FINAL 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 DETECTION TESTS FOR CLUBROOT

Final report 2007

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Signed on behalf of: Warwick HRI

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FV 259: BRASSICAS: DEVELOPMENT AND VALIDATION OF DETECTION TESTS FOR CLUBROOT

GROWER SUMMARY

Headline

Rapid tests for clubroot (lateral flow devices) have been successfully constructed and used for detection of clubroot resting spores in water and directly in soil samples. Lateral flow devices have been compared to other methods of detecting clubroot resting spores.

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 dependent 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:

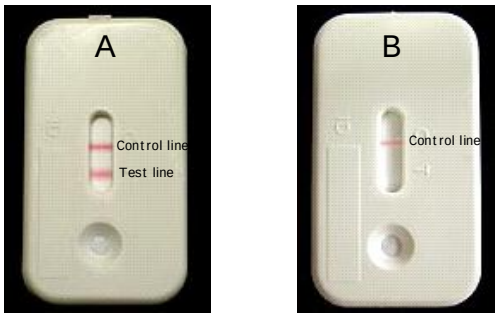
- Better detection of clubroot in the field before disease is visible in the crop.
- Detection tests which can be used “in field” to determine the level of risk to the brassica crops posed by clubroot resting spores.
- Determining the level of risk of crop loss in soils before crops are planted.
- Monoclonal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

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. The competitive lateral flow test format was successful in detecting 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.

Figure 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 extracts, water) 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 (as 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 will also form showing that the test has been completed satisfactorily.

Detection threshold of the competitive lateral flow for clubroot resting spores in soil

The competitive lateral flow format could be used as a rapid test for clubroot resting spore detection. The format was clearly able to detect clubroot spores at close to epidemiological significant levels (1×10^4 spores gram^{-1} of water). At an optimal 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 1a and b). The device can be used in both soil and water. The accuracy and variability of the test line would be lower in water and could be used to check irrigation systems within propagation.

Table 1a: Optical density values of the test line at varying clubroot resting spore concentrations in buffer

Sample concentration (Clubroot resting spores gram^{-1})	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

Table 1b: Optical density values of the test line at varying clubroot resting spore concentrations in soil

Sample concentration (Clubroot resting spores gram^{-1})	Optical Density value
0	3.6
1×10^3	4.1
1×10^4	3.2
1×10^5	1.5
1×10^6	0.8
1×10^7	0.6

However the working test will require further optimisation and investigation in different soil types. The detection threshold of this test will need further adjustment. A visualisation of the test line (negative result) would be required at around the 1×10^4 spores $gram^{-1}$ of soil or below. At this level the test could prove useful in designating the regimes required for clubroot control. Tests using the device directly with soil samples also gave satisfactory results (Table 1b).

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.

Action points for growers

Specific action points for growers are:

- Limited further testing of LFD devices will require grower cooperation during 2008. Growers wishing to cooperate to contact Roy Kennedy at Warwick HRI, Wellesbourne.
- Molecular tests (laboratory based) for clubroot detection in soil and water samples will be available for growers during 2008
- 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.

SCIENCE SECTION

INTRODUCTION

The clubroot pathogen infecting vegetable Brassicas

Clubroot caused by the protist *Plasmodiophora brassicae* Woronin is one of the most intractable plant pathological problems of cultivated cruciferous crops world-wide. Despite such significance the disease has resisted many attempts to develop economic and environmentally safe agrochemical controls. 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. Once land is infested with the clubroot organism eradication is extremely difficult due to the persistence of viable resting spores in the soil.

Biology of clubroot on vegetable Brassica crops

The process of infection and subsequent growth and development of the pathogen within host tissues is still not completely understood. Infection occurs through the root hairs by the infective agent, the zoospore. Development within the root is ascribed to uninucleate and multinucleate plasmodia (Ingram and Tommerup, 1972). Plasmodia form secondary zoosporangia which liberate secondary zoospores. These are liberated into the soil from the root hairs where they migrate to the base of the root hair and re infect. Secondary plasmodial structures are formed in re-infected tissues and become multi-nucleate to form galls on the root within which resting spores are formed. 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. Infected plants and soil are transported widely within modern vegetable production and this could contribute to spread of disease to new areas of production.

Control of clubroot on vegetable Brassica crops

Traditional control measures include improving drainage, liming to raise soil pH and rotation of susceptible crops with non-cruciferous crops (Karling, 1968). Other treatments tested include partial soil sterilisation, pre-planting incorporation of fungicides into soil and fungicide treatment of seed, transplant roots or plant-raising modules. Partial soil sterilisation is effective (White & Buczacki, 1977) but the treatments are costly and hence their use has mainly been restricted to plant raising beds. Chemicals which in the past have given partial control as soil incorporations include a thiabendazole/iodophor complex, benomyl, thiophanate methyl, carbendazim, calcium cyanamide, dazomet, manganese-zinc-iron-dithiocarbamate, a phenolic mixture, sodium tetraborate and thiophanate-methyl (Buczacki, 1973; Buczacki, Cadd, Ockenden & White, 1976; Dixon & Wilson, 1983, 1984, 1985). However, many of these chemicals no longer hold approval for use on vegetable Brassica crops. Partial disease control has been reported with slurry or dip applications to the roots of transplants with thiophanate-methyl, Hg_2Cl_2 or benomyl (Jacobsen & Williams, 1970; Dixon et al., 1972; Buczacki, 1973). The latter chemical was ineffective when formulated as a seed pellet (Buczacki, 1973). The increased use of modular systems for raising brassicas has presented the opportunity to incorporate chemicals into the module compost or to apply treatments to the modules before planting. Dixon & Wilson (1985) tested thiophanate-methyl as pre-planting soaks of the module compost. The latter chemical reduced disease severity but thiophanate-methyl did not. Ann, Channon, Melville & Antill (1988) obtained good protection against clubroot by incorporating mercurous chloride (Hg_2Cl_2) into the module compost before sowing. Useful control was also obtained with a pre-planting compost drench with a mixture of thiophanate-methyl and captafol however all of these chemicals have now lost their approval for control of clubroot in vegetable Brassica crop productions because most are highly persistent or contribute to human and animal health problems due to their toxicity. Currently only two chemicals (cyazofamid – Ranman and fluazinam – Shirlan) approved for control of disease in potato crops have been demonstrated to have any potential for controlling clubroot in the field. Ranman does not hold approval for control clubroot in vegetable brassicas as their efficacy against clubroot has not yet been demonstrated. Alternative control measures are still urgently needed. In glasshouse tests clubroot symptoms on cabbage plants were suppressed by several surfactants and fungicides (Humpherson-Jones, 1989).

Effect of inoculum concentration on clubroot development

It has been reported that calcium, pH and inoculum concentration function interactively affecting processes such as infection, colonisation and symptom formation (Webster & Dixon, 1991). Other studies have shown greater percentages of clubbed plants as spore concentrations increased (Macfarlane, 1952, Jones, 1981). In these studies a linear relationship was demonstrated between spore concentration and root hair infection. The slope of the line was influenced by resting spore age. Studies conducted by Webster and Dixon (1991) confirmed these findings however they also reported that the slope of the line was reduced when environmental factors do not favour infection. Macfarlane (1952) also reported a positive correlation between root hair infection level and the percentage of clubbed plants. 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. There would obviously be considerable differences between this epidemiological threshold and that considered as an economic threshold for clubroot development.

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 (Samuel & Garrett, 1945). Other studies have also indicated that while liming an infected field may control the disease if the spore load is low, heavy applications of lime or other control methods may not be effective if the soil is heavily contaminated.

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 samples. 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. Additional information is also required on sampling regimes required to detect different quantities of resting spores within fields.

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 *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.

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 2). 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 fails 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.

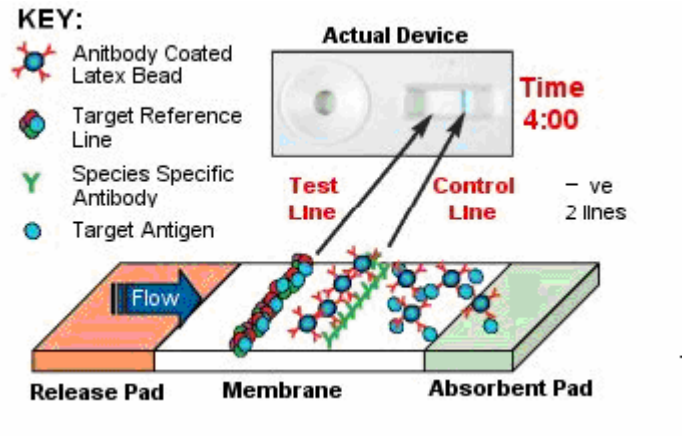


Figure 2: The Competitive lateral flow assay format

The Non-competitive assay format: In a non-competitive assay format the test line generally comprises of an antibody complex which if present, target antigen in the test sample will bind to. The control line consists of a complex matrix which binds material within the sample flow to indicate successful test execution. The release pad and membrane are assembled as descried 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. As 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. Use of reader technology allows the line intensity to be recorded, and therefore level of particulate accumulation to be calculated using reflectance photometry. A number of readers can now be made available for use under field conditions. Some are already commercially available (e.g. EVL reader) but require attachment to a lap top computer. By introducing internal control latex particles to the assay, a standard control line can be produced for use as a reference against the test line intensity. Variations in line intensity can be more reliably distinguished using a reader, facilitating the differentiation between target concentrations.

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

If accurate 'in field' tests for clubroot resting spores are to be constructed they will require specific antibodies that can differentiate between different types of pathogenic spores. 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 which react with the clubroot resting spores. The development and testing of clubroot specific monoclonal antibodies has been an important part of the work. 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 the soil environment. 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. The level of reactivity of the antibody is also important as this can be used to quantify the number of clubroot resting spores in soil samples. In the first year of the work these aspects in the development of the detection system for clubroot are required.

MATERIALS AND METHODS

Production of monoclonal antisera to *Plasmodiophora brassicae*

Production of *P. brassicae* immunogen for antibody production

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 16/14ºC 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 16/14ºC 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 of plants used in each experiment. Plants were inoculated using a 5 ml suspension of clubroot resting spores harvested from field grown cauliflower plants grown in contaminated soil in a quarantine area (QF field) at Warwick HRI Wellesbourne.

Collection of *P. brassicae* resting spores from roots

Washed clubbed root galls (20g fresh weight) from *Brassica napus* (grown in the QF field) were homogenised in 100 ml phosphate buffered saline (PBS) pH 7.2 and filtered through four layers of butter muslin. The filtrate was centrifuged for 5 mins at 2000 x g in an MSE Chilspin (MSE, Crawley, UK). Clubroot resting spores, identified as a distinct layer in the pellet, were collected and resuspended in 20ml PBS. The resulting spore suspension was centrifuged twice (as above) before being resuspended in PBS to a final volume of 20ml. To remove *Brassica napus* host and root contaminants the spore suspension was passed through a range of Spectra Mesh filters (300 µm to 5µm pore size: NBS Biologicals Ltd, Huntingdon, Cambridge, UK; Cat. No. 145/). The collected aqueous phase was then passed through a Millipore filter of 3µm pore diameter (Milliopore Corp., USA; Cat. No. SSWP02500), which retained the resting spores of *Plasmodiophora brassicae* (clubroot) but not the *Brassica napus* host material (cell wall debris). The collected resting spores were resuspended in 10ml of chilled sterile distilled water and adjusted to a concentration of 1×10^9 spores ml. The resulting spore suspension was agitated on a wrist action shaker for 24hrs and then ultracentrifuged at 1500 x g for 15 mins. The spore pellet was discarded and the soluble fraction concentrated by freeze-drying (Modulyo 4K, Edwrds, Crawley, UK) over a 48 hr period. The sample was rehydrated in 1.6 ml PBS.

