ANNUAL REPORT

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BRASSICAS: DEVELOPMENT AND VALIDATION OF DETECTION TESTS FOR CLUBROOT

HDC PROJECT FV 259

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Commercial - In Confidence

Grower Summary

FV 259

BRASSICAS: DEVELOPMENT AND VALIDATION OF TESTS FOR CLUBROOT

Annual report 2006

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Signed on behalf of: Warwick HRI

Signature:…… ***Date***: …….
Name: Professor Simon Bright
Director and Head of Department

The results and conclusions in this report are based on an investigation conducted over one year. The conditions under which the experiment was carried out and the results obtained have been reported with detail and 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.

CONTENTS PAGE

1. GROWER SUMMARY

1.1 Headline

A rapid test for clubroot (lateral flow devices) has been successfully constructed. This will be used to detect clubroot contamination in soil samples in the field. Further work is required to optimize this device and test it on contaminated soil samples.

1.2 Background and expected deliverables

Clubroot caused by the protist *Plasmodiophora brassicae* Woronin is one of the most intractable plant pathological problems of cultivated cruciferous crops world-wide. Symptoms are characteristically galls or clubs formed on the roots which reduce plant vigour and yield. Infection leads to wilting, death and total crop loss especially when young plants are invaded. Older plants may produce limited yields but plant maturity will be erratic and harvesting schedules disrupted. Traditional control measures include improving drainage, liming to raise soil pH and rotation of susceptible crops with non-cruciferous crops. Previous studies have indicated that symptom expression is dependant on the presence of a threshold level of infection. Inoculum concentration plays an important part in the incidence and severity of clubroot development within crops. In reality these thresholds are determined by environmental and control measures operating within the crop. Minimal spore concentrations and a pH of 7.2 inhibited root hair infection whereas clubs developed at higher inoculum densities at the same pH. Threshold levels of infection are required for maximum symptom development however severity of clubbing was shown to be proportional to increased spore concentration and total root hair infection. Above this threshold increasing spore concentration may generate greater root hair infection but this does not lead to further increases in symptom development. Other studies have also indicated that liming an infected field may produce effective control of the disease. Chemical control is not an option at present as there are no chemicals which hold approval for clubroot control in vegetable brassicas. Alternative control measures are thus urgently needed.

The expected deliverables from this project are:

- Better detection of clubroot in the field before disease is visible in the crop.
- Detection tests which can be used “in field” to determine the level of risk to the brassica crops posed by clubroot resting spores.
- Determining the level of risk of crop loss in soils before crops are planted.
- Monoclonal antibodies which recognise resting spores of clubroot.

1.3 Summary of second year work on FV 259

1.3.1 Development of a field based test format for use in the detection of clubroot resting spores.

Competitive lateral flow test

The lateral flow device is a detection test which can be used under field conditions to detect target particles. It works in a similar way to human pregnancy test kits however in the development of these tests for clubroot, soil samples would be used and the clubroot resting spore would be the target particles that the device would react to. A format for the test is required and in the clubroot work in year two of the project a competitive lateral flow test format was successful. This format can be used to detect clubroot resting spores. In this lateral flow format the absence of a test line represents a positive result. An additional control line is also used in the device to demonstrate successful test operation.

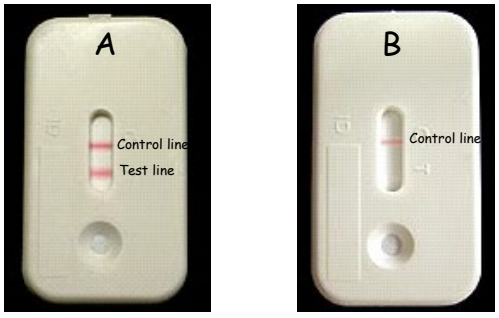


Plate 1. Competitive lateral flow assay showing a positive sample result (B) and (A) a negative sample result.

- Negative sample = Observation of the test line and the control line
- Positive sample = Observation of the control line. No test line formation

The sample extract (soil or soil extract) is applied to a release pad within the device, facilitating specific antibody bound coloured spheres to flow on the nitrocellulose membrane. Reaction of these particles with clubroot material (sprayed lines) on the membrane gives rise to a visible pink line on the membrane. The presence of this line for each sample indicates a negative result conversely the absence of the test line indicates a positive result for clubroot in the sample. A clear visible red control line (second line) will form showing that the test has been completed satisfactorily.

1.3.2 Detection threshold of the competitive lateral flow for clubroot resting spores

The competitive lateral flow format shows great promise as a rapid test for clubroot resting spore detection. The format was clearly able to detect clubroot spores at close to epidemiological significant levels (1 x 10^4 spores $gram^{-1}$ of soil). An antibody/coloured conjugate volume of 27µl (within the device) was optimal for line detection on the lateral flow device both visually and using a Bio-dot lateral flow reader electronic optical equipment. There is potential for semi-quantitative analysis employing an electronic reader when optical density values are calculated (Table 1).

Table 1. Optical density values of the test line at varying clubroot resting spore concentrations

Sample concentration (Clubroot resting spores)	Optical Density value
0	9.9
1 x 10^3	8.0
1 x 10^4	7.6
1 x 10^5	6.7
1 x 10^6	2.4
1 x 10^7	1.3

However the working test will require further optimisation and methods for rapid extraction of clubroot spores from soil will also be required. The detection threshold of this test will need further adjustment. A visualisation of the test line (negative result) would be required at around the 1 x 10^4 spores $gram^{-1}$ of soil or below. At this level the test could prove useful in designating the regimes required for clubroot control.

1.4 Action points for growers

There are no specific action points for growers at this stage in the project. However in the future

- Growers can use the “in field” test together with an approved sampling procedure to determine the clubroot status of their Brassica production areas.
- The system will be able to indicate appropriate control measures based on the level of clubroot contamination.

1.5 Anticipated practical and financial benefit

- The usage of the “in field “tests for clubroot will improve information on the potential control options in the field.
- Appropriate control treatments can be applied to fields on the basis of clubroot risk. This will improve the economics of production by promoting targeted clubroot control.

SCIENCE SECTION

2. INTRODUCTION

2.1 The clubroot pathogen infecting vegetable Brassicas

Clubroot (caused by the protist *Plasmodiophora brassicae* Woronin) is a major pathogen affecting the production of vegetable brassicas in the UK. Symptoms are characteristically galls or clubs formed on the roots which reduce plant vigour and yield. Infection leads to wilting, death and total crop loss especially when young plants are invaded. Older plants may produce limited yields but plant maturity will be erratic and harvesting schedules disrupted.

2.2 Clubroot symptom development in vegetable Brassica crops

Once land is infested with the clubroot organism eradication is extremely difficult to due to the persistence of viable resting spores in the soil. Resting spores give rise to zoospores which are the primary source of transmission and infection of vegetable brassica root hairs. The process of infection, subsequent growth and development of the pathogen within host tissues is still not completely understood however resting spores are formed within infected roots. When the root decays these resting spores are liberated into the soil where they may germinate immediately, or in the absence of a host remain viable for up to 8 years (Jorstad, 1923). Disease spread has been attributed to several factors. Feeding infected plants to livestock is one method of spread now widely accepted as contributing to transmission of disease. Movement of surface water and transfer of contaminated soil are major sources of potential disease transfer. Additionally the use of transplants in vegetable Brassica production can also be an important source of disease. Infected plants and soil are transported widely within modern vegetable production and this could contribute to spread of disease to new areas of production. It has been reported that calcium, pH and inoculum concentration function interactively affecting processes such as infection, colonisation and symptom formation (Webster & Dixon, 1992). Other studies have indicated that symptom expression is dependant on the presence of a threshold level of infection. Webster and Dixon (1992) reported that under environmentally unlimiting conditions and below a threshold level of infection for maximum symptom development severity of clubbing was proportional to increased spore concentration and total root hair infection. Above this threshold increasing spore concentration may generate greater root hair infection but this does not lead to further increases in symptom development. This indicates that inoculum concentration plays an important part in the incidence and severity of clubroot development within crops. It also indicates that there is a threshold of inoculum below which clubroot although present will not develop although there would obviously be considerable differences between this epidemiological threshold and that considered as an economic threshold for clubroot development.

2.3 Methods for determining the risk of clubroot in soil samples

The environmental conditions which favour clubroot development are poorly understood. In general there are few reliable methods which can be used for forecasting soil borne pathogens based on environmental criteria. For this reason many studies have attempted to develop methods to detect and quantify resting spores of *P. brassicae* in the soil as a means of determining risk of clubroot infection and development. The concentration of infestion of the soil by resting spores has been shown to directly affect the degree of clubroot infection (Buczacki & Ockendon, 1978). Resting spore concentrations in excess of 10^{5} g $^{-1}$ soil are required for severe and uniform disease expression on test plants. However additional factors such as the conducive or suppressive nature of the soil may also influence the concentration required (Rouxel *et, al.*, 1988). As the pathogen only grows within living tissues it is not possible to use standard dilution plating techniques to quantify numbers of pathogenic propagules within soil. Resting spores can be observed directly in soil samples using microscopy however this can be very inaccurate, requires specialist knowledge and is highly labour intensive. Some studies have shown that stains can be used to differentiate resting spores from soil and determine their viability (Takahashi & Yamagichi, 1989). However the accepted approach developed by Melville & Hawken (1967) relies on the observation of gall formation on bait plants exposed to standard quantities of test soil. This type of test has the disadvantage of being labour intensive, costly and slow. It has also a considerable drawback in that it requires larger numbers of plants with greater numbers of observations to detect lower numbers of resting spore concentrations. Even when this approach is employed it can still be in accurate as by chance low levels of resting spores may not result in quantifiable infection on roots. For these reasons more rapid approaches that are not laboratory based are urgently required for clubroot resting spore quantification in soils. These tests should be simple and easy to use by untrained operators. Such tests are already used in a great number of areas for example the pregnancy test kit (based on immunological lateral flow assays) are widely used. To develop these approaches requires the use of existing techniques which can accurately quantify low numbers of resting spores in soil samples. Only detection techniques based on molecular approaches have the ability to do this.

2.4 Molecular tests for clubroot resting spores in soil.

Molecular detection methods for *P. brassicae* based on PCR have been developed for conserved sequences in the 18S-like ribosomal RNA gene and the internal transcribed spacer (ITS) regions of clubroot DNA sequences (Chee *et al*., 1998, Faggian, *et al*., 1999). Specific detection has also been achieved using primers derived from the sequence of the isopentyltransferase gene (Kim and Lee, 2001, Ito, *et.al*., 1999). Up to 1000-fold increases in the sensitivity can be achieved when some molecular methods are used (Wallenhammar and Ardwisson 2001). Other workers reported levels of spore detection of one spore per gram of soil in potting mix using single primer pairs (Faggian *et al*., 1999). With the exception of a single isolate, none of these primers has been tested against of *P. brassicae* isolates of UK origin. Recently another type of PCR test has been developed (real time PCR) in which PCR amplification is monitored in real time. The accumulation of PCR product can be compared to a set of standards which enables sample inoculum concentrations to be determined. Inoculum concentration can be related to disease severity in the field. A protocol to quantify the amount of clubroot inoculum in a sample was developed by Faggian and Parsons (2002). However this technique could not be successfully used on all soil types to predict severity of disease (Donald *et al*., 2003) in Australia. Further work is currently being carried out to improve the assay.

2.5 Immunological tests (Lateral Flow Devices)

Lateral flow assays are only one type of rapid assay which can be employed to quantify target particles or molecules. However they are now commonly and widely used for detection purposes. They rely upon the competition for binding sites by sensitised coloured particulates. Antibodies (polyclonal or monoclonal) raised to a specific target spore, are bound by passive or covalent means to these coloured particles. These sensitised particles (latex or immunogold are generally used) are then applied using an immersion procedure on to a release pad, to produce a stable particle reservoir for release on to a nitro-cellulose-based membrane. Two lines of reagents are immobilised on to the membrane using a sophisticated reagent dispenser. The constituents of these lines will vary from test to test but commonly only two types of formats are adopted.

The Competitive assay format: In a competitive assay format the test line comprises of homologous antigen (clubroot spore components) and the other, the control, is a line of anti-species antibodies. The release pad and membrane are assembled together with an absorbent pad into a plastic housing as illustrated below (Figure 1). Three drops of sample are added to the well, releasing the specific antibody bound coloured particles, which then begin to flow across the membrane. If the target antigen is present in the sample extract, antibody binding will occur to produce a coloured particulate conjugated antibody -antigen complex. Any antibody conjugated coloured particles that fail to bind to an antigen will attach to the immobilised test line as they traverse the membrane; thus producing a visible line of deposited coloured particulates at the test line. The anti-species antibody will capture excess

sensitised antibody / coloured particles to produce an internal control line, providing a visible confirmation of antibody / particulate flow. Sufficient target presence *i.e.* of clubroot spores, would induce complete inhibition of antibody attachment to the test line, a result that is indicated by a single line of coloured particle deposition (the control line). Two lines of equal colour intensity indicate a negative result.

