Developing on-farm diagnostic kits for brassica diseases

Robert Faggian
VIC Department of Primary Industries

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Level 1
50 Carrington Street
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Telephone: (02) 8295 2300
Fax: (02) 8295 2399
E-Mail: horticulture@horticulture.com.au

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On-farm diagnostic kits for brassica diseases.

Final report for project VG04059

Horticulture Australia Project No: VG04059

Project Leader: Dr Robert Faggian

Contact Details:
Department of Primary Industries,
Primary Industries Research Victoria - Parkville Centre
32 Lincoln Square Nth, Carlton
PO Box 4166, Parkville Vic 3052
Ph: +61 3 8341 2414
Fax: +61 3 9347 6056
E-mail: Robert.Faggian@dpi.vic.gov.au

Project Team:
Principal Researcher: Dr Vijay Kaul[1]
Project team member: Dr Caroline Donald[1]
Project team member: Dr Ian J Porter[1]

Collaborators:
Dr Roy Kennedy[2]
Dr Alison Wakeham[2]

Addresses:
1. Department of Primary Industries, Primary Industries Research Victoria, Knoxfield Centre. Private Bag 15 Ferntree Gully DC, Victoria 3156
2. Warwick HRI (Wellesbourne). Wellesbourne, Warwick, Warwickshire. CV35 9EF United Kingdom.

Purpose of project:
This project report details the outcomes of a 2-year study to develop new on-farm diagnostic test kits for soil-borne brassica diseases, using clubroot as a case-study example.

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We would like to acknowledge and thank Horticulture Australia Limited, the U.K. Horticultural Development Council, the Australian Vegetable Industry, the British Growers Association and the Department of Primary Industries Victoria for financial support.

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General Introduction

Plasmodiophora brassicae, which causes the clubroot disease of vegetable brassicas, is an obligate pathogen that is responsible for up to 10% of crop losses each year in Australia. It has a long-lived resting spore stage that is impervious to many pesticides, so an important control measure is preventing the spread of *P. brassicae*. This can be achieved by planting in areas where clubroot is not present and by using plants and growing media that are free of contaminating *P. brassicae* inoculum. A sensitive and reliable technique for the detection of clubroot inoculum is therefore essential in preventing the spread of *P. brassicae*.

The only commercial methods available to Australian growers for testing soil or water for the presence of *P. brassicae* at present is a plant bioassay or a laboratory-based molecular assay. The detection of *P. brassicae* with a plant bioassay is time-consuming, labour-intensive and not sensitive enough to detect low concentrations of resting spores (< 1000 spores). On the other hand, the molecular assay is extremely sensitive (<10 spores) but is also very expensive at approximately $450 per test. Neither is therefore useful as a supporting mechanism for routine soil testing to improve farm hygiene and prevent the spread of clubroot. Where the molecular test is most useful though is for the quantification of soil inoculum loads. Previous HAL-funded research (VG99008) developed a quantitative molecular assay for use in soil, and allowed growers for the first time to make informed disease management decisions through the selection of clubroot control measure that matched their particular level of soil infestation. The test has also proven invaluable as a research tool enabling quantitative assessments of the impact of control measures on clubroot populations. In terms of commercial use, the assay has found a niche in seed testing where the high sensitivity has proven to be useful in tracing the source of new outbreaks.

Ideally though, a diagnostic assay needs to be inexpensive, rapid and simple enough that it can be used on-farm by growers, or other non-specialist personnel. This would encourage the more widespread use of diagnostics as a pre-planting and predictive tool, to help prevent the spread of disease to clean areas, and to reduce chemical use by providing growers with the information necessary to make informed disease management decisions.

This report outlines a two-year study that was conducted in collaboration with leading international scientists from the United Kingdom (from Warwick HRI) and supported, financially, by British growers (through the U.K. Horticultural Development Council). The aim of the study was to develop a simple, inexpensive and rapid on-farm diagnostic test that could be packaged into a kit for use by growers on their own farms. The collaborative link was essential to the success of the concept, since it required both Australian molecular expertise and British serology expertise.

This report is organised into three sections that represent the three major themes of research activities.

1. DNA extraction from soil and optimization of molecular test.
2. Production and testing of monoclonal antibodies.
3. Optimisation and validation of on-farm kit.

Media Summary

Australian vegetable growers will soon be able to rapidly assess the threat of a major plant disease, using a home pregnancy-style testing kit.

The Victorian Department of Primary Industries (DPI) has joined forces with leading scientists from the United Kingdom to develop a fast, cheap and easy-to-use on-farm diagnostic kit for the devastating soil-borne disease known as clubroot.

Clubroot causes an estimated $16 million worth of damage and lost production in Australian brassica crops (broccoli, cabbage, cauliflower, Brussels sprouts) each year. The gross annual value of Australian brassica production is around $160 million.

DPI scientist Dr Rob Faggian said that the on-farm kit, which is similar in concept to home pregnancy testing kits, will be inexpensive ($10 per test) and produce a result in two minutes.

“Growers will be able to test their soil, water or plant material, and use the results to estimate the level of clubroot contamination,” said Dr Faggian.

The on-farm kit works using antibodies raised to specifically detect the fungus which causes clubroot.

Previous DPI research resulted in the world’s first rapid DNA-based test for clubroot. That test, which is now offered commercially by DPI’s Crop Health Services unit, is the most reliable and accurate, but can only be carried out by specialised staff in a laboratory and costs around $450.

“We were looking for a test that was both inexpensive and sufficiently accurate for crop management purposes,” said Dr Faggian.

“At the same time, researchers at Warwick HRI in the United Kingdom had developed tests capable of detecting clubroot in soil and water, but these were also technically demanding.”

“The two groups are now working together and combining their technologies in a jointly-funded project to develop the on-farm diagnostic kit for clubroot.”

Dr Faggian said that clubrooot detection had come a long way in recent years.

“Before the use of DNA technologies, the only way to detect clubroot was to grow susceptible plants for eight to ten weeks and look for symptoms.”

“That was too long to be useful for growers, who need to make management decisions based on the test results.”

The project team has developed a fully working prototype of the kit that has undergone significant field-testing in Australia. Validation of the test will continue as

the project, and collaboration, extends into a second phase, with a view to making the kit available to growers on a commercial basis in 2008.

“This joint project is a great example of two international vegetable communities coming together to tackle a problem common to both countries,” said Dr Faggian.

The research is supported by both British and Australian industry groups, as well as by the UK Horticulture Development Council and Horticulture Australia.

Technical Summary

Plasmodiophora brassicae, which causes the clubroot disease of vegetable brassicas, is an obligate pathogen that is responsible for up to 10% of crop losses each year in Australia. It has a long-lived resting spore stage that is impervious to many pesticides, so an important control measure is preventing the spread of *P. brassicae*. This can be achieved by planting in areas where clubroot is not present and by using plants and growing media that are free of contaminating *P. brassicae* inoculum. A sensitive and reliable technique for the detection of clubroot inoculum is therefore essential in preventing the spread of *P. brassicae*. Laboratory-based techniques such as PCR and ELISA assays already exist, and the aim of this study was to develop a simple, inexpensive and rapid on-farm diagnostic test that could be packaged into a kit for use by growers on their own farms. To achieve this, a collaborative link was established with leading international researchers at Warwick HRI (U.K.) and the research was financially supported by both British and Australian growers.

The study focussed on three areas:

1. DNA extraction from soil and optimization of molecular test.
2. Production and testing of monoclonal antibodies.
3. Optimisation and validation of on-farm kit.

The first part of the study developed various means to improve the reliability of the molecular diagnostic assay, with a view to using it to calibrate the prototype on-farm kit. The results demonstrated that commercially available kits for the extraction of soil DNA have improved significantly in recent years and are already effective in a range of soil types. The commercially available kits may produce DNA that is PCR-amplifiable without the need for pre- or post-extraction purification steps. This was the case in those soils tested in the U.K. However, for 'recalcitrant' soils, we trialed a number of methods to facilitate the extraction of PCR-amplifiable DNA, including:

- A DNA extraction additive (skim milk) to increase DNA yields from soil.
- A PCR reaction additive (T4 Gene 32 Protein) to reduce the inhibitory effects of humic/fulvic acids.
- Methods to adjust the sensitivity of the PCR (single-step nested, Sybr-Green1, TaqMan)

The second part of the study focussed on the development and testing of monoclonal antibodies for the specific serological detection of *P. brassicae*. This component of the work was carried out by the project's U.K. collaborators at Warwick HRI and resulted in the assembly of a prototype lateral flow device that enabled the positive diagnosis of clubroot resting spores in solution, using a monoclonal antibody (3A5). This is one of the few examples where this format of test has been applied successfully to a non-viral pathogen in plant pathology.

The third and final part of the study focussed on the establishment of the U.K.-developed antibody-based assays in Australia, and also on the validation of the on-farm kit prototype. The monoclonal anitbody 3A5 was able to detect Australian isolates of *P. brassicae* in soil in an ELISA plate test, and also as part of a prototype lateral flow device. Spore age and viability did not impact on the efficacy of the test, but the situation is expected to be different in the soil environment where biological

degradation will, presumably, ensure that dead or non-viable spores do not increase the risk of false positives. The prototype worked well in the case-study soil samples used throughout this project. Sensitivity was 10^5 spores per gram of soil, which is the threshold level for yield losses and therefore ideal for an on-farm decision-support tool. However, some inter-batch variability was observed between lateral flow devices that dropped sensitivity to almost 10^6 – quality control will therefore be a major issue in Phase II of the project, which will follow on from this project.

In summary, the project has developed a range of antibody-based diagnostic assays for the detection of *P. brassicae* in soil, as well as a series of improvements to the molecular assay to ensure it is robust and reliable. A prototype on-farm kit has also been developed that allows the detection of *P. brassicae* in soil – the test is simple and rapid, although there are a number of issues to be resolved before it can be offered commercially. Primarily, these are around test interpretation (removing subjectivity) and inter-batch quality control.

On-farm diagnostic kits for brassica diseases.
Final report for project VG04059

Part 1. DNA extraction from soil and optimization of molecular test.

Introduction

The success of molecular detection from environmental samples such as soil and water strongly depends on the quality and quantity of nucleic acid, which in turn is reliant on the method of extraction and purification. The improvement and optimization of DNA extraction methodology is therefore crucial to the advance of DNA-based approaches. Two factors that can complicate DNA extraction from soils are acidity, and soil-DNA interactions. Many protocols for DNA extraction from soil have been developed (Robe, et al, 2003; Schneegur et al, 2003), both for diagnostic purposes and to facilitate a better understanding of soil microbiology at the molecular level. However, these methods do not necessarily apply to all types of soil and modifications are often required to adjust to different soil types. Any modifications, however, need to be mindful of the intended use of the DNA – if for research purposes, then more complex and time-consuming procedures may be acceptable. On the other hand, if the DNA is for diagnostic purposes, modifications must be inexpensive, quick, and result in improved yields of DNA and improved quality of DNA.

For instance, Takada-Hoshino & Matsumoto (2005) were able to significantly improve the yield of DNA from problem volcanic ash soils simply by adding skim milk to the extraction buffer.

Our overall objective of this part of the study was to ensure the previously-developed clubroot molecular diagnostic assay was robust in a range of soil types (including U.K. soils) such that it could be used to a) quantitate clubroot inoculum accurately in both Australian and U.K. soils and b) calibrate the prototype on-farm kit so that it only detects economically relevant inoculum levels. The specific aims were to 1) investigate simple means to improve DNA recovery (quality and quantity) from soil that avoid complex or extensive additional purification steps, and 2) strike a balance between PCR detection sensitivity and the reliability and practical relevance of diagnostic test results by employing various pre- and post extraction purification steps.

To this end, soil samples were collected that represented contrasting soil types and were used for the majority of optimization and validation experiments throughout this study (listed below). Noteworthy is a rich Tasmanian soil with high organic matter content (>9%) - this soil had proved problematic for DNA extraction in past HAL-funded research. A range of other soils were also tested, and in particular those obtained through a collaborative link with Elders, who are assisting the project team to assemble a soil library from all brassica-growing regions around Australia. The completed molecular protocol was also tested on a range of U.K. soils.

Soils:

1) Knoxfield, Victoria (clay-loam; used as a negative control from non-brassica growing region and for spiking experiments)
2) Werribee, Victoria (clay soil; one source, from a known infested site, was used as a positive control, while another source, from a known non-infested site, was used in spiking experiments)
3) Forth, Tasmania (peat-loam with high organic-matter content (>9%); used as a positive control from a known infested brassica growing area)
4) Cranbourne/Sommerville, Victoria (sand; used as a positive control from known infested brassica growing region)

Note on DNA extraction procedure:
Unless specified otherwise, all DNA extractions from soil were carried out using the FastDNA SPIN Kit for soils (QBiogene, CA, USA). DNA extraction was performed according to the manufacturer's protocol but is essentially a sodium dodecyl sulphate-based extraction procedure that uses mechanical disruption to lyse cells and glass milk to recover DNA.

Briefly, 0.5g of soil was combined with buffer and surfactant and processed in a tissue matrix tube (containing glass beads) in a BIO101 FastPrep® FP120 instrument or QIAGEN TissueLyser manufactured by Retsch. After a protein precipitation step, a binding matrix (glass milk) was used to bind the DNA. The bound DNA was purified using a kit supplied SPIN filter. The DNA was eluted from the filter using nuclease free-water. The eluted DNA was subjected to further column purification through a PVPP column as described by Cullen and Hirsch (1998), only if humic acid was co-extracted, thereby giving it a light brown colour. Extracted DNA samples were stored at 4^0C. The purity of soil DNA was assessed spectrophotometrically by calculating both A260/A230 and A260/A280 ratios. Qualitative evaluation of possible DNA fragmentation was carried out by gel electrophoresis.

Optimisation of Molecular Diagnostic Assay:

1. Conventional two-step nested PCR clubroot diagnostic test.

The first significant finding of the study was simply the result of repeating the previously developed diagnostic protocol with updated versions of the commercially available kits. Whereas in 2001 it was not possible to reliably amplify DNA from Tasmanian soil without lengthy post-extraction purification procedures, in 2006/2007, with updated commercial DNA extraction kits, it was possible to PCR amplify DNA from the same Tasmanian soils. Amplification products were not always visible when run on agarose gels (Fig 1) (i.e. suggesting occasional low yields or the continuing presence of PCR inhibitors), but this is still a major improvement given no additional purification steps were used. This finding was supported by parallel work in the U.K. that found PCR-amplifiable DNA could be extracted reliably from a range of soils around the Lancashire brassica growing region using only the commercial kit and no pre- or post-extraction purification steps.

