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GROWER SUMMARY

Headline

A detection test developed for the detection and measurement of clubroot spores in soil has been evaluated for use by UK growers.

A Brassica disease forecast is under development to evaluate the potential to generate risk assessments for clubroot disease based on soil type, crop and clubroot resting spore numbers.

Background and expected deliverables

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

Once soil has been contaminated, clubroot spores remain viable for up to 18 years. Information on the presence or absence of clubroot in soils has been difficult to obtain because the traditional methods cannot detect low levels of clubroot in soils. These methods are based on using the reaction of bait plants, however, large numbers of plants are required in these tests if small amounts of clubroot were to be detected. With the development of new detection methods based on molecular approaches the presence or absence of clubroot can be determined in most soil samples. These tests are laboratory based but require a high degree of precision by the operator.

In this project, a competitive lateral flow device has been developed and is under validation for use in UK commercial soils for the rapid testing and detection of the clubroot resting spores. This test can identify very low levels of clubroot in the soil (10000 spores/gram of soil).

The lateral flow device can be used in soil (field growers) and the potential for use in water based systems such as reservoirs and irrigation lines (vegetable Brassica propagators). A quantitative measurement of clubroot resting spore infestation can be made using the lateral flow test device when used in conjunction with a lateral flow reader device and standard

curve data. This means that a prediction on whether the crop is at risk can be determined and at what level i.e low, medium and high risk.

Determining the clubroot resting spore number in soils using either a molecular or lateral flow test is an essential component in the development of an integrated disease management programme. Currently only two chemicals (cyazofamid – Ranman and fluazinam – Shirlan) approved for control of disease in potato crops have been demonstrated to have any potential for controlling clubroot in the field. However both these chemicals do not hold approval for clubroot control in vegetable Brassicas as their efficacy against clubroot has not yet been demonstrated. Alternative control measures are still urgently needed. Within the current project alternative products are assessed for application to clubroot infested soils to assist in the control of the disease. A Brassica disease forecast is under development to evaluate the potential to generate risk assessments for clubroot based on soil type, crop and clubroot resting spore numbers.

The expected deliverables from this project are:

- Better detection of clubroot in the field before planting the crop.
- Detection tests which can be used “in field” to determine the level of risk to the vegetable Brassica crop posed by clubroot.
- Investigation of alternative products for clubroot control in the field.
- Investigation of the economics of Brassica production under different levels of clubroot risk.

Summary of results and main conclusions

Years 2 and 3

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

The current study has evaluated two diagnostic tests for the measurement of clubroot in soil. The molecular test has provided the measure/yardstick to evaluate the antibody based test against (lateral flow test). Throughout the process the lateral flow test prototype has evolved to provide a field test which shows potential for the semi-quantitative estimation of clubroot resting spores in UK commercial soils. In Year 3 of the project, 53 commercial soils were assessed by each test process and 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 (qPCR) test. Ranking both sets of results into low, medium and high disease risk categories however improved the correlation further.

Variation in the level of clubroot infestation in soils between the qPCR test and the lateral flow may result from the storage process of the soils ahead of testing. Dr Robert Faggian (DPI, Melbourne, Victoria: personal communication) reported that storage conditions effect clubroot resting spore DNA extraction level. Within one month at 4ºC storage, the DNA test signals of clubroot infested soils were seen to decline and the resultant qPCR value generated was significantly below that originally observed. This was observed in soils collected in 2007 and 2010 (series 7 and 10) which were initially stored at 4° and then -20°C, prior to re evaluation in 2011. Soil type is also important in the context of test disease estimation. It is known that the soil type can influence DNA extraction and subsequently the outcome of PCR amplification due to the presence or absence of inhibitory substances (Lloyd-Jones and Hunter, 2001). This has also been noted for immunological tests (antibody tests i.e lateral flow device) and with particular reference to soils high in humic and fulvic acid.

Soil textural parameters could also be linked to differences in test results. Soils collected in 2007 were assessed for soil textural type and approximate proportion of sand, silt and clay composition. Following analysis by qPCR it was determined that the silt content was significant in reducing test sensitivity in naturally infested soils. This study was extended in Year 3 to newly collected soils which were identified as clubroot disease free. These soils were then artificially inoculated with clubroot disease over a concentration range of high to low. The study revealed that although silt was significant in reducing the qPCR signal in naturally infested soils the effect of sand and clay and, their interaction, was significant. Further analysis of the soils from these two data sets will provide greater information on the significance of these factors for the accurate estimation of clubroot resting spores in the soil. Future studies should address this area not only with qPCR but inclusion of the lateral flow device. Peat based soils found in the low lying sea areas of West Lancashire and Lincolnshire should also be included within the study. This may prove important in the

performance of the lateral flow test and should not be limited to the effect of soil textural type but include analysis of humic and fulvic acid content along with pH. Each of which could have a significant effect on the lateral flow assay when predicting the risk of clubroot disease occurrence at or below the generally accepted disease threshold spore load of 100000 spores gram of soil.

The development of the two test systems (laboratory test: *molecular qPCR* and field based grower test: *lateral flow device*) has provided the UK horticultural industry with the ability to assess fields for clubroot disease and measure disease potential i.e the number of disease propagules (resting spores) in the soil. Ultimately however the development and expression of the disease will be dependent on a number of factors : the resting spore concentration, the conducive or suppressive nature of the soil type, the environmental conditions over the growing season and the Brassica cultivar planted. For this part the project has in Years 2 and 3 examined the potential for development of an integrated clubroot field risk assessment which is based on knowledge of the soil resting spore concentration and the environmental parameters to predict crop response.

The prototype clubroot disease forecast model considers known crop responses to environmental parameters, within additional factors incorporated to cover spore responses. The basis for the clubroot model is derived from N_ABLE. This was initially developed by Greenwood and Draycott in 1989 and has since been well researched and documented, and further developed into an integrated N,P,K model by combination with other Potassium and Phosphorus crop response models. In the development of the clubroot disease forecast model the nitrogen component was considered to be the most important, as inclusion of other parameters would have added unnecessary complexity at this stage (and possibly not been that important to clubroot disease development). The inclusion of pH, Calcium and Magnesium however are included given their significance in the development of the disease to gall formation. Initial investigations have generated a model capable of predicting environmental parameters important to the development of clubroot disease. Further trials are required to establish whether the significant factors have a direct or indirect effect on clubroot disease potential. The ability of the model to predict clubroot disease is at this point limited by the ability to predict weather patterns. Nevertheless the model is applicable to different soils so it will be possible to work quickly towards generating risk assessments for clubroot disease based on soil type, crop and pre planting clubroot resting spore levels

In terms of clubroot control, the project also investigated the use of Limex as a potential control measure. The results confirmed the findings carried out in 2009 in Scotland that

Limex could be used to control clubroot in heavily infested land while maintaining marketable yields of Broccoli crops. The optimal application rate was between 7.5–10 tons Limex/ha. The results demonstrate that the application rate could be reduced without a detrimental effect on broccoli yields. The results show that the highest levels of clubroot were recorded in plots which were treated with standard lime or were untreated. Standard lime and Limex did not have a significant effect on the pH of the plots although high rainfall was recorded at the time of application. The pattern of clubroot development in the plots differed between 2009 and 2010 reflected in differences in rainfall between the two seasons.

Anticipated practical and financial benefit

- The usage of the detection tests for risk assessment for clubroot will improve the control of this pathogen.
- Generation of a clubroot disease forecast model based on soil type, crop and soil disease level will assist knowledge on planting risk and subsequent disease management strategies.
- New information will be available on an integrated management programme for predicting disease risk and strategies for clubroot control

Action points for growers

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

- Growers can have their soils tested for clubroot disease inoculum concentration ahead of testing.
- Limex can be used to control clubroot in affected land however it will not reduce clubroot risk in subsequent seasons.
- The optimal level of Limex required for clubroot control is 7.5 – 10 tons Limex/ha.

SCIENCE SECTION

Year 2 – Project Summary

Compare diversity sets from 2007 and 2010 field collections using LFD devices and qPCR tests

Introduction

Quantitative PCR has been an established procedure in the quantification of disease pathogens in many horticultural systems, including in the detection and quantification of the clubroot disease pathogen *Plasmodiophora brassicae*. The ability to quantify *P. brassicae* in field soils has useful applications in assessing the risk of clubroot disease ahead of planting Brassica crops and in management of the disease. In UK soils, Buczacki (1978) reported that a concentration of 100000 ($1x10^5$) clubroot resting spores /g soil are required for severe and uniform disease expression on plants. Although it is important also to consider environmental factors such as moisture, temperature and soil pH which will affect infection level irrespective of spore density i.e. depending on the suppressive or conducive nature of the soil to the disease

Technology allowing lateral flow devices (LFDs) to quantify clubroot resting spores in soil has also been developed. This is a significant step forward for growers in the quantification of *P. brassicae* spore load, providing a rapid diagnostic tool which is portable and can be carried out quickly and relatively inexpensively in their own fields by non-scientific staff. Using both quantitative PCR (qPCR) and the lateral flow technology, soils collected in 2007 and 2010 from around the UK were tested for clubroot infestation. The two test procedures were evaluated for their use in determining clubroot disease risk.

Materials and Methods

Assessment by lateral flow of soil diversity sets for P. brassicae infestation

Soil samples

A total of 37 soil samples collected from various locations around the UK were used in this small comparative study. Twenty three soils were identified and collected from Brassica cultivated sites across the UK in 2007 (series 7) and a second set of 17 soils were collected in 2010 (series 10). Each soil was dried then ground and milled using gradients of wire mesh sized down to 2mm before weighing into 0.26g aliquots in preparation of clubroot resting spore soil analysis. A soil, identified with no previous record of Brassica cropping history and which had tested negative for *P. brassicae* both by molecular detection and bait planting, was included in the study as a negative control. The soil was prepared for analysis as described previously.

Lateral flow construction

Competitive lateral flow devices comprised a 5mm membrane pad made up of a Millipore 180 Hi flow^{TM} cellulose ester membrane cast onto 2ml Myler membrane backing (Millipore Corp., Billerica, MA.), an absorbent pad (Whatman Ltd. Maidstone, Kent) to absorb any excess liquid, a filter pad (Whatman Ltd. Maidstone, Kent) to act as a pre-filter for the sample moving onto the membrane pad, and a conjugate pad for retaining the antibody. The membrane pad construction is seen in Figure 1 and was described previously *(in* Annual Report FV349, August 2010).

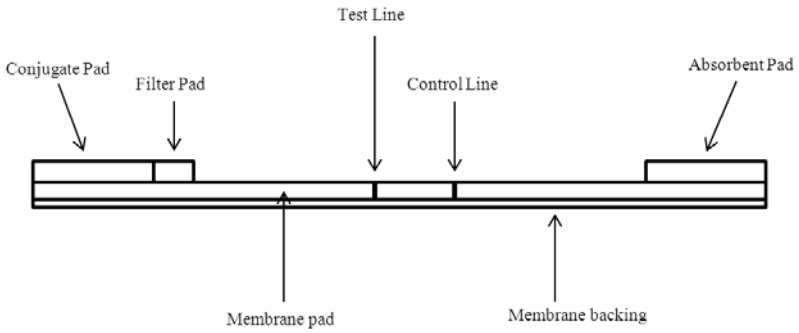


Figure 1. Lateral flow strip construction.

Prior to full lateral flow construction, a test line of *P. brassicae* spore suspension was applied to the membrane pad. A 5µl gold anti mouse IgM solution (BA GAMM 40, British Bio cell International, Cardiff, UK) was mixed with 30µl UW 249 (1:40 dilution in conjugate buffer (0.0025M PBS, 2% trehalose, 2% BSA, 2% sucrose)) and applied to another separate pad, termed the conjugate pad. The conjugate pad was laid horizontal and air dried at 35^{o}C for a period of 20 mins. until dry, then placed onto the membrane pad. The completed LFD membrane (also referred to as the lateral flow strip) was then placed inside the plastic casing (LFD cassette) ahead of application of the test soil sample.

Standard curve

A standard curve was created for each batch of lateral flow devices prepared. A soil which had previously been identified as free of clubroot spores was artificially inoculated to provide a 10 fold serial dilution of *P. brassicae* resting spore concentrations ranging from 1x10^{9} to 1x10^{3} resting spores g^{-1} soil. A control soil of zero resting spores was also included. These samples were processed by lateral flow assay, as described below, to provide a standard curve.

Lateral flow assay

For each soil, 0.26g was weighed into an individual microtube and 400µl of B2 buffer was added. The soil was shaken vigorously and the suspended soil particulates then allowed to settle over a 1 min. period. After which a 100µl aliquot of the liquid phase was removed and transferred to a lateral flow device. A reading of optical density (OD) was taken after 30 minutes using an ESE reader device. The OD values recorded for each of the artificially infested soils were then used to generate a standard curve. By referencing the lateral flow OD values of the soils collected in 2007 and 2010 to the artificially infested soil calibration curve the resting spore concentration for each soil set was determined.