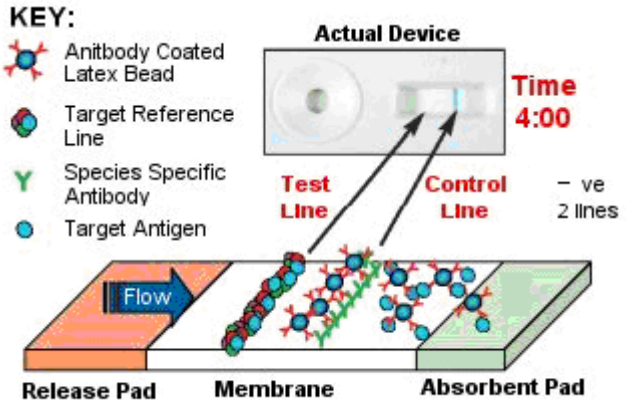


Figure 1. The Competitive lateral flow assay format.

The Non-competitive assay format: In a non-competitive assay format the test line generally comprises of an antibody complex which if present, will bind to the target antigen in the test sample. The control line will consist of a complex matrix which will bind material within the sample flow to indicate successful test execution. The release pad and membrane are assembled as described above. Three drops of sample are added to the well, releasing the specific antibody bound coloured particles, which then begin to flow across the membrane. If the target antigen is present in the sample extract (clubroot), antibody binding will occur to produce a coloured particulate conjugated antibody -antigen complex. This target complex passes over the test line enabling capture of the antigen to occur immobilising the antibody coated coloured particulates; thus producing a visible line of deposited coloured particulates at the test line. Excess coloured particulate material is captured at the control line, providing a visible confirmation of antibody/ particulate flow. Two lines of equal intensity indicate a positive result . Both assay formats can produce a semi-quantifiable test.

2.6 Developing 'in field' tests for detecting the presence or absence of clubroot spores in soil

If specific antibodies can be raised then the development of rapid assays such as lateral flow tests which incorporate them can proceed. No antibodies are commercially available for the clubroot pathogen. For this reason most of the first and some second year work in the project must be devoted to the development and testing of clubroot specific monoclonal antibodies. Reactive cell lines in mice must be raised which produce specific antibodies. These are then tested for their specificity to clubroot resting spores and their reactivity to a range of common soil-borne fungi. This would include other pathogens which are common in the soil in vegetable Brassica crops notably *Botrytis cinerea* and *Rhizoctonia solani*. It must also include the full range of soil-borne organisms such as *Penicillium sp*., *Aspergillus sp*., and *Fusarium sp*. Many of these pathogens survive on debris in the soil or are found on leaves on Brassica crops. Consequently tests with clubroot resting spores in soil should not react with the conidia of other pathogens and saprophytes common in soil environment crops. If the antibody does react with conidia of other fungi found in the soil then it cannot be used for the development of “in field” tests. In the first year of the work these aspects in the development of the detection system for clubroot are required. Once suitable antibodies have been developed prototype lateral flow devices which detect clubroot resting spores can be developed. This work was carried out within the second year of the project.

3. SELECTION OF SPECIFIC MONOCLONAL ANTIBODY CELL LINES FOR DETECTION OF CLUBROOT SPORES

3.1 Selection of monoclonal antisera to *Plasmodiophora brassicae*

3.1.1 Introduction

A full description of the production of cell lines for screening and further selection is detailed in HDC First year annual report for FV259. The clubroot pathogen (*Plasmodiophora brassicae*) is an obligate parasite and must be isolated and routinely maintained on susceptible plants for there to be a ready supply of clubroot resting spores. Resting spores used for screening were produced by routinely culturing the clubroot pathogen on roots of Brussels sprout plants (cv. Golfer). Four hybridoma cell lines previously selected for their recognition characteristics to *P. brassicae* resting spores (clubroot) were twice cloned and coded EMA 3A5, 3A12, 2D6, 2H4 respectively . These antibody cell lines were tested for their specificity to *P. brassicae* both by plate trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) and immunofluorescence (IF).

3.1.2 Materials and Methods

3.1.2.1 Immunization of mice with *P. brassicae* resting spores

Four female Balb C mice (coded 9011, 9012 9013 and 9014) were immunised (by intraperitoneal injection) each with 100µl of the spore washing preparation mixed with an equal volume of Titermax adjuvant. The mice were immunised twice more at 2 weekly intervals. Following tail bleeds and, employing a plate trapped antigen ELISA (PTA-ELISA), a mouse was identified which exhibited a high level of sensitivity to soluble material associated with *P. brassicae* resting spores. The selected mouse was immunized twice more at 2 weekly intervals. Four days after the final immunization, a terminal bleed (polyclonal antiserum) was collected and the spleen removed. A fusion (antibody producing B cells are isolated from the spleen and fused in vitro with a lymphoid tumour cell (myeloma)) was carried out at Warwick HRI Wellesbourne. Hybridoma tissue culture supernatants were screened both by immunofluorescence (IF) and by PTA ELISA (plate-trapped antigen enzyme-linked immunosorbent assay) 14 days after cell fusion for the presence of antibodies which recognised components associated with the resting spores of *P. brassicae* (clubroot inoculum). This process was repeated for each of the mice.

3.1.2.2 Monoclonal Antibody Screening

Plate trapped antigen ELISA (PTA ELISA)

One hundred µl of *P. brassicae* resting spore soluble root extracts in 0.01M Phosphate buffered saline, pH 7.4, were aliquoted in to each of 96 polysorp microtitre wells in strips (Nunc, Roskilde, Denmark; Cat. No. 469957). The strips were then incubated overnight in an enclosed chamber at 18ºC. After which unbound material was removed and the microtitre wells were washed once with 200 µl PBS. The microtitre wells were blocked with 200 µl of 1 % Casein buffer (1 % (w/v) casein PBS) and incubated at 37 º C for 45 min. Residual blocking buffer was removed and wells were washed four times for one min each with 200 µl PBS, 0.05 % Tween 20 and 0.1% Casein (PBSTw C). After which each well received 100 µl of fusion hybridoma tissue culture supernatant mixed with PBS TwC. Following incubation in a Wellwarm shaker incubator (30° C) for a period of 45 mins as above, wells were washed three times for one min each with 200 µl PBSTincTw. A DAKO duet amplification system was then used (DAKO Ltd, Angel Drive, Ely, Cambridge, UK; Cat no. K0492) to amplify the signal generated by bound tissue culture supernatant antibodies. Wells were washed as described above and to each well 100µl of 3, 3',5,5'- tetramethylbenzidene substrate (Sigma, Poole, Dorset UK; Cat. No. T-3405 and P-4922) was then added. The reaction was stopped by adding 25µl of a 20% 1M H_2S0_4 solution to each well. Absorbance at 450nm was determined with a Biohit BP800 ELISA plate reader (Alpha Laboratories, 40 Parham Drive, Eastleigh, Hampshire, UK).

The ELISA process was repeated but on subsequent occasions wells were coated with non-soluble *P. brassicae* resting spore / root components. Hybridoma antibody tissue culture supernatants which were identified as positive to either of these microplate well coatings were selected and screened for reactivity against 'non-infected clubroot' plant root material.

Immunofluorescence

Twenty µl of a 10^4 spores ml $^{-1}$ *P. brassicae* spore suspension was aliquoted to individual multiwell glass slides (Cel-Line/eries Scientific Corp, USA; Cat No. 10-3404). Following air drying any unbound spore material was removed with a PBSTwC wash. Material remaining bound to the multiwell glass slides was incubated with 20µl of hybridoma tissue culture supernatant antibodes (TCS) for a period of 30 minutes at room temperature. A counter stain was incorporated within the TCS antibody suspension to quench *P. brassicae* spore autofluorescence. The multiwell slide received a wash as described above and following air drying were incubated with an anti-mouse antibody which had been conjugated to fluoroscein isothanyacyte dye. A counter-stain was again included to ensure quenching of resting spore autofluorescence. Incubation was carried out at room temperature in darkness to prevent photo-bleaching of the conjugated antibody. The processed microwells received a final wash of PBSTwC and after air drying were mounted and viewed by episcopic fluorescence for the presence of antibody / flourescein tagged resting spores of *P. brassicae*.

Hybridoma antibody tissue culture supernatants which were identified as positive to *P. brassicae* resting spore material employing PTA ELISA and IF were selected and expanded in to 24 well plates for further testing.

3.1.2.3 Production of *P. brassicae* infected plant roots

Seeds of cauliflower cv. Belot were sown (one seed per cell) in a mixture of 70:30 Fisons F2 compost and sand contained in Hassey 307 units. Plants were grown in a 16/14 ° C day/night temperature regime. Plants were repotted into FP9 pots (one seedling per pot) at the third true leaf stage and grown for a further 2 weeks at 16/14 ° C day night temperature regime in the glasshouse. Plants were placed in trays prior to inoculation and unhealthy or atypical plants removed to ensure the uniformity plants used in each experiment. Plants were inoculated using a 5 ml suspension of clubroot resting spores harvested from field grown cauliflower plants (see section 6.2) grown in contaminated soil in a quarantine area (QF field) at Warwick HRI Wellesbourne.

3.1.2.4 Production of clubroot resting spores material for cross-reactivity studies

Washed clubbed root galls (20g fresh weight) from *Brassica oleracea* were homogenised in 100ml phosphate buffered saline (PBS) pH 7.2 and filtered through four layers of butter muslin. The filtrate was centrifuged for 5 mins at 2000 xg. Clubroot resting spores were collected and resuspended in 20ml PBS. The resulting spore suspension was centrifuged twice more before being resuspended in PBS (0.02% sodium azide) to a final volume of 20ml. To remove *Brassica oleracea* host and root contaminants the spore suspension was passed through a range of mesh filters (300 to 5µm pore size). The collected aqueous phase was then passed through a filter of 3µm pore diameter, which retained the resting spores of *Plasmodiophora brassicae*. The collected resting spores were resuspended in 10ml chilled PBS (0.02% sodium azide) and adjusted to a concentration of $1x10^4$ spores ml^{-1}.

3.1.2.5 Production of fungal spores and bacterial colonies for cross-reactivity studies

Spores of *Spongospora subterranean* (potato scab) were supplied by Dr. John Walsh (Warwick HRI) (0.3 g *S. subterranean* spore balls) hydrated in 1ml PBS. A range of fungal plant pathogens: *Rhizoctonia solani*, *Phoma betae*, *Sclerotinia sclerotiorum*, *Sclerotium cepivorum*, *Trichoderma* sp., *Botrytis cinerea*, *Fusarium culmorum*, *Verticillium dahliae* and *Pythium sylvaticum*. were inoculated on to an agar medium which had been pre-covered with a PN6026 Supor 450 90mm diameter membrane (Gellman Sciences Cat. No. 60206). When full radial growth of each fungus had occurred, the membranes were removed and 5ml PBS was added to each. Plate surface washings were taken by gently stroking the surface of the membrane with a sterile glass rod. The surface washings were centrifuged in a lab top microfuge at 13,000 rpm for 5 mins. The aqueous phase was retained and stored on ice whilst the fungal pellet of each isolate was re-suspended in to 2x 1ml aliquots of PBS.

For each fungal isolate one 1ml aliquot was retained on ice whilst the contents of the other were disrupted using a Fast Prep device. Bacterial colonies isolated from soil and clubbed roots of *Brassica napus* were selected and purified on nutrient agar plates. Following 3 to 7 days growth at room temperature bacterial suspensions were then prepared at 1 x 10^7 cells ml^{-1} in PBS. All spore material was retained at a temperature of 0 - 4°C before ELISA and IF studies were conducted.

3.1.2.6 Reactivity of selected monoclonal antibodies to a range of soil fungi and bacteria

Plate trapped antigen ELISA

Samples were loaded (100µl per well) in to paired wells of a polysorp microtiter well strip (Nunc, Roskilde, Denmark; Cat No. 469957) and incubated overnight at 4°C. Unbound material was then removed by inverting the strips over a sink and after slapping directly down on to absorbent towelling. The microtiter wells were then washed once with 200µl PBS and blocked with 200µl of 1% Casein (1% (w/v) casein PBS) for 45 minutes at 37°C. Residual blocking buffer was removed as described above and then wells were washed once with 200µl PBS, 0.05% Tween 20 and 0.1% Casein (PBSTw C). After which each well received immune sera of EMA 3A5 at a dilution of 1 in 1600 PBSTw C. Following incubation in a Wellwarm shaker incubator at 30°C for a period of 45 minutes, wells were washed three times for one minute each with 200µl PBSTw C. A DAKO duet amplification system was then used (DAKO Ltd, Angel Drive, Ely, Cambridge, UK; Cat no. k0492) according to manufacturers guide lines to amplify the signal generated by bound monoclonal antibodies. Wells were then washed as described above and after which 100µl of 3,3',5,5'-tetramethylbenzidene substrate (Sigma T-3405 and P-4922) was then added to each well. The reaction was stopped by adding 25µl of a 20% $1MH_2SO_4$ solution to each well. Absorbance at 450nm was determined with a BioHit BP800 ELISA plate reader (Alpha Laboratories, 40 Parham Drive, Eastleigh, Hampshire, UK)

The ELISA procedure was repeated out for each of the four selected monoclonal antibody cell lines listed below and at the dilutions shown in Table 2.