Fig.1. PCR amplification, in duplicate, of *P. brassicae* DNA extracted from 3 soils (K = Knoxfield, W = Werribee, T = Tasmania, N = Negative control and P = Positive control)

2. Two-step nested PCR vs single step nested PCR.

The second significant finding of this study was that the previously-developed two-step nested PCR protocol could be converted to a single-step protocol without losing sensitivity.

Methodology

Two Step PCR

A 5uL volume of soil DNA was mixed with 45uL of PCR reaction mix, which included 30ng of each *P. brassicae* specific primer (PbITS1 and PbITS2, Table 1) and 0.5 units of Qiagen HotStar DNA polymerase with associated proprietary buffers. Reactions were carried out in duplicate in thin-walled PCR tubes using a Hybaid PCR Express thermal cycler. To ensure reagents were free from contamination, negative controls were run with each PCR consisting of the same reaction mix but using 5 μL nuclease-free water instead of DNA template. A positive control using 2 μL purified *P.brassicae* DNA as template was also run concurrently. All reagents were dispensed in a laminar flow cabinet with aerosol-resistant filter tips.

The temperature cycling conditions for the first PCR were an initial denaturation at 94^0C for 5 min, followed by 7 cycles of : denaturation at 94^0C for 30 s, primer annealing at 62^0C for 30 s (with temperature decreasing 1^0C per cycle for 7 cycles), and a 30 s extension at 72^0C. This was followed by 30 cycles of: 30 s denaturation at

94^0C, 30 s annealing at 55^0C and 30 s of extension at 72^0C. At the end of cycling, the reactions were cooled to a holding temperature of 15^0C until ready for analysis.

For the nested stage, the reaction components were as described above, with the exception of primers and template DNA. Primers PbITS1 and PbITS2 were replaced by Pb8 and Pb9 (Table 1) and 2 μL of PCR product from the first PCR was used as template DNA. The cycling conditions for step 2 were the same as for step 1.

One step PCR

Both sets of primers, PbITS1/PbITS2 and Pb8/Pb9 were added to the same tube together with the same reagents as in Step 1 (described above).

Gel electrophoresis

The samples were loaded onto a 1% w/v agarose gel (GibcoBRL Life Technologies, Mulgrave,Vic) with 500 ng/mL EtBr. Molecular weight marker X (1 kb) (6 μL) (Roche Diagnostics, Nundwading, Vic) was added to a separate well as an indicator of PCR product size. Each gel was placed in an electrophoresis apparatus (BioRad, SubCell) with a running buffer of 0.5x TBE (0.045 M tris-borate, 0.001 M EDTA, pH 8.0) and a current of 100 V was applied until bands were well separated. The gel was visualised on a UV transilluminator.

Results and Discussion

The single step protocol resulted in detection of P. brassicae template DNA down to 100 fg, compared to 10 pg for the conventional nested protocol (Fig 2).

This single-step nested protocol has a number of advantages over conventional nested PCR. The first is its greater sensitivity – 100-fold over its predecessor. The second is the reduced risk of false positives through cross contamination – there is no need to open tubes between steps and release contaminating amplicons into the testing laboratory. The third and perhaps most significant is the reduction in the time required to conduct the test. The cost of a diagnostic PCR for clubroot, as quoted by the Department of Primary Industries' Crop Health Services, is $450. The greatest contributor to the cost of this, or any, commercially available PCR test is labour (reagents represent less than 5% of the total cost). Reducing the protocol from a two-step to a single-step PCR eliminates at least one hour of labour and therefore results in a significant reduction to the overall cost of testing.

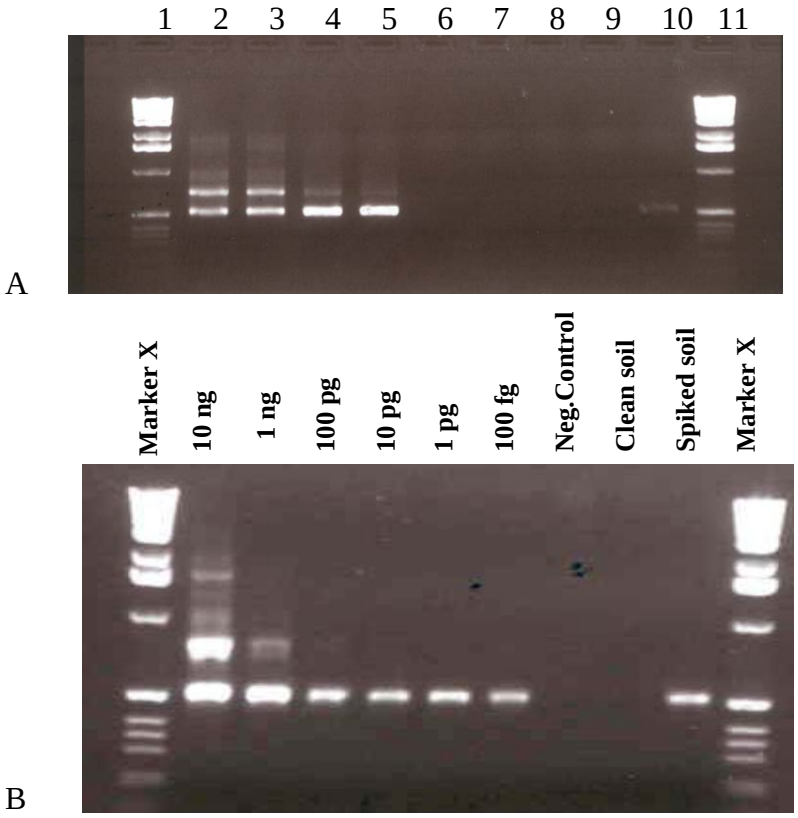

Fig. 2. Ethidium bromide-stained 1% agarose ge with (A) two-step nested and (B) single-step neste polymerase chain reaction products from a 10-fol serial dilution of *P. brassicae* DNA (lanes 2-7) water negative control (lanes 8), clean soil negativ control (lane 9) and positive control (lane 10).

Table 2: Primer sequences and expected product size after PCR amplification from *Plasmodiophora brassicae* DNA template (Faggian *et al.*, 1999)

Primer	Sequence	Product size
PbITS1	5' ACT TGC ATC GAT TAC GTC CC 3'	1100
PbITS2	5' GGC ATT CTC GAG GGT ATC AA 3'	
PbITS6	5' CAA CGA GTC AGC TTG AAT GC 3'	620
PbITS7	5' TGT TTC GGC TAG GAT GGT TC 3'	
Pb8	5' CCC CAT GTG AAC CGG TGA CG 3'	350
Pb9	5' TGC CGC AGC AAA GCT CAT TGT 3'	

3. Real-time PCR – TaqMan chemistry vs Sybr-green chemistry

Given the success of the single-step conventional PCR, similar gains were sought in real-time PCR. Real-Time PCR provides fast, precise and accurate quantitative estimates of starting template DNA, which can then be extrapolated back to estimate

inoculum loads of clubroot in soil. It is the quantitative nature of the clubroot diagnostic assay that has made it such a useful research tool.

Experiments were conducted to compare the performance of real-time amplification and quantification of *P. brassicae* DNA in a soil samples, using either Sybr-Green1, an intercalating dye that binds non-specifically to double stranded DNA (therefore as more double stranded amplicons are produced, the Sybr-Green dye signal will increase in proportion with increasing amount of amplified product), or a TaqMan hybridization probe. TaqMan chemistry utilizes a sequence-specific probe hybridizing between the forward and reverse PCR primers and is labeled with a fluorescent dye to detect the formation of amplicons during PCR amplification.

Compared to TaqMan probes, Sybr-Green1 is inexpensive and requires less optimization and pre-reaction preparation.

Methodology

Clean soil was spiked with clubroot resting spores to achieve concentrations ranging from 10^7 – 10^3 spores per gram. DNA was extracted from 0.5g sub-samples of the spiked soil using the FastPrep Spin Kit as described earlier.

PCR was performed in a real-time thermal cycler (Corbett Rotogene) using either Sybr-Green1 dye or a TaqMan probe. The primers used for the Sybr-Green1 reaction were PbITS7 and PbITS7, and the temperature cycling regime was the same as for the conventional PCR described above. The primers used for the TaqMan reaction were Pb8 and Pb9 and the temperature cycling regime was as specified by the probe manufacturer.

Results and Discussion

Both TaqMan and Sybr Green1 chemistry allowed reliable quantification of *P. brassicae* spore dilutions in soil down to at least 10^5 spores per gram (Fig 3 and Fig 4). TaqMan chemistry gave better separation of 10-fold dilutions and therefore allowed better discrimination of inoculum loads in soil. TaqMan was more sensitive, especially when DNA from sources other than soil were used (eg. Pure *P. brassicae* DNA, or spores diluted in water) – under these circumstances it allowed quantification of *P. brassicae* spore dilutions down to 10^2 spores per gram (Fig 4). However, after prolonged use of the quantitative protocol in conjunction with TaqMan chemistry, persistent problems with false negatives were encountered. Repeated efforts to eliminate the problem eventually helped us to determine that continuous use, in the one location, can lead to amplicon contamination of the laboratory. The high sensitivity of the assay made it particularly susceptible to this sort of contamination and thus false positives.

Given that the intention of the molecular assay, and indeed the on-farm kit which it will support, is to detect clubroot inoculums levels that are likely to result in economic losses (ie. 10^5 spores per gram of soil in most soil types), high sensitivity is not a requirement for routine soil testing. As such, Sybr-Green1 was selected and optimized for soil testing, and TaqMan chemistry was reserved for cases where high-sensitivity was required, such as seed and water tests.

Standard curves were subsequently developed for the routine quantification of *P. brassicae* spores in soil using Sybr-Green1 chemistry in conjunction with the conventional PCR primers mentioned earlier (Fig 5). Excellent correlations between DNA concentration and cycle number were achieved (R^2>0.99) (Fig. 6), indicating that the assay could be used to reliably quantitate spore numbers in soil.

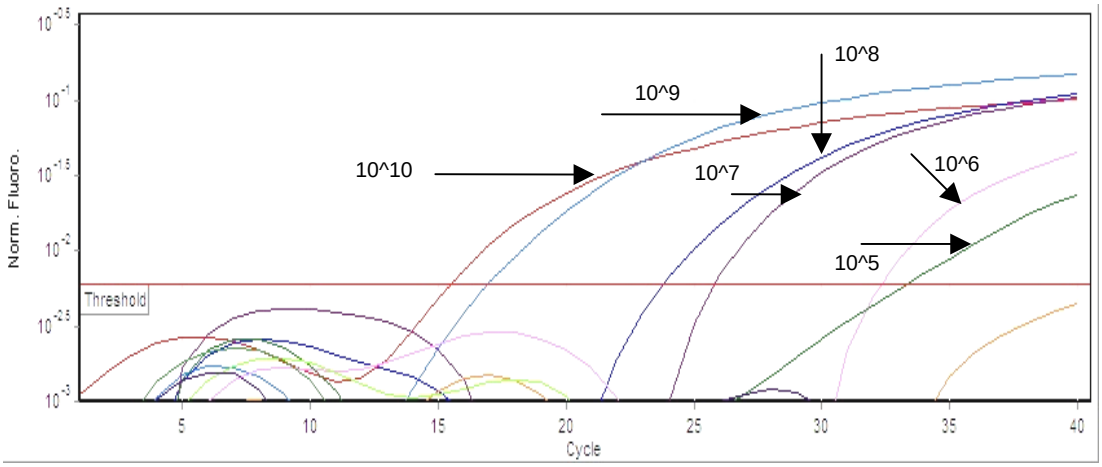

Fig. 3 Amplification plot of serially diluted *P. brassicae* spores in soil using TaqMan Chemistry.

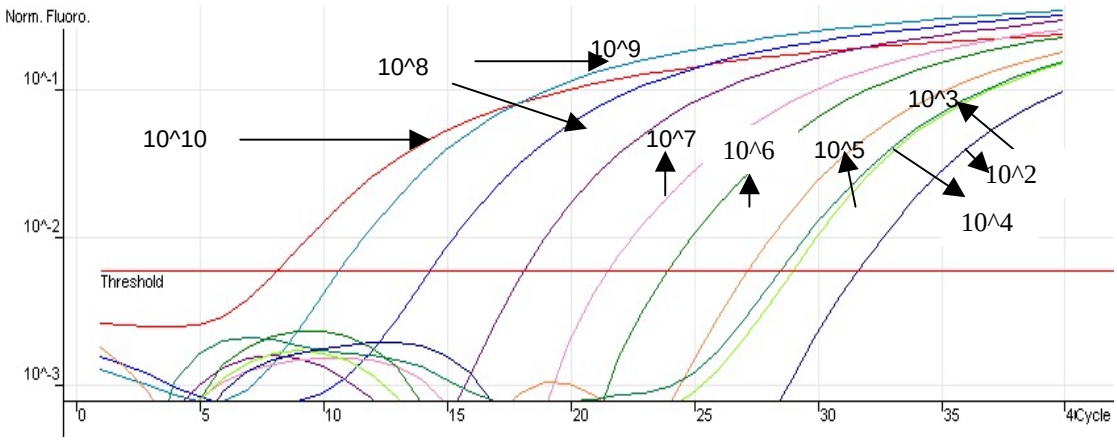

Fig. 4 Amplification plot of serially diluted *P. brassicae* DNA using TaqMan chemistry.

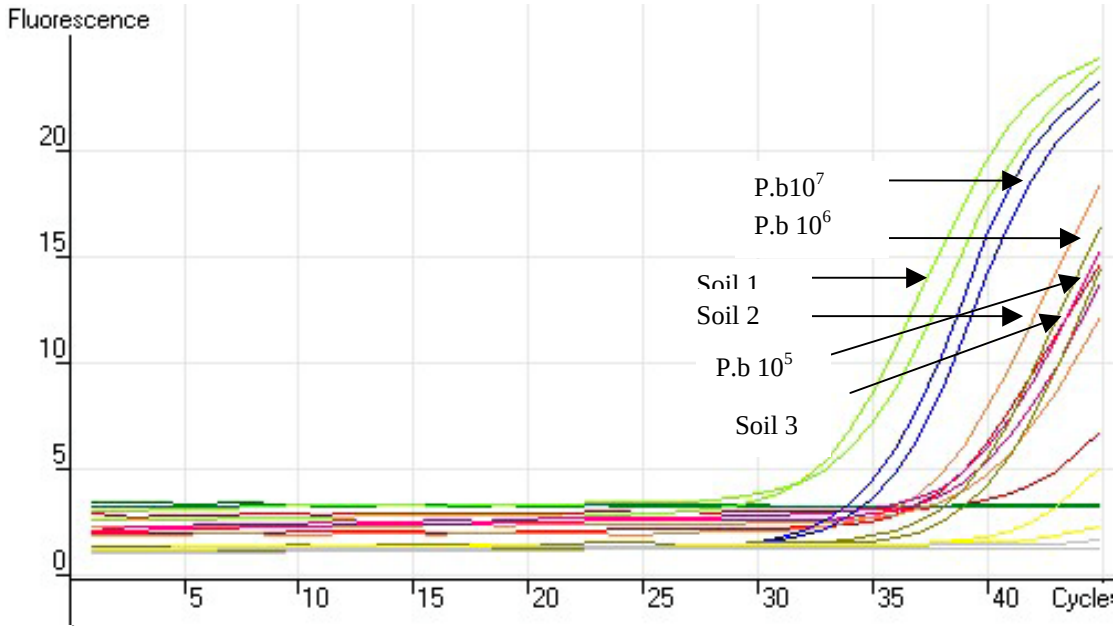

Fig. 5 Amplification plot of serially diluted *P. brassicae* spores in soil using Sybr-Green1 Chemistry. Soil 1 = DNA from spiked (10^7) Knoxfield soil, Soil 2 = DNA from Werribee (Unknown inoculum level), Soil 3 = DNA from Tasmania (Unknown inoculum level).

