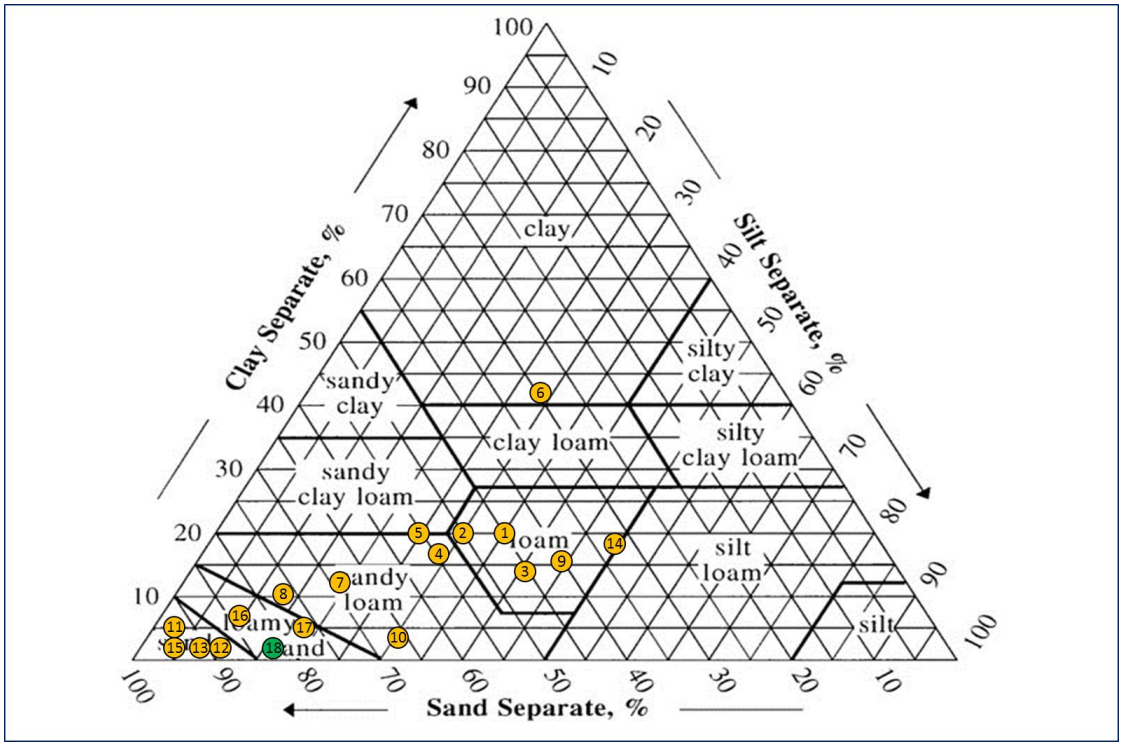


Figure 12a Textural triangle positioning of the Lincolnshire group soils as based on the soil classification system of the Soil Survey of England and Wales.

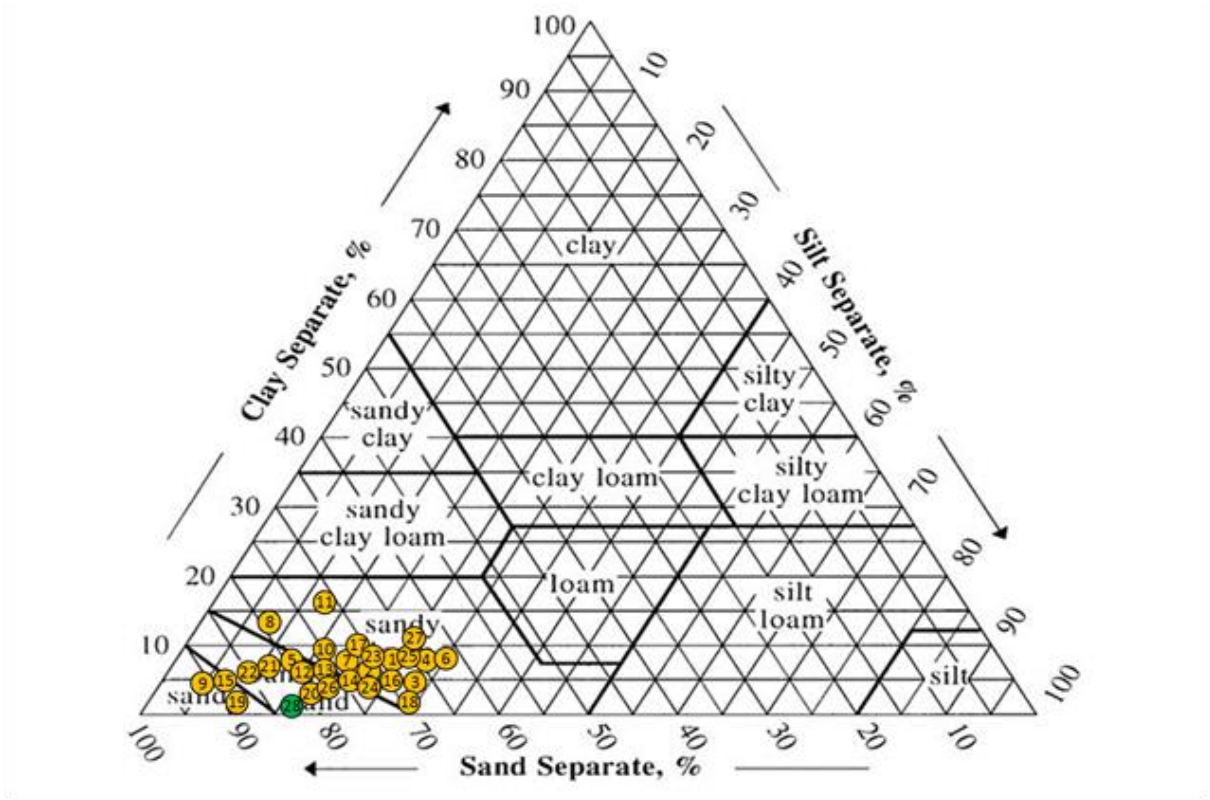


Figure 12b Textural triangle positioning of the Scottish group soils as based on the soil classification system of the Soil Survey of England and Wales.

