Project title:	Brassicas: further development of “in field” tests for resting spores of clubroot and the development of clubroot control based on detection.
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Signed on behalf of: **NPARU, University of Worcester**

Signature: …… Date: ……

Name: Professor Roy Kennedy
Director of NPARU

The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

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Grower Summary

Headline

Limex applied at 7.5 – 10 tonnes Limex/ha controlled clubroot in broccoli crops planted on heavily infected land and resulted in higher levels of marketable yield.

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 the swelling of the root tissue causing galls and club shaped structures. Clubroot resting spores are capable of inducing disease in vegetable Brassica crops years after initial infestation.

Once the soil has been contaminated, clubroot spores remain viable for up to 18 years. In the past, information on whether clubroot is present or absent in the soil has been difficult to obtain because the 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 were 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 and require a high degree of precision by the operator.

However, a competitive lateral flow device for rapid testing and detection of clubroot resting spores in the field has been developed. The device was clearly able to detect clubroot spores at close to epidemiological significant levels (10,000 spores/gram of soil). The device can be optimised for use in soil (for field growers) and in water based systems such as reservoirs and irrigation lines (for vegetable Brassica propagators). The accuracy of the test device can be increased if used in conjunction with a lateral flow reader device. This means that it could detect clubroot at very low levels.

Determining the number of clubroot resting spores in a soil test (either molecular or lateral flow based detection) would be an essential component if control regimes for clubroot were to be successfully developed. Cyazofamid (Ranman) and fluazinam (Shirlan) which are approved for disease control in potato crops have been demonstrated to have some potential for controlling clubroot in the field. However, neither are currently approved for clubroot control in vegetable Brassicas as their efficacy has not yet been demonstrated. Alternative control measures are still urgently needed.

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 the project and main conclusions

Year One

Detection Tests

A competitive lateral flow has been reconstituted which can determine resting spore numbers in the liquid phase. Visual discrimination is limited to determining resting spore presence when in excess of 10,000 resting spores/ml. Quantitative measurement of resting spore numbers is possible using an electronic reader, in conjunction with a standard curve. However, the current test is limited by the instability of the test line antigen. This means that the test results could change depending on when the tests were used. Studies currently in progress are looking to resolve this problem. The development of an alternative protocol for the test which incorporates a *P. brassicae* polyclonal antibody (PAb) at the test line has enabled a detection sensitivity comparable to that observed with the competitive lateral flow. However, in the soil, this approach has yet to prove effective. An innovative procedure to extract resting spores directly from soil is currently being investigated and incorporated within the lateral flow system to allow soil analysis to be carried out in the field.

Field Trials for Clubroot Control

The results of field trials at Crail (East of Scotland Growers) in 2009 demonstrated that Limex can be used to control clubroot in heavily infested land while maintaining marketable yields of broccoli crops. This was because the plots treated with Limex reduced the clubroot gall formation compared with the untreated plots. The optimum application rate was observed between 7.5 – 10 tonnes of Limex/ha (Figure 1) although this would need to be confirmed in additional trials located in other production areas.

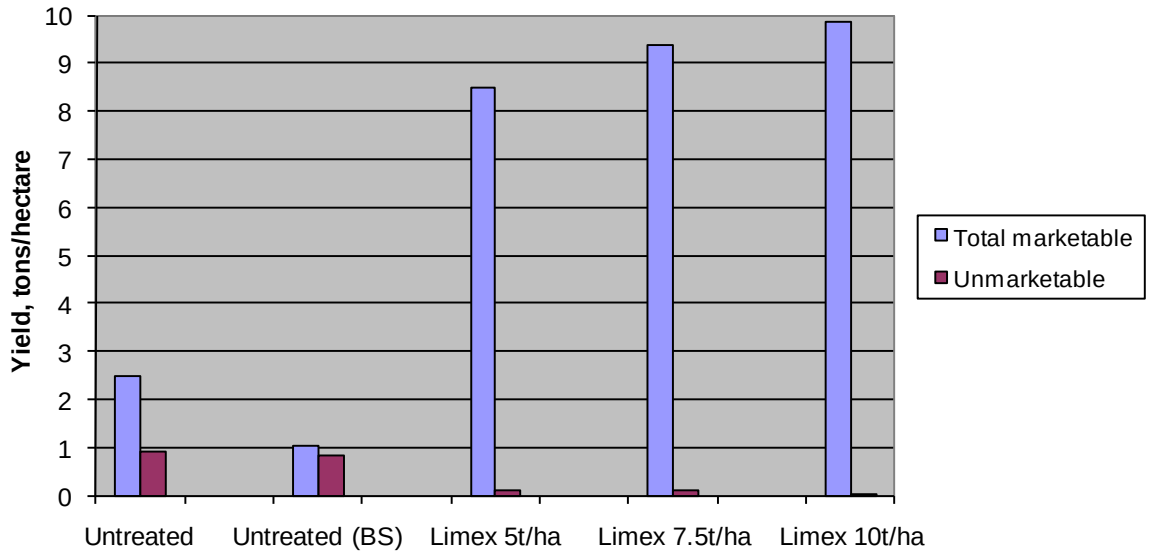


Figure 1: Marketable yield of broccoli grown in heavily clubroot infested soil at Crail 2009 treated with Limex

However, there was no effect of Limex on clubroot resting spore content of plots in comparison to untreated plots. Clubroot numbers in all plots decreased after transplanting before increasing towards the end of the growing season at harvest. The results suggest that the clubroot could possibly be migrating between plots. Limex treated plots had higher amounts of clubroot present during November 2009 (end of the trial period) in comparison to untreated control plots. Overall, the clubroot content in the plots increased over time from the time of planting to the time of harvest in October/November 2009.

Anticipated practical and financial benefit

- The use of the detection tests for risk assessment for clubroot will improve the control of this pathogen.
- More information will be available on the appropriate timing and rate of Limex for clubroot control with different planting dates and in different soils in the next year of the project.

Action points for growers

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

- Growers can use laboratory molecular tests for clubroot to determine the initial risk from the pathogen in fields until the “on site” test is available.
- Limex can be used to control clubroot in affected land. However, it will not reduce clubroot risk in subsequent seasons.
- The optimum level of Limex required for clubroot control appears to be 7.5 – 10 tonnes of Limex/ha.