Molecular quantification of P. brassicae in the collected soils

DNA extraction from soil samples

Soil DNA was extracted 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. A Polyvinylpolypyrrolidone (PVP) DNA cleanup was subsequently performed on all DNA extracts (Klemsdal *et al*., 2008) and DNA was stored at -20^{o}C.

Quantitative PCR was performed using set of primers PbITS3 and PbITS4 and as a 20µl reaction mix comprising 10µl Platinum® SYBR® Green qPCR SuperMix-UDG w/ROX (Invitrogen Ltd. Carlsbad, CA), 0.4µl each primer (final concentration 4nM), 7.7µl ROH_20 and 1.5µl DNA. To generate standards for real time quantification, total DNA was extracted and cleaned from eight 0.26g samples of a soil which had tested negative for clubroot disease by PCR and bait planting. A further aliquot of this soil was inoculated with 1x10^{9} spores g^{-1} *P. brassicae* suspension prior to DNA extraction and clean up. The spore suspension used for inoculating soil was obtained from gall tissue by filtration under vacuum. A dilution series of the 1x10^{9} spores g^{-1} *P. brassicae* soil DNA extract was then made into the uninoculated soil DNA extracts, providing a set of standards for quantification. Absolute quantification analysis based on the second derivative maximum method was used to quantify the levels of *P. brassicae* DNA by qPCR (VanGuilder *et al.*, 2008). Using this method the generation of the standard curve is based on non-linear regression.

Comparison of different primers for quantification by qPCR

A total of 37 soil samples collected from various locations around the UK were used in this small comparative study (soil series 7 and 10). Each soil was dried then ground and milled using gradients of wire mesh sized down to 2mm before weighing into 0.26g aliquots in preparation for DNA extraction.

Soil DNA was extracted and cleaned up as described above and standards for quantification by qPCR were as previously described. A second set of *P. brassicae* specific primers were developed called PbML1F and PbML1R and these were supplied by Sigma, and were designed to amplify within 18S and ITS1 regions of *P. brassicae* rDNA .Tailored quantitative PCR reactions were carried out for each primer set.

Evaluation of P. brassicae soil quantification by lateral flow device and qPCR.

Soils from series 7 and 10 were assessed by both immunological (lateral flow device) and molecular (qPCR) techniques for *P. brassicae* resting spore concentration. In order to assess the immunoassay data provided by the lateral flow device readouts, the 4 parameter logistic nonlinear regression model was applied to the standard curves to predict the response variables. The distribution of results from each quantification method and subsequent data transformations were assessed using Shapiro-Wilk normality test. The Wilcoxon test was used to compare the medians of the data pairs, and correlation between the two techniques was assessed using Pearson's product moment correlation coefficient. Only the soils which had tested positive for *P. brassicae* above the detection threshold of $1x10^3$ spores g^{-1} soil in at least one of the quantification methods were compared. A series of artificially *P. brassicae* infected soils (prepared as described above in section 1.2.2) were run as standards and at three times for both qPCR and LFD. The resulting outputs were plotted against each other and modelled using 4 parameter logistic regressions to generate a regression line highlighting the relationship between the two techniques.

Determining the lower limits of quantification

The MoBio DNA extraction kit has shown itself to be extremely effective at isolating DNA from soil but due to the amount of starting material incorporated per isolation (0.25g soil – following manufacturer's recommendations) this ultimately places limitations on the lower levels of detection of *P. brassicae* spores in soil that could be reported on. Moreover by incorporating just 0.25g of soil inoculated with 1000 spores g^{-1} of soil, only the equivalent of two hundred and fifty spores are actually being extracted from. Final elution volume of 100ul

allows a maximum average theoretically of two and a half spores per microlitre, though one should bear in mind a small percentage loss due to the extraction and clean up procedures. To follow on only two microlitres of the resultant 100µl elution volume per sample is incorporated into each qPCR reaction. While it may be possible to detect a minimum of approximately $2x10^2$ spores g^{-1} soil, the reliability of quantification is lower at this level due to the high number of qPCR cycles required. To increase sensitivity it would be necessary to either increase the size of the reaction or increase testing frequency, each of which would increase the cost. As below $1x10^3$ spores g^{-1} would generally be expected to indicate a low risk of severe and uniform clubroot disease occurrence the 'not detected' threshold is used to describe levels of *P. brassicae* DNA that fall at or below $1x10^3$ spores g^{-1} of soil.

Results

Standard curve for quantification of P. brassicae in artificially infested soils by LFD

A correlation (r^2=0.987) was observed between the optical density values of the lateral flow test device (LFD) readings and the number of clubroot resting spores in the artificially inoculated soils.

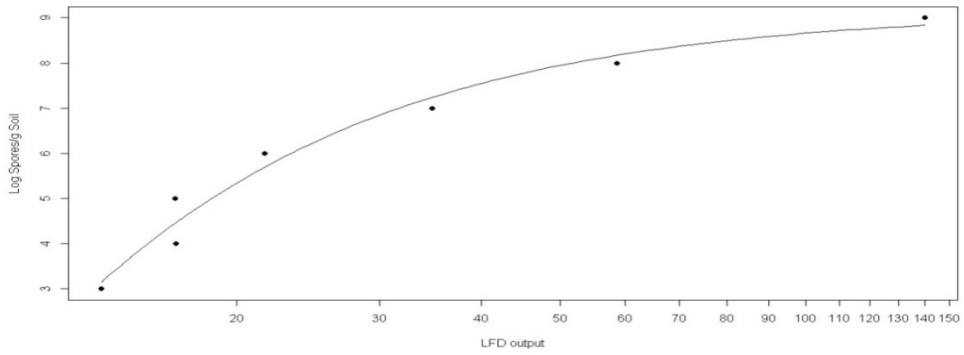


Figure 2. Standard curve generated using soil samples inoculated with known concentrations of *P. brassicae* resting spores g^{-1} soil.

Lateral flow quantification of soil diversity sets

The results of the LFD values for the 7 series soil samples are seen in Figure 3. Five of the soil samples fall below the lower limit of detection (1, 5, 6, 7, and 15) and were considered as not detected. Three samples yielded results >1x 10^6 spores g^{-1} soil. Eight samples

yielded results between 10^4 and 10^6 spores g^{-1} of soil with sample 7.21 being the highest of these. The remaining six samples yielded results between 10^3 and 10^4 spores g^{-1} of soil.

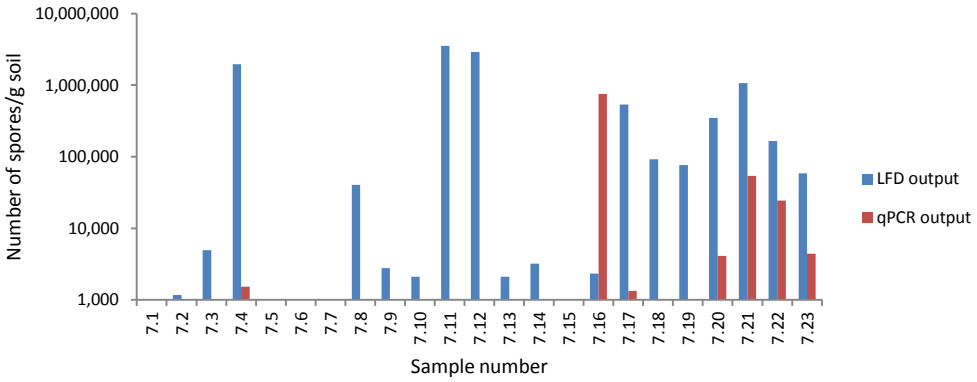


Figure 3. Lateral flow and qPCR results for the "series 7" collection of 23 UK soil samples.

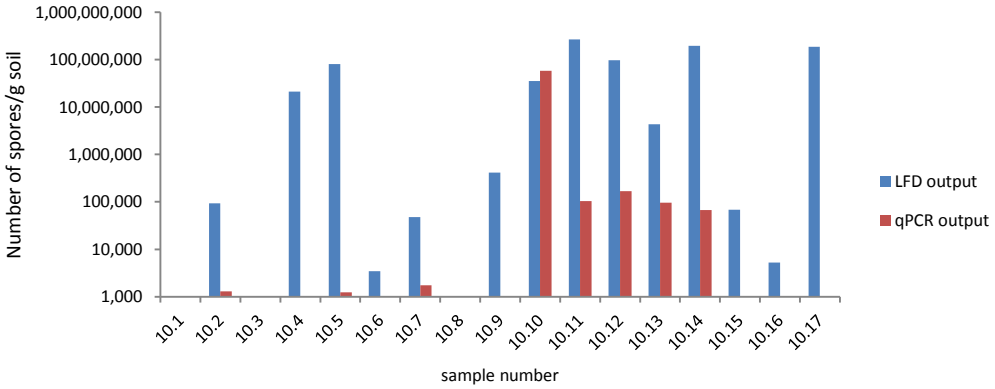


Figure 4. Lateral flow and qPCR results for the "series 10" collection of 21 UK soil samples.

The results for the 'series 10' samples are seen in Figure 4. Three samples fell below the lower limit of detection and were considered free / low risk of clubroot (samples 10.1, 10.3 & 10.8). Eight of the samples recorded resting spore concentration at >1x10^6 spores g^{-1} of soil (high risk of severe and uniform clubroot disease). Four samples yielded results between 1x10^4 and 1x10^6 spores g^{-1} of soil (medium to high risk). Two samples yielded results between 1x10^3 and 1x10^4 spores g^{-1} of soil (low risk of severe and uniform clubroot disease).

Molecular quantification of P. brassicae in the soil diversity sets

The amount of *P. brassicae* detected in 'Series 7 and 10' soils is shown in Figures 3 and 4. In 'Series 7', three soils were below the detection threshold and therefore considered not

detected. Thirteen 'Series 7' soils did not show any amplification at all and were considered negative for *P. brassicae* spores giving a total of 16 soils in which *P. brassicae* was not detected (Figure 3). Of the positive soils the highest levels of *P. brassicae* resting spore concentration was in sample 7.16 and recorded at $7.56x10^5$ spores g^{-1} soil.

Of the 17 soils in Series 10, eight were identified as positive for *P. brassicae* infestation. The highest concentration was recorded in soil 10.10 ($5.80x10^7$ spores g-1) and at the lowest level value of $1.23x10^3$ spores g-1 in soil 10.5.

Comparison of different primers for quantification by qPCR

Out of the 37 soil samples tested, 16 showed successful amplification of *P. brassicae* DNA, whilst 21 of the samples fell below the detection threshold suggesting no presence or very low levels of *P. brassicae*. Matching results were observed for 19 of the 21 samples with both the PBITS3/4 and the PBML1F/R primers. Both primer sets indicated some soils with *P. brassicae* levels below the detection threshold, however only two samples gave a difference between a positive compared to a below threshold result. Of these two, one sample suggested a result of just below the cut-off threshold indicating a borderline result and the other gave an increased deviation in the cross-over point (Cpd) value suggesting that with an increase in replicates these results may be more defined.

For the remaining 16 samples showing positive values for both primer sets, The Welch two sample t-test showed no significant difference between the means of the samples from the PbML1F/R and PbITS3/4 primer sets, with t= 0.2615, df = 29.733 and p-value = 0.7955. Overall R=0.0995, thus showing a strong correlation between primer sets PbML1F/R and PbITS3/4 when using Pearson's Product Moment Correlation Coefficient (Figure 5).

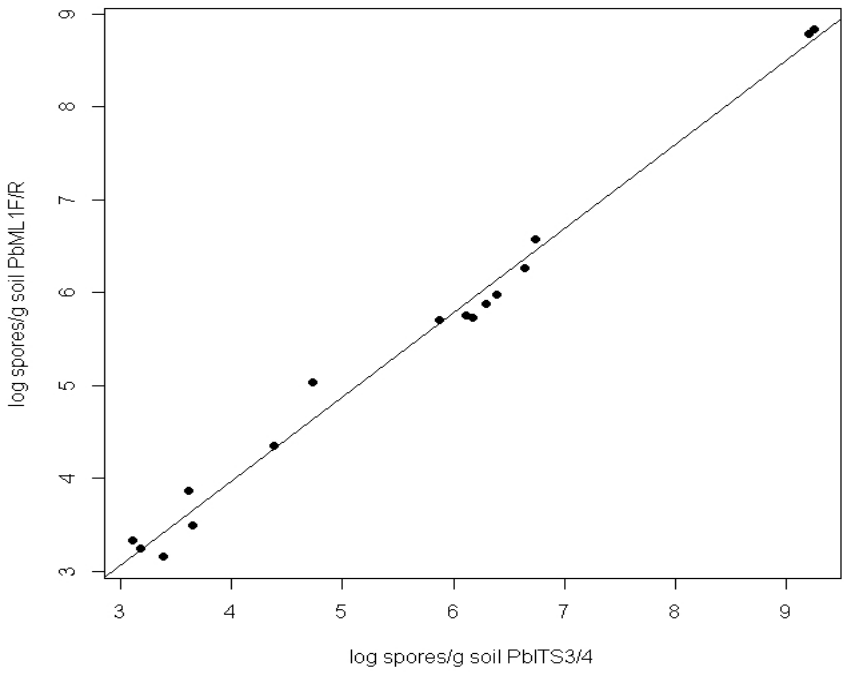


Figure 5. Correlation of *P. brassicae* quantification from soil by PbITS3/4 and PbML1F/R.

