**Project title:** Brassicas: Further development of infield tests for resting spores of clubroot and the development of clubroot control based on detection

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| **Project number:** | FV 349 |
| **Project leader:** | R.Kennedy |
| **Report:** | Final report, May 2013 |
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| **Location of project:** | Worcester University |
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| **Date project commenced:** | 1st April 2009 |
| **Date project completed (or expected completion date):** | 31st May 2013 |

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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

Progress has been made towards an in-field clubroot test for Brassica growers.

## Background

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 traditional methods cannot detect low levels of clubroot in soils. These methods were based on using the reaction of bait plants, however, large numbers of plants are required in these tests if small amounts of clubroot are 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. However, a competitive lateral flow device (lab on a stick) has been developed and evaluated for use in UK commercial soils for the detection of clubroot resting spores. The device was able to detect clubroot spores at close to epidemiological significant levels (10000 spores/gram of soil) in artificially infested soils.

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

Determining the clubroot resting spore number in soils using either a molecular or lateral flow test is an essential component in the development of an integrated disease

management programme. Currently only two chemicals (cyazofamid – Ranman and fluazinam – Shirlan) approved for control of disease in potato crops have been demonstrated to have any potential for controlling clubroot in the field. However both these chemicals do not hold approval for clubroot control in vegetable Brassicas as their efficacy against clubroot has not yet been demonstrated. Alternative control measures are still urgently needed. Limex, a by-product of the British sugar industry (www.limex.co.uk) was found in trials carried out over three consecutive years of the project to reduce the effect of clubroot infestation in soils on Brassica crop production. This was ahead of any other of the treatments used. An applications rate of Limex at or above 10 tons Limex ha-1 was found to be optimal in reducing clubroot disease.

## Summary of the project and main conclusions

A Brassica disease forecast has been under development to evaluate the potential to generate risk assessments for clubroot based on soil type, crop and clubroot resting spore numbers. In the final phase of the project the forecast has focused on three main environmental parameters: pH, calcium and magnesium. Each of which are considered significant due to their relationship in development of the disease to gall formation in the plant root. Further trials are required to establish whether the significant factors have a direct or indirect effect on clubroot disease potential. However 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 likely to be 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.

The deliverables from this project are:

- Better detection of clubroot in the field before planting the crop.
- Detection tests which have been 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.
- Initial development of a disease risk forecast for clubroot

## Conclusions

- A simple ‘lab on a stick’ detection test, developed for the detection and measurement of clubroot spores in soil, has been evaluated in UK soils for use by the industry. A restricted UK Soils testing programme for measurement of clubroot contamination risk is offered in 2013 and 2014 to HDC members.

- A Brassica disease forecast to evaluate the potential to generate risk assessments for clubroot disease based on soil type, crop and clubroot resting spore numbers has been reviewed. Findings suggest that a risk matrix based on soil pH, Calcium, Magnesium and spore concentration could provide a useful measure of clubroot disease risk to susceptible crops ahead of crop planting.

- Limex was found in each year of the conducted field trials to improve the control of clubroot in heavily infested land and maintain marketable yields of Broccoli crops. This was ahead of any other of the treatments used. An applications rate of Limex at or above 10 tons Limex $ha^{-1}$ was found to be optimal.

### Financial Benefits

- 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

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 assist control clubroot in affected land however it will not reduce clubroot risk in subsequent seasons.
- The optimal level of Limex required for clubroot control was found to be 10 tons Limex $ha^{-1}$

# SCIENCE SECTION

## Introduction

In the first three years, the project evaluated two diagnostic tests for the measurement of clubroot in soil. The molecular test has provided the measure/yardstick to develop and evaluate an antibody-based lateral flow test (lab on a stick). The lateral flow test 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 (q PCR) test. Ranking both sets of results into low, medium and high disease risk categories improved the correlation.

In Year 4, study approaches for the integrated control of clubroot continued: the use of an environmental models to assist in determining disease risk, the measurement of soils for clubroot resting spore concentration and efficacy of the ascribed control methods. These processes are described below and listed as under Year 4 project milestones.

## Methods

### Milestone 1. Complete tests using typed isolates of clubroot using LFD test kits

#### *Background*

To establish the lateral flow test as a robust assessment of clubroot resting spores in soil, it is important that the technology is capable of detecting different pathotypes or races of the clubroot disease pathogen, *Plasmodiophora brassicae*.

The existence of different *P. brassicae* pathotypes is well documented and conventionally determines pathotypes based on the ability to infect and produce disease in a range of brassica cultivars which have varying degrees of susceptibility (Buczacki *et al*. 1975, Toxopeus *et al.* 1986). The objective of this experiment was to establish whether the lateral flow device was capable of detecting different typed isolates of *P. brassicae*.

#### *Method*

*Lateral flow construction:* Competitive lateral flow devices comprised a 5mm membrane pad made up of a Millipore 180 Hi flow™ 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).

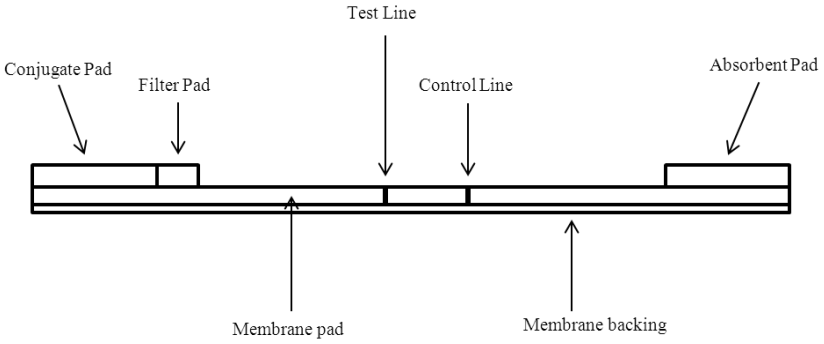



**Figure 1.** Lateral flow strip construction

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 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 race sample.

*Typed isolates.* Within Europe it would be ideal to use typed isolates based on the ECD set. However due to the complexity and levels of resources needed to type isolates in that manner it is difficult to either source ECD typed isolates or perform typing of this nature. Rather than using the ECD set commercial companies frequently type isolates based on their ability to overcome resistance genes in their own specific Brassica cultivars. Gall tissue from six isolates typed in this manner were kindly provided by Syngenta and referred to as Race 1 to Race 6. Of these six isolates three were capable of overcoming resistance (gene breakers – races 1 to 3) and three were not (races 4 to 6).

*Sample processing:* For each of the six race samples, the galls were macerated in a blender at maximum speed for 5 minutes along with 100mL purite $H_2O$. The resulting suspension was filtered through four-fold muslin and divided equally across two 50mL tubes which were centrifuged at 2500rpm for ten minutes. The supernatant was discarded and the

spore layer on the top of the pellet collected and resuspended in purite $H_2O$ with 0.01% sodium azide. Counts of spore concentration were performed using an improved Neubauer haemocytomer and suspensions adjusted to provide ten-fold serial dilutions from $10^8$ spores $mL^{-1}$ down to $10^2$ spores $mL^{-1}$. Each sample was vortexed and 100µL aliquots applied to the LFD conjugate pad. Optical density (OD) was read at 30 minutes using an ESE reader device. Samples were run in triplicate and the mean OD values over the spore concentration ranges for each race were plotted (Figure 2).

