ANNUAL REPORT

To:
Horticultural Development Council
Bradbourne House
Stable Block
East Malling
Kent, ME19 6DZ

BRASSICAS: DEVELOPMENT AND VALIDATION OF DETECTION TESTS FOR CLUBROOT

HDC PROJECT FV 259

R Kennedy and A J Wakeham

Warwick HRI
Wellesbourne, Warwick, CV35 9EF

September 2005

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Grower Summary

FV 259

BRASSICAS: DEVELOPMENT AND VALIDATION OF TESTS FOR CLUBROOT

Annual report 2005

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Project title:	Brassicas: development and validation of detection tests for clubroot
Project number:	FV 259
Project Leader:	Dr R Kennedy
Annual report:	First Year Annual report
Previous reports:	None
Key workers:	Dr R Kennedy Ms A Wakeham
Location of project:	Warwick HRI, University of Warwick, Wellesbourne, Warwick
Project co-ordinators:	Mr F Tyler, Mr A Ewan
Date project commenced:	1 June 2004
Date project completed:	31 May 2007
Key words:	clubroot, *Plasmodiophora brassicae*, disease prediction, detection, rapid tests, disease control, disease, brassica

Signed on behalf of: Warwick HRI

Signature: …………………………………………

Name: Professor Simon Bright
Director and Head of Department

Date: …………………………………………

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

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1. GROWER SUMMARY

1.1 Headline

A laboratory based molecular test for clubroot detection has been successfully used in the UK for the detection of clubroot resting spores in naturally and artificially infected UK soils. Specific antibodies which recognize clubroot resting spores have also been raised, which is the first stage to the development of a field-based prototype kit.

1.2 Background and expected deliverables

Clubroot caused by the protist *Plasmodiophora brassicae* Woronin is one of the most intractable plant disease problems of cultivated brassica 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-brassica crops. Previous studies have indicated that symptom expression is dependant on the presence of a threshold level of infection. Inoculum concentration plays an important part in the incidence and severity of clubroot development within crops. In reality these thresholds are determined by environmental and control measures operating within the crop. Minimal spore concentrations and a pH of 7.2 inhibited root hair infection whereas clubs developed at higher inoculum densities at the same pH. Threshold levels of infection are required for maximum symptom development however severity of clubbing was shown to be proportional to increased spore concentration and total root hair infection. Above this threshold increasing spore concentration may generate greater root hair infection but this does not lead to further increases in symptom development. Other studies have also indicated that 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. 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.

1.3 Summary of first year work on FV259

1.3.1 Development of specific antibodies for detecting and quantification of clubroot resting spores

By employing an immunodiagnostic test (ELISA), 35 cell lines were identified as producing antibodies which recognised components associated with clubroot infected plant root material. By screening these cell lines against ‘non-clubroot’ infected plant material only five were shown to react to resting spores of *Plasmodiophora brassicae*. These five hybridoma cell lines are currently being multiplied and will ultimately be tested for specificity and suitability for inclusion within rapid test assay formats for detection and quantification of *P. brassicae* resting spores in infested soils. By using immunofluorescence (another type of microscopic based immunodiagnostic test) 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.* Antibodies which recognise components of resting spores or of clubroot infected plant material will be taken forward for further testing against a range of soil borne organisms. A polyclonal antiserum was also raised to *Plasmodiophora brassica.*

1.3.2 Validation of molecular tests for detecting clubroot in soil in the field

Molecular detection and diagnosis is now routinely used in many areas of biology including medicine, forensic pathology. The polymerase chain reaction (PCR) is now a key tool in molecular biology. It is a rapid and powerful technique for the *in vitro* amplification of DNA sequences. If DNA sequences (primers) which are unique to a particular organism are used in it can be a reliable and sensitive technique for determining the presence or absence of a target organism or in its quantification (real-time PCR). Using primers (developed by the Department of Primary Industries , Melebourne, Victoria, Australia) specifically to regions of *Plasmodiophora brassicae* DNA, the presence of the clubroot pathogen was readily detected at a low level of infection when assayed. Initial results suggest that the primers used are able to discriminate between non-infected and infected clubroot material from vegetable brassica crops and will prove useful in detection of clubroot resting spores in soil. The test was used on several soil samples of unknown clubroot contamination status to determine if it could be used to detect clubroot resting spores. Soil samples were

subjected to the MoBio DNA soil extraction process which was effective in the removal components which could interfere with the test. The molecular test was able to discriminate the presence of resting spores in a soil sample (from Lancashire) which had previously been considered to be clubroot free.

1.4 Action points for growers

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

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

1.5 Anticipated practical and financial benefit

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

SCIENCE SECTION

2. INTRODUCTION

2.1 The clubroot pathogen infecting vegetable Brassicas

Clubroot caused by the protist *Plasmodiophora brassicae* Woronin is 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 to eradicate it due to the persistence of viable resting spores in the soil.

2.2 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. Additionally the use of transplants in vegetable Brassica production can also be an important source of disease. Infected plants and soil are transported widely within modern vegetable production and this could contribute to spread of disease to new areas of production.

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2.3 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 crops 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. However both these chemicals do 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).

2.4 Effect of inoculum concentration on clubroot development

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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 although 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.

2.5 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.

2.6 Molecular tests for clubroot resting spores in soil

Molecular detection methods for *P. brassicae* based on PCR have been developed for conserved sequences in the 18S-like ribosomal RNA gene and the internal transcribed spacer (ITS) regions of clubroot DNA sequences (Chee *et al*., 1998, Faggian, *et al*., 1999). Specific detection has also been achieved using primers derived from the sequence of the isopentyltransferase gene (Kim and Lee, 2001, Ito, *et.al*., 1999). Up to 1000-fold increases in the sensitivity can be achieved when some molecular methods are used (Wallenhammar and Ardwisson 2001). Other workers reported levels of spore detection of one spore per gram of soil in potting mix using single primer pairs (Faggian *et al*., 1999). With the exception of a single isolate, none of these primers has been tested against of *P. brassicae* isolates of UK origin. Recently another type of PCR test has been developed (real time PCR) in which PCR amplification is monitored in real time. The accumulation of PCR product can be compared to a set of standards which enables sample inoculum concentrations to be determined. Inoculum concentration can be related to disease severity in the field. A protocol to quantify the amount of clubroot inoculum in a sample was

developed by Faggian and Parsons (2002). However this technique could not be successfully used on all soil types to predict severity of disease (Donald *et al*., 2003) in Australia. Further work is currently being carried out to improve the assay.