Comparison of methods for quantification of soil resting spore concentration.

The null hypothesis of the qPCR and LFD data being normally distributed was tested by Shapiro-Wilk (Table 1). All data transformations applied showed probability of less than 0.05 (95% confidence level) meaning that the null hypothesis was rejected. The presence of zero results in the population made the Log_{10} transformation inappropriate.

Table 1: The amount of *P. brassicae* detected in 'Series 7' and 'Series 10' soils

Transformation	qPCR		LFD	
	W	p-value	W	p-value
None	0.1515	5.07E-14	0.4354	3.17E-11
Square Root	0.2154	1.85E-13	0.5635	1.03E-09
Cube Root	0.3447	3.73E-12	0.6722	3.51E-08
Fourth Root	0.4828	1.07E-10	0.7597	1.08E-06

To test whether there is significant difference between the medians of the results from the two techniques a Wilcoxon test for matched pairs was applied to the untransformed data. The null hypothesis was no difference existed between the medians of the samples comprising the two data sets and this was rejected due to the resultant low p-value ($5.157x10^{-5}$).

Pearson's product moment correlation coefficient was assessed to determine whether there was any correlation between the qPCR and the lateral flow results however the low R value (-0.04 to 2dp) suggested there was no correlation and this was unlikely to be due to randomness (t = -0.2208, df = 38, p-value = 0.83). The correlation between the detection and absence of *P. brassicae* by the two methods was higher at R = 0.41 (to 2 dp) (t = 2.7568, df = 38, *P* = 0.008919), and correlation between the two methods when risk of clubroot disease was ranked (1 = low risk $<10^3$ spores g^{-1} soil, 2= medium risk 10^3-10^5 spores g^{-1} soil and 3= high risk $>10^5$ spores g^{-1} soil) gave an R value of 0.43 (to 2 dp) (t = 2.9358, df = 38, *P* = 0.005621).

When the artificially infected clubroot soil standards were modelled using 4 parameter logistic regression the relationship between the qPCR output and LFD output was visible (Figure 6). The graph shows the relationship between LFD optical density (OD) output and qPCR crossing point (Cp) value. The LFD OD is the output of the LFD reader and the Cp of qPCR is the value at which quantification is performed. This comparison was based on three standard curves from each technique applied to soil extracts used as standards in the assays.

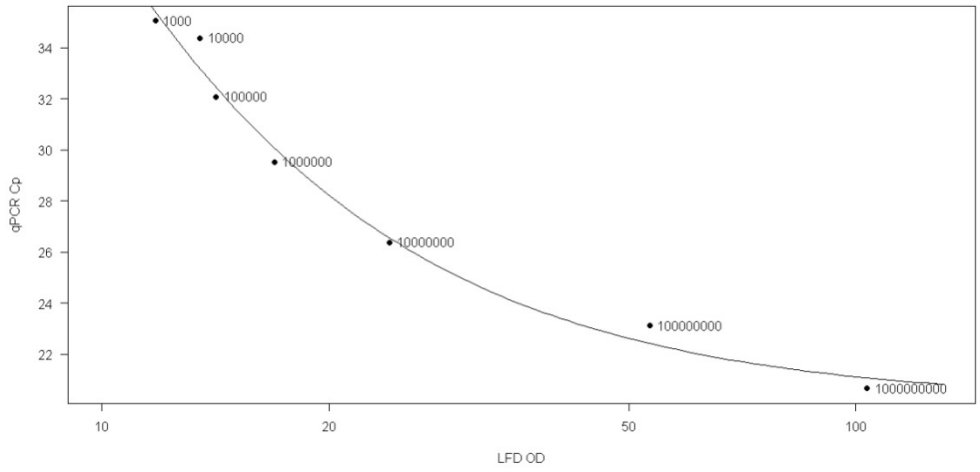


Figure 6 . Relationship between LFD quantification and qPCR quantification in soil standards. Points relate to the number of clubroot spores g^{-1} soil.

Summary

In the comparison of different primers for quantification by qPCR the results indicate the R value to be well above the 0.05 level, showing that there is no significant difference between DNA levels obtained for these samples using primer set PbML1F/R compared to using the primer set PbITS3/4. Thus it can be confidently concluded from this set of data that for QPCR implication of *P. brassicae* DNA concentration levels will be consistent across the two primer sets.

When the 'Series 7 and 10' soils were considered for predicted clubroot infestation a poor correlation between the data sets (lateral flow and qPCR) was observed. To determine if the lateral flow devices and the qPCR are detecting *P. brassicae* in the same soil samples a correlation based purely on presence or absence of *P. brassicae* was performed and, while this showed improved degree of relatedness between the samples, the correlation was still below half. Ranking both sets of results into low, medium and high disease risk categories improved the correlation further.

The variation observed between qPCR and lateral flow may however result from the extended storage time of the soils. Faggian (DPI, Melbourne, Victoria: personal communication) noted that storage conditions effect clubroot resting spore DNA extraction level. Within one month at 4^0C storage, the DNA test signals of clubroot infested soils were seen to decline and the resultant qPCR value generated was significantly below that originally observed. The soils collected in 2007 and 2010 (series 7 and 10) were stored at -20^0C prior to evaluation in 2011.

In the comparison of different primers for quantification by qPCR the results indicate the R value to be well above the 0.05 level, showing that there is no significant difference between DNA levels obtained for these samples using primer set PbML1F/R compared to using the primer set PbITS3/4. Thus it can be confidently concluded from this set of data that for

QPCR implication of *P. brassicae* DNA concentration levels will be consistent across the two primer sets.

When the ‘Series 7 and 10’ soils were considered a difference between the populations, as highlighted by the difference between the medians of the results from each technique, was observed. This difference was also apparent by virtually no correlation between the data sets. To determine if the lateral flow devices and the qPCR are detecting *P. brassicae* in the same soil samples a correlation based purely on presence or absence of *P. brassicae* was performed and, while this showed a greater degree of relatedness between the samples, the correlation was still below half. Ranking both sets of results into low, medium and high disease risk categories improved the correlation further.

When the relationship between the artificially infected soil standards was examined the initial plot suggested that the relationship between the two outputs is non-linear, instead following a typical biological response curve which can be modelled by 4 parameter logistic regression. This would explain the lack of correlation observed as correlation was assessed on the basis of a linear relationship. Alternative methods of assessing the relationship and generating confidence intervals for the regression analysis are needed to allow determination of whether the relationship remains the same when applied to naturally infected soil samples, such as those used in series 7 and 10. Increasing the number of samples will also contribute to increased robustness of the relationship.

Commence development of lateral flow optimisation for problem soil types

Introduction

Due to the complexity of the soil environment and the longevity of *P. brassicae* within the soil, the interaction of resting spores with their environment is important to consider. The soil environment does not remain constant throughout different seasons, and there are many factors which could potentially have an effect on the resting spores, such as temperature, moisture content, soil composition (both nutritionally and structurally). It is well established that soil type can also affect the detection and quantification of organisms by the molecular methods of PCR and qPCR. Immunological based techniques (such as LFD quantification) are also reported to be affected by soils types. Otten (1997) observed antibody retention in some soil types (especially in clay) which proved problematic in quantifying fungal pathogens in soil. Researchers have identified various compounds in soil which are referred to as inhibitory substances (Lloyd-Jones and Hunter 2001, Thakuria *et al* 2008) and although found in many environmental samples, prove particularly problematic in a complex soil matrix (Menking *et al.* 1999).

Materials and Methods

Textural analysis and quantification to determine problematic soils

The 'series 7' soils were profiled (LandLook UK Ltd.) to give soil textural type and approximate proportion of sand, silt and clay composition. Due to the robustness of *P. brassicae* quantification by qPCR, the values given by this molecular approach (Figure 3) were used to assess whether soil type influenced the level of *P. brassicae* quantified.

Results

Textural analysis to determine problematic soils in lateral flow optimisation

Textural analysis of the 'series 7' soil collection showed a range of soil types were present (results not shown). The majority of samples were clay-loams or sandy-loam derivatives. Clay, sandy-clay, silty-clay or silty-loam soils were not well represented in this collection, however the

distribution of soil types which were tested displayed an even distribution of *P. brassicae* (χ^2= 11.6364, df = 9, *P* = 0.2346). From the collection of 23 soils, eight soils displayed ≥ 10^2 spores g^{-1}. The greatest number of spores g^{-1} from a soil was $7.6x10^5$. Figure 7 shows the distribution of the soils where *P. brassicae* was, or was not detected. For the soils that tested positive for *P. brassicae* DNA, one-way ANOVA was performed on each soil component against log transformed *P. brassicae* DNA levels. No significance of percentage clay content to number of spores was observed. Sand appeared to have a slight influence on the number of spores detected, but this was not significant (df=1, F value = 4.5055, *P* = 0.078). The silt content of the soil was significant at *P* = 0.04 (df=1, F value = 6.8374).

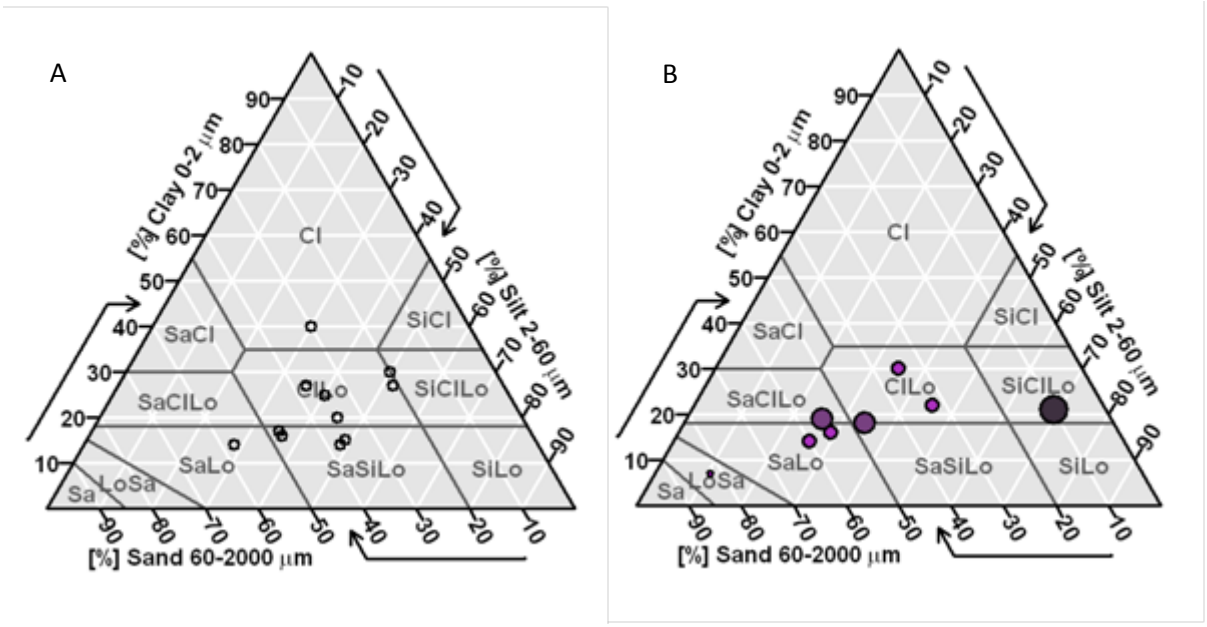


Figure 7 The distribution and concentration of *P. brassicae* across a range of soil types as detected by qPCR. A = *P. brassicae* not detected, B = detected with PbITS3 and PbITS4. Increasing circle size depicts increasing *P. brassicae* levels.

Summary

As qPCR is currently the most well tested and robust quantification method it was used as the method by which problem soil types may be identified within the existing sample sets. While the data shows that silt in particular may show a relationship with *P. brassicae* levels, the different textures present in the collection of soils are not completely representative of all possible soil textural classifications, and the results lead to the conclusion that soil type and

its effect on *P. brassicae* prevalence is an area worthy of further investigation. While using qPCR to identify problem soil types is helpful in determining soil properties which may be problematic, an assumption is made that the LFD will be inhibited by the same soil parameters as qPCR. The general properties which inhibit extraction of DNA (thus affecting qPCR) are chelating properties, high organic matter concentrations and clay content (Otten et al 1997, Menking et al 1999).

Year 3 – PROJECT SUMMARY

Complete initial testing of simple models for clubroot risk assessment (June 2011)

Introduction

Assessing the risk of Clubroot disease in a crop cannot be achieved without first understanding the interaction between the main factors; soil, crop and meteorological. The ability to provide a risk assessment for clubroot disease requires knowledge of both the crop and the spore responses to environmental conditions (soil and weather). The crop response is important as the health of the plant influences its ability to resist disease, and the spore response is important as it influences the survival of spores within the soil along with the germination of zoospores which are needed for plant infection to occur.