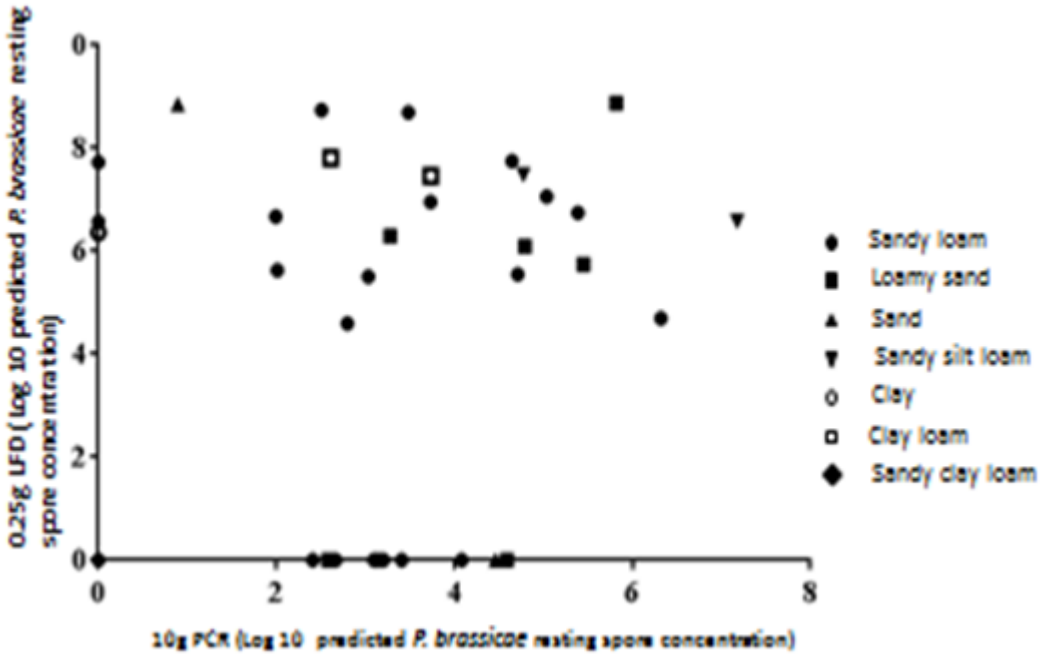


Figure 13. Relationship between the predicted risk of *Plasmodiophora brassicae* by lateral flow (0.25g soil tested) and the 10g molecular test.

Too few results are available (qPCR, LFD, bait test, textural analysis) for a comprehensive study (Tables1 a, b and 2). However, sandy loam soils and those in association with clay may associate with high positive results for the lateral flow. Soils which contain more components of loam associated with clay might tend towards false positive results (L1 and L2). Loam associated with sandy soils might result other false positive results (S10, S17 and S11). However, very sandy soils appear to be associated with false negative results using the bait test (S9 and S16) and the lateral flow device (S19 and S21). Further analysis would be required using a greater number of vegetable brassica growing soil types from sandy and sandy/loam soil growing areas.

Liaise with Growers to determine crop outcome based on club-root disease prediction with each of the systems tested.

Within the project remit (four weeks of receipt of soils) the results were reported back to the two sector leads (Lincolnshire and Scottish soils co-ordinators).To facilitate this the two sector co-ordinators had been provided with log-in details to access an interactive club-root soils database. The database provided information on soil sample receipt at NPARU and the result outcomes. Of the spring soil samples tested the results were available on the

database within five and 21 days of sample receipt at Worcester. Of the soils received in the autumn turn-around of results was within 12 days. An exception to this was for the 23 soil samples that had been received to assess the usefulness of GPS mapping of a soil for club-root potential. These soils were identified as a research exercise and fell outside the 'commercial testing' process.

The table below presents a history of club-root field disease history and grower supplied crop outcome for the soils tested, prediction of club-root disease using molecular (DNA PCR), lateral flow (in field antibody based test) and bait test. Where data is not available the following symbol is used *.

Table 2 Club-root soils database for 2014

Soil Code	Club-root Field History	Molecular DNA(0.25g) club-root spore concentration / g soil	Molecular DNA (10g) club-root spore concentration / g soil	Lateral flow test club-root spore concentration / g soil	Bait Test (% risk)	Grower outcome
S1	No symptoms when last cropped	4750	245000	11606130	*	Severe symptoms across the whole field with an average of 50% yield loss some areas worse some better
S2	No severe symptoms observed	5740		5613494	*	Severe symptoms across the whole field with an average of 50% yield loss some areas worse some better
S3		0	256	<10000	*	There was no signs of infection in any of these fields with 100% crop yield
S4		0	1600	<10000	*	There was no signs of infection in any of these fields with 100% crop yield
S5		0	0	426609	0	Full crop with no evidence of infection

Soil Code	Club-root Field History	Molecular DNA(0.25g) club-root spore concentration / g soil	Molecular DNA (10g) club-root spore concentration / g soil	Lateral flow test club-root spore concentration / g soil	Bait Test (% risk)	Grower outcome
S6					0	
S7					48.3	
S8		0	1080	320617	0	1st early crop where no instance of club-root was observed; 2nd crop there was instances of infection that contributed to yield loss
S9		0	0	<10000	78.3	1st early crop where no instance of club-root was observed; double cropped and no symptoms observed in the 2nd crop
S10		0	0	549521300	1.7	No evidence of club-root
S11		0	3040	499492800	8.3	No evidence of club-root
S12		0	0	<10000	0	
S13		0	0	39646	0	
S14					0	
S15		0	0	715749600	0	No evidence of club-root
S16	Suspected areas of club-root	1105	0	<10000	45.8	Crop to full potential with very low instances of club-root.
S17		0	0	53840800	13.3	Crop to full potential with very low instances of club-root
S18		0	5147	356652	13.3	Crop to full potential with very small instances of club-root appearing.
S19	Suspected	0	18930	<10000	21.7	Moderate symptoms with a some yield loss
S20		70880	283000	556113	28.3	Very low instance of club-root which didn't contribute to yield loss and across the

Soil Code	Club-root Field History	Molecular DNA(0.25g) club-root spore concentration / g soil	Molecular DNA (10g) club-root spore concentration / g soil	Lateral flow test club-root spore concentration / g soil	Bait Test (% risk)	Grower outcome
						whole field
S21		0	39333	<10000	21.7	instance of club-root which didn't contribute to yield loss this was across the whole field
S22		13584	659667	760723000	96.1	Very low instance of club-root with no effect on yield loss. Across the whole field.
S23					0	
S24		0	62533	1000000		No sign of Club-root
S25		0	1910	<10000	*	No sign of club-root infection
S26		0	1400	1945773	*	No sign of club-root infection
S27	Increased evidence of club-root	13240	1210000	3629298	*	
Sa		0	0	62598	*	No sign of infection and 100% crop yield
Sb		0	0	100133400	11.7	Full crop with no evidence of infection
Sc	Suspected	0	652513	961626600	38.3	Moderate symptoms with a bit of yield loss
Sd	More evidence of infection	14160	731000	1065835	*	95% crop infected and with a budget yield
GPS					83.3	
GPS					100	
L1	Suspected due to poor crop	0	5467	28656580	*	No club-root suspected
L2	Suspected due to poor crop	0	≤1000	64113240	*	No club-root suspected
L3	Severe	90860	15140000	3832005	*	Patches of severe club-root and crop loss
L4	Patches, suspected club-root	354800	2103333	49788	*	Areas of complete crop loss - severe club-root
L5	Poor crop frame - suspected club-root	0	0	<10000	*	No club-root suspected