Science Section One (Field Trials)

BACKGROUND

Clubroot caused by the fungus *Plasmodiophora brassicae* Woronin is one of the most important plant pathogens of cultivated cruciferous crops world-wide. Traditional control measures include improving drainage, liming to raise soil pH and rotation of susceptible crops with non-cruciferous crops. Other treatments tested include partial soil sterilisation, pre-planting incorporation of fungicides into soil and fungicide treatment of transplant roots or plant-raising modules.

Mild clubroot infections lead to slowed growth, lack of uniformity in crops, delayed harvesting and yield loss. Severe infections result in total crop failure. Once soil has been contaminated 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 could not detect low levels of clubroot in soils. These methods were based on using the reaction of bait plants however large numbers of plants were 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.

MATERIALS & METHODS

Soil Nutrient Measurements and physical parameters taken at Crail

The following measurements were taken at the site over the course of the trial:

1) pH of sampled soil
2) Moisture content (taken by drying samples)
3) Exchangeable calcium
4) Exchangeable magnesium
5) Chloride and sulphate content

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, 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

Initial dry weight in t/ha whole plant except fibrous roots was taken at planting. The fertilizer and crop protection inputs for the crop were as for commercially produced crops in adjacent fields.

Clubroot Trial Design

Two clubroot areas (Block W and Y) were used in at the Crail trial site. In initial tests these areas had different levels of clubroot resting spore contamination.

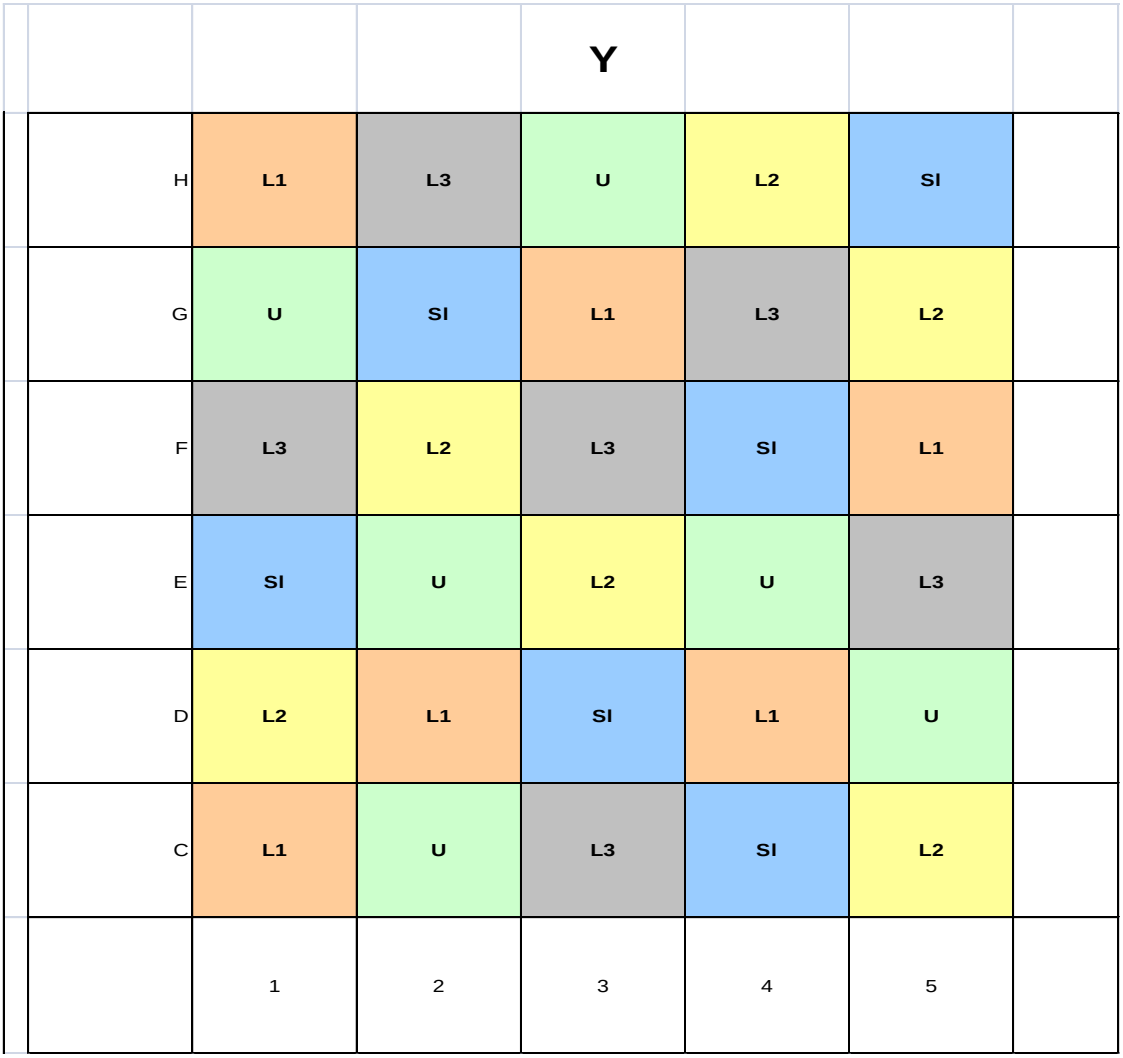
Area W

			W			
H	L1	U	L3	SI	U	
G	L2	L3	SI	L1	L3	
F	SI	L1	L2	U	L1	
E	L3	L2	L1	L3	L2	
D	U	SI	U	L2	SI	
C	L2	L3	SI	U	L1	
	1	2	3	4	5	

Treatments (Block W)
L1 = Limex 7.5 tons/ha
L2 = Limex 10 tons/ha
L3 = Limex 12.5 tons/ha

Figure 2: Randomisation of Limex treatments in Block W at Crail

Area Y



Treatments (Block Y)
L1 = Limex 5 tons/ha
L2 = Limex 7.5 tons/ha
L3 = Limex 10 tons/ha

Figure 3: Randomisation of Limex treatments in Block Y at Crail

Field Plantings and treatment application methods

Field planting took place on 4 June 2009 and each plot of 10m^2 comprised of five beds of 10 metre length, with three rows per bed, and plants spaced at 44cm. Limex treatments were applied by hand on 16 June 2009 and after mechanical incorporation commercially raised broccoli cultivar Parthanon was planted on 25 June 2009.