### *Results*

All races were tested for a significant effect of spore concentration on OD output by one-way ANOVA. All except race 5 showed a significant effect (at least 95% confidence interval), spore concentration was only significant to OD output at the 90% confidence interval in this exception (Table 1)). When the mean ODs of all three races capable of overcoming resistance were compared to the mean ODs of those not capable of overcoming resistance, a paired t test showed there was no significant difference in OD between the two types of races (t = 6.2674, df = 7, p-value = 0.0004171).

**Table 1.** Analysis of Variance F values and probabilities for the six *P. brassicae* races

| Race | F value | Probability |
|---|---|---|
| 1 | 16.904 | 0.006276 |
| 2 | 40.207 | 0.0007204 |
| 3 | 13.033 | 0.01123 |
| 4 | 13.832 | 0.009863 |
| 5 | 5.3815 | 0.05946 |
| 6 | 10.401 | 0.01802 |

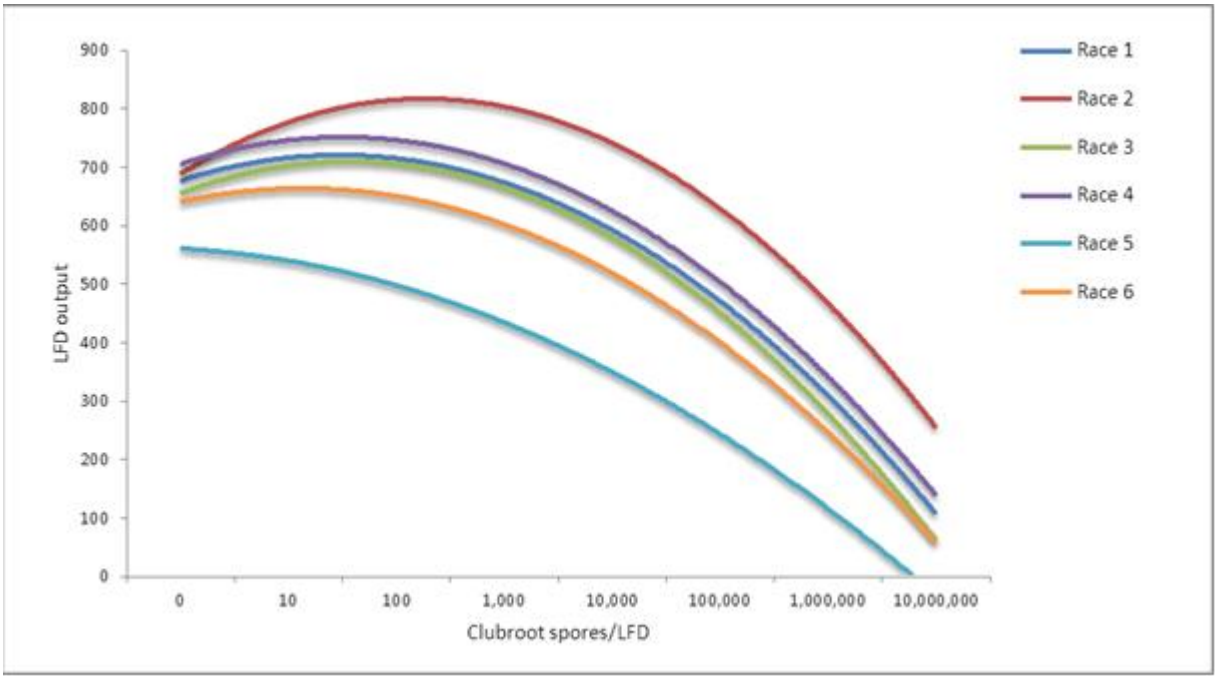



**Figure 2.** Graph to show the Optical Density (OD) recorded for six of the typed *P. brassicae* races over a range of spore concentrations

### *Conclusion*

All six races tested could be detected by the lateral flow devices, supporting the effectiveness of this assay against different pathotypes. The difference in mean optical density between the two types of races (those capable of overcoming resistance and those incapable) was not significant. Nevertheless the variation observed between the races may cause issue in providing truly quantitative results, notably observed with race 5. This could mean it would be hard to rely on the lateral flow as a completely accurate quantitative measure without further knowledge of the predominant pathotype. The relationship of the biological systems involved i.e. soil and meteorological parameters may also prove influential. Nevertheless, the results of this pathotype study supports the potential of the lateral flow test to provide a semi-quantitative estimation of clubroot resting spores in UK commercial soils.

**Milestone 2. Complete field experiments in Lancashire (changed from Scotland) to investigate integrated clubroot control.**

### *Background*

Further to the field trial briefly outlined under Year 3, milestone 2 in HDC Project FV349 Annual Report 2011 (p26) the established field trial in West Lancashire investigating integrated control measures for clubroot disease was completed. The trial was similar in design to previously detailed trials and incorporated four treatments; two lime based treatments and two biological control agent treatments. The effect of these as integrated control measures for clubroot disease is discussed below.

### *Methods*

*Clubroot Trial Design.* Following a preliminary soil sampling in late May 2012 to assess the level of clubroot spores, the experiment was established at a site with a history of brassica cropping near Hooton, Wirral, on a typical stagnogley soil of the Clifton series. From assessment by the Soil Survey of England and Wales this series comprises slowly permeable seasonally waterlogged reddish fine and coarse loamy soils, and similar soils with slight seasonal waterlogging. Some deep coarse loamy soils seasonally affected by groundwater are also included

*Soil physical parameters taken at Hooton*. Soil was air dried and milled 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 air temperature, humidity, leaf wetness, soil temperature and rainfall were collected at 30 minute intervals from when the logger was sited at the experimental area, using an Aardware Smaartlogger. Measurements were downloaded by GSM portable phone Link (Aardware, Kingston upon Thames). The logger was powered by a 12 V battery. Soil pH, moisture content, mineral nitrogen and cation exchange capacity were taken from three deep core soil samples collected across the experimental site.

*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 treatment plot, nominally of area 50 square metres, comprised three beds of 15 metre length, with two plant rows per 56 inch bed, and plants spaced at 18 inches apart. The test lime product and standard lime were applied by hand on 28 June 2012 and after mechanical incorporation commercially raised cauliflower cultivar Boris F1 was planted on 5 July 2012. Transplants treated by drenching were planted by hand the same day. Treatment layout is shown in figure 3.

Drenched transplants were treated with Serenade ASO (BASF) at 10 litres of product per hectare in the minimum volume of water for spraying (400 litres per hectare). Transplants in Hassy trays were steeped for one hour in a solution of sufficient depth to cover the Hassy trays.

Where appropriate, plots planted with drenched transplants were sprayed with Serenade ASO at a rate of 10 litres of product per hectare in 400 litres water per hectare. Application was by conventional knapsack sprayer with a twin nozzle lance.