2.7 Immunological tests (Lateral Flow Devices)

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

The Competitive assay format: In a competitive assay format the test line comprises of homologous antigen (clubroot spore components) and the other, the control, is a line of anti-species antibodies. The release pad and membrane are assembled together with an absorbent pad into a plastic housing as illustrated below (Figure 1). Three drops of sample are added to the well, releasing the specific antibody bound coloured particles, which then begin to flow across the membrane. If the target antigen is present in the sample extract, antibody binding will occur to produce a coloured particulate conjugated antibody -antigen complex. Any antibody conjugated coloured particles that fail to bind to an antigen will attach to the immobilised test line as they traverse the membrane; thus producing a visible line of deposited coloured particulates at the test line. The anti-species antibody will capture excess sensitised antibody / coloured particles to produce an internal control line, providing a visible confirmation of antibody / particulate flow. Sufficient target presence *i.e.* of clubroot spores, would induce complete inhibition of antibody attachment to the test line, a result that is indicated by a single line of coloured particle deposition (the control line). Two lines of equal colour intensity indicate a negative result.

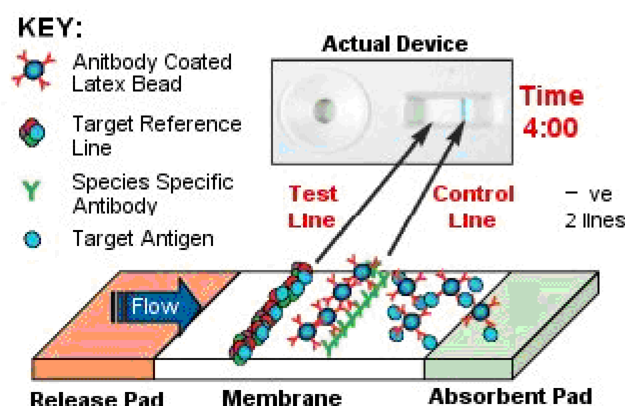

Figure 1. The Competitive lateral flow assay format.

The Non-competitive assay format: In a non-competitive assay format the test line generally comprises of an antibody complex which if present, target antigen in the test sample will bind to. The control line will consist of a complex matrix which will bind material within the sample flow to indicate successful test execution. The release pad and membrane are assembled as 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 are now available for use under field conditions. 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 distinguished using a reader, facilitating the differentiation between target concentrations.

2.8 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 for the clubroot pathogen. For this reason most of the first year work of the project must be devoted to the development and testing of clubroot specific monoclonal antibodies. Reactive cell lines in mice must

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be raised which produce specific antibodies. These are then tested for their specificity to clubroot resting spores and their reactivity to a range of common soil-borne fungi. This would include other pathogens which are common in the soil in vegetable Brassica crops notably Botrytis *cinerea* and *Rhizoctonia solani*. It must also include the full range of soil-borne organisms such as *Penicillium sp*., *Aspergillus sp*., and *Fusarium sp*. Many of these pathogens survive on debris in the soil or are found on leaves on Brassica crops. Consequently tests with clubroot resting spores in soil should not react with the conidia of other pathogens and saprophytes common in soil environment crops. If the antibody does react with conidia of other fungi found in the soil then it cannot be used for the development of "in field" tests. 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.

3. PRODUCTION OF ANTISERA

3.1 Production of monoclonal antisera to *Plasmodiophora brassicae*

3.1.1 Introduction

The clubroot pathogen (*Plasmodiophora brassicae*) is an obligate parasite. The clubroot pathogen must be isolated and routinely maintained on susceptible plants

for there to be a ready supply of clubroot resting spores. The pathogen infects root material and is favoured by high moisture levels in the compost.

3.1.2 Materials and Methods

3.1.2.1 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 16/14 C day/night temperature regime. Plants were repotted into FP9 pots (one seedling per pot) at the third true leaf stage and grown for a further 2 weeks at 16/14 C day night temperature regime in the glasshouse. Plants were placed in trays prior to inoculation and unhealthy or atypical plants removed to ensure the uniformity plants used in each experiment. Plants were inoculated using a 5 ml suspension of clubroot resting spores harvested from field grown cauliflower plants (see section 3.1.2.2) grown in contaminated soil in a quarantine area (QF field) at Warwick HRI Wellesbourne.

3.1.2.2 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.

3.1.2.3 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 HRI Wellesbourne. Hybridoma tissue culture supernatants were screened both by immunofluorescence (IF) and by PTA ELISA (plate-trapped antigen enzyme-linked immunosorbent assay) 14 days after cell fusion for the presence of antibodies which recognised components associated with the resting spores of *P. brassicae* (clubroot inoculum). This process was repeated for each of the mice.

3.1.2.4 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 then incubated overnight in an enclosed chamber at 18ºC. After which unbound material was removed and the microtitre wells were washed once with 200 µl PBS. The microtitre wells were blocked with 200 µl of 1 % Casein buffer (1 % (w/v) casein PBS) and incubated at 37 º C for 45 min. Residual blocking buffer was removed and wells were washed four times for one min each with 200 µl PBS, 0.05 % Tween 20 and 0.1% Casein (PBSTw C). After which each well received 100 µl per 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*.