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 of plants used in each experiment. Plants were inoculated using a 5 ml suspension of clubroot resting spores harvested from field grown cauliflower plants grown in contaminated soil in a quarantine area (QF field) at Warwick HRI Wellesbourne.

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}.

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 1×10^7 cells ml^{-1} in PBS. All spore material was retained at a temperature of 0 - 4°C before ELISA and IF studies were conducted.

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 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 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

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.

Monoclonal Antibody Screening

Plate trapped antigen ELISA (PTA ELISA)

Employing polysorp microtitre well strips (Nunc, Roskilde, Denmark; Cat. No. 469957), 100µl of *P. brassicae* resting spore soluble root extracts in 0.01M Phosphate buffered saline, pH 7.4, were aliquoted in to each of 96 wells. The strips were incubated overnight in an enclosed chamber at 18ºC. 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 37ºC 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). Each well received 100 µl of fusion hybridoma tissue culture supernatant mixed with PBS, 0.05 % Tween 20 and 0.1% Casein. Following incubation in a Wellwarm shaker incubater (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 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. A final fusion (mouse 9014) is currently underway and *P. brassicae* positive hybridomas will be tested and screened as described above.

Determination of clubroot resting spore concentration in soil using molecular methods

Isolation of DNA from *Brassica oleracea* root material

Employing a DNA Easy Plant DNA extraction kit (Qiagen Ltd, Crawley UK; Cat No. 69106) DNA was isolated from the root system of plants which had been assessed for severity of clubroot infection. Plants were assessed using an arbitrary system of disease indices as described by Buckzacki *et al*., (1975). Root material from a healthy plant, scored as 0 (no infection observed), and plant roots exhibiting a score of 1 (low level infection) and 3 (severe clubbing) were chosen (Figure 3). A 5µl volume of isolated DNA from each root sample was adjusted to 5ng, 0.5ng and 0.05ng and each was mixed with 15µl of PCR Master mix (11.35µl H_20, 2.5µl 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; Primer 1 CGCTGCATCCCATATCCAA; Primer 2 TCGGCTAGGATGGTTCGAAA). A number of negative controls were included within the PCR process (Molecular grade water + PCR Master mix and Primers; Plant DNA and Master mix alone). Employing a Hybrid PCR thermal cycler machine the production of sample amplicon products (amplified specific sequence of sample DNA) was facilitated and if present, visualised by Agarose Gel separation. A 2.5% Metaphor Agar Gel was used and, for each PCR sample product, 3 µl was mixed and loaded with 3µl of Bromophenol blue buffer. Molecular weight markers (phix 174) were applied to the outer wells of the gel, at a 1µl sample volume. Electrophoretic separation of sample components was at 150v for approximately 45 mins and, in a continuous ethidium bromide buffer. Employing a transilluminator, emiting light at 302nm, the fluorescent yield of ethidium bromide – DNA amplicon complex was determined and a photographic image recorded (Figure 3).

Figure 3: Seedlings of *Brassica napus* showing clubroot severity ratings of 0, 1, 2 and 3 as indicated

Evaluation of molecular methods for quantification of clubroot resting spores in soil

Isolation of DNA from UK soils

Soil Samples

Control soils: Two soil types, both considered to be free of clubroot disease were identified. A sandy soil, which had not been exposed to vegetable brassica or cruciferous weed growth in the past twenty years and a peat based soil, located at a commercial growers holding site, were collected and air-dried at 50°C for a 24 hour period. The soil samples were adjusted to a moisture contents of 20% using sterile distilled water.

Artificially infested soils: A suspension of *P. brassicae* resting spores was prepared ($5x10^6$ spores ml^{-1}) in sterile distilled water. Each of the above described soil types had ,0.2 ml of the prepared clubroot spore suspension added (to 0.8 g of soil) and, using a roller board, mixed overnight. Prior to DNA extraction the control and artificially infested clubroot soils were air-dried at 50°C for a 24 hour period.

Extraction of DNA from soils

Employing a MOBiol UltraClean Soil DNA Isolation kit 0.3g of each control and artificially infested soil type was processed according to manufacturers instructions (MoBio Laboratories, POBox 606 Solana Beach, California). To maximise DNA isolation yields the alternative protocol, as described in the MOBiol UltraClean Soil DNA Isolation kit product guidelines, was followed. For each sample, the collected DNA was in a total volume of 50µl S5 buffer. A 10µl aliquot of each DNA soil sample extract was retained and stored at -20°C. Of the remaining 40µl DNA volume, samples were divided in to 2x 20µl lots. One of these aliquots was processed using a method supplied by Dr R. Faggian (DPI, Australia) whilst a QIAquick PCR Purification Kit (Cat. No. 28106) was employed for the remaining 20µl aliquot (Table 4).

Table 4: DNA soil extracts as isolated for PCR (polymerase chain reaction)

DNA extraction method	HRI DNA soil extract	HRI clubroot artificially infested DNA soil extract	Lancashire DNA soil extract	Lancashire clubroot artificially infested soil DNA extract
MOBiol UltraClean Soil DNA Isolation kit	10µl	10µl	10µl	10µl
MOBiol UltraClean Soil DNA Isolation kit & DPI protocol	20µl	20µl	20µl	20µl
MOBiol UltraClean Soil DNA Isolation kit & QIAquick PCR Purificatino Kit	20µl	20µl	20µl	20µl
QBiogene Fast DNA Spin Kit	20µl	20µl	20µl	20µl
QBiogene Fast DNA Spin Kit & DPI protocol	40µl	40µl	40µl	40µl
QBiogene Fast DNA Spin Kit & QIAquick PCR Purification Kit	40µl	40µl	40µl	40µl

The soil DNA extraction process was repeated with each of the above soil types but this time a QBiogene Fast DNA Spin Kit (BIO101, 2251, Rutherford Road, Carlsbad, California CA92008) was used for extraction. For each soil sample a 50µL DNA soil extract was collected, 20µl was retained and stored at -20°C whilst the remaining 80µl volume was divided in to 2 x 40µl lots and processed as described above (Table 1). For each collected DNA soil extract (Table 1) 10 fold dilutions to 1 in 100 were made in TE buffer(10mM Tris-Cl,1Mm EDTA, pH8.0).

Preparation of DNA sample extracts for PCR

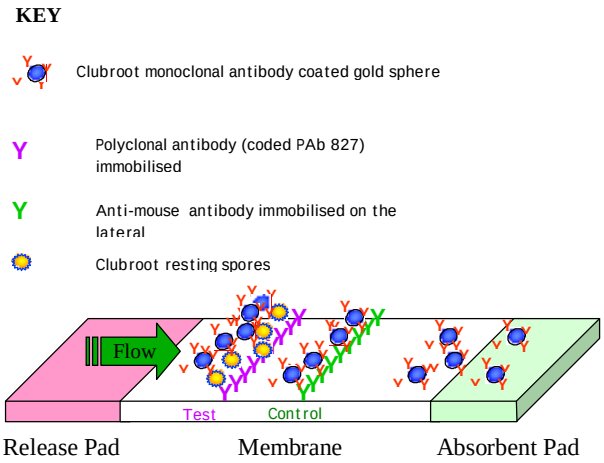
A 5µl volume of isolated DNA from a soil sample extract was mixed with 15µl of PCR Master mix (11.35µl H_20, 2.5µl PCR buffer 10x, 0.75µl 50mg (1.5mM) Mg, 0.2µl DNTP, 0.2µl DNase) and 30ng of *P. brassicae* specific primers (Faggian *et al*., 2003; Primer 1 CGCTGCATCCCATATCCAA; Primer 2 TCGGCTAGGATGGTTCGAAA). A negative control of molecular grade water + PCR Master mix and Primers was included. A positive control of 0.05ng *P. brassicae* DNA root extract mixed with PCR Master mix and primers was also 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 3% Metaphor Agar Gel was used and, for each PCR sample product, 5 µl was mixed and loaded with 3µl of Xylene cythanol buffer. A Molecular weight marker (phix 174) was applied to the gel at a 1µl sample volume. In a continuous Ethidium bromide buffer electrophoretic separation of sample components was at 150v for 25 minutes. Employing a transilluminator, emmiting light at 302nm, the fluorescent yield of ethidium bromide – DNA amplicon complex was determined and a photographic image recorded.

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

Double antibody sandwich test construction and procedure

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 4). 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 4: Schematic drawing of a Double Antibody Sandwich (DAS) lateral flow device *(Positive result shown)*

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 (Figure 5). 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.

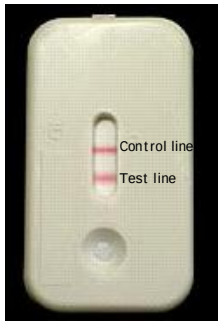


Figure 5: DAS lateral flow test exhibiting a positive result with visualisation of test and control lines

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 5).

Table 5: 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.

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 5) 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.

Competitive assay test construction and procedure

Interpretation of results using a competitive lateral flow test format is the opposite to that expressed using the DAS lateral flow format (Figure 5). 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.

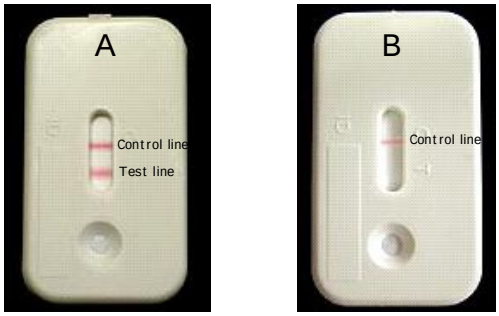


Figure 6: Competitive lateral flow assay showing a positive sample result (B) and (A) a negative sample result

- 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.

Competitive lateral flow assay format

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.

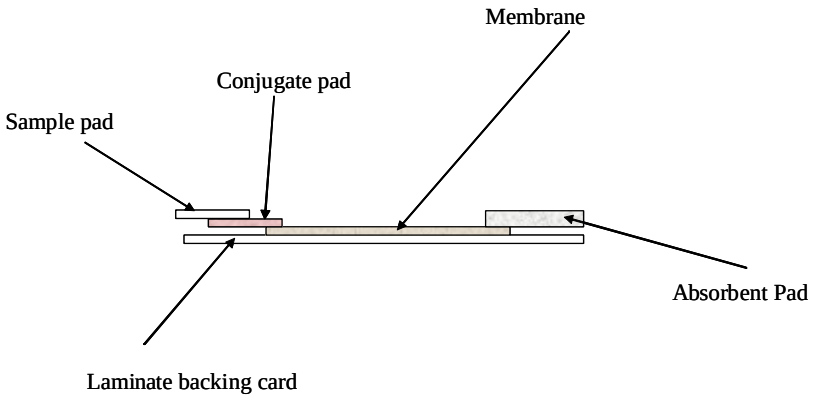


Figure 7: Sectional view of a lateral flow for use in test studies for rapid detection of clubroot resting spores

Membranes and buffers used

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.