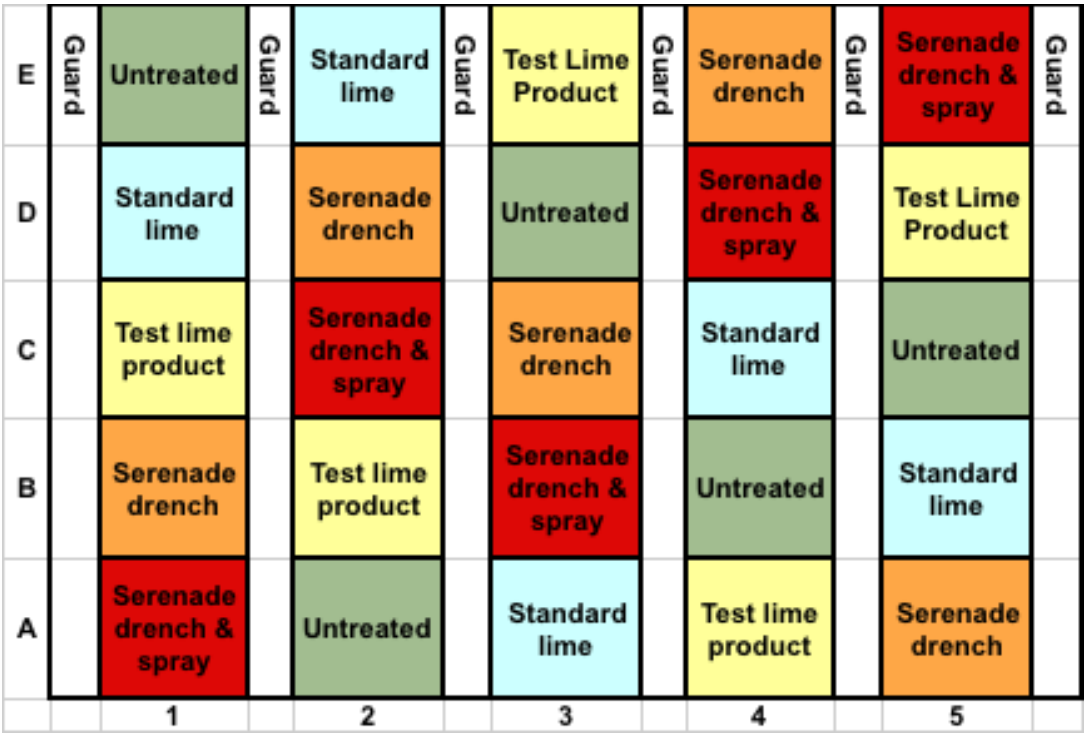



**Figure 3.** Randomisation of plot treatments at Hooton. Treatments were; Untreated, Standard lime, Limex, Serenade drench, Serenade drench & spray

*Clubroot Gall assessment*. The numbers of clubroot galls on plant roots in each trial plot were assessed on one occasion 7 weeks after transplanting. Plants were chosen at random from each treated area of each plot. Root systems were removed intact and after careful washing the numbers of clubroot galls on the roots of two plants per plot were counted.

*Determination of clubroot resting spore concentration in soil using molecular methods.* Soil DNA was extracted from sieved air-dried soil samples using the MOBiol UltraClean Soil DNA Isolation kit according to the manufacturer's instructions (MoBio Laboratories, POBox 606 Solana Beach, California). The vortex stage was replaced by three steps on a FastPrep® Instrument (QBiogene, Irvine, CA) at speed 5.5 for 25 seconds with samples resting on ice between steps. A polyvinylpolypyrrolidone (PVP) DNA cleanup was made on all DNA extracts using a protocol modified from Klemsdal *et al.*, (2008), where BioRad spin columns were placed in 2ml Starstedt tubes and filled to the shoulder with PVP. The PVP was rehydrated with 500µl molecular grade $H_2O$) and left to hydrate at room temperature for 5 minutes. Columns were then centrifuged for 3 minutes at 1500 x g. Flow through was discarded, and the rehydration and centrifugation steps then repeated. A further centrifugation of 1 minute at 4500 x g was subsequently made and the columns placed in clean 1.5ml low-bind Eppendorf tubes. Aliquots of 100µl of DNA extract were applied to the surface of the PVP and incubated at room temperature for 5 minutes. A final centrifugation step of 3500 x g for 3 minutes eluted the DNA.

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

### *Results*

*Rainfall at Hooton 2012*. The rainfall recorded at the Hooton trial site is shown in Figure 4. Rainfall was high during the cropping period. From 12th July to the end of the month a total of 64.2mm of rain was recorded. A further 90mm of rain fell during the month of August. From the beginning of September until crop harvest on 25th September a total of 144mm of rain fell.

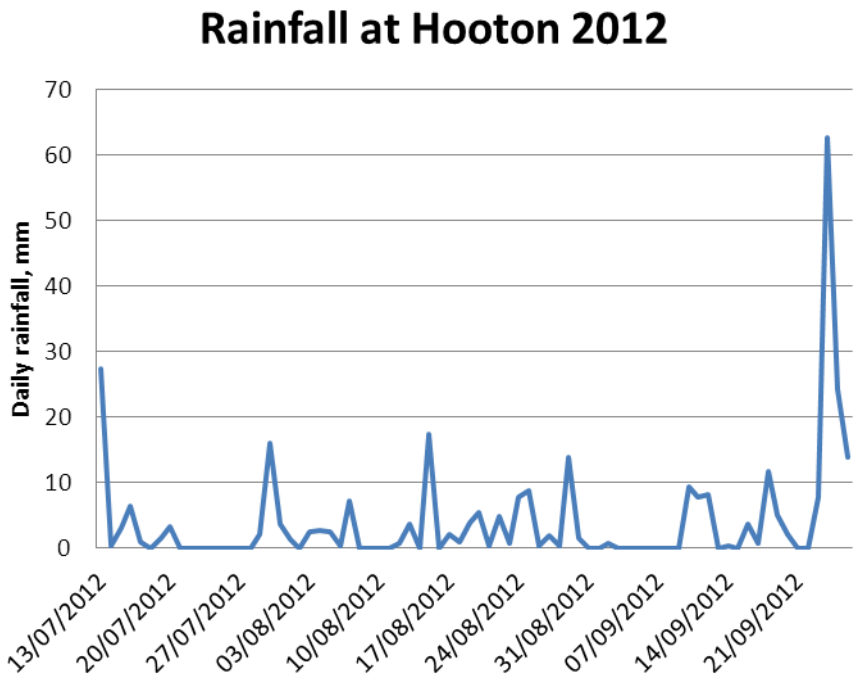



**Figure 4.** Rainfall at Hooton 2012 (mm)

*Numbers of clubroot galls recorded in treated plots.* The results in Figure 5 show the number of clubroot galls on plants 7 weeks after transplanting. Galls were counted and means calculated from the results per plant in each treatment. High average numbers of galls were recorded on plant roots, reflecting the inherent high level of soil infestation by clubroot spores and the unusually wet growing season. Nevertheless, the numbers of visible galls in plots treated with Limex at 10t/Ha were reduced by almost 50% compared to the untreated plots. The standard lime treatment gave a small reduction in gall numbers, in the region of 20%, although this treatment was applied in sub-optimal conditions for this type of liming material. A mean clubroot gall numbers of 16 were observed on plants from the untreated plots. Of significant, Serenade when used as a drench alone, reduced galls per plant to 6.