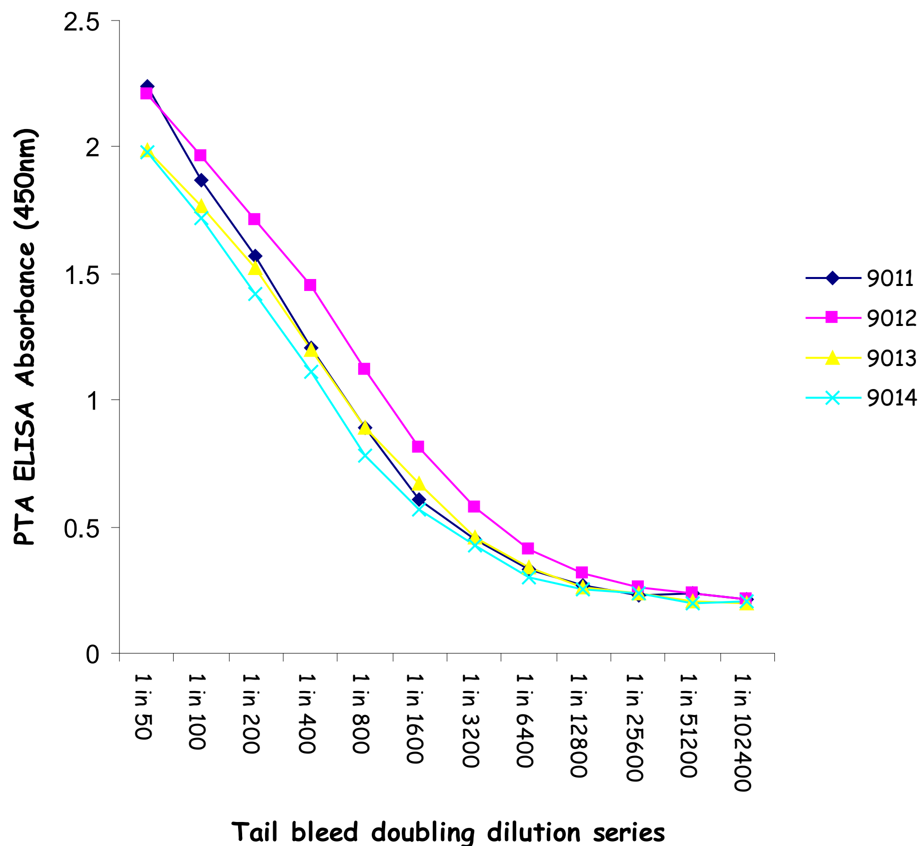
Hybridoma antibody tissue culture supernatants which were identified as positive to *P. brassicae* resting spore material employing PTA ELISA and IF were selected and expanded in to 24 well plates for further testing. A final fusion (mouse 9014) is currently underway and *P. brassicae* positive hybridomas will be tested and screened as described above.

3.1.3 Results

3.1.3.1 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 (Fig. 1). Employing immunofluorescence antibodies were identified which recognised components of the resting spore wall of *P. brassicae*

Figure 2 Response of tail bleeds of 4 Balb C mice immunised with *P. brassicae* resting spore washings to homologous antigen by PTA ELISA.



3.1.3.2 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* (Plate 1). A proportion of antibody producing hybridoma cell lines recognised bacteria and plant root material. These were excluded from any further studies.

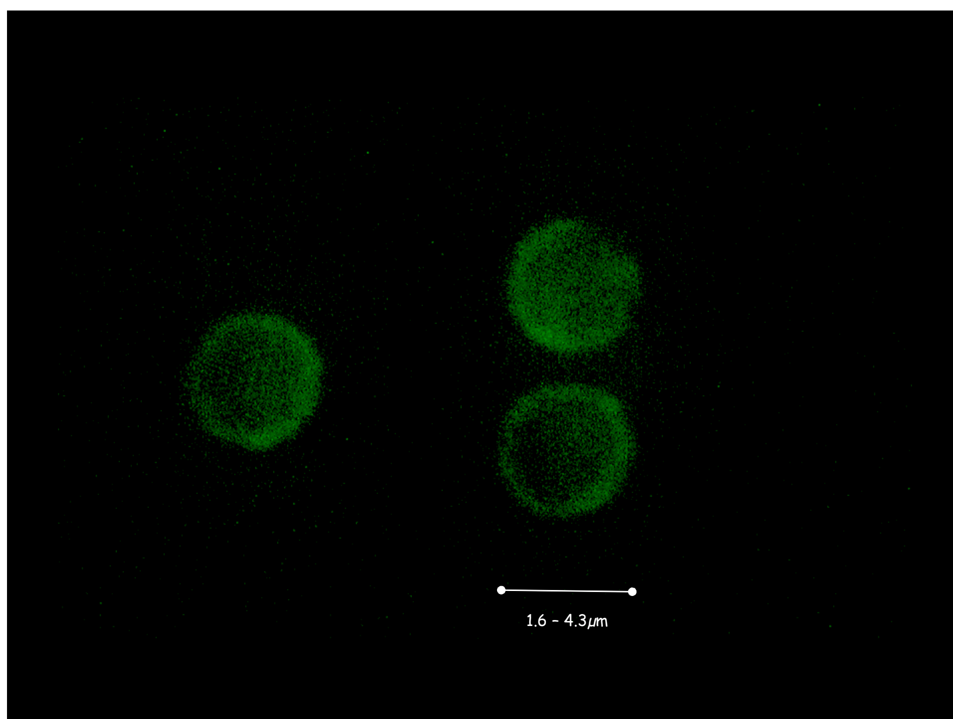


Plate 1. *Plasmodiophora brassicae* resting spores labelled with monoclonal antibodies conjugated to a fluorescein tag.

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3.1.4 Conclusion

The production of a specific monoclonal antibody to a fungal plant obligate parasite is difficult compared to organisms that grow culture media. However the extraction process used proved adequate in isolating *P. brassicae* resting spores from infected host material whilst retaining key immunogenic target determinants. A previous study reported improved specificity of polyclonal antisera to clubroot spores when spore soluble washings were used as immunogen (Wakeham & White, 1996). The collection of a 'pure *P. brassicae* soluble immunogen' proved problematical due to low level retention of bacteria (present initially in infected host material). Nevertheless the *P. brassicae* immunogens selected, and the intensive immunization schedule, resulted in the production of antibodies to clubroot resting spores. A comprehensive and strategic screening system has enabled the identification and isolation of a number of hybridoma spleen cells which produce antibodies to *P. brassicae*. Hybridoma selection has resulted in the selection of antibodies which are targeted to both soluble antigens and retained spore wall components of *P. brassicae* which will enable increased flexibility in future rapid test development. Selected hybridoma cells are currently being expanded, cloned and characterised.

3.2 Production of polyclonal antisera to *Plasmodiophora brassicae*

3.2.1 Materials and Methods

Production of *P. brassicae* immunogen for polyclonal antibody production and collection of *P. brassicae* resting spores from roots are as detailed in sections 3.1.2.1 and 3.1.2.2.