Determination of the detection threshold of the competitive lateral flow test

A series of lateral flows were prepared as shown in Figure 7 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.

Determination of clubroot resting spore concentration in artificially infected soil using molecular methods

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).

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, PO Box 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).

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.

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

History of commercial soil samples used in comparative studies in 2006

The history of cropping of all soil samples used in comparative tests is shown in Table 6. 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.

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

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 year of carrots. Very high population of shepherds purse	Medium/High clubroot levels.
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

History of commercial soil samples used in comparative studies in 2007 trials

The origin of soil samples used in comparative tests in 2007 is shown in Table 7. Soil samples were air dried on arrival, before bar-milling through 3mm nominal diameter mesh to improve the uniformity of samples.. Samples had been collected using two methods. A general sample was taken across the field at regular intervals and pooled. In the same field samples were taken from any high risk areas of the field. These could be areas of compaction or poor drainage. These were treated as separate samples.

Table 7: Origin of commercial soils used in diagnostic studies in 2007

Sample Ref	Farm	Field	Received	Field	Pooled Sample (P) Spot Sample (S)
1	Emmett Wales Ltd	Cae Garw	27/06/2007	Pyle Bridge	P
2	Emmett Wales Ltd	Cae Garw	27/06/2007	Sam Lean 1	P
3	Emmett Wales Ltd	Cae Garw	27/06/2007	Five Acre A	P
4	Emmett Wales Ltd	Cae Garw	27/06/2007	Five Acre B	
5	Emmett Wales Ltd	Cae Garw	27/06/2007	Bryngarn2	
6	Emmett Wales Ltd	Cae Garw	27/06/2007	Stones A	
7	Emmett Wales Ltd	Cae Garw	27/06/2007	Stones B	
8	Emmett Wales Ltd	Cross	27/06/2007	Road 1	
9	Emmett Wales Ltd	Cross	27/06/2007	Quarry 1	
10	Emmett Wales Ltd	Cross	27/06/2007	Quarry 2	
11	Emmett Wales Ltd	Comeston	27/06/2007	18 C	S
12	Emmett Wales Ltd	Comeston	27/06/2007	C23 A	P
13	Emmett Wales Ltd	Comeston	27/06/2007	C23 B	P
14	Andrew Buchan		27/06/2007		
15	Andrew Buchan		27/06/2007		
16	Fred Tyler	Lancashire	27/06/2007	Lancashire 1	P
17	Fred Tyler	Lancashire	27/06/2007	Lancashire 2	P
18	Ellis Luckhurst	Mike Waters	04/07/2007	MW12	
19	Fred Tyler	Lancashire	03/07/2007	Cooksons Barn	
20	Fred Tyler	Lancashire	03/07/2007	Hornby	
21	Fred Tyler	Lancashire	03/07/2007	Hunters Lane 2	
22	Fred Tyler	Lancashire	03/07/2007	Special Moss	S
23	East Scotland Growers	Fife	09/07/2007		P
24	East Scotland Growers	Fife	09/07/2007	Dam Park/ Barnsmuir	P
25	East Scotland Growers	Fife	09/07/2007	East Seafield/ Kirkmay	P
26					

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 (Figure 8).

Figure 8: Seedlings of *Brassica napus* showing clubroot severity ratings of 0,1,2 & 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

Baiting Test used for detection of clubroot in commercial soils in 2007

Chinese cabbage cv. Granaat was sown in sterile Vermiculite (sieved 3mm diameter round hole sieve) and in 1 cm modules. Seeds were watered with sterile RO water. Four 7cm square pots were filled with air dried soil for each of the 26 samples (2 pots only for samples 14 & 15 as insufficient soil delivered).Two week old seedlings were transplanted from the vermiculite modules to the pots of soil. Two seedlings were used per pot with four replicates for each sample were placed in a half tray (no holes) to keep the samples isolated (ie. no randomisation). Watering was from the bottom only.

After 6 weeks growth in a cool glasshouse compartment, the seedlings were carefully removed from the soil and the roots washed in running water. Roots were assessed according to the scale below :

0 Normal roots (for size of plant).
1 Galling on stem base, minor thickening of roots away from stem
2 Clubbed roots (away from stem)
3 Severely clubbed roots or root system destroyed (used where root system absent, - this category only assigned when galling was evident).

All plants photographed before 'harvesting', example root systems also photographed.

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

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.

DNA Extraction and Amplification by molecular PCR assay

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

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.

Effect of cropping on the infestation of soil by clubroot

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 9) 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.

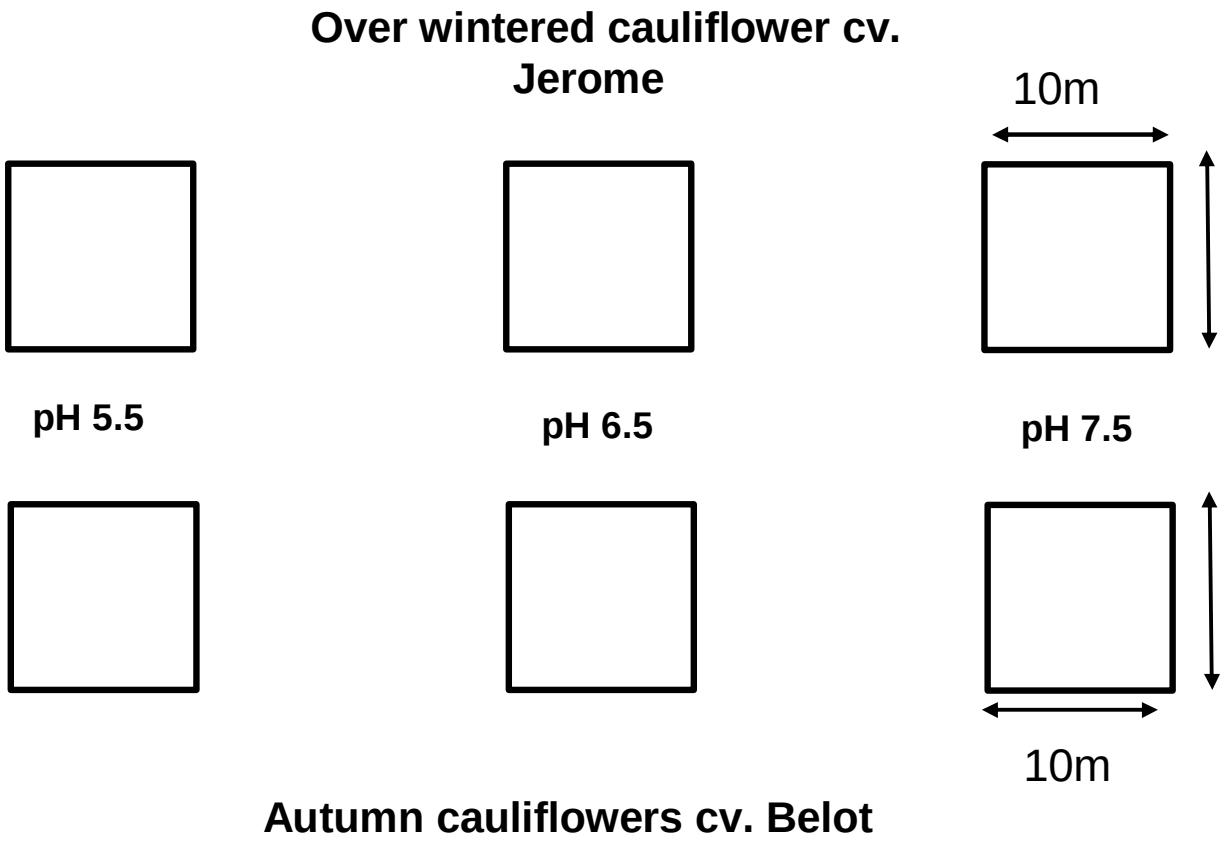


Figure 9: Experimental design of cauliflower clubroot inoculum variation trial area (Wellesbourne 2005)

RESULTS

Production of monoclonal antisera to *Plasmodiophora brassicae*

Immunization

For each of the immunized mice, tail bleeds taken at day 38 in the immunization schedule, showed a good immune response to the homologous antigen (soluble *P. brassicae* resting spore surface washings) when tested by ELISA (Figure 10). Employing immunofluorescence antibodies were identified which recognised components of the resting spore wall of *P. brassicae*

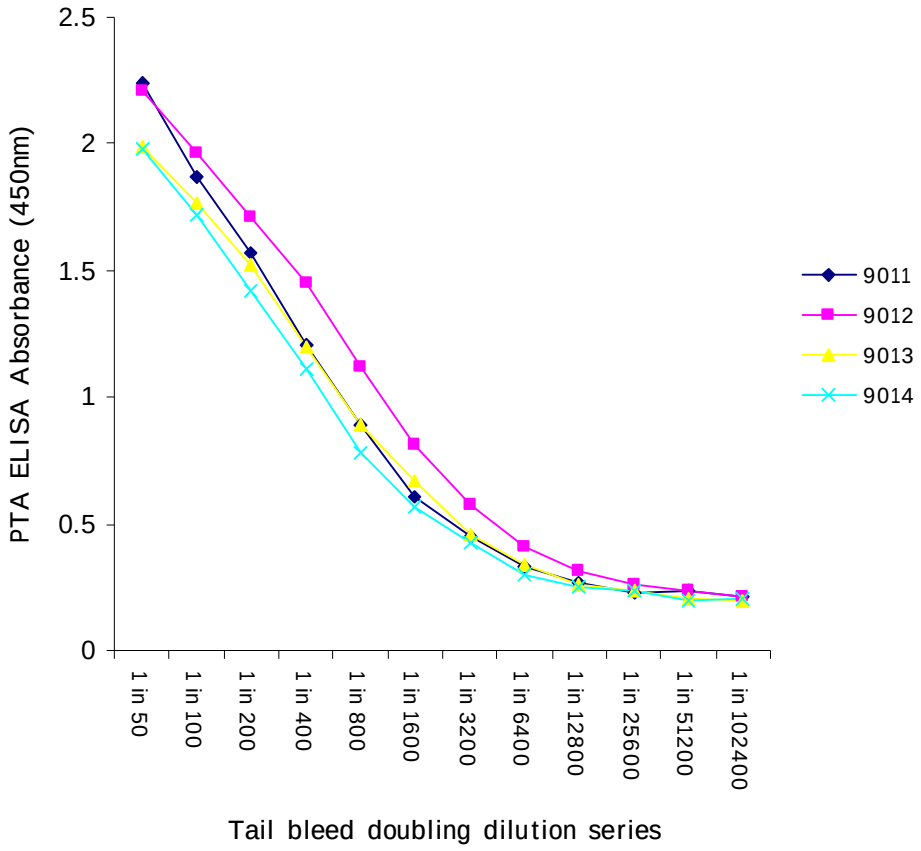


Figure 10: Response of tail bleeds of 4 Balb C mice immunised with *P. brassicae* resting spore washings to homologous antigen by PTA ELISA

Monoclonal Antibody Screening

Plate trapped antigen ELISA (PTA ELISA)

Employing a PTA ELISA, 35 hybridoma cell lines were identified as producing antibodies which recognised components associated with clubroot infected plant root material. Screening against 'non-clubroot' infected plant material identified that of these 35 lines, only five were targeted to *Plasmodiophora brassicae* (causative agent of clubroot). These five hybridoma cell lines are currently being expanded, cloned and will ultimately be tested for specificity and suitability for inclusion within a rapid test assay format for detection and quantification of *P. brassicae* in infested soils.