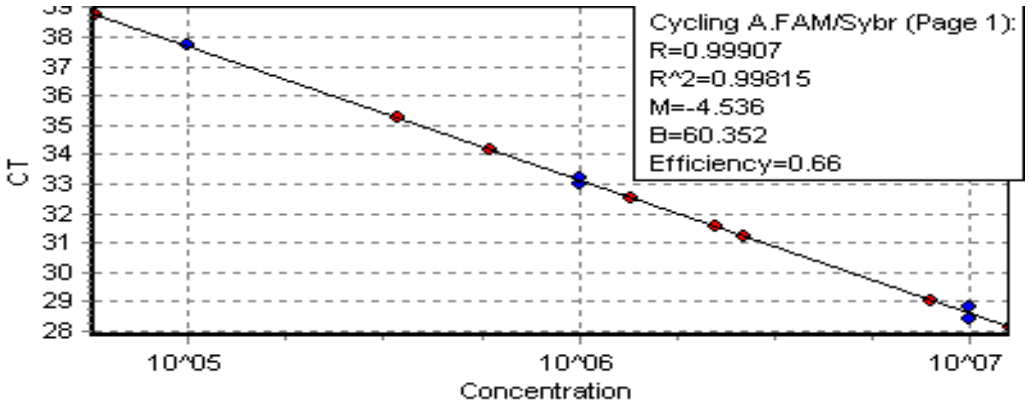

Fig. 6. Standard curve of serially spiked soil. Correlation coefficient (R-value) of R= 0.99 (where 1 is a perfect fit).

4. Pre-treatment (Calgon) vs reaction additive (T4 Gene 32 Protein)

Calgon vs T4 Gene 32 Protein:

Two of the most well-publicized problems associated with microbe DNA extraction from soil is the presence of humic substances like humic and fulvic acids, and DNA-soil or cell-soil interactions.

Generally, humic acids, which are often co-purified with DNA from soil, interfere with PCR by directly inhibiting DNA polymerases. Soil-DNA interactions, however,

or soil-microbe interactions, result in DNA or microbes binding to soil (usually clay, in the case of DNA) and therefore giving reduced yields of DNA.

Donald *et al*. (2004) (VG03022 HAL final report) suggested a soil pre-treatment with 2% Calgon (sodium hexametaphosphate) to overcome the potential binding of clubroot resting spores to soil particles, and thus make them more available for downstream DNA extraction procedures. The method, while improving DNA yields from problem soils such as those from Tasmania, is lengthy and involves several long incubation periods with careful pH adjustments.

An alternative method of dealing with the PCR-inhibitory substances present in soil DNA was trialed that challenges the premise of soil-spore interactions. The T4 Gene 32 Protein was added to PCR reactions on the assumption that, as has been reported in the literature many times, the major cause of PCR failures when using soil DNA is co-purified humic and fulvic acids. The T4 Gene 32 Protein increases the minimum-inhibitory-concentration of humic/fulvic acids such that DNA polymerases can function in the presence of higher concentrations of these PCR inhibitors (Tebbe & Vahjen, 1993).

Methodology

A sample of particularly problematic soil (from Howqua in Victoria's high country – selected for its potential for PCR inhibition via high clay and humic acid content) was divided into two sets of subsamples and spiked with clubroot resting spores. One set was subjected to Calgon treatment followed by routine DNA extraction and PCR procedures. The other received no pre-treatment and instead T4 Gene 32 Protein was added to PCR reactions as per Tebbe and Vahjen (1993).

Results and Discussion

The results of quantitative real-time PCR show that both Calgon pre-treatment (Fig. 7a) and T4 Gene 32 Protein treatment (Fig. 7b) enabled the amplification of clubroot DNA from soil. However, T4 Gene 32 Protein resulted in significantly earlier detection of clubroot DNA at cycle 28 instead of 33, which represents approximately ten-fold greater level of sensitivity.

Treatment with T4 Gene 32 Protein is a post-extraction procedure that involves the addition of protein to the PCR reaction mix. It is much quicker and simpler than Calgon treatment, and more effective. It also has a completely different mode of action, since it acts directly on the DNA polymerase and enables the enzyme to function in the presence of inhibitors. Calgon, on the other hand, is supposed to interfere with soil-spore interactions.

However, the T4 Gene 32 Protein results, and the finding that updated soil extraction kits were able to extract DNA from soil more reliably than previous iterations, suggested that the mechanism of action of Calgon may not involve the soil-spore interaction at all. Instead, the more likely scenario is that the pH adjustments inherent in Calgon treatment somehow reduce the co-purification of humic/fulvic acids during downstream DNA extraction procedures.

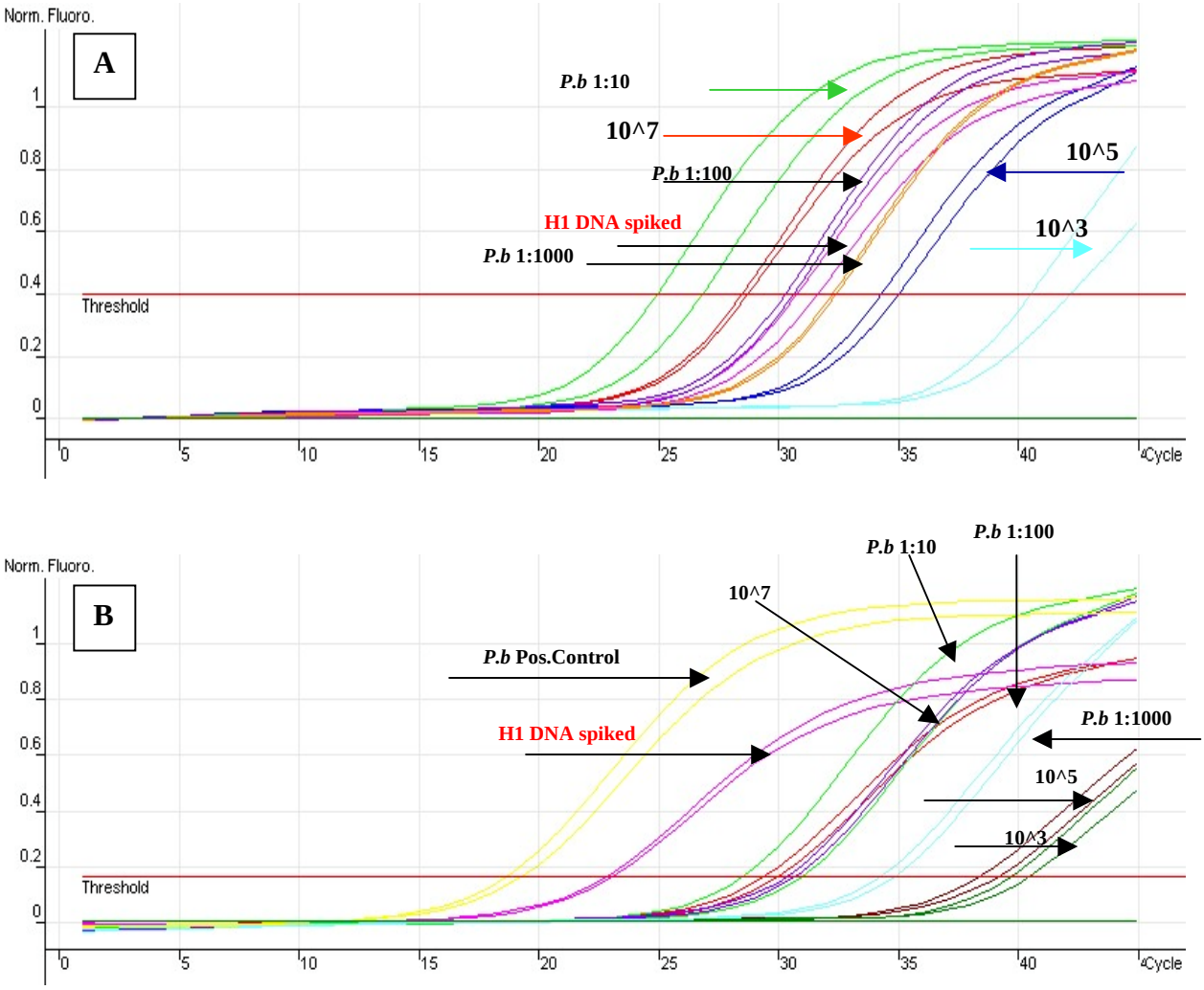

Fig. 7. Comparison of real-time quantitation of *P. brassicae* DNA in soil following pretreatment with a) Calgon and b) no-pretreatment plus T4 Gene 32 Protein.

5. DNA-Soil interactions

As mentioned, soil-DNA interactions can reduce the yield of DNA from soil, but Calgon may not in fact address this. There are two options – separation of microbes from soil (eg. via differential centrifugation, or similar techniques), or the addition of substances to the extraction buffer that block DNA binding sites on soil. One of the most commonly reported additives is skim milk, and it has been reported to improve the yield of DNA extraction from soil.

Methodology

Ten milligrams of skim milk powder was added to soil, then subjected to routine DNA extraction procedures. The resultant DNA was run on agarose gels to determine the quantity and quality of DNA.

Results and Discussion

DNA extraction from soil using FastDNA Spin Kits with the addition of skim milk powder resulted in the extraction of large quantities of high molecular weight DNA (Fig. 8).

The addition of additives like skim milk, or other nucleic acids (RNA) act as adsorption competitors that reduce the number of binding sites on soil. With fewer binding sites, particularly on clay particles, more DNA can be purified from soil. Hoshino and Matsumoto (2005) have previously demonstrated that skim milk is effective in increasing the yield of DNA from 'recalcitrant' Japanese soils with high clay contents, and that it does not increase the yield of DNA from sandy soils.

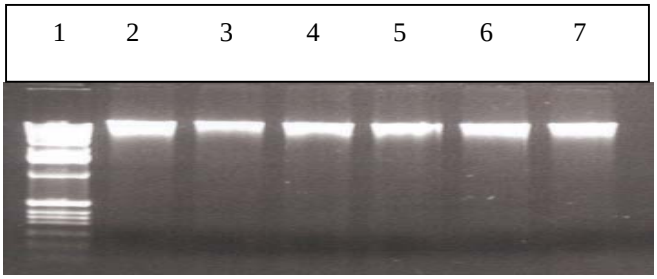

Fig. 8. Ethidium bromide-stained 1% agarose gel of high molecular soil DNA extracted with the addition of skim milk.

Summary

This portion of the study focused on means to improve the reliability of the molecular diagnostic assay, with a view to using it to calibrate the prototype on-farm kit. The results demonstrated that commercially available kits for the extraction of soil DNA are effective in a range of soil types and may produce DNA that is PCR-amplifiable without the need for pre- or post-extraction purification steps. This was the case in those soils tested in the U.K. However, the fact that problem soils were not encountered in the U.K. but have been in Australia is probably more a factor of the scale of clubroot testing that has occurred in Australia (due to previous HAL-funded research), and not because there are no problem soils in the U.K. As such, we investigated a number of options to enable molecular diagnosticians to overcome potential problems associated with DNA-based molecular assays for soil-borne pathogens. These included:

- A DNA extraction additive (skim milk) to increase DNA yields from soil.
- A PCR reaction additive (T4 Gene 32 Protein) to reduce the inhibitory effects of humic/fulvic acids.
- Methods to adjust the sensitivity of the PCR (single-step nested, Sybr-Green1, TaqMan).

These options should be added to those previously developed, such as post-extraction purification through ion-exchange chromatography columns (VG99008), Calgon treatment (VG03022) and spore separation methods (VG99008). In total, these tools form a large part of the molecular diagnostician's arsenal, enabling reliable and robust detection of pathogens in soil using PCR.

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On-farm diagnostic kits for brassica diseases.
Final report for project VG04059

Part 2. Production and testing of monoclonal antibodies

Introduction

Antibodies are molecules that can bind with high affinity to antigens. They are produced by mammalian immune systems where they are used to help identify invading organisms or substances. Researchers have over the years devised methods to manipulate the immune/antibody response to produce a wide range of specific, high-affinity antibody molecules for use in diagnostic assays, including against plant pathogens. The use of antibody technology in this way is well established, particularly in medical diagnostics, and interest in its use in plant pathology has been increasing over the last decade. Methods for producing antibodies for plant pathogen diagnostics and their use have been reviewed by several authors (Werres & Steffens 1994; Dewey & Thornton, 1995; Dewey et al., 1997; Torrance, 1998).

Diagnostic methods based on antibodies, such as ELISA, are robust and generally can be used in at least a semi-automated fashion. However, one of its drawbacks is specificity. Although excellent for viral pathogens, the production of antibodies has been less successful for more complex organisms such bacteria and fungi; it is difficult to find antigens, against which to raise antibodies and which are specific to the pathogen in question. The second problem is sensitivity. ELISA is often not sensitive enough to reliably pick up small amounts of pathogen. Thus, the popularity of DNA-based diagnostic techniques continues to grow.

The relative lack of sensitivity means that there is a possibility of ELISA failing to detect low pathogen levels and therefore result in false negatives. However, there are advantages, including the low cost of antibody tests, the scope for automation and high-throughput sample processing, and the ability to transfer assays onto simple platforms such as Lateral Flow Devices, which allow diagnostics to be carried out by non-specialist staff out in the field.

With respect to *P. brassicae*, Arie *et al*. (1988) developed an immunofluorescence detection method for use with soil samples. However, the cross-reactivity of the antiserum with other organisms was not tested, and detection limits were not discussed. Lange *et al*. (1989) prepared a polyclonal antiserum for use in a dot immunobinding protocol to detect *P. brassicae*. The antiserum did not cross-react with *Polymyxa graminis*, another plasmodiophorid, or the common root pathogens *Rhizoctonia solani*, *Pythium ultimum* and *Fusarium oxysporum*. The technique allowed detection of *P. brassicae* in infected plant tissue up to a dilution of 1 in 2048, prompting the authors to state "The sensitivity obtained was within the range permissible for a routine test." The method was not used for soil detection, although it was suggested that a fluorescent antibody technique be developed to detect *P. brassicae* in soil samples.