Table 2 Antibody dilutions used to screen *P. brassicae* monoclonal antibody cell lines using PTA ELISA

Antibody cell line	Antibody dilution in PBSTw
3A5	1:1600
3A12	1:1600
2D6	1:64
2H4	1:3

Immunofluorescence

For each fungal spore / mycelial suspension 20 µl was aliquoted to each of 8 wells of a multiwell glass slide. Following air drying any unbound spore material was removed with a PBSTwC wash. Material remaining bound to the multiwell glass slides was incubated with 20µl of the immune sera diluted in PBSTw C as described in Table 3

Table 3. Antibody dilutions used to screen *P. brassicae* monoclonal antibody cell lines using IF

Antibody cell line	Antibody dilution in PBSTw
3A5	1:800
3A12	1:800
2D6	1:32
2H4	1:2

Incubation was within a humid environment and in darkness for a 30 minute period (minimum). A counterstain (Evans blue / Eriochrome black) was incorporated within each of the antibody suspensions to quench spore autofluorescence. The multiwells were washed as previously described and following air drying were incubated with an anti-mouse antibody which had been conjugated to fluorescein isothanyacyte dye (Sigma F0257 HRI 1 in 80, Lot 092K9153 (Sigma working dilution of 1 in 125). A counterstain was again included to ensure quenching of resting spore autofluorescence. Incubation was carried out at room temperature in darkness to prevent photo-bleaching of the conjugated antibody. The processed microwells received a final wash of PBSTwC and after air-drying were mounted and viewed by episcopic fluorescence for the presence of antibody / fluorescein tagged spores

3.1.3 Results

PTA ELISA

Plate trapped antigen ELISA results established a low level of reactivity of any of the monoclonal antibodies tested to a wide range organisms whether present in a particulate or soluble format. For each monoclonal (EMAs : 3A12, 3A5, 2D6 and 2H4)a high level of affinity was observed to clubroot resting spore components whether in a soluble or particulate format (Figs. 2a and b).

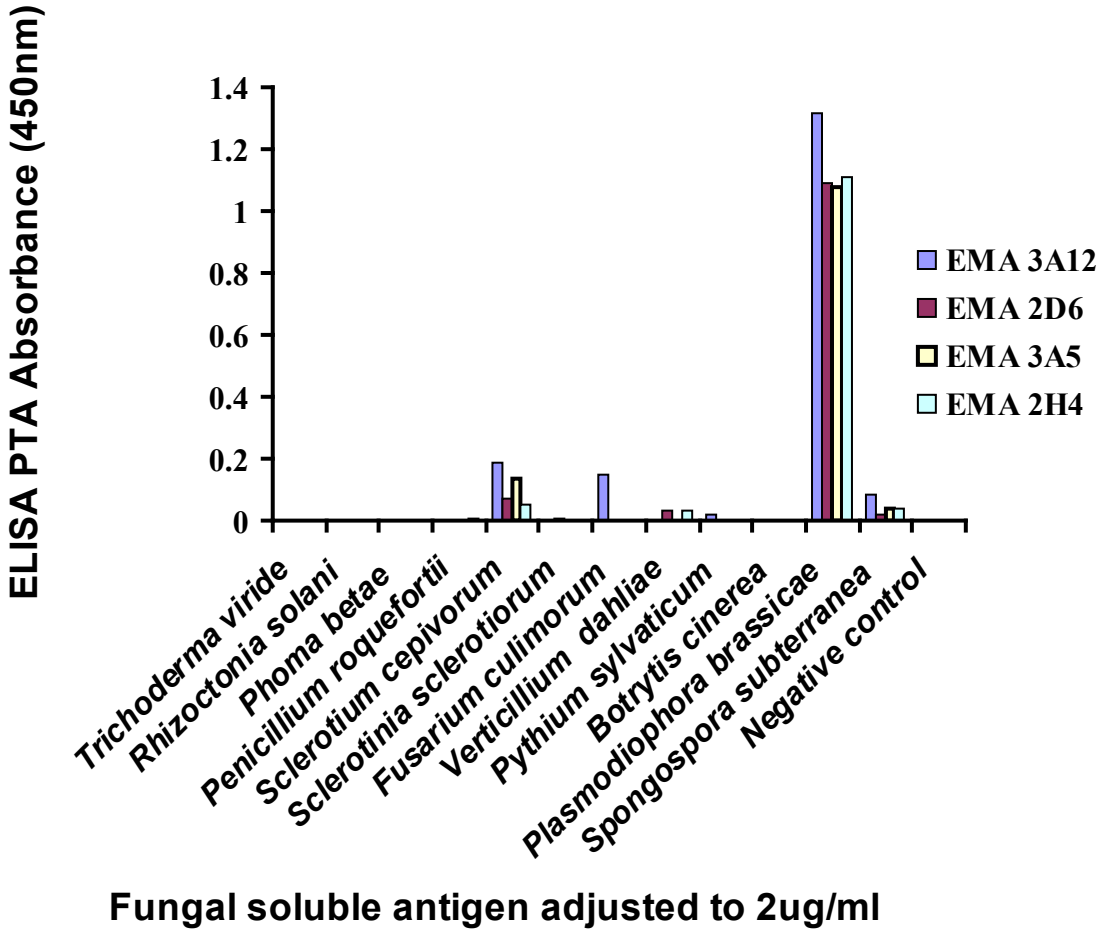


Figure 2a Reactivity of *P. brassicae* monoclonal antibodies (EMA 3A5, 3A12, 3A5 and 2H4) to soluble material from a range of soilborne fungi as tested by PTA ELISA

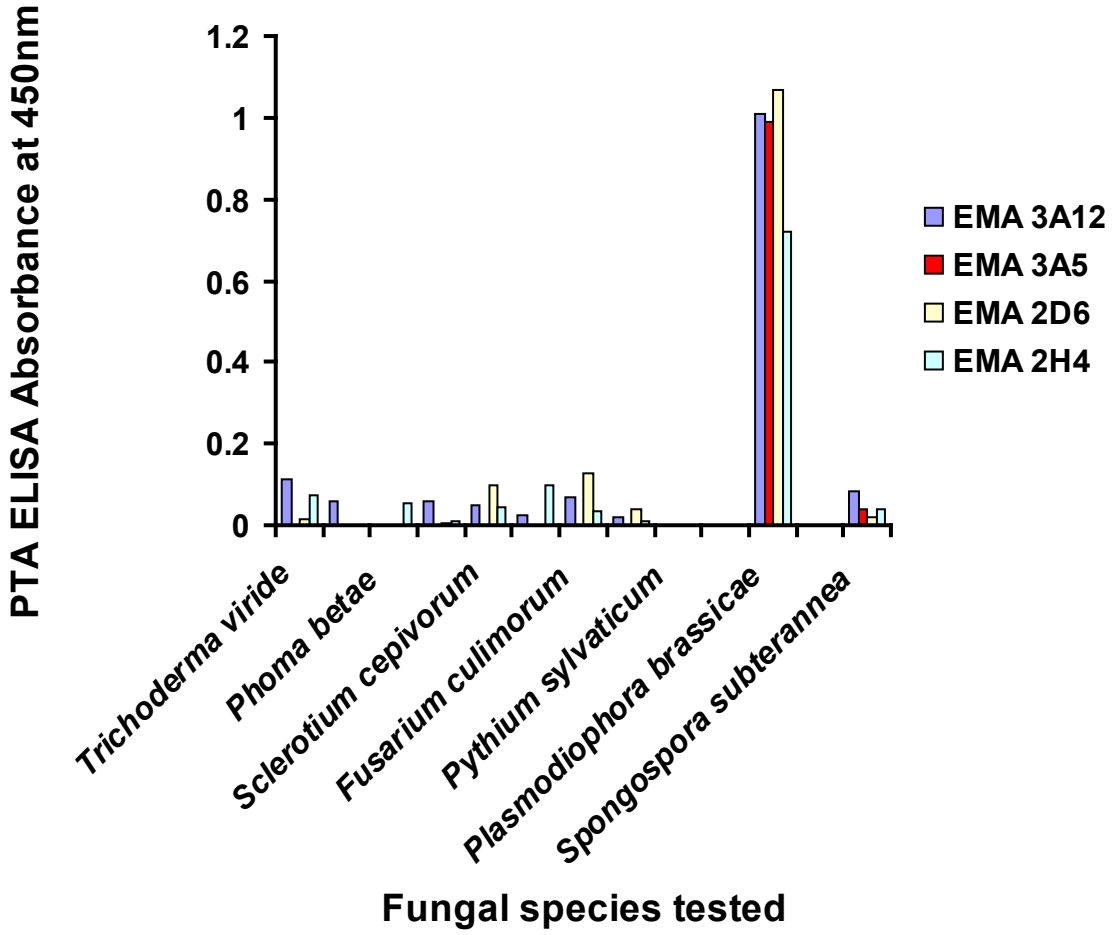


Figure 2b. Reacitivity of *P. brassicae* monoclonal antibodies (EMA 3A5, 3A12, 3A5 and 2H4) to particulate material from a range of soilborne fungi as tested by PTA ELISA

Immunofluorescence

For each of the four monoclonal antibodies tested a high level of antibody binding was observed to components of the resting spore wall of the clubroot pathogen *Plasmodiophora brassicae* (Plates 2a – d). Monoclonal antibodies EMA 3A5 and 3A12 demonstrated a high level of specificity whilst EMA 2H4 showed weak binding to particlate material of *Phoma betae* and strong recognition to a budding yeast cell contaminant. EMA 2D6 exhibited a level

of recognition to hyphal fragments of *Sclerotium cepivorum*. No reactivity was observed to the bacterial isolates tested.

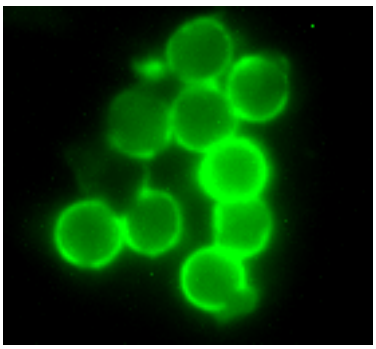


Plate 2a. Reactivity of *P. brassicae* EMA 3A5 monoclonal antiserum to resting spores of clubroot as viewed by immunofluorescence microscopy

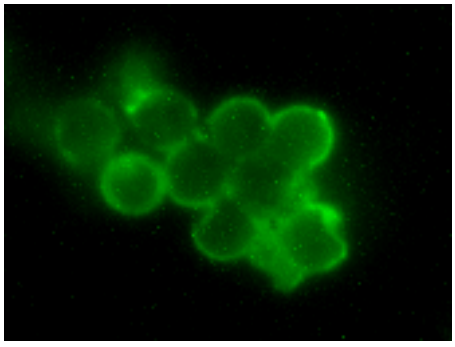


Plate 2b. Reactivity of *P. brassicae* EMA 3A12 monoclonal antiserum to resting spores of clubroot as viewed by immunofluorescence microscopy

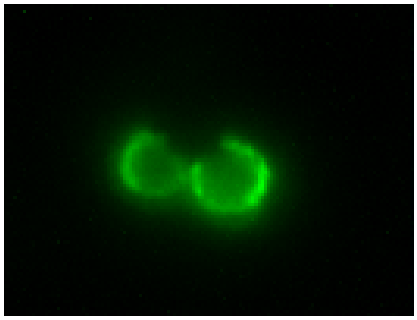


Plate 2c. Reactivity of *P. brassicae* EMA 2D6 monoclonal antiserum to resting spores of clubroot as viewed by immunofluorescence microscopy

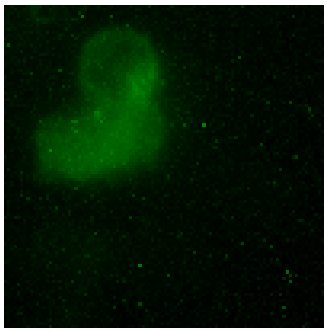


Plate 2d. Reactivity of *P. brassicae* EMA 2H4 monoclonal antiserum to resting spores of clubroot as viewed by immunofluorescence microscopy

3.1.4 Conclusions

Reactivity tests both by ELISA and immunofluoresence determined that two monoclonal antibody cell lines (EMA 3A5 and 3A12) exhibited a level of sensitivity and specificity for use in developmental studies of a lateral flow for the specific detection of clubroot spores. The other antibodies used in these tests were considered unsuitable for use in lateral flow development. Antibody cell line EMA 2D6 was more reactive than other cell lines tested to *S. cepivorum* and *Fusarium culmorum*. Although reactivity was low to these two fungal species they are commonly found in soil and could produce inaccuracy if used within a lateral flow device for clubroot detection in soil. Antibody cell line EMA 2H4 was not used in further clubroot lateral flow development because it was not reactive enough to clubroot resting spores.