Clubroot Gall assessment

The numbers of clubroot galls were assessed at two 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 manufacturers 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

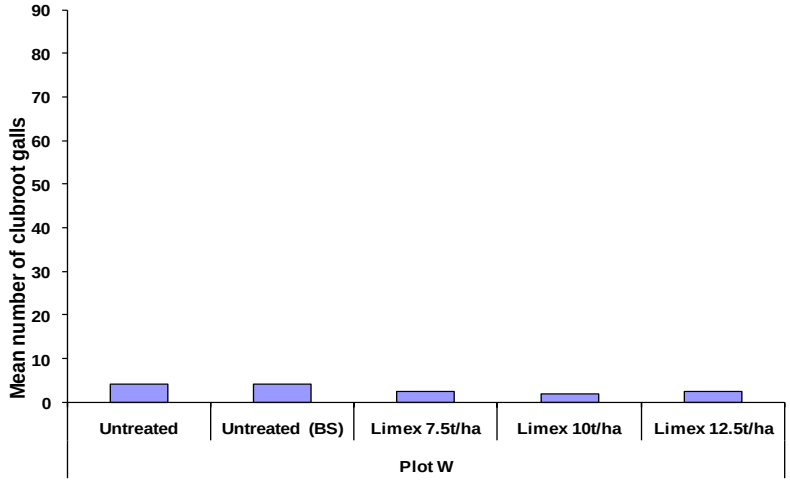
A 5µl volume of isolated DNA from a soil sample extract was mixed with 15µl of PCR Master Mix (11.35µl H_2O, 2.5µ PCR buffer 10x, 0.75µl 50mg (1.5mM) Mg, 0.2µl DNTP, 0.2µL DNase) and 30ng of *P. brassicae* specific primers (Faggian et al., 1999). A negative control of molecular grade water + PCR Master 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

Numbers of clubroot galls recorded in Limex treated plots

The results in Figure 4 show the number of clubroot galls on plants 8 weeks after transplanting. Galls were counted and means calculated from the results per plant in each treatment. There were very few differences in the numbers of clubroot galls in each treatment. Less than 10 clubroot galls per treatment were observed on plants. Lower numbers of clubroot galls were observed on Plot Y (Figure 4B) in comparison to Plot W (Figure 4A) regardless of Limex treatment. The numbers of visible clubroot galls in plots at Crail 11 weeks after transplanting is shown in Figure 5. Numbers of visible galls increased to approximately 90 in untreated plots. However plots treated with Limex showed reduced numbers of galls present on roots.

A)



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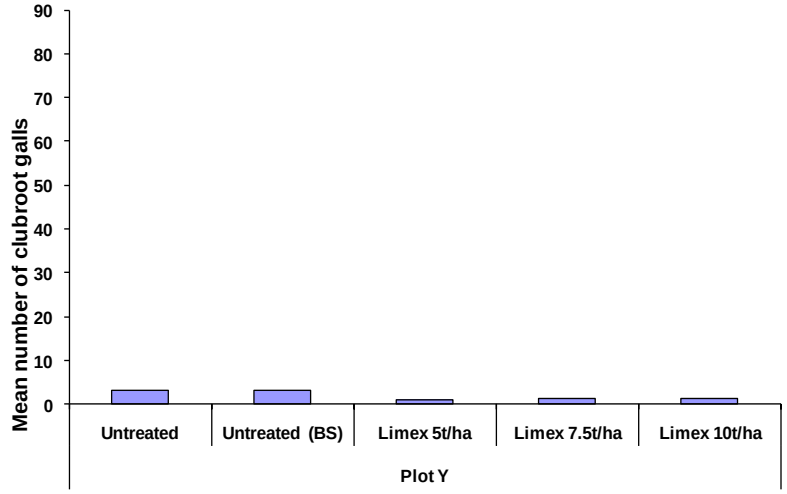

Figure 4: Mean number of clubroot galls per treatment 8 weeks after transplanting in A) W plots and B) Y plots at Crail 2009

The results show that there was lower clubroot gall formation in treatments where Limex had been applied regardless of application rate 8 weeks after transplanting in comparison to untreated control areas. Untreated areas had fewer than ten galls per treatment with Limex treated areas having approximately half this number.

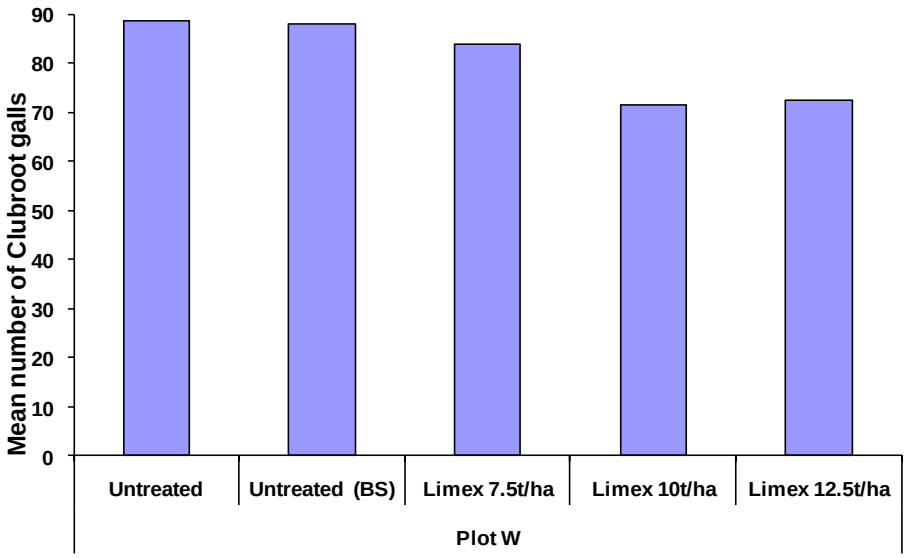

Figure 5: Mean number of clubroot galls per treatment 11 weeks after transplanting in W plots only at Crail 2009

However eleven weeks after transplanting (Figure 5) the number of clubroot galls in untreated plots had risen to approximately 90. Lower numbers were observed in Limex treated plots especially those treated with 10 and 12.5 tons ha^{-1} of Limex. Lower numbers of clubroot galls were recorded in Y plots but with the same overall pattern (data not presented).