Planting Brassica crops stimulates the germination of resting spores within infected soils and the molecular method of qPCR can be applied to soils, therefore making it a suitable technique for quantifying the movement of *P. brassicae* DNA within the soil as spores germinate and enter the host plant. Subsequently environmental parameters can be predicted using crop response models (Pers. Comm., Dr. D. Greenwood, University of Warwick; Greenwood and Draycott 1989a, b; Greenwood et al 1996; Zhang et al. 2007) to allow the determination of the key environmental factors that influence *P. brassicae* within its natural environment. Therefore a model was developed based on known crop responses to environmental parameters, within additional factors incorporated to cover spore responses. The basis for the clubroot model is a model called N_ABLE. This was initially developed by Greenwood and Draycott in 1989 and has since been well researched and documented, and further developed into an integrated N,P,K model by combination with other Potassium and Phosphorus crop response models.

For the purposes of developing this clubroot model the nitrogen component was considered to be the most important, as inclusion of other parameters would have added unnecessary complexity at this stage (and possibly not been that important to clubroot disease development). However, some other factors were included as they were considered to potentially be very significant to clubroot disease (pH, Calcium and Magnesium).

Materials and Methods

Experimental sites

Initial experimental sites for testing a simple model for Clubroot risk assessment were established in two 0.58Ha trial locations (Scotland and Warwickshire). Each site had previous history of Brassica cropping and was known to contain *P. brassicae* levels of approximately $>1x10^5$ spores g^{-1} soil. Replicated experiments were performed at each site starting with core sampling to determine soil chemical composition.

Cropping procedure

Each site was cropped with *Brassica oleracea* cultivars and meteorological conditions were recorded throughout the growing season using an environmental monitoring station (Skye Instruments Ltd. Llandrindod Wells, Wales). At the Warwickshire site two transplantation dates were implemented (June and August) across three different soil pH's (due to an existing soil pH gradient across the site). The Scottish site transplantation was in June, and at this site the plots covered two soil pH levels. Each site followed commercial protocols for fertilization and crop protection measures and soil cores were taken to a depth of 15-25cm at weekly intervals throughout the growing season. Soil cores were then processed by drying and milling before DNA extraction and quantification by qPCR as previously described.

Modelling P. brassicae responses to environmental conditions

An existing nitrogen, phosphorus and potassium model (Greenwood and Draycott 1989a, b; Zhang *et al*. 2007) was adapted to the purpose of Clubroot risk assessment by removal of phosphorus and potassium outputs and the incorporation of predictive calcium, magnesium

and pH components (Pers. Comm. Dr. D. Greenwood, University of Warwick) as shown in Figure 8.

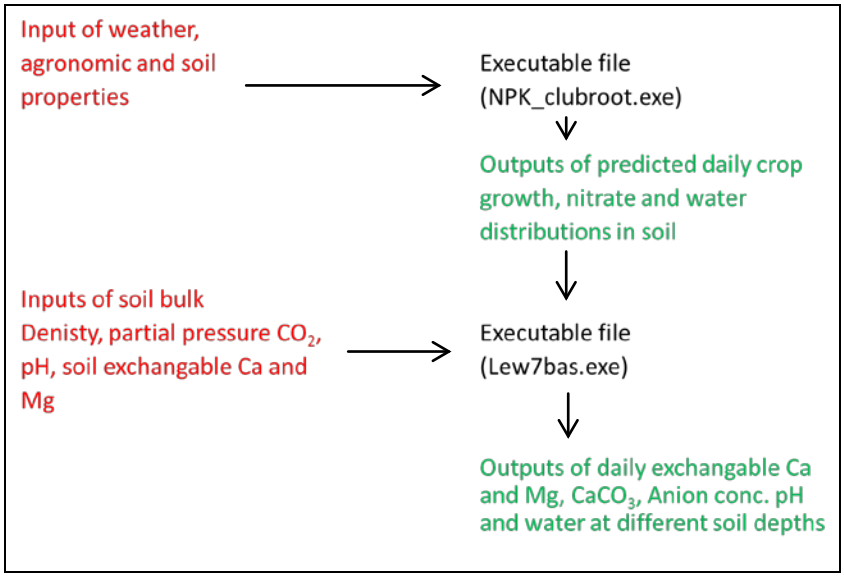


Figure 8 Clubroot risk assessment model incorporating nitrogen, calcium, magnesium and pH components.

Data collected from the weather stations along with soil chemical parameters provided the model inputs, and running the model generated predictive data of environmental conditions throughout the growing season. These outputs were then assessed against *P. brassicae* spore numbers determined by qPCR from the soil samples, allowing trends between the modelled changes in environmental factors and *P. brassicae* levels to be analysed. Data was log transformed and assessed by linear regression and ANOVA.

Results

Results from these trials showed that pH, calcium, magnesium and the interaction between them had a significant effect on the number of *P. brassicae* spores detectable in the soil. At the Warwickshire site one way ANOVA showed a statistically significant effect of pH on spore numbers (at the 99% confidence level) (df =1, F value = 11.151, p=0.001005) and this effect was also apparent at the Scottish site (df = 1, F value = 17.819, p = 7.175×10^{-5}). Exchangeable calcium and magnesium were shown to be more significant than total calcium and magnesium at the Scottish site as shown in Table 2, but this was not as distinct at the

Warwickshire site. Anion concentration, soil water, rainfall, temperature were among the other factors which showed significance at least one of the two sites (Table 3).

Table 2: Exchangeable and total calcium and magnesium concentrations at the Scottish site.

	F value	P-value
Exchangeable calcium (mEq g^{-1})	18.1290	$6.305x10^{-5}$
Exchangable magnesium mEq g^{-1})	18.1290	$6.305x10^{-5}$
Calcium mEq g^{-1}	3.0729	$8.398x10^{2}$
Magnesium mEq g^{-1}	3.0029	$8.752x10^{2}$

Table 3: Anion concentration, soil water, rainfall, temperature showing significance to *P. brassicae*.

	F value	P-value
Soil Temp 100cm (^{o}C)	10.734	$1.725x10^{-3}$
Pan Evaporation	5.0485	$2.805x10^{-2}$
Mean Daily Temp (^{o}C)	11.690	$1.089x10^{-3}$
% N Plant	10.512	$1.873x10^{-3}$
Spread Roots	10.410	$1.964x10^{-3}$
Depth Roots	9.975	$2.407x10^{-3}$
% N Soil 90cm	7.654	$7.368x10^{-3}$
NO3_N	10.375	$1.997x10^{-3}$
Water 0-90cm	5.122	$3.384x10^{-2}$
Leaching 35cm	4.190	$4.472x10^{-2}$
SMD	4.808	$3.191x10^{-2}$
Anion (mid 30cm)	9.669	$5.111x10^{-3}$

Summary

Using the simulated values of the field parameters generated from the model it was possible to examine which factors were having the greatest influence on the number of spores within the soil. In agreement with the existing literature (Myers and Campbell 1985, Webster and Dixon 1991) pH, Calcium and Magnesium and the interaction between them all had a significant effect on the number of *P. brassicae* spores in the soil. The work presented here provides extra detail to these relationships by establishing the effect of these factors at

different soil depths and the greater significance of exchangeable calcium and magnesium levels than total calcium, magnesium and anion levels. It is also demonstrated that there was a relationship between soil water and *P. brassicae* spore numbers although the mechanisms behind water movement and availability within the soil would need to be elucidated to determine the effect on spore numbers due to the different significances recorded of SMD, rainfall and percentage soil water over the two different trial sites. It is unsurprising that soil temperature was shown to be significant. The daytime temperature ranges within the UK, particularly in summer time, are not too dissimilar to the optimum temperatures recorded both in this study and by others (Buczacki 1983; Myers *et al* 1983).

Initial investigations have generated a model capable of predicting environmental parameters important to the development of clubroot disease. Further trials using unplanted soil would be able to establish whether the significant factors have a direct or indirect effect on clubroot spores. The ability of the model to predict clubroot disease is limited by the ability to predict weather patterns - however the model is applicable to different soils, thus it will be possible to work towards generating risk assessments for clubroot disease based on soil type, crop and initial clubroot spore levels.

Establish a field experiment to investigate integrated clubroot control using successful treatments from years 1 and 2.

Materials and Methods

Clubroot Trial Design

One clubroot trial area was used in at the Crail trial site in 2010. This was situated in a different area of production in comparison to the 2009 trials. Initial tests were conducted in this area to ascertain the levels of clubroot resting spore contamination before the trial was sited.

Treatments

L1 = Limex 7.5 tons/ha **L3 = Limex 12.5 tons/ha**

L2 = Limex 10 tons/ha **Standard = Standard Lime treatment**

Figure 9: Randomisation of Limex treatments at Crail in 2010

Soil physical parameters taken at Crail in 2010

Soil was air dried and milled soil before wetting with water and the pH measured electrometrically. Soil (10g) was placed into a 50ml container with 25ml distilled water. The suspension was shaken immediately and at frequent intervals for 15 minutes. A pH meter electrode calibrated with standard buffers was lowered into the suspension to a depth of 1cm and the pH recorded after 30 seconds. Rainfall (mm) was also recorded at the trial site.

Measurement of Environmental Conditions

Measurements of temperature, humidity, leaf surface wetness and rainfall were collected at 30 min intervals from when the logger was sited in the vegetable Brassica crop using a SKYE Datahog II 7 channel logger. Measurements were collected by GSM portable phone Link (Skye Instruments Ltd, Llandrindod Wells, and Powys). The logger was powered by a 12 V battery. Moisture content, exchangeable calcium, exchangeable magnesium, chloride and sulphate content were taken from soil samples collected during the trial period from each plot.

Crop and Planting information

The fertilizer and crop protection inputs for the crop were as for commercially produced crops in adjacent fields.

Field Plantings and treatment application methods

Each plot of $10m^2$ comprised of five beds of 10 metre length, with three plant rows per bed, and plants spaced at 44cm apart. Limex treatments were applied by hand on 15 July 2010 and after mechanical incorporation commercially raised broccoli cultivar ‘Parthenon’ was planted on 20 July 2010.

Clubroot Gall assessment

The numbers of clubroot galls were assessed at one sampling periods after transplanting in each trial plot. The numbers of clubroot galls on two plants per plot were counted on roots. Plants were chosen at random from each treated area of each plot.

Determination of clubroot resting spore concentration in soil using molecular methods

Extraction of DNA from soils

Employing a MOBiol UltraClean Soil DNA Isolation kit 0.3g of each control and artificially infested soil type was processed according to manufacturer's instructions (MoBio Laboratories, POBox 606 Solana Beach, California). To maximise DNA isolation yields the alternative protocol, as described in the MOBiol UltraClean Soil DNA Isolation kit product guidelines, was followed. For each sample, the collected DNA was in a total volume of 50µl S5 buffer. A 10µl aliquot of each DNA soil sample extract was retained and stored at -20°C. Of the remaining 40µl DNA volume, samples were divided in to 2x 20µl lots. One of these aliquots was processed using a method supplied by Dr R. Faggian (DPI, Australia) whilst a QIAquick PCR Purification Kit (Cat. No. 28106) was employed for the remaining 20µl aliquot

Preparation of DNA sample extracts for PCR

A 5µl volume of isolated DNA from a soil sample extract was mixed with 15µl of PCR Master Mix (11.35µl H_2O, 2.5µ PCR buffer 10x, 0.75µl 50mg (1.5mM) Mg, 0.2µl DNTP, 0.2µL DNase) and 30ng of *P. brassicae* specific primers (Faggian et al., 2003). A negative control of molecular grade water + PCR Master mix and Primers was included. Employing a hybrid PCR thermal cycler machine the production of sample amplicon products (amplified specific sequence of sample DNA) was produced and, if present, visualised by agarose gel separation. A 2.5% Metaphor gels was used and, for each PCR sample product, 8µl was mixed and loaded with 3µl of Xylene cyanol buffer. A molecular weight marker was applied to the gel at 1µl sample volume. In a continuous ethidium bromide buffer electrophoretic separation of sample components was at 150v for 25 mins was achieved. The fluorescent

yield of ethidium bromide - DNA amplicon complex was determined using a transilluminator, emitting light at 302nm, and a photographic image recorded.

Results

Rainfall at Crail 2010

The rainfall recorded at a site close to the Crail trial site is shown in Figure 10. There was approximately 60 mm (2 inches) of rain at the trial site during mid July when the Limex was applied. Incorporation of Limex within the soil may have been affected by rainfall.