Wakeham and White (1996) prepared several polyclonal antisera against *P. brassicae*, and used them in the development of soil diagnostic tests in the form of western-blots, dip-sticks,

dot-blots, immunoblotting assays, indirect enzyme-linked immunosorbent assays (ELISA) and indirect immunofluorescence assays. A range of soil fungi, including the closely related plasmodiophorid, *S. subterranea*, generally showed low cross-reactivity with the antisera. A detection limit of 100 spores per gram of soil was achieved with one of the polyclonals, PAb 15/2, when used in the dip-stick method, indirect ELISA and immunofluorescence.

Generally, the specificity of antisera raised against fungal plant pathogens can vary (Hardham *et al*., 1986; Savage & Sall, 1981), and as polyclonal antisera cannot be reproduced (and are therefore limited in quantity), their usefulness as diagnostic tools is limited. It is therefore imperative that any antibody-based diagnostic technique has at its core a specific monoclonal antibody.

Here we report on the production of several monoclonal antibodies and their testing for specificity to *P. brassicae* and sensitivity. All work in this part of the report (part 2) was carried out in the U.K. by Warwick HRI staff members Dr Alison Wakeham and Dr Roy Kennedy.

Methodology

1. Production of *P. brassicae* immunogen for antibody production

Washed clubbed root galls (20g fresh weight) from *Brassica napus* 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 to a final volume of 20ml. To remove *Brassica napus* 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 *P. brassicae*. The collected resting spores were resuspended in 10ml chilled sterile distilled water and adjusted to a concentration of $1x10^9$ spore ml^{-1}. The 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. The sample was rehydrated in 1.6ml PBS.

Immunization of mice with P. brassicae resting spore washings

A female 6 week old Balb C mouse (coded 9013) was immunized by intraperitoneal injection with 100µl of the spore washing preparation mixed with an equal volume of Titermax adjuvant (www.sigmaaldrich.com, H4397). At two weekly intervals the mouse was immunized a further four times.

Monoclonal antibody production

Four days after the final immunization a terminal bleed was collected and the spleen of the mouse was removed. Antibody producing B cells, isolated from the mouse spleen, were fused in vitro with Ag-SP2 carcinoma cells (www.immunesystems.co.uk, ISP1/SP2) at Warwick HRI (Wellesbourne) according to the method of Kennett *et al*., 1980. Two weeks after cell fusion the cell cultures were screened both by immunofluorescence (IF) and by PTA ELISA (plated trapped antigen enzyme-linked immunosorbent assay) for the presence of antibodies which recognised components associated with the resting spore of

P. brassicae. Four P. *brassicae* positive cell lines (coded: 3A12 H8 H9, 3A5 B6 C7, 2D6 G7 E2 and 2H4 C10) were selected, twice cloned and expanded in Dulbecco's modified Eagles medium (DMEM) (www.sigmaaldrich.com, D5796) 10% Foetal Calf Serum (FCS) (www.immunesystems.co.uk, T31/FCS).

Collection of monoclonal antibodies
Monoclonal antibodies were collected in the tissue culture supernatant medium (TCS) (DMEM, 10%FCS) with cell debris removed by centrifugation at 1200g for 10 minutes. To inhibit microbial growth 0.04% sodium azide (NaN_3) was added to each collected TCS medium. Each monoclonal antibody TCS was stored at -20°C in 200µl aliquots prior to use.

Harvested monoclonal antibody TCS's were coded : 3A12, 3A5, 2D6 and 2H4.

2. Antibody reactivity testing

Production of P. brassicae antigen
Washed clubbed root galls (20g fresh weight) from *Brassica napus* 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 napus 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 *P. brassicae*. The collected resting spores were resuspended in 10 ml chilled PBS (0.02% sodium azide) and adjusted to a concentration of 1x108 spore ml-1.

Spongospora subterranea
John Walsh (Warwick HRI) supplied 0.3g S. subterrane spore balls which were hydrated in 1ml PBS.

Production of 'non-obligate' fungal plant pathogen antigenic material
Selected fugal isolates (*Trichoderma* sp., *Rhizoctonia solani*, *Phoma betae*, *Sclerotium cepivorum*, *Phoma sp.*, *Sclerotinia sclerotiorum*, *Fusarium culmorum*, *Verticillium dahliae*, *Pythium sylvaticum*, *Botrytis cinerea*, *Plasmodiophora brassicae* and *Spongospora subterranea*) were inoculated on to an agar medium that had been precovered with a PN6026 Supor 450 90mm diameter membrane (Gellman Sciences Cat. No. 60206). When full radial growth of the 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 aqeous phase was retained and stored on ice whilst the fungal pellets were resuspended in 2ml PBS.

Spore disruption
0.5ml aliquots of each of the resuspended fungal suspensions (contains spore and / or mycelial fragments) was dispensed into Eppindorfs containing 0.5g of 0.5mm diameter bollotini beads (Jencons-PLS, Leighton Buzzard, UK. Cat. No. 136-015). Using a Fast

Prep device (QBiogene) and, operating at a speed setting of 5 for each of three 20 second periods, collected spore suspensions were disrupted. Following each 20 second disruption phase spore samples were retained on ice for 5 minute periods. The aqueous phase of each fungal spore type was then collected and centrifuged in a lab top microfuge at 13,000 rpm for 5 mins. The soluble fraction of each fungal spore type was retained and combined with the homologous soluble phase of the previous extraction and, stored on ice. The fungal pellet for each spore type was rehydrated in 1ml PBS. The protein concentration of each spore fraction was determined using a Bio Rad Protein assay (Bio-Rad, Cat No. 500-0006) and adjusted to 2µg -1 ml.

ELISA

Fungal 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 (see plate layout). Unbound material was then removed by inverting the strips over a sink and then slapping them directly down on to absorbent towelling. The microtiter wells were then washed once with 200µl PBS. The microtiter wells were blocked with 200µl of 1% Casein (1% (w/v) casein PBS) and incubated at 37°C for 45 minutes. Residual blocking buffer was removed and the wells were washed once with 200µl PBS, 0.05% Tween 20 and 0.1% Casein (PBSTw C). After which each well received immune sera diluted in PBSTw C.

Following incubation in a Wellwarm shaker incubator (30°C) for a period of 45 min. wells were washed three times for one minute each with 200µl PBSTw C. A DAKO duet amplification system was then used (DAKO Ltd, Angel Drive, Ely, Cambridge, UK; Cat no. k0492) to amplify the signal generated by bound tissue cuture supernatant antibodies. Wells were washed as described above and to each well 100µl of 3,3',5,5'-tetramethylbenzidene substrate (Sigma T-3405 and P-4922) was then added. The reaction was stopped by adding 25µl of a 20% 1MH2SO4 solution to each well. Absorbance at 450nm was determined with a BioHit BP800 ELISA plate reader (Alpha Laboratories, 40 Parham Drive, Eastleigh, Hampshire, U.K.)

Immunofluorescence

Each monoclonal antibody was tested against *P. brassicae* in an immunofluorescence assay. A 20 µl suspension of resting spores 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.

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

Results and Discussion

The results of specificity testing show that 5 monoclonal antibodies were specific for *P. brassicae*, with little cross-reactivity against a range of non-obligate plant fungal pathogens (Figs 8a & 8b), as well as the closely related *S. subterranea* (Fig 10). Monoclonal 3A5 showed virtually no cross-reactivity. Also, the preparation of antigen (ie. whether soluble fraction or particulate matter) did not impact on specificity, but soluble components did, in general, elicit a stronger reaction, which will have implications for the extraction procedures used in the final on-farm kit that is released to growers.

Monoclonal antibody 3A5 was selected for further analysis and incorporation into a lateral flow device for the first on-farm kit prototype, due to its low cross-reactivity with other soil organisms and high reactivity with *P. brassicae.*

For comparison purposes, and to highlight the usual problems associated with the development of antibodies against antigens from complex organisms such as fungi, the results of several monoclonals with unacceptable levels of cross-reactivity are also presented (Fig. 9a and 9b).

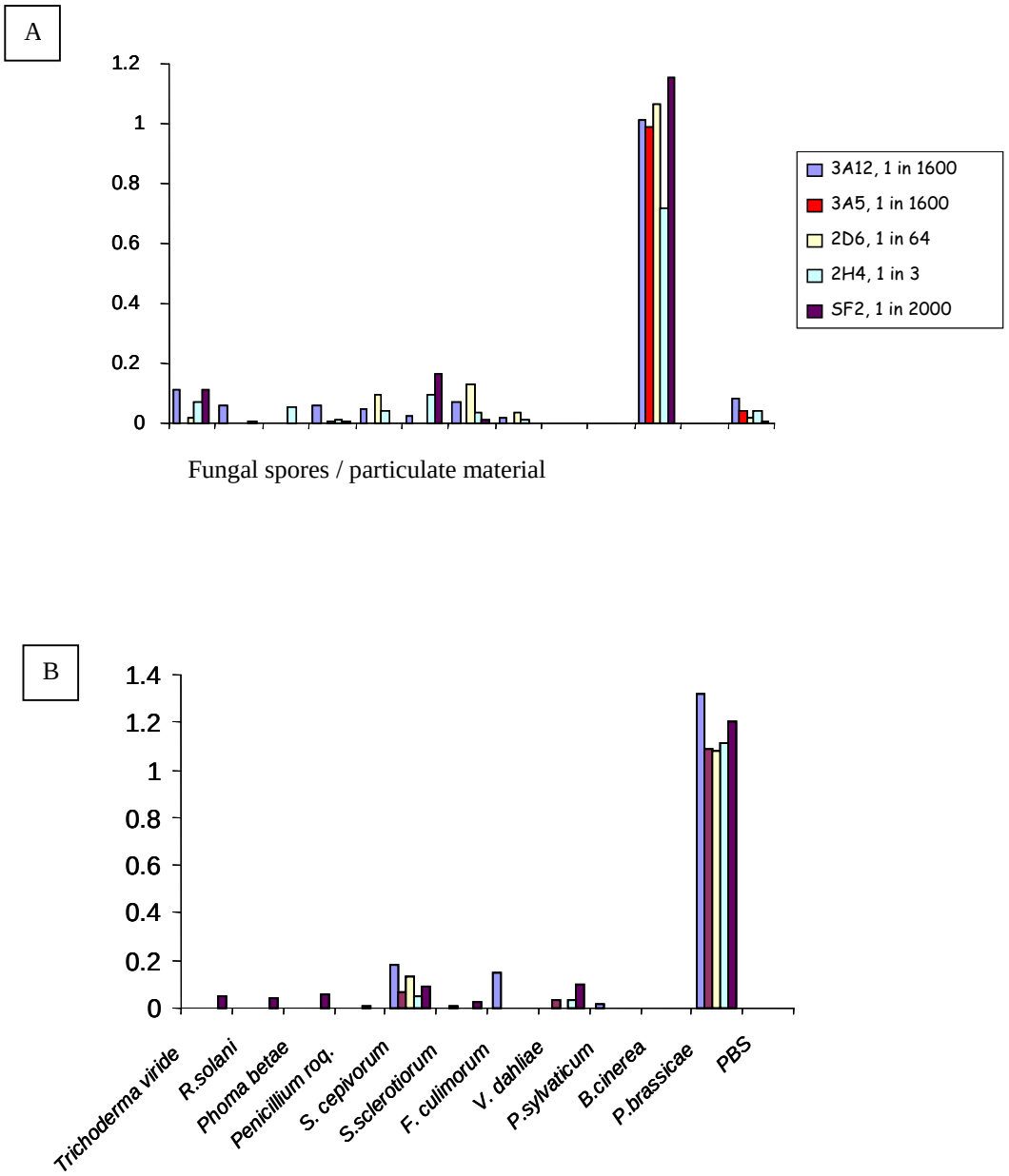

Fig. 8. Absorbance values produced by PTA ELISA using 5 *P. brassicae*-specific monoclonal antibodies against a) particulate and b) soluble antigenic components of selected fungal species.

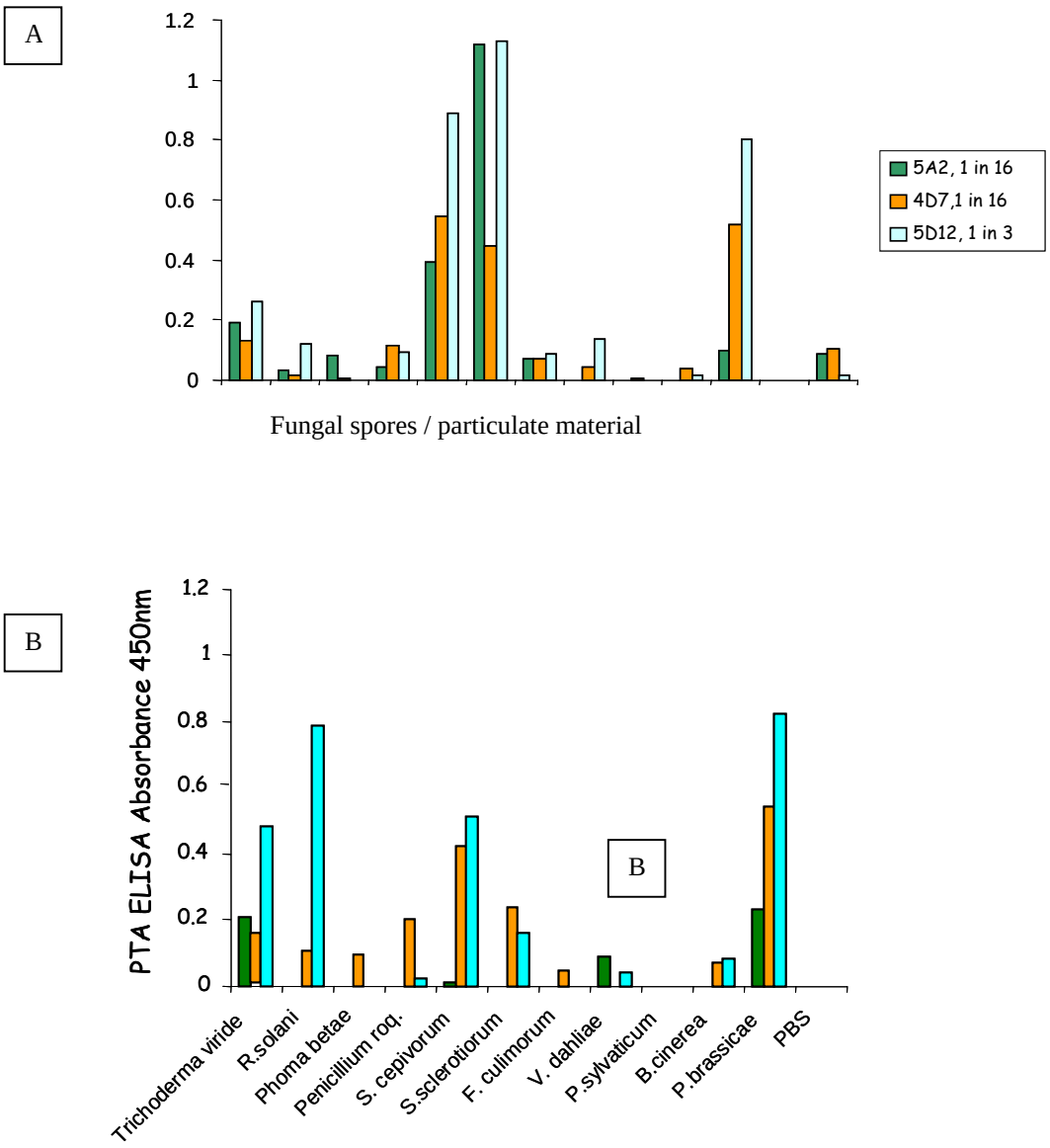

Fig. 9. Absorbance values produced by PTA ELISA using 3 non-specific *P. brassicae* monoclonal antibodies against a) particulate and b) soluble antigenic components of selected fungal species.