4. DEVELOPMENT AND OPTIMISATION OF LATERAL FLOW FOR DETECTION OF CLUBROOT

4.1 Selection of lateral flow format for rapid detection of *Plasmodiophora brassicae*

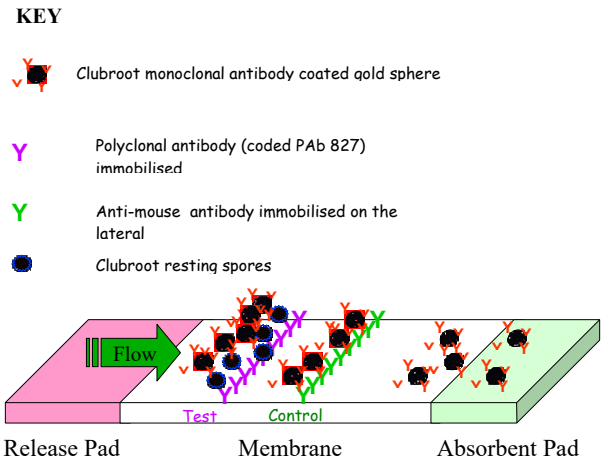
4.1.1 Introduction

Lateral flow devices (Lfd's) offer the potential to deliver to the end user a diagnostic test which can identify the presence / absence of specific target analytes associated with the presence of a pathogen. The lateral flow test although simple to use incapsulates a complex biological operating system of interacting reagents and materials. Determining optimal reagents and materials and optimising the operating system, where these interact, involves considerable investigation. This section of the report details the development of a working lateral flow format which can detect clubroot resting spores, and its optimisation to quantify resting spore concentrations.

4.1.2 Materials and Methods

4.1.2.1 Development of Double Antibody Sandwich (DAS) test format for use in the detection of resting spores of *P. brassicae*

Two sources of antibody are required within the double antibody sandwich LFD test format, which can comprise either a pair of the same or two different antibody types. One antibody type is bound to the nitrocellulose membrane (test line) the other is labelled with a visual marker (in all tests listed below gold microspheres are used) which are held within a release pad within the device (Figure 3). To ascertain successful test operation a control line comprising of an anti-mouse antibody was prepared and sprayed on the same membrane as the test line to capture unbound gold particles. In these tests a monoclonal antiserum which recognises clubroot resting spores was bound to gold microspheres and these are retained in a release pad. Once the test sample is applied to the lateral flow pad the specific gold conjugated antibodies are released and if present can bind to target sample resting spore antigens.



- Negative sample = Observation of the control line.
- Positive sample = Observation of the test line and the control line

Figure 3. Schematic drawing of a Double Antibody Sandwich (DAS) lateral flow device *(Positive result shown)*

4.1.2.2 DAS lateral flow test procedure

When a few drops of the clubroot resting spore test sample is applied to the lateral flow release pad the gold sphere conjugated clubroot monoclonal antibodies are released in to solution and flow with the sample laterally towards the antibody test line. If the target antigen (clubroot resting spores) is present within the sample the specific clubroot antibody conjugated gold spheres bind to form a complex.

As this complex flows over the test line, it is captured by the immobilised test line antibody. This reaction is visualised by the formation of a red line (Plate 3). If no target antigen (clubroot resting spore component) is present within the sample the antibody conjugated gold spheres are not captured at the test line and no line is visible. In either situation, excess antibody conjugated gold spheres will become immobilised at the control line and a clearly visible red line will form showing that the test has operated satisfactorily.

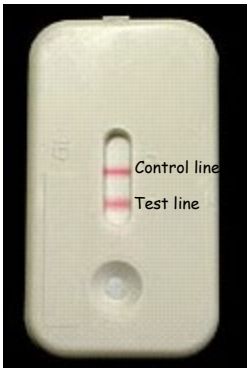


Plate 3. DAS lateral flow test exhibiting a positive result with visualisation of test and control lines.

4.1.2.3 Capture and detector antibodies for inclusion within a DAS lateral flow device

To determine the applicability of the DAS lateral flow format for the detection of clubroot resting spores the selected antibody combinations below were examined (Table 4).

Table 4. Antibody combinations used in the DAS lateral flow assessment

Gold sphere conjugated antibody	Test line antibody	Control line antibody
EMA 3A5	PAb 827 IgG purified	Anti-mouse IgM
EMA 3A12	PAb 827 IgG purified	Anti-mouse IgM
PAb 827 IgG purified	EMA 3A5	Anti-rabbit IgG
PAb 827 IgG purified	EMA 3A12	Anti-rabbit IgG

These tests were conducted by applying the chosen antibody in the form of a test line on the membrane. Known clubroot spore concentrations could be placed on the release pad containing the gold conjugated antibody type which creates a flow producing. If successful, a visualisation of the control line results from this flow.

4.1.2.4 Membranes and buffers used

All tests were carried out using lateral flows comprised of a Millipore 180 HiFlow™ cellulose ester membrane direct cast on to 2ml Mylar backing (Cat No. SHF2400225, Millipore Corp, USA.), absorbent pad (Cat No. GBOO4, Schleicer and Schuell, Germany) and a sample pad (Cat No. T5NM, Millipore Corp., USA). The specified test line antibodies (Table 4) were applied directly to the membrane in Phosphate buffered saline solution, pH 7.5 (PBS) employing a flat bed air jet dispenser (Biodot Ltd, The Kingley Centre, West Sussex, UK). Sprayed membranes (with lines) were air-dried at 20°C for a period of 24 hours. The antibody labelled lateral flow membranes were cut in to 5 mm strips and labelled. The gold conjugated antibody complex and the test antigen (*P. brassicae* resting spores $1x10^5$ spores / ml) were mixed and then applied to the sample pad. The lateral flow device strips were viewed 5 minutes post sample application.

4.1.3 Results

All antibody combinations (shown in Table 4) gave negative results i.e. no test line development was observed. However control line development was noted to confirm that the test had run with antibody / gold flow (Plate 4).

Plate 4 DAS lateral flow format showing a negative result.

4.1.4 Conclusions

The double antibody sandwich lateral flow test, in the format used, was unsuccessful for the detection of *P. brassicae* resting spores. The failure of the double antibody labelling system is not uncommon in fungal / protist immunological assays. Problems associated with stearic hinderence may prevent the two antibody systems from binding simultaneously to the target analyte. More often a single type specific capture antibody is used for these assay systems. Additionally the antibody type may have resulted in a poor flow of the antibody conjugate inhibiting reactions on the test line.

4.2 Development of a competitive assay test format for use in the detection of resting spores of *P. brassicae*

4.2.1 Competitive lateral flow test procedure

Interpretation of results using a competitive lateral flow test format is the opposite to that expressed using the DAS lateral flow format. The absence of a test line represents a positive result. As in the DAS format a control line is observed and demonstrates successful test operation .

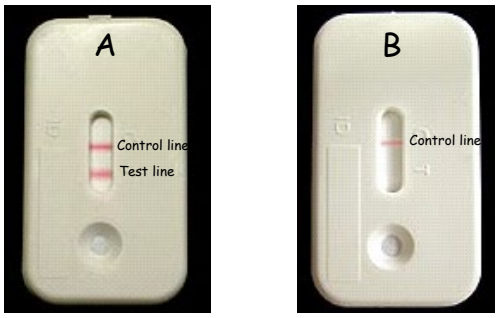


Plate 5. Competitive lateral flow assay showing a positive sample result (B) and (A) a negative sample result.

- Negative sample = Observation of the test line and the control line
- Positive sample = Observation of the control line. No test line formation

Test operation is as described for the DAS lateral flow format. The sample extract is applied to the release pad, facilitating the 'immobilised' specific antibody bound gold spheres to flow on the nitrocellulose membrane. However in this format the test line is composed of soluble components of disrupted *P. brassicae* clubroot resting spores at a protein concentration of 500 ug ml^{-1} PBS. As the sample extract and the specific antibody bound gold spheres flow laterally though the membrane there is potential for binding between the target antigen (*P. brassicae*) and specific gold labelled antibodies. The formation of a target antigen / gold conjugated antibody complex inhibits capture at the test line. With sufficient target antigen present (*P. brassicae* resting spores) complete inhibition occurs and no test line is observed. In a negative sample (target *P. brassicae* resting spores absent) the antibody conjugated gold spheres remain unbound and are captured at the test line to produce a visible line. In either situation, excess antibody conjugated gold spheres will become immobilised at the control line. The control line is composed of an anti-species antibody which will react with the gold/antibody complex flowing from the sample pad. A clear visible red control band will form showing that the test has been completed satisfactorily.

4.2.1.1 Competitive lateral flow assay format construction for the detection of *Plasmodiophora brassicae* resting spores

Preliminary tests were carried out using lateral flows comprised of a Millipore 240 HiFlow™ cellulose ester membrane direct cast on to 2ml Mylar backing (Cat No. SHF2400225, Millipore Corp, USA.), an absorbent pad (Cat No. GBOO4, Schleicer and Schuell, Germany) and a sample pad (Cat No. T5NM, Millipore Corp., USA). Following lateral flow construction (Figure 4) control lines of an anti-mouse serum in PBS was sprayed directly on to the membrane surface using a flat bed air jet dispenser (Biodot Ltd, The Kingley Centre, West Sussex, UK). A collected resting spore preparation of *P. brassicae*, prepared as described in Section 3.1.2.4 was collected in PBS and adjusted to a concentration of $1x10^8$ resting spores ml^{-1} PBS. The resting spore solution was aliqoted (0.5ml) in to eppindorf vessels each containing 0.5g of 0.5mm diameter Ballotoni Beads (Jencons-PLC, UK). Using a Fast Prep device (Q Biogene) at an operating speed of 5 metres sec^{-1} for three consecutive 20 second periods resting spores were disrupted. The resting spore soluble fraction was retained and applied directly, using a flat bed air jet dispenser, to the lateral flow membrane to produced a test line. Membranes were air dried at 18° C for a period of 24 hours. The test and control line labelled lateral flow membrane cards were cut in to 5 mm strips.

4.2.1.2 Competitive lateral flow assay format procedure

A 10µl volume of British Biocell gold anti-mouse was pre-mixed (conjugated) with 50µl EMA 3A5 (working Ab dilution of 1 in 100 in 0.05M TrisHCL, 0.15M NaCl, 0.4% Triton X100, 0.2% Tween 20, 0.2% BSA (HRI buffer 1) for a 20 min. period on a Wellwarm shaker incubator. The conjugated solution was applied directly to the sample pad dropwise. The lateral flow device (*c lfd*) was viewed 5 minutes post sample application for the formation of a test and control line. Variable antibody concentrations of EMA 3A5 ranging from 1 in 100 to 1 in 1600 were used in these tests.

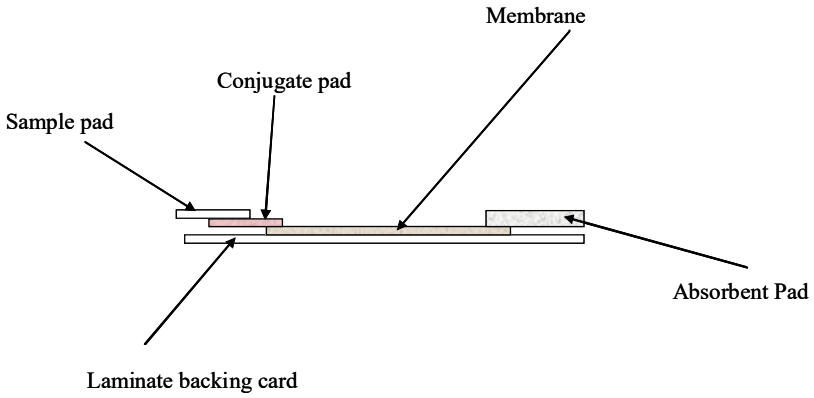


Figure 4. Sectional view of a lateral flow for use in test studies for rapid detection of clubroot resting spores.

4.2.2 Results

When the specific *P. brassicae* monoclonal antibody (EMA 3A5) was present at all but its weakest concentration (1 in 1600) a test line was observed to form on each of the lfds tested (Plate 6). The test line was at its strongest at the lowest antibody dilution (1 in 100) and reduced in strength as specific antibody concentration decreased.

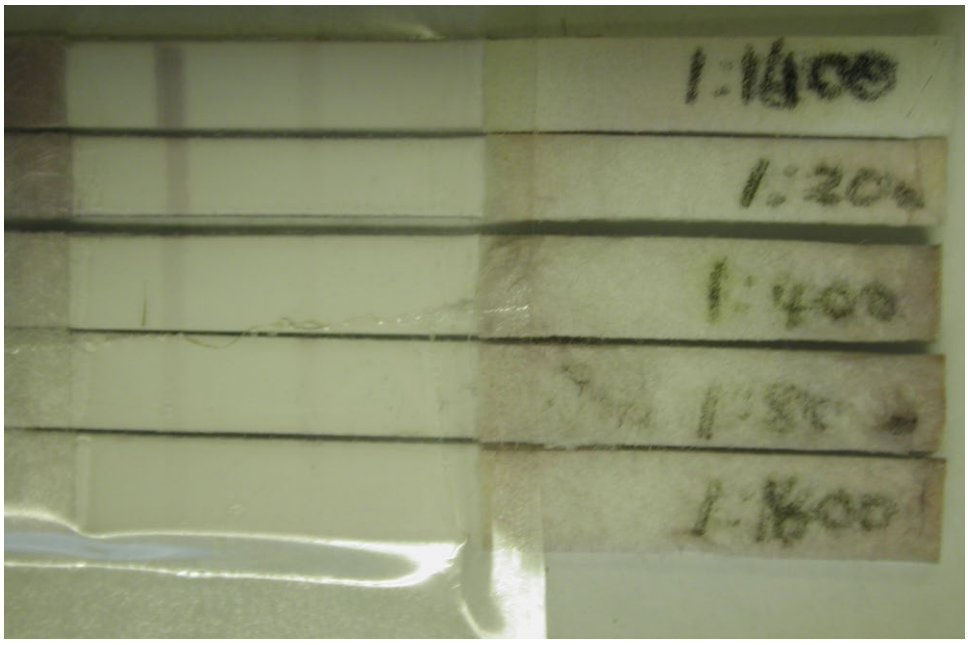


Plate 6. Determining monoclonal antibody activity of EMA 3A5 for optimal binding potential at the test line in a competitive lateral flow format.