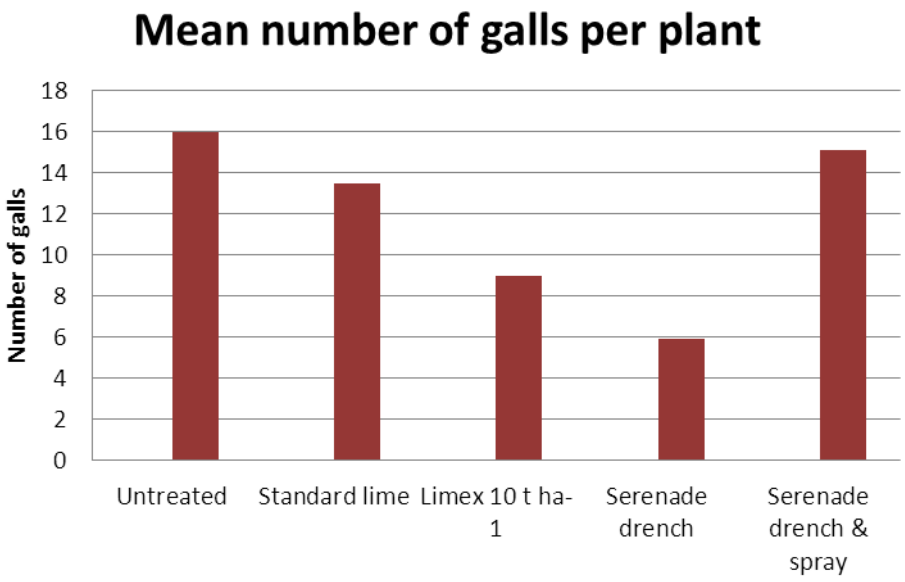



**Figure 5:** Mean number of clubroot galls per plant 7 weeks after transplanting at Hooton 2012

*Estimation of pH in trial plots at Hooton 2012*. Application of Serenade to plants as a drench or as a combined drench and spray had little effect on the mean pH value in comparison to the untreated control throughout the trial. Standard lime and Limex treatments of plots increased the mean pH observed in soil samples when compared to samples from the untreated plots (Figure 6). The Limex treatment maintained the increased pH for the period.

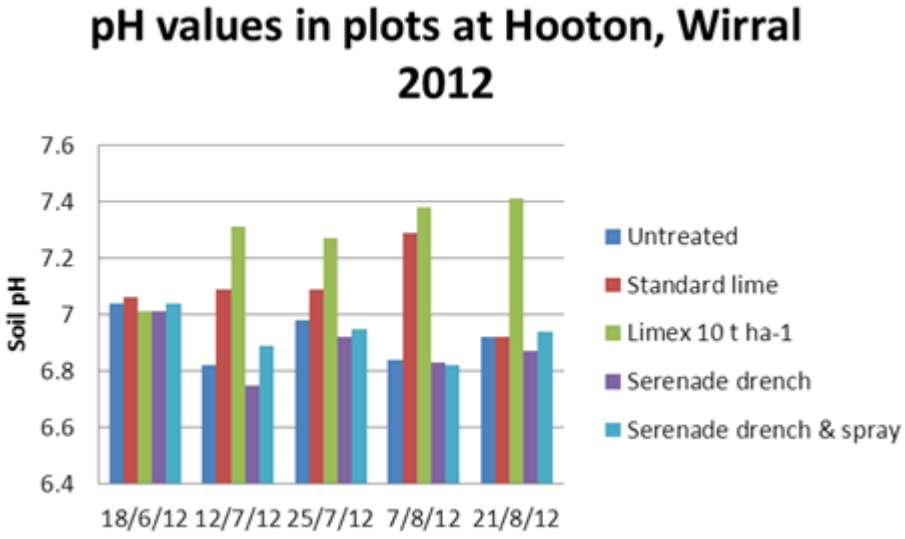



**Figure 6.** The pH values recorded in soils at Hooton 2012

*Estimation of clubroot spore numbers in trial plots at Hooton 2012.* The numbers of clubroot spores per gram of soil in the plots was very high at the start of the trial (Figure 7). This

reflected the very favourable conditions (wet and mild) and the intensive previous cropping pattern of the trial plot used in 2012. At the 18th June both the standard lime treatment and the Limex treatment reduced the initial incidence of clubroot in the soil after planting. However by the 12 July 2012 sampling period the soil samples taken from both the standard lime treatment and the Limex treatment showed little difference in clubroot in comparison to the untreated controls and serenade treatments. The amount of clubroot in the soil increased significantly when soils were tested on the 18 June 2012. At the next sampling date (25 July 2012), the clubroot concentration in the standard lime and serenade drench/spray soil samples continued to increase whilst those in the Limex, untreated and Serenade drench treatments decreased.

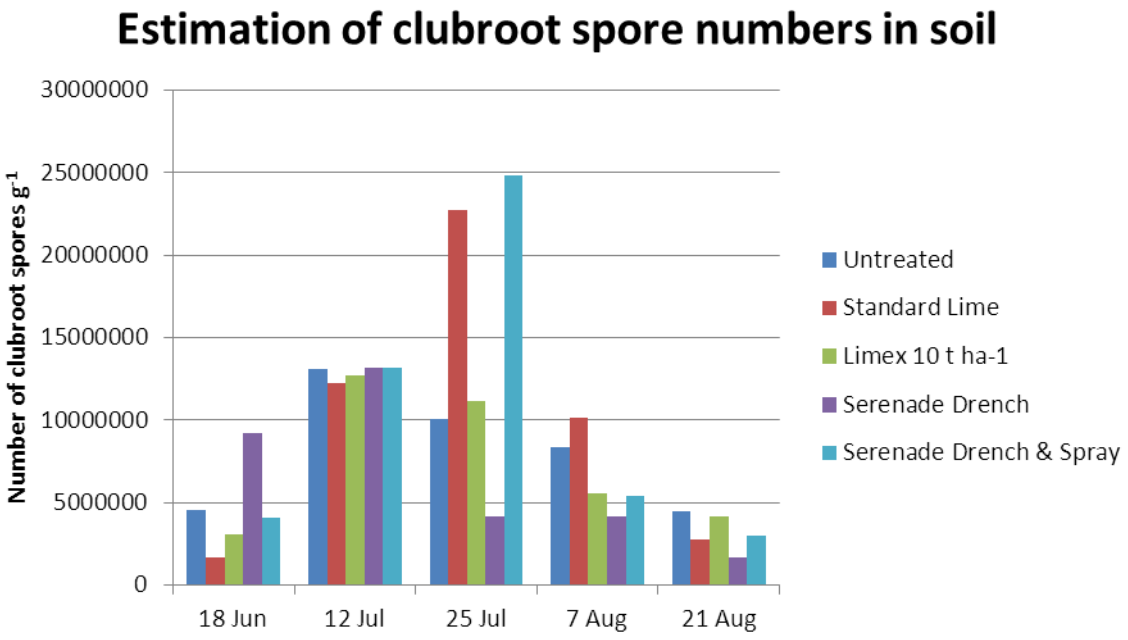



**Figure 7.** Clubroot resting spores per gram of soil in field trial at Hooton 2012

*Marketable Yield of Cauliflower at Hooton 2012*

Figure 8 shows the marketable yield of cauliflower in experimental plots at Hooton in 2012. There was no marketable yield of cauliflowers in treatments other than those given standard lime or Limex. The number of plants surviving at 18 September, as a percentage of the number of plants established by 7 August (25 days post planting) is shown in Figure 9. The presence of clubroot affected the survival of transplants used in each treatment.