Soil Code	Club-root Field History	Molecular DNA(0.25g) club-root spore concentration / g soil	Molecular DNA (10g) club-root spore concentration / g soil	Lateral flow test club-root spore concentration / g soil	Bait Test (% risk)	Grower outcome
L6						
L7	Suspected - crop losses in patches	0	≤1000	4720468	*	No club-root suspected
L8	Suspected - crop losses in patches	≤1000	0	3766903	*	Area left unplanted in 2014
L9	Patches of crop loss - suspected club-root	2794	59867	30579890	*	Patches of crop loss - severe club-root (winter cauliflower)
L10	Patches of crop loss - suspected club-root	≤1000	44700	56338020	*	Patches of crop loss - severe club-root (winter cauliflower)
L11	Unknown	n/a	10283	<10000	*	
L12	Unknown	n/a	25300	<10000	*	
L13	Unknown	n/a	56100	975891	*	
L14	unknown	n/a	17000	610580	*	
L15	Unknown	n/a	16500	1591861	*	
L16	Severe clubbing observed	n/a	15240	<10000	*	
L17	Unknown	n/a	9723	<10000	*	

*No bait test available

Detection sensitivity of the lateral flow at > 10000 (medium to high risk of club-root). All results below this concentration are recorded at <10000 (low to zero risk).

Tables 3 and 4, are derived from the data set recorded in Table 2. Only soils that include a bait test are included or where moderate to severe yield loss was observed. The rationale for this is that resting spore concentrations in excess of 10,000 spores per g of soil are considered required for disease expression to be observed within a crop. It is likely that below this concentration resting spores could be present but no above ground visible signs apparent on an infected host. Results for the DNA test combine both the 0.25 and 10g test. Negative or positive relates to the growers observation of club-root in the crop.

Table 3. Comparison of the three tests for prediction of soils club-root infestation as compared to grower disease observation.

	Lateral flow (0.25g)	PCR (0.25 combined with 10g)	Bait
False positive	S5,**S10***,**S11***, S15, S13,**Sb***	**S11***	S9,**S10***,**S11***,**Sb**
False negative	S16*,S21,S19	S17	S8
True positive	Sc, S1,Sd,L3,L4,L9 S2,S8,S17,S18,S20, S22, S27,L10	S20,Sd,Sc,S21,S22,L3,L4,L9, L10,S1, S8, S2, S16 S18, S19,S27	S17,S18,Sc,S19,S20, S21,S16,S22
True negative	S3,S9,S12,L5	S5,S10,S3,S9,S13,S12,S15,Sb, L5,	S5,S15,S12,S13

- Lateral flow (low to 0 risk ≤10000)
- DNA PCR test (low to 0 risk <1000)

Bait tests S10 provided a positive club-root test but at a low level (<10% value). The lateral flow predicted moderate to high disease risk. No above ground symptoms were observed in the crop. The molecular test recorded a club-root negative at 0.25 and 10g. For this reason the molecular test was treated as true negative according to the criteria of the test (grower observations)

*Each of the three tests (PCR, lateral flow and bait) recorded club-root positive for S11 soil. No above ground symptoms were recorded in the crop so all tests were recorded as false positive

Table 4. Comparison of the three tests for prediction of soils club-root infestation as compared to grower disease observation

	Lateral flow (0.25g)	PCR (0.25 combined with 10g)	Bait
False Positive	6 (22%)	1 (4%)	4 (23.5%)
False Negative	3 (11%)	1 (4%)	1 (6%)
True Positive	14 (52%)	16 (59%)	8 (47%)
True Negative	4 (15%)	9 (33%)	4 (23.5%)
Total	27 (100%)	27 (100%)	17 (100%)

Optimisation of the magnetic fishing process (IMF) for the isolation, collection and concentration of resting spores of P. brassicae from soils.

UW 249 (P. brassicae monoclonal antibody) digestion and separation of IgM fragments: Successful digestion and separation of IgM monomer and Fab2 was confirmed by Western Blot and determination of protein concentration in separated fractions. Fractions produced by gel filtration were assessed for protein content (Figure 14).

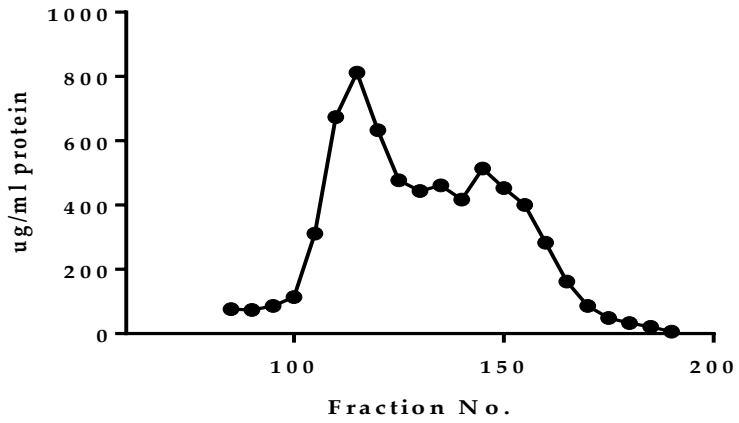


Figure. **14** Protein concentration of fractions collected

The first peak represents a fraction that is mainly IgM, the second minor peak is mostly IgM monomer and the third peak is a fraction containing mostly Fab2.

Fraction representing peaks one and three were used in Western blot (Figure 15). Intact IgM has a molecular weight of around 950kDa, so appears above the molecular weight markers. IgM monomer appears as an approx. 180 kDa band in lanes 3 and 4, while Fab2 appears at approx. 100kDa, predominantly in lane 4. Pepsin digestion can also result in the production of slightly smaller fragments as seen in Figure 15, which are likely to be a smaller version of Fab2. All fractions containing predominantly Fab2 and/or IgM monomer, but no intact IgM, were pooled and used for coupling to beads. Ability of fractions to bind to antigen was confirmed via dot blot (results not shown) before pooling.