4.2.3 Conclusions

In the DAS lateral flow format it had not been possible to develop a system for test line visualisation with the antibody/antigen combinations investigated. This is not an uncommon phenomenon whereby small molecules and or those with a single antigenic determinant are unable to bind to two antibodies simultaneously. Problems associated with stearic hindrance may prevent binding of target molecules using these antibody formats. As a result adoption of a competitive assay system which uses only a single specific antibody type to label and identify the target antigen was successfully investigated. Other lateral flow techniques for fungi have also used this type of format. For example the dark leaf spot lateral flow device (FV233) also adopted this type of approach in the rapid detection of dark leaf spot spores.
In the competitive lateral flow device the level of binding of target spores in a test sample to the specific gold conjugated specific antibody is reflected in the concentration of captured specific gold conjugated antibody at the test line. In these tests no target spores were applied to the LFD sample pad and as a result the specific gold conjugate present in the sample pad was free to bind to the test line. With increasing levels of specific gold conjugated antibody test line strength developed accordingly. Nevertheless in the development of a competitive lateral flow device assay it is important to establish the quantitative limits of the test to provide a meaningful result to the end user. The competitive lateral flow is a dynamic biological system and optimisation of these elements is critical.

4.3 Detection threshold of the competitive lateral flow (*clfd*) for resting spores of *Plasmodiophora brassicae*

4.3.1 Introduction

Investigating the detection threshold of any successful lateral flow format is another step in the development of a rapid test for a target organism. In the competitive lateral flow format a visualisation of the test line was achieved however this requires adjustment to the detection threshold for practical usage. Previous reports indicated that resting spore concentrations in excess of 10^5 g $^{-1}$ soil are required for severe and uniform disease expression on test plants. Therefore it would be necessary for the lateral flow device to be optimised at or below this concentration of clubroot resting spores. Additionally the extraction of resting spores from the soil would also require investigation.

4.3.2 Materials and Methods

A series of lateral flows were prepared as shown in Figure 4 using a Millipore HiFlow240 membrane base system. The sample pad of each assembled lateral flow antibody concentrations of an EMA 3A5 gold conjugate (at a dilution of 1 in 177) had previously been applied in sample application buffer and air-dried. Sample pads were put within a series of lateral flow devices. Approximately 80µl test samples of clubroot resting spores ranging

from 1 x 10^3 spores ml^{-1} to 1 x 10^8 spores ml^{-1} was aliquoted to each flow device. After a 20 minute development time test line development was confirmed using a BioDot lateral flow reader. A negative control of lateral flow running buffer alone (0 resting spores) was also included within theses tests.

4.3.3 Results

4.3.3.1 Effect of antibody/conjugate concentration on test line development

When a negative sample (0 spores) was applied strong test line development was observed only when a dried gold conjugated specific antibody dilution of EMA 3A5 at 1 in 200 had been applied at or above 24µl in test volume to the sample application pad. When specific antibody volume exceeded 37µl in application buffer to the sample pad test line formation was observed for both negative and positive samples alike. Between these antibody concentrations of gold conjugated EMA 3A5 (24 to 37µl sample pad application) a difference in the development of test line strength was observed (Table 5).

Table 5 Reaction of the test line of the Millipore HiFlow™ Membrane 240 at different concentration of antibody conjugate to a dilution series of clubroot resting spores

	No *P. destructor* spores in sample						
EMA 3A5 Ab dilution at 1 in 200	0	1x10^3	1x10^4	1x10^5	1x10^6	1x10^7	1x10^8
24 µl applied	✓✓	✓✓	✓✓	✓×	×	×	×
27 µl applied	✓✓✓	✓✓✓	✓✓✓	✓	×✓	×	×
30 µl applied	✓✓✓✓	✓✓✓✓	✓✓✓✓	✓✓	✓×	×	×
33µl applied	✓✓✓✓✓	✓✓✓✓✓	✓✓✓✓✓	✓✓✓	✓✓	×✓	×
37µl applied	✓✓✓✓✓	✓✓✓✓✓	✓✓✓✓✓	✓✓✓	✓✓	×✓	×

✓✓✓✓✓ Intense test line development
✓✓✓✓ Strong test line development
✓✓✓ Clear test line development
✓✓ Visible test line development
✓ Poor test line development
×/✓ Weak test line development (barely visible)
× No test line development

Using a Bio-dot lateral flow reader a 27µl volume of EMA 3A5 1 in 200 gold conjugate applied and dried to a competitive lateral flow device sample pad proved optimal in the current assay format for a semi-quantitative assay for resting spores of *P. brassicae* (Figure 4).

4.3.3.2 Optical density measurements

By decreasing the concentration of the detector antibody (conjugated gold EMA 3A5) test sensitivity for *P. brassicae* resting spores increased (reaction visualised by reduction in test line formation). However test line development strength for negative samples was compromised accordingly with no test line formation for any samples observed when antibody volume was below 24µl / sample pad. In the current format an optimal antibody volume of 27µl is recommended for the detection of resting spores of *P. brassicae* when read both visually and using electronic optical equipment. The potential for semi-quantitative analysis employing an electronic reader shows potential when optical density values are calculated (Table 6).

Table 6. Optical density values of the test line at varying clubroot resting spore concentrations

Sample concentration (Clubroot resting spores)	Optical Density value
0	9.9
1×10^3	8.0
1×10^4	7.6
1×10^5	6.7
1×10^6	2.4
1×10^7	1.3

In the tests carried out a HiFlow 240 membrane was used. This has a nominal capillary flow rate of 240 s / 4cm travel and is currently the slowest running membrane available for lateral flow construction. A faster run time is considered commercially desirable and to be less prone to background streaking. Nevertheless test line reaction kinetics are key to development of a successful lateral flow device. A membrane with a slower travel time across the test line can prove more sensitive. The reaction rate at the test line capture point decreases with the square of the increase in flow rate. Conversely for a competitive assay this may prove useful and in future tests a HiFlow 180 membrane will be assessed (180s/4cm travel). Ultimately however it is a critical balance of the activity level of the antibody detection molecule and, assay time across the test line that will result in a meaningful test.

4.3.4 Conclusions

The competitive lateral flow format shows great promise as a rapid test for clubroot resting spore detection. The format was clearly able to detect clubroot spores at close to epidemiological significant levels. However the working test will require further optimisation and methods for rapid extraction of clubroot spores from soil will also be required. Further optimisation will be required to address two points. Firstly the cost of the test will need to be examined i.e. reagent costs (detection antibody) and required run time by the end user so that it can be used commercially. Additionally the detection threshold of this test will need further adjustment. A visualisation of the test line (negative result) would be required at around the 1 x 10^4 spores $gram^{-1}$ of soil. At this level the test could prove useful in designating the regimes required for clubroot control. However it is interesting to note that differences in concentration of clubroot resting spores could be detected at a low level using a lateral flow reader device in the absence of the soil matrix.

5. DETECTION OF CLUBROOT SPORES IN ARTIFICIALLY INFESTED SOIL USING POYMERASE CHAIN REACTION (PCR)

5.1 Introduction

There are several challenges to developing a successful PCR assay. It is important that the assay is absolutely specific and of a suitable sensitivity to provide useful information to the end user. The assay system developed must prove infallible within the environment it is designed for use. Efficient DNA extraction protocols are now being used routinely for extraction of DNA from soil yet the complex nature of the soil environment proves challenging in developing reliable and robust systems for detection and quantification of soil borne organisms.

5.2 Determination of the detection sensitivity of the PCR Molecular test

5.2.1 Materials and Methods

5.2.1.1 Soils used in tests

Control soil

A sandy soil, (collected at Warwick HRI, Wellesbourne, Warwickshire) which had not been exposed to vegetable brassica or cruciferous weed cultivation in the past twenty years, was identified and considered to be free of clubroot disease. Soil samples were collected and air-dried at 50°C for a 24 hour period. The soil samples were combined, mixed and then adjusted to a moisture content of 20% using sterile distilled water.

Artificially infested soils

Suspensions of *P. brassicae* resting spores in sterile distilled water were prepared. These ranged from $5x10^6$ spores ml^{-1} through to $5x10^2$ in ten fold dilution steps. To 10g of air dried control soil 2ml spore suspensions were added in ten fold aliquots. Using a roller board the soils were mixed overnight and then air-dried at room temperature (18-22°C).

5.2.1.2 DNA Extraction

A M0Bio UltraClean Soil DNA isolation kit was used with a 0.3g sample of each control and artificially infested soils and processed according to manufacturers instructions (MoBio Laboratories, POBox 606 Solana Beach, California). To maximise DNA isolation yields the alternative protocol was followed and, a Fast Prep device (Qbiogene, Carlsbad, California, USA) was used according to the manufacturer's guidelines. For each soil a 50µl DNA volume was purified using a method supplied by Dr R. Faggian (DPI, Australia). For each extracted DNA soil extract 10 fold dilutions to 1 in 100 were made in TE buffer (10mM Tri-Cl,1Mm EDTA, pH 8.0).

5.2.1.3 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.

5.2.2 Results

A 1 in 10 dilution of the extracted DNA soil sample proved optimal with amplicon product detected in each of the artificially infested soils (1 x 10^5 to 1x10^3 spores g^{-1} soil) (Plate 9) . Using the extracts neat reduced sensitivity to a detection level of 1x10^4 spores g^{-1} soil whilst a dilution factor of 1 in 100 detected a clubroot amplicon product in samples in excess of 1x10^7 g^{-1} soil.

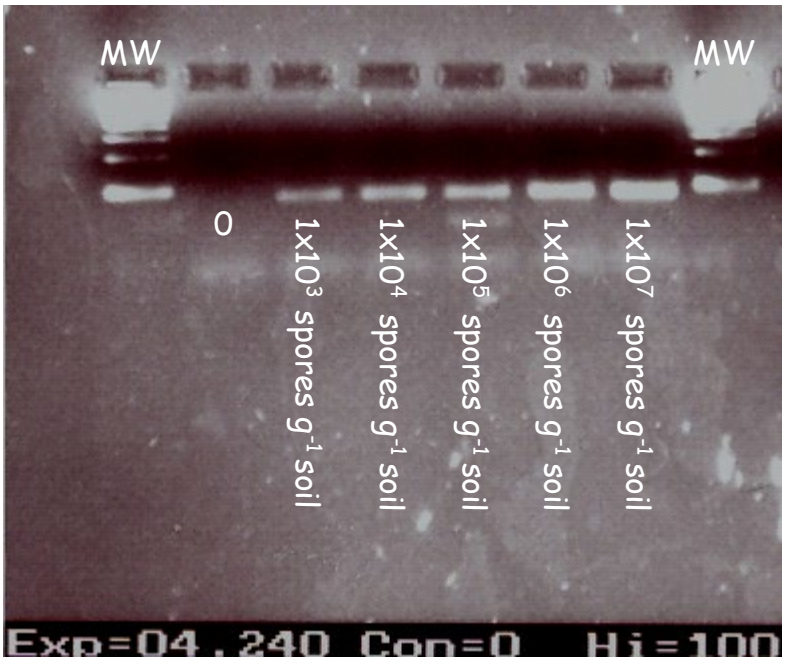


Plate 7. PCR detection of clubroot spore concentrations in artificially infested soil

5.2.3 Conclusions

The molecular test proved highly sensitive in detecting DNA of clubroot resting spores in soil at the lowest level of infestation tested ($1x10^3$ resting spores g^{-1} soil). Detection below this level of contamination was not tested due to the small numbers of resting spores this equated to. However it is likely that the test could detect clubroot at $1x10^2$ resting spores g^{-1} soil. Detection below these levels would be difficult to test because of the dilution factor of clubroot spores in soil. The amplicon product for each soil infestation level appeared quantitative to the initial level of clubroot spores inoculated (Plate 7). Nevertheless the soil tested comprised of a sand base and this may influence the level of resting spores detected in comparative terms with other soil types. Previous studies using the PCR test have reported that there is variation in detection depending on soil type tested (Faggian & Donald, 2005). It is unclear why this variation exists and further studies are being conducted by the DPI to determine the effect of soil type on PCR detection. Further studies will be needed in the UK to determine affected soil types. A 10 fold dilution of the DNA extract proved optimal for detection of clubroot spores of *P. brassicae* for this soil extraction process.

6 COMPARATIVE STUDIES OF THE DETECTION OF CLUBROOT IN NATURALLY AND ARTIFICIALLY INFESTED SOILS USING MOLECULAR, ANTIBODY AND CONVENTIONAL BAIT TESTS.

6.1 Comparisons of methods for assessment of clubroot resting spore contamination in soil

6.1.1 Introduction

To date a range of tests have been developed for the detection of clubroot spores in infested soils. Conventional methods of detecting the pathogen involve a lengthy indirect test based on observation of gall development on bait plants grown in test soil. The test takes around 6 weeks and the severity of clubroot infestation is based upon the disease expression of the baited plants. As a routine test, although useful, it is laborious, requires considerable amounts of glasshouse space and time, and would prove costly to offer as a practical diagnostic assay for large numbers of field samples.

