Project title:	Development and testing of single and multiplex diagnostic devices for rapid and precise early detection of oomycete root and collar rot pathogens for disease avoidance, management and control
Project number:	**CP 136**
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Report:	Final report, August 2018
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Location of project:	University of Worcester
Industry Representative:	Russ Woodcock, Bordon Hill Nurseries Ltd
Date project commenced:	01/06/2015
Date project completed	31/08/2018

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

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

[Name]

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GROWER SUMMARY

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Headline

- A representative cross-sector collection of 124 identified isolates of oomycete plant pathogens and 'background' species, plus records of a further 180+ representative isolations assembled, and selections made for raising antibodies and testing their cross-reactivity.
- Out of 50+ antibodies raised, five were selected for developing lateral flow devices (LFD) to detect the following: oomycetes in general; *Phytophthora* spp.; pathogenic *Phytophthora* clades 7/8; *Pythium* spp.; and viability probes.
- Sensitivity of LFD tests was down to 10 and often fewer zoospores – a level that is useful for determining disease risks.
- Field efficacy of the LFD tests has been assessed in 1021 individual tests on 647 field samples of infected & healthy plants, growing media, water, baits & swab tests, and mycelium, taken from across horticultural sectors. These tests have shown that the *Phytophthora*-specific antibody (3H7) is robust and has very good specificity and that the *Phytophthora* pathogenic clades 1/7/8-specific antibody (3C4) has so far (in 307 tests) consistently detected pathogenic *Phytophthora* species, whilst in a comparatively smaller number of field-sample tests (67), one of the *Pythium* antibodies (4B5) has given very good results.
- Two immunodiagnostic test approaches have been found to discern between live and dead oomycete spores, but these both require an incubation period of several hours to 24h+ and require further development.

Background

Oomycetes and crop disease. Worldwide, oomycete diseases cause significant losses across a range of agricultural and horticultural commodities. The diseases they cause include seedling blights, damping-off, crown and root rots, foliar blights and downy mildew. Of the Oomycetes (a group of fungus-like organisms), *Pythium* species are well known for causing damping-off and seedling rots. *Pythium* related disease epidemics are also synonymous with root rots on newly emerged or more mature plants and soft rots of fleshy fruit. Likewise, the aptly named *Phytophthora* genus (*Phyto* (plant) *phthora* (destroyer)) cause significant damage worldwide on a broad range of crop types from trees to annual flowers. Some *Phytophthora* species are associated with diseases of above ground plant parts i.e. shoot

apex, leaf, stem and fruit rots whilst other groups within the genus cause serious root, crown and collar rots.

Detection and diagnosis. Reliable and affordable detection and diagnosis are key to effective oomycete disease management. With increasing globalization, travel and the international trade in plants the risk of disease through inadvertent introduction is exacerbated. A classic example of this was reported by White (HDC PC 97) with widespread dissemination of *Pythium* species across UK nurseries *via* Danish trolleys. Early diagnosis can provide growers with vital information regarding the effectiveness of nursery sanitization processes, source contaminants, control measures to prevent spread, disease containment or eradication, varietal selection, harvest date and post-harvest handling. Information on pathogen presence prior to the possibility of infection can be used to highlight where and when treatments are needed, potentially thereby reducing disease epidemics significantly. Classical methods for the isolation and identification of oomycete crop pathogens are commonly only deployed after disease symptoms are observed and take valuable time to implement. Current best practice diagnostic tests for *Pythium* and *Phytophthora* take upwards of 24 hrs with bait tests and between 3 and 10 days by conventional agar methods.

Even with identification to genus, the choice is still between taking further time to carry out a pathogenicity test if the potential pathogen has been isolated or the application of immediate control measures. Immediate control measures would likely be recommended in the event of detecting *Phytophthora* sp., whereas the immediate response to a *Pythium* sp.-positive test would be more ambivalent unless this was clearly linked to plants showing unequivocal symptoms. This is because most *Phytophthora* species currently known are plant pathogens, and whilst different species have different host preferences and host ranges, it is assumed that mere presence of detectable inoculum is an indication of potential trouble. On the other hand, a large proportion of the 200 or so species of *Pythium* are saprophytic or certainly not known to be pathogenic to any horticultural crop, and four species are known to be mycophagous (breakdown other fungi), some have the capacity to elicit disease resistance mechanisms in plants (Vallance *et al*., 2009) and therefore potentially beneficial. Rapid *in situ* diagnosis to genus level is currently possible using commercially-available ELISA-based LFD test kits (e.g. Alert LF™ kits, Adgen Phytodiagnostics and Pocket Diagnostic® kits, Forsite Diagnostics). Whilst of some help, these tests are unfortunately limited by their lack of specificity and the potential cross-reaction of the antibodies used with some non-target species of closely related oomycete genera leading to some ‘false-positives’.

Project aims. This AHDB-funded project (CP 136) is aimed at improving this situation by developing new monoclonal antibodies for LFD kits, raised to selected specific species (or groups within species known as clades) of *Phytophthora* and *Pythium*. With these more

specific tests it is hoped that a better idea of potential disease threats can be quickly obtained on site. In addition to greater specificity of on-site test tests, this project had the ambitious aim of developing a reliable test for pathogen viability in an on-site kit format. Such a test would be very useful in monitoring the efficacy of treatment systems for the elimination of plant pathogens from irrigation water.

Summary

From regular visits to nurseries and field sites throughout the project, as well as clinic samples and donations from colleagues (especially from James Townsend at STC), a representative cross-sector collection of 124 identified isolates of *Phytophthora*, *Pythium* and other oomycete species from plant roots, collars and crowns as well as compost and water samples has been assembled. This collection consists of both pathogens and commonly-seen 'non-target' species, including a small selection of important non-oomycete species. In addition, a further 180+ oomycete isolates were identified, and recorded but not kept. All isolates were identified based on their morphology, with key isolates having their identifications confirmed by PCR and ITS DNA sequencing. These collections and records were important for three reasons:

1) to make sure that the antibodies raised in the project were to isolates/species that represent current disease threats to the UK industry;
2) for realistic cross-reactivity testing of antibodies raised;
3) for testing the efficacy and sensitivity of the antibody tests subsequently developed.

Key to the success of this study has been the extensive cross-reactivity testing carried out. It is especially important to select antibodies that show minimal cross-reactivity with non-target organisms as this will result in the potential for 'false positive' tests and high levels of cross-reactivity have been problematic with some currently-available antibody tests for oomycete pathogens.

Over 50 antibodies were raised to *Phytophthora* and *Pythium* isolates selected from the culture collection and these were subjected to an extensive cross-reactivity testing program. From this work antibodies have been selected and successfully developed in lateral flow devices (LFD) to detect the following:

a) Oomycetes in general (for use in general viability tests, see below 'oomycete PAb' {UW 548})
b) *Phytophthora* identification (MAb 3H7)
c) *Phytophthora* clades 1/7/8 (predominantly pathogenic specifies) (MAb 3C4)

d) *Pythium clades F/G/I* (detects *Pythium irregulare, P. violae, P. ultimum & P. nunn*) (MAb 4B5)

The sensitivity of the LFD tests described above was assessed against dilutions of *Phytophthora* and *Pythium* zoospores in sterilised and untreated 'raw' reservoir ('pond') water. Zoospore dilutions were made in 1 litre volumes as this is the standard volume for water tests for these pathogens. The spores were then extracted from the water by filtering onto 3µm mesh membrane filters and LFD tests were carried out on these membranes. These assessments consistently showed that tests could detect down to 10 and often fewer, spores per litre (i.e. per filter membrane) – a level of sensitivity that is useful for determining potential disease risks.

To assess the efficacy of the LFD tests developed, samples have been taken at production facilities across horticultural sectors. Several types of sample were tested:

- direct samples of plant material or growing media – examples of both healthy and unhealthy material containing suspected infections;
- water samples - membrane-filtered (3µm pore size) and the filter membranes tested;
- swab tests were taken from some nurseries;
- plant tissue baits placed in water systems, puddles and wet areas, as well as 'wash-through' tests of growing media;
- mycelial colonies growing on conventional agar plates of all above sample types.

So far, 647 samples have been tested out of which 332 tested positive for *Phytophthora* sp. with antibody 3H7, a general *Phytophthora* test. All except six of these tests were confirmed as '*Phytophthora* positive' by conventional isolation techniques and observing morphological markers. All six 3H7 positive samples where *Phytophthora* could not be confirmed by culturing techniques had been chlorinated with sodium hypochlorite solution and contained no detectable viable oomycete propagules. Of the 647 samples, 307 were also tested with antibody 3C4 for *Phytophthora* clades 1/7/8, and 177 tested positive. The majority (132) of these positive results were confirmed by morphological tests. The remaining 25 tests could not be confirmed positive by conventional methods within reasonable time limits. *Pythium* tests using antibody 4B5 have been applied to the 67 most recently collected samples, of these 33 have given positive reactions and all of which have been confirmed as *Pythium* sp. by conventional plating methods. Using these antibody tests in combination has been found to be very useful with some samples, for example in one case of anemones showing root and collar rot symptoms. These plants tested positive for *Phytophthora* using 3H7 but negative

for clades 1/7/8 with 3C4, they also tested positive for *Pythium* with 4B5. Plating these infected tissues confirmed the presence of *Phytophthora gonapodyides* (a species in clade 6 that is commonly found in pond, river and nursery run-off samples, and, although not a serious pathogen of horticultural crops, is occasionally seen infecting woody hosts, often in association with other oomycete species in an 'infection complex' causing symptoms of decline), *Pythium irregulare* and one other *Pythium spp.* Another sample of similar plants from a different nursery tested positive for *Phytophthora* with both 3H7 and 3C4 and negative for *Pythium* with 4B5 and was confirmed to be infected with *Phytophthora cryptogea* (a pathogenic clade 8 species) by conventional plating and ITS sequencing.

Developing a test for the viability of detected oomycetes, or more specifically differentiating between live from dead (killed) zoospores, was a highly ambitious objective and this part of the study has focussed on three approaches:

1) the use of antibody probes developed to specific proteins/glycoproteins CBEL and BCAT that are thought to be of key importance in developmental processes in the early stages of zoospore germination/infection;
2) screening the full spectrum of antibodies produced in this study just in case one of them has developed to an antigen that can act as a marker for viability;
3) and zoospore trapping immunoassay (ZTI) – an assay that relies on specifically visualising germinated (i.e. live) zoospores after an incubation period.

None of these approaches was able to provide an instant answer on viability, but ZTI and, to a limited extent, use of the CBEL probe can both discern between live and dead samples after an incubation period to allow live spores to germinate. For ZTI this takes between 3-5h or overnight, whereas CBEL takes 24h+.

Financial Benefits

Reliable and affordable detection and diagnosis are key to effective oomycete disease management. Pathogen detection prior to infection or the development of symptoms invariably improves the efficacy of timed control measures and can significantly reduce disease epidemics and control treatment inputs. This project provides considerable scope for benefit in terms of early detection and targeted treatments (sanitization programs, biological and / or chemical control). The introduction of tests will also assist disease certification schemes.

The use of rapid and accurate diagnostic tests will provide a significant step forward in the development of lower-input farming systems and help minimize the number and volume of fungicide interventions by detecting problems early and removing the need for prophylactic ‘insurance’ applications.