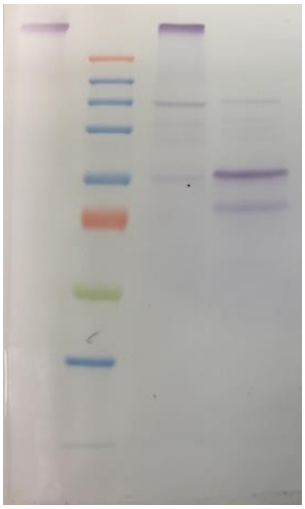


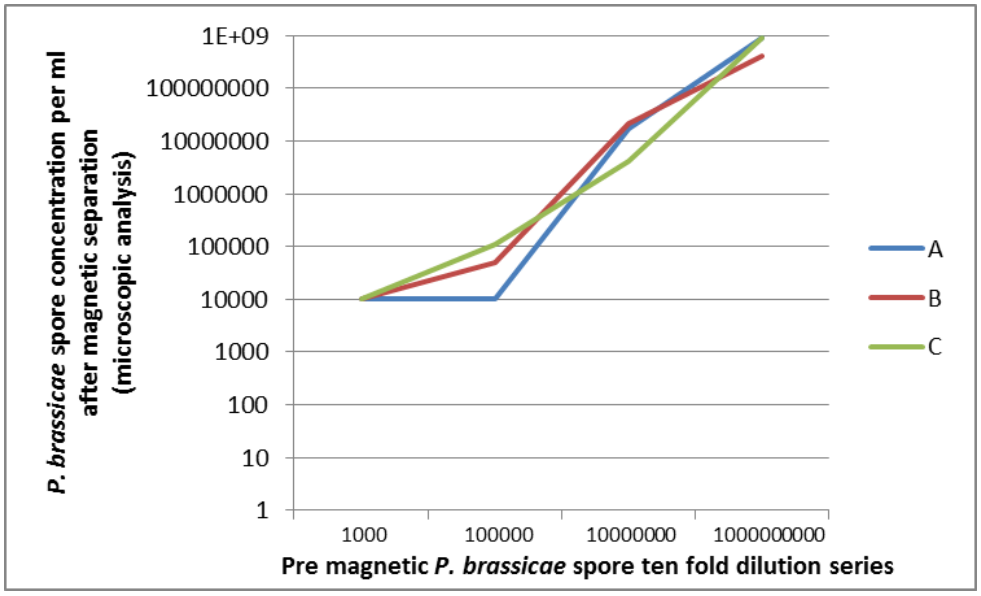
Figure 15. Western Blot: Lane 1, Undigested IgM; lane 2, Multicolour High Range Protein Ladder; lane 3, digested IgM early fraction; lane 4, digested IgM, later fraction.

Coupling of UW 249 IgM fragments to super paramagnetic beads. After coupling fragmented antibody on to magnetic microspheres the quantity of antibody in the supernatant was measured. From a starting concentration of 2mg mL^{-1} antibody, a mean of 0.097mg mL^{-1} antibody remained in solution after coupling. This would suggest that if the protein measured was antibody the conjugation had been successful. However two way ANOVA on the control experiment was less conclusive.

Assessment of the conjugated beads to isolate and concentrate P. brassicae resting spores in solution. By bright field microscopy, little or *no* difference was observed between UW 249 antibody coated super paramagnetic beads (IgM pentamer and Igm fragments) or control beads (no antibody conjugation) to isolate P*. brassicae* resting spores from a spore dilution series (Figure 16). Although, beads coated with whole IgM and antibody fragments ,(B,C) showed an increase in spore collection at a *P. brassicae* resting spore starting

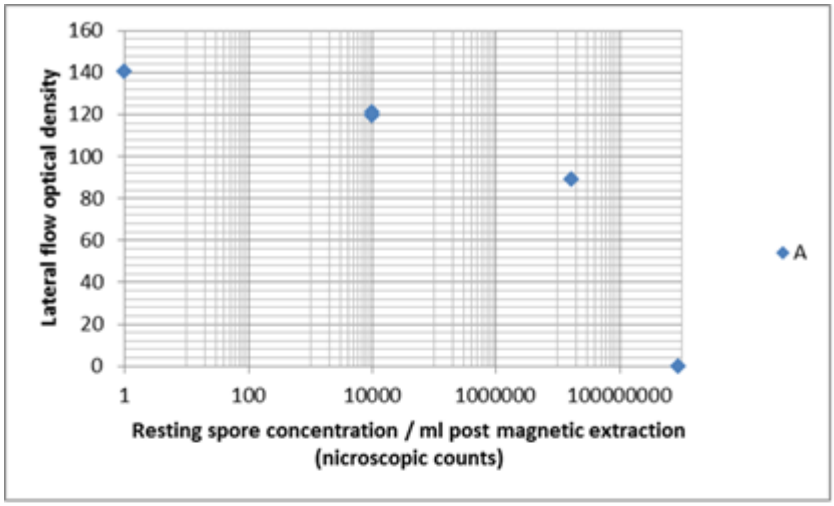
concentration of 100000 ml^{-1} (C, 10 fold increase in collection of spores post magnetism). This trend may apply to the lowest spore concentration of 1000 ml^{-1} as the accuracy of microscopic analysis at this concentration is questionable.

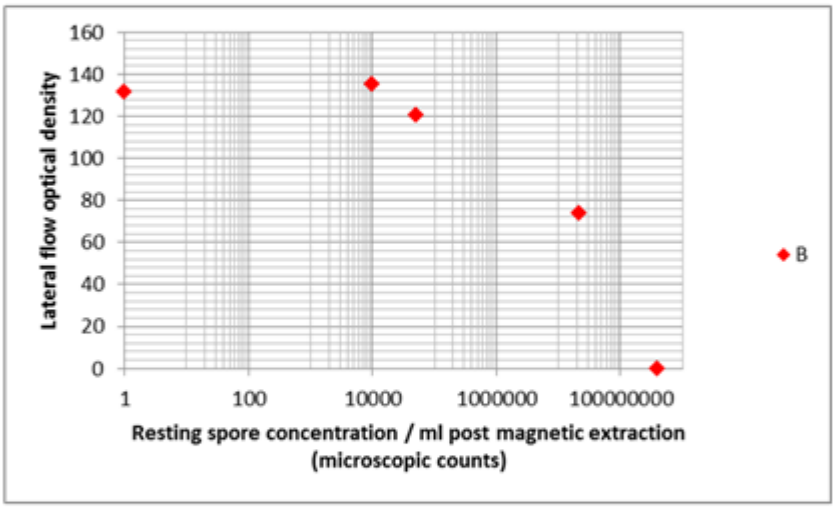
Following removal of the beads (of bound club-root spores / antigen) from each of the three solutions (A, B and C), availability of *P. brassicae* resting spore antigen in solution was tested by lateral flow device. In effect, this provides a competitive analysis to test antibody activity on the conjugated beads for a target (resting spore / soluble antigen). A trend of reduced test sensitivity for *P. brassicae* was observed across the three parameters (A – C). This would suggest that the antibody coated beads (B, C) may have bound and retained some club-root antigen which would not have been available for the lateral flow test. However these preliminary results would suggest this was not at an activity or concentration to prove efficient for spore extraction over a concentration range that would be expected in field soils.