More recently workers (Faggian *et al*., 1999, Faggian & Parsons, 2002) in Australia have developed a molecular diagnostic test to detect and quantify the presence or absence of *P. brassicae* resting spores directly from soil. This test has been used nationally although a recent study (Donald et al.. 2004) has determined that for a specific soil type the test has proved inaccurate returning false negative results. Other workers have used this molecular assay to test infected root hairs of bait plants grown in commercial soils. However quantification will prove difficult as *P. brassicae* on initial infection of a root hair is uninucleate rapidly develops within the host cell and becomes multinucleate. As a result disease infestation levels can be exaggerated far beyond the level of actual risk.

In this study we have compared techniques for the detection of clubroot spores in soil and examine below the comparative merits of this, using antibody, conventional baiting and molecular based tests for the detection of clubroot spores both in artificially and naturally infested soils.

6.1.2 Materials and Methods

6.1.2.1 History of commercial soil samples used in comparative studies

The history of cropping of all soil samples used in comparative tests is shown in Table 7. Soil A had been taken from a site which had over successive plantings become heavily compacted with poor drainage. In these tests all soils were aerated prior to transplanting. Soil A was high in organic matter (20%) which would assist in the retention of moisture. Cropping history would have suggested increased resting spore concentrations and disease in this soil. The nature of the soil type and the high level of compaction may prove conducive to disease development. Soil 516 had been sampled from a site which in 2000

had a medium to high level of clubroot disease within the crop planted. However grower trials from this site demonstrated that high levels of disease were in areas where increased soil impaction had occurred.

Soil Code	pH	Organic matter (%)	Cultivation History	Clubroot Disease history
A	6.2	20	Cultivation of Brassicas Imperfectly drained soil heavy compaction .	High levels of clubroot in past years
B	7.5	3	Cauliflowers for most years over last 40 years. Some years twice cropped.	Clubroot when present restricted to peat transplant blocks. High level of shell components within soil matrix.
C	6.7	11	No history of Brassicas	No clubroot observed
D	7.1	6	Brassica cultivation	Clubroot present
E	6.5	5	Brassica cultivation	Clubroot present
F	NotTested	Not Tested	Sample taken from Glasshouse	High level of clubroot observed in seedlings
106	5.8	4.92	Broccoli in 2004 and 2 crops in 2005, previous to that it was double cropped in either 1999 or 2000 Rotation of fodder beet and grass.	Clubroot first seen in the first crop in 2005. High level of clubroot in second crop.
206	5.9	4.38	6 year rotation of cereals, broccoli and cauliflower, potatoes	No clubroot seen in 2004 but medium to high levels about 1998
306	5.7	6.76	5 year rotation of cereals, broccoli, cauliflower, potatoes	No clubroot seen in 2006 (put in early specifically to reduce clubroot risk, levels probably not seen due to dry conditions ?).Medium clubroot in 2001 and patches in 1996
406	5.9	4.62	6 year rotation of cereals, broccoli, cauliflower, potatoes	No clubroot seen in 2000
506	6.3	6.82	Broccoli at least 3 years in the past 10 with at least 2 of those being double cropped. Rotation of cereals, broccoli, cauliflower, potatoes and one	Medium/High clubroot levels.

			year of carrots. Very high population of shepherds purse	
606	6.3	6.61	Cereals and potatoes, maybe the odd crop of oilseed rape.	No history of clubroot
706	6.3	4.93	probably due to the early planting of this field. Rotation cereals, broccoli, cereals, broccoli etc	High clubroot levels in 1998/9 but since then medium levels.
516	6.0	6.47	Rotation of cereals, potatoes and veg.	High levels in large areas in 2000

Table 6 Structure, disease and cropping history of commercial soils used in diagnostic studies

6.1.2.2 Conventional bait testing

Control soils

A sandy soil, which had not been exposed to vegetable brassica or cruciferous weed growth in the past twenty years, was identified and considered to be free of clubroot disease. Soil samples were collected and air-dried at 50°C for a 24 hour period. The soil samples were combined, mixed and weighed in to 80g lots. After which each was adjusted to a moisture content of 20% using sterile distilled water. Soils were added with a 1/12 dilution of sand and then evenly distributed between 8 FP9 pots. Three clubroot free *Brassica napus* transplants were added to each pot. Following a six week period in a glasshouse at 18°C, seedlings were examined visually for incidence and severity of clubroot symptoms (Plate 10).

Plate 10. Seedlings of *Brassica napus* showing clubroot severity ratings of 0,1,2, and 3.

Commercial soils
Fourteen commercial soil samples were collected and air-dried at 50°C for a 24 hour period. Each soil sample was mixed thoroughly and weighed in to 80g lots. After which each was adjusted to a moisture content of 20% using sterile distilled water. Soils were added with a 1/12 dilution of sand and then evenly distributed between 8 FP9 pots. Three clubroot free *Brassica napus* transplants were added to each pot. Following a six week period in a glasshouse at 18°C, seedlings were examined visually for incidence and severity of clubroot symptoms

6.1.2.3 Standard PCR soil molecular test (*Faggian* et al*., 1999*)

6.1.2.3.1 Soils used in tests

Control soil
A sandy soil, which had not been exposed to vegetable brassica cultivation or cruciferous weed growth in the past twenty years, was identified and considered to be free of clubroot disease. Soil samples were collected and air-dried at room temperature (18-22°C). Each soil sample was mixed thoroughly, combined and weighed in to 0.3g lots.

Artificially infested soils
Suspensions of clubroot resting spores in sterile distilled water were prepared. These ranged from $5x10^6$ spores ml^{-1} through to $5x10^2$ in ten fold dilution series. Ten grams of air dried control soil were added to a 2ml spore fraction in ten fold aliquots. Using a roller board

the soils were mixed overnight and then air-dried at room temperature (18-22°C). Each soil sample was mixed thoroughly and weighed in to 0.3g lots.

Commercial soils
Fourteen commercial soil samples were collected and air-dried at 50°C for a 24 hour period. Each soil sample was mixed thoroughly and weighed in to 0.3g lots.

6.1.2.3.2 DNA Extraction and Amplification by molecular PCR assay

Extraction of DNA and processing by PCR was as described in Section 5.2.1. A 1 in 10 dilution of each soil extracted DNA sample proved optimal for amplicon product visualisation by agarose gel.

6.1.2.4 Soil Immunfluorescence

Control, artificially infested and 14 commercial soils were prepared as described in section 3.1.2.2 and each weighed in to 3 sets of 0.3g lots. To each soil lot 500µl Phosphate buffered saline (PBS) was added and the samples then agitated to ensure thorough mixing. For each soil 8 x 20 µl was aliquoted to each of 8 wells of a multiwell glass slide. Following air drying any unbound material was removed with a PBS, 0.05% Tween 20 and 0.1% Casein wash (PBSTw C). Material remaining bound to each of the multiwell glass slide wells was incubated with 20µl of clubroot specific monoclonal antibody (EMA 3A5) at a working dilution of 1 in 200 diluted in PBSTw C. Incubation was within a humid environment and in darkness for a 30 minute period (minimum). A counterstain (Evans blue / Eriochrome black) was incorporated with the antibody suspension to quench spore autofluorescence. After the incubation period the multiwells were washed as previously described and, following air drying, were incubated with an anti-mouse antibody which had been conjugated to fluorescein isothanyacyte dye (Sigma F0257 Lot 092k9153 (Sigma working dilution of 1 in 125)) The working dilution of the FITC conjugated antimouse antibody was at 1 in 64 and diluted in PBSTw C . A counterstain was again included to ensure quenching of resting spore autofluorescence. Incubation was carried out at room temperature and in darkness to prevent photo-bleaching of the conjugated antibody. The processed microwells received a final wash of PBSTwC and after air-drying were mounted (DAKO fluoroscein mounting fluid) and viewed by episcopic fluorescence for the presence of antibody / fluorescein tagged spores.

6.1.3 Results

6.1.3.1 Assessment of clubroot resting spore concentration in soil using bait tests

No clubroot symptoms were recorded on the plants grown in the non-infested control soils. Clubroot ratings of 1, 2 and 3 were seen on some plants grown in commercial soils however considerable variation in disease incidence between soil types was observed (Table 8). Six soils were identified where roots were predominantly healthy with a small percentage of roots with marginal thickening (score rating of 1). Soil E, and soil 106 exhibited a high level

of clubroot disease incidence with an excess of 70% plants with a clubroot score rating of 2 or 3. Soil sample 706 also exhibited a high level of clubbed transplants with an excess of 30 % of transplants affected at score ratings of 2 or 3.

	% Healthy	% Clubroot Incidence		
Commercial soils	0	Clubroot disease index 1	Clubroot disease index 2	Clubroot disease index 3
A	90	5	2.5	2.5
B	80	20	0	0
C	95	5	0	0
D	92.5	5	2.5	0
E	20	7.5	10	62.5
F	79	13	8	0
106	0	0	29	71
206	92	8	0	0
306	71	21	4	4
406	92	8	0	0
506	92	4	4	0
606	95	4	0	0
706	46	21	12	21
516	92	8	0	0

Table 8 Percentage healthy plants and those showing clubroot root infection

6.1.3.2 Assessment of clubroot resting spore concentration in soil using PCR tests

At a 1 in 10 dilution of the extracted DNA soil optimal amplicon product was detected semi-quantitatively in each of the artificially infested soils tested (1 x 10^7 to 1x10^3 spores g^{-1} soil) (Plate10a). Clubroot amplicon product was observed for most of the soils tested but with the exception of Soils C, 206, 606 and 516. (Plate 10 a,b,c).

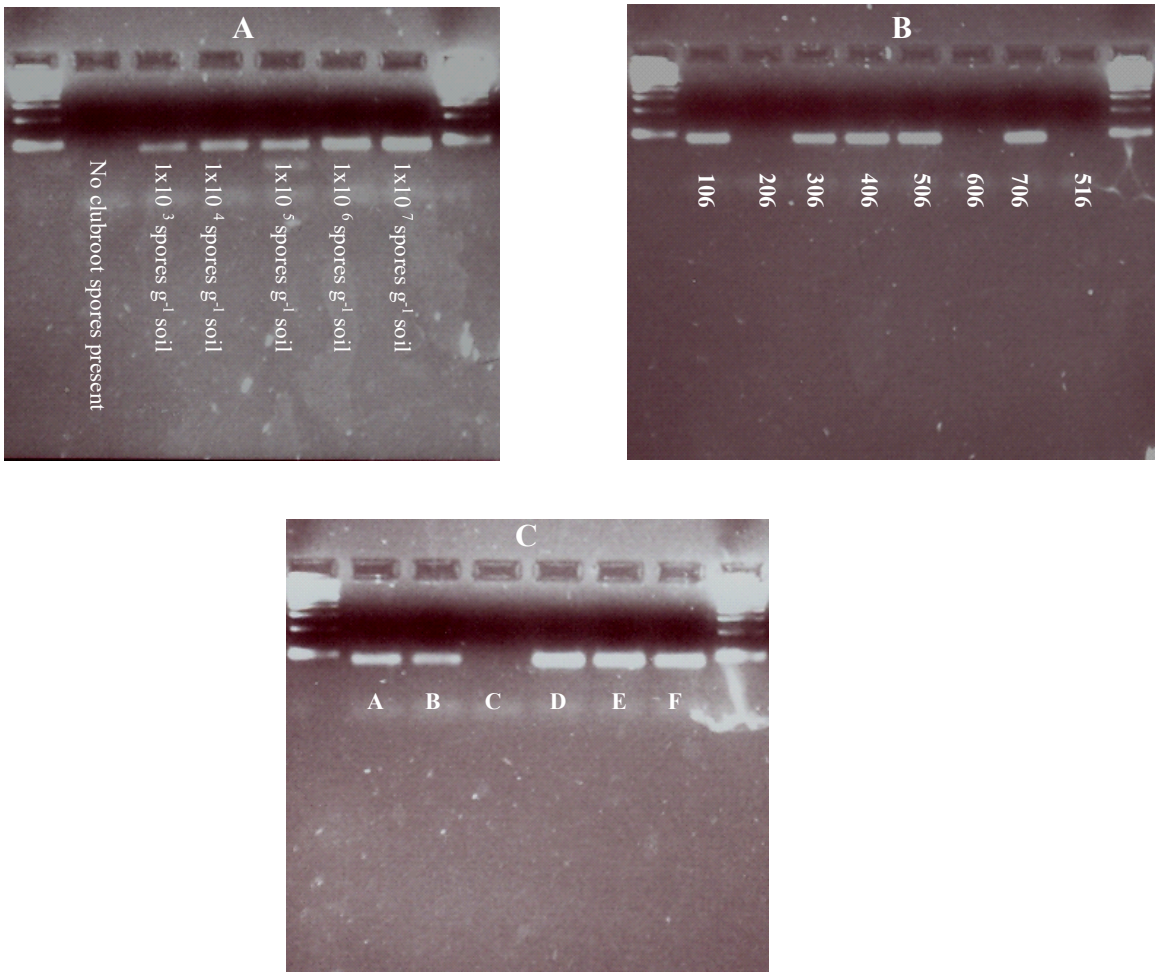
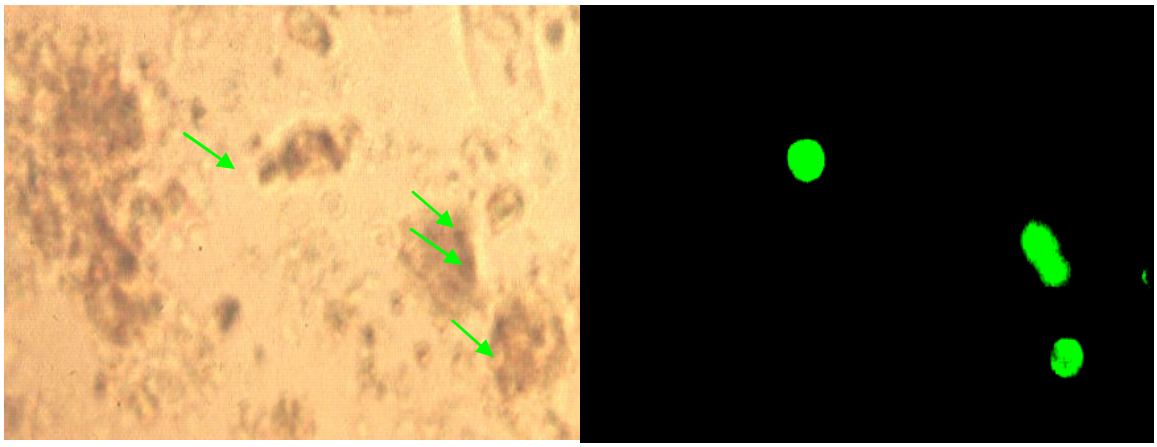


Plate 8 **PCR molecular tests of soil samples inoculated with dilutions of clubroot resting spores (a), and PCR tests of commercial soil samples (b, c).**

6.1.3.3 Assessment of clubroot resting spore concentration using soil immunfluorescence

Resting spores of clubroot were visualised readily in both artificially (Plate 9) and commercial soils.