Estimation of pH and calcium levels in Limex treated W plots at Crail 2009

Analysis was carried out on W treated plots only. These plots had the highest levels of Limex applied indicating that trends in W treated plots would also apply to those plots in Y treated areas. The effect Limex on pH of W plots at Crail is shown in Figure 6. The results indicated that Limex has an impact on pH of the soil over longer periods of time and certainly over the life of the crop. The pH in untreated areas was consistently lower than that observed in areas treated with Limex regardless of the rate applied. The calcium concentrations found in plots within the W area are shown in Figure 7.

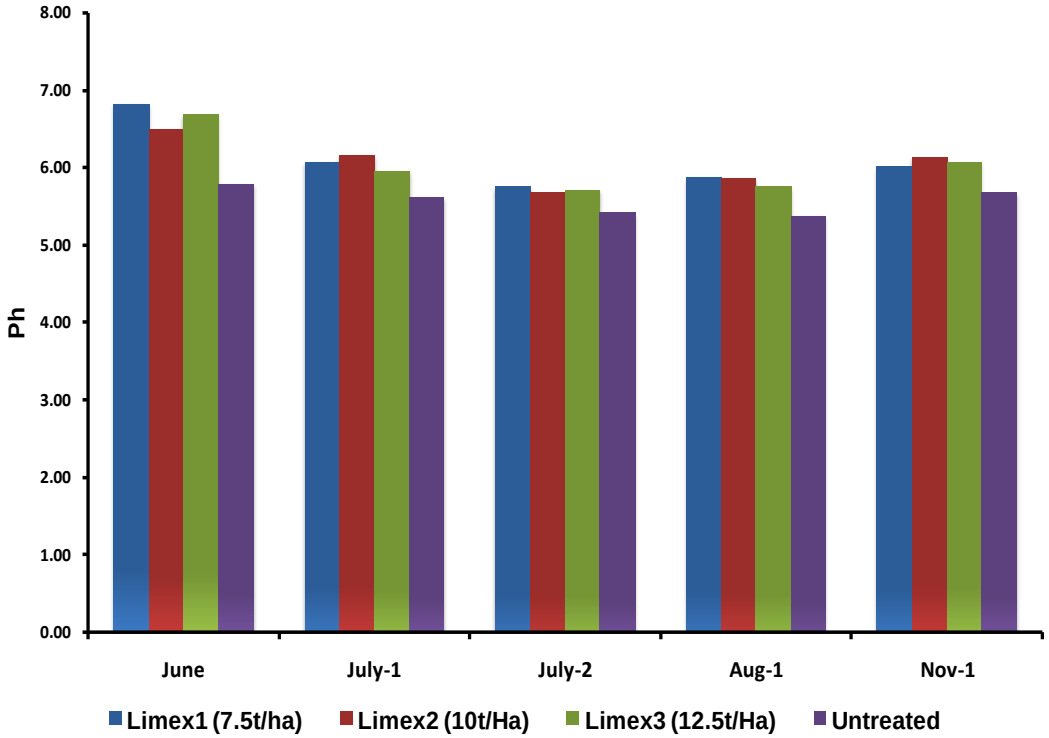

Figure 6: pH values in W plots at Crail 2009 after application in June

All Limex rates increased the calcium concentrations in soils over the duration of the trial regardless of rate applied. Untreated plots had consistently lower levels of calcium in the soil.

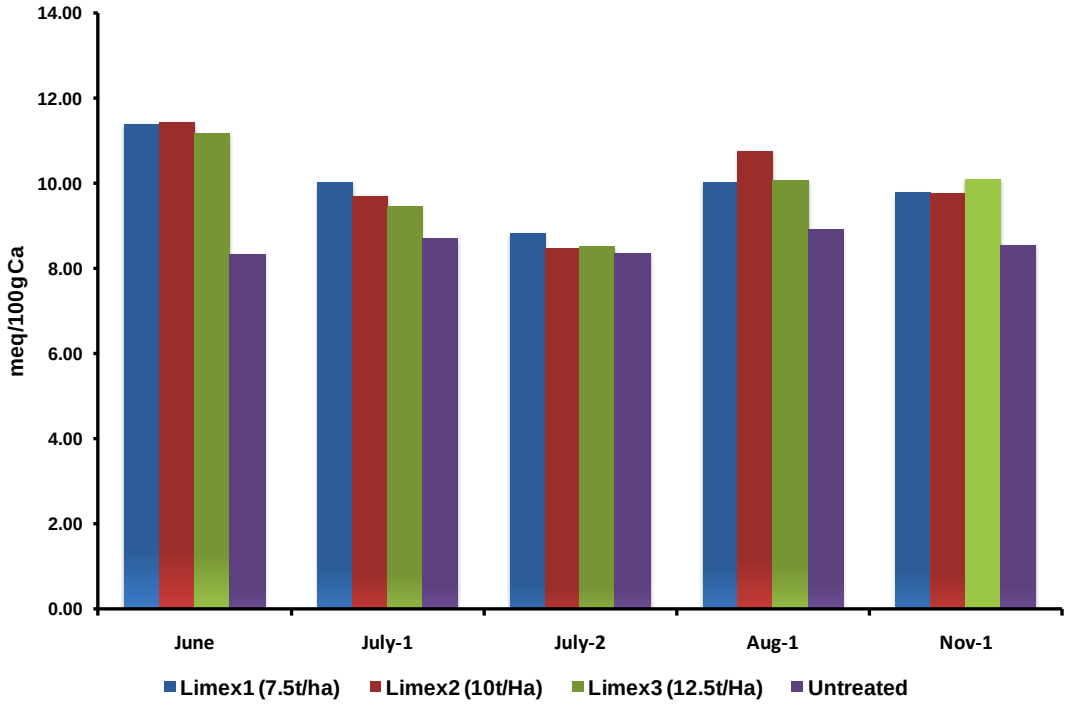

Figure 7: Calcium concentration (meq 100 g Ca^{-1}) values in W plots at Crail 2009 after application in June

Estimation of clubroot concentration in Limex treated W and Y plots at Crail 2009

Levels of clubroot detected within field plots during the trial period are shown in Figure 8 (W plots) and 9 (Y Plots). Results were expressed in relation to clubroot resting spore number. There was no effect of Limex on clubroot content of plots treated with Limex in comparison to untreated plots. Clubroot numbers in plots decreased after transplanting but increased towards the end of the growing season at harvest. The results suggest that there is the possibility of migration of clubroot between plots. Limex treated plots had higher amounts of clubroot present during November 2009 in comparison to untreated control plots. Limex treated W plots had higher numbers of plants and root material present during November 2009 in comparison to untreated plots. Overall clubroot content in plots increased from that observed at time of planting until harvest during October/November 2009.