3.2.1.1 Collection of *P. brassicae* resting spores from roots

3.2.1.2 Immunization of mice

Four Female Balb C mice (coded 8272, 8273, 8274 and 8275) were each immunised a total of 5 times by intraperitoneal injection (see section 3.1.2.3) and at 2 weekly intervals. A terminal bleed was made 4 days post the final immunization. The collected polyclonal antiserum from each mouse was in sodium heparin and stored at 4°C

3.2.1.3 Antibody Screening

The collected polyclonal antisera were screened by immunofluorescence (IF) and by PTA ELISA (plate-trapped antigen enzyme-linked immunosorbent assay) against resting spores of clubroot as detailed in section 3.1.2.4 .

3.2.2 Results

For each polyclonal antiserum a good immune response was observed to its homologous *P. brassicae* antigen with a titer end point of 1 : 819,200 recorded for each when employing PTA ELISA (Figure 3). Using IF each of the polyclonal antiserum was observed to bind to whole *P. brassicae* resting spores (Plate 2).

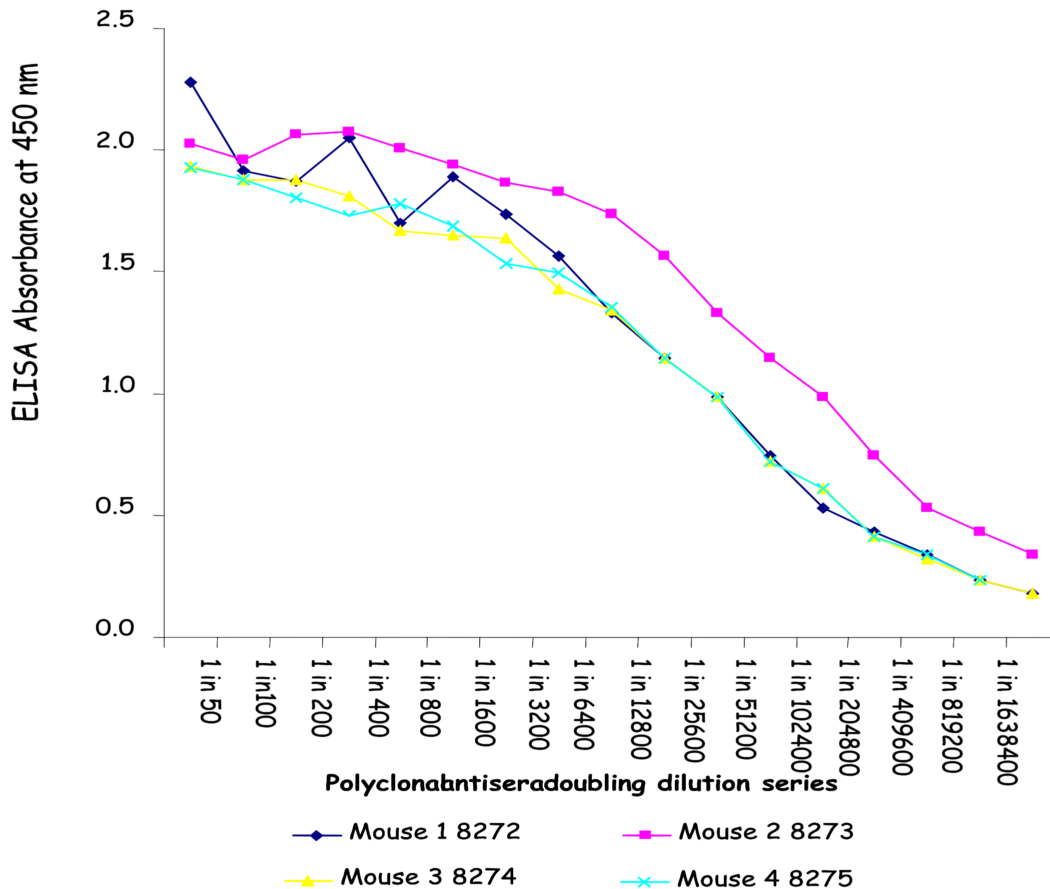

Figure 3. Response of collected polyclonal antisera of 4 Balb C mice immunised with *P. brassicae* resting spore components to homologous antigen by PTA ELISA.

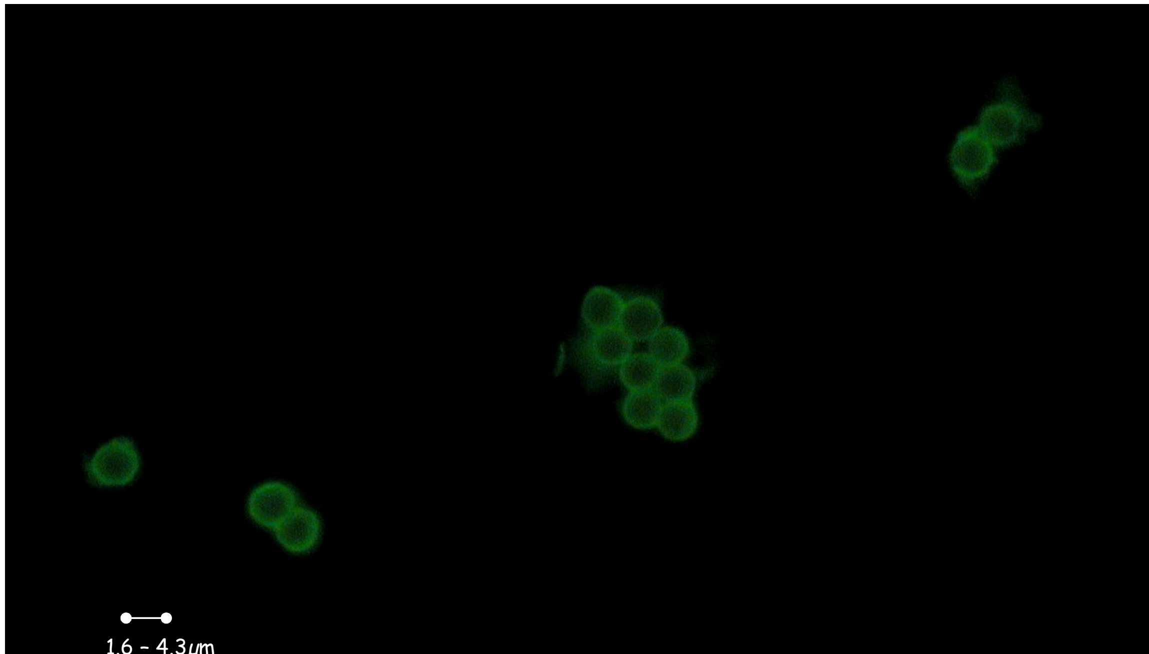

Plate 2 . Resting spores of *P. brassicae* labelled with a polyclonal antiserum and visualised with an anti-mouse flouroscein conjugate

3.2.3 Conclusion

The production of a pool of mouse polyclonal antisera which are highly reactive to clubroot spore material may prove important in the development of a non-competitive lateral flow assay format; hydrogen bonds associated with the Fc / FAb rabbit antibody have been attributed with non-specific reactions in lateral flow development (Klause Hochleitner, 2004; Theory and Practice of Rapid Immunodiagnostics Test Development and Manufacture). If the polyclonal is used in the development of a rapid assay format for *P. brassicae* in soil, the purification of the IgG fraction of this polyclonal antisera will be considered.