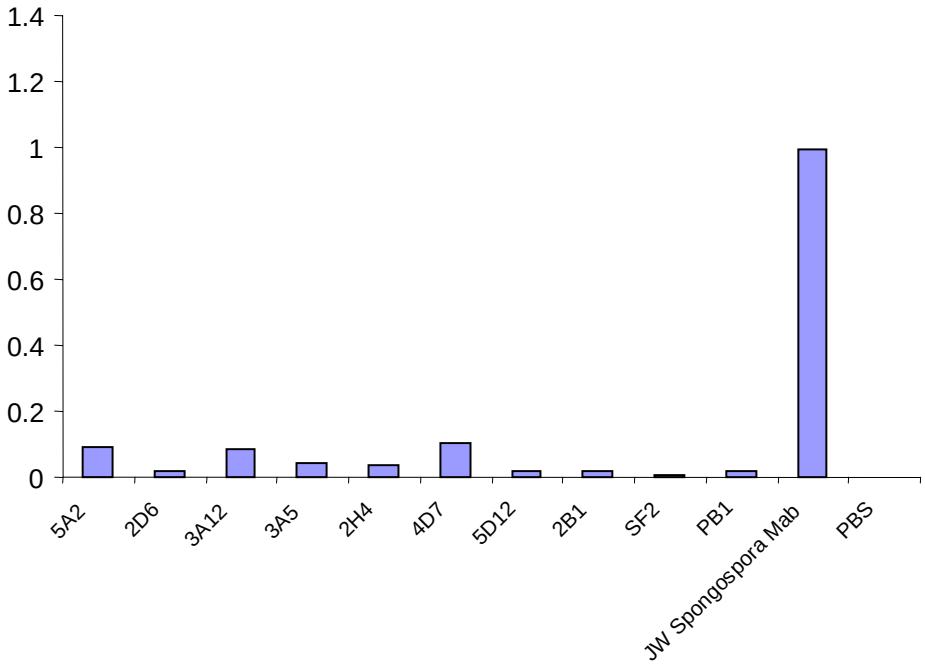

Fig. 10. Absorbance values produced by PTA ELISA for *S. subterranea* using all monoclonal antibodies raised against P. brassicae.

The results of the immunofluorescence assay (Fig. 11) support those of the PTA ELISA – that is, highly specific monoclonals such as 3A5 gave a strong immunofluorescence reaction, and those showing high-cross reactivity were poor, such as 5A2. This assay also provides a useful and very visual means of assessing the binding of antibody to antigen, and therefore an additional means of quantifying spore numbers in soil.

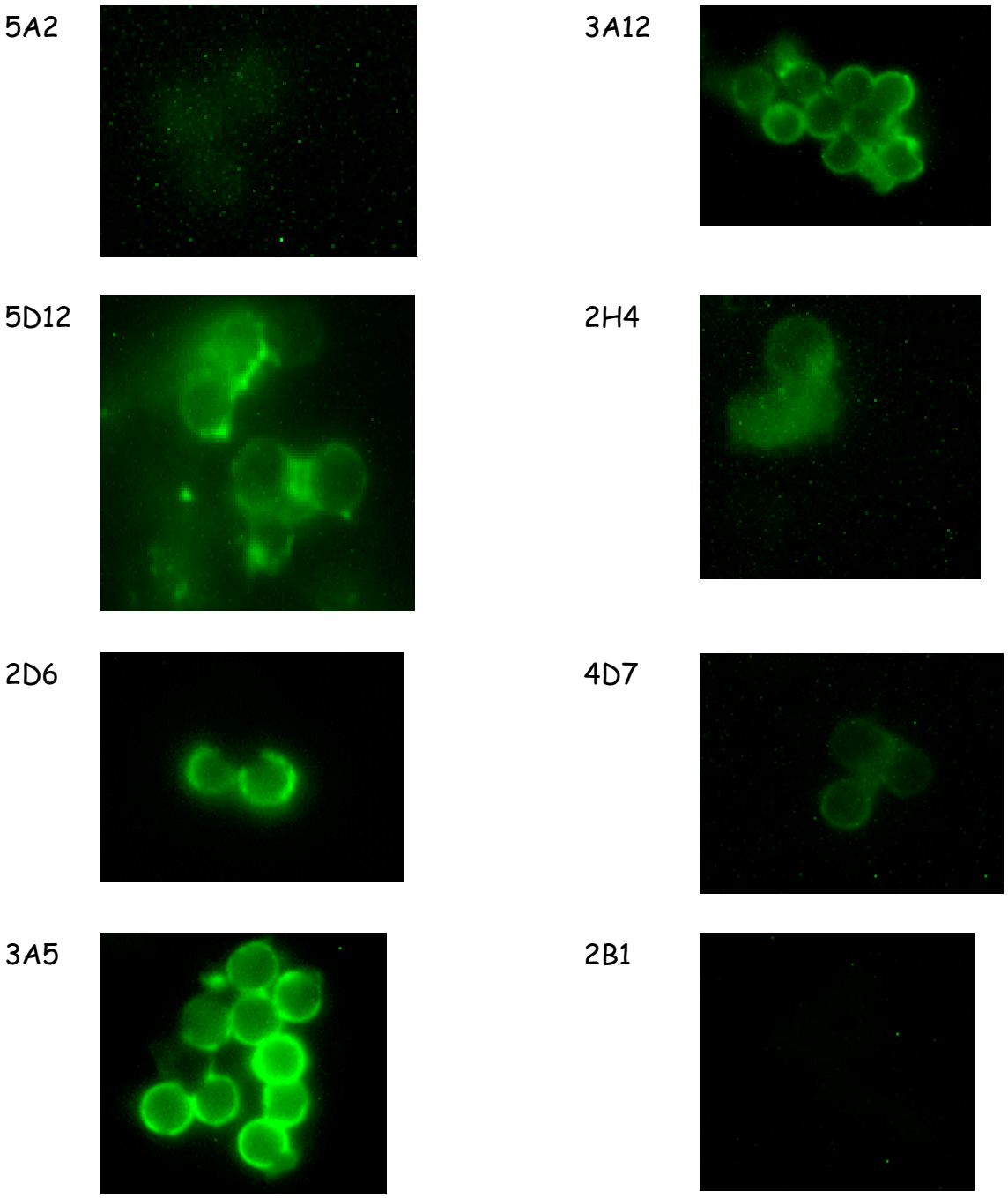

Fig. 11. Immunofluorescence photos showing the reactivity of different monoclonal antibodies to *P. brassicae* resting spores.

3. Clubroot lateral flow prototypes

Assembly of lateral flow prototype

The on-farm kit prototype consisted of a membrane onto which antigen (for a control test line) was applied, a conjugate pad that contained antibody conjugated to gold label and a sample pad to accept extracted test samples (spores, soil, etc.) (Fig. 12).

Test extract is applied to the sample pad. This pad filters out particulate matter and allows the antigen to migrate to the conjugate pad underneath. The antigen and *P. brassicae*-specific gold-labelled antibody bind on the conjugate pad and migrate down the membrane where the antibody-antigen complex will encounter either a) the test strip or b) the control strip. Generally, the test strip on lateral flow devices contains target-specific antibody that traps antigen-antibody complexes and results in the development of a visible line. The control strip traps surplus antibodies that are not complexed to antigen, also resulting in the development of a line and thus indicating that the assay has worked.

However, as this is a prototype, and for the sake of simplicity of production, the strips were assembled with a *competitive* ELISA assay. The competitive assay prototypes contained no control lines – therefore, the presence of cubroot resulted in the gradual disappearance of a test line, whereas the absence of clubroot resulted in a strong test line.

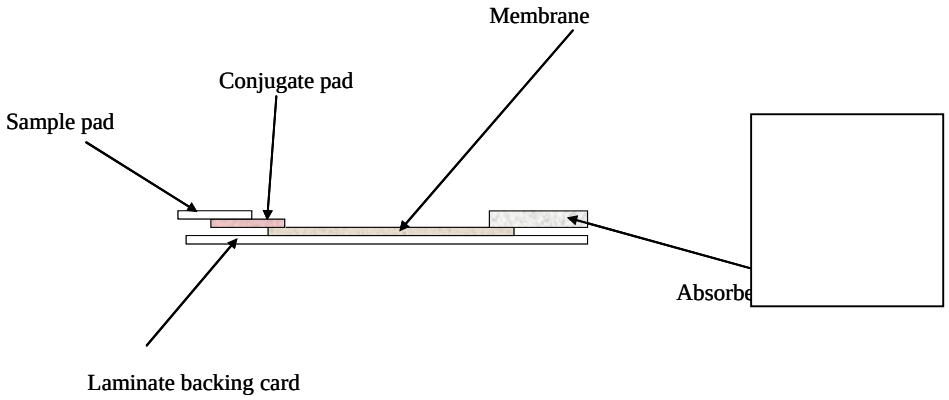

Fig. 12. Components of the prototype lateral flow device.

Production of lateral flow test line

Washed clubbed root galls (5g fresh weight) from *Brassica napus* were homogenised in 30ml 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 10ml PBS. The resulting spore suspension was centrifuged twice more before being resuspended in PBS to a final volume of 10ml. To remove *Brassica napus* 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 5ml PBS and adjusted to a concentration of $1x10^8$ spore ml^{-1}. 0.5ml aliquots of the resting spore solution was dispensed into eppindorfs containing 0.5g of 0.5mm diameter bollotini beads (Jencons-PLS, Leighton Buzzard, UK. Cat. No. 136-015). Using a Fast Prep device (QBiogene) and, operating at a speed setting of 5 for three consecutive 20 second periods, resting spores were disrupted. The soluble fraction, collected post centrifugation at 10,000 xg, was then striped directly on to the membrane surface of the prepared lateral flow using a flat bed air jet dispenser (Biodot Ltd). The membrane was air-dried overnight at 18°C and cut in to 4mm strips.

Application of gold conjugated monoclonal antibody to the lateral flow sample pad

A 10µl aliquot of monoclonal antibody 3A5 TCS was mixed with 375µl goat anti-mouse IgM 40nm gold conjugate (Code BA GAMM 40, www.british-biocell.co.uk) in 2 ml Phosphate buffered saline buffer (PBS) and incubated on a roller incubator for 3 hours. To remove unbound monoclonal antibody 3A5, the sample was then centrifuged at 4000 x g and the aqueous phase discarded. The gold conjugate pellet was gently resuspended to a final volume of 1.625ml in HRI application buffer (20mM Sodium phosphate buffer, 100Mm Sodium Chloride, 0.25% Trehalose, 0.1% Sucrose, pH 7.2). To each sample pad of a lateral flow device 60µl of the antibody gold conjugate solution was aliquoted and then air dried at 27ºC. Following air-drying the lateral flows devices were mounted within a lateral flow plastic housing device (www.schleicher-schuell.com).

Results and Discussion

The assembly of a prototype lateral flow device enabled the positive diagnosis of clubroot resting spores in solution when applied to the test pad and allowed to migrate to, and react with, monoclonal antibody 3A5 (Fig. 13). This is one of the few examples where this format of test has been applied successfully to a non-viral pathogen in plant pathology.

A number of antibody-based assays have been developed for the detection of plant viruses (Sward & Eagling, 1995; Torrance, 1995), but much fewer for fungi and bacteria (Dewey & Thornton, 1995; Spire, 1995; Dewey et al., 1997). In fact, several companies now specialise in the supply of antibodies and kits for the detection of viral plant pathogens, e.g. Adgen, Agdia, Bioreba, Loewe and Sigma. Some are simple assay formats that are available for antibody-based detection, and semi-quantification of the target pathogen is possible. The tests work well with viruses due to the prevalence of antibodies and the ease with which antigen recognition can be achieved, but unfortunately it is often difficult to achieve the required specificity for more complex organisms such as bacteria and fungi.

Furthermore, lateral flow devices are not new, and are particularly prevalent in the medical field (e.g. home pregnancy test kits). However, their use for the detection of soil-borne pathogens is new and represents a significant advance. The successful development of a simple, rapid lateral flow device such as the prototype shown in this study would allow growers to conduct diagnostic tests themselves at low cost. It is estimated that the prototype could be produced for less than $10AUD, compared to $450+AUD for molecular tests.

The following section will describe our attempts to optimise the prototype for use with soil extracts and to calibrate it against the molecular test to allow quantification of soil-borne inoculum.

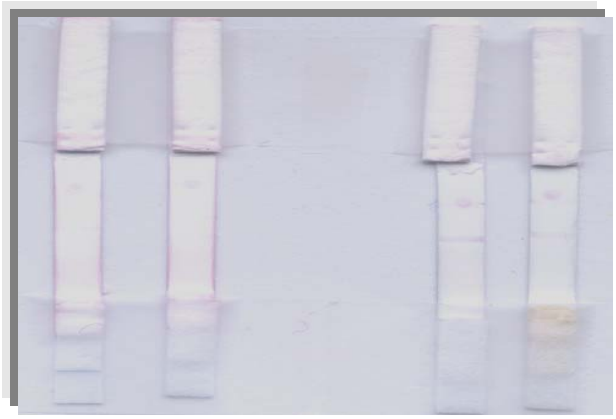

Fig. 13. Prototype lateral-flow device showing positive reaction with *P. brassicae* (two left-most test strips with no test line) and negative reaction with control solution (two right-most test strips with test line).

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On-farm diagnostic kits for brassica diseases.
Final report for project VG04059

Part 3. Optimisation and validation of on-farm kit.