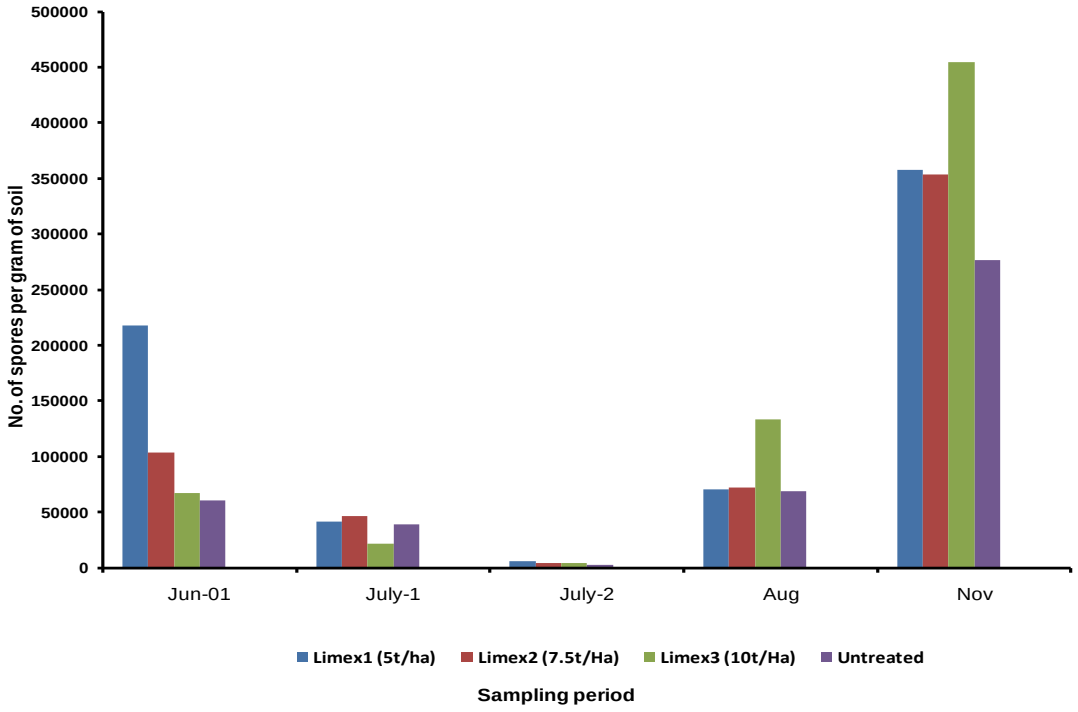

Figure 8: Clubroot concentration in W plots at Crail 2009

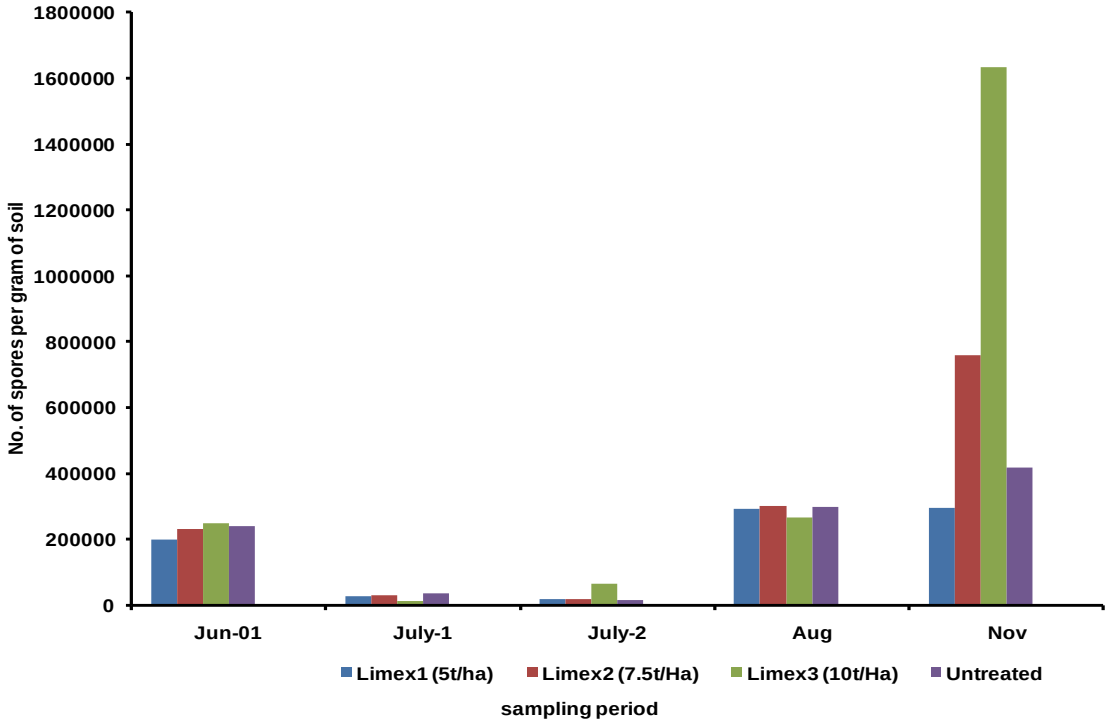

Figure 9: Clubroot concentration in Y plots at Crail 2009

Higher amounts of clubroot were recorded in Y plots (Figure 9) in comparison to W plots. There was no effect of Limex on clubroot content (lower amounts of Limex had been applied to these plots). Clubroot content within plots was significantly lower after planting (July) in comparison to other sampling times. Plots treated with Limex at 7.5 and 10 tons ha^{-1} had higher amounts of detectable clubroot during November 2009 than untreated plots.