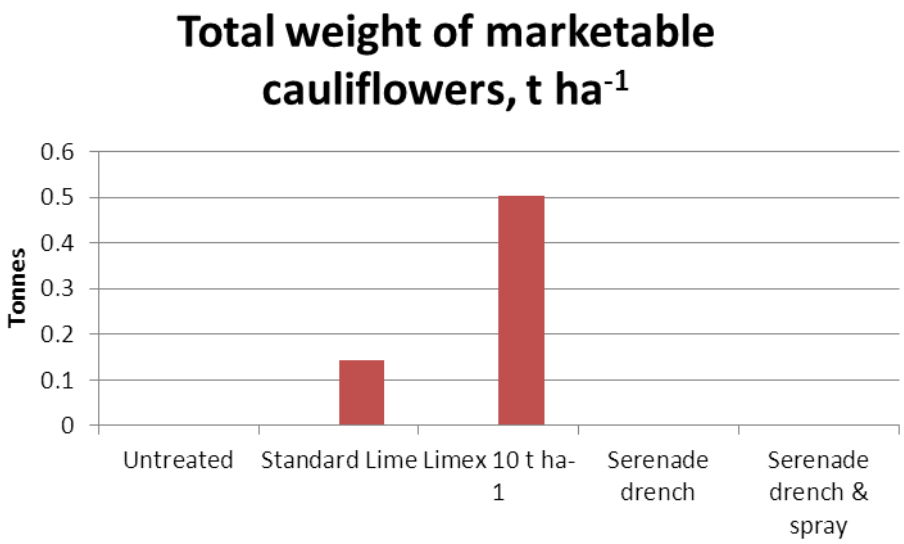



**Figure 8.** Marketable yield of Cauliflower in plots at Hooton 2012

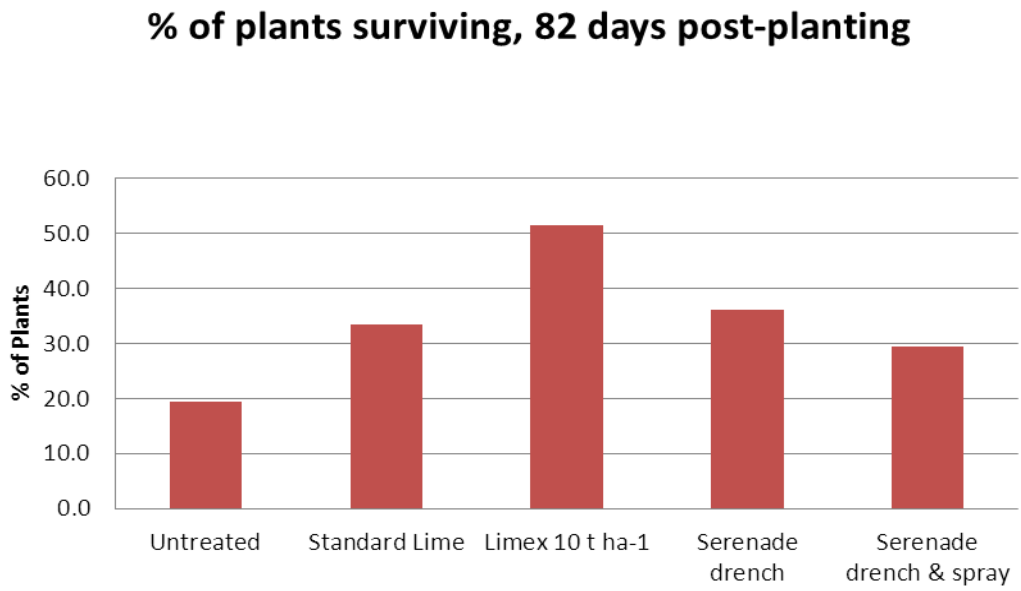



**Figure 9.** Percentage of plants surviving, 82 days post-planting at Hooton 2012

### *Discussion*

A high level of clubroot was observed at the start of the trial at Hooton, Lancashire. All plants were affected by clubroot and significant numbers died during the cropping season. The application of Serenade as a drench significantly reduced the incidence of clubbing when galls were assessed at seven weeks after transplanting. However only the Lime and Limex treatment provided some marketable plants.

The results confirm the findings reported for the clubroot trials conducted in 2009 and 2010 at Crail in Scotland. Of the treatments used, Limex proved most efficient in reducing the impact of clubroot disease on plants sown in heavily infested soils. In each of the trials (2009 – 20012) the application of Limex raised the pH of soils but to a level which would be acceptable for clubroot infection and development. This would indicate that other factors within the product application provide an inhibitive action towards disease progression.

**Milestone 3. Produce risk assessments for soil types based on optimised models and initial clubroot resting spore contamination in vegetable Brassicas and OSR.**

### *Background*

Previous work had investigated the effect of environmental factors on the number of *P. brassicae* spores in soil before and during cropping. From this it had been established that DNA levels indicate resting spore numbers. Fluctuation of levels during the growing period were most likely to be due to germination of *P. brassicae* zoospores from the existing resting spore population and the subsequent infection of the host brassica root tissue. Factors which showed significant relationship with the spore numbers had been investigated with linear regression, however here further work was performed by the collation of additional data and further examination of the relationships of the significant environmental factors with particular focus on soil pH, calcium and magnesium levels.

### *Method*

*Data collection and sites.* Previous data had been collected from two experimental sites, each of 0.58 Ha, as detailed in HDC CP45 Final Report (2010). One of these sites was Wellesbourne, Warwickshire (52°12'N 1°36'W) and the other Crail, Scotland (56°14'N 2°39'W). Both had a recent history of Brassica growing, and were known to contain significant levels of *P. brassicae.* Further data was collected from the site at Hooton, Wirral (2012) and was run in conjunction with the field experiment investigating clubroot control detailed in milestone 2. At all sites measurements of air temperature, humidity, leaf wetness, soil temperature and rainfall were collected at 30 minute intervals from when the logger was sited at the experimental area, using either an Aardware Smaartlogger or environmental monitoring station (Skye Instruments Ltd.). Measurements were downloaded by GSM portable phone Link (Aardware, Kingston upon Thames). The loggers were powered by 12 V batteries. Soil pH, moisture content, mineral nitrogen and cation exchange capacity were taken from three deep core soil samples collected across the sites. At the Wellesbourne site soil temperature and soil moisture were also measured every half an hour using a 10k thermistor temperature sensor and an SM200 theta probe (Delta-T Devices Ltd.)

*Quantification.* To quantify spore numbers *P. brassicae* DNA was extracted from soils using the MOBIO UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Inc.) following the manufacturers protocol. A Polyvinylpolypyrrolidone (PVP) DNA clean up was then performed (Klemsdal *et al* 2008). qPCR was performed using primer set PbML1F (GCC AAG TCC TAC GTC GAA TC) and PbML1R (GAA TAC AGC GAC ACG CAA GA). The master mix for this primer set was 10µl Lightcycler® 480 Sybr Green I Master (Roche Diagnostics), 2µl each primer (0.5µM final concentration), 4µl $ROH_20$ and 2µl DNA. The cycling parameters were pre-incubation at 95°C for 15 mins, followed by 55 cycles of 95°C for 10 s, 65°C for 20 s and 72°C for 20 s. Acquisition of the fluorescence measurement took place during the 72°C step. A subsequent melting curve was generated by 1 cycle of a hold at 95°C for 5s, 65°C for 1 min, and an increase to 97°C of 0.05°C/s, recording fluorescence 10 times/°C. Finally the samples were cooled to 40°C and held for 10 s. Amplification was performed in 384 well plates (Applied Biosystems).

*Statistical analysis*. Previous work (data not shown) had suggested pH, calcium and magnesium as among the significant factors to the number of spores in the soil. The relationships were examined in greater detail than previous work and a function of each factor determined, based on polynomial regression. Each factor could then be assessed in terms of the number of spores in the soil and from this it was possible to predict the number of spores that would be present in a soil with particular concentrations of the factors. By knowing the relationship between initial spore numbers and disease severity the risk of the soil to clubroot disease could then be determined.

## *Results*

*The relationship between spore numbers and soil pH*. A scatterplot of the spore numbers detected in soil across the pH range of all sites revealed an apparent unimodal distribution, which suggested polynomial regression would be the most appropriate form of regression to perform. Polynomial functions of the standard form were determined up to the 4th degree ($k = 4$).