A Super paramagnetic beads alone
B Super paramagnetic beads conjugated to UW 249 FAb_2
C Super paramagnetic beads conjugated to UW 249 IgM

Figure 16. Assessment of *P. brassicae* antibody conjugated beads (UW 249) and uncoated beads (control) for the isolation of *P. brassicae* resting spores from a tenfold resting spore dilution series.





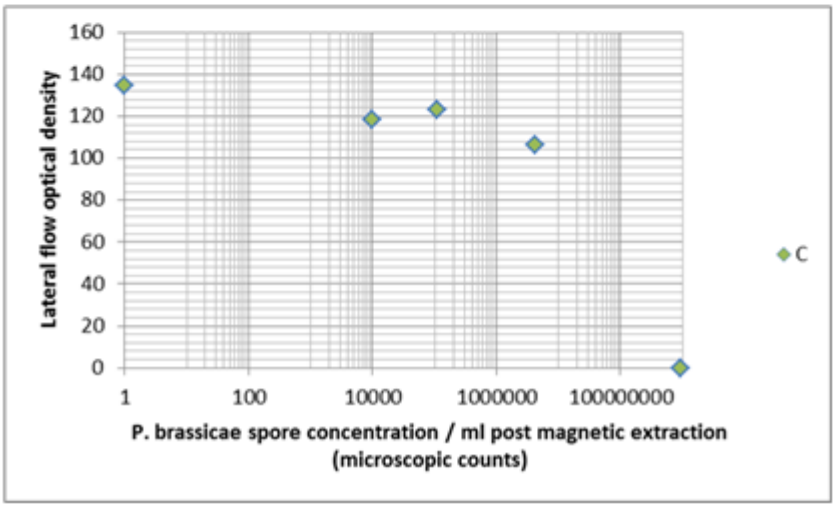


Figure 17 . Following magnetic extraction and in the absence of superparamagnetic spheres the measurement of 'free' *Plasmodiophora brassicae* antigen by lateral flow devices

Conclusions

Club-root 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 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 club-root 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 club-root 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 MoBio 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 increased the concentration of inhibitors and affected the elution efficiency of the final step. For this reason the samples were diluted and a substantial number of soils previously scored as club-root free / low risk were found to have epidemiologically significant number of resting spores present.

The second area which had been raised as an area 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 saturated on arrival at NPARU due to the wet conditions at sampling. Air drying the soils at room temperature took upwards of three to four weeks in some cases. To ensure uniformity of the club-root resting spores in the soils, an extensive grinding and sieving process was required prior to the DNA extraction process, whilst the DNA extraction and quantification by qPCR was short (1.5 days for all samples). In comparison to the length of time taken here, a Canadian commercial testing laboratory offers club-root 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 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 provide 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 likely to 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.

Based on the results observed in Year 1 it was decided that for the 2015 period, all soils would be assessed on arrival for soil moisture content and then adjusted to 50% using molecular grade water. Most soils in 2015 were recorded to have a moisture content of 15% at arrival to NPARU. Thereafter two DNA extraction processes were adopted (10g and 0.25g soil) and by lateral flow (0.25g and 7.5g soil) for the assessment for club-root resting spores in the soil. Of the soil samples tested (41) in spring the results were available between five and 21 days of sample receipt at NPARU. In the autumn the turnaround of results was within 12 days. An interactive web based club-root soils database was developed by the IT department at the University of Worcester and made live from 2015 for dissemination of results to the participating 'grower' sector co-ordinators.

Assessment of the molecular (DNA PCR) test for the measurement of club-root in cultivated horticultural soils indicates a test accuracy of 92% when compared with grower observation of club-root symptoms in the following cropping period. However, this accuracy should be recorded at 96%, as each of the tests (molecular 10g, bait and lateral flow) recorded *P. brassicae* positive for a 'club-root negative' soil (S11). No above ground disease symptoms were observed in the crop however it has been well documented that >10000 resting spores g^{-1} is required before uniform disease expression is observed on infected plants. The PCR test for this soil type was 3040 spores g^{-1} soil and is indicative of the healthy crop outcome observed. However, in this field soil successive plantings of Brassicas are likely to result in the occurrence of club-root disease symptoms as the resting spore concentration is close to the disease threshold. By adopting a four year rotation period the concentration of resting spores could be reduced by up to 50% and maintain resting spore concentration at well below the threshold level.

The 10g soil DNA extraction measured an increased concentration of *Plasmodiophora brassicae* resting spores in the soils tested when compared with the 0.25g test. However on one occasion the 0.25g test provided a positive test that was missed by the 10g test. A low level of disease was observed in the crop. This inaccuracy is likely a result of the sampling process rather than indicative of the test. Resting spores of club-root disease are reported as of patchy distribution within the soil medium. Conversely, when the same comparison was made with the lateral flow test, it was determined that the mean number of club-root resting spores detected were higher when the 0.25g lateral flow method was adopted.

Compared with the grower observations for club-root disease, a 67% test accuracy rate was recorded for the lateral flow test (negative to low risk<10,000 spores g soil; ≥10,000 medium to high risk club-root). However as for the molecular test, instances were recorded where the lateral flow test recorded positive for disease and this was subsequently confirmed by bait test. Examples of this are recorded for soils S10, S11 and Sb. Using the same analogy as described for the molecular test, an accuracy of 79% could therefore be recorded. Also, S16 soil sample was recorded as a false negative result (0<1000 spores g^{-1} soil recorded) but where the grower observed low instances of disease in the crop. The result may reflect the sample process (0.25g) and patchy nature of the disease at this low level rather than the test accuracy. The molecular test recorded a resting spore concentration of 10,000 spores g^{-1} soil which is at or near the cusp of disease expression in the field. Also, only one of the two molecular tests identified disease presence. Similarly, for soil S21 (low level of club-root disease observed and patchy) a negative result was recorded by the lateral flow device whilst the molecular test recorded in only one of the tests applied (10g, 30000 spores g^{-1} soil).

Based on the data set provided the bait test provided an accuracy of 70.5% compared to the grower outcome. However where no disease was observed in the crop (i.e. resting spores concentration was below the disease threshold for above ground symptom expression) the bait test detected disease presence but only at a low level (S10, S11, Sb: < 12%). However, S9 remains as a false positive, the bait test reported 78.3% risk where the molecular, lateral flow and a double cropped Brassica reported no disease. The bait test when compared to the other diagnostic tests could record an accuracy of 88.5%.

Textural Analysis. In earlier studies (FV 349), a difference of spore concentration on the lateral flow test read out (optical density) had been observed for different *P. brassicae* pathotypes, in particular race 5, although this pathotype was not considered a gene breaker i.e. able to overcome Brassica club-root resistant varieties. Studies have shown that within a single club-root gall multiple pathotypes can be present. Differences observed by the lateral flow could in part result from the P. brassicae pathotypes present in the soils. However it is more likely that the textural structure of the soil type and the small sample size will have influenced the accuracy of the test.