4. DETERMINATION OF CLUBROOT RESTING SPORE CONCENTRATION IN SOIL USING MOLECULAR METHODS

4.1 Isolation of DNA from *Brassica oleracea* root material

4.1.1 Introduction

Molecular methods are now routinely used in many areas of biology including medicine and forensic pathology. Multiplication of specific target DNA sequences by polymerase chain techniques (PCR) (Saiki et al., 1988) has been widely applied within biological systems, including downy mildews (Wiglesworth, et al. 1994, Alvarez et al., 1994). The polymerase chain reaction is now a key tool in molecular biology. It is a rapid and powerful technique for the *in vitro* amplification of DNA sequences. PCR has great potential for diagnosis and identification of plant pathogen interactions and can be particularly useful for identification of soil borne pathogens. The technique requires pure DNA from the test sample which act as a template and together with oligonucleotide primers result in synthesized DNA. The synthesised DNA can be amplified to form a measurable product which can be detected by gel electrophoresis as a visual signal. Molecular detection methods for clubroot based on PCR have been developed for conserved sequences in the 18S-like ribosomal RNA gene and the internal transcribed spacer (ITS) regions by the Department of Natural Resources and Environment (DNRE) Agriculture, Victoria, Australia. Specific detection using molecular techniques and has been shown to produce up to 1000-fold increase in the sensitivity of clubroot detection. Using these methods spore detection of $1x10^3g^{-1}$ and below has been reported.

4.1.2 Material and Methods

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 (Plate 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 (Plate 4).

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

4.1.3 Results

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 (Plate 4 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.

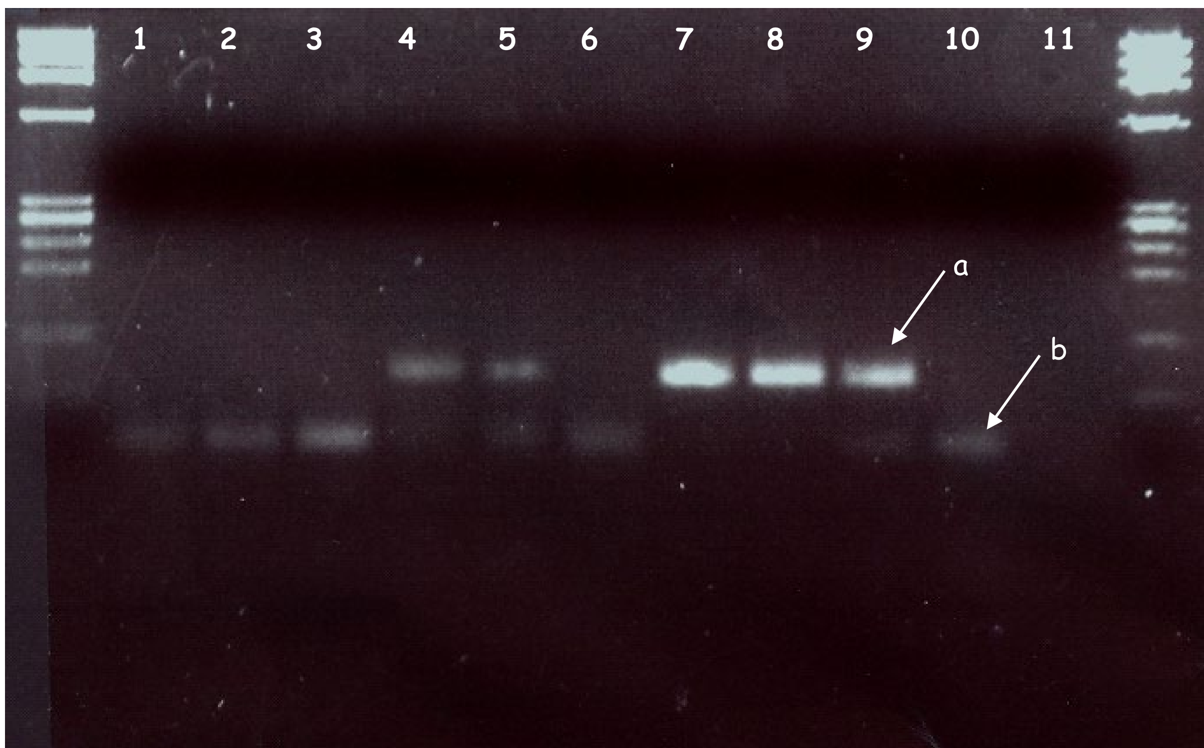

Plate 4 Amplicon products derived from control and clubroot infested root material employing polymerase chain reaction.

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)

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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.

4.1.4 Conclusion

Using primers developed specifically to regions of *Plasmodiophora brassicae* DNA, the presence of the pathogen was readily detected at a low level of infection when assayed under the conditions described above. Initial results suggest that the primers used are able to discriminate between non-infected and infected clubroot material of *Brassica oleracea* and may prove useful in quantitative infection studies

4.2 Preparation of clubroot infected field site for investigation of clubroot sampling regimes

4.2.1 Introduction

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

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4.2.2 Material and Methods

Treatment areas and plantings

Three pH areas were situated according to a soil sample taken in the quarantine field during May 2004. These were based on field areas created during trials conducted in 1996. Two plots were sited in each pH area each measuring 10 x 10 m with a 50 cm spacing between plants. Autumn cauliflowers cv. Belot were planted in one plot in each pH area (see Figure 4) 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.