Immunofluorescence

Eighteen cell lines were identified as producing antibodies which recognised components found within clubroot infected root material. Of these, three were identified as targeted to components of the resting spore wall of *Plasmodiophora brassicae* (Figure 11). A proportion of antibody producing hybridoma cell lines recognised bacteria and plant root material. These were excluded from any further studies.

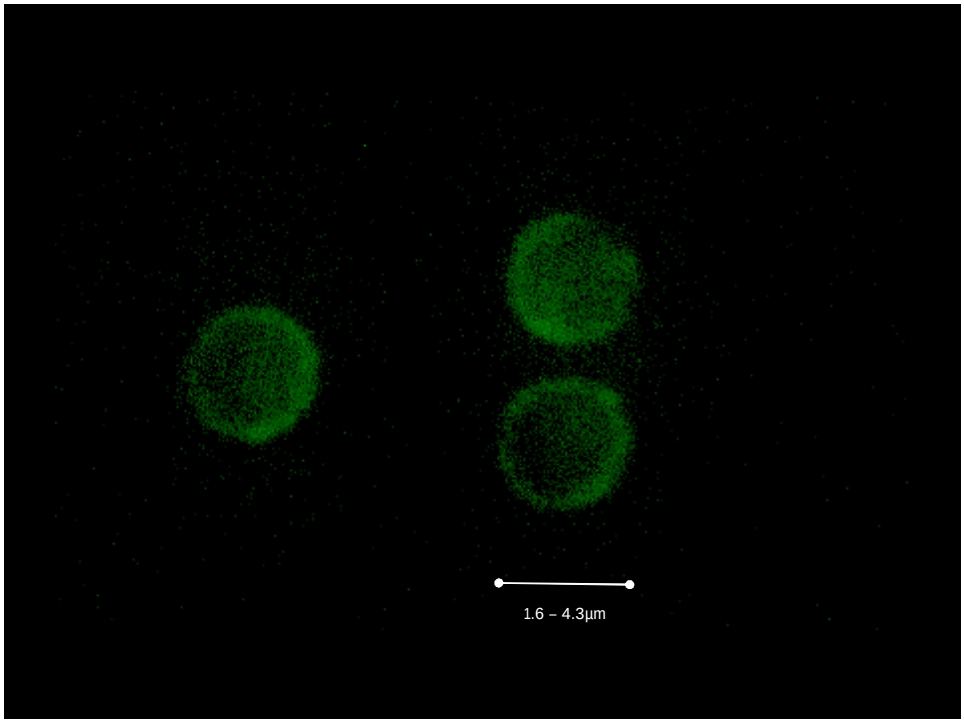


Figure 11: *Plasmodiophora brassicae* resting spores labelled with monoclonal antibodies conjugated to a fluorescein tag

Estimation of Clubroot resting spore concentration in soil samples using molecular techniques

No amplicon product relating to *P. brassicae* (clubroot) was visualised in the healthy plant root samples. The production of a primer-dimer (production of small strands of DNA which are unrelated to specific target DNA amplicon product) was observed in each of the control samples. In the plant root which visually exhibited a low level of clubroot infection (clubroot score rating 1), when isolated DNA (host and or pathogen) was at or above 0.5ng, an amplicon product associated with the target DNA of *P. brassicae* was observed when visualised by gel electrophoresis. The banding intensity observed was weak in comparison to amplicon products produced when DNA isolated from a plant exhibiting severe clubroot infection (plant score rating 3) was processed (Figure 12 treatment 7, 8, 9). In the heavily infected root material, amplicon products associated with the clubroot pathogen were detected from a sample containing 5 picrograms of initial DNA extract. The banding observed was of increased intensity to those observed in the most concentrate DNA extraction of the mildly infected clubbed root. The formation of primer dimer increased as clubroot target DNA decreased. Primer dimers were not formed in samples taken from a heavily infected clubbed root when initial DNA extracts were at or above 0.5ng DNA.

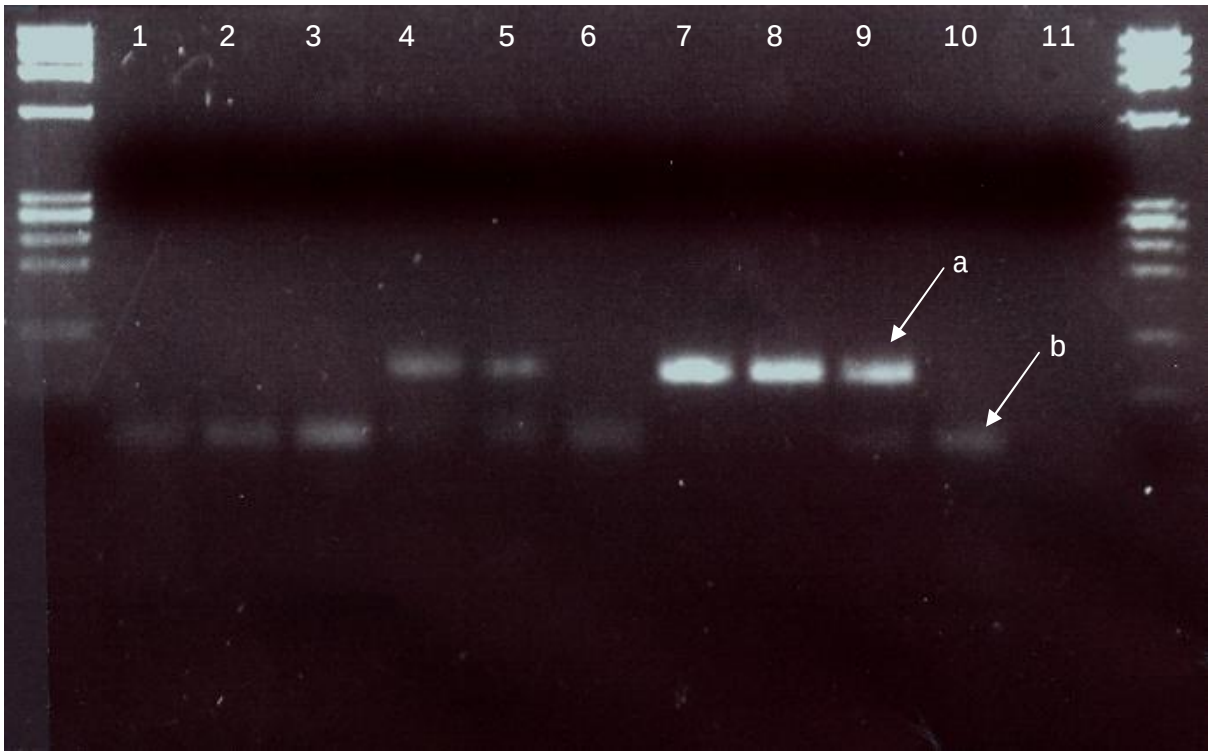


Figure 12: Amplicon products derived from control and clubroot infested root material employing polymerase chain reaction

DNA isolated from:

1. Healthy plant root material (adjusted to 5ng DNA prior to PCR)
3. Healthy plant root material (0.05ng)
4. Low level clubroot infested root material (adjusted to 5ng DNA prior to PCR)
5. Low level clubroot infested root material (0.5ng DNA prior to PCR)
6. Low level clubroot infested root material (0.5ng DNA)
7. High level clubroot infested root material (adjusted to 5ng DNA prior to PCR)
8. High level clubroot infested root material (0.5ng DNA prior to PCR)
9. High level clubroot infested root material (0.05ng DNA prior to PCR)
10. Molecular grade water + PCR Master mix and Primers
11. Healthy Plant DNA root material (adjusted to 5ng DNA prior to PCR) and Master mix alone

a – *P. brassicae* amplicon product at approximately 100 base pairs.

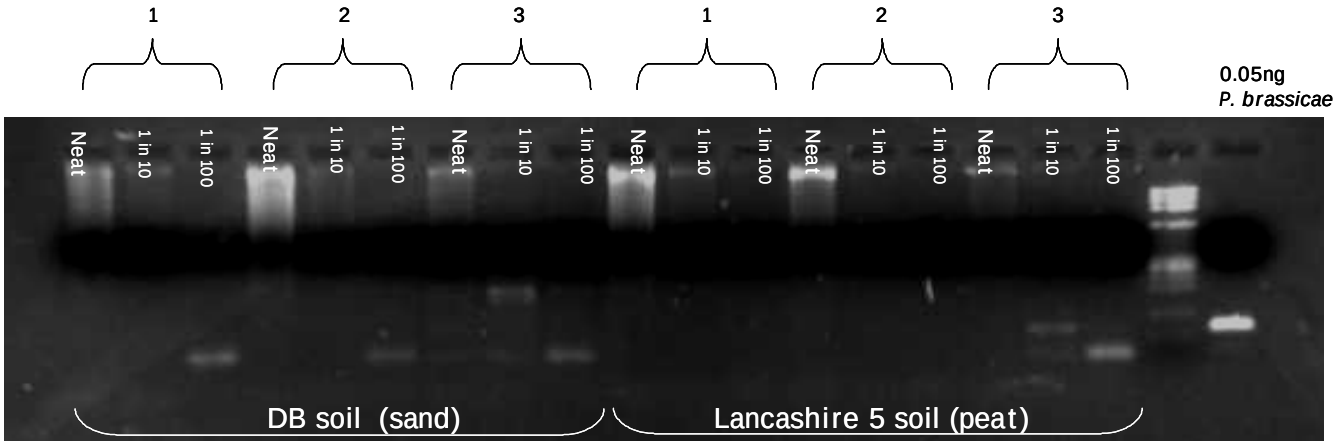
b – Primer dimer product at 40 -50 base pairs.