*Standard form*

1) $$\hat{y}=b_0 + b_1X_i \ldots\ldots b_{k-1}X_i^{k-1} + b_kX_i^k$$

For the 2nd degree and 3rd degree polynomial functions the coefficients were significant (table 1), and had the following functions;

*2nd degree*

2) $f(x) = -125.5078 + 40.8183x - 3.1797x^2$

*3rd degree*

3) $f(x) = 609.6546 - 314.5526x + 53.7818x^2 - 3.0266x^3$

For the 2nd degree polynomial function Multiple R-squared: 0.4246, Adjusted R-squared: 0.4123, F-statistic: 34.68 on 2 and 94 DF, p-value: 5.236e-12. For the 3rd degree polynomial function Multiple R-squared: 0.5096, Adjusted R-squared: 0.4938, F-statistic: 32.21 on 3 and 93 DF, p-value: 2.282e-14. The increase in multiple $R^2$ value for the 3rd degree model suggests a higher level of model accuracy. This was tested by ANOVA between the two models and the p value below 0.05 suggests that there was a significant increase in accuracy in the 3rd degree model compared to the 2nd. As the 3rd degree model showed the best fit with the data the confidence and prediction intervals were determined for the predicted fit

*The relationship between spore number and exchangeable calcium.* A scatter plot of the data again suggested a unimodal distribution of spore numbers against exchangeable calcium within the soil, and so polynomial functions were tested as for soil pH. Only the 2nd degree model showed significance of the coefficients.

*2nd degree*

4) $f(x) = -12.15977 + 2.80718x - 0.10638x^2$

For the 2nd degree polynomial function Multiple R-squared: 0.4174, Adjusted R-squared: 0.405, F-statistic: 33.67 on 2 and 94 DF, p-value: 9.379e-12

*The relationship between spore number and exchangeable magnesium.* Magnesium did not follow such an easily determined distribution as Calcium and pH. Initial plotting of the points showed an increasing distribution. Polynomial regression was applied and both 1st and 4th degree functions showed significance of coefficients.

*1st degree*

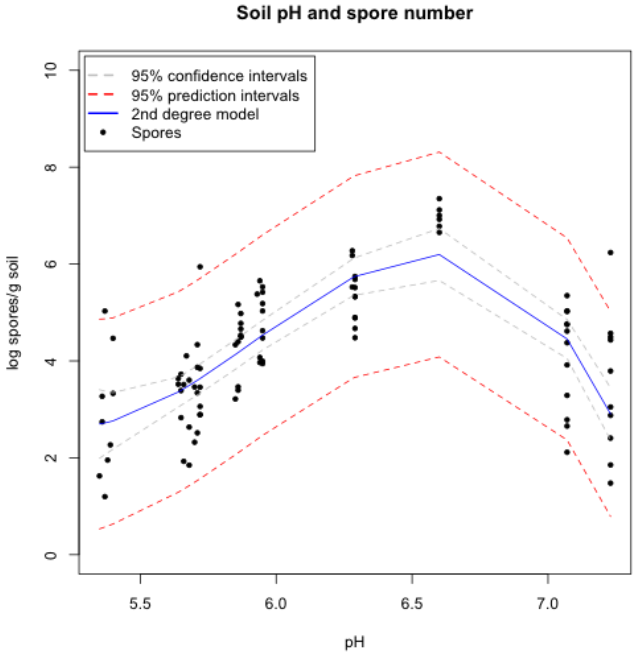
5) $f(x) = 2.5270 + 1.1123\ x$

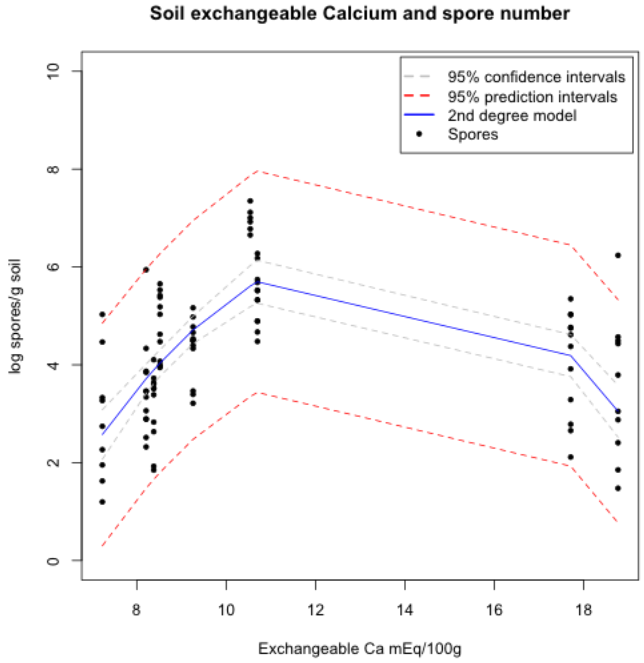
*4th degree*

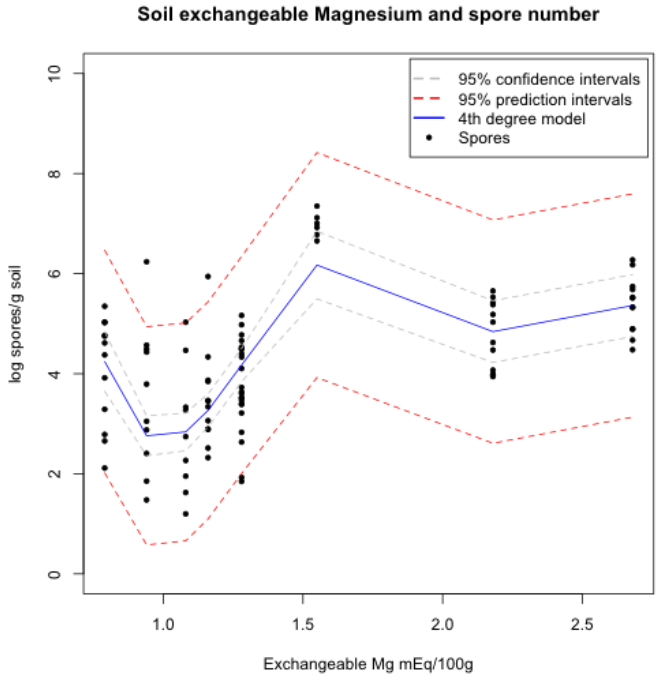
6) $f(x) = 81.471 - 221.430\ x + 219.104x^2 - 89.345\ x^3 + 12.860x^4$

For the 1st degree polynomial function multiple R-squared: 0.2187, adjusted R-squared: 0.2105, F-statistic: 26.59 on 1 and 95 DF, p-value: 1.368e-06. For the 4th degree polynomial function multiple R-squared: 0.4677, adjusted R-squared: 0.4445, F-statistic: 20.21 on 4 and 92 DF, p-value: 5.702e-12

The increase in multiple $R^2$ value for the 4th degree model suggests a higher level of model accuracy. This was tested by ANOVA between the two models and the p value below 0.05 suggests that there was a significant increase in accuracy in the 4th degree model compared to the 1st






*Relationship between spore levels and disease severity*. Much of the experimental data on the dose response of clubroot disease levels has been based on an exponential dose response curve. Collated information from published studies and previous experimentation

show that even at low spore levels it is possible to get a substantial level of disease, although data relating the level of disease to loss of yield was not assessed in this study.