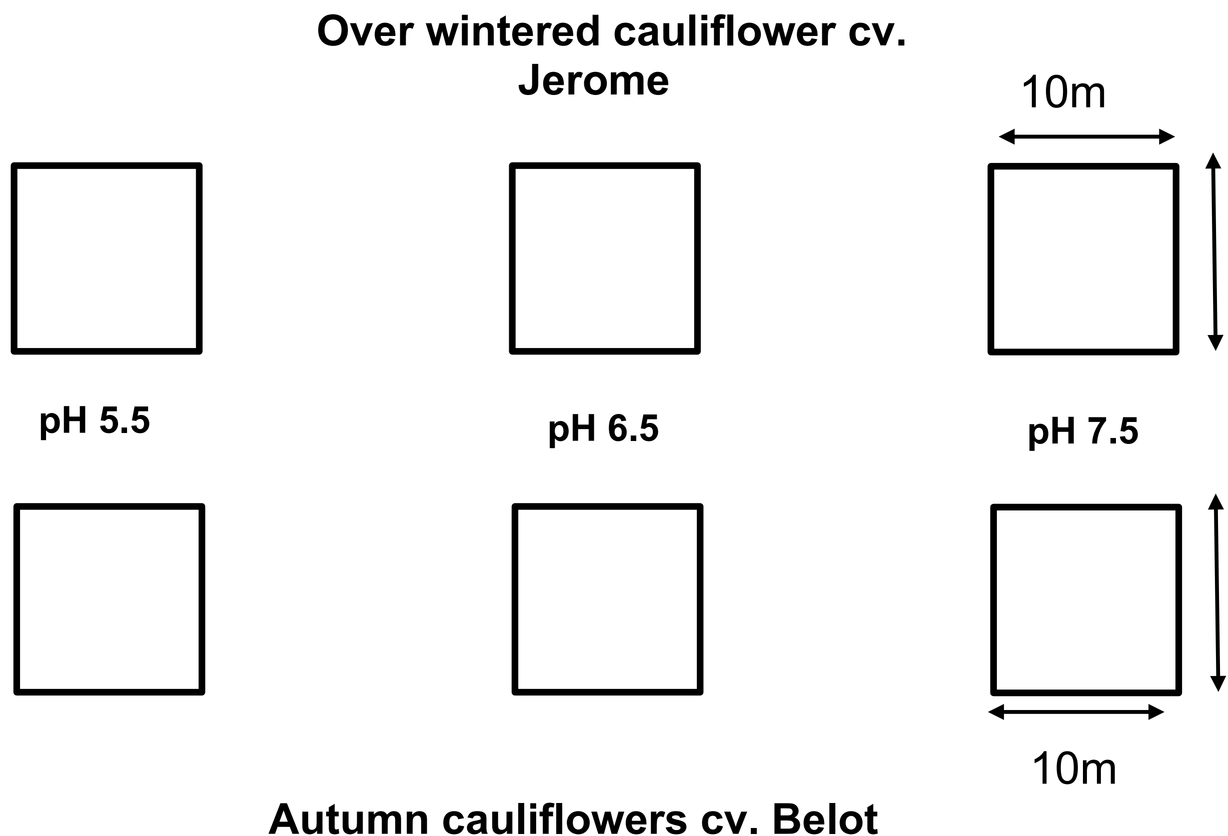


Figure 4 Experimental design of cauliflower clubroot inoculum variation trial area (Wellesbourne 2004)

4.2.3 Results and Conclusions

High levels of clubroot infection were observed on all autumn cauliflowers cv. Belot except I in plots at pH 7.5. However there was little clubroot infection on over wintered cauliflowers cv. Jerome regardless of pH. The arrangement of the plots with each pH area was sufficient to give variability in clubroot inoculum concentration over the course of the project. This was observed in the pattern of symptom expression in plots of both autumn and over-wintered cauliflowers. The level of symptom expression was highest in 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 (autumn or over-wintered) planted in the pH 7.5 area.

5.0 EVALUATION OF MOLECULAR METHODS FOR QUANTIFICATION OF CLUBROOT RESTING SPORES IN SOIL

5.1 Isolation of DNA from UK soils

5.1.1 Introduction

There are several challenges to developing a successful PCR assay. It is important that assays are absolutely specific. Efficient DNA extraction protocols are required from infected plant material and in some instances from isolated spores (Schaad & Frederick, 2002). Reliable methods for extracting target DNA from soil are critical since the presence of small amounts of contaminating DNA can render the PCR in accurate. For this reason methodologies must be used which reduce this possibility. The soil environment is complex and there is considerable variation in soil organic matter. For this reason universal methodologies are required which will give quantifiable DNA extraction regardless of soil type.

5.1.2 Materials and Methods

5.1.2.1 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.

5.1.2.2 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 1).

Table 1. DNA soil extracts as isolated for PCR (polymerase chain reaction)

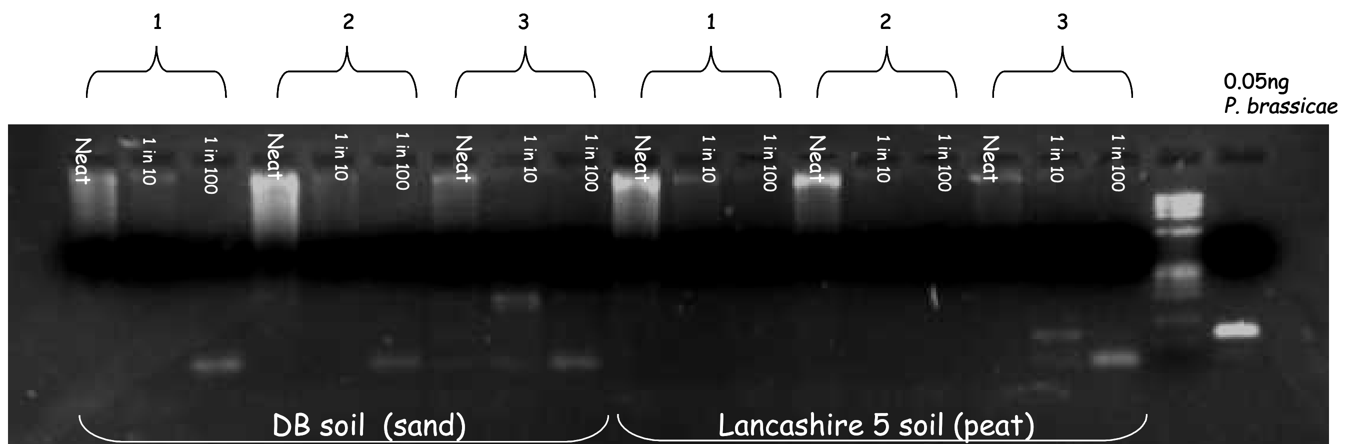
DNA extraction method	HRI DNA soil extract	HRI clubroot artificially infested DNA soil extract	Lancashire DNA soil extract	Lancashire clubroot artificially infested soil DNA extract
MOBiol UltraClean Soil DNA Isolation kit	10µl	10µl	10µl	10µl
MOBiol UltraClean Soil DNA Isolation kit & **DPI protocol**	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