Molecular detection of clubroot resting spores in soil

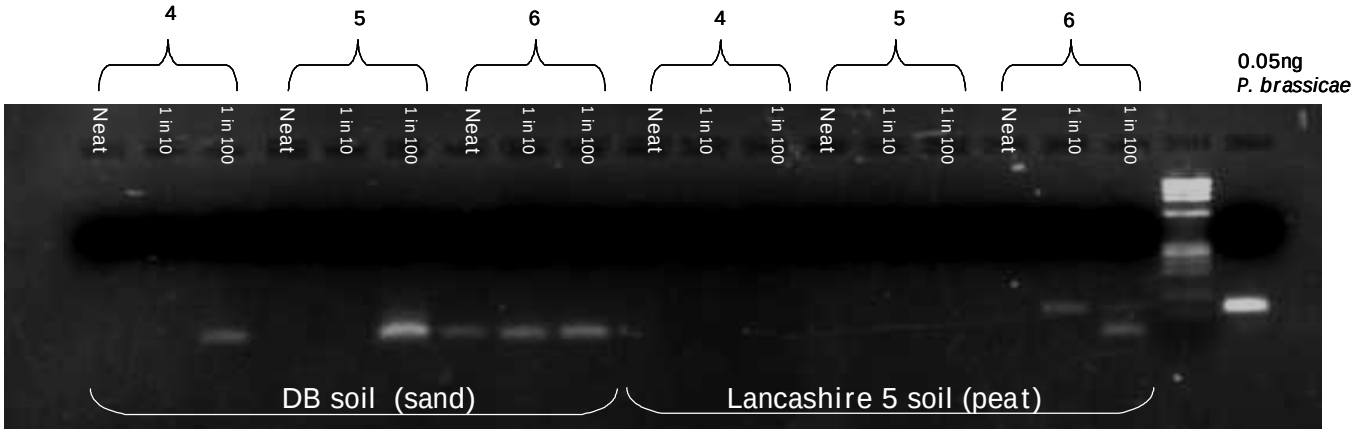
Using the Q Biogene soil extraction process (Q Bio) high molecular weight smearing was observed in all 'neat' sample extracts irrespective of the soil type processed. This non-specific reaction was reduced by sample dilution. Smearing was not observed in soils extracted using the MoBio Ultra Clean soil extraction system (MoBio). Low molecular weight primer dimer formation was observed in each of the control sand based soil extracts at one or more of the dilution factors assayed employing both the QBio and MoBio DNA soil extraction systems.

In wells which received sample amplicon product from an initial PCR mix containing 0.05ng *P. brassicae* DNA root extract a single intense band was observed (Figures 13a,b). This band, with the exception of the MoBio Ultra Clean system when used alone to extract DNA from artificially infested Lancashire peat soil, was clearly identified in all *P. brassicae* artificially infested soils at some dilution extract (Figure 13a). The use of the QIA quick purification system in conjunction with either the MoBio or QBio extraction system improved identification of *P. brassicae* presence in the artificially infested soils. However across the two soil types the DPI final purification step was superior when used in combination with either the Qbio or the MoBio extraction process. This was observed also in the Lancashire control soil where, at a reduced intensity, an amplicon product band

associated with *P. brassicae* was clearly identifiable when either the MoBio or QBio soil extraction kit was used in conjunction with the DPI extraction process (Figure 13b).



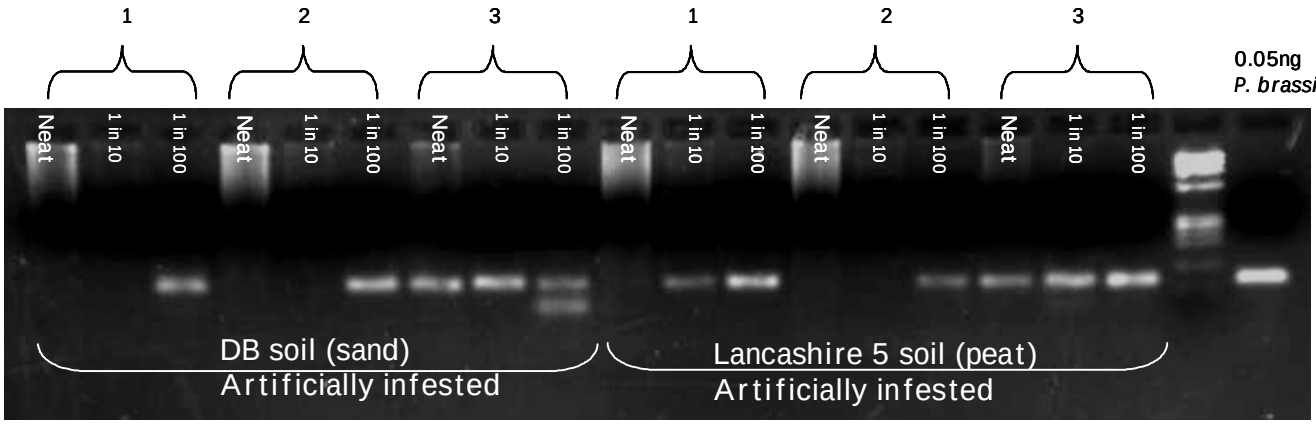
Q Biogene Fast Soil DNA Isolation Kit



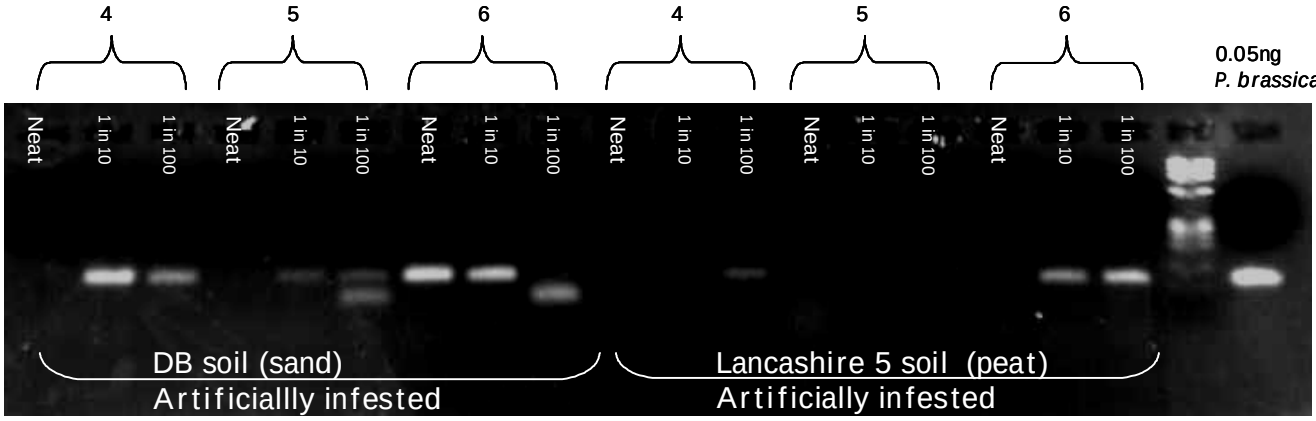
MoBio UltraClean Soil DNA Isolation Kit

1. QBiogene Fast DNA Spin Kit & QIAquick PCR Purification Kit
2. QBiogene Fast DNA Spin Ki alone
3. QBiogene Fast DNA Spin Kit & DPI protocol
4. MOBiol UltraClean Soil DNA Isolation kit & QIAquick PCR Purificatino Kit
5. MOBiol UltraClean Soil DNA Isolation kit alone
6. MOBiol UltraClean Soil DNA Isolation kit & DPI protocol

Figure 13a: Determination of the presence of *P. brassicae* inoculum in two UK soil types employing a range of DNA extraction processes and visualisation by polymerase chain reaction technology



Q Biogene Fast Soil DNA Isolation Kit



MoBio UltraClean Soil DNA Isolation Kit

1. QBiogene Fast DNA Spin Kit & QIAquick PCR Purification Kit
2. QBiogene Fast DNA Spin Ki alone
3. QBiogene Fast DNA Spin Kit & DPI protocol
4. MOBiol UltraClean Soil DNA Isolation kit & QIAquick PCR Purificatino Kit
5. MOBiol UltraClean Soil DNA Isolation kit alone
6. MOBiol UltraClean Soil DNA Isolation kit & DPI protocol

Figure 13b: Molecular detection of *P. brassicae* inoculum in two artificially infested UK soils employing a range of DNA extraction processes

Selection of specific monoclonal antibody cell lines for detection of clubroot spores

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 (Figure 14a and b).

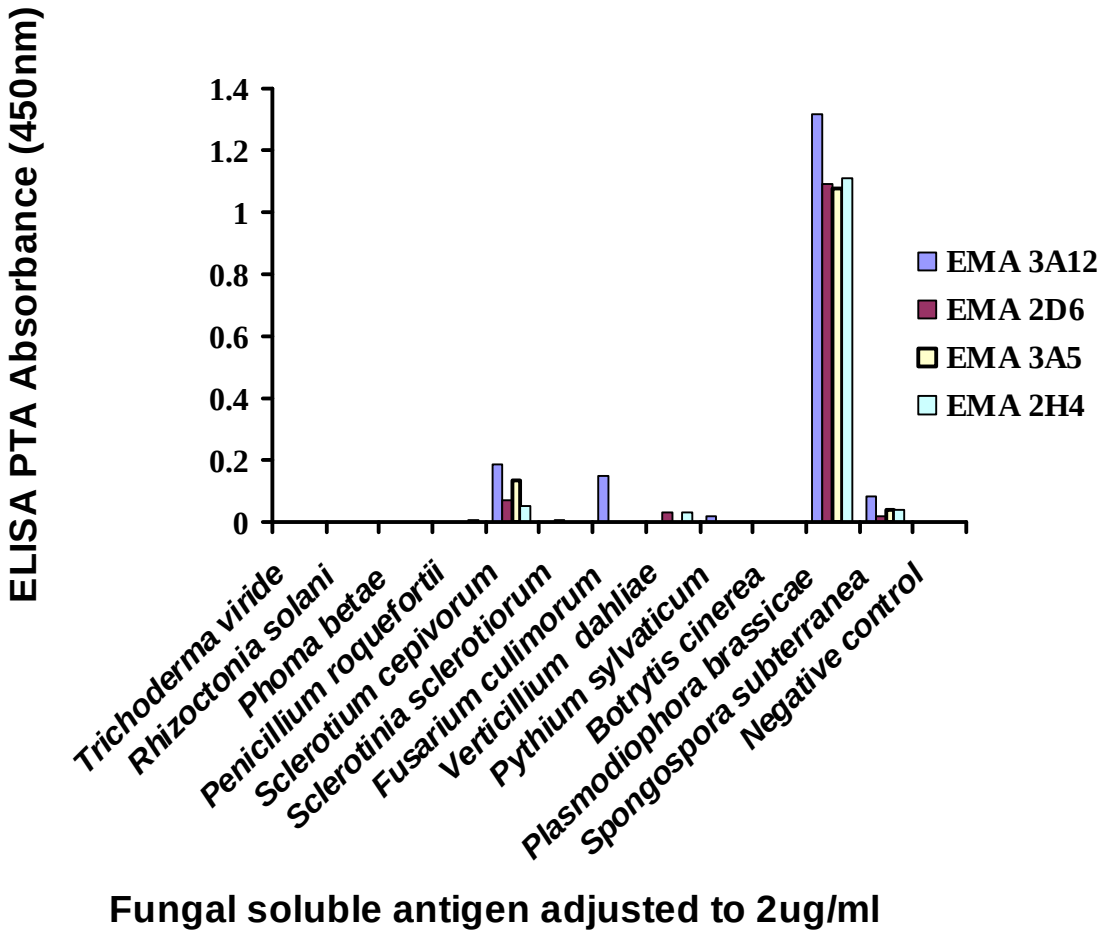


Figure 14a: Reactivity of *P. brassicae* monoclonal antibodies (EMA 3A5, 3A12, 3A5 and 2H4) to soluble material from a range of soilborne fungi as tested by PTA ELISA