Data from glasshouse and field trials using known inoculum density and disease severities were divided between into low, medium and high risk categories based on the number of spores present and the % disease severity observed (Tables 2 and 3) (low risk = $<1.0x10^3$ spores g-1, medium risk = $1.0x10^3$ and $9.9x10^4$ spores g-1 soil, and high risk = $>1x10^5$ spores g-1 soil). Only three out of the total 29 samples predicted a lower risk of disease than was observed (samples 19, 21 and 25). In 11 samples the disease severity observed was lower than the predicted risk based on these definitions.

**Table 2.** Assigned number of spores and estimated disease risk category

| Spore Number $g^{-1}$ soil | Estimated infection level and risk categorisation |
|---|---|
| $<1x10^3$ | LOW risk. Unlikely to get more than 33% infection. |
| $10^3$ and $10^4$ | MEDIUM risk. Likely to get between 33% and 66% infection. |
| $>1x10^5$ | HIGH risk. Likely to get >66% infection. |

**Table 3**. Data from this study and others regarding the number of spores and observe disease index

| Sample | Soil load | Approx. Disease Index (2dp) | Reference |
|---|---|---|---|
| 1 | $1x10^3$ | 0.00 | This study |
| 2 | $1x10^1$ | 0.80 | Cao *et al* 2007 |
| 3 | $1x10^2$ | 1.40 | Cao *et al* 2007 |
| 4 | $<1x10^3$ | 1.67 | This study |
| 5 | $5 x10^3$ | 5.40 | Webster and Dixon 1991 |
| 6 | $<1x10^3$ | 6.67 | This study |
| 7 | $1x10^3$ | 11.0 | Cao *et al* 2007 |
| 8 | $<1x10^3$ | 12.33 | This study |
| 9 | $1x10^4$ | 13.0 | This study |
| 10 | $<1x10^3$ | 13.67 | This study |
| 11 | $<1x10^3$ | 14.0 | This study |
| 12 | $3.58x10^4$ | 19.0 | This study |
| 13 | $1x10^4$ | 24.4 | Cao *et al* 2007 |
| 14 | $2.31x10^4$ | 28.67 | This study |
| 15 | $1x10^5$ | 33.0 | This study |

| | | | |
|---|---|---|---|
| 16 | $5.09x10^4$ | 34.67 | This study |
| 17 | $1x10^5$ | 41.8 | Cao *et al* 2007 |
| 18 | $2.15x10^5$ | 42.0 | This study |
| 19 | $<1x10^3$ | 44.33 | This study |
| 20 | $1x10^6$ | 53.0 | This study |
| 21 | $<1x10^3$ | 62.0 | This study |
| 22 | $5x10^7$ | 66.9 | Webster and Dixon 1991 |
| 23 | $5x10^5$ | 67.9 | Webster and Dixon 1991 |
| 24 | $1.02x10^5$ | 89.33 | This study |
| 25 | $5x10^3$ | 90.0 | Webster and Dixon 1991 |
| 26 | $5x10^5$ | 90.0 | Webster and Dixon 1991 |
| 27 | $5x10^7$ | 90.0 | Webster and Dixon 1991 |
| 28 | $1x10^7$ | 93.0 | This study |
| 29 | $4.59x10^6$ | 100.0 | This study |

It is worth noting that Webster and Dixon (1991) employed a different permutation of disease index calculation to the one used in this study, and also in Cao *et al* (2007), Hwang *et al* (2010), Tsushima *et al* (2010), and Murakami *et al* (2002). Voorips (1996) based disease index on percentage of infected plants and did not take into account disease severity.

When the dose response curves from previous studies were examined an approximation of spore numbers relating to 33%, 66% and 99% disease severity were obtained. Without precise data from the studies spore numbers were roughly assigned to the risk groups based on visual assessment of the curves and colour coded accordingly (Table 4). Here three samples underestimated the medium disease severity risk (samples 3, 6, 14) with spore numbers $<1x10^3$ spores $g^{-1}$ soil giving approximately 66% disease severity, while 7 underestimated the high disease severity risk (samples 1, 3, 4, 6, 14, 17, 18). Across all disease risk categories there was a degree of overestimation of risk as also observed for the previous table.

*Relationship between pH, calcium and magnesium and disease severity.* Using the predicted fit of the models it is possible to see that at pH 6.6 the model predicts that 95% of spore levels in soil is between 1.2 $x10^4$ and $2.1x10^8$ spores $g^{-1}$, and the mean number of spores is $1.6x10^6$ $g^{-1}$ (95% confidence interval). This means that the predicted spore levels would fall into medium and high risk categories. At pH 5.5 95% of spore levels in soil is between 7.44 $x10^0$ and $1.1x10^5$ spores $g^{-1}$, and the mean number of spores is $9.1x10^2$ $g^{-1}$ (95% confidence interval). This means that half of the predicted number of spores in the soil

is below the $1x10^3$ spores $g^{-1}$ threshold for medium risk. A similar situation is observed at higher pH, at pH 7.3 95% of spore levels in soil is between 0 and $1.6x10^4$ spores $g^{-1}$, and the mean number of spores is $1.1x10^2$ $g^{-1}$ (95% confidence interval).

Using the predicted fit of the models it is possible to see that 13mEq/100g calcium the model predicts that 95% of spore levels in soil is between $2.3 x10^4$ and $2.4x10^9$ spores $g^{-1}$, and the mean number of spores is $7.4x10^6$ $g^{-1}$ (95% confidence interval). This means that the predicted spore levels would fall into medium and high risk categories. Between calcium levels of 5 to 8 and 19 to 21 mEq/100g soil the predicted mean number of spores is below $1x10^3$ $g^{-1}$ and therefore low risk.

A risk assessment table was created to enable rapid determination of the approximated risk of disease based on the number of spores present at the different concentrations of nutrients (table 5). With the model response of spore numbers to magnesium concentration it is possible to see potentially less of an effect of magnesium as risk could be considered medium to high over the majority of magnesium concentrations responses were predicted for. Between 0.9 and 1.1mEq magnesium/100g soil the predicted number of spores falls into the low risk category. Due to the nature of the polynomial function, the low coefficient of determination ($R^2 = 0.2187$) and the restricted range over which a difference in potential risk can be seen it is likely that the relationship of magnesium to spore numbers would require greater investigation over a larger range of magnesium concentrations.

**Table 5.** Predicted risk assessment based on the predicted number of spores at each concentration of nutrient.

| pH | Lower prediction limit (95%) | Mean prediction limit | Upper prediction limit (95%) | Ca mEq /100g | Lower prediction limit (95%) | Mean prediction limit | Upper prediction limit (95%) | Mg mEq /100g | Lower prediction limit (95%) | Mean prediction limit | Upper prediction limit (95%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 5 | | | | 0 | | | | 0.5 | | | |
| 5.1 | | | | 1 | | | | 0.6 | | | |
| 5.2 | | | | 2 | | | | 0.7 | | | |
| 5.3 | | | | 3 | | | | 0.8 | | | |
| 5.4 | | | | 4 | | | | 0.9 | | | |
| 5.5 | | | | 5 | | | | 1 | | | |
| 5.6 | | | | 6 | | | | 1.1 | | | |
| 5.7 | | | | 7 | | | | 1.2 | | | |
| 5.8 | | | | 8 | | | | 1.3 | | | |
| 5.9 | | | | 9 | | | | 1.4 | | | |
| 6 | | | | 10 | | | | 1.5 | | | |
| 6.1 | | | | 11 | | | | 1.6 | | | |
| 6.2 | | | | 12 | | | | 1.7 | | | |
| 6.3 | | | | 13 | | | | 1.8 | | | |
| 6.4 | | | | 14 | | | | 1.9 | | | |
| 6.5 | | | | 15 | | | | 2 | | | |
| 6.6 | | | | 16 | | | | 2.1 | | | |
| 6.7 | | | | 17 | | | | 2.2 | | | |
| 6.8 | | | | 18 | | | | 2.3 | | | |
| 6.9 | | | | 19 | | | | 2.4 | | | |
| 7 | | | | 20 | | | | 2.5 | | | |
| 7.1 | | | | 21 | | | | 2.6 | | | |
| 7.2 | | | | 22 | | | | 2.7 | | | |
| 7.3 | | | | 23 | | | | 2.8 | | | |
| 7.4 | | | | 24 | | | | 2.9 | | | |
| 7.5 | | | | 25 | | | | 3 | | | |