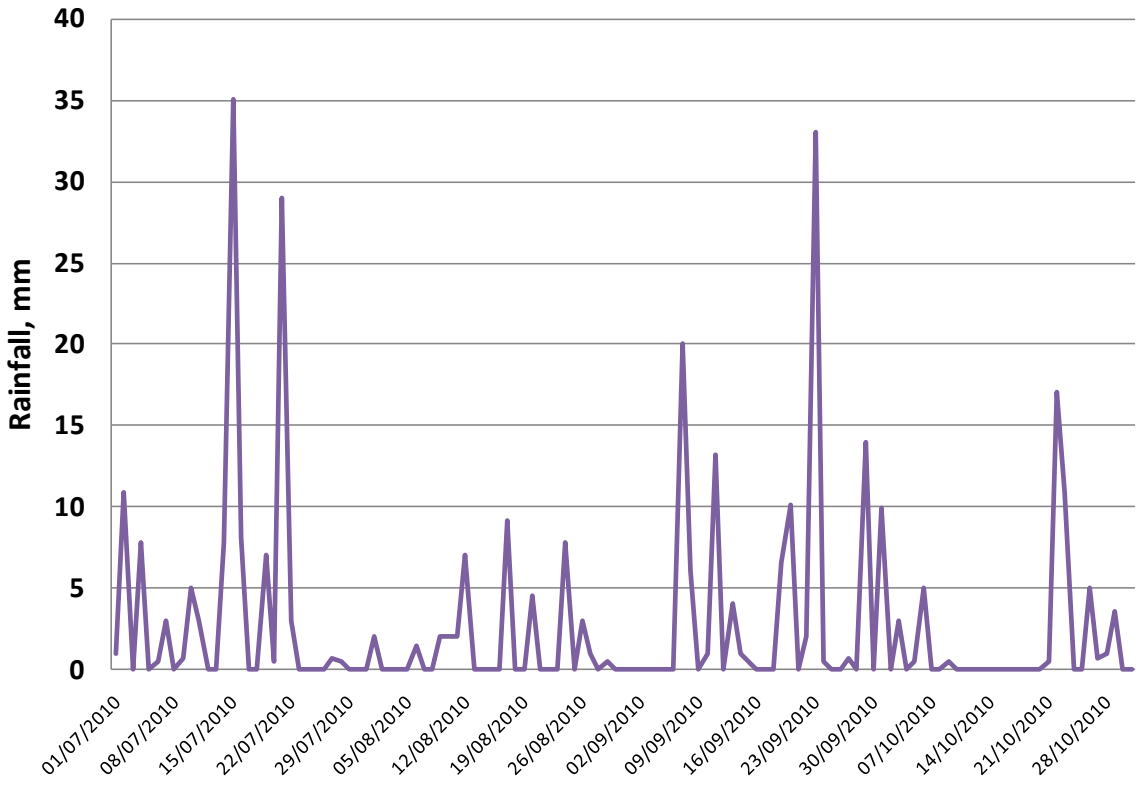


Figure 10: Rainfall at Crail 2010 (mm)

Numbers of clubroot galls recorded in Limex treated plots

The results in Figure 11 show the number of clubroot galls on plants 4 weeks after transplanting. Galls were counted and means calculated from the results per plant in each treatment. Low numbers of clubroot galls were recorded on treatments (less than 1 per plant). However untreated plots and those treated with standard lime had higher mean gall

numbers in comparison to 7.5 and 12.5 tons ha^{-1} Limex treated plots. The numbers of visible galls in plots treated with Limex at 10 tons ha^{-1} was higher.

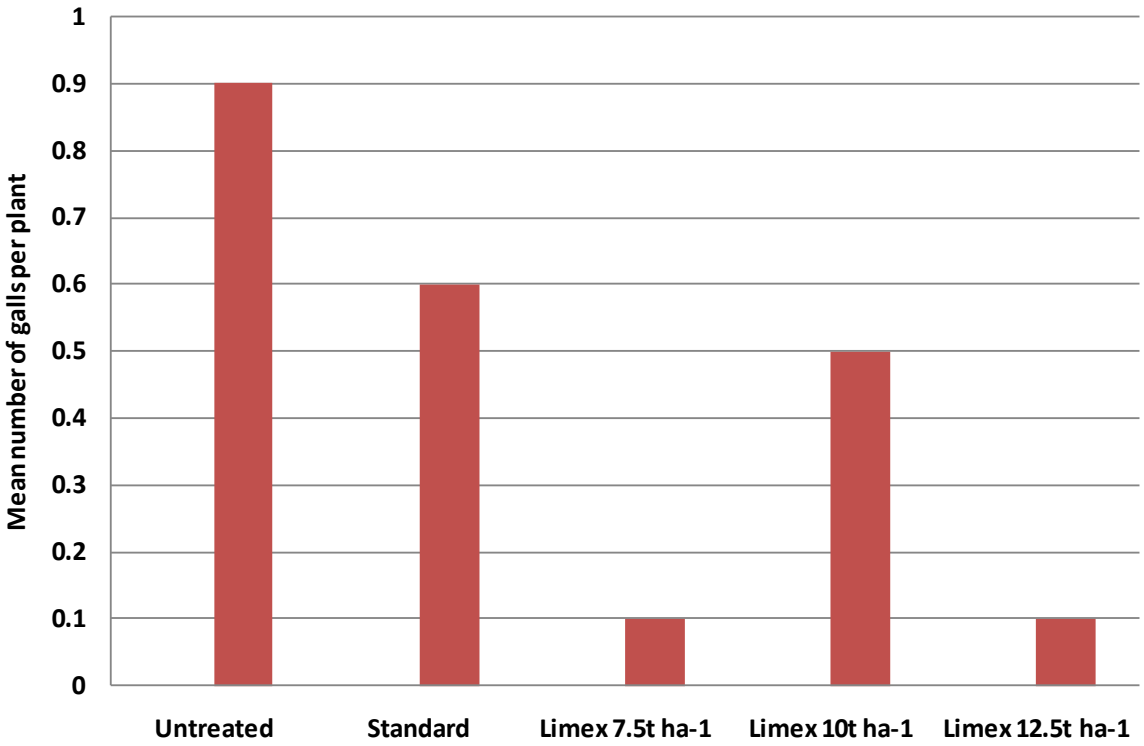


Figure 11: Mean number of clubroot galls per treatment 4 weeks after transplanting at Crail 2010

Estimation of pH in trial plots at Crail 2010

Application of Limex to plots increased the mean pH value in comparison to the untreated and standard lime treated plots. The effect was evident 2 weeks after application of Limex regardless of rate of application (Figure 12). However at this time higher pH values were observed in plots treated with 10 and 12.5 tons $hectare^{-1}$. Observations in mid August 2010 showed a reduction in pH in all treatments however the effect was less pronounced in the 10 and 12.5 tons Limex $hectare^{-1}$ treatments.

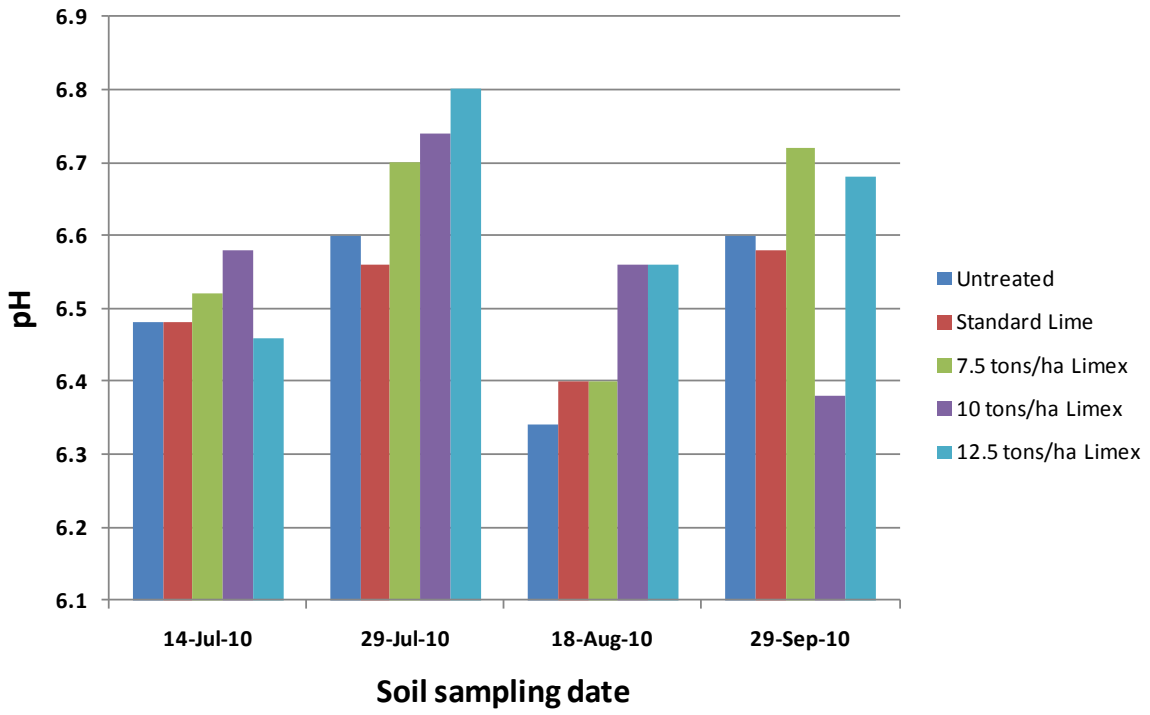


Figure 12: pH values in plots at Crail 2010 after Limex application in July 2010

Estimation of clubroot concentration in Limex treated plots at Crail 2010

Numbers of clubroot resting spores per gram soil^{-1} are shown in Figure 13. There were in excess of 1,000, 000 clubroot resting spores per gram of soil at the trial site before planting occurred in July 2010 (Figure 13). This reflects the production of a broccoli crop on the trial area before the trial was planted. The numbers of clubroot resting spores recorded were above the threshold for clubroot infection of the roots and should have resulted in widespread stunting of growth and crop failure. After planting, numbers in all treatments (except the plots treated with 12.5 tons/ha Limex) increased. Significantly especially in soil samples from the untreated and standard lime treated plots. All Limex treatments gave lower numbers of clubroot spores in samples during Late July 2010. However clubroot resting spore numbers increased in plots treated with 7.5 and 10 tons hectare $^{-1}$. Plots treated with 12.5 tons per hectare^{-1} had little increase in clubroot resting spore concentration in soil over the duration of the trial. Resting spore concentration in untreated and standard lime treated plots declined at harvest during October 2010.

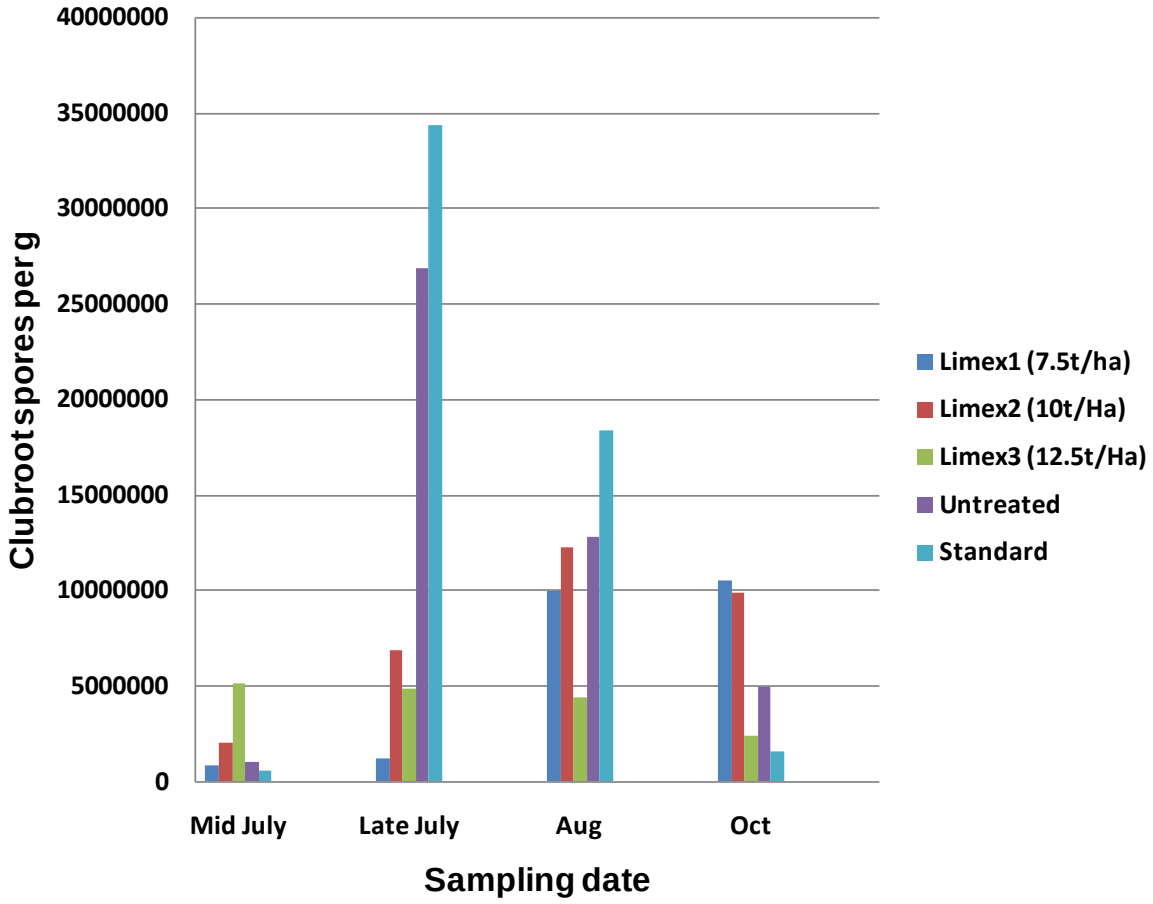


Figure 13: Clubroot resting spore concentration per gram of soil in Field Trial at Crail 2010

Marketable Yield of Broccoli at Crail 2010

The marketable yield of Broccoli in plots is shown in Figure 14 at Crail in 2010. All Limex treated plots gave high marketable yields (approximately 5 - 6 tons ha^{-1}) of Broccoli regardless of Limex application rate. Untreated plots yielded only 2 tons ha^{-1} which was similar to that observed in the 2009 trials (Figure 6). However the equivalent harvested weight of Broccoli was lower in untreated plots in comparison to Limex treated plots (approximately 2000 – 3000 grams). This was due to the large number of missing plants in the untreated plot and to a lesser extent in the standard lime treated plots. (Figure 15).