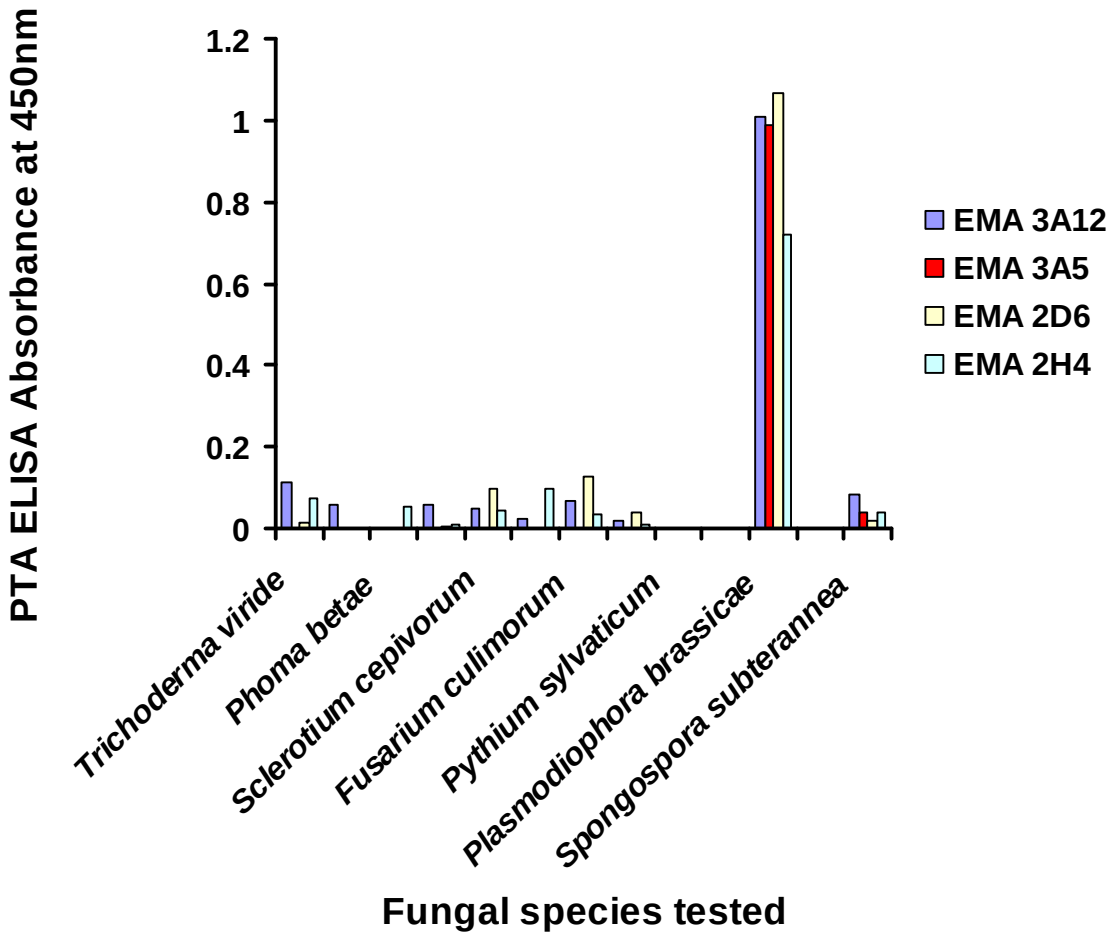


Figure 14b: Reacitivity of *P. brassicae* monoclonal antibodies (EMA 3A5, 3A12, 3A5 and 2H4) to particulate material from a range of soilborne fungi as tested by PTA ELISA

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* (Figure 15a – 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.

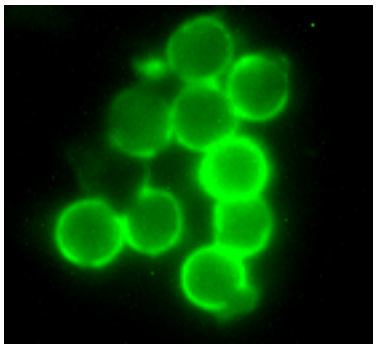


Figure 15a: Reactivity of *P. brassicae* EMA 3A5 monoclonal antiserum to resting spores of clubroot as viewed by immunofluorescence microscopy

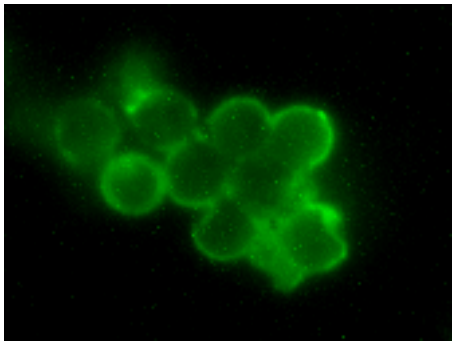


Figure 15b: Reactivity of *P. brassicae* EMA 3A12 monoclonal antiserum to resting spores of clubroot as viewed by immunofluorescence microscopy

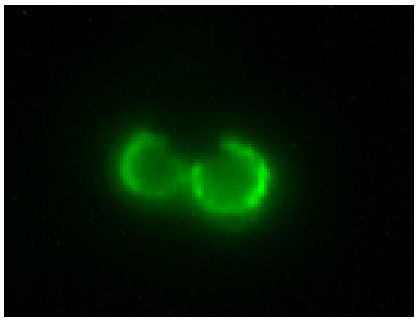


Figure 15c: Reactivity of *P. brassicae* EMA 2D6 monoclonal antiserum to resting spores of clubroot as viewed by immunofluorescence microscopy

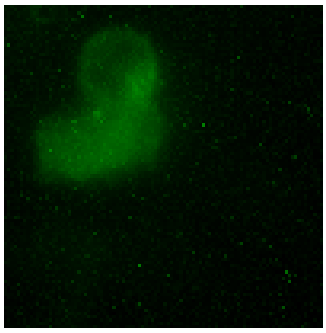


Figure 15d: Reactivity of *P. brassicae* EMA 2H4 monoclonal antiserum to resting spores of clubroot as viewed by immunofluorescence microscopy

Double antibody sandwich test for rapid detection of *Plasmodiophora brassicae*

All antibody combinations (shown in Table 5) 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 (Figure 16).

Figure 16: DAS lateral flow format showing a negative result

Competitive format test for rapid detection of *Plasmodiophora brassicae*

User of Competitive lateral flow format test

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 (Figure 17). The test line was at its strongest at the lowest antibody dilution (1 in 100) and reduced in strength as specific antibody concentration decreased.

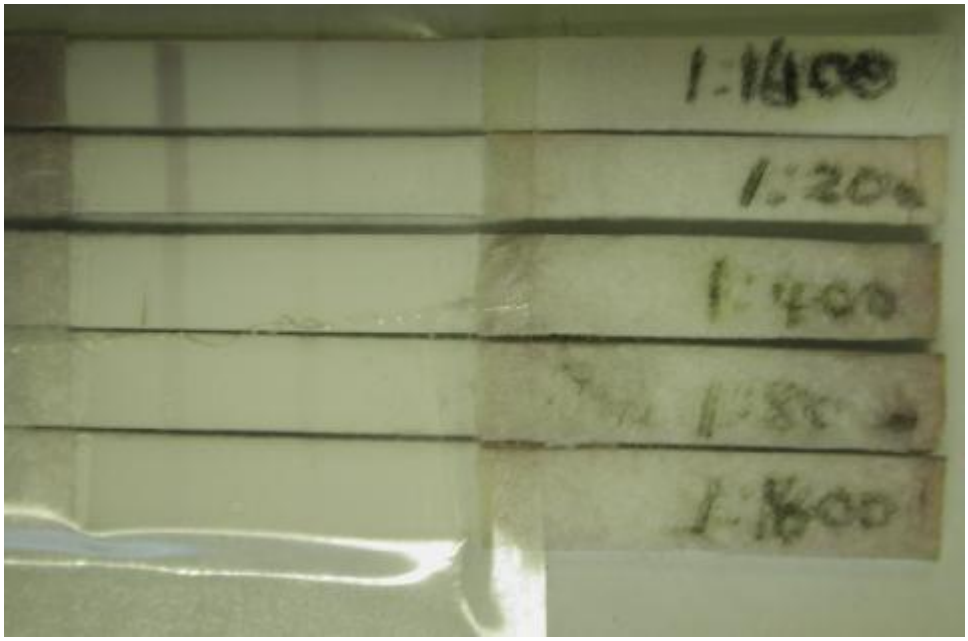


Figure 17: Determining monoclonal antibody activity of EMA 3A5 for optimal binding potential at the test line in a competitive lateral flow format

Detection threshold of the competitive lateral flow test for resting spores of *Plasmodiophora brassicae*

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 8).

Table 8: 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	**üü**	**üü**	**üü**	**üû**	**û**	**û**	**û**
27 µl applied	**üüü**	**üüü**	**üüü**	**ü**	**ûü**	**û**	**û**
30 µl applied	**üüüü**	**üüüü**	**üüüü**	**üü**	**üû**	**û**	**û**
33µl applied	**üüüüü**	**üüüüü**	**üüüüü**	**üüü**	**üü**	**ûü**	**û**
37µl applied	**üüüüü**	**üüüüü**	**üüüüü**	**üüü**	**üü**	**ûü**	**û**

üüüüü Intense test line development
üüüü Strong test line development
üüü Clear test line development
üü Visible test line development
ü Poor test line development
û/ü Weak test line development (barely visible)
û 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 17).

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 9).

Table 9: 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.

Determination of clubroot resting spore concentration in artificially infected soil using molecular methods

A 1 in 10 dilution of the extracted DNA soil sample proved optimal with amplicon product detected in each of the artificially infested soils (1×10^5 to 1×10^3 spores g^{-1} soil) (Figure 18). Using the extracts neat reduced sensitivity to a detection level of 1×10^4 spores g^{-1} soil whilst a dilution factor of 1 in 100 detected a clubroot amplicon product in samples in excess of 1×10^7 g^{-1} soil.

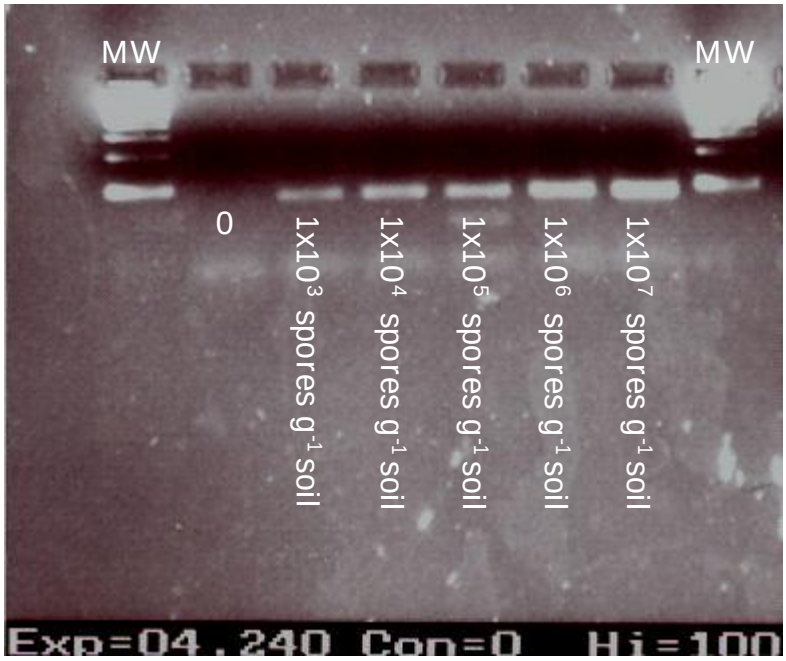


Figure 18: PCR detection of clubroot spore concentrations in artificially infested soil

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

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 10). 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.