Fungicide usage is costly and can be one of the major inputs in crop production after fuel and labour. Also, the availability of effective fungicides is becoming increasingly restricted and the flow of new active ingredients onto the market significantly reduced. Targeted application of control measures will help delay the onset of pathogen resistance to currently available fungicides, thus prolonging their useable life. The cost of diagnostic tests must be compared with a typical spend per hectare for materials and labour for a single fungicide treatment. Ultimately, financial benefit will be gained through improved quality and improved control procedures.

SCIENCE SECTION

Introduction

Oomycetes cause significant losses across agricultural commodities worldwide. They are a large group of fungus-like microorganisms, with representatives in virtually every terrestrial, marine and freshwater habitat. A significant proportion of oomycetes are parasitic, colonising and causing disease in a very diverse range of organisms from other protists to higher plants and animals (Beakes *et al*., 2012). Both *Pythium* and the closely related genus *Phytophthora*, are economically important plant pathogens with disease generally favoured by wet soil conditions. Their rapid dispersal is often achieved by asexual, flagellate zoospores. Both genera are commonly detected in contaminated irrigation water supplies and can rapidly spread in hydroponically grown crops or in situations where irrigation water is being recycled.

Economic losses resulting from disease development can be reduced by early detection and identification of pathogens. The latter being essential for the selection of appropriate control/management measures and timings, whilst rapid detection improves the efficacy of treatments and can allow interception and avoidance strategies to be effectively deployed. Conventional techniques for pathogen detection involving isolation, culturing and microscope work are reliable but require laboratory facilities and skilled staff, and tend to be too slow for effective disease interception, being more often deployed once disease symptoms have started to appear (i.e. rather too late). The use of molecular and immunoassay-based techniques offers the promise of increased speed of diagnosis, often accompanied by improved sensitivity, and there are increasing possibilities for deploying these techniques in a field setting (Wakeham & Pettitt, 2017). Immunodiagnostic tests for *Phytophthora* species have been successfully used in epidemiological studies (Hardham, 2005), and more recently in screening for *Phytophthora ramorum* infections with field tests using the lateral flow device (LFD) format (Lane *et al*., 2007). A limitation to the use of immunodiagnostic tests is the specificity of the antibodies used and when antibodies 'cross-react' with non-target organisms they can give 'false positive' tests.

The central aim of this project was to raise new antibodies to disease-causing *Pythium* and *Phytophthora* species prevalent in UK horticulture and rigorously test them for cross-reactivity and develop them for use in the LFD format for possible use to improve early on-nursery pathogen detection. The overall aims of this project as originally set up are reviewed in detail in the year 1 annual report (Wakeham *et al*., 2016, pp. 8-12). The aims of the 3rd and final year of the project were:

1) To contribute, where relevant, to the largely 'complete' culture collection created for sensitivity and cross-reactivity testing of antibodies and prototype tests.

2) Complete the last fusions, and complete the remaining cross-reactivity tests – taking forward any further promising antibodies to LFD.

3) Continue efforts to develop a general oomycete viability test for field-testing treatments applied against potential oomycete pathogens – for example testing water treatment efficacy.

4) Complete agreed further work on the multiplex testing of samples with MagPix technology, investigating the possible use of restriction enzymes to cut pathogen sequences to a size that can be detected by the system.

5) Complete a series of nursery visits and field tests comparing prototype tests with conventional sampling and testing techniques to assess plant tissue, growing media and water samples – looking where possible, at on-site protocol development.

Materials and methods

Completion of isolations, clean-up, identifications and collection of representative isolates of *Pythium* and *Phytophthora*:

Clinic samples continued to be screened for the purposes of collecting representative isolates of oomycete pathogens for testing antibody probes throughout year 3 of the project. Isolates were also taken from samples collected during nursery visits. Isolations, clean-ups and identifications, and collections continued, following the procedures described in detail in the year 1 annual report (Wakeham *et al*., 2016. pp. 13-16).

Completion of new antibody preparation and cross-reactivity testing:

The five last batches of fusions (21 in total) were completed in year 3. These were raised to: *Pythium* clade A (*Pythium aphanidermatum*); *Phytophthora* clade 1 (*Phytophthora cactorum*); *Phytophthora* clade 8 (*Phytophthora cryptogea*); *Pythium* clade F (*Pythium sylvaticum*); '*Pythium* general' (*Pythium aphanidermatum*, *P. lutarium*, *P. intermedium, P. ultimum*). Antibody preparations from all fusions were subjected to cross-reactivity tests, carried out against mycelial extracts prepared from cultures of a representative range of 45 target and non-target oomycete, and non-oomycete species following the procedures established in year 2 of the project (Pettitt *et al*., 2017, pp. 12-13).

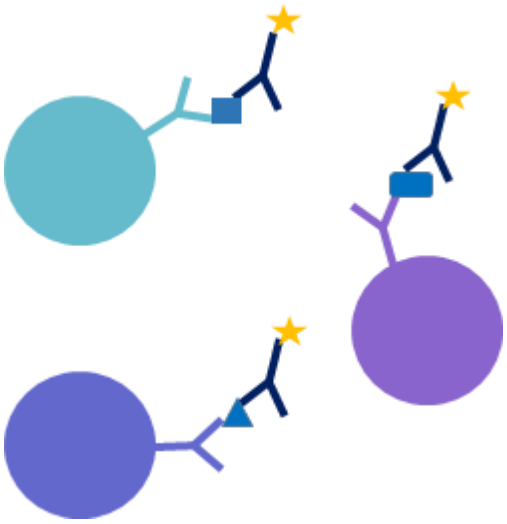
Investigating the development of multiplexing platform for detection of oomycete pathogens using the MAGPIX® system:

In year 2, the proposed detection of signature amplified ITS DNA fragments by the MAGPIX® system proved unsuccessful. It is thought this was because the relevant fragment lengths were too long, resulting in the distance between the fluorescent labels and the coloured magnetic beads being too great for detection in the X-map system. As a consequence, two lines of research were agreed and followed in year 3 of the project to try to overcome this obstacle to developing a MAGPIX®–based multiplex diagnostic system for oomycete pathogens.

The first line of research involved an attempt to exploit some of the antibodies developed in year 2 to detect 'signature' proteins rather than using oligonucleotides to detect amplified ITS DNA fragments. This approach investigated using the sandwich principle, using two antibodies - each detecting a different epitope of the signature antigen protein. The aim was to use the generic oomycete polyclonal antibody, labelled with a fluorescent marker on one part of the antigen in combination with the relevant differentiating monoclonal antibody coupled with a coloured magnetic bead attaching to a different epitope of the same antigen (Figure 1). To achieve this dot-blot assays were carried out to confirm that the antibodies

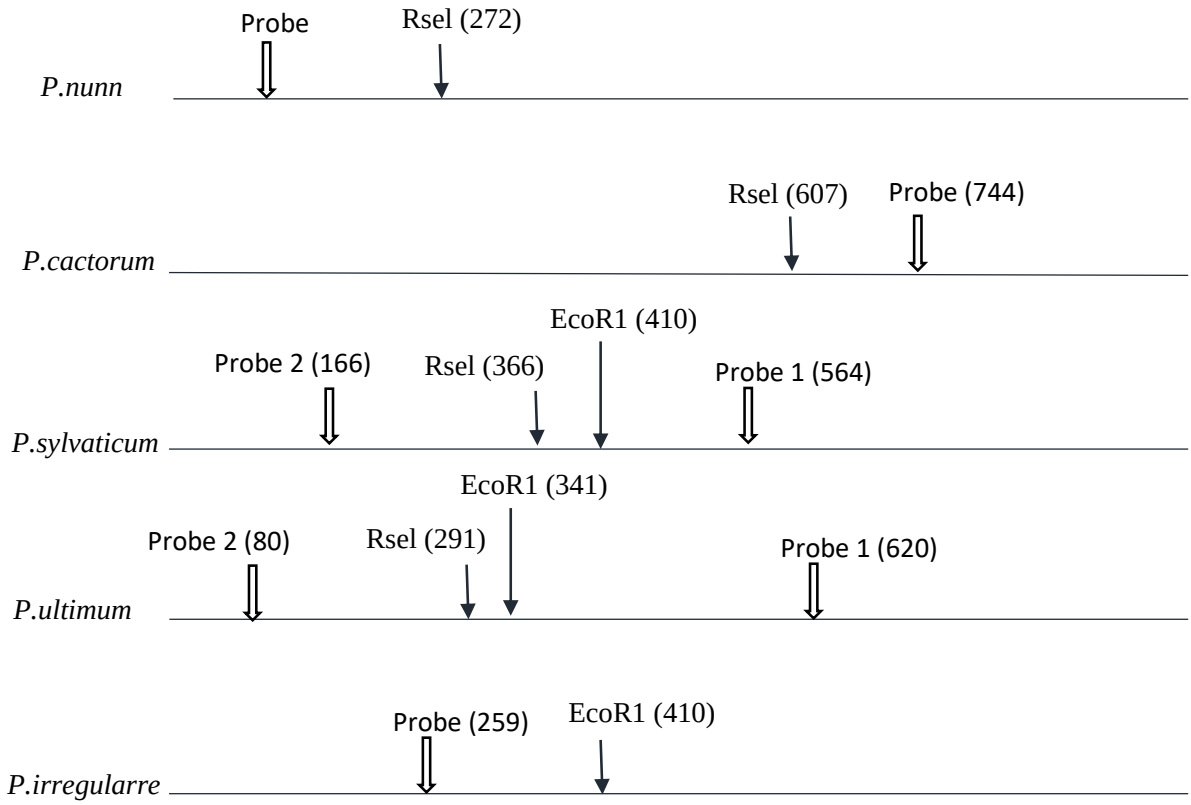
proposed were actually competing for different epitopes and that no undesired binding would occur between the different proposed components of the sandwich that would result in the overall assay giving false positive readings.

Figure 1: Schematic of the use of antibodies in the X-map (Luminex) system. Monoclonal antibodies with different specificities are coupled to magnetic beads of different colours. These capture antigen in solution. Captured antigen is detected by a fluorescently labelled polyclonal antibody.



Year 2 work showed that the ITS DNA amplicons used for oomycete diagnoses were probably too long (around 1000bp) for the MAGPIX® instrument to detect (recommended detection optimum 100-300bp). The possibility of using restriction digest enzymes to digest these amplicons was therefore investigated in year 3. The appropriate DNA sequences were identified and maps were generated for the species *Pythium nunn, Phytophthora cactorum, Pythium sylvaticum, Pythium ultimum* and *Pythium irregulare* and the locations of potential restriction endonuclease sites were identified using Mega7 software (Kumar *et al*., 2016). Restriction enzymes were selected based on their potential cleavage sites being between the forward primer site and a species specific probe site. Two restriction enzymes were selected: RsaI and EcoR1, as their restriction sites were present on the ITS sequences of the majority of the oomycete species investigated. The probe binding sites and restriction sites identified are shown in Figure 2. The probe binding sites for *P. sylvaticum* and *P. ultimum* were too far from the restriction site, so different probes were coupled to magnetic bead, for these species and the sites of these are also shown in Figure 2 as identified as 'probe 2'.

Figure 2: Schematic of DNA sequences of *P. nunn, Ph. cactorum, P. sylvaticum, P. ultimum* and *P. irregulare* amplified using ITS4 and ITS6 primers, showing the relative locations of probe binding and restriction sites. A second identification probe (labelled 'probe 2') was used for *P. sylvaticum* and *P. ultimum* as the potential binding site for the probe used for the other three species was too far from the biotin-labelled site. Numbers in brackets are the number of base pairs from the start of the amplicon.