(A) (B)

Plate 9. Clubroot resting spores in artificially infested soil labelled with specific monoclonal antiserum EMA 3A5 and tagged with fluorescein isothanyacate as seen under (a) bright field and (b)ultra violet light.

In soils considered to be free of clubroot no resting spores were detected. Generally low background fluorescence attributable to soil particulates or other components of the soil microflora was observed. The exception to this was for commercial soil 516 where particulate specific fluorescence material was observed. Resting spore fragmentation was considered as a result. From the visual identification of resting spores of *P. brassicae* in artificially infested soils it was possible to predict resting spore numbers in the commercial soils assayed (Table 8).

Soil	Mean No. resting spores / microtiter well	Predicted spore numbers g^{-1} soil.
A	20	1.3×10^3
B	8	1×10^3
C	0	0 or $< 1 \times 10^3$
D	68	3.6×10^5
E	708	8.8×10^6
F	Not tested	-
106	773	9.6×10^6
206	37	9.2×10^4
306	96.5	5.1×10^5
406	34	6×10^4
506	40	1×10^5
606	0	0 or $< 1 \times 10^3$
706	381	4×10^6
516	*	*

*** damaged / fragmented resting spore material**

Table 9 Clubroot Immunofluoresecence counts in commercial soil samples

	Clubroot resting spore number

Artificially infested soil sample (Clubroot resting spore density)	estimated by Immunofluorescence
0	0
1 x 10^2	0
1 x 10^3	7
1 x 10^4	15
1 x 10^5	40
1 x 10^6	148
1 x 10^7	801

Table 10 Clubroot Immunofluoresecence counts in artificially infested soil samples ($1x10^3$ equates to 1000 spores g^{-1} soil, $1x10^4$ equates to 10000 spores g^{-1}soil, $1x10^5$ equates to 100000 spores g^{-1} soil, $1x10^6$ equates to 1000000 spores g^{-1} soil, $1x10^7$equates to 10000000 spores g^{-1} soil)

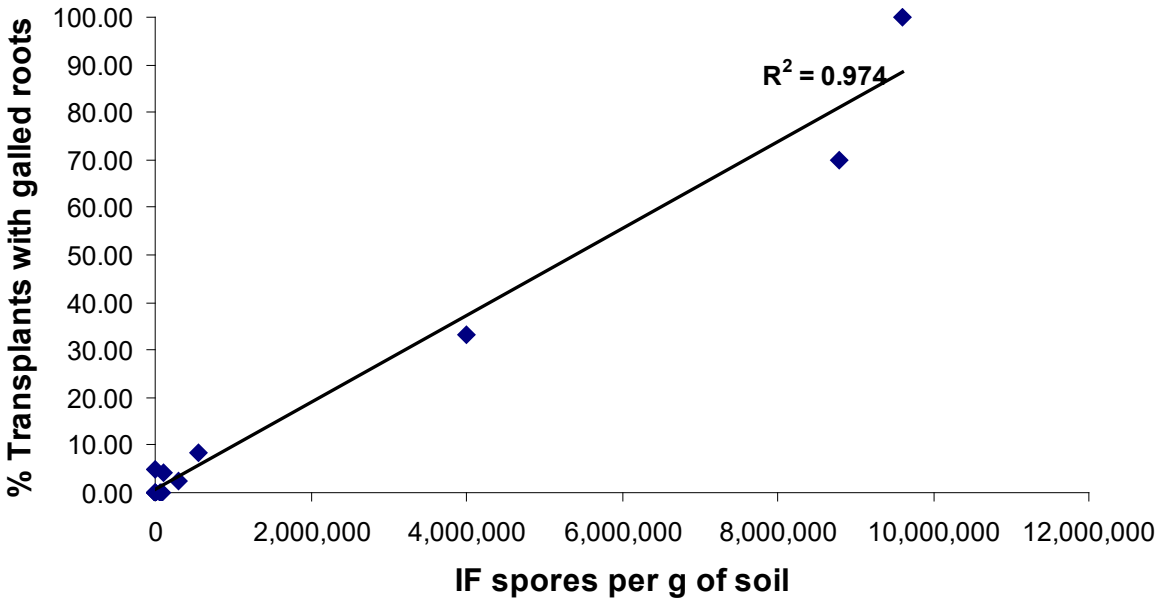


Figure 5. Relationship between Immunfluoresence predicted resting spores levels in commercially tested soils and galled roots of soil exposed bait transplants.

A strong linear correlation between intact whole resting spores in soil, as identified by immunofluorescence (monoclonal antibody fluorescein labelling of spores in soil), and disease development on transplants was observed for each of the commercial soils tested (Figure 5). Nevertheless for disease development to occur uniformly on transplants very high levels of resting spores in soil were required (>5,000,000 resting spores / g soil). At resting spore concentrations below 1x 10^6 spores /g soil (< 1,000,000) the soils tested showed only a few plants infected and showing clubroot symptoms. At these levels there would be little or no economic impact of the disease.

6.1.3.4 Analysis of commercial soil samples using Immunoflorescence, baiting test and PCR molecular tests

Two soils were identified by all three clubroot diagnostic tests as negative for clubroot these were soil C and soil 606 (Table 7). Soil C had been taken from a garden where ornamental plants had previously been grown and had no known history of cruciferous cultivation. Soil 606 originated from land that had previously been cropped with cereals and potatoes. The soil may in the past have been exposed to an oilseed rape crop although this could not be confirmed. Soil A had relatively low numbers of resting spores as detected both by PCR and immunofluorescence (1300 spores g^{-1} soil) and 95% of transplants were recorded as healthy.

With the exception of soil 206, the molecular PCR test identified all commercial soils that had been predicted by the immunfluorescence (IF) test to have intact clubroot resting spore presence. Where low soil infestation was observed by IF (less than $1x10^4$ resting spores g^{-1} soil) reduced amplicon product was noted for the PCR test. For soils of high resting spore concentration, both by IF and bait tests intense amplicon product was noted indicating the potential for quantitive analysis. Work is currently in progress to assess a real time quantitative PCR test (Faggian and Parson, 2002) where for these soils actual spore concentrations can be estimated instead of visual amplicon product. In examining soil 206 both the transplant bait test and the PCR test returned a negative result. The antibody IF test however identified a resting spore concentration of 9.2×10^4 resting spores g^{-1} soil. At this spore concentration, in relation to the results observed with the other soils, little or no disease would be expected on the exposed transplants. The variability that exists between the soil samples tested and the small volumes assessed by PCR and IF (0.3g) may explain the differences observed at this potentially low spore concentration. The cropping history for this soil showed a medium to high level of clubroot on cropped plants in 1998 but in 2004 plants appeared healthy.

Two soils were identified (106 and D) by bait transplant testing as being of high clubroot risk. Six soils were seen to have no disease risk. Using the PCR test soils could be separated in to high, medium, low and 0 risk dependent on amplicon product and intensity (Plate 8a,b,c) . Eight of the fourteen soils (D,E,F,106,306,406,506 and 706) were identified as high or at medium risk. The immunofluorescence test proved useful in predicting spore numbers in the soil samples assayed. This information and allied with subsequent transplant disease data identified two soils at immediate risk of the disease (106 and D), both at a predicted excess of $1x10^6$ resting spores g^{-1} soil. However the ability to identify spore numbers below this disease inoculum threshold should prove useful in cropping and disease management strategies to control the disease already present in those identified soils. Nevertheless work in progress using the modified PCR test will also enable determination of spore numbers. Future work should however examine the potential of both the PCR and IF tests to differentiate between viable and non-viable resting spores in soil

over time. This of importance given the rotation periods used and the decline in disease observed at some of the sites tested (Table 7).

Soil 516 identified by IF as having a high level of damaged/fragmented resting spore material produced healthy plants by the bait test method and, using the molecular PCR assay, was predicted as being free of the disease. It is probable that damaged resting spore material in soil and over time would not provide DNA for identification purposes by PCR molecular detection. Examining the cropping history of this commercial soil showed that in 2001 when this field was last cropped with Brassicas a high level of clubroot was observed. At a concentration of $1x10^5$ intact resting spores g^{-1} soil over 95% of the transplants were recorded as healthy.

6.1.4 Conclusions

The bait test for clubroot detection is in practice impractical for use in detecting low levels of clubroot contamination as large numbers of bait plants would be required to detect the possibility of one or two clubs arising from a soil sample with low clubroot contamination. Additionally bait tests using directly sown seedlings may give varying results when compared to transplants produced under different modular regimes. Transplants are used commercially for vegetable brassica production. There would be variance in the interpretation of results from different types of bait test when using these to determine control regimes. The degree of accuracy of bait tests therefore depends on the methodology used to operate the bait test. Despite this bait tests were compared to other methods of detection. The immunofluorescence and PCR techniques gave very useful results and were able to detect clubroot resting spores at relatively low levels in soil samples. Additionally the immunofluorescence technique gave a strong relationship between the number of resting spores detected by this method and the number of plants with clubroot symptoms. This information will be very useful in determining the threshold level of resting spore contamination of soil samples which the lateral flow test should give line visualisation at. Of those soils which produced an amplicon product (denotes clubroot DNA presence), when visually compared with the artificially infested soils, inoculum levels would appear to be in excess of $1x10^4$ spores g^{-1} soil. Nevertheless for truly predictive quantification of resting spores levels in field soils the use of real-time PCR (Faggian & Parsons, 2002) must be used. This work is currently in progress and will be evaluated within the year three work in FV259.

6.2 Effect of cropping on the infestation of soil by clubroot

6.2.1 Introduction

Clubroot severity is affected by soil type and soil environmental factors such as pH and soil water potential. Producing gradations of clubroot inoculum and soil environmental factors is not practical at commercial sites where crop rotations are practiced together with clubroot control treatments. Additionally clubroot severity on plants could not always be measured under commercial production constraints. A field site where different levels of inoculum could be produced under different soil conditions was required. This technique could also be used to investigate the effect of cropping regime. Warwick HRI has an clubroot quarantine facility where approaches can be utilised to produce infected area of soil with differing gradients of inoculum. Additionally soil factors such as pH, moisture content and cropping pattern can be varied to produce variable conditions in which clubroot diagnostic tests can be investigated and optimised. This area can also be used to investigate soil sampling regimes.

6.2.2 Material and Methods

Treatment areas and plantings

Three pH areas were situated according to a soil sample taken in the quarantine field during May 2004. These were based on field areas created during trials conducted in 1996. Two plots were sited in each pH area each measuring 10 x 10 m with a 50 cm spacing between plants. Autumn cauliflowers cv. Belot were planted in one plot in each pH area (see Figure 6) during May 2004. Over-wintered cauliflowers cv. Jerome were planted in the other remaining plot in each pH area during August 2004. Plants were harvested in each area and the plant material was mulched into the planting area. New cauliflower transplants were replanted within each plot during 2005.