Table 10: Percentage healthy plants and those showing clubroot root infection

	% 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

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 (Figure 19a,b,c).

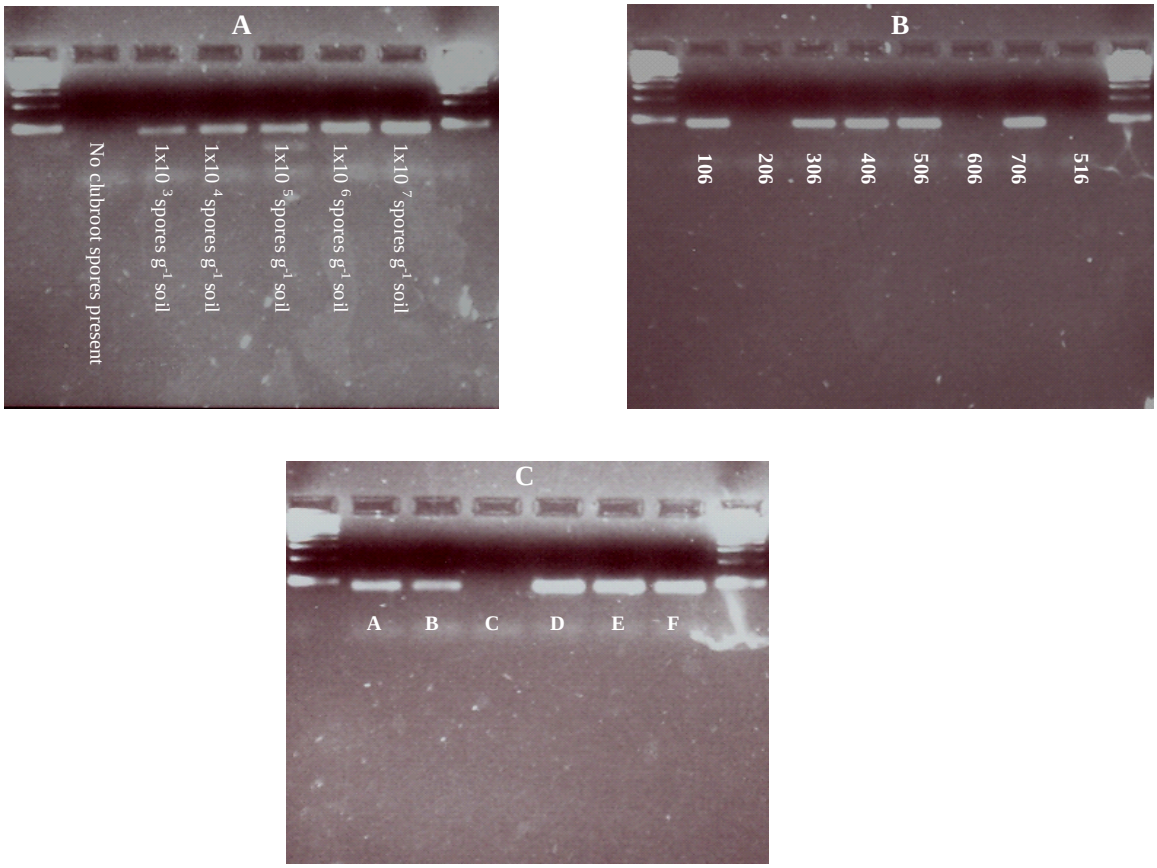
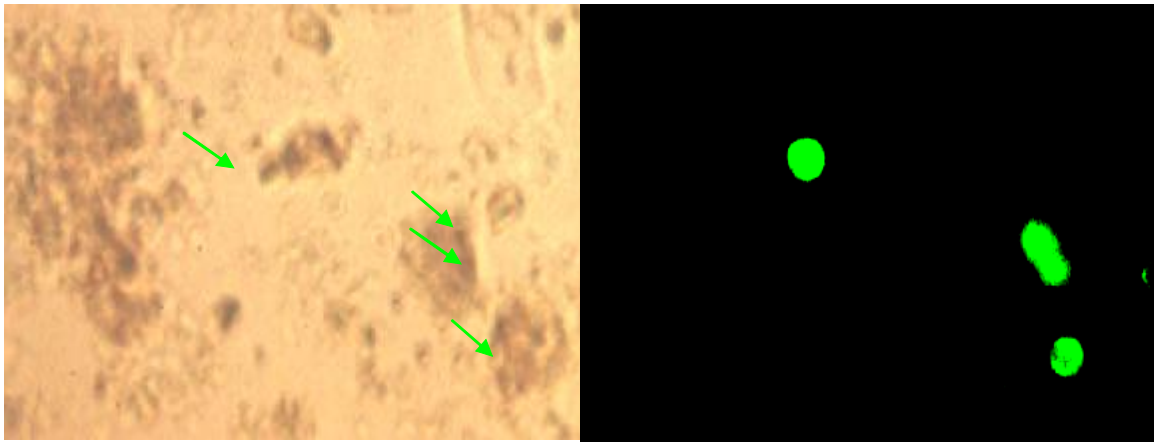


Figure 19: PCR molecular tests of soil samples inoculated with dilutions of clubroot resting spores (a), and PCR tests of commercial soil samples (b, c)

Assessment of clubroot resting spore concentration using soil immunfluorescence

Resting spores of clubroot were visualised readily in both artificially (Figure 19) and commercial soils.



(A) (B)

Figure 20: 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 11).

Table 11: Clubroot Immunofluoresecence counts in commercial soil samples

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 12: 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)

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

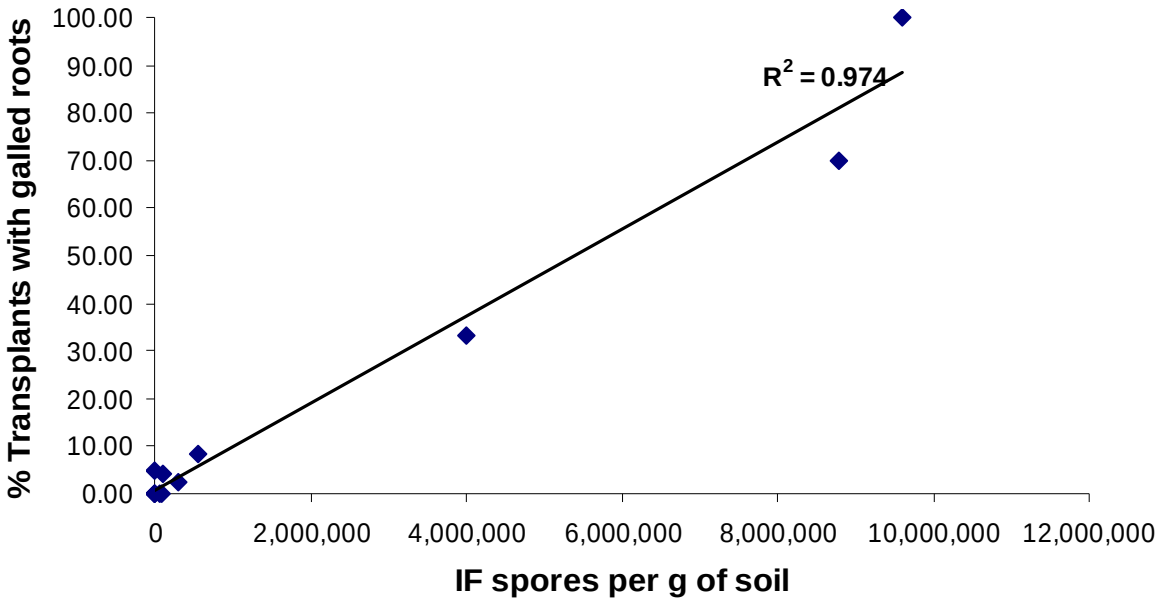


Figure 21: Relationship between Immunfluoresence predicted resting spores levels in commercially tested soils and galled roots of soil exposed bait transplants

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 21). 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.

Analysis of commercial soil samples using Immunoflorescence, baiting test and PCR molecular tests in 2006

Two soils were identified by all three clubroot diagnostic tests as negative for clubroot these were soil C and soil 606 (Table 6). 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 (Figure 18a,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 6).

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.

Analysis of commercial soil samples using Immunoflorescence, baiting test and PCR molecular tests and lateral flow tests in 2007

Bait Tests

Table 13: Clubroot infection on bait plants in samples taken in 2007

Sample Number	Total clubs recorded/site	Plants Recorded	Mean clubs/plant
1	3	7	0.43
2	9	7	1.29
3	11	7	1.57
4	7	8	0.88
5	18	8	2.25
6	2	8	0.25
7	1	7	0.14
8	0	7	0.00
9	0	8	0.00
10	1	8	0.13
11	0	8	0.00
12	0	8	0.00
13	0	8	0.00
14	0	4	0.00
15	6	4	1.50
16	0	7	0.00
17	2	8	0.25
18	21	3	7.00
19	4	7	0.57
20	0	8	0.00
21	0	6	0.00
22	0	8	0.00
23	17	5	3.40
24	9	3	3.00
25	3	8	0.38
26	0	6	0.00

Chinese cabbage cv. Granaat was used in bait plants tests in 2007 as this variety was considered to be completely susceptible to clubroot infection. In each sample tested a total of 8 plants were assessed for clubroot infection. Samples 14 and 15 used only 4 plants as insufficient soil was delivered to carry out tests with larger plant numbers. The results shown in Table 13 indicate that samples 8, 9, 11, 12, 13, 14, 16, 20, 21, 22, and 26 were free of clubroot contamination. No clubroot galls were observed on plants harvest from these samples. Low levels of clubroot contamination were observed in samples 1, 6, 7, 19 and 25 indicating a low level of potential clubroot contamination. However samples 5, 18, 23 and 24 were the most heavily clubroot contaminated samples according to these bait tests. Sample 18 was the most heavily contaminated of those tested.

Immunoflorescence

The results of assessing a 0.3 g soil sample of soil by immunofluorescence for soil samples collected in 2007 is shown in Table 14. Large numbers of clubroot resting spores were observed in sample 18. However in contrast samples 5, 6, 7 and 22 gave negative results for clubroot resting spores as assessed by immunofluorescence. Several other samples including sample 20 had relatively low numbers of resting spores. However sample 20 had mostly broken or fragmented resting spores suggesting that resting spores had either germinated or were destroyed in some way. Samples 1, 2, 14, 15, 17, 19, 23 and 25 had relatively low numbers of resting spores (in the tens of thousands). Samples containing approximately 10000 resting spores per gram of soil could result in some visible symptoms if vegetable brassicas were grown on the land where the sample originated. However brassica plants grown in soils where sample 18 had originated would have been heavily infected with clubroot. Sample 20 contained no whole resting spores and only fragments were observed. This soil has not shown any clubroot symptoms despite continuous cultivation of vegetable brassicas. There was no indication of the likely cause of the fragmentation of resting spore like bodies however sample 4 also had fragmented resting spores.