Studies reported in Year 1 showed that silt and the effect of sand and clay and, their interaction was significant in reducing the capability of the q PCR molecular test for measurement of *P. brassicae* in soil. This effect on the lateral flow had not been considered. In variation to the PCR test, a complex extraction process is not followed or the removal of inhibitors made. Instead a simple flotation process is adopted where the larger soil particles

are allowed to settle out of solution. This is required to prevent the pores of the lateral flow (nitrocellulose membrane) from blocking and inhibition of the test. It is likely that this 'settle' process provides opportunity for resting spores of *P. brassicae* to be taken out of solution. Also the positively charged resting spore has been reported to adhere to clay particles which are flat, plate-like and negatively charged. Too few results were available for a comprehensive study of soil textural significance on the efficiency of lateral flow test. However sandy loam soils and those in association with clay might lend towards false positive results. Gold complexes (to include colloids used in the lateral flow) have been shown to absorb to organic matter, clays and iron. Each of these factors could affect the accuracy of the test. For this purpose, studies in year 2 continued to focus on isolation of the resting spores from the soil by immuno-magnetic extraction.

Immuno-magnetic study. Immuno-magnetic fishing has been used in the food industry to isolate and concentrate target bacteria. Club-root resting spores are of a similar size. Studies at NPARU have shown that *P. brassicae* specific antibody coated magnetic beads can label and isolate resting club-root spores directly from a range of soil types. This process has provided capability for the concentration of resting spores from collected soil samples of 50g lots. In these studies the club-root resting spores were directly labelled with UW 249 monoclonal antiserum and then indirectly captured with anti-mouse IgM super paramagnetic particles BE-M03/0.3 (Merck Chimie SAS). These particles were of suitable size for lateral flow test development (300nm). In Year 1 of the project, the super paramagnetic particles were withdrawn from the market place as a standard product. Availability was limited to a specialist production order at a provided ball park figure in excess of £5000:00 for a 20 ml volume. For this reason it was decided to invest time at Worcester to develop a conjugation process of UW 249 antibody directly to 300nm super paramagnetic beads. Magnetic labels have compared to gold colloids shown to provide increased levels of sensitivity when used in assay format. This has the potential to improve the detection limit of the assay to 100 spores per g^{-1} 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 lateral flow test format.

For this purpose the *P. brassicae* IgM antibody (UW249) has been purified and digested by pepsin, trypsin and papain for production of antibody fragments which retain activity of the complementarity determining regions (CDRs) for *P. brassicae* recognition and binding. In Year 2 of the project this process has been achieved with the production of IgM monomer

and Fab_2 fragments. Unfortunately, the conjugation of these fragments to the super paramagnetic microspheres (300nm) has not proved efficient for the isolation and extraction of resting spores from solution or soil. Optimisation of the conjugation process may improve this efficiency. Where conjugation of IgG isotypes is routine and standard protocols developed the application of this procedure for IgM antibodies is not well reported. This area of work is likely to require significant investment of time to develop suitable protocols.

Commercialisation of the molecular and lateral flow tests for the UK Horticultural Industry.

Molecular DNA test: The UK horticulture industry now has an optimised and validated molecular *q PCR* test for the assessment of field soils for club-root disease. This test can be carried out quickly (within days of laboratory receipt) and of increased accuracy compared to the industry standard (bait test). HDC reports (FV259, 349 and CP099a) provide detailed protocol development of the soil DNA extraction test process and the q PCR test parameters. The clubroot primers used were developed by Faggian and Parsons (2002).

Primer 1 CGCTGCATCCCATATCCAA
Primer 2 TCGGCTAGGATGGTTCGAAA

At this time it is unclear whether the University of Worcester would be able to facilitate a commercial clubroot soils molecular test for the UK horticultural industry. The HDC may wish to offer this service through plant clinics UK wide.

Lateral flow test: Unfortunately, the lateral flow test remains problematic for commercialisation at this stage. The test suffers in accuracy as a result of the varied soil matrix and the 0.25g sample size. To address these issues the following suggestions are made:

1. Continuation of studies to develop an immuno-magnetic fishing test for the isolation of clubroot resting spores directly from soil. During this project good progress has been made to purify and fragment the clubroot IgM antibody (IgM monomer and Fab_2). Conjugation of these fragments to super paramagnetic spheres has shown some promise for the isolation of clubroot resting spores directly from soil. However, the method developed at this time provides a poor collection efficiency. Only a ten-fold increase was observed at the lower resting spore concentrations (≤100,000 spores). Future work should look to improve the IgM conjugation process to the spheres and thereafter apply to soils for the isolation and concentration of resting

spores from soil. Once this conjugation process has been developed and fully optimised it should be applicable to other IgM antibodies within the HDC diagnostics portfolio providing improved diagnosis of plant pathogens in a wide variety of environmental samples i.e. seeds, soil and water. For example, the isolation and concentration of target disease will help to negate issues of sample size and the low test extract volume used.

Interestingly, medical research has only recently discovered the use of IgM monoclonal isotypes for clinical application. However, as we have found, difficulties of working with this isotype are reported (Gautam and Loh, 2011; Sherif, 2012). To help solve these issues for clinical test application an annual International conference was set up in 2013: The new ParadIgM - IgM from bench to clinic (3rd Workshop: The New ParadIgM –**IgM** from **Bench to Clinic** April 21–22, 2015). It is likely that with continued effort in this area, advances will be made for this isotype in pharmaceutical test application. Where we have made good progress in this project (purification and fragmentation of IgM), clinical test applications and tailored IgM commercial products may in the future come on stream. However, some caution should be maintained as this will likely be at a cost given the pharmaceutical application that they will be derived from.

For this reason, focus should remain on improvement of assay formats which rely on IgM diagnostic probes for test delivery in crop protection. Antibody fragmentation and the development of label conjugation protocols for the clubroot IgM isotype would prove highly translational to the other IgM antibodies that have been developed and used in HDC studies. For example, the monoclonal antibodies raised to *Mycosphaerella brassicicola*, *Didymella bryoniae* (*Mycosphaerella melonis*), *Peronospora destructor*, *Erysiphe cruciferarum*, *Albugo candida* and *Pyrenopeziza brassicae* are all characterised as IgM isotype. The immunoassay tests developed for each of these antibody probes rely on the indirect attachment of markers for quantitative measurement of the target disease. This requires additional steps in the assay process and extra cost for the purchase of anti-species labels i.e. goat anti-mouse IgM gold spheres for lateral flow application (British Biocell Solutions, http://www.bbisolutions.com). Solving the IgM conjugation issue with direct attachment to markers would be useful across crop protection test applications. However, given the fundamental aspects of study required, would best suit a PhD. Below an approach is described for the clubroot IgM antibody:

- Optimise conjugation of fragmented IgM to super-paramagnetic spheres for the efficient isolation and concentration of clubroot resting spores from water and soil.
- Validation of this approach using the soils collected and stored in CP 099a and compared with the bait and molecular DNA test results.
- Progression of the clubroot lateral flow with magnetic sphere captured resting spores.
- Exploration of this technology with smart phone lateral flow readers for quantitative measurement of clubroot.
- Evaluate field sampling processes with focus towards GPS mapping of soils for pH, magnesium, calcium, nitrate and clubroot resting spore concentration. Studies would assess the soil sample volume, sample collection period and application of the test results within an integrated disease management system for the targeted control of clubroot.
- For improved efficiency of the test integrate the magnetic clubroot 'fishing' stage with a simple pre soil 'exclusion' process (see below).