Investigating the development of diagnostic probes for oomycete spore viability.

Probes to potentially diagnostic proteins:

Five monoclonal antibodies were available with specificity to BCAT1 (branched-chain amino acid aminotransferase). It was noted in another unrelated study that the epitope(s) for some of these antibodies appeared to degrade as it became oxidised. Since the majority of sterilants used against plant pathogens operate by oxidation, it was considered possible that a BCAT1 antibody might be a useful marker for oxidation and possibly therefore loss of viability. Initially the five BCAT1 antibodies (2C4, 5C7, 5D7, 3A7 and 2H9) were tested for cross-reactivity with soluble mycelial extract of *Phytophthora cryptogea* by ELISA. From this initial screen, two antibodies (3A7 and 2H9) were selected for further assessment. In the second experiment BCAT1 antibodies 2H9 and 3A7, 3H7 (*Phytophthora* general) and 3C4 (*Phytophthora* clades 1/7/8) were compared by ELISA against live *Phytophthora cryptogea*

zoospores and similar zoospores killed with either 3% potassium hypochlorite, 0.05% or 0.1% silver-stabilised hydrogen peroxide.

A polyclonal antibody was successfully raised to the cellulose-binding elicitor lectin (CBEL) in the second year of this study (Pettitt *et al*., 2017, pp. 21-24). The CBEL glycoprotein is important in pathogenic oomycetes and expressed in the early stages of zoospore encystment and germination and was considered a good candidate as an indicator of spore viability (Wakeham *et al*., 2016, pp. 16-19). Activity of a dilution series of the CBEL antibody was assayed against live *Phytophthora cryptogea* zoospores by ELISA in comparison with similar zoospores killed by UV treatment (λ= 254nm, 900 mJm^{-2}, 10 min), chlorination (1% v/v sodium hypochlorite) or heat (60^{o}C, 5 min). The effects of dilutions of hypochlorite (10,1, 0.1, and 0.01% v/v) on the CBEL ELISA were further investigated firstly against zoospores of *P. cryptogea*, and then against a purified CBEL glycoprotein solution.

The possibility of CBEL accumulation in and around viable oomycete spores during germination being exploited as an indicator of viability was investigated by filtering 10 ml aliquots of a zoospore suspension on 47mm 3µm cellulose nitrate membrane filters. Filters bearing filtered spores were placed in 9 cm Petri dishes maintained at 100% RH with filter paper soaked in water and placed in the lids and were wetted with 300µl 30mM glucose solution to encourage spore germination and germ tube growth. Filters were sampled at intervals (0, 4, 8 and 24h) and sub-samples were assessed for germination under the microscope. Sampled filters were placed in 1 ml phosphate buffered saline plus 0.5g glass beads in 2ml non-stick, screw cap microcentrifuge tubes and homogenised by three rounds in a FastPrep homogeniser at setting 6.5 for 40 seconds. Homogenised samples were centrifuged at 3000 xG for 10 minutes and the supernatant assayed by ELISA with the CBEL antibody.

Screening project antibody collection for potential probes:

In addition to looking at the use of antibodies raised to specific proteins, a selection of 17 of the antibodies raised during this study, most of which had been put to one side as they cross-reacted with too many non-target species, were assessed for their potential as viability markers. This was assessed by measuring the sensitivity of antibody dilutions by ELISA against live- and heat- treated (100^{o}C, 5 min.) *Phytophthora cryptogea* zoospores.

Assessing the potential of zoospore trapping immunoassay (ZTI) for determining viability:

The possibility of using the 'oomycete general' polyclonal antibody raised in this project in conjunction with the normally more species/genus-focussed zoospore trapping immunoassay (ZTI) as a method of obtaining a generic measure of spore/inoculum viability was investigated

using non-sterile pond water samples 'spiked' with known dilutions of *Phytophthora cryptogea* zoospores. The ZTI procedure had to be optimised for use with the oomycete antibody although the protocol developed was based on the procedure used in the HRI Efford plant clinic (Pettitt *et al*, 2002), adapted from the method designed by Wakeham *et al*., (1997). Water samples were filtered through 47mm, 3µm cellulose nitrate membrane filters. Filters were incubated in 100% RH at room temperature to encourage spore germination as described above after being wetted with a 30mM glucose solution containing 1 in 100 BNPRA antibiotics concentrate (Pettitt & Pegg, 1991) added to inhibit the growth of non-oomycete species in the sugar solution. After 3-5h germination, membranes were air dried and either processed immediately or stored in the refrigerator for processing the next day. Entire membranes can be processed (especially when the concentration of spores in a sample is low), but to conserve reagents and membranes during the method development, small samples (25mm^2) were cut from membranes for processing allowing 'useful membranes to be stored and re-sampled for cross-comparison purposes. Membrane pieces were placed in a small shallow dish and were blocked with 1.5ml 5% rehydrated skimmed milk in tris-buffered saline plus Tween 20 (TBST) for 1h, after which they were washed three times for 5 minutes in 1.5ml TBST. Oomycete polyclonal antibody diluted in TBST to 1/500 was added in a 1.5ml aliquot and incubated with gentle agitation at room temperature for 1h before a further three washes in TBST followed by addition of 1.5ml secondary antibody solution (Anti-Rabbit IgG conjugated to alkaline phosphatase; diluted to 1/6000 in TBST) and a second 1h incubation under the same conditions. Filter pieces were then given a final three washes in TBST after which 350µl of Fast red TR/Naphthol AS-MX mix was added to develop colour in spores and structures labelled by the antibodies. Once colour was developed, the reaction was stopped by rinsing in distilled water and drying the filters after which they were assessed under the microscope (this can be done immediately with wet membranes too), and lysed, germinated and un-germinated spores were found and counted.

Efficacy testing of prototype LFDs *in vitro* and on field samples:

Following cross-reactivity testing, the antibodies selected for use in LFDs were subjected to three levels of assessment:

1) *In vitro* sensitivity assays, mostly in the LFD format, against dilution series of pure extracts of target species' mycelium and of zoospores;
2) In LFD tests against field samples spiked with target organism propagules (mostly zoospores)

3) LFD-testing of field samples alongside conventional diagnostic and quantitative techniques (selective agar-plating, baiting, and microscopy - in some instances complemented by DNA sequencing).

Lateral flow assembly:

The lateral flow devices used in this study comprised of a Millipore 180 HiFlow™ cellulose ester membrane direct cast onto 2 ml Myler backing (Cat no. SHF2400225, Millipore Corp, USA), with an absorbent pad (Cat no GB004, Schleicer and Schuell, Germany) at one end to absorb the excess liquid that passes through the device, and a filtration and sample (conjugate) pad (Cat no. T5NM, Millipore Corp, USA) placed at the other (Figure 3a). The lateral flow membrane and backing comes as a sheet onto which the pads are attached as strips and a line of *Phytophthora* or *Pythium* antigen (consisting of mycelial extract in a mixture of sucrose, trehalose, isopropanol and sodium azide) is applied directly onto the membrane sheet surface using a flatbed air jet dispenser (Biodot Ltd, The Kingsley centre, West Sussex, UK). The lateral flow sheet is then air dried at 37°C for a period of 24 hours before cutting into 5mm wide strips.

For use in the conjugate pad, 5µl of goat anti mouse IgM gold conjugate (BA GAMM 40, BBI, Cardiff) is added to 30µl of the relevant monoclonal antibody/conjugate buffer (2% sucrose, 2% trehalose, 2% Bovine serum albumin (BSA) in ¼ strength phosphate-buffered saline (PBS)). 30 µl of this mixture was transferred to conjugate pads, dried for 35 mins at 27°C before transferring the pads to LFD strips which were then inserted housing devices (Figure 3b) in preparation for use in tests. The housing device facilitates the addition of a 100µl aliquot of test extract and the reading of the intensity of the reaction lines using an ESE reader (see Wakeham *et al*. 2016).

Figure 3a: Side elevation, longitudinal cross-section of a test strip from a lateral flow device as used in this study for semi-quantitative diagnostic testing for *Phytophthora* and *Pythium* spp.

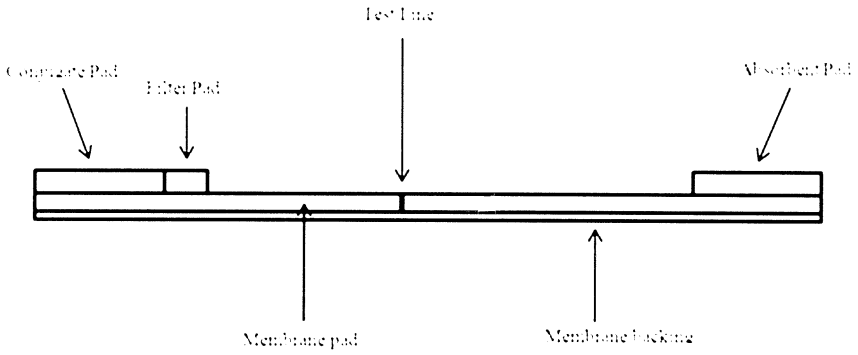
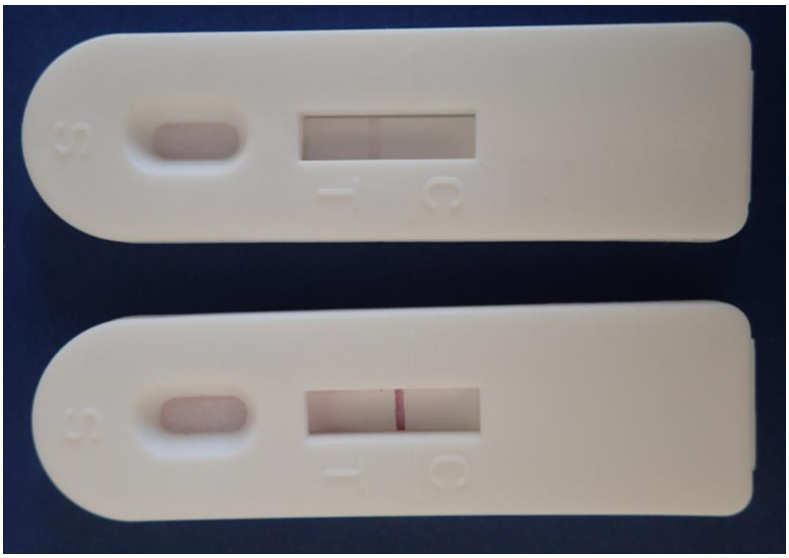


Figure 3b: LFD test strips installed in housings – the strips illustrated here have been used to test samples and the top strip shows a strong positive response for target pathogen presence, with the intensity of 'test line' being considerably weaker than the bottom strip which is showing the intense colour indicating a negative response.



Sample preparation for LFD testing

Extracts of target oomycete species were prepared in 'B2' buffer solution (0.25% polyvinyl pyrrolidone (PVP), 0.4% casein, 0.05% sodium azide dissolved in phosphate-buffered saline (PBS) which gives a pH of 7.4). Pre-weighed fresh mycelium (approx. 0.2g) was added to 1.5 ml B2 buffer plus 0.5g of glass beads and homogenised in 2 ml screw-capped vials on a

FastPrep 24™ benchtop homogeniser using 3 x 30 second rounds at setting 6. The homogenate was centrifuged at 3000 x g for 10 minutes and the supernatant collected and taken through dilution series in further B2 buffer, dilutions being recorded and applied directly to LFD tests. LFD test responses were measured by the intensity of colour developed in LFD test bands in comparison with 'negative' controls.