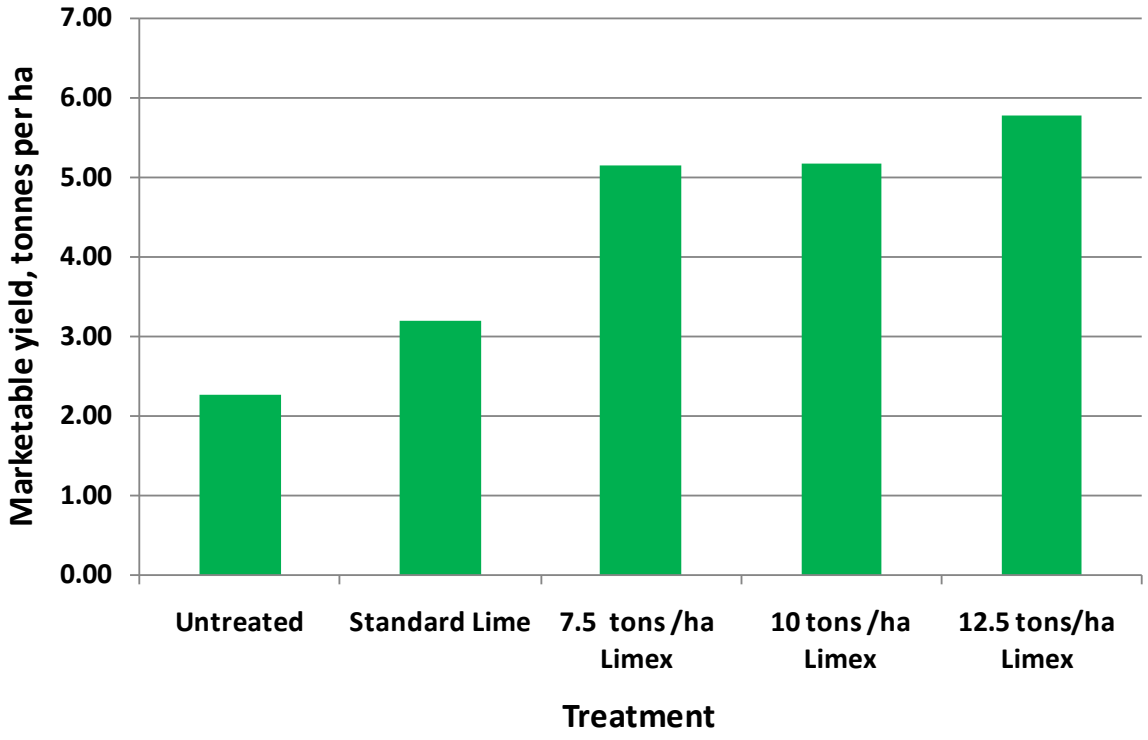


Figure 14: Marketable yield of Broccoli in plots at Crail 2010

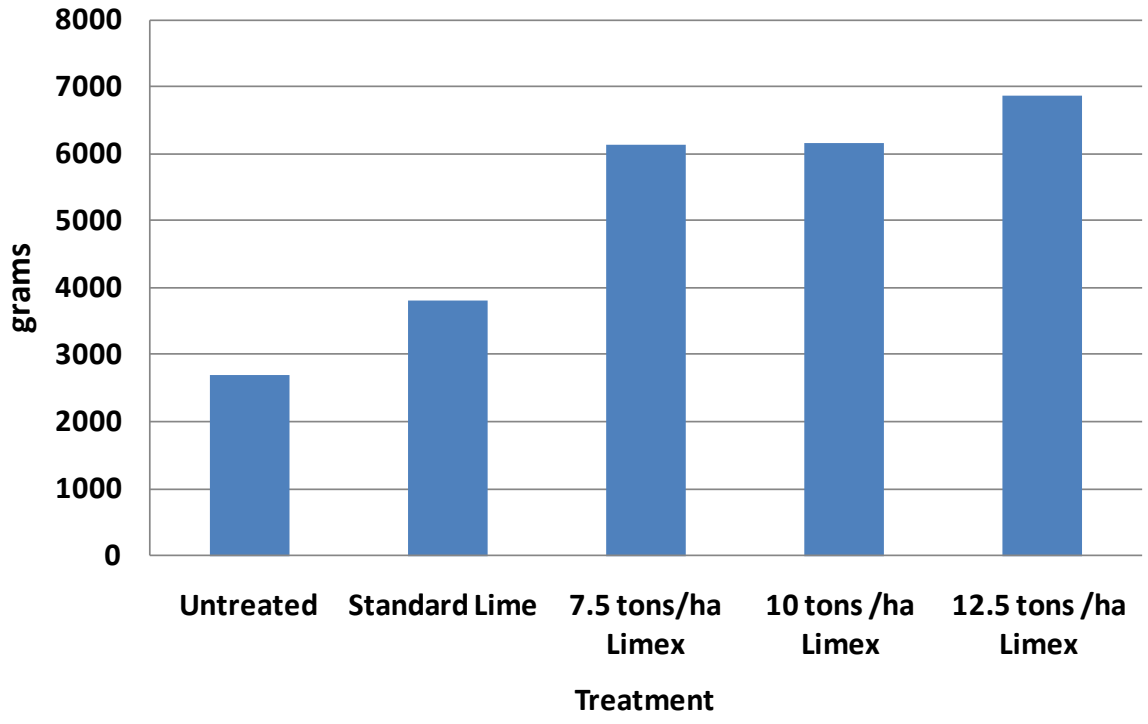


Figure 15: Total yield of harvested Broccoli in plots at Crail 2010

Conclusions

The results confirm the findings reported in clubroot control trials conducted in 2009 at Crail in Scotland using Limex. Limex can be used to control clubroot in heavily infested land while maintaining marketable yields of Broccoli crops. The optimal application rate was also observed to be between 7.5 – 10 tons Limex ha^{-1}. The results demonstrate that the application rate could be reduced without a detrimental effect on broccoli yields. The results show that the highest levels of clubroot were recorded in plots which were treated with standard lime or were untreated. Standard lime and Limex did not have a significant effect on the pH of the plots although high rainfall was recorded at the time of application. The pattern of clubroot development in the plots differed between 2009 and 2010. In 2009 there were drier conditions during the trial period. However in 2010 there was higher rainfall and higher soil moisture consequently the amount of clubroot activity increased during the trial. During 2009, clubroot activity decreased, reflecting the lower soil moisture content. If these results were replicated in soils with lower contamination levels it would explain the patchy distribution of clubroot activity. Information on resting spore content of soils cannot therefore be assumed because it will vary between seasons and locations. Tests would need to be conducted prior to planting to determine the clubroot contamination. The high levels of soil contamination by clubroot reflect the cropping of broccoli on the site in that the trial was planted on land which had already produced a crop of broccoli in that season. Limex raises the pH in treated plots although the effect on plant growths and marketable yield does not appear to be pH mediated as the increased levels of pH recorded were still optimal for clubroot infection and development.

Complete development of lateral flow device optimisation for problem soil types and distribute prototypes

Introduction

Following on from the preliminary development of LFD optimisation for problem soil types a further range of soils with known characteristics was identified. Alongside this, work was also started looking at increasing sampling efficiency using immuno-magnetic separation (IMS). IMS provides targeted separation of materials, for example spores from environmental samples, based on the ability to bind target specific antibodies to a magnetic particle which can then be removed from the starting material, such as soil. This technology has been applied in several organisms (Fu *et al*, 2005, Parham *et al* 2003) but has not yet been extensively tested in the extraction of *P. brassicae* spores from the soil matrix. If successful it is possible that larger soil samples could be used which would help to increase sensitivity of LFD quantification.

In tandem with this approach, methods of simple extraction of resting spores in soils were explored to enable growers to quickly sample and evaluate field soils for clubroot infestation. Prototype lateral flow devices were then distributed to a Brassica grower to trial the system. The grower was supplied with an ESE reader and a simple protocol for processing of the collected field soils by lateral flow. The soils collected by the grower (and identified as Series 12) were sent to the National Pollen and Aerobiology Research Unit for independent assessment of *P. brassicae* resting spore concentration by qPCR and lateral flow test. The results of the study were compared and the two techniques evaluated for the detection and quantification of resting spores in soil.

In addition, studies were carried out to examine and strengthen the shelf life of the developed lateral flow test. In the previous year's testing it was observed that over time assay sensitivity could be compromised. Two areas of concern were highlighted: stability of

the immunological complex retained in the lateral flow conjugate pad and application of the antigen at the test line (Figure 1).

Materials and Methods

Further determination of problematic soil types

A range of soil types within the Worcestershire area were identified using the Soil Survey of England and Wales (Palmer, 1982). Locations corresponding to each soil type were identified and sampled using a soil corer to a depth of approximately 20-25cm. A maximum of five cores were taken at each site and pooled together.

Soils were then weighed and air-dried, before milling and sieving to 2mm. A spore suspension containing $1x10^9$ resting spores ml^{-1} was diluted in Purite water to provide a range of $1x10^8$, 10^6 and 10^4 spores ml^{-1}. Each soil was weighed into four aliquots of 4g each. Each aliquot was poured into a sterile 9cm petri dish and gently shaken to distribute across the surface of the dish. Each aliquot of the soil was then inoculated with 4ml (in a dropwise manner) of either $1x10^8$, 10^6, 10^4 or 0 spores ml^{-1}. The inoculated soils were then left to air dry for three days before DNA extraction and qPCR quantification using primers PbML1F and PbML1R as previously detailed.

Immunomagnetic separation of spores from soil to improve sampling capacity

Previous work (data not shown) had demonstrated that it was possible to remove spores from soil using spores bound to antibody on magnetically coated particles. This work was further optimised to increase the sample volume from which spores could be extracted.

Initially one gram of artificially inoculated soil containing $1x10^5$ spores g^{-1} was weighed into a 30ml glass universal and suspended in 10ml of either 5, 10, 15 or 50% sucrose solution. Magnetic fleas were added and the suspension stirred for 20 min before the large soil particles were allowed to settle and the supernatant decanted into further glass universals.

To each sample 40µl *P. brassicae* monoclonal antibody UW249 was added and the universals were sealed and placed on a MultiMix roller for 30 min, after which 40µl of antibody coated magnetic particles were added and mixed for a further 30 mins. Magnetic extraction was then performed by opening the lid and resting the universal within a magnetic field for 2 mins. The liquid component was removed using a 3ml pastette and the bead extracted particles washed from the side of the universal. This was performed in triplicate and the DNA extracted from the resultant samples using the MoBio PowerSoil DNA extraction kit as previously detailed. The level of spores extracted by IMS was quantified by qPCR using primers PbML1F and PbML1R as detailed.

Eight soils from 'series 7' were then run through IMS using an increased sample volume of 50g in 100ml 15% sucrose solution within a 1L Duran. Due to the increased sample size the period of time stirring occurred was increased to 2h. The liquid component was decanted as before but run through soil sieves to 180µM, and then distributed equally between two 50ml centrifuge tubes. The specific monoclonal was then added at 0.4% and the tubes were agitated on a MultiMix Roller for 1h before activated magnetic particles were added (0.4%) and agitated again for 1h prior to magnetic separation as before. It was found that some soil particles were not removed on the initial pass through the magnet, and so any remaining particles were washed and the sample passed across the magnet for a further 2mins. The number of washes required varied with the soil type, and is indicative of differences in the soil compositions. The final rinse volume of 1ml to collect the magnetically bound spores was then transferred to a 1.5ml microfuge and centrifuged at 5000rpm for 1min. The supernatant was discarded and the pellet was suspended in liquid from the MoBio PowerSoil bead tubes before DNA extraction and quantification as previously described.

Evaluation of Series 12 UK commercial soil samples (2012) by grower distributed lateral flow devices, laboratory qPCR and an evaluation of the two methods.

Lateral flow device testing: An ESE Reader, the computer software and a 2012 batch of optimised LFDs were sent to the East of Scotland Growers for use in the evaluation of commercial soil samples for clubroot resting spore infestation. A simple protocol was followed: individual collected soil samples were each shaken in a small volume of lateral flow buffer, allowed to stand for 1 minute and a small volume of the liquid phase of each field sample transferred to the sample pad of individual lateral flow devices. A negative (soil with 0 resting spores) and positive samples (calibration series of artificially infested soil) were also processed by LFD and for each OD measurements were made. In total, 53 'grower' commercial field soils were tested for clubroot resting spore presence and the subsequent OD values generated were compared and evaluated against laboratory generated qPCR results.