Introduction

For an on-farm diagnostic test kit, or any diagnostic test, to be a success and form the basis of grower decision-support systems, it must be infallible. Above all, it should never give a false-negative result. False negatives could result in significant financial loss to a grower who has used a test result to omit disease control measures from his agronomic practices. In addition the test must be easy to interpret and ideally would only give a positive result at inoculum levels that are known to be at or above the threshold level where economic loss begins to be a consideration. Growers will often be content to tolerate diseased crops provided the pathogen population in question is being managed and that the crop will still produce good yields of marketable produce. The test should also be semi-quantitative to enable growers to match disease management strategies to inoculum loads – therefore, if inoculum loads are low, in the case of clubroot, it might be sufficient to adjust soil pH with lime prior to planting and not take any other action; if inoculum loads are high, the situation may call for soil pH adjustments in addition to other soil amendments or even soil fumigation.

Diagnostic tests must therefore be validated, through laboratory and field experiments, to certify their status as 'fit-for-purpose'. This is a particularly important step in the case of a technology like the lateral flow device presented here, as it is new to the Australian vegetable industry. Lateral flow devices are well-established in the U.K. where they are reducing chemical usage and disease (for example, Brassica spot disease). A hasty release of an improperly validated lateral flow device in Australia could jeopardise the adoption of the technology in this country and deprive industry of the potential benefits.

This section of the report therefore reports on experiments to establish the U.K.-developed antibody-based diagnostic tests in Australia and to validate the tests in soil.

1. ELISA plate-based testing protocol for clubroot detection.

The monoclonal antibodies discussed in Section 2 of this report were developed using *P. brassicae* isolates from the U.K. The first step in validating the assay was therefore to determine whether the antibodies would also recognize Australian isolates and to determine their level of activity (ie. to establish working dilutions for each antibody).

Methodology:

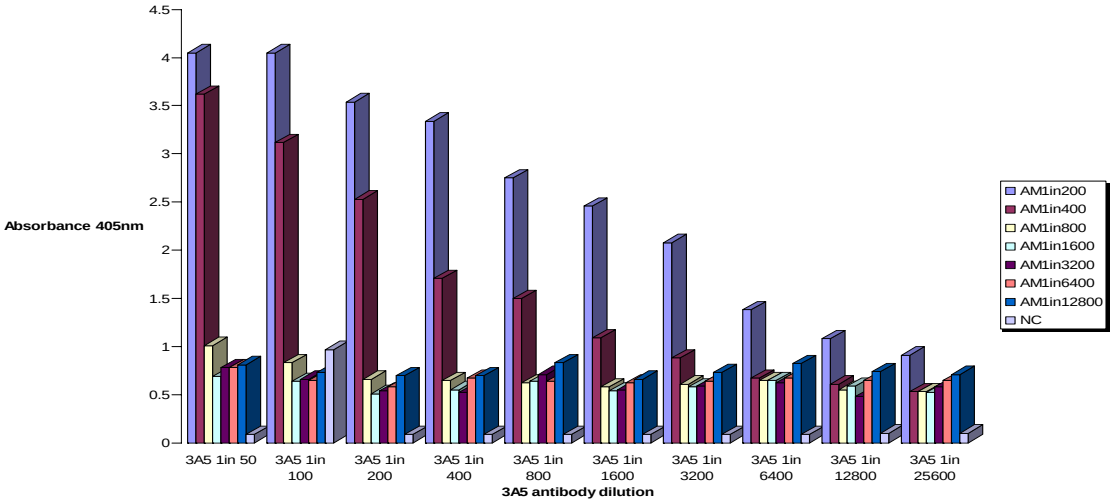
Four monoclonal antibodies (coded: 3A12, 3A5, 2D6 and 2H4) were tested for their ability to recognize an Australian isolate of *P. brassiaae* using a plate-based ELISA format (see PTA ELISA described in Section 2). In brief, a virulent Victorian pathotype of *P. brassicae* resting spore preparation was adjusted, in PBSr, to 10^7 spore mL^{-1} and 100µL was loaded in to Polysorp microtiter 96-well plates (Nunc. Roskilde, Denmark). The plate was incubated at 4^oC overnight. A serial dilution of both primary and secondary

antibodies was then prepared in PBS with 1% Casein and added in a matrix to the wells to identify the optimum titres for routine ELISA. Following incubation at 37^{O}C for 45 min in a rocking incubator, each well was washed gently three times for one minute with 200µL PBSTwC. Each well then received 100 µL of secondary antibody (Sigma anti-mouse alkaline phosphatase conjugate) and incubated for a further one hour at 37^{o} C. The wells were again washed three times with PBSTwC before being developed with a Sigma-Fast P-Nitrophenyl phosphate solution (Sigma N2770) as per the manufacturer's instructions. After 45 minutes the plates were read on a microplate reader at 405nm.

Having determined the optimum concentrations of antibody, the PTA-ELISA was repeated for each monoclonal to compare sensitivity of detection.

Results and Discussion:

The PTA-ELISA results demonstrated that a) the U.K.-developed antibodies were able to detect Australian isolates of *P. brassicae*, and also determined the optimal titres of both primary and secondary antibodies for use in routine ELISAs (Fig. 14). The optimum primary/secondary antibody combinations were selected for all monoclonal, although antibody 3A5 was the most sensitive at the target absorbance of approximately 1.5 (1:200 in combination with AM at 1:400), followed by monoclonal antibody 2D6 (Fig. 15). Monoclonal 3A5 also performed well in the U.K. and this result determined that 3A5 would be used in the on-farm kit. This finding means that the U.K.-developed monoclonal antibodies can be used as the basis of a rapid ELISA-based assay to confirm the presence of *P. brassicae* in clean substrates such as water or roots.



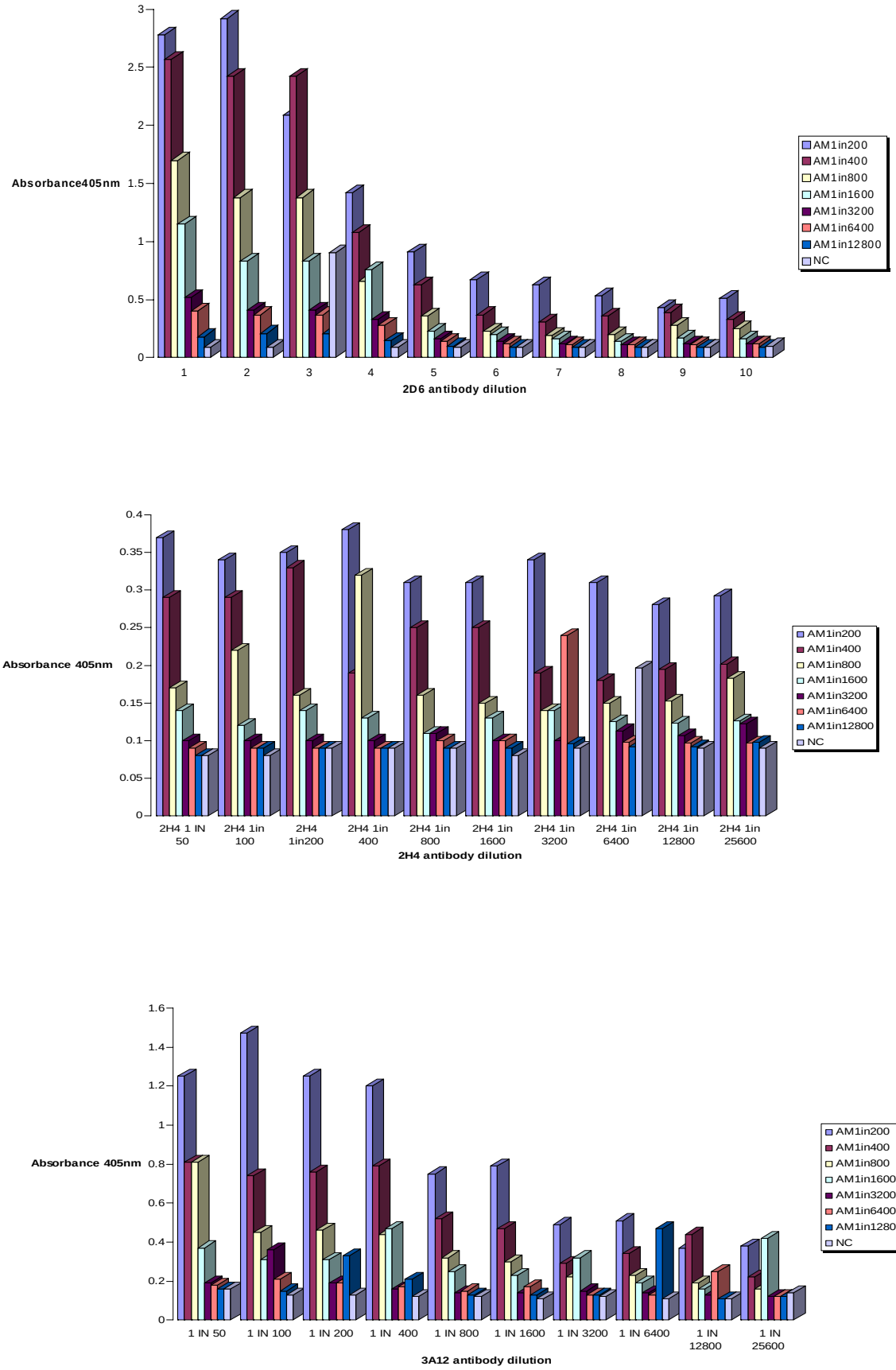


Fig. 14. PTA ELISA absorbance values of four different monoclonal antibodies at differing levels of primary and seconday antibodies, against *P.brassicae* resting spores (10^7) as antigen).

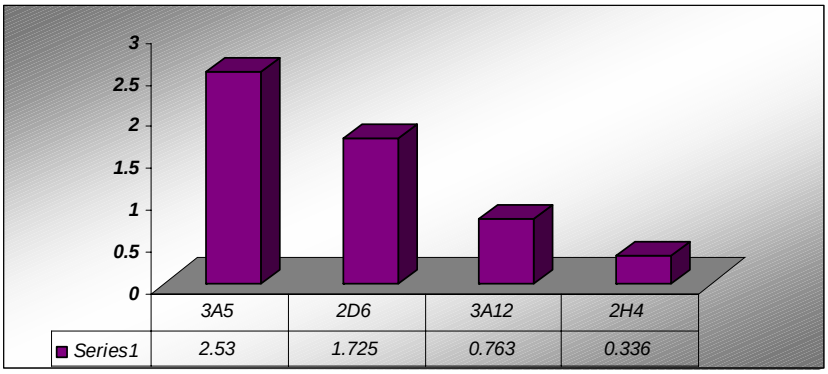


Fig. 15. Comparison of PTA ELISA absorbance values of four different monoclonal antibodies at their optimum concentration against *P.brassicae* spore dilution 10^7.

2. PTA-ELISA in soil

The PTA-ELISA/monoclonal antibody assay was also tested to determine whether it could effectively detect clubroot in soil, since some antigen targeted by antibodies are known to degrade rapidly in soil (Otten *et.al*., 1997), thereby rendering the assay useless for such applications.

Methodology

Clean (clubroot free according to the molecular test) soil was spiked with 10^8 clubroot resting spores and a soil slurry prepared by mixing 1g spiked soil with 2 mL of PBS. Soil slurries were also prepared with 60% LUDOX – LUDOX is a viscous and inert silica-based solution that the project team have used in the past to quickly separate resting spores from soil based on specific gravity. Negative controls were also prepared with un-spiked soil. One-hundred microlitre sub-samples of soil slurry were added to wells in a polysorp microtiter strip, incubated overnight at 4^OC and then the same procedure as described above was carried out.

Results and Discussion

PTA-ELISA successfully detected *P. brassicae* in soil slurries. This is a significant finding that indicates soil will not adversely interfere with antibody-based assay. Untreated, spiked soil gave significantly better results than spiked soil with LUDOX (Fig. 16). Without knowledge of the antigenic target that the antibody is recognizing, it is impossible to know the reason for this, but we can speculate that either LUDOX interferences with the solubility of the antigen, or it increases retention of antigen in soil particulate matter, or it simply does not effectively separate spores from soil. Regardless, the PTA-ELISA worked well in untreated soil slurries, which will facilitate the development of the assay on the lateral flow platform.

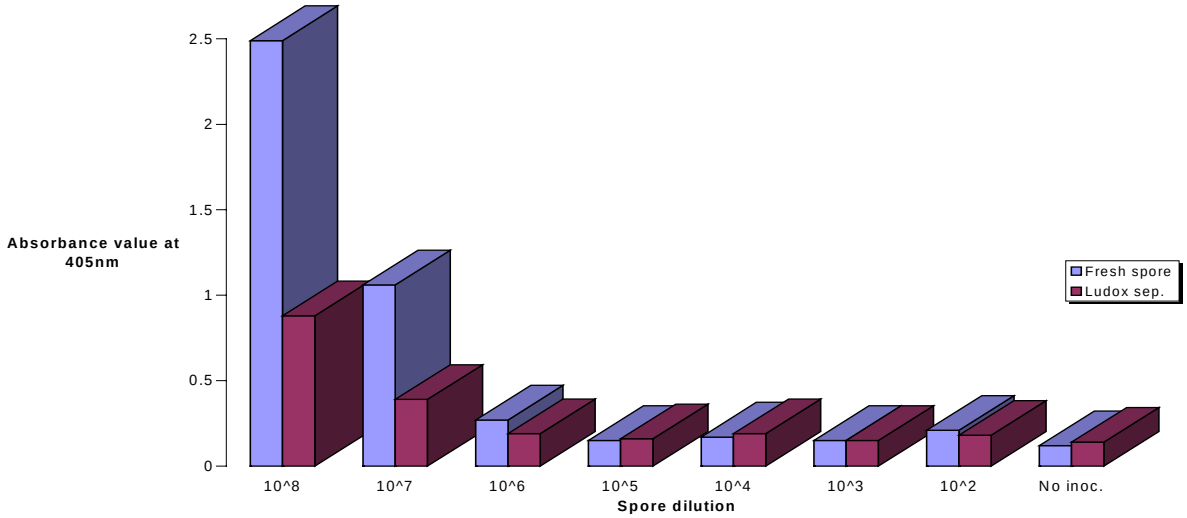


Fig. 16. Comparison of PTA ELISA absorbance values of soil spiked with serial dilutions of *P. brassicae* resting spores (10^8 to 10^2) and the same spiked soil treated with LUDOX to separate spores.

