# Developing on-farm diagnostic kits for brassica diseases

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VIC Department of Primary Industries

Project Number: VG04059

**VG04059**

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**Final report for project VG04059**


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**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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# Contents

## 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.

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## 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 $4^0$C. 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.

**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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## 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.

## 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.