Marketable Yield of Broccoli in W and Y plots at Crail 2009

The marketable yield of Broccoli in Y plots is shown in Figure 10 at Crail in 2009. All Limex treated plots gave high marketable yields (approximately 9 tons ha^{-1}) of Broccoli regardless of application rate. Untreated plots yielded only 2 tons ha^{-1}. Similar results were obtained in harvests from W plots (Figure 11). However the marketable yield of Broccoli was lower in these plots (approximately 5 – 7 tons ha^{-1}). Untreated plots in the W area had lower marketable yields compared to equivalent plots in Y areas.

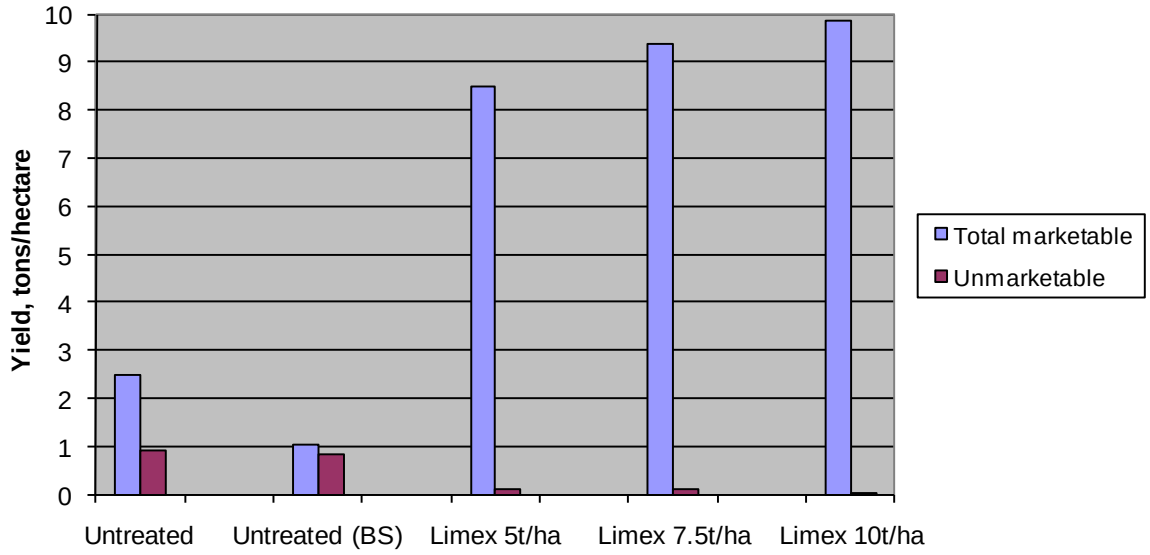

Figure 10: Clubroot concentration in Y plots at Crail 2009

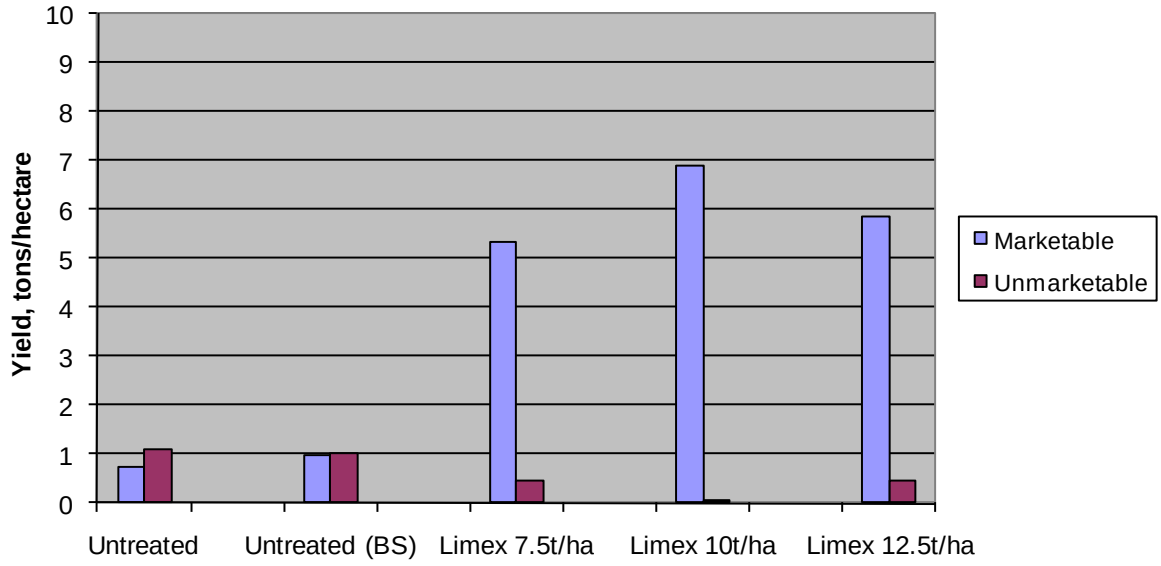

Figure 11: Clubroot concentration in W plots at Crail 2009

Conclusions

The results demonstrate that Limex can be used to control clubroot in heavily infested land while maintaining marketable yields of Broccoli crops. The optimal application rate was observed between 7.5 – 10 tons Limex ha^{-1} although this would need to be confirmed in additional trials located in other production areas. The results show that the highest levels of clubroot were recorded in plots which had the highest yields. This suggests that the degree of

root growth determines the amount of clubroot present in the plot at the end of the growing season. An alternative explanation may be that there is also a large amount of cross contamination between plots resulting in higher activity in plots supporting higher root growth. The cross contamination in the trial at Crail would have been lower because larger plots were used however smaller plot sizes would have been more prone to this effect. Treatment with Limex did not control the amount of clubroot in the plot compared to untreated controls. Limex appears to raise the pH and calcium levels in treated plots although the effect diminished with time. However this initial effect would appear to have been sufficient to result in high marketable yields.