This route provides a robust but a long term approach towards the development of a system to isolate and extract clubroot resting spores from soil. In the short term, it may prove possible to isolate clubroot spores partially free of soil by using simple low cost size exclusion columns.

2. Column size exclusion study. To test this theory a preliminary study has been made with the filtration of six commercial soils (Table 5) through china clay sand columns. The soils had previously been evaluated for clubroot infestation either in Years 1 or 2 of CP 099a by molecular PCR (10g test) and lateral flow (0.25g). Each of the china clay columns were prepared within a disposable 50ml syringe (Figure 18) and pre-washed with a blocking agent and a surfactant.

Figure 18. A disposable 50ml syringe packed with a china clay sand mix prior to soil filtration (soil size exclusion)

Method: The soils were prepared in 10g lots and to each 40 ml of filtration solution was added. The soils were vigorously shaken and by gravitation allowed to filter through a china clay column. The filtrate of each soil solution was collected and by bright field microscopic examination an attempt was made to estimate the resting spore concentration. The filtrate was then applied (100µl) directly to a clubroot lateral flow and a reading made after 15 minutes. To act as a standard control a soil designated by PCR as free of clubroot disease and an artificially infested soil series (100, 1000 to 100000000 resting spores g^{-1} soil) were processed in the same way.

Results: Clubroot resting spores in the artificially infested soil were only visible in the filtrate by bright field microscopic examination when concentrations of ≤ 1,000,000 spores g^{-1} soil were used in the study. The volume of liquid used to bring the soil into suspension and the filtration process provided a reduction of resting spores of between 10 and 100 fold when assessed by bright field examination (Table 5). Examination of the commercial soils post filtration identified clubroot resting spores in two of the soils. When compared to the artificially infested soils, these results indicate soil D (Lincolnshire Glass1) prior to the filtration process was at a minimum clubroot infestation level of 1,500,000 spores g^{-1} soil and soil F

(G3DFFA012) 6,000,000 resting spores g^{-1} soil. Resting spores may have been present in the other commercial soils but at a concentration below the level of detection by bright field microscopy.

Table 5. Measurement of clubroot resting spores in natural (commercial soils) and artificially infested soil following a column size exclusion study

Soil code	Soil identifier	Microscope counts (clubroot resting spores ml^{-1} filtrate)
A	G3Conb 073 (2013)	0
B	Hard 1 (2014)	0
C	Kit Kat 1	0
D	Glass 1	150,000
E	2013MBH 1122 (2013)	0
F	G3DFFA 012	600,000
0	Clubroot free soil	0
1000	Clubroot artificially infested (spores g^{-1} soil)	0
10000	Clubroot artificially infested (spores g^{-1} soil)	0
100000	Clubroot artificially infested (spores g^{-1} soil)	0
1000000	Clubroot artificially infested (spores g^{-1} soil)	100,000
10000000	Clubroot artificially infested (spores g^{-1} soil)	800,000
100000000	Clubroot artificially infested (spores g^{-1} soil)	5,000,000

The limit of detection by microscopic examination was deemed to be 1,000,000 resting spores g^{-1} soil

When the soil filtrates were processed by lateral flow (detection limit of 10,000 spores g^{-1} soil) soils B, C and E (Hard 1 2014, Kit Kat 1 and MBH1122 2013) fell at or below the limit of detection i.e. low to negative risk. Soil A was determined to be at risk to clubroot with 25,900 spores g^{-1} soil identified, soil D at 32,700 and F at 126,000 spores g^{-1} soil. These values were generated from a standard curve (clubroot free soil and an artificially infested soil filtrate series).

The grower observation for each of the soils is shown in Table 6 along with the molecular 10g test result, lateral flow 0.25g and the lateral flow soil filtrates.

Table 6. Assessment of natural soils (commercial soils) for clubroot infestation

Soil code	Molecular 10g test	Lateral flow 0.25g test	Pre soil sieve / filtrate extraction lateral flow	Grower observation
A	7250	*<1000	25,900	Patches severely damaged with crop losses
B	<1000	508,034	*<1000	No clubroot observed
C	<1000	1,238,334	*<1000	No clubroot observed
D	15,140,000	847,656	32,700	Patches severely damaged with crop losses
E	0	*<1000	* 10000	No clubroot observed
F	NT	*<1000	126,000	Severe clubroot leading to crop loss

***limit of detection so value is 0 to ≤10000 spores**

NT – soil not tested in 2013 with 10g test but a resting spore value of 46,300 g^{-1} soil when assessed by the 0.25g molecular PCR test.

It should be noted that the soils used in the lateral flow tests had been air dried and ground (as per Year 1 CP 099a soil preparation).

Conclusion.

- From a small pilot study, the filtration of soils by size exclusion provides potential for clubroot resting spore recovery. Soil contaminants remained visible in the filtrate but at a reduced concentration. Some loss of resting spores was observed during the filtration process for the artificially infested soils (between 10 and 100 fold). This is likely the case for the naturally infested soils.

- The loamy sand of the artificially infested soil left no visible layer at the surface of the size exclusion column (china clay sand mix) and the filtration process was rapid. For the commercial soils (natural soil) sedimentation at the column surface was at times observed, the amount variable and the filtration time varied. The removal of sedimented soil material by size exclusion is likely to prove very important in the ability of the lateral flow test to run successfully. During this study (CP 099a) many of the natural soils tested have been observed at the outset of the assay to block the lateral flow membrane. This will likely restrict the flow of the clubroot antibody and

provide a false positive result. The pre filtration step used in this pilot study looks promising in reducing this effect.

- The assessment of other size exclusion formats (i.e. china clay sand gradient and other mixes or cellulose based) should be investigated. It may be possible to improve the removal of soil contaminants and increase resting spore recovery. Following the filtration process, the use of immunomagnetic separation could be used to concentrate resting spores and provide a final sample clean ahead of lateral flow application. This should increase the sensitivity of the test and reduce inhibition of the lateral flow assay by soil contaminants or inhibitors, if still present.

References

Andrew, S & Titus, J. (2000). Fragmentation of Immunoglobulin G. In. Current Protocols in Cell Biology. Unit number 16.4.