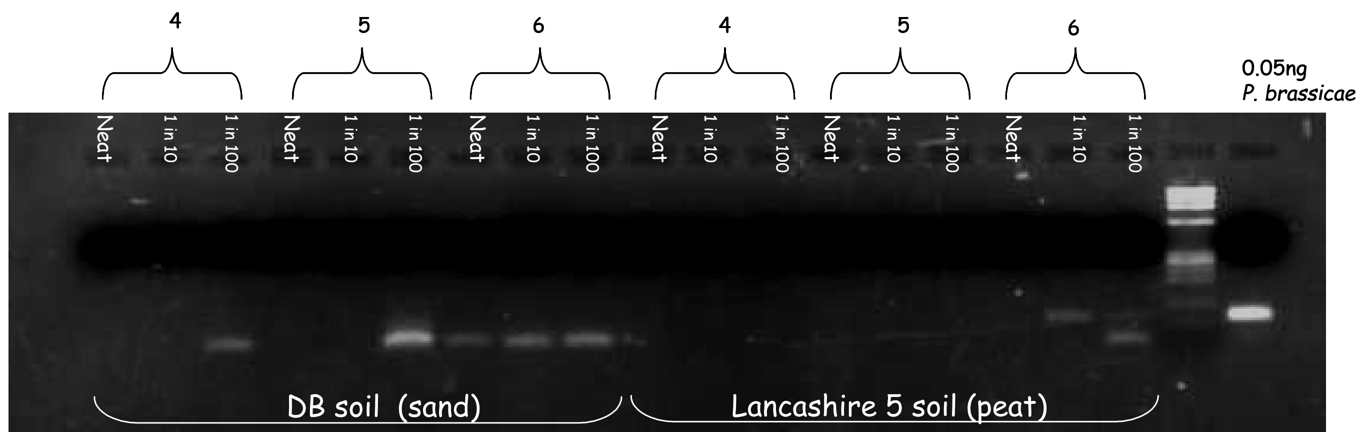
80µl volume was divided in to 2 x 40µl lots and processed as described above (Table 1). For each collected DNA soil extract (Table 1) 10 fold dilutions to 1 in 100 were made in TE buffer(10mM Tris-Cl,1Mm EDTA, pH8.0).

5.1.2.3 Preparation of DNA sample extracts for PCR

A 5µl volume of isolated DNA from a soil sample extract was mixed with 15µl of PCR Master mix (11.35µl H_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 (Plates 5a,b).



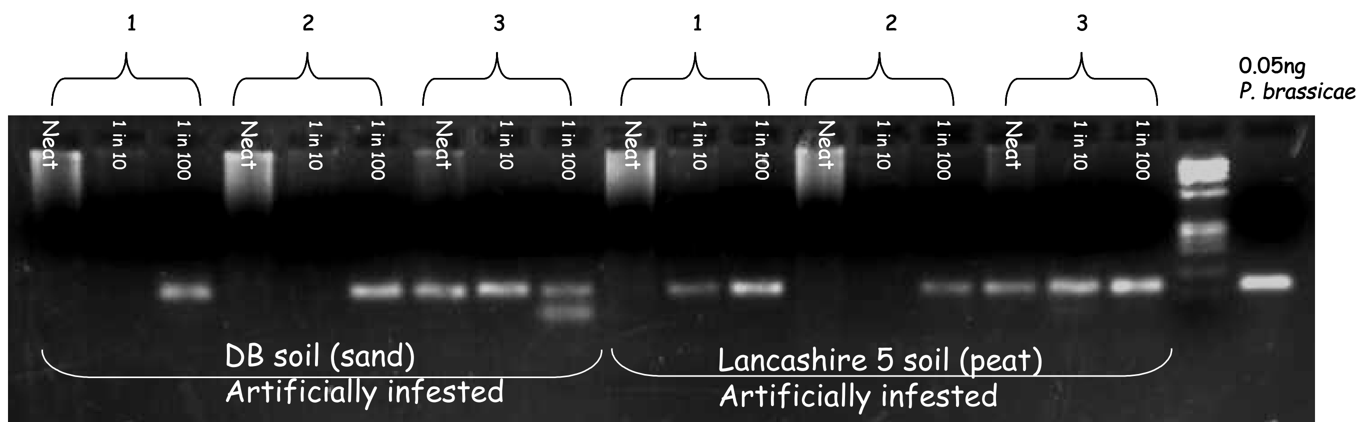
Q Biogene Fast Soil DNA Isolation Kit



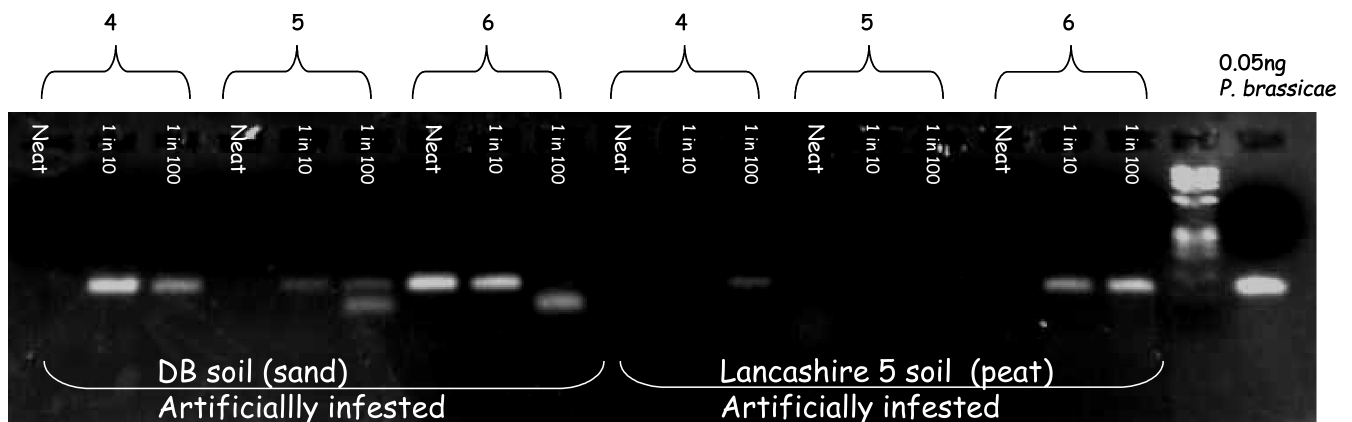
MoBio UltraClean Soil DNA Isolation Kit

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

Plate 5a 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

Plate 5b Molecular detection of *P. brassicae* inoculum in two artificially infested UK soils employing a range of DNA extraction processes