Science Section Two (Laboratory clubroot Test Development)

BACKGROUND

Existing lateral flow technology (LFD's) for the detection of resting spores of clubroot (FV259) have been produced but require adaptation. A range of new components for the test will be required. The stability of the lateral flow test line will also need to be improved. Lateral flow test line variations will need to be developed and tested over the first year of the project and under different storage conditions to improve the storage time of tests. Tests will be required to determine test sensitivity and specificity of lateral flow format variation in relation to soil types where the output from the lateral flow test is different from that obtained using the qPCR clubroot molecular test.

Production of new *Plasmodiophora brassicae* antibody conjugates

Method

A clubroot lateral flow test, based on the protocol developed in HDC project FV259, was constructed. This time however a 5 x 5 mm sample filtration pad (www.whatman.co.uk) was inserted between the conjugate and membrane to inhibit blocking by soil particles (Figure 12.). A control line of anti-mouse serum (0.5mg ml^{-1}) and a test line of a *Plasmodiophora brassicae* soluble antigen were applied to the membrane as described in HDC project FV259. Four batches of 50 conjugate pads each were prepared using Monoclonal antiserum EMA 3A5 (Batch 4) at a dilution of 1 in 200 in gold Warwick HRI conjugate buffer. To each 5 x 5 mm conjugate pad a 27 µl aliquot of the antibody bound gold buffer was applied. Following air drying each pad was incorporated in to a lateral flow device. Ten fold concentrations, ranging from $1x10^8$ to $1x10^2$ resting spores of *P. brassicae* in lateral flow

sample buffer (Kennedy & Wakeham, 2008), were applied in 100µl aliquots to sample pads of seven constructed lateral flows. After ten minutes optical density readings (OD) were made using a Quadscan lateral flow reader (www.biodot.com). A control of extraction buffer alone was added to an additional lateral flow at 100 µl volume. This process was repeated for each of the prepared conjugate batches.

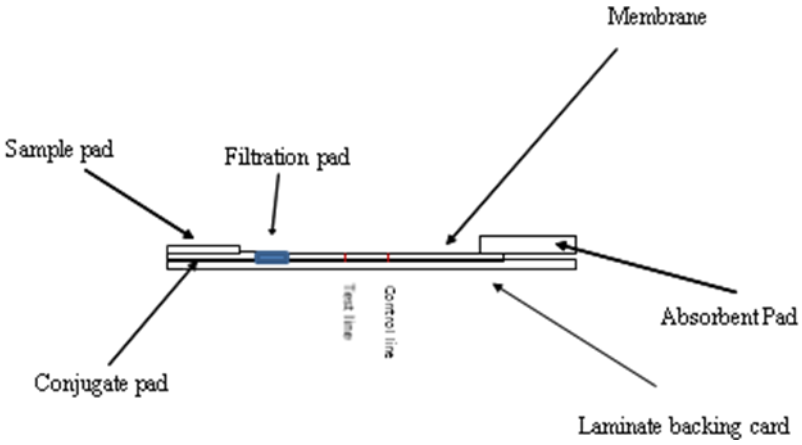

Figure 12: Lateral flow device for semi-quantitation of clubroot resting spores

Results

A linear correlation of 0.9878 was recorded between the *P. brassicae* resting spore numbers applied and the corresponding competitive lateral flow competitive lateral flow device (*clfd*) OD value generated (Figure 13). Visual observation of each of the *clfd* conjugate batches tested, recorded test line depletion of the *clfd* to occur in the region of $1x10^5$ resting spores ml^{-1}.

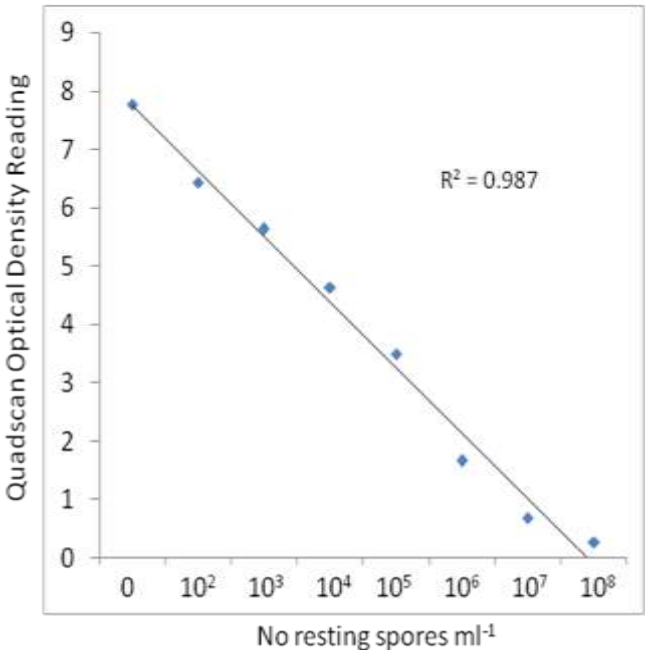

Figure 13: Lateral flow device for semi-quantitation of clubroot resting spores in liquid phase

Conclusion

Conjugate pads prepared as determined in HDC project FV259, proved optimal for semi-quantitative detection of *P. brassicae* in liquid phase, using an electronic reader. Variability between the prepared batches was minimal and, the addition of a pre-filtration step in the lateral flow device did not affect test sensitivity. Monoclonal antiserum EMA 3A5, stored in 100µl lots at -20°C, retained an activity observed in previous studies (FV259).

Investigation of LFD test line stability

Method

A 10ml filtered 1x10^8 *P. brassicae* resting spore suspension in chilled phosphate buffered saline ((PBS) 0.01M phosphate buffer, 0.0027M potassium chloride, 0.137M sodium chloride, pH 7.4), was processed with Ballotini glass beads in a Fast Prep 120 machine (www.qbiogene.com), at a speed setting of 5 for 30 seconds. The sample was held on ice for 5 min. and the process was repeated twice. The liquid phase was microfuged at 5000 rpm for 5 min. and the pellet discarded. The retained liquid phase was adjusted to 10mg ml^{-1}

in PBS and a series of lateral flow test line samples were prepared (Table 1). Each test line solution contained 1mg ml^{-1} of *P. brassicae* soluble resting spore fraction.