3. Effect of age on resting spore age/viability

A limitation for DNA-based tests is that they cannot discriminate if the pathogen is alive or dead. It is often assumed that serological assays are superior because, theoretically, they recognize surface proteins and other antigens that degrade as cells die. Therefore they should be able to distinguish between viable and non-viable cells. Experiments were conducted to determine whether clubroot resting spore age/viability affected the sensitivity of the ELISA test, in comparison to the molecular test.

Methodology

A number of clubroot resting spore solutions, which had been stored at 4^o C from 1996 through to 2006, were resurrected. Subsamples of each were taken, concentrations adjusted to 10^7 spores per mL, and viability status assessed using staining methods (Donald *et al*., 2002). Each spore suspension was then subjected to PTA-ELISA or real-time PCR, as per the techniques in Sections 1 and 2 of this report.

Results and Discussion:

The viability of resting spore suspensions, according to staining methods, showed a general trend of decreasing viability with age, from a high of 90% in 2006 through to 10% in 1996 (if one ignores the seemingly anomalous result of the 2005 spore suspension, which was 20%) (Fig. 17).

In contrast, there was no real trend observed with either the PTA-ELISA (Fig. 18) or real-time PCR (Fig. 19), other than for both, the 2006 spore suspension was the most

easily detected. Therefore, age does not appear to impact on the ability of antibody- or DNA-based assays to detect *P. brassicae*. This does not take into account, however, the likelihood that dead spores will be quickly degraded in the soil environment. In the absence of historical soil samples though, it is not possible to test this theory with any certainty. Previous HAL-funded research (VG99008) indicated that spores that had been killed by autoclaving and added to non-autoclaved soil decreased quickly in numbers over a matter of days, presumably as a result of the degradative processes driven by other soil microbes.

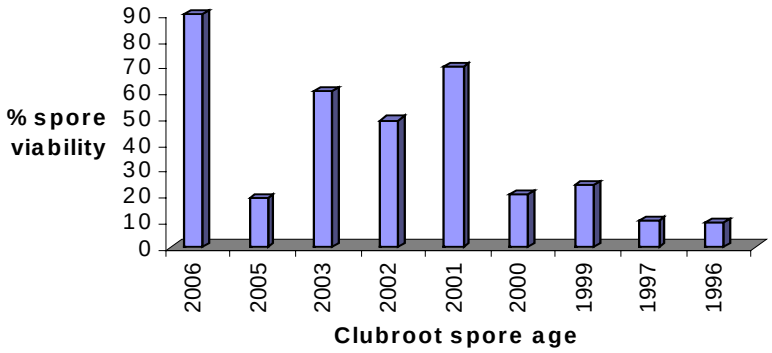


Fig 17. Percentage viability of resting spore suspensions of different ages (1996 to 2006).

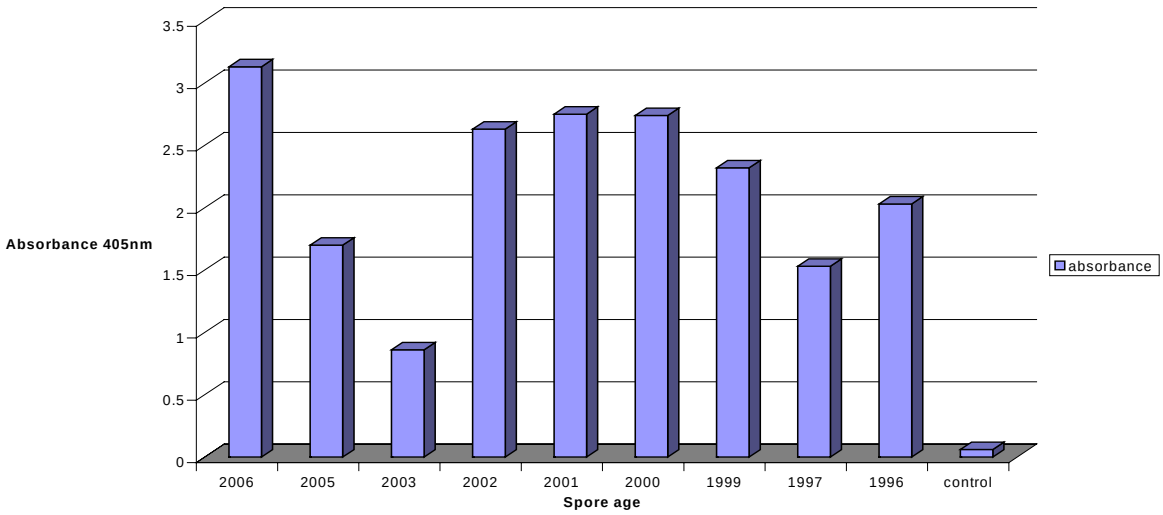


Fig 18. PTA-ELISA absorbance values from clubroot resting spores of varying age (1996 to 2006).

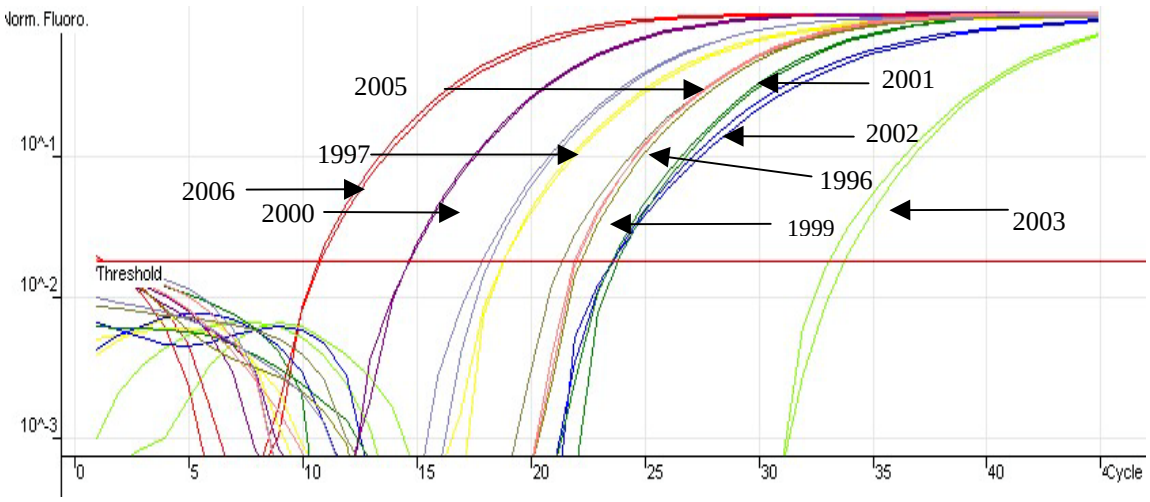


Fig. 19. Real-time amplification plot of *P. brassicae* spore suspension DNA from samples of different ages (2006 to 1996).

4. Optimisation of kit extraction buffer

Introduction

Having gained a better understanding of the performance of the antibody-based assay through laboratory experiments – that is, under ideal conditions using the PTA-ELISA – the next challenge is to determine how it performs in its final lateral-flow format. As previously mentioned, lateral flow devices are not new and have been used for the detection of viral plant pathogens (Danks and Barker, 2000; Salomone *et al*., 2002). However, applying soil as a test substrate would be a first. Here we have trialed the prototype with spiked soil, to determine its sensitivity (and validated this with PTA-ELISA and with the molecular assays) and with naturally infested soil, and also investigated the only variable that we have control over, which is the extraction buffer.

Methodology

The main components of the LFD extraction buffer are Polyvinylpyrrolidone 40 (PVP40), casein and tween20, all of which impact on antibody stability and the efficiency of the migration of antibodies/antigens through the prototype's membranes. The concentration of individual components of the buffer were varied in a matrix to determine the optimum ratio's for detection of resting spores in suspension. Spore suspensions (10^5- close to the expected detection limit of the LFD's), in PBS, were pelleted in a centrifuge and resuspended in extraction buffer. Approximately three drops of the spore/extraction buffer slurry was applied to the sample pad (in the case of naked test strips) or sample well (where strips had been mounted in to a plastic housing). Drops were applied slowly, to allow the solution to absorb into the sample pad, through the conjugate pad and along the main membrane strip. Care was taken to ensure the well was not overfilled so as to prevent the solution from saturating the membrane and then migrating backwards. The LFD was then left at room temperature for between 10-15 minutes to allow the test line to develop.

The sensitivity of the assay for the detection of spores in solution was then determined using the optimum buffer concentrations. This was followed by experiments to determine the sensitivity of detection using spiked soil.

Results and Discussion.

To achieve good detection (and as described earlier, this is indicated by the disappearance of a test line), buffer components concentrations of 0.25% PVP40, 0.4% Casein and 0.25% Tween20 were used. Fig. 20 shows the range of serological reactions elicited by different extraction buffers on several of the prototype tests. The decreasing intensity of a test-line indicates increasing strength of the reaction. Therefore, the complete absence of a line indicates the greatest reaction efficiency (i.e. saturation of antibody/antigen complexes) and is equivalent to the reaction that is observed when high inoculums loads are encountered. On the other hand, an increase in line intensity (up to a maximum that is indicated by controls), indicates an excess of unbound antibody and therefore is equivalent to low inoculums loads or levels of inoculums below detectable levels. This is somewhat counter-intuitive but necessary if we are to incorporate the more sensitive and robust competitive-ELISA on to the LFD format.

The sensitivity of the prototype, of resting spores in solution, was 1 x 10^5 spores per mL (Fig. 21). Similar levels were observed in spiked soil. Given that symptoms are not observed until inoculum concentrations reach 10^4, and 10^5 is where yield will begin to be impacted by disease, 10^5 is an acceptable detection limit – a positive result therefore means that growers need to take action, while a negative reaction, and therefore no action, will not result in yield loss.

Fig. 20. Optimisation of buffer concentrations using a fixed concentration of clubroot resting spore suspension – sample of LFD results across 9 different concentration ratios of PVP40 and Casein.

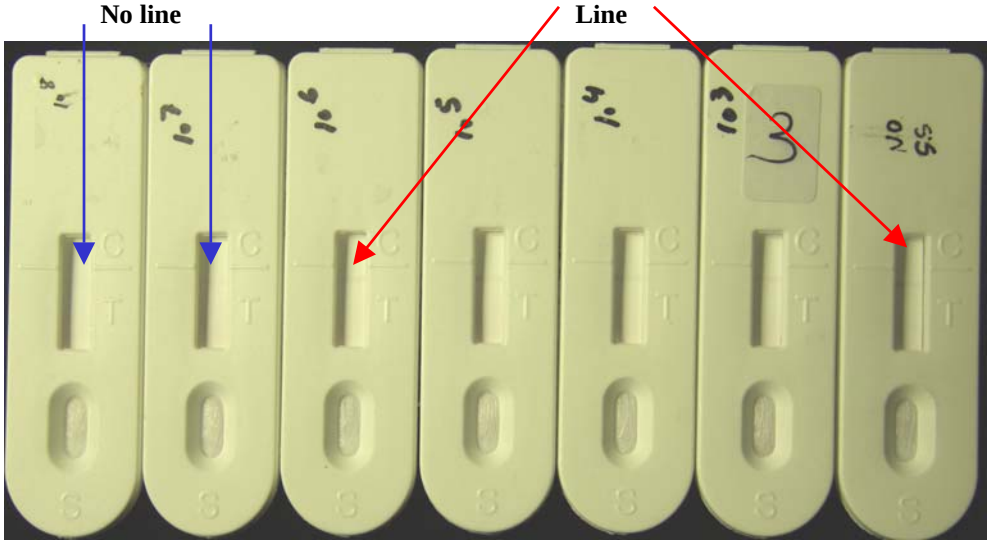


Fig. 21. Sensitivity of LFD for detection of P. brassicae determined using spore supensions ranging from108 - 103 spore/ mL

5. LFD detection of clubroot in soil.

Introduction:

The prototypes were next tested against several field soils from known infested sites. All tests that were carried out with LFD's were also carried out with PTA ELISA and with real-time PCR, in order to validate the LFD results. These tests were carried out late in the project at which time new prototypes were available, this time with control lines (which appear as pink circles on the membrane of an LFD that has been used).

The effect of pooling soil samples was also investigated using real-time PCR.

Methodology

Soil was sampled from infested sites in Werribee, Cranbourne and Tasmania (as described in Section 1), as well as spiked soil from Knoxfield, and tested with the LFD as described above. The same soils were also subjected to testing by PTA ELISA and to real-time PCR.

To investigate the effect on detection of pooling soil samples, a one gram sample of soil was spiked with clubroot resting spores to a concentration of 10^7. The spiked sample was diluted successively with one gram samples of clean soil, and tested at each point using LFD's and real-time PCR. This was designed to simulate a transect sampling regime across a paddock with a spot infection, to determine how many samples could be pooled before the spot infection is no longer detectable.

Results and Discussion

All naturally infested soil samples tested positive for the presence of clubroot with the LFD's (Fig. 22). The control soil sample from Knoxfield was negative. The sample wells quickly became clogged with soil slurry, but this did not impair the functioning of the LFD's in the case of the three case-study soil types. The results were supported by PTA-ELISA and real-time PCR (Fig. 23).

The project team did notice however, that the speed with which soil slurries migrated to, and through, LFD membranes varied from soil type to soil type. This was also observed during the course of additional experiments using other soil types – there is a risk that the migration of soil slurry on LFD's for some soil-types, will be too slow to allow the serological reactions to take place efficiently. The project team also noticed that migration times varied between batches of LFD's – for one batch, the variation resulted in a drop of sensitivity down to 8×10^5 spores per gram of soil. As sensitivity reaches 10^6, the value of the on-farm kit drops since at such inoculums levels yield losses could be significant.

This reveals two problems. The first is quality control. In a research project aiming to produce a 'prototype' on-farm kit, emphasis was placed on the development process and the various factors that could impact on detection such as the specificity and sensitivity of antibodies, the composition of the extraction buffer and comparisons with results from established tests such as the molecular assay. When the on-farm kit is ready for commercial release however, quality control will become the most important issue. Intra- and inter-batch consistency must be within acceptable limits, which would be 1×10^5 to 2×10^5. Sensitivity levels approaching 10^6 will result in false negatives - a single false negative that results in bad publicity could permanently harm the prospects for adoption of the test.

The second problem is the subjectivity of test result interpretation. Relying on personal determinations of what constitutes a line and when the line is considered to have vanished completely is not acceptable. Project staff, for instance, could clearly see lines where their colleagues (who were not familiar with the test) could not. An immediate solution would be to accredit only certain individuals, such as agronomists, to perform the test on behalf of growers. This would ensure that there is some level of consistency with regard to result interpretation, and consistency with regard to the disease control advice that is given in response to the 'quantified' soil inoculums levels. A longer term and better option would be the development of a hand-held colorimetric reader that analyses the line intensity and converts the transforms the data according to a numerical scale that corresponds to inoculums levels. The project's U.K. collaborators routinely used a laboratory colorimetric reader (see example of output – Fig. 24), and take this instrument out into the field to conduct tests for growers (by connecting the instrument to a car battery). Similar readers are not available in Australia, but the project team did discuss the development of hand-held readers with representative from the CRC for Microtechnology. According to the CRC, it would be possible to develop a hand-held readers and they suggested distribution to growers could be modeled on mobile-phone contracts – that is, in return for agreeing to conduct 100 LFD tests, the grower is given a hand-held reader for free.