5.1.3 Results

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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 (Plates 5a,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 (Plate 5a). 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 (Plate 5b).

5.1.4 Conclusion

For both soil types the MoBio DNA soil extraction process was effective in the removal of non-specific interactions which resulted in high molecular weight smearing, as viewed in all 'neat' QBio extracted samples (Plate 5a, b). The QBio system however proved effective in removal of inhibitors specifically related to peat based soils (Lancashire sample), enabling the subsequent development and visualisation of *P. brassicae* amplicon product in artificially infested soils (Plate 5a). For each extraction process (MoBio and QBio) the use of an additional purification step enabled visualisation of *P. brassicae* amplicon product when artificially infested soils were probed. Nevertheless and, when viewed in conjunction with the control soils (low level clubroot infestation in Lancashire control soil), the use of the DPI system proved superior to that of the QIAquick PCR purification system. In comparison to the QIAquick PCR purification system the use of the DPI extraction process also proved optimal on a cost basis / test.

6.0 DISCUSSION

6.1 Developing rapid techniques for determining contamination of soil by pathogen organisms

Detection of soilborne pathogens has always been difficult because of soil properties and the sporadic occurrence of inoculum. However 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 have been developed by the Department of Natural Resources and Environment (DNRE), Victoria, Australia which could be used to detect clubroot resting spores in soil. In part of this project the validation of these tests and their adaptation for use for UK strains of the clubroot pathogen has been investigated. 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. With validation of these methods for UK clubroot isolates it will then be possible to investigate the usage of rapid “in field “ based techniques for clubroot detection. Using results from molecular tests across soil types will provide information on the accuracy and sensitivity of rapid “in field” immunological tests.

The project also requires a suitable sampling protocol for detecting low inoculum levels for use in the field. With more reliable information on the quantification of clubroot further investigation of appropriate control treatments can also be undertaken. Assessment of the effectiveness of clubroot control treatments has been problematical due to the lack of information on the initial infestion of soils by resting spores.

6.2 Validation of molecular tests for clubroot in UK soils

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Using the methods developed by the DPI, Victoria, Australia, tests on the presence or absence of clubroot resting spores were used on soil samples taken from UK vegetable Brassica production areas. Molecular tests confirmed the presence of clubroot at low levels in soil samples from Lancashire which was previously thought to be clubroot free. Although initial tests were limited to only a few samples further molecular tests are being conducted using a range of soil samples from all areas of vegetable production in the UK. At the moment the test can only discriminate between the presence and absence of clubroot in the soil (there is no quantification). However as lateral flow development depends on the detection of clubroot above and below economic or epidemiological thresholds it is likely that the present test can be utilised for this purpose. The molecular test will therefore be useful for lateral flow validation. Molecular tests are also being conducted to determine the limit of clubroot detection in each soil type artificially infested with clubroot resting spores at a range of concentrations. Additionally a collection of clubroot isolates from the UK are being investigated using the molecular test to determine if they can be detected or have differences in the limit of detection. When these investigations are complete it will then be possible to modify the molecular test or soil sample extraction methods so that greater accuracy can be obtained.

6.3 Producing antibodies for use in rapid immunodiagnostic tests

Within this project monoclonal and polyclonal antibodies have been successfully raised which selectively recognise components of the resting spore of *P. brassicae*. The antibodies raised to clubroot resting spores have not been extensively tested against other soil organisms. These tests are on going at the time of writing of this report. However initial results indicate that they are highly specific to the clubroot resting spore. Immunoflorescence tests conducted using the monoclonal antibody indicate that it reacts with the resting spore wall. Some of the antibodies raised appear specific enough be used in lateral flow test kits to detect clubroot. Further tests are required which investigate the sensitivity of these antibodies to clubroot resting spore concentrations. In other experiments the specificity of antibodies already raised will be tested against a range of common soil borne bacteria and fungi. If reactivity to other 'non-target' fungal/bacterial species is observed further antibodies may have to be raised to overcome this problem. Using simple indirect ELISA tests where the microtitre wells are directly coated with the test sample (PTA ELISA) the monoclonal's raised in this project appeared to be extremely sensitive to the presence of the clubroot resting spore. However the obligate nature of the clubroot pathogen and the binding step used in PTA ELISA's can give false positive results due to contamination. This means that the results must be confirmed using immunoflorescence techniques. Additionally these techniques can indicate what

each antibody is reactive to and the results can be used to improve the assay. If for example antibodies react to glycoproteins and carbohydrates (cell walls) also may cross reactive to proteinaceous antigens that can be precipitated from the sample by heat treatment before the antigen is coated to the well. Proteinaceous antigens are commonly found in soil samples.

6.4 Sampling regimes used for quantification of clubroot resting spores spatially

Developing and testing sampling regimes which can be used in combination with "in field clubroot tests" is an important part of the current project. Information on the number of samples, the bulking of samples and the number of tests required to be performed on each bulked sample would be important parts of any protocol for the detection clubroot in field soils. Sampling pattern adopted for infected soils would also be important and this may need to be varied depending on the level of clubroot which was required to be detected. 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. Sampling regimes could be tested which gave predicted distributions of clubroot which were not statistically different from the known distribution within the quarantine field. In this way the relative importance of sampling pattern, bulking procedures and test procedures can be ascertained. If these patterns were successfully applied to commercial crops future trials outside the scope of this study might investigate the association between levels of detection and control treatments which could be applied that were economic. Control treatments could involve 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.

6.5 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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