Table 1 Test line solution types for lateral flow application

Test line
P. brassicae soluble antigen in 0.0025M PBS
P. brassicae soluble antigen in 0.0025M PBS, 1mg ml^{-1} Poly-L-Lysine
1mg ml^{-1} Poly-L-Lysine in 0.025M PBS overlaid with *P. brassicae* soluble antigen in 0.0025M PBS
P. brassicae soluble antigen in 0.0025M PBS, 0.5% Bovine Serum Albumin
P. brassicae soluble antigen in 0.0025M PBS, 0.5% Casein

A control line of an anti-mouse serum at 0.5 mg ml^{-2} (www.sigmaaldrich.com) was sprayed directly on to the membrane surface of a lateral flow (Figure 12) using a flat bed air jet dispenser (Biodot Ltd,West Sussex, UK) at a constant rate of 50 m/s. The *P. brassicae* (clubroot) test line fractions were individually applied to lateral flow membranes (Figure 12) at a rate of 25 m/s. The lateral flow membranes were air-dried overnight at 18 to 20° C, sectioned in to 5mm strips and a conjugate pad inserted before being individually housed in a plastic case (EVL, Netherlands). Using ten fold concentrations of *P. brassicae* resting spores in sample buffer, 100µl aliquots were applied to the competitive lateral flows (*clfd*) of each test line type. Optical density readings were made 10 min. after application using a Quadscan lateral flow reader. A control comprising of extraction buffer alone was included for each test line type at 100 µl volume. This process was repeated twice.

Results

Addition of test line material and / or pre striping the *clfd* membrane with Poly-L-Lysine did not improve the interaction of monoclonal antibody / antigen binding at the clubroot *clfd* test line (Figure 14a). In addition, these components gave no benefit in retaining test line activity over time (Figure 14b). Degradation of the test line was observed for all *clfd* test line applications over time. Sensitivity and quantitative loss of the test was observed three months post test line application.

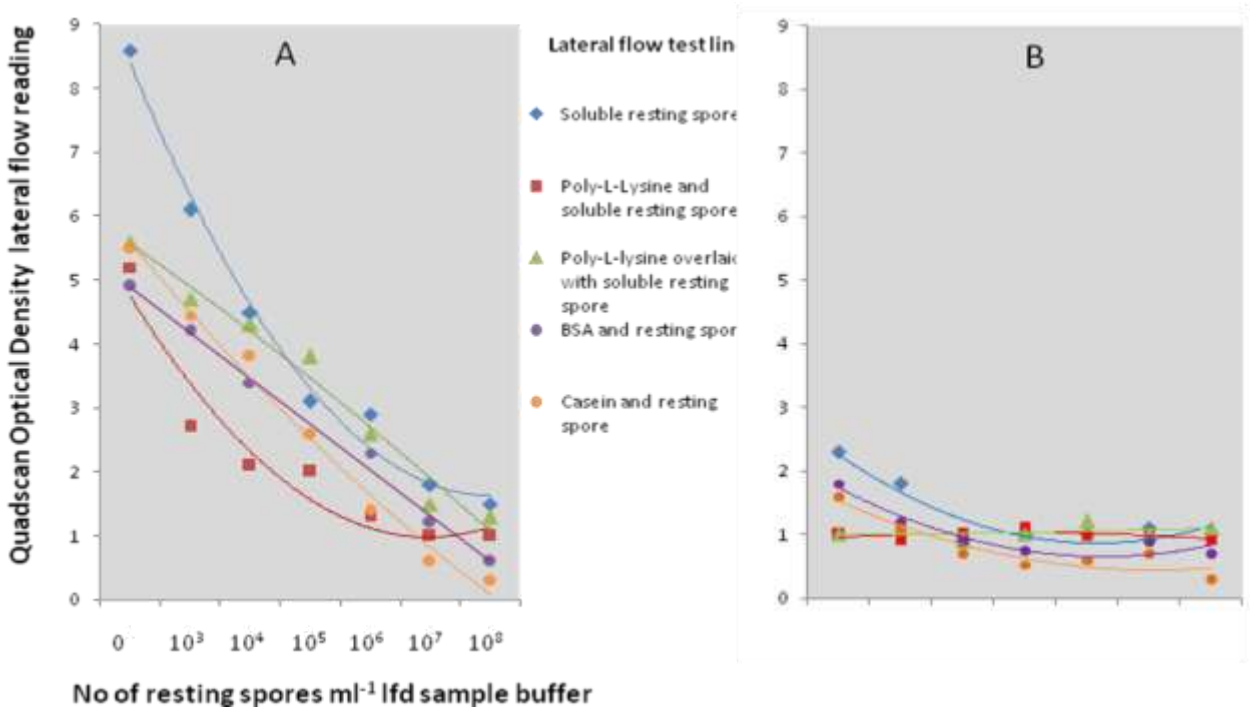

Figure14: Quantitative measurement of clubroot resting spores using lateral flows prepared 48 hours after test line application (A) and at three months (B)

Conclusion

To date stability of the clubroot *clfd* test line antigen has proved difficult. The addition of components to the test line fraction, prior to clfd membrane striping, has shown no benefit in attaining a robust test over time. In addition, competition in binding of the clubroot antigen and the incorporated proteins to the membrane was observed and, test line optical density values (OD) reduced accordingly. Poly-L-Lysine, a positively charged polymer, routinely used as a binding support for biological material, did not enhance *P. brassicae* binding or retention to the membrane test line application area. Additional studies since have however determined that heat and protease affect the resting spore test line antigen and antibody binding. No effect was observed when a DIG glycan differentiation kit was used indicating that a glycoprotein is not involved in the complex binding between the *P. brassicae* antigen and the monoclonal antibody (MAb). Generally it has been observed that where resting spore fungal surface washings have been used to induce an immune response, the resultant antibodies have bound to glycoprotein fungal antigenic determinants (Macdonald *et al*., 1989, Werres & Steffens, 1994). Analysis of the chemical composition of *P. brassicae* resting spore wall (Moxham & Buczacki, 1983) found no evidence of glycoprotein

complexes. Future work should investigate incorporation of a component to attain protein stability within the test line.

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