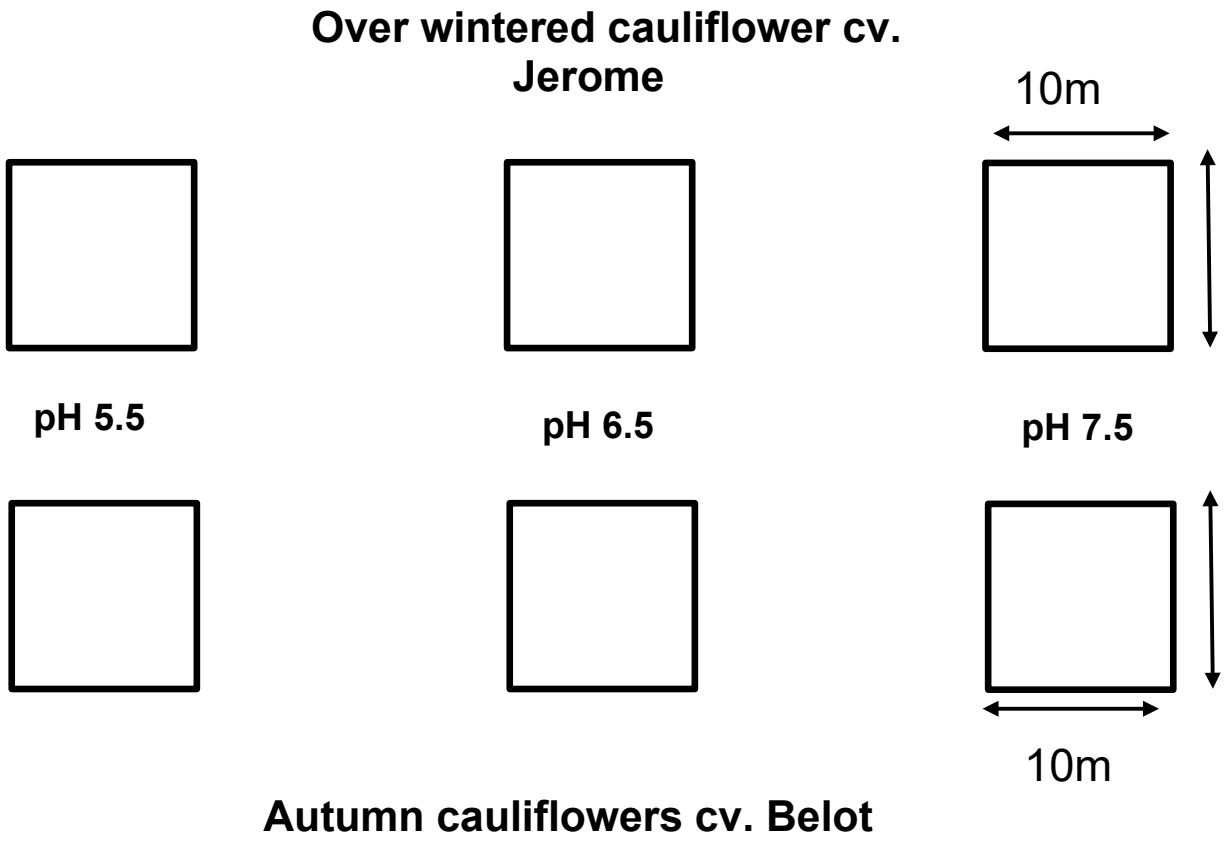


Figure 6 Experimental design of cauliflower clubroot inoculum variation trial area (Wellesbourne 2005)

6.2.3 Results and Conclusions

The level of symptom expression was highest in summer/autumn cauliflower plots planted within the pH 5.5 and 6.5 areas of the quarantine field. There was no symptom expression in either cauliflower plot (summer/autumn or over-wintered) planted in the pH 7.5 area. Soil samples tested for the level of clubroot resting spore contamination by immunoflorescence showed that all areas regardless of PH had high levels of clubroot spores in the soil. Some evidence of higher levels from plots where over-wintered cauliflowers had been grown was observed but it could not be ascertained if this was significant. The results show that summer and winter cauliflower, under equal soil clubroot levels, will develop differential amounts of clubroot infection. The plots will be planted with the same cauliflower varieties in year three of the project and measurements of the level of resting spores in soil samples will be taken. It will be interesting to ascertain if these two methods of production result in increasing divergence in clubroot resting spore contamination of soil.

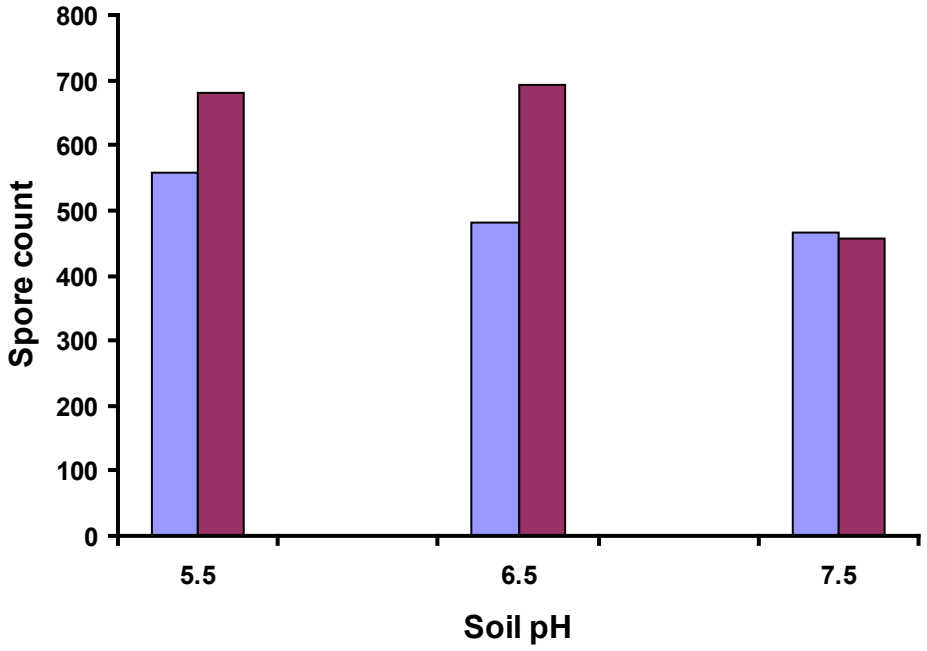


Figure 7 Immunoflorescent clubroot resting spore counts from plots cultivated with summer (■) and over winter cauliflowers (■) under differing soil pH regimes.

7.0 DISCUSSION

7.1 Antibody detection of clubroot in soils

This project has successfully developed two highly specific monoclonal antibodies which recognise clubroot resting spores. These antibodies recognise cell wall components of the resting spore of *P. brassicae*. The antibodies raised to clubroot resting spores have been tested for their cross-reactivity with other major soil borne fungal species. Although these tests have not been completely extensive they indicate that the antibodies do not cross react with other organisms tested and are of sufficient specificity to be used in the development of rapid immunodiagnostic tests. Immunofluorescence tests conducted using the monoclonal antibody indicates that they react with the resting spore wall and can be used directly in soil. Using the immunofluorescence test, which is laboratory based, a resting spore concentration in soil from 1x 10^3 to 1x 10^7 could be quantified and this is in the concentration range which will result in economic losses to vegetable brassica growers. This type of test is extremely sensitive for clubroot resting spores and is useful for determining the accuracy of other clubroot diagnostic tests. At these levels of sensitivity it is close to that achieved using the PCR molecular test.

7.2 Using molecular tests for clubroot in UK soils

The molecular tests used in this report have been developed by the DPI, Victoria, Australia. Molecular tests can confirm the presence of clubroot at low levels in soil samples. In previous work (Fv 259 Annual report 2005) the test was successfully validated using clubroot contaminated and uncontaminated soil samples from Lancashire. The use of this test has been successfully extended for use on soil samples from other brassica growing areas. Molecular tests have been conducted to determine the limit of clubroot detection in soil artificially infested with clubroot resting spores at a range of concentrations. The molecular test was not used to detect clubroot contamination below 1 x 10^3 resting spores per gram of soil. Detection below this level of contamination was not tested due to the small numbers of resting spores this equated to per gram of soil. However it is likely that the test could detect clubroot at 1x10^2 resting spores g^{-1} soil. Detection below these levels would be difficult to test because of the dilution factor in soil. Low numbers of resting spores within dilutions are difficult to replicate and so samples may not be of insufficient accuracy to ensure that they were contaminated at 1 x 10^2 or below.

7.3 Developing lateral flow detection tests for clubroot resting spores in soil.

One of the key issues in determining the occurrence of the clubroot pathogen is due to the lack of availability of a reliable method for detecting the pathogen. Classical methods of detection have used lengthy plant assays, but this has limited the number of samples that can be processed and lacks the sensitivity to detect very low levels of inoculum reliably. To identify both the source of inoculum in field soils, which leads only to sub-clinical levels of disease, demands the use of highly sensitive and quantitative systems for the detection of *P. brassicae*. These techniques need to be capable of handling a reasonably large number of samples from the field to ensure comprehensive testing and, more or less, guaranteed freedom from clubroot inoculum or the presence of inoculum below a detectable threshold. Molecular techniques described above have been developed by the DPI, Victoria, Australia which could be used to detect clubroot resting spores in soil. However using these tests to detect UK strains of the pathogen would also be useful. Clubroot strains found in the UK appear to be able to tolerate significantly higher soil PH values and may have adapted to UK production techniques. Validation of these molecular methods for UK clubroot isolates has within the second year of the programme enabled the investigation and the usage of rapid "in field " based techniques for clubroot detection. Using results from molecular tests across soil types has provided a basis on which the accuracy and sensitivity of rapid "in field" immunological tests used on soil samples could be tested.

7.4 Optimisation of lateral flow device for clubroot resting spores

Development of lateral flow tests has been undertaken in year two of the project. The competitive lateral flow format proved to be the most successful for detection of clubroot resting spores. Several formats were tested however lateral flow formats based on the movement of resting spore material up the lateral flow membrane were not successful (DAS formats). However the competitive lateral flow assay for clubroot resting spores proved very sensitive in its reaction to epidemiologically significant numbers of clubroot resting spores within test samples. These tests were carried out on relative clean concentrations of clubroot resting spores isolated from infected clubbed roots. The lateral flow device when used on these samples could detect by the visualisation of the test line a concentration of approximately 1×10^5 spores $gram^{-1}$ of soil. In this format the presence of a test line indicates the absence of clubroot resting spores in a sample. These levels of clubroot resting spores are significant epidemiologically and commercially within vegetable brassica production. It is also interesting to note that resting spore concentration below that giving a line visualisation could be estimated using a lateral flow reader device. With this facility the test becomes semi quantitative in nature. However further optimisation of the device is planned within the year three work in FV 259 to explore these possibilities further.

7.5 Practical usage of the clubroot resting spore lateral flow test under field conditions

Validation of the lateral flow device in the field on soil samples is necessary if the test is to be useful. It is unlikely that soil solutions could be used directly within the device due to the potential for clogging of the sample pad. The soil sample would be required to be collected and treated to separate the clubroot resting spore containing fraction from other soil materials. Soil extraction processes which could be used under field conditions to separate clubroot resting spores in soil are currently under investigation by the DPI, Victoria, Australia. Substances are available which can achieve separation of soil fractions by specific gravity gradient. Using this material differential separation in soil might be achieved by adding these substances directly to the soil sample within a vial. The contents with vial with the added gradient separation material would be shaken and a specific amount of the required fraction removed and placed in buffer before transfer on to the sample pad of the fully optimised clubroot resting spore lateral flow device. If this technique was successful the results of the test would be visible as lines on the lateral flow device. This would indicate the presence of clubroot resting spores above a specific concentration in the sample. By changing the format (run time) the sensitivity of the device can be adjusted however for initial field trials investigating the usage of the test the current sensitivity may be advantageous to establish a base line reaction. The use of a reader device would appear to make this test semi-quantitative. By using the existing sensitivity, the system has the potential of detecting the clubroot contaminated soil. The grower/consultant could use this test to ascertain the need for control treatments. It is hoped that the system will improve the options for control of clubroot.

7.6 Clubroot control criteria based on inoculum detection

Information on the presence of threshold levels of clubroot inoculum would be particularly useful in reaching decision about clubroot control in areas where the clubroot status of production areas is unknown. The use of the test may also be worthwhile in preventing disease transfer between clubroot infected areas and those which are clubroot free. Some studies with the clubroot pathogen have shown that pathogenic inoculum needs to be above critical levels before disease problems occur. It is likely that this also applies to the likelihood of clubroot transmission from one area to another. This means that damaging disease outbreaks can be predicted accurately before they affect the crop giving time for the grower to take action to control potential transmission or disease outbreaks. Clubroot disease predictions will therefore be based on the presence or absence of damaging levels of clubroot resting spores in standard soil samples. Knowledge of the level of clubroot within the field would be very important in informing the grower about which clubroot control treatments might be successful. This in many years would be determined by the economics of crop production. Some treatments might be relatively uneconomic unless certain control criteria were implemented. To facilitate this type of information in year one the clubroot infected field at Warwick HRI was planted with different cauliflower crops. These were placed in different areas building on earlier trials where differing pH treatments (produced by liming) were introduced into the field. The effect of continuous cropping of vegetable brassicas on the build up of clubroot resting spores in soil can be investigated. This might produce information (models) on when cropping patterns would produce economically

important levels of resting spores in soils. It might also inform the use of control treatments involving the use of effective chemicals such as cyazofamid (Ranman) and fluazinam (Shirlan), if approved, in combination with pH amendments and other cultural control methods. It is unclear at this point whether the test would be best deployed to determine within or between field variation. However these considerations would have a significant impact on options for control.

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