Molecular qPCR: DNA extraction and qPCR analysis were carried out on all 53 commercial as described previously. The results from each assay were compared using 4 parameter logistic regression to model the relationship between them, and the fit of predicted spore g^{-1} soil values generated from LFD quantification were tested against the actual spore g^{-1} soil values obtained by qPCR.

Lateral flow shelf life: Antibody and antigen stability

For each conjugate pad, a solution containing 30µl of 1:40 UW249 monoclonal antiserum in conjugate buffer (0.0025M PBS, 2% trehalose, 2% BSA, 2% sucrose) with 5µl anti-IgM gold beads) (BBI International) was prepared. To this solution, different concentrations of sucrose (0%, 5%, 10%, 15% & 20%) were then added. The pads were dried at 37°C for 20 minutes. After which the conjugate pads were stored in a dry state at room temperature prior to assessment at twelve consecutive time points. At each selected time point three conjugate pads were removed from storage and each inserted within an LFD complex (Figure 1). To

each LFD 100µl B2 buffer was then added and an ESE reading was taken after 30 mins run time.

A solution of whole spores of *P. brassicae* resting spores ($1x10^9$ spores ml^{-1} water) were prepared in a) water b) 0.05% trehalose and 1% isopropanol and c) 0.05% trehalose , 1% isopropanol and 2% sucrose. These solutions were applied as antigen test lines to a series of lateral flow membranes at a speed of 10mm s^{-1}, and allowed to air-dry at room temperature overnight. The membranes were sectioned in to 5mm strips and assembled within a LFD format. The LFDs were stored at room temperature in foil pouches. At selected time intervals the LFDs were opened and, using newly prepared conjugate pads, a solution of *P. brassicae* resting spores was aliquoted to each sample pad and OD readings were taken after 30 min LFD run time.

Application of the control line to the LFD devices

A test line of *P.brassicae* was applied to a series of lateral flow membranes as described previously. A second line (control) containing IgG purified *Alternaria brassicae* (0.6mg/ml) was applied to the same membrane at a speed of 10mm sec^{-1} (Fig. 1). The membrane was allowed to air dry overnight. Three solutions: a) 33µl conjugate buffer with 7µl Protein A gold beads (BBI International) b) 28µl 1:40 UW249 and 5µl anti mouse gold beads with 7µl conjugate buffer c) 28µl 1:40 UW249 and 5ul Anti-mouse gold beads with 7µl protein A gold beads were applied to LFD conjugate pads and air-dried at 37^oC for 20 mins. After which the conjugates were incorporated in to the LFD structure with the test and control lines applied. A 100µl aliquot B2 buffer was then applied to each LFD and the ESE output read after 30 mins.

Extraction of clubroot resting spoils from soils using a sucrose gradient.

Six tubes containing 0.26g of clubroot free soils (negative control), and a further 6 tubes containing 0.26g of artificially infested resting spores at $1x10^9g^{-1}$ soil received 400µl aliquots of B2 buffer in 0%, 5%, 10%, 15%, 20% or 25% sucrose. The tubes were vortexed briefly

and allowed to settle for 1 min. Afterwhich 100µl of the surface solution was removed from each of the tubes and transferred to individual LFD devices. An ESE reading was taken at 30 minutes and the results evaluated.

Results

Further determination of problematic soil types

Of the soils which were not artificially infested with a clubroot resting spore suspension, four showed amplification of *P. brassicae* DNA above the cut-off limit for detection ($3x10^3$ spores g^{-1}). One of these soils provided a potential moderate to high clubroot disease risk of $7.8x10^5$ spores g^{-1} soil. These soils were eliminated from further analysis due to their potential for existing *P. brassicae* populations. Soils which were artificially inoculated with $1x10^4$ spores g^{-1} soil were processed by qPCR and the effect of inhibitors in sand, silt or clay content were assessed. Only one of these soils 'a high clay' content proved significant ($F = 6.7839$, $df = 1$, $P = 0.025$) in inhibiting the qPCR process for quantification of P. brassicae resting spore concentration. None of the other soil factors proved significant at any of the other inoculation concentrations. When the interaction between factors was considered a two-way ANOVA showed that clay, sand and the interaction between them was significant at $1x10^4$ spores g^{-1}, (Table 4) but silt was not and neither was there any effect at the other spore inoculation levels.

Table 4: Two way ANOVA interaction between factors to determine problematic soil types

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
clay	1	8.0873	8.0873	18.869	0.001867 **
sand	1	4.3074	4.3074	10.050	0.011363 *
clay:sand	1	4.9487	4.9487	11.546	0.007898 **
Residuals	9	3.8574	0.4286		

Immunomagnetic separation of spores from soil to improve sampling capacity:

The number of *P. brassicae* resting spores extracted using the different concentrations of sucrose did not vary greatly with the lowest being $6.2x10^4$ spores g^{-1} and the highest being $3.5x10^5$ spores g^{-1}, which was achieved with 15% sucrose. The number of spores extracted from the naturally infested soils was increased in the 50g soil samples compared to the standard 0.26g soils from which DNA would routinely be extracted. Pearson's product moment correlation coefficient showed a strong correlation between the qPCR results from 0.26g soil and 50g soil (R = 0.997 to 3dp).

Evaluation of 2012 soil samples for P. brassicae resting spore concentration by using lateral flow devices and qPCR and comparisons between the two methodologies:

Lateral Flow: Of the 53 commercial field soils tested 32 would be considered at high risk for sown Brassica crops developing severe and uniform disease expression when grown under environmental conditions conducive to the disease development. All soils for this group were recorded at a P*. brassicae* resting spore concentration of $1x10^6$ g^{-1} soil or greater. Twenty three of these soils recorded resting spore concentrations between $1x10^7$ and $1x10^9$ resting spores g $soil^{-1}$ which in terms of disease risk is exceptionally high. Six field soil samples (16, 17, 32, 38, 49 and 50) were considered to be at moderate to high risk of the disease with resting spore concentration circa $1x10^4$ to $1x10^5$ resting spores g^{-1} soil. Soils samples which recorded a resting spore concentration between $1x10^3$ and $1x10^4$ resting spores g^{-1} soil were graded at low to moderate risk of clubroot disease development (Soils 1,2,3,19,29). The remaining five samples recorded values of less than $1x10^3$ spores g^{-1} and are considered to be free / low risk of the disease (4,5,26,27,41,45,46,47,51). Ultimately the development and expression of the disease 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.

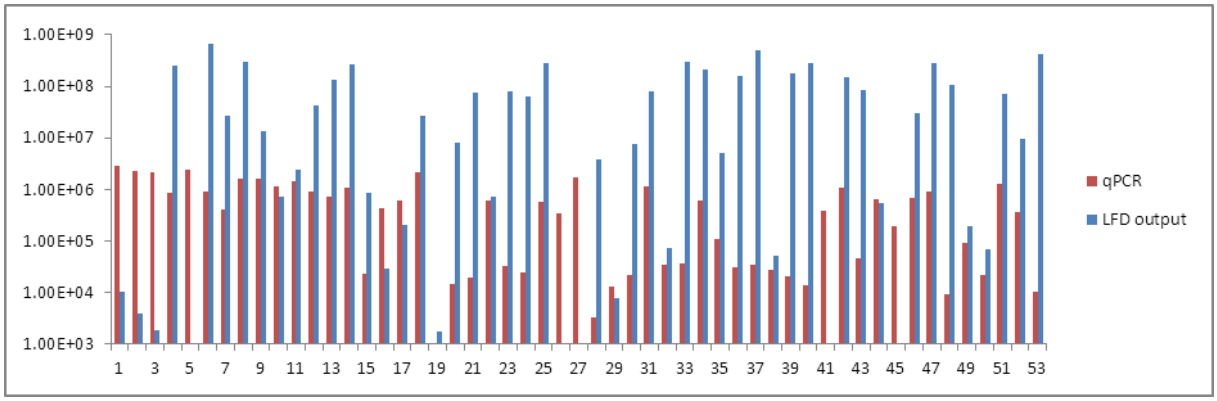


Figure 16: Lateral flow and qPCR results for the "Series 12 collection of 23 UK soil samples

qPCR: None of the 53 commercial field soils when tested by Molecular quantification were considered at or >1x10^{7} resting spores g^{-1} soil (very high clubroot risk). This is at variance to the lateral flow test which identified 23 soils at or above this level. Nevertheless 29 soils were recorded to have resting spore concentrations at circa 1x10^{6} resting spores g^{-1} soil which would place sown Brassica crops at high risk of developing severe and uniform disease expression when grown under environmental conditions conducive to the disease development. A further 20 soils were identified as moderate to high risk with resting spore concentrations circa 1x10^{4} to 1x10^{5} resting spores g^{-1} soil. A single field soil (28) was identified at low risk to the clubroot disease. Only one of the samples tested was determined to be below the detection threshold of 1x10^{3} spores g^{-1} soil and was rated as free / low risk to the disease (soil 19) .

When the distribution of measured sample qPCR spores g^{-1} soil was plotted against the confidence limits of the predicted qPCR spores g^{-1} (generated from regression of the LFD OD values), 17 (36.6%) fell outside the confidence intervals while the remaining 30 were inside (64%) (Figure 17).

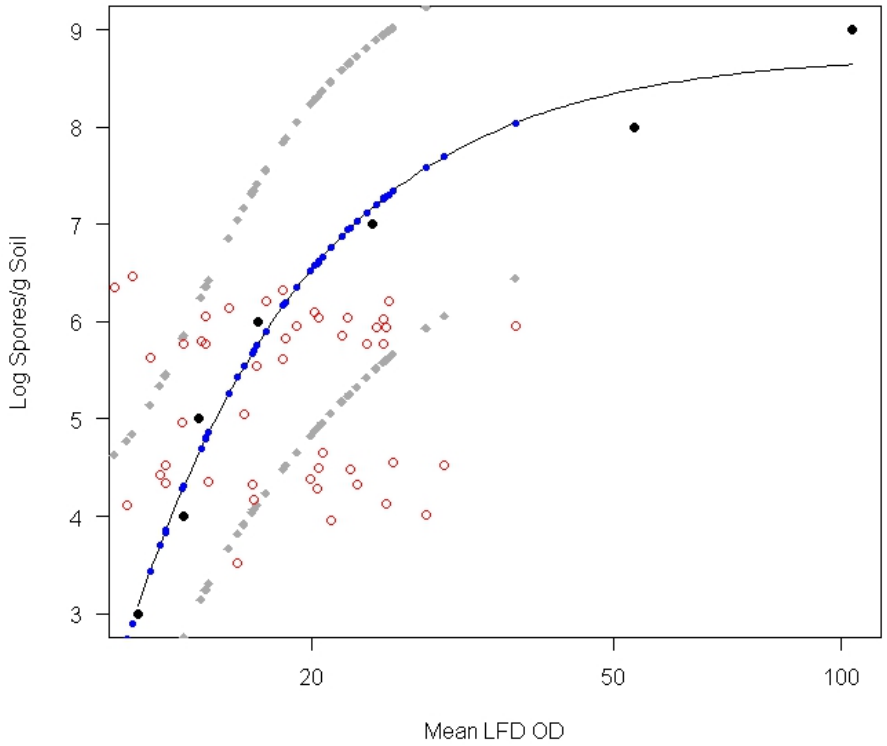


Figure 17: Predicted and actual log spores g^{-1} soil from LFD and qPCR. Black points and line = 4 parameter logistic regression of LFD and qPCR standards, blue points = predicted output based on LFD OD, open red circles = actual LFD against qPCR results, grey points = confidence intervals for predicted outputs.

Antibody and antigen lateral flow device stability studies

With the exception of the conjugate pads which received a sucrose concentration of 10% or greater, the immune-reactivity of each was compromised over time when compared to a conjugate pad which had been prepared within 1hr of production (*i.e.* fresh / no storage). This confers with other studies which report improved immune-stability when samples were stored at a 2% sucrose concentration.