## Discussion

The effectiveness of the model to generate data that could be applied to a risk matrix was tested based on three factors, which vary across soil types. While the amount data available to relate number of spores to disease indices and (in particular) yield loss was limited, what data was available fell into a discernible pattern from which was possible to approximate risk based on assumed spore numbers. The concept of using models to predict the likely range of spores which can be present in a particular soil type is useful as it can allow a quick assessment of whether a soil is likely to carry a high clubroot resting spore load (such as a soil within pH 6 with calcium within the range of 16mEq/100g soil) or a low load (eg. pH 5.3 with <7mEq/100g calcium). Obviously the relationship between calcium and pH will prove to be important in this relationship and it may be that further refinement would allow only one of these factors to be considered. It is to be expected that this very preliminary risk assessment data shown here will be able to be greatly refined over time as more and more data becomes available, particularly now that a degree of routine molecular quantification is planned within the UK brassica industry, as long as disease index data and/or yield data is also available and can be coupled with at least a basic knowledge of the soil parameters. This type of assessment of risk has the potential to also be applied to other outputs of N_ABLE (Greenwood and Draycott, 1989a, b) along with weather predictions. At the

present time the predicted values from the polynomial models encompasses spore level fluctuations across the growing season – it has been observed that DNA levels fluctuate within the soil during plant growth, likely to be due to the movement of zoospores into the root tissue and the subsequent release of secondary zoospores and then the release of resting spores back into the soil – however in time it may be possible to use spore numbers quantified prior to planting along with data regarding the soil type and nutrient status to provide a more accurate risk matrix as a useful tool to brassica growers.

## Conclusions

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 (q PCR) 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 q PCR 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 q PCR 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 q PCR 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 q PCR 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.

To establish the lateral flow test as a robust assessment of clubroot resting spores in soil, it is important that the technology is capable of detecting different pathotypes or races of the clubroot disease pathogen, *Plasmodiophora brassicae*. This study had previously been carried out for the molecular test. The existence of different pathotypes is well documented. Pathotypes are conventionally based on the ability to infect and produce disease in a range of Brassica cultivars which have varying degrees of susceptibility (Buczacki et al. 1975, Toxopeus et al. 1986). In Year 4 of the project, studies were carried out to establish whether the lateral flow device was capable of detecting different typed isolates of *P. brassicae*. Six race types were supplied for testing from a commercial company. Three of which were capable of overcoming clubroot disease. Some variability in the sensitivity of the test for the different races was observed over the disease concentration gradient tested. However on analysis this was not deemed as significant. Nevertheless the variation observed may cause issue in providing truly quantitative results, and the relationship of the biological

systems involved i.e. soil and meteorological parameters, may also prove influential. Nevertheless, the results of this pathotype study supports the potential of the lateral flow test to provide a semi-quantitative estimation of clubroot resting spores in UK commercial soils.

The development of the two test systems (laboratory test: *molecular q PCR* 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 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. However in the development of the clubroot disease forecast model, the inclusion of pH, Calcium and Magnesium are considered significant due to their relationship in development of the disease to gall formation. The effectiveness of the model to generate data that could be applied to a soil risk matrix was therefore based heavily on these three factors. Each of which will vary across soil types. In addition, resting spore concentrations vary across the season. This is likely to be due to the movement of zoospores into the root tissue and the subsequent release of secondary zoospores and then the release of resting spores back into the soil. As such, the development of the risk model focuses on the three main environmental parameters and the initial clubroot resting spore concentration prior to planting. Over time this may be refined as disease index data and/or yield data is made available and can be coupled with at least a basic knowledge of the soil parameters. This type of assessment of risk has the potential to also be applied to other outputs of N_ABLE (Greenwood and Draycott, 1989a, b) along with weather predictions. 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

## Knowledge and Technology Transfer

### *Publications*

LEWIS, M., WAKEHAM, A.J. & KENNEDY, R. (2012) The Effect of Micronutrients on the Germination and Infection of *Plasmodiophora brassicae*. *In* 6th International Symposium on Brassica and 18th Crucifer Genetics Workshop' in Catania, Italy. 12-16th Novemeber 2012 (*in press*)

LEWIS, M., WAKEHAM, A.J. & KENNEDY, R. (2012) A Molecular Based Seedling Assay System to Study the Germination and Primary Infection by *Plasmodiophora brassicae. In* 6th International Symposium on Brassica and 18th Crucifer Genetics Workshop' in Catania, Italy. 12-16th Novemeber 2012 (*in press*)

WAKEHAM, A.J., KEANE, G., PROCTOR, M. & KENNEDY, R. (2012). Monitoring infection risk for air and soil borne fungal plant pathogens using antibody and DNA techniques and mathematical models describing environmental parameters. Microbes in Applied Research: Current Advances and Challenges p 152-156. Editor: A. Mendez-Vilas, World Scientific Publishing Co., Ltd ISBN: 978-981-4405-03-4.

### *Seminars*

WAKEHAM, A. J. (2012). Monitoring infection risk of soilborne plant pathogens. West Midlands Fresh Produce Forum at Pershore College on 7th November, 2012

KENNEDY, R. (2012). Detection, forecasting and control of vegetable diseases. West Midlands Fresh Produce Forum at Pershore College on 7th November, 2012.

WAKEHAM, A.J. (2012). Development of 'in field' tests for resting spores of clubroot and the clubroot control based on detection. Syngenta : Brassica Marketing and Development Meeting on 10-11th July, Enkhuizen, The Netherlands

KENNEDY,R. (2012). Brasica Disease Forecasting. HDC Brassica Technical Seminar, on 3rd July, 2012.

KENNEDY, R. (2012). Detection and control of clubroot and other diseases. Alphagrow Growers Evening Meeting, Lancashire on 14th February, 2012

KENNEDY, R. (2012). Vegetable, Salad & Herb Growers Technical Update meeting, 7th February 2012, Pershore College. UK.

WAKEHAM, A.J. (2012). Detection and control of airborne / soil borne diseases of Brassicas. Getting to the Heart of Horticulture 'Opportunities and challenges for the horticulture and potato sectors in the West Midlands' at The Civic Centre, Pershore on 13th January, 2012.

WAKEHAM, A.J. (2012). RD- 2008-3525 Brassicas: Further development of "in field" tests for resting spores of clubroot and the development of clubroot control based on detection. HGCA Oilseed Rape Disease Monitoring meeting at Broom's Barn Research Centre on 11th January 2012.

WAKEHAM, A.J. & R. KENNEDY (2009). Determining risk of clubroot in UK soils. Brassica Association Growers Meeting, Lancashire.

### *Demonstrations*

G. KEANE, 2011. Demonstration of lateral flows and reader for quantitative measurement of clubroot spore infestation in soil samples. East of Scotland Growers, July 26th.
G. KEANE, 2012. Demonstration and assessment of commercial soils for clubroot spore infestation, East of Scotland Growers, February

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