With regards to soil sampling, there are two soil-sampling regimes already in use for clubroot diagnostics. The first is that recommended by the Scottish Agricultural College (SAC). SAC recommends that for vegetable brassicas, the sampling area should be 2 hectares and that 50 soil cores be taken in a 'W' pattern across the area, to a total of 4.5 kg of soil. The soil is pooled, mixed and tests are carried out on the entire sample using plant bioassays. The second soil-sampling regime is that recommended by DPI Victoria in the IPM Brassicas CD by Caroline Donald. DPI recommends that a paddock (size not specified) be sampled by taking small trowel-fulls of soil every few steps in an 'X' pattern. The soil is pooled, mixed and a one kilogram sub-sample taken for molecular testing and plant bioassays.

These regimes need to be considered with two scenarios in mind for sampling for a soil-borne disease: 1) relatively uniform soil infestation where a pathogen population has become established in a paddock and 2) 'spot' infestations in new outbreaks. The existing sampling regimes are more suited to new infestations since the large number of samples increases the likelihood of detecting a small 'spot' infection. However, neither sampling regime is practical for routine testing by growers where the pathogen has become established and population management is more important than detection sensitivity.

The soil-dilution experiments revealed that the limit of detection when pooling soil is 6 samples. That is, 5 dilutions (one gram of infested soil plus 5 one-gram samples of clean soil) can still be detected, but 1 more dilution makes the entire sample return a false negative result.

This indicates that pooling samples, as well as being impractical and time-consuming for growers, increases the risk of false-negatives due to dilution, especially where clubroot infestation is not uniform. Soil sampling should therefore be more strategic and focussed on areas where clubroot is most likely to be found, such as at paddock gates and depressions in the landscape where water may pool, or areas where samples are likely to representative of the paddock as a whole. This reduces the need for large numbers of samples and for pooling of samples.

The issue of sampling, as well as test interpretation and quality control, will be addressed in Phase II of the project.

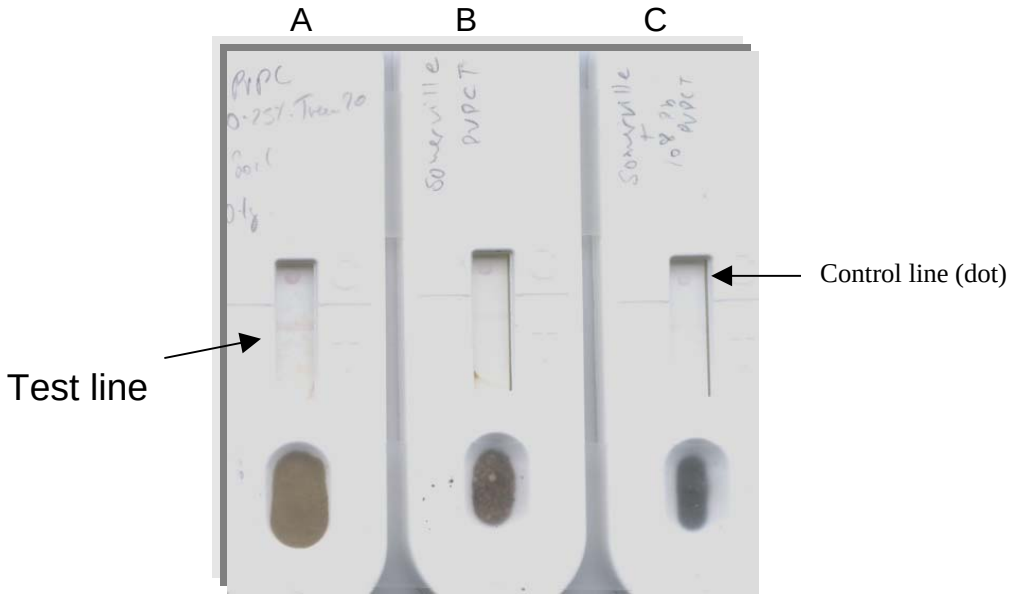


Fig. 22 Sample of LFD results on naturally infested field soils (B & C) and one control soil (A).

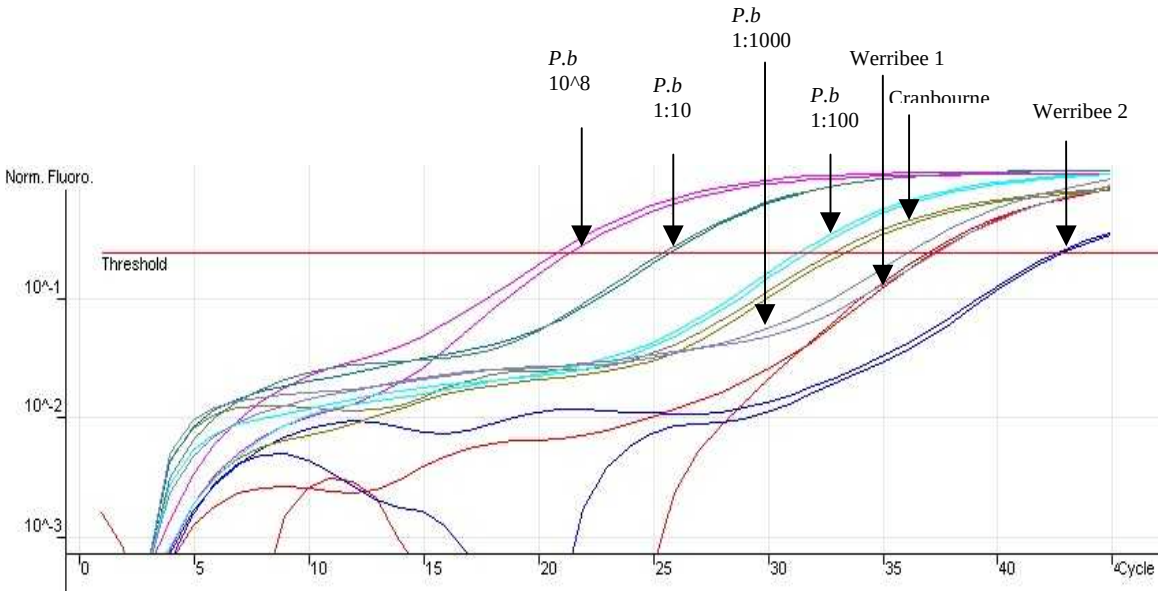


Fig.23 Sample amplification plots of from clubroot-infested field soils.

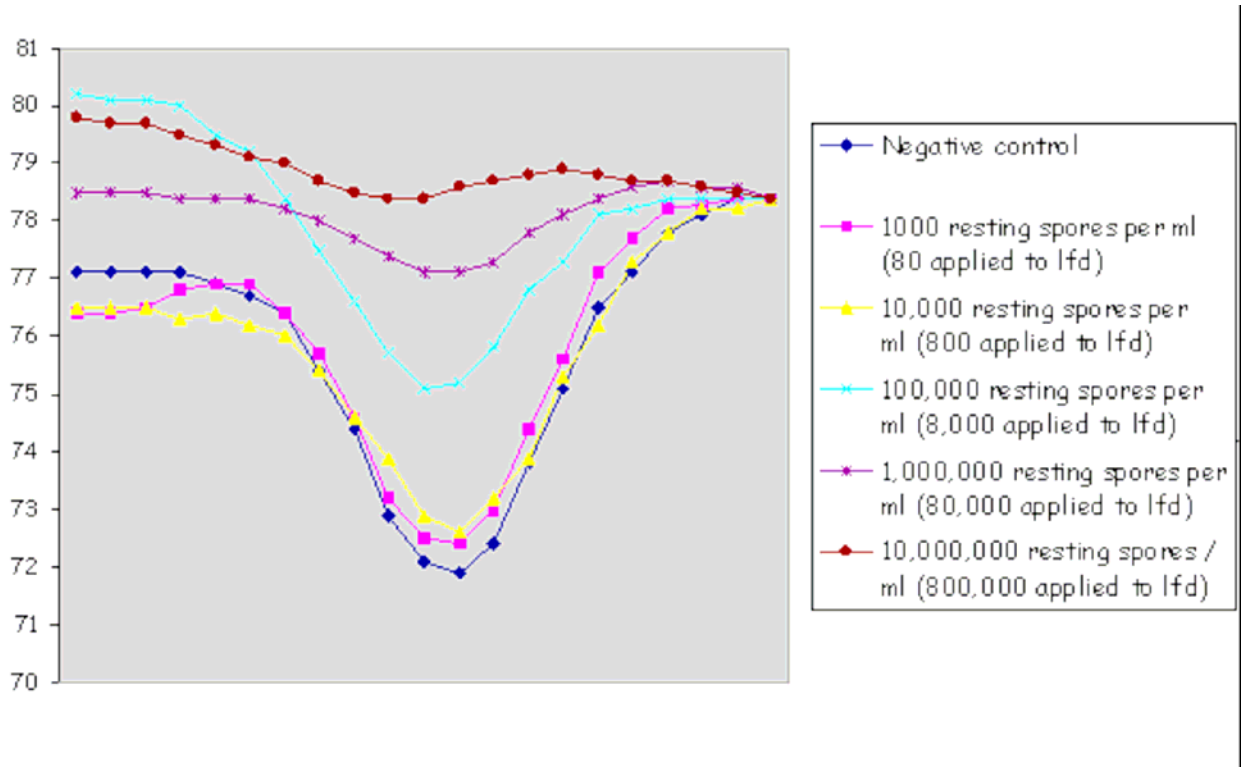


Fig. 24. Sample output of LFD colorimetric reader showing the improved sensitivity (down to 8 x 104 spores per gram, that can be achieved by assessing the results via a non-subjective measure.

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Otten W, Gilligan CA, Thornton CR. (1997) Quantification of fungal antigens in soil with a monoclonal antibody-based ELISA: analysis and reduction of soil-specific bias. Phytopathology. 87, 730–736.

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Recommendations

This joint project with the Horticultural Development Council (U.K.) has developed a prototype on-farm diagnostic kit for the detection of clubroot, based on a specific monoclonal antibody and lateral flow technology. It has been tested in Australian and U.K. soils and was supported by a DNA-based quantitative assay that allowed the accurate validation of the on-farm kit. The project has also made a series of improvements to the existing molecular assay to facilitate its use as a reliable research tool and sensitive alternative diagnostic test.

The specific recommendations of the project are:

1) In order for the on-farm kit to be successful, the subjectivity must be eliminated from test result interpretation. The development of an electronic hand-held colorimetric reader is therefore seen as crucial to the eventual commercialisation of the test – various options for the cost associated with a hand-held reader to be significantly reduced through a commercial partner have been explored.

2) The best model for adoption of the on-farm kit in the early stages of its release would be for agronomists, crop consultants and extension staff be accredited to interpret the test results, and for growers to seek their expert advice when using the kit as a decision-support tool. This would build confidence in the kits over time and increase the prospects for the technology to a) be adopted widely and impact on the spread and prevalence of clubroot and b) be applied to other pathogens and other crops.

3) That Australian and British growers commit to developing and using the on-farm diagnostic kit as a routine decision-support tool to help reduce chemical usage and minimise the spread of clubroot to clean areas. Similar tests are already in use in the U.K. (for air-borne brassica pathogens) and form the basis for disease prediction services that are reducing chemical usage.

4) That Horticulture Australia and the Australian Vegetable Industry support an extension to this research to ensure a fully validated and 100% reliable on-farm diagnostic test is released commercially and thus paves the way for the development of similar tests for other pathogens. British growers have already flagged their intention to support an extension to this research.

5) That the Australian Vegetable Industry continue to explore ways to link with overseas scientific agencies, and in particular with agencies in the U.K., who have shown exceptional good will to largely fund this research and give freely of their technology. Other advantages a collaborative link with a country such as the United Kingdom is that they do not compete for the same markets with Australian growers yet they share common problems, and therefore by co-investing, research and development becomes more affordable for both parties.

6) That the Australian Vegetable Industry continue to look to other fields of research for new technologies that will advance Australian Horticulture. The technology behind this on-farm kit was developed in the medical field where it forms the basis of home pregnancy test kits – there are numerous instances where seemingly unrelated or ‘blue sky’ concepts could make significant impacts on Australian vegetable production if given the chance through brave investment. For instance, the project’s U.K. collaborators are using sophisticated, in-field spore-trapping devices that are helping to automate the diagnosis and predictive processes for a number of air-borne brassica diseases – this technology could be immediately applied in Australian vegetable production with some baseline validation.

Technical Transfer

A number of technology-transfer activities have been undertaken, with the proviso that the on-farm kit should not be released to the vegetable industry until it is 100% reliable – communications have therefore been geared towards gaining support for the research rather than to drive adoption.

1) **Steering Committee meetings** – the project partnered with Dr Liz Minchinton's White Blister research project (VG04013) and used their regular (6-monthly) White Blister Steering Committee meetings as a forum to increase grower awareness of the on-farm kit project and its progress.
2) **Werribee Vegetable Expo** – the project had a presence at both the 2005 and 2007 Werribee Vegetable Expo's, with fliers and information sheets available at the DPI Victoria pavilion. Additionally, the project was publicised at the 2005 Expo via a presentation in the Victorian Vegetable Idustry Development Officer's pavilion.
3) **AusVeg Conference** – A poster was presented at the 2006 AusVeg conference and fliers were distributed.
4) **Brassica IPM National Newsletter** – a project update was published in the Brassica IPM National Newsletter on August 2006 (Issue 9).
5) **Brassica IPM Seminar Series** – project updates were provided in Victoria's Werribee and Cranbourne brassica growing regions in June 2007 through the Brassica IPM Seminar Series
6) **VegCheque** - a project update was published in the VegCheque newsletter (February 2007). The newsletter was also used to request soil samples from growers and proved to have excellent coverage across the Victoria vegetable industry.
7) **Victorian Vegetable IDO Newsletter –** A story detailing the benefits of the collaborative link with British growers was published in the January 2007 edition of the Victorian Vegetable IDO newsletter.
8) **British Growers Association –** an overseas visit to the U.K., to conduct key pieces of project laboratory work, was used as an opportunity to visit British growers and provide updates on the project work. Amongst other communication activities, on-farm presentations were given to growers in Lancashire district.
9) **Scientific Community –** a scientific paper that outlines the advances this project has made in clubroot soil diagnostics is in the final draft stage and will be submitted to Australasian Plant Pathology in July.