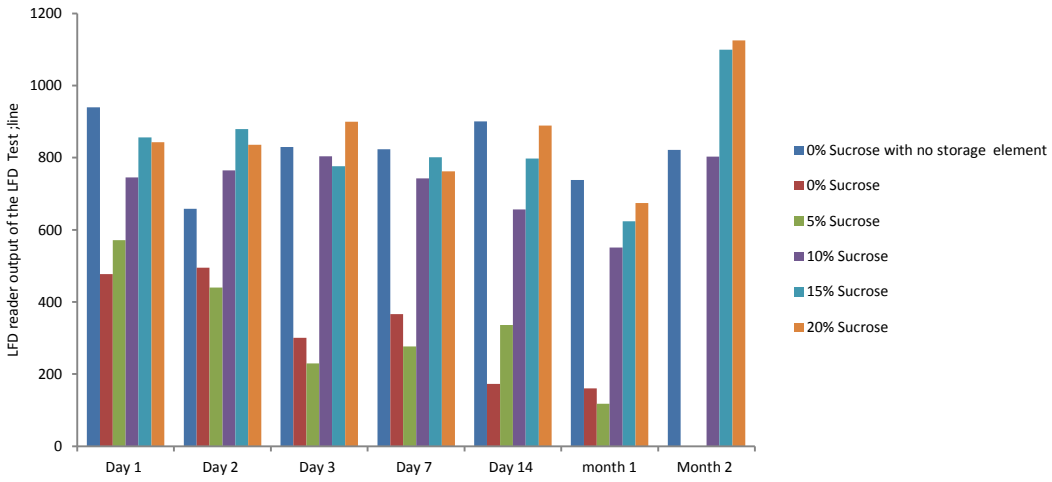


Figure 18: Time course study to determine the immune-reactivity of the air-dried conjugate pads over time

Over an eight month period the addition of both Trehalose and Sucrose to the standard test line application enabled both visual and OD test stability to be retained and at a sensitivity similar to that recorded at the initial time of application (Fig. 19). In contrast the standard test line application gave rise to a 50 % reduction in test line signal after a two month storage period. This equated to near or no test line visability when viewed by visual eye recognition. An OD reading however could still be generated using an ESE reader but a reduced signal. After two months the OD reading was at 0 for both the standard test line application (no sucrose applied) and at a 5% sucrose application.

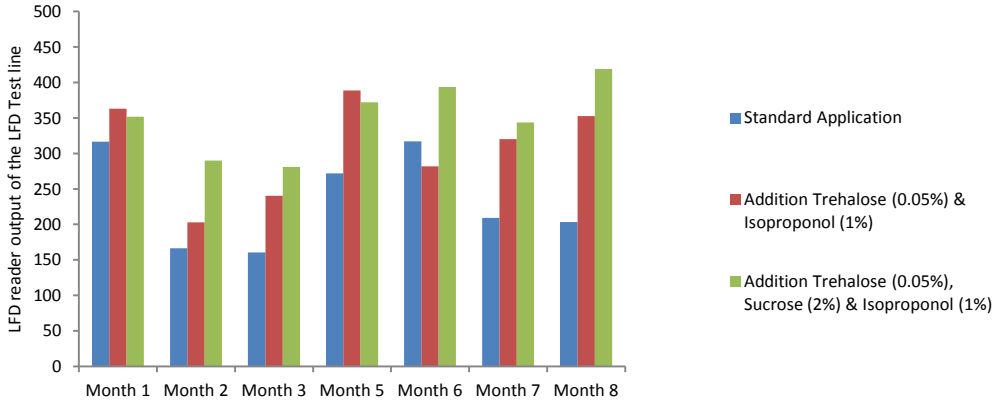


Figure 19: The effect of Trehalose, Sucrose and Isopropanol on the stability of *P. brassicae* resting spores when applied as a test line to an LFD membrane and monitored over time

The results in Figure 20 show that the disruption of P. *brassicae* resting spores to release soluble antigen prior to test line application has no effect on LFD test line stability when applied in a 10% sucrose application buffer

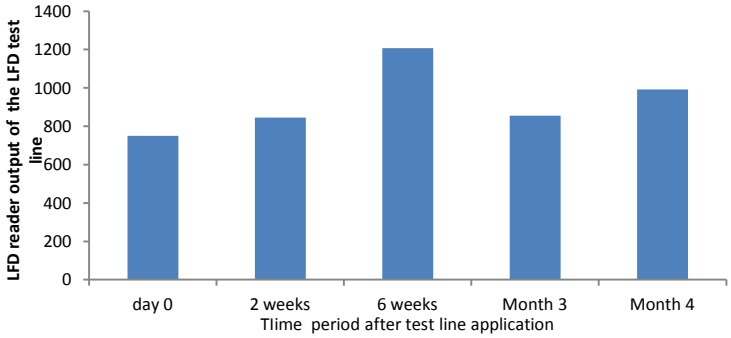
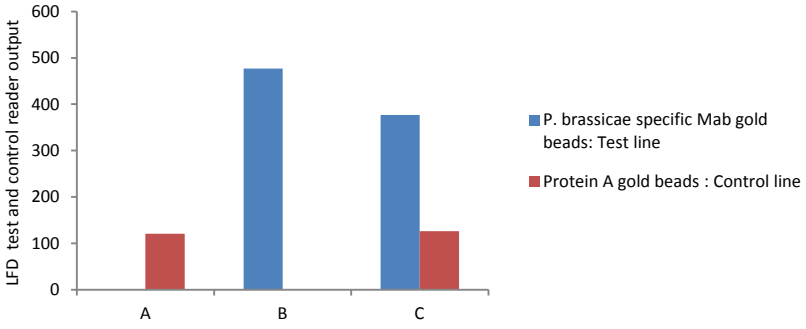


Figure 20: Stability of disrupted *P. brassicae* resting spores in a 10% Sucrose, 0.05% trehalose, 1% isopropanol and 0.05% sodium azide application buffer when applied to a LFD membrane and the test line measured over time.

Introduction of a control line to the LFD devices.

When Protein A gold beads alone were held within the conjugate pad only a control line reading was observed on the LFD device after test application (Figure 21 (A)). Conversely in the absence of Protein A but with the addition of a specific *P. brassicae* gold conjugated antiserum a test line was this time observed but with no control line (Figure21 (B)). When both the Protein A conjugated gold beads and the *P. brassicae* specific conjugated beads were applied to the conjugate pad a test and control line were observed after test run completion (Figure 21(C)).



Test and Control line development

Figure 21: Investigation to determine the reactivity of Protein A gold beads and MAb conjugated anti-mouse gold beads to the test and control lines of a clubroot LFD test.

Extraction of clubroot resting spores from an artificially infested soil using a sucrose gradient:

The use of a sucrose gradient to extract the resting spores from the soil matrix ahead of application to the lateral flow did not prove useful (Figure. 22).

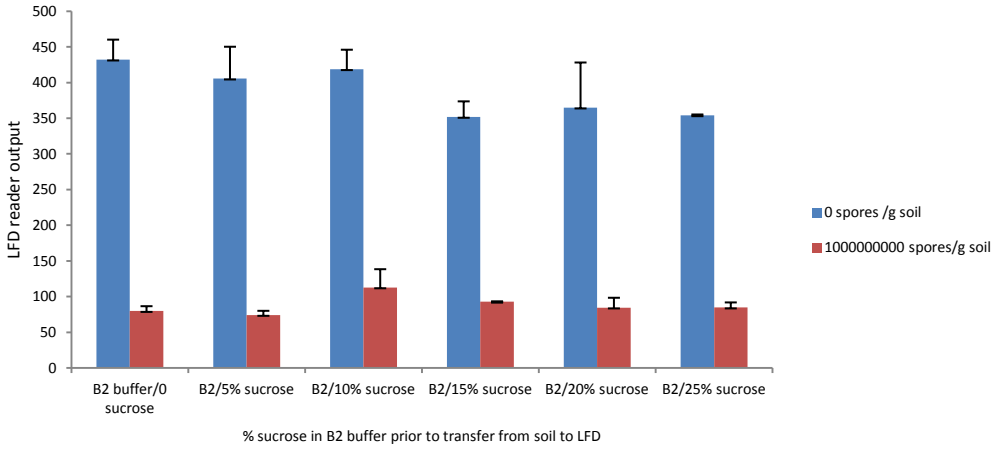


Figure 22 The use of sucrose as a gradient to separate resting spores from the soil matrix.

Summary

From the results, soil textural parameters become more significant in the detection sensitivity of *P. brassicae* as detected by qPCR when at lower spore numbers. This could be important to predicting the risk of clubroot disease occurrence at spore levels just below the generally accepted disease threshold spore load of $1x10^5$ spores g^{-1} soil, as it may indicate either the quantification of spore numbers is not completely representative of the spore load at low levels in certain soil types, or that some soil types retain different spore loads to others.

Soil type is also important in the context of molecular studies as it is known that the soil type may influence DNA extraction and subsequently the outcome of PCR amplification due to the presence or absence of inhibitory substances (Lloyd-Jones and Hunter, 2001). There are different ways these compounds can interfere with PCR however precise mechanisms have yet to be fully determined. It is thought that these compounds may affect cell lysis, thus

hindering the extraction of DNA from cells, they may also bind to DNA or induce DNA degradation, and there may also be a negative effect on DNA polymerase activity thus reducing the capability of annealing and extension of primers used for the PCR (Wilson, 1997). Although DNA clean-up was performed to help remove such substances, the complete removal cannot be guaranteed. Thus until further analysis is performed with LFD quantification it is hard to say whether the levels of *P. brassicae* quantified are affected by PCR inhibition or other factors that directly affect the spores themselves.

Interestingly the parameters of significance in this experiment are sand and clay (and their interaction), however the previous data suggests that silt may be a significant factor. The main difference between these two experiments was that silt was shown to be significant in naturally occurring soils, while sand and clay where shown to be significant in artificially occurring soils. There are several reasons why these results have been observed; firstly the soil dynamics and complex interactions within naturally occurring soils affect the binding of spores to the soil and, artificially infected soils (while the closest approximation possible) do not fully represent such relationships. In the strictest sense it would not accurate to compare the two sets of data, and further experimentation should be based on the findings of both experiments independently. Secondly it is possible that increasing the soil type representation affects the proportion of soils within each type thus influencing the significance of each factor. It is possible that by artificially infecting different soil types, soils which would not naturally carry infection may become included in the analysis and skew the results. Thirdly it is possible, if not probable, that other factors within the soils have a strong influence on spore load and the risk of clubroot occurrence. Further analysis of the soils from these two data sets will provide greater information on more significant factors.

The quantification by qPCR of *P. brassicae* from eight soil types using either standard DNA extraction or IMS extraction of spores from soils shows promising correlation between the data sets. This is an exciting find as it will be able to reduce the detection threshold allowing

determination of whether soils are most likely to be free from *P. brassicae*, or whether the spores are present at very low levels. A greater sample volume also means that the samples can be more representative of large areas without the need for further labour intensive sampling and, providing a sufficient number of samples are tested, this will help to remove variation that can occur within a field due to localised regions of higher or lower spore concentrations. It is possible that this technique could be applied to even greater quantities of soil however the cost effectiveness of larger sample volumes would have to be calculated. The results of this experiment support accuracy of results given by the current sampling practise and DNA extraction quantities, however there are many benefits of being able to extract from larger amounts of soil, at least reassurance to growers that the sampling is representative of their entire field. In addition the use of immuno-magnetic separation could provide a 'clean sample' free of soil inhibitors. This could prove useful not only for qPCR but also in the use of field soils for lateral flow assessment. Future work should assess this.

From the quantification results of the qPCR and LFD on the 'series 12' samples it was possible to determine which soil samples fall outside the predicted output range when the actual qPCR results are compared against the LFD results. It appears that the majority of outliers are at higher LFD OD readings but lower spore numbers, suggesting that the LFD predicts slightly higher than the qPCR. It may be that a particular soil type influences the results and that there may be inhibitory factors which will contribute to the presence of outliers. Further analysis of these outlying soil samples and comparison to the others will allow determination of any inhibitory factors and whether the impact of such factors affects the quantification by qPCR or by LFD.

The relationship between quantification by LFD and qPCR is non-linear. There are several hypothesised reasons for this, the first being that the qPCR measured actual DNA and the expectation would be one genome per resting spore. The LFD measures the amount of antibody binding to the resting spore walls and soluble proteins associated with the resting

spore. It is possible that the increased signal of the lateral flow relates to resting spore viability and /or raised soluble proteins levels as a result of suspension in the lateral flow extraction buffer prior to test application. The IMS process confirmed that resting spores were bound by the specific monoclonal antibody as it is possible to detect DNA from the resultant samples of magnetically extracted particles. Therefore it is likely to be a combination of resting spores and soluble components which are acting as the target analyte in the LFD protocol.

It is not known at this stage whether the amount of soluble antigen would be consistent across different *P. brassicae* populations, and there may be an influence of *P. brassicae* race/ pathotype on the level of soluble antigen released. Also it is possible to observe 'empty' resting spores within samples in which the nucleic acids are fluorescently stained (data not shown), and in such a scenario the 'empty' spores would not be detected by qPCR as they do not contain DNA. However using an antibody based system, the resting spore wall may provide a binding site for the antibody whether or not it contains DNA. Thus the viability of the spores could be important in the comparison of the two assay systems, and again this is something that could be influenced by the predominant race/pathotype of *P. brassicae* present.

Test line and activity of the dried antibody conjugates in the LFD prototype show an improved and retained level of stability when sucrose was incorporated as an additive. The concentration of sucrose application proved important in retaining test sensitivity. Sucrose is a known stabiliser and during drying provides a structure around the antibody to assist retention of protein conformation and in doing so maintain the chemical stability, resist denaturation, aggregation and the loss of biological activity of the antibody. Previous studies (Andy *et al*., 2003 and Johnson *et al*., 2003) both purport the use of sucrose in stabilising antibody structure by covering hydrogen bonding sites to inhibit aggregation and providing a durable 'cake' support for protein stability.

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