Antibody sensitivity tests and initial LFD assessments:

Zoospores of target species were produced by the procedure described by Pettitt *et al*. (2002). Concentrations of zoospores were determined by haemocytometer and appropriate dilution series set up in sterile pond water (SPW). Aliquots of dilutions were assayed directly in ELISA wells or applied to LFDs, or dilutions in larger volumes of SPW (i.e. >100 ml) were membrane filtered (47 mm diameter, 3µm cellulose nitrate membranes, under vacuum and mounted in Nalgene reusable bottle-top funnels), the membranes being collected and extracted in 1.5 ml B2 buffer and processed by LFD as described for mycelium samples above.

Assessing spiked field samples:

Following on from initial sensitivity tests, known numbers of target zoospores were added to otherwise untreated field samples in 'spiking' treatments prior to extraction and LFD testing to give an idea of sensitivity in presence of potential test inhibitors and natural populations of non-target microorganisms. Large (>6 litres) samples of river water and raw reservoir water were collected and divided into 500ml aliquots in freshly-emptied polyethylene terephthalate (PET) carbonated water bottles (Pettitt, 2015). After addition of 10 ml aliquots of SPW containing pre-determined numbers of zoospores, these bottles were gently swirled to assist mixing, then left to stand for a few minutes before membrane filtration, extraction and testing by LFD as described above. In addition to spiking water samples, target pathogen inoculum was added to plant tissues prior to extraction and testing by LFD. Plant tissue extractions for these assessments were much the same as the mycelial extractions described above, with approximately 0.2 g of plant tissue being added to 1.5 ml B2 buffer containing 0.5 g glass beads. Zoospore inoculum was added in 50 or 100 µl aliquots to the B2 extraction buffer prior to homogenisation.

Field tests of LFDs in comparison with conventional diagnostic methods:

To assess the efficacy of the LFDs developed in this study for field testing, samples of plant tissues, water, growing and support media, swabs, and mycelium of isolated 'suspect' oomycetes where collected from 64 nurseries, fields and production facilities from across the protected edibles, protected ornamentals, hardy nursery-stock, soft fruit and field crop sectors. Samples were obtained following procedures developed for conventional diagnostics and all were assessed by both LFD test and conventional plating methods.

Plant tissues:

Plant tissue tests entailed cutting small pieces of surface-sterilised tissue (preferably from a lesion edge), and plating these onto oomycete-selective agar (BNPRA, Pettitt & Pegg, 1991 modified from Masago *et al*., 1977), and quarter-strength potato dextrose agar (¼PDA) whilst floating some pieces in SPW overnight. Suspected oomycetes on agar plates were sub-cultured, cleaned and identified based on morphological characters/microscopic structures (especially sporangia, chlamydospores, mycelium characteristics, hyphal swellings, appressoria and oospores – using the wider literature and useful descriptions and keys in Van der Plaats-Niterink, 1981; Dick, 1990; Ribiero, 1978; Ho, 1981; Stamps *et al*., 1990; Bush *et al*., 2006) seen on the original SPW floats and in cultures grown on ¼PDA, pea broth agar, sterilised grass blades in SPW in addition to mycelial cultures grown in clarified V8 broth, washed three times and transferred to 'starvation' in SPW. This detailed work was necessary to provide a reasonable basis for both 'positive' and 'negative' conventional diagnoses, bearing in mind that the latter are difficult to prove conclusively. For LFD tests, similar pieces of plant tissue were taken (prior to the application of surface sterilisation treatments when these were used for plating) and extracted in 1.5 ml B2 buffer as described above, before application to appropriate LFD test strips. In later tests, some simple alterations to extract preparation were used as part of the development of simplified approaches/protocols for field testing, and these are outlined below.

Water samples:

Water samples were tested by membrane filtration and by baiting assays. Membrane filtration was carried out using 47 mm diameter, 3 µm cellulose nitrate membranes, following the procedure outlined above but filtering larger (measured and generally ranging from 250-1000ml) volumes depending on how much sample was available and its 'filterability'. Membranes were divided, with half being extracted in B2 buffer and tested by LFD as described above and the other half being mixed with resuspension medium and plated out for detection and enumeration and identification of oomycete colony forming units (CFU i.e. mostly spores, 'propagules' such as mycelial fragments or small pieces of infected detritus), following the procedures described by Büttner *et al*. (2014). Baiting procedures are widely used to detect the presence of oomycete species, especially those that produce zoospores, in water and involve the use of plant tissues such as leaves, pieces of leaves, fruits or in some cases seedlings, to attract and 'capture' infective propagules widely dispersed in water. The choice of baiting material is the subject of much discussion in the literature and to a large extent is influenced by the target species or group being baited (Werres *et al*., 2014). For the current study a fairly generic bait was required and cut leaf pieces (approximately 3 x 3 mm) of four different plant species; strawberry, rhododendron, tomato and chrysanthemum, were

used either singly or in mixed baits. The leaf pieces were cut and stored refrigerated in 95% ethanol in 20 ml universal bottles. Baits were deployed in two ways; 1) *in situ*, involving leaving the baits, in a cotton muslin bag, suspended in the water source (pond, tank, root zone etc.) being tested, or more frequently 2) *ex situ*, where baits were placed in a water sample and incubated in the lab. Although the baiting period used can be varied, for this study most baits were left in the water being tested for 12-24 h before removal. For each bait assay approximately 30-40 leaf pieces were placed in the water, following removal these were blotted dry on sterile tissue paper and 10 were plated onto ¼PDA+BNPRA for conventional isolations and diagnoses, whilst a further 10 were extracted in B2 buffer as described for plant tissue extractions above and tested by LFD, with the remaining surplus being discarded.

Growing media:

Two approaches were used to test growing media: 1) direct using plating/extraction, and 2) indirect using flooding, baiting and/or membrane filtration prior to plating/extraction. The direct plating method used in this study was a version of the Warcup (1950) procedure; a weighed aliquot of growing medium/soil (approximately 0.5 g or less) was added to 10 ml sterile distilled water, containing 0.1 ml BNPRA oomycete selective antibiotics concentrate (SDW+), mixed for 30 s on a vortex mixer and taken through a 1 in 10 dilution series in SDW+. Aliquots (0.5ml) of the 1:10, 1:100 and 1:1000 dilutions were pipetted into 9 cm Petri dishes to each of which 10 ml of molten (45^{o}C) ¼PDA + BNPRA was added and gently agitated to achieve even distribution before solidifying. Subsequent CFU appearing were categorised by colony morphology, the different categories counted and examples sub-cultured for identification following the basic procedures outlined above.

Swab tests:

Swab tests were carried out on a small number of nurseries. These tests use an absorbent cotton wool swab impregnated with a 0.1% agar solution containing BNPRA antibiotics. Swabs are each contained in a sterile 2ml Eppendorf vial and are removed from this and held with sterile forceps or a sterile cocktail stick. A sample is collected by gently rubbing the moist swab over the surface being tested (e.g. surfaces on Danish trolleys, staff foot-ware, surfaces on machinery, matting and other plastic surfaces etc.) – preferably swabbing an area of approximately 100 cm^{-2}. When this operation is completed, the swab is returned to its vial and stored for testing. From each swab, two plates are taken. The first is a form of streak plate, where the used swab is gently moved over the agar surface in a zig-zag pattern, the plate is then left to dry in a flow hood for about 1 minute. In the second plating procedure, the swab is place in an empty 9 cm Petri dish to which 15 ml of molten ¼PDA+BNPRA is added and gently swirled in a way similar to the Warcup (1950) procedure described for

growing media samples above. Extraction of swabs for LFD testing was essentially the same as described above for filter membranes and plant tissues, except for the addition of a tungsten carbide pellet in addition to the sand to help with the sample homogenisation process.

Mycelia and CFU:

To investigate their potential to enhance conventional diagnostic procedures, LFD tests were carried out on CFU from soil and water tests and on mycelium growing out of swabs, plant tissues and growing media samples. When CFU and mycelial cultures were tested, a small piece of each tested colony was sub-cultured to fresh agar media and the sub culture identified by procedures outlined above. Meanwhile the remainder of the colony (normally a piece of agar plus mycelium approximately 10mm x 10mm square) was extracted in B2 buffer plus 0.5g of glass beads as described above and the clarified extract tested by LFD.

Results

Completion of isolations, clean-up, identifications and collection of representative isolates of *Pythium* and *Phytophthora*:

The main body of the culture collection work was completed in years 1 and 2 of the project and a culture collection already established (Pettitt *et al*., 2017, pp. 14-21). However, with the intensive sampling and back-up agar culture work involved with the antibody field testing in year three, it was inevitable that further isolates would be added to the culture collection. Details of these isolates are given in (Table 1).

Table 1: Isolates of *Pythium* and *Phytophthora* collected, cleaned, characterised and stored in year 3 of this study.

Culture identifier	Taxonomic identification	Source	Verified by ITS sequences	Confirmed by morph-ology
Oomycete species				
C517-4	*Pythium dissotocum*	Fuchsia growing medium	+	(+)
C525-3	*Pythium* HS	Lettuce	-	(+)
C522-8	*Pythium irregulare*	Asparagus – soil	+	+
C511-2	*Pythium oligandrum*	Tomato roots	+	+
C350-4	*Pythium violae*	Carrot taproot	-	+
C492-1	*Phytophthora cactorum*	Strawberry crown necrosis	+	+
C503-5	*Phytophthora cactorum*	Heathers – media	+	+
C503-7	*Phytophthora chlamydospora*	Greenhouse puddle HNS	+	+
C513-T3	*Phytophthora cryptogea*	Anemone collar rot	+	+
C525-1	*Phytophthora cryptogea*	Lettuce root rot	+	+
C503-4	*Phytophthora gonapodyides*	Woodland stream (bait)	+	+
C512-5A	*Phytophthora gonapodyides*	HNS runoff	+	+
C511-14	*Phytophthora infestans*	Tomato stem	+	+
C350-2	*Phytophthora porri*	Leek	-	+

Completion of new antibody preparation and cross-reactivity testing:

A single fusion was carried out from mice immunised with mixed *Pythium* mycelial extracts (*Pythium aphanidermatum*, *P. lutarium*, *P. intermedium, P. ultimum*), and one new cell line established as detailed in Table 2. A further three fusions were completed with mice immunised with *P. aphanidermatum,* and two with mice immunised with *P. sylvaticum* (clade F), with respectively four and five new cell lines established and these are described in Table 2, including their isotypes and unique identification codes.

Table 2. Details of cell lines raised as potential (a) *Pythium* sp. general, (b) *Pythium* clade A, and (c) *Pythium* clade F specific antibodies.

a) ***Pythium*** **sp. general**, raised to a preparation of mixed mycelial extracts from cultures of *Pythium aphanidermatum* (Clade A), *P. lutarium* (Clade B), *P. intermedium* (Clade F) and *P. ultimum* (Clade I)

Cell line	Isotype	Unique Code
3H10 E1 E1	Not discerned	UW 428

b) ***Pythium*** **clade A specific**, raised to a preparation of mycelial extracts from cultures of *Phytophthora aphanidermatum*

Cell line	Isotype	Unique Code
4G2 A2 B1	IgM	UW 406
4F3 A1 A2	IgM	UW 407
4D4 B1 A1	IgM	UW 408
4F2 E3	IgM	UW 409

c) ***Pythium*** **clade F specific**, raised to a preparation mycelial extracts from cultures of *Pythium sylvaticum* (UW 046, CBS 633.67).

Cell line	Isotype	Unique Code
4H11 G1	IgM	UW 422
3B7 B2 E3	IgM	UW 423
4C11	IgM	UW 424
5B10	IgG2b	UW 425
1E4	IgG2b	UW 426

Single fusions were completed with mice immunised with *Phytophthora cactorum* (clade 1) and with *Phytophthora cryptogea* (clade 8) and five new monoclonal cell lines were

established from each. Details of isotype and unique codes for these 10 cell lines are given in Table 3.

Table 3. Details of cell lines raised as potential (a) *Phytophthora* Clade 1 specific and (b) *Phytophthora* Clade 8 specific antibodies.

a) ***Phytophthora*** **Clade 1 specific**, raised to a preparation of mycelial extracts from cultures of *Phytophthora cactorum* (CBS 231.30)

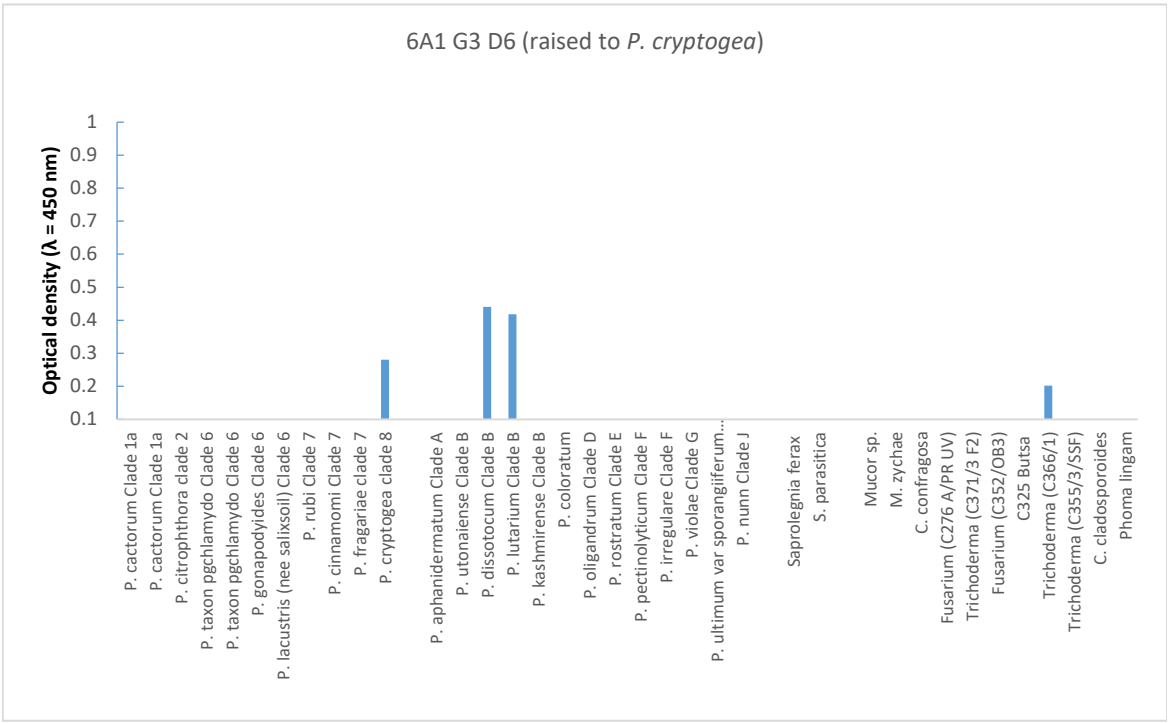
Cell line	Isotype	Unique Code
3B5 B1 D2	IgM	UW 417
5B12 E1 B2	IgM/IgG3	UW 418
5D7 B1 F1	IgM	UW 419
6A3 F1 H1	Not discerned	UW 420
6H11 C1 B2	IgM/IgG3	UW 421

b) ***Phytophthora*** **Clade 8 specific** antibodies; raised to a preparation of mycelial extracts from cultures of *Phytophthora cryptogea* (E563)

Cell line	Isotype	Unique Code
6D11 D2 B3	IgM	UW 412
3F4 G3 B2	IgM	UW 413
6A1 G3 D6	IgM	UW 414
2H2 H4 C4	IgM	UW 415
5G5 B4 B4	IgM	UW 416

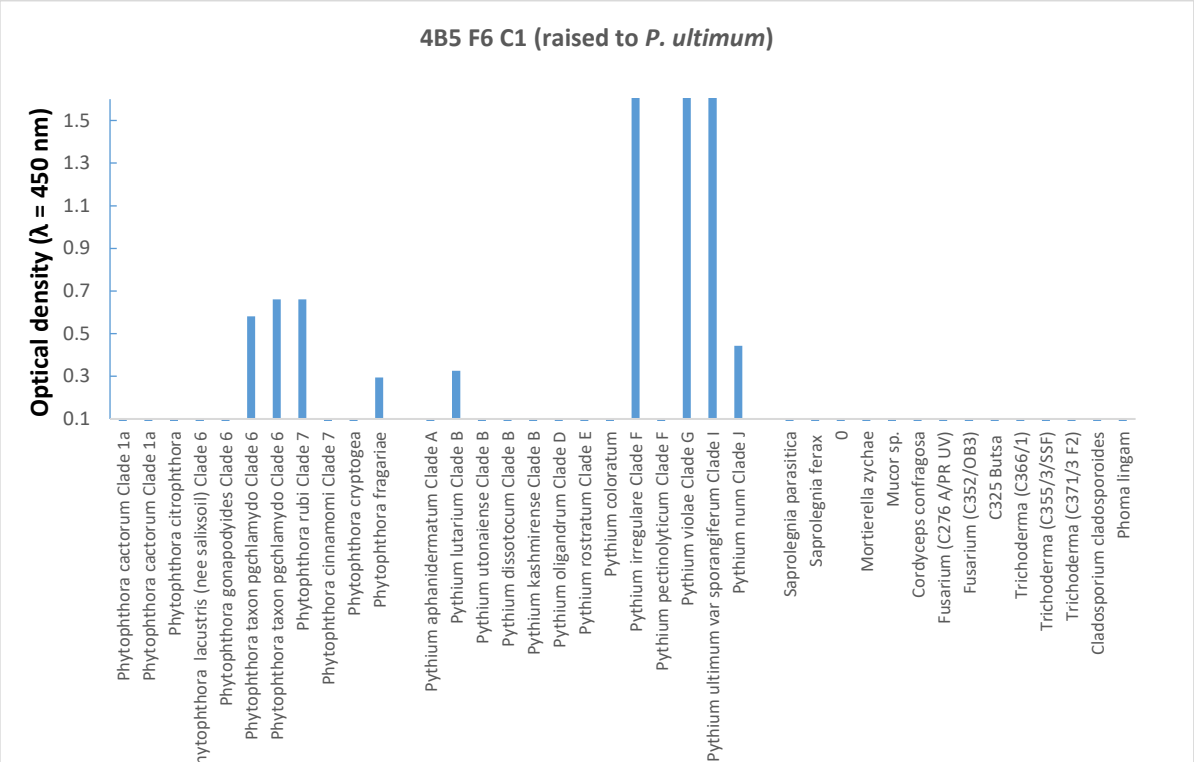
The antibodies from all 20 cell lines were subjected to cross-reactivity tests and all except one (6A1 G3 D6, UW 414, raised to *Phytophthora cryptogea*), showed little specificity and cross-reacted with most species in the test panel (Figure 4 and Appendix Figures 1a-j, 2 & 3). Antibody 6A1 showed strong reactivity to *P. cryptogea* but also to clade B *Pythium* species and a slight reaction with *Trichoderma*. This identifies it as a potentially useful antibody to be used in conjunction with others to discern between pathogen groups for example if used with antibody 3C4 and 3H7, it might be possible to discern *Phytophthora* clade 7 which should give 3H7+ve, 3C4+ve and 6A1-ve. This approach will be considered further in the Discussion section below.

Figure 4: Results of cross-reactivity tests showing results for antibody 6A1 (UW 414), showing potentially useful specific reactions to *Phytophthora cryptogea* and Clade B *Pythium* species.



Cross-reactivity tests were also carried out on six antibody cell lines raised in year two of the project to mycelium of *Pythium ultimum* var. *sporangiferum* (Clade I). Of these, five showed very broad reactivity and were thus of not much further interest. However, one antibody (4B5 UW 402 {IgG1}), showed strong activity for *Pythium* Clades F, G and I plus a small amount of activity with *Phytophthora* clades 6 and 7 (Figure 5). Antibody 4B5, together with two antibodies identified in year 2 of the project (3H7 {UW 375, *Phytophthora* genus-specific} and 3C4 {UW 387, specific to *Phytophthora* clades 1/7/8}), was selected for deployment in LFD test devices for field testing (see section: 'Efficacy testing of prototype LFDs *in vitro* and on field samples' below).

Figure 5: Results of cross-reactivity tests showing results for antibody 4B5 (UW 402), showing potentially useful specific reactions to *Pythium* clades F, G and I and *Phytophthora* clades 6 and 7.



Investigating the development of multiplexing platform for detection of oomycete pathogens using the MAGPIX® system:

In Table 4 the series of dot-blot assays is outlined giving (A), desired results of each blot if the antibodies were only binding with oomycete material and (B) the actual results observed. The dot-blot assays indicated that the rabbit ('oomycete general') polyclonal antibody bound to the mouse monoclonal antibody. It is theoretically possible that the monoclonal mouse antibody was cross-reacting with the rabbit antibody, although this was considered unlikely. The next step was to attempt to remove anti-IgM specific antibodies from the polyclonal antibody preparation by pre-incubation with an excess of mouse antibody, followed by filtration to remove the antibody complexes generated, and thus produce a non-mouse-specific polyclonal antibody filtrate. However, even after this pre-incubation step, the rabbit ('oomycete general') antibody remained with affinity for mouse antibody.

Table 4: Dot-blot assays of the proposed component antibodies for the double antibody sandwich. Capture antibody was coated onto nitrocellulose, before other components in rows were added sequentially with wash steps between each application. (A) Desired results indicate how the antibodies should behave if only binding oomycete antigen. (B) Actual results have been highlighted in red where they disagree with desired.

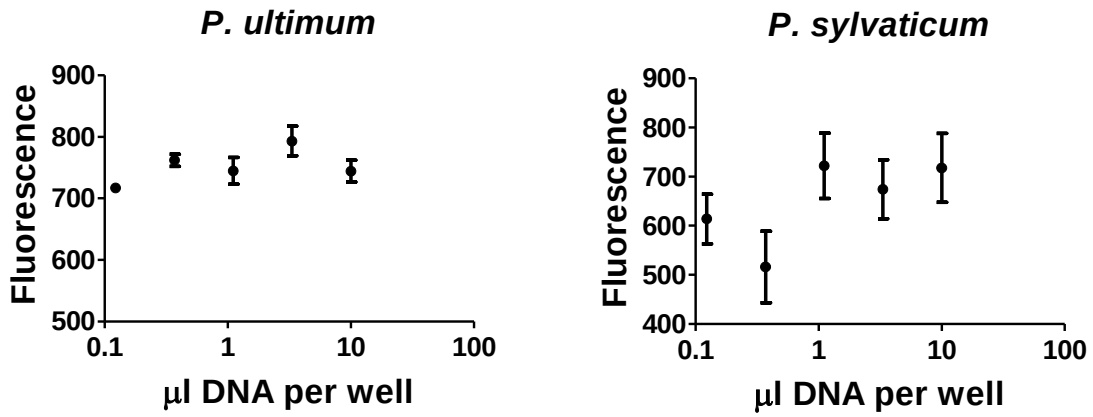
Capture antibody	Antigen	Detection antibody	A Desired result	B Actual result
Mouse IgM anti-oomycete	Yes	Rabbit anti oomycete	+	+
None	Yes	Rabbit anti oomycete	-	-
Mouse IgM anti-oomycete	Yes	None	-	-
None	Yes	None	-	-
Mouse IgM anti-oomycete		Rabbit anti oomycete	-	+
None		Rabbit anti oomycete	-	-
Mouse IgM anti-oomycete		None	-	-
None		None	-	-
Rabbit anti oomycete	Yes	Mouse IgM anti-oomycete	+	+
None	Yes	Mouse IgM anti-oomycete	-	-
Rabbit anti oomycete	Yes	None	-	+
None	Yes	None	-	-
Rabbit anti oomycete		Mouse IgM anti-oomycete	-	+
None		Mouse IgM anti-oomycete	-	-
Rabbit anti oomycete		None	-	+
None		None	-	-

Next the mouse-monoclonal antibody was digested and separated, to try to identify a fragment which could still recognise target antigen, but which was not recognised by the rabbit polyclonal antibody. Mouse IgM antibody was digested using a 2000x molar excess of pepsin, overnight at pH 4.5 – a procedure known to produce multiple fragments of antibody of different sizes. Digested antibody was passed through a Sephacryl 200 column to separate fragments based on molecular weight. Unfortunately all fragment sizes which came off the column were recognised by the rabbit polyclonal antibody, showing that the combination of antibodies available for this approach could not be developed in a double antibody sandwich as proposed.

Restriction digests were successful, with resulting fragments of amplicon being of appropriate size matching sizes indicated in the schematic (Figure 6). Digests were purified using QuiAmp DNA purification kits, and were used for MAGPIX® experiments. A threefold dilution series, staring at 10µl of amplicon per reaction was used, and each amplicon digest ample was tested against its specific probe labelled with an appropriate coloured magnetic bead. Amplicon digests for *P. sylvaticum* and *P. ultimum* were assessed first, since these were

considered to be the most appropriately-sized fragments with the best chance of working. However, the dilution series showed no indication that magnetic bead probe was binding to DNA (Figure 6). Furthermore, the complete absence of any DNA showed a similar frequency of fluorescing beads for *P. ultimum* and *P. sylvaticum* (708 and 584 respectively) as all other concentrations of DNA.

Figure 6: Digested DNA volume vs frequency of coloured fluorescent beads measured by the Luminex MAGPIX® X-map system for restriction digests of ITS amplicons of *Pythium ultimum* and *Pythium sylvaticum*. Bars show mean and standard error of the mean.

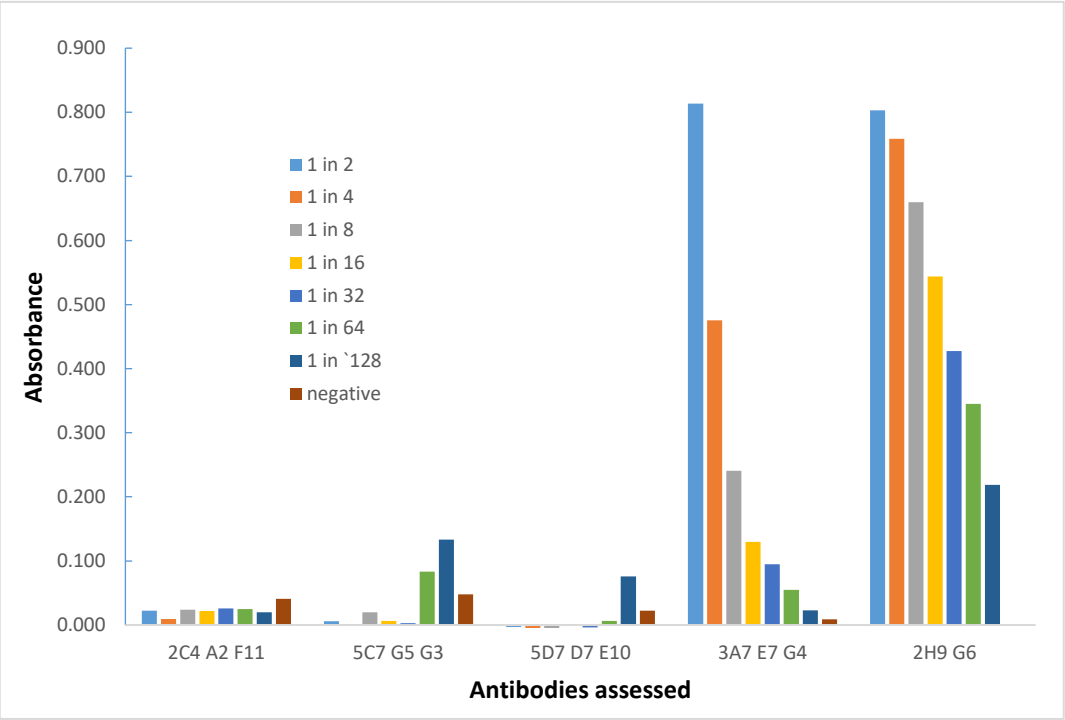


Investigating the development of diagnostic probes for oomycete spore viability

Probes to potentially diagnostic proteins:

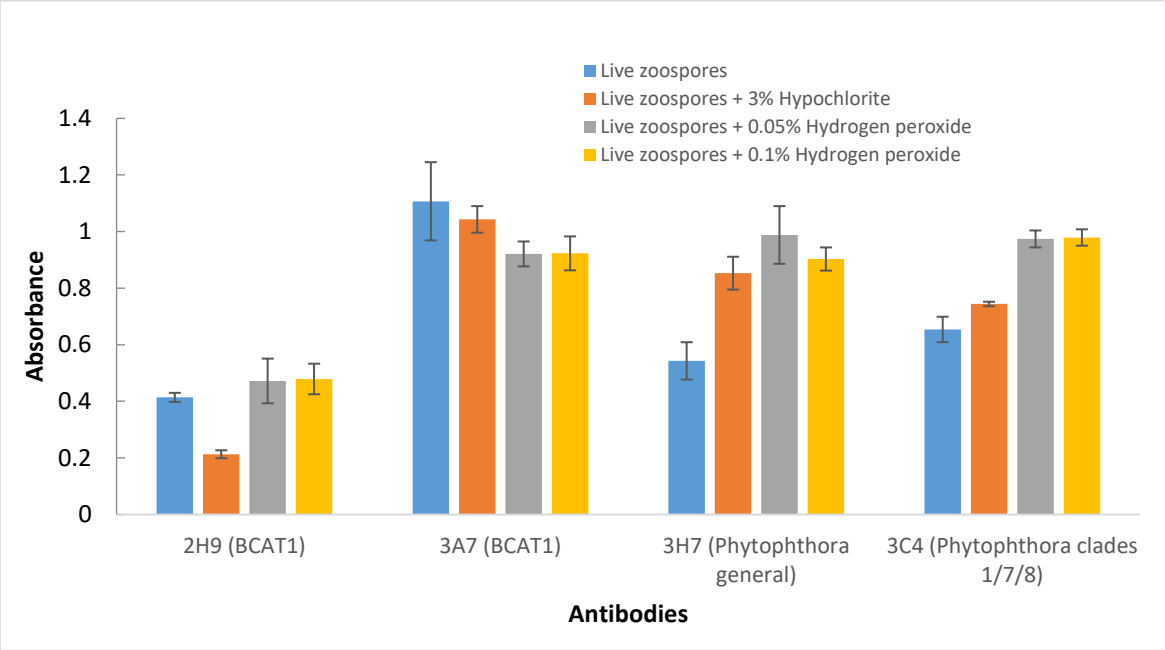
ELISA of five BCAT1 monoclonal antibodies against dilutions of soluble mycelium extract of *Phytophthora cryptogea* in B2 buffer revealed two antibodies that gave strong reactions indicating that *Phytophthora* may contain a related protein with epitopes in common with BCAT1 (Figure 7). These two antibodies, 3A7 and 2H9, were selected for further investigation.

Figure 7: Cross-reactivity of BCAT1 monoclonal antibodies with soluble mycelium extract of *Phytophthora cryptogea*.



Zoospores of *Phytophthora cryptogea* (isolate E563) were subjected to lethal doses of either sodium hypochlorite (3% v/v) or silver-stabilised hydrogen peroxide (Intra Hydrocare Quill Productions, UK: 0.05% and 0.1% peroxide). These four suspensions of live and 3 of killed zoospores were assayed by ELISA with the two selected BCAT1 antibodies, 3A7 and 2H9, and compared with 3H7 (*Phytophthora* general) and 3C4 (*Phytophthora* clades 1/7/8) specific antibodies (Figure 8). This assay showed that there was no effect of the kill treatments on the response of antibody 3A7 or of the two *Phytophthora* antibodies 3H7 and 3C4. There was a significant reduction in the response of antibody 2H9 to the zoospore suspension treated with 3% sodium hypochlorite, although the lack of response to the peroxide treatments indicates that this reduction is unlikely to be a marker of a loss of viability and is possibly indicative of epitope destruction/disruption by the hypochlorite.

Figure 8: Testing potential of two BCAT1 antibodies (2H9 & 3A7) for their potential as viability markers in comparison with 3H7 (*Phytophthora* general) and 3C4 (*Phytophthora* clades 1/7/8) antibodies.



The sensitivity of the CBEL antibody did not appear influenced by cell viability as there were barely perceptible differences between the ELISA results for untreated, fully viable zoospores of *Phytophthora cryptogea* and similar spores killed by high doses of UV irradiation and by prolonged lethal temperatures (Figure 9). Nevertheless, chlorination with 1% v/v sodium hypochlorite did have a profound effect on CBEL antibody sensitivity and this effect was examined further in two experiments assessing the impact of dilutions of sodium hypochlorite on the CBEL antibody's binding capacity. Binding to either *Phytophthora* zoospores or the purified CBEL glycoprotein antigen remained largely unaffected by hypochlorite concentrations of 0.01% and 0.1%, but started to significantly decline at 1% and still further at 10% v/v (Figure 10). Although there appeared to be little immediate effect of heat and UV kill treatments on the expression of the CBEL glycoprotein relative to viable treatments, viable spores might be expected to continue generating CBEL if encouraged to germinate and therefore generate a difference in CBEL concentration to killed control treatments. This possibility was assessed by measuring the potential build-up of the glycoprotein over a short incubation/germination period. Germination of *Phytophthora cryptogea* zoospores on nitrocellulose membranes did increase the amount of CBEL detected over a 24h incubation period with the fastest rate of expression occurring over the first 10-12h (Figure 11). Nevertheless, the build-up was relatively small and this phenomenon needs more

optimisation work to generate reliably measurable differences between samples containing viable and non-viable spores in field tests.

Figure 9: Sensitivity of dilutions of CBEL polyclonal antibody in ELISA against live and killed zoospores of *Phytophthora cryptogea*

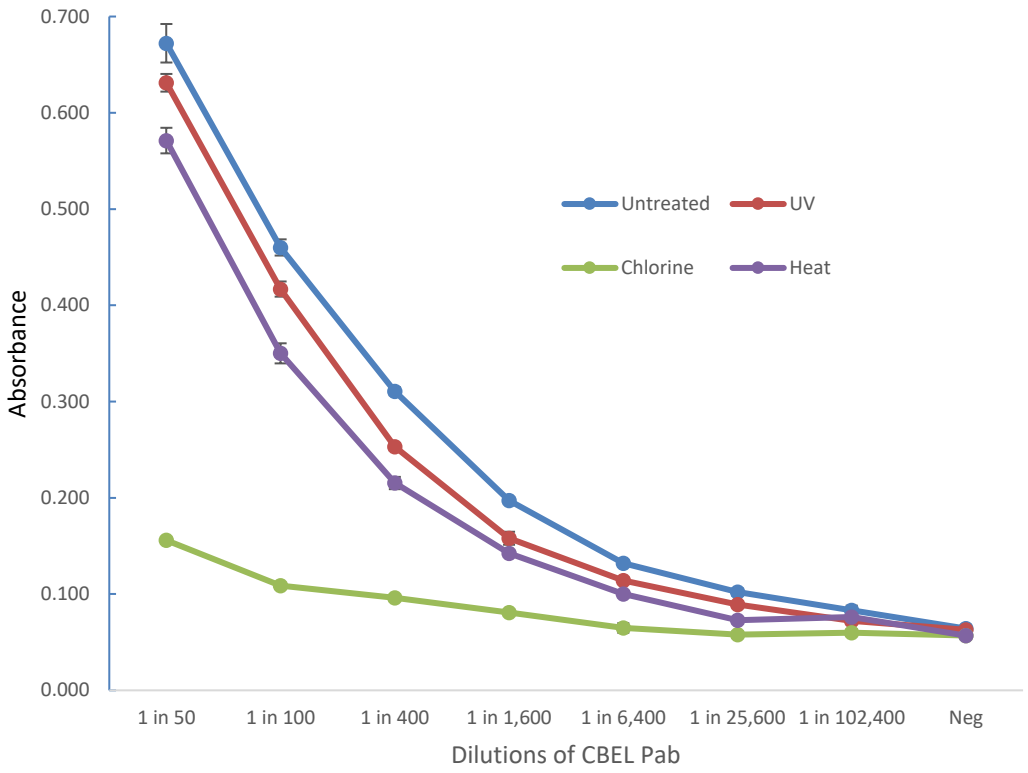
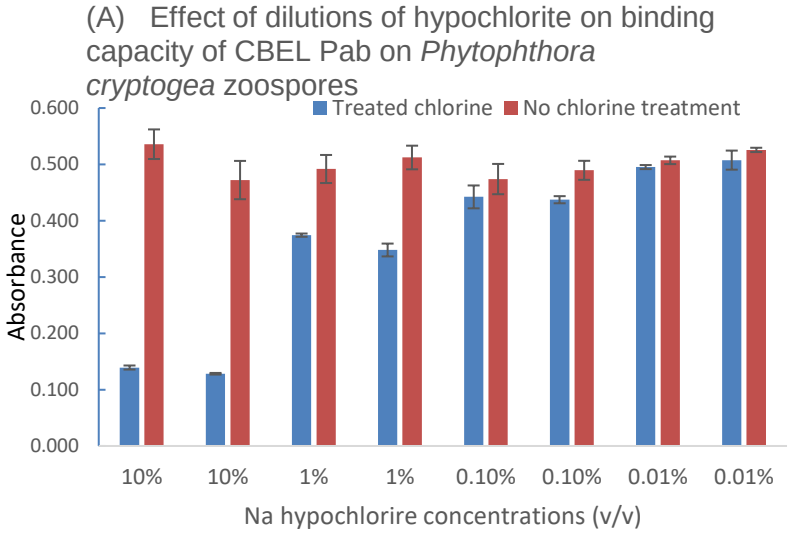


Figure 10: Effects of sodium hypochlorite concentration on the binding capacity of the CBEL antibody against (A) *Phytophthora cryptogea* zoospores and (B) purified GBEL glycoprotein.



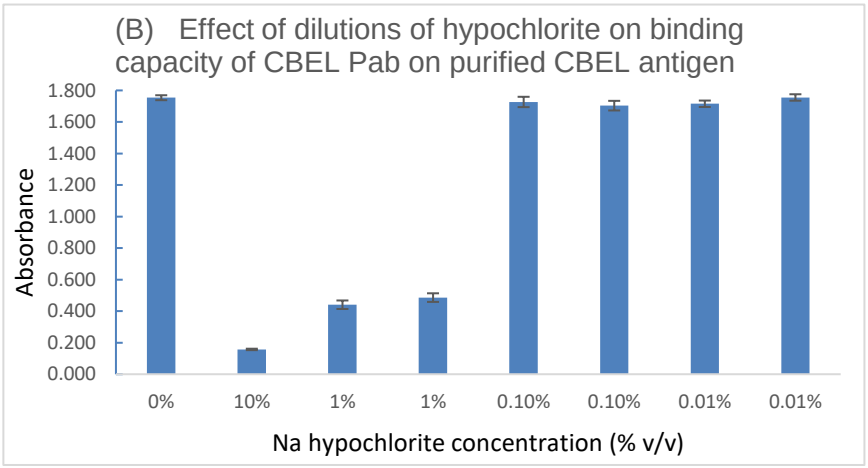
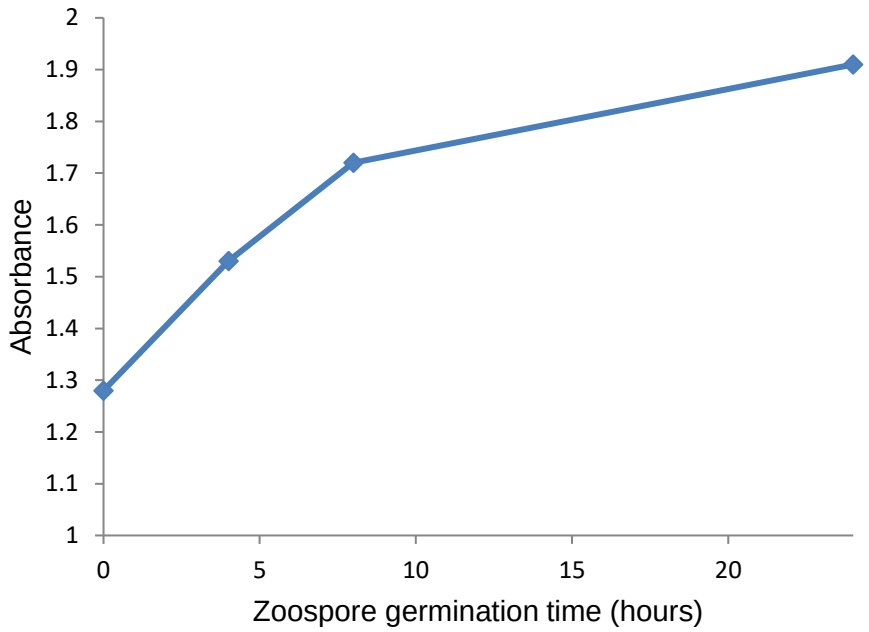


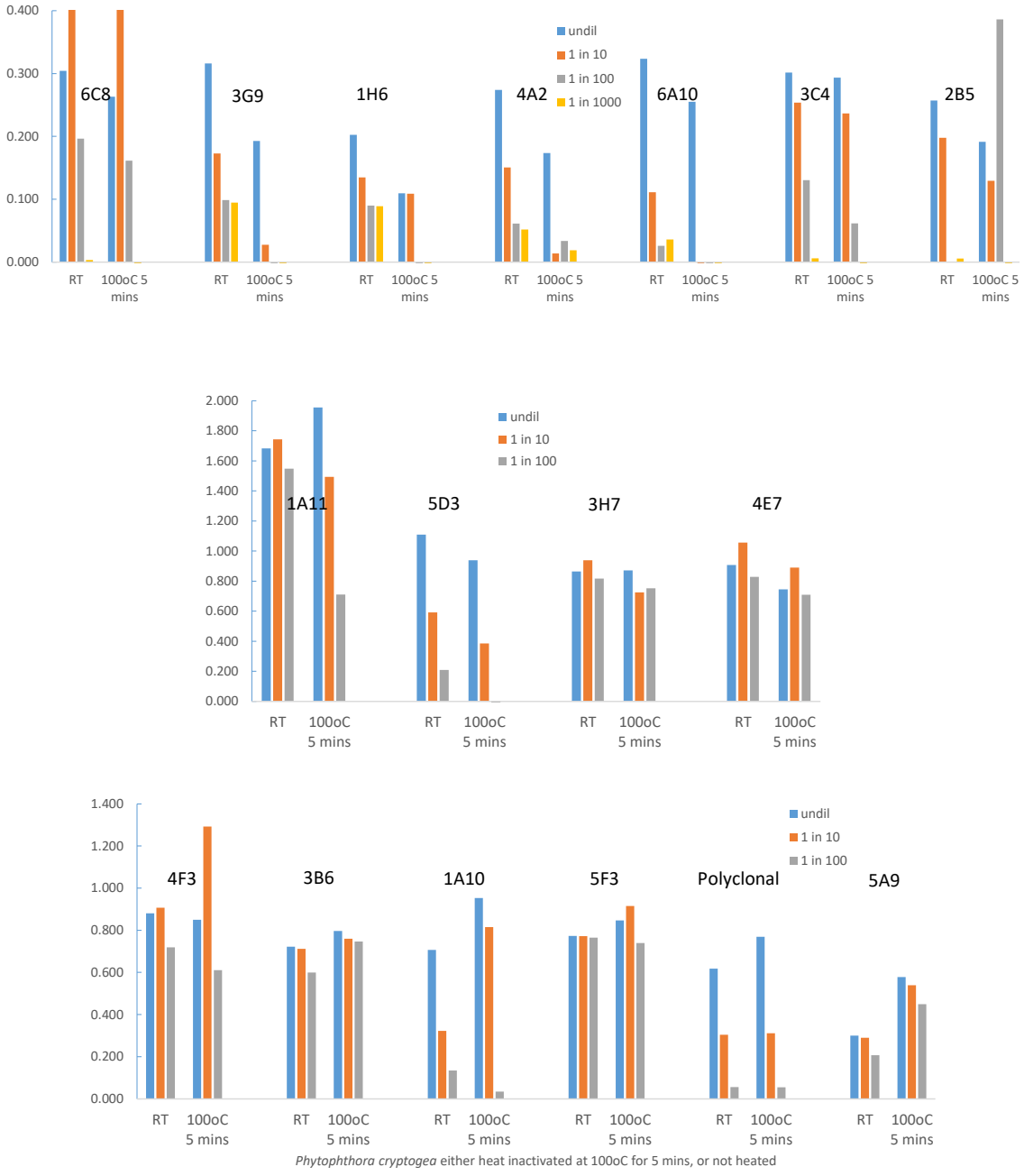
Figure 11: Increasing expression of CBEL glycoprotein in the early stages of *Phytophthora cryptogea* zoospore germination as measured by ELISA with CBEL polyclonal antibody.



Screening project antibody collection for potential probes:

The 17 antibodies assessed for potential as viability markers were: 6C8, 3G9, 1H6, 4A2, 6A10, 3C4, 2B5, 1A11, 5D3, 3H7, 4E7, 4F3, 3B6, 1A10, 5F3, 5A9, oomycetes general polyclonal. Of the 17 antibodies tested, only three gave some indication of reduced response to heat-killed spores: 3G9, 1H6 and 6A10 and these only at the lower dilutions and against a back drop of an overall weak ELISA response (Figure 12). On further investigation, unfortunately none of these three antibodies gave a suitable response.

Figure 12: Assessment of 17 antibodies (antibody codes label the bars) for their potential as viability markers by ELISA comparisons the sensitivity of antibody dilutions to live and killed *Phytophthora cryptogea* zoospores.



Assessing the potential of zoospore trapping immunoassay (ZTI) for determining viability:

ZTI using the oomycete general polyclonal antibody proved very effective at picking out germinated zoospores of *Phytophthora cryptogea* in non-sterile pond water samples. This was in part due to the fact that this antibody tends to bind very well to mycelium, resulting in relatively clearly-stained germ tubes, making these straightforward to identify under the microscope and even under a x20 hand-lens (Figure 13). In studying dilution series of *Phytophthora* zoospores made in non-sterile pond, river and reservoir water sources, dilutions down to 10 spores per membrane filter were readily discerned by ZTI and in zoospore 'kill' experiments using 1 minute microwave or applications of 3% sodium hypochlorite viable spores could be discerned from non-viable using ZTI by the presence of stained germ tubes in the latter (Figure 14).

Figure 13: Example of ZTI-stained germinated *Phytophthora* zoospores

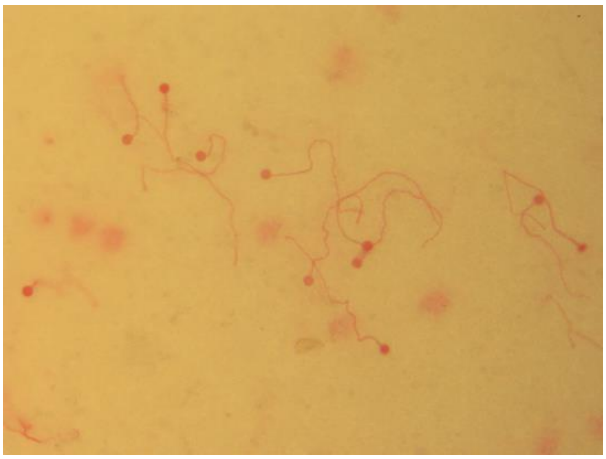
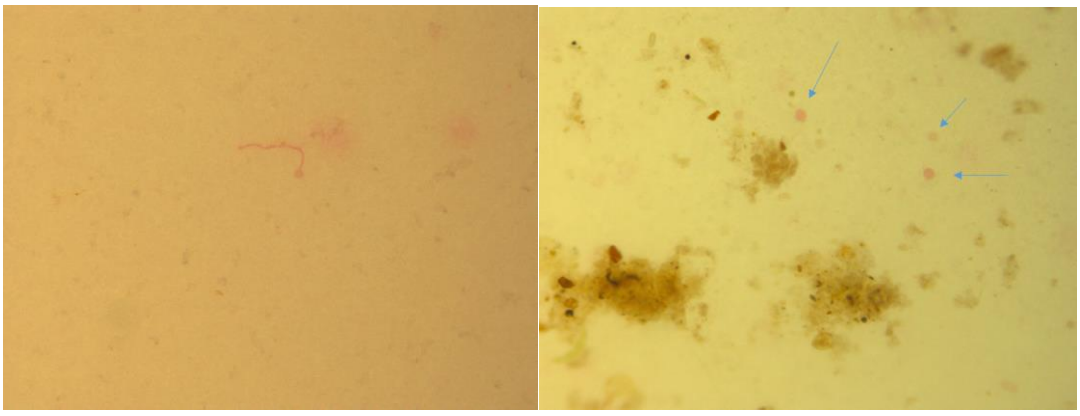


Figure 14: Comparison between (left) stained viable spore (at concentration of 10 spores per membrane which is equivalent to 10 spores per litre of filtered sample) and (right) non- viable spores (indicated by blue arrows).

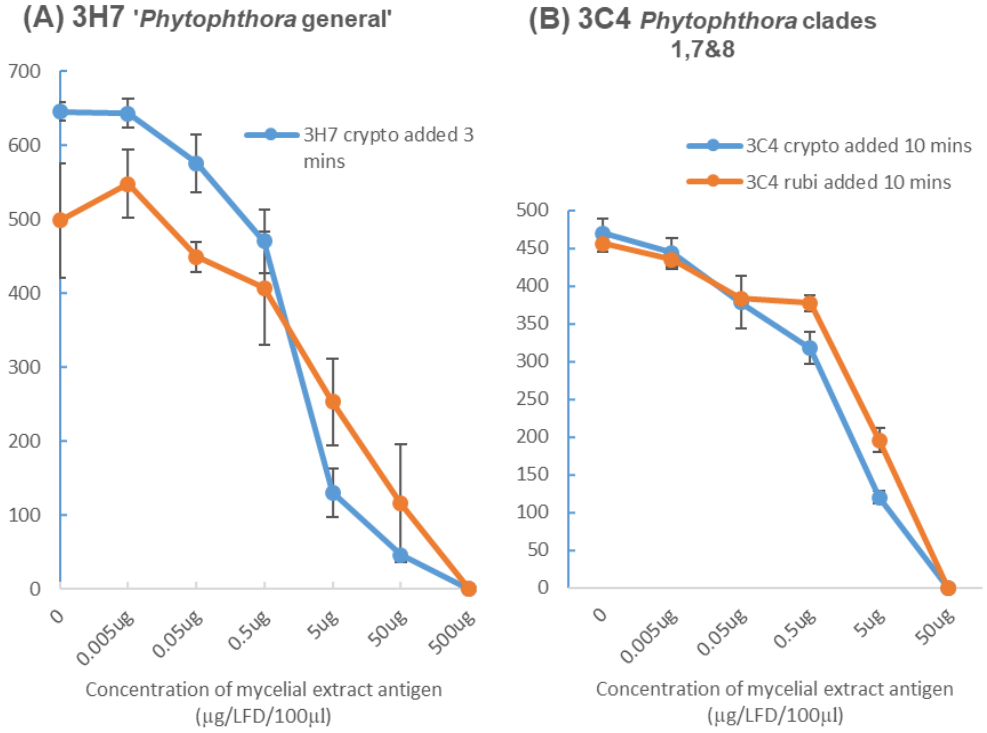


Efficacy testing of prototype LFDs *in vitro* and on field samples:

Antibody sensitivity tests and initial LFD assessments:

Three antibodies have so far been tested extensively on field samples using an LFD format. These are 3H7 (UW 375) originally from a batch of cell lines raised to mixed encysted zoospores of *Phytophthora cinnamomi, P. citrophthora* and *P. cryptogea* as potential viability markers (Year 2 report, Table 3 (Pettitt *et al*., 2017)); 3C4 (UW 387) originally raised to mixed mycelial extracts from cultures of *Phytophthora cactorum, P. rubi* and *P. cryptogea* as a potential *Phytophthora* genus-specific marker (Pettitt *et al*., 2017, Table 4); and 4B5 (UW 402) originally raised to mycelial extracts of *Pythium ultimum* var. *sporangiferum* as a

Figure 15. Detection of dilutions of *Phytophthora cryptogea* and *P. rubi* mycelium extracted in B2 buffer by (A) 3H7 *Phytophthora* general' antibody and (B) 3C4 *Phytophthora* clade 1/7/8 specific antibody in prototype LFD tests.

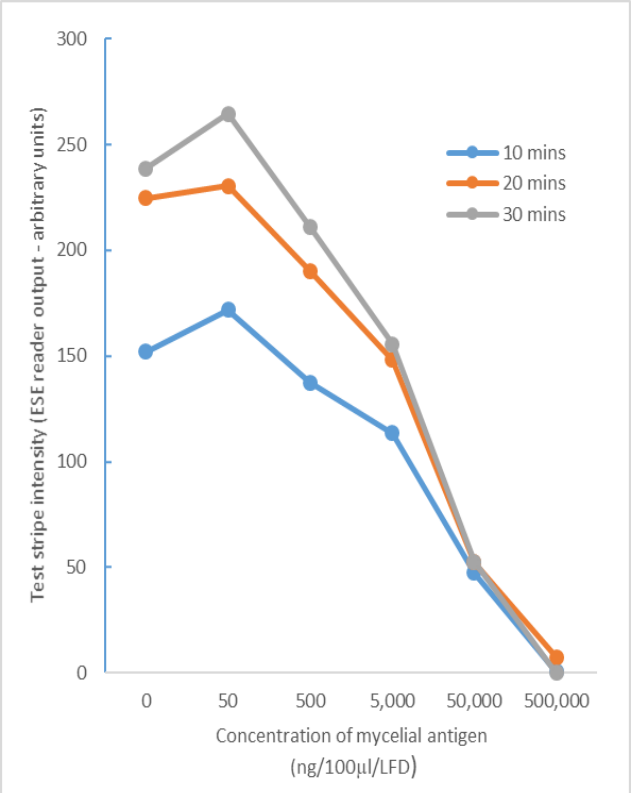


potential *Pythium* clade I marker (Pettitt *et al*., 2017, Table 6). Cross-reactivity testing in year 2 of this study showed that 3H7 was an excellent genus-specific marker for *Phytophthora* species, although it did also react strongly to the plant pathogenic *Pythium* species *P. ultimum* var *sporangiferum* (Pettitt *et al*., 2017, Figure 6), and that 3C4 reacted strongly with members of *Phytophthora* clades 7 and 8 (*Phytophthora rubi, P. cinnamomi*, and *P. cryptogea*) and did not react with any other oomycetes. Antibody 3C4 has subsequently been shown to react

with clade 1 species *P. cactorum* and *P. infestans* and also gave a strong positive reaction to a freeze-dried extract of mycelium of clade 7 species *P. fragariae* kindly supplied by Dr David Cooke of the James Hutton Institute (Invergowrie, Dundee, Scotland). Usefully, in cross-reactivity tests 3C4 did not appear to react with any of the *Pythium* species seen in UK field samples. In year three cross-reactivity tests antibody 4B5 showed strong specificity to *Pythium* clades F, G and I (see cross-reactivity testing section & Figure 5 above).

The sensitivity of antibodies 3H7 and 3C4 was initially determined against dilution series of *Phytophthora cryptogea* and of *P. rubi* mycelium extracted in B2 buffer. Both 3H7 and 3C4 cell lines produce IgM antibodies both of which recognise *P. cryptogea* and *P. rubi*. Initially separate batches of LFDs were prepared in which the antigen line (homogenised and