Bifulco, J.M. & Schaefer, F.W. (1993). Antibody-magnetic method for selective concentration of Giardia lamblia cysts from water samples. Appl. Environ. Microbiol. 59: 772–776.

Buckzacki, S.T, Ockendon, J.G. 1978. A method for the extraction and enumeration of resting spores of *Plasmodiophora brassicae* Woronin from infested soils. Ann. Appl. Biol. 88, 363-376.

Cai, P., Huang, Q.Y. & Zhang, XW. (2006). Interactions of DNA with clay minerals and soil colloidal particles and protection against degradation by DNase. Environ. Sci. Technol. 40, 2971-2976.

Cao, T., Tewari, J., Strelkov, S.E., (2007). Molecular detection of Plasmodiophora brassicae causal agent of club-root of crucifers, in plant and soil. *Plant Dis.* 91, 80-87.

Erdner, D.L., Percy, L., Keafer, B., Lewis, J. and. Anderson, D.M. (2010). A quantitative real-time PCR assay for the identification and enumeration of *Alexandrium* cysts in marine sediments. Deep Sea Res (2) Top Stud Oceanogr. 57, 279–287.

Gallaher, S. (2006). One dimensional SDS Gel Electrophoresis of proteins. Curr Protoc Mol Biol. Unit 6.1.

Hwang, S.F., Ahmed, H.U, Strelkov, S.E., Gossen, B.D., Turnbull, G.D, Peng, G., Howard, R.J (2011). Seedling aga and inoculum density affect club-root severity and seed yield in canola. *Can J Plant Sci.* 91:183-190.

Jorstad, I. (1923). Hvorledesskan man bekaempe klumproten paa vore kaalvekster? Norsk Havetia, 39: 126 – 127.

Kemp, B.M., Monroe, C., Judd, K.G., Reams, E. and Grier, C. (2014). Evaluation of methods that subdue the effects of polymerase chain reaction inhibitors in the study of ancient and degraded DNA. J. Archaeol. Sci. 42, 373-380

Kemp, B.M., Monroe, C. and Smith, D.G. (2006). Repeat silica extraction: a simple technique for the removal of PCR inhibitors from DNA extracts. J. Archaeol. Sci. 33, 1680-1689.

Klemsdal, S.S., Herrero, M-L., Wanner, La., Lund, G. and Hermansen, A. (2008). PCR based identification of *Pythium* spp. causing cavity spot in carrots and sensitive detection in soils. *Plant Pathology* 57: 877-886.

Kong, P., Hong, C.X., Jeffers, S.N., Richardson, P.A (2003). A species specific polymerase chain reaction assay for the presence of *Phytophthora nicotianae* in irrigation water. Phytopath. 93, 822-831.

Liu, C. & Jia, Q. (2011). Lateral flow immunochromatography assay for sensitive pesticide detection by using Fe_3O_4 nanoparticle aggregates as colour reagents. Anal Chem. 83, 6778-84

Lombard, N., Prestat, E., Dirk van elsas, J. & Simonet, P. (2011). Soil-specific limitations for access and analysis of soil microbial communities by metagenomics. FEMS Microbiol. Ecol. 78: 31-49.

Luk, J.M.C. & Lindberg, A.A. (1991). Rapid and sensitive detection of *Salmonella* by immunomagnetic monoclonal antibody-based assays. J. Immunol. Methods. 137: 1-8.

Lloyd-Jones, G. and Hunter D.W.F. (2001) Comparison of rapid DNA extraction methods applied to contrasting New Zealand soils. Soil Biol. Biochem. 33, 2053–2059.

Mansfield, L.P. & Forsythe, S.J. (1993). Immunomagnetic separation as an alternative to enrichment broths for *Salmonella* detection. Lett. Appl. Microbiol. 16: 122-125.

Mullins, P.H., Gürtler, H. & WellingtoN, E.M.H. (1995). Selective recovery of *Streptosporangium fragile* from soil by indirect immunomagnetic capture. Microbiol. 141: 2149-2156

Murakami, H., Tsushima, S., Shishido, Y., (2002). Factors affecting the pattern of the dose response curve of club-root disease caused by *Plasmodiophora brassicae*. *Soil. Sci Plant. Nutr.* 48, 421-427.

Plaut, A.G., & Tomasi, T.B. (1970). Immunoglobulin M: pentameric Fcmu fragments released by Trypsin at higher temperatures. Proc Natl. Acad. Sci. 65, 318-22.

Rouxel, F., Briard, M. & Lejeune, B. (1988). Studies of soil receptiveness to club-root caused by *Plasmodiohora brassicae*: experiments on responses of a series of vegetable soils in Brittany. Proceedings of the CEC/IOBC Experts' group meeting, Rennes, 20-22nd November 1985, 145-152.

Stewart-Wade, S. M. (2011). Plant pathogens in recycled irrigation water in commercial plant nurseries and greenhouses: Their detection and management. Irrigation Sci. 29, 267-97.

Theron, J. & Cloete, T. E. (2004). Emerging microbiological detection techniques. In Microbial Waterborne Pathogens (Eds. Cloete, T. E., Rose, J., Ford, N. and Ford, T.) IWA Publishing London. ISBN 843390558: 155-186

Tsushima, S., Murakami, H., Akimoto, T., Katahira, M., Kuroyanagi, Y., Shishido, Y., (2010), *JARQ.* 44, 383-390.

VanGuilder, H.D., Vrana, K.E., Freeman, W.M. (2008). Twenty-five years of quantitative PCR for gene expression analysis. *Bio Techniques* 44: 619-626.

Voorrips, R.E., (1996). A one hit model for the infection of club-root-susceptible cabbage (*Brassica oleracea* var. *capitata*) by *Plasmodiophora brassicae* at various inoculum densities. *Eur. J Plant. Path.* 102, 109-114.

Webster M.A., and Dixon G. R. (1991) Calcium, pH and inoculum concentration influencing colonisation by *Plasmodiophora brassicae*. *Mycological Research* 95: 64-73.

Wilson, I. G. (1997). Inhibition and facilitation of nucleic acid amplification. Appl. and Environ. Micro. 63, 3741–3751

Knowledge and Technology Transfer

Publications

Alison Wakeham May 2013. Final Report HDC Project FV349 entitled “Further development of an in field tests for resting spores of club-root and the development of club-root control based on detection

Seminars

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

Winter cauliflower and spring cabbage trials Open day and Conference, January 21st 2015. Club-root roadshow at Trevarnon farm, Gwithian, and at Tregenna castle, St. Ives. (38 soils sampled on site using the lateral flow).

UK Brassica and Leafy Salad conference and trade exhibition, Wednesday 28th January 2015, Peterborough, Lincolnshire
Club-root LFD roadshow (11 soils sampled on site using lateral flow).