Table 14: Clubroot spore number in 0.3 g of soil from samples taken in 2007 assessed by Immunoflorescence

Soil sample	No of resting spores/ g soil
1	80000
2	28571
3	131429
4	251428.5 **
5	0
6	0
7	0
8	108571
9	222857
10	297143
11	217143
12	977143
13	NA
14	51429
15	97143
16	137143
17	62857
18	5845714
19	40000
20	2857.1 **
21	148571
22	0
23	40000
24	120000
25	68571
26	280000

** Spore fragments , NA – Not available

PCR Molecular tests

The results of assessing a 0.3 g soil sample of soil by polymerase chain reaction (PCR) for soil samples collected in 2007 is shown in Table 15. There were no detectable clubroot resting spores in samples 4, 5, 6, 7, 8, 10, 12, 13, 20 and 22 (Table 15). These were confirmed by repeating the test using these soil samples but spiking the sample with a known quantity of clubroot resting spores. The retested sample for each of these soils gave positive results using the spiked sample. This indicated that there was nothing in the soil samples which would have resulted in a false negative test for each of these samples. Sample 18 gave the highest estimated clubroot resting spore contamination. Several samples (9 and 16) had negligible estimated levels of clubroot contamination indicating either recent transmission of clubroot into these fields or the lack of brassica production for a considerable time period. All soil samples supplied from Scotland had high levels of clubroot resting spore contamination. Sample 19 had estimated clubroot resting spore concentrations per gramme of soil of 580,000 (Table 15) indicating a high risk of clubrrot if vegetable brassica plants were transplanted into this field.

Table 15: Clubroot resting spore number in 0.3 g of soil from samples taken in 2007 assessed by PCR

		No of resting spores/ g soil	
Soil sample	**Mean clubs per plant**	**Immunoflorescence**	**Molecular PCR Test**
1	0.43	80000	124216
2	1.29	28571	75106
3	1.57	131429	101743
4	0.88	251428.5 **	0
5	2.25	0	0
6	0.25	0	0
7	0.14	0	0
8	0.00	108571	0
9	0.00	222857	300
10	0.13	297143	0
11	0.00	217143	17776
12	0.00	977143	0
13	0.00	NA	0
14	0.00	51429	217223
15	1.50	97143	142992
16	0.00	137143	407
17	0.25	62857	19949
18	7.00	5845714	669021
19	0.57	40000	580426
20	0.00	2857.1 **	0
21	0.00	148571	32321
22	0.00	0	0
23	3.40	40000	188623
24	3.00	120000	166261
25	0.38	68571	331119
26	0.00	280000	138808
Control	0.00	NA	NA

Lateral flow tests with soil samples in 2007

The results of lateral flow tests using 0.3 g samples of soil collected during 2007 is shown in Table 16. Tests used soil directly applied to the lateral flow device. The results are expressed as optical densities on the test line. The control sample which did not contain clubroot gave an optical density reading of approximately 4.5. The control sample does not necessarily reflect a non contaminated reading for each of the soils tested since the soil samples vary in their component parts and are non uniform in composition and clubroot contamination. The most heavily contaminated soil (sample number 18) gave a reading on the test line of 0.5 indicating a positive result. There was no visible test line on the lateral flow tests (see Figure 22a). A very faint line was observed when the lateral flow device was tested using sample 17 which had lower levels of clubroot contamination (Table 16, Figure 22a). However this sample had clubroot resting spore numbers of approximately 20000 per gram (molecular test) and 60000 per gram of soil (immunofluorscence test) of soil which is

below that at which uniform disease symptoms would be observed in the field. However there were soil samples which gave false positive estimates of clubroot contamination using the LFD device. Samples 5 and six gave low optical density readings (a positive test) but had zero numbers of resting spores in the soil sample as estimated by both the molecular and immunofluorscence tests (Table 16). Some samples gave LFD results which followed the molecular test rather than the immunofluorscence test result. Samples 12 and 20 gave an optical density reading suggesting a negative result (Figure 22b Table 16). The molecular test was negative for both these samples and positive for the immunofluorscence test. There were some false negative samples recorded. Samples 3, 21, and 25 gave on the balance of probabilities negative test results (optical densities of these three tests ranged from 3.6 – 6.6). However each of these three samples gave positive results as observed using molecular or immunoflorescence. It would be useful to ascertain what component parts of the soil sample influenced the binding of the antibody/conjugate complex to the test line.

Table 16: Mean optical density of lines on lateral flow tests from soil from samples taken in 2007

		No of resting spores/ g soil		
Soil sample	**Mean clubs per plant**	**Immunoflorescence**	**Molecular PCR Test**	**Optical Density LFD Test**
1	0.43	80000	124216	0.5
2	1.29	28571	75106	1.1
3	1.57	131429	101743	3.6
4	0.88	251428.5 **	0	1.7
5	2.25	0	0	0.6
6	0.25	0	0	1.1
7	0.14	0	0	1.9
8	0.00	108571	0	1.8
9	0.00	222857	300	2
10	0.13	297143	0	0.8
11	0.00	217143	17776	2.8
12	0.00	977143	0	5.9
13	0.00	NA	0	NA
14	0.00	51429	217223	3.1
15	1.50	97143	142992	1.3
16	0.00	137143	407	2.3
17	0.25	62857	19949	1.4
18	7.00	5845714	669021	0.5
19	0.57	40000	580426	3.6
20	0.00	2857.1 **	0	6.1
21	0.00	148571	32321	6.6
22	0.00	0	0	3.2
23	3.40	40000	188623	2.9
24	3.00	120000	166261	1.4
25	0.38	68571	331119	6.1
26	0.00	280000	138808	1.4
Control	0.00	NA	NA	4.6

(a)

(b)

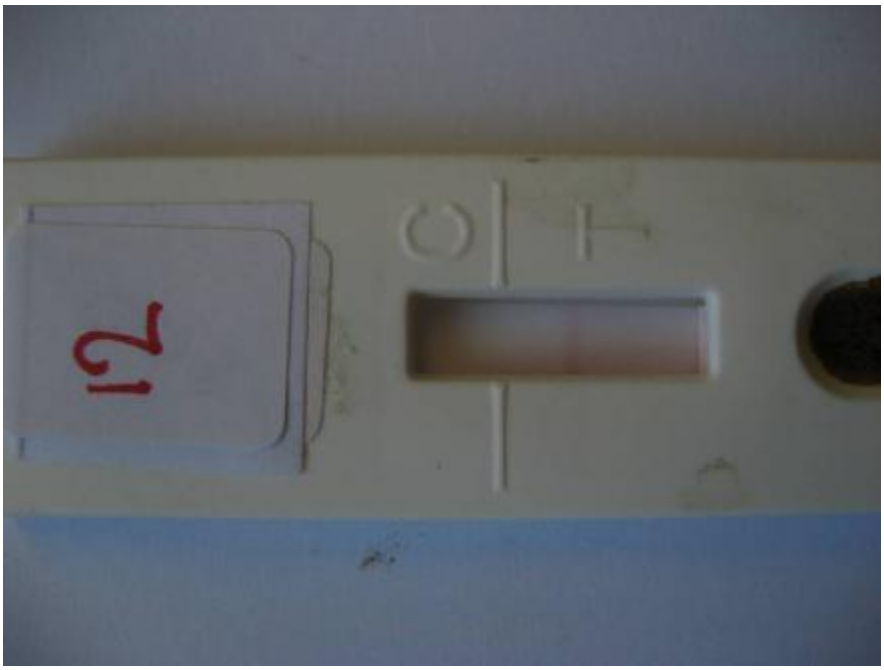


Figure 22: LFD tests with commercial soil samples 12, 17, and 18 in 2007

Optical density values of the test line at varying clubroot resting spore concentrations in soil

Optical densities of lateral flow tests using control soils spiked with known quantities of clubroot resting spores is shown in Table 17. The optical density where zero resting spores were present was approximately 3.5. However this dropped with increasing clubroot spore number. One million clubroot resting spores (1×10^6) gave an optical density of approximately 0.8 . No line visualisation on the device was observed indicating a positive test. Use of an electronic reader device would enable the precise clubroot contamination to be estimated. However the device could be calibrated to give a line visualisation at 1×10^5 (100,000 resting spores).

Table 17: Optical density values of the test line at varying clubroot resting spore concentrations in soil

Sample concentration (Clubroot resting spores)	Optical Density value
0	3.6
1×10^3	4.1
1×10^4	3.2
1×10^5	1.5
1×10^6	0.8
1×10^7	0.6

Effect of cropping on the infestation of soil by clubroot

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 (Figure 23). 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.

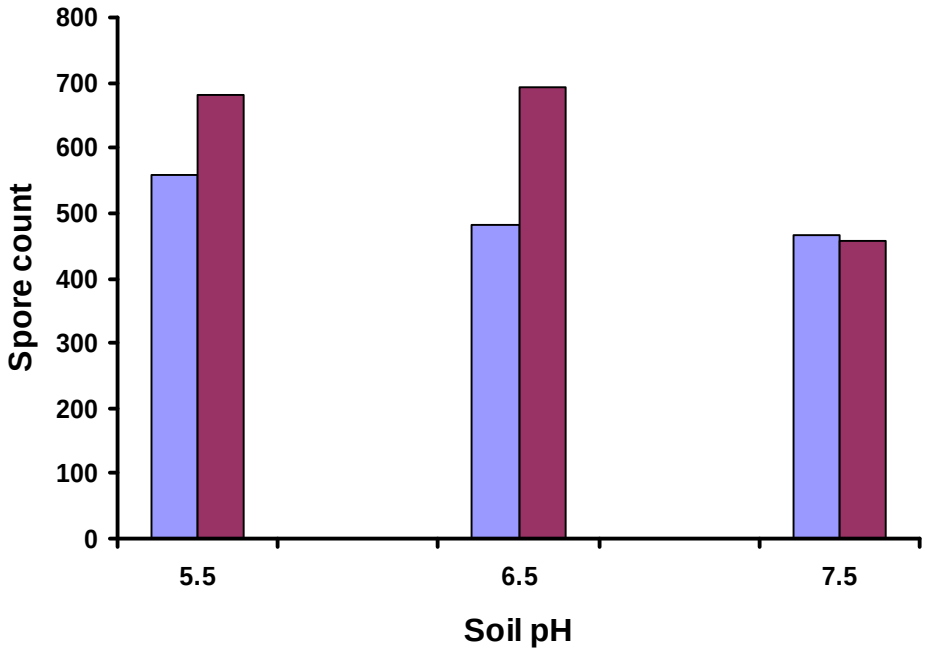


Figure 22: Immunoflourescent clubroot resting spore counts from plots cultivated with summer (■) and over winter cauliflowers (■) under differing soil pH regimes

DISCUSSION

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.

Validation of molecular tests for clubroot in UK soils in 2006 and 2007

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 2006) 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 1×10^3 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 1×10^2 or below.

Developing lateral flow detection tests for clubroot resting spores in soil and water

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.

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 across a range of soil types is planned within a new project which could commence during 2008.

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.

Further development of LFD tests for clubroot 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.

Clubroot forecasting criteria based on inoculum detection

Tests which, can be conducted in the field are necessary if information on clubroot inoculum concentration is to be of more practical value. As tests for pathogenic inoculum can be carried out in the field (by using lateral flow devices) the system meets the criteria necessary for its uptake by the brassica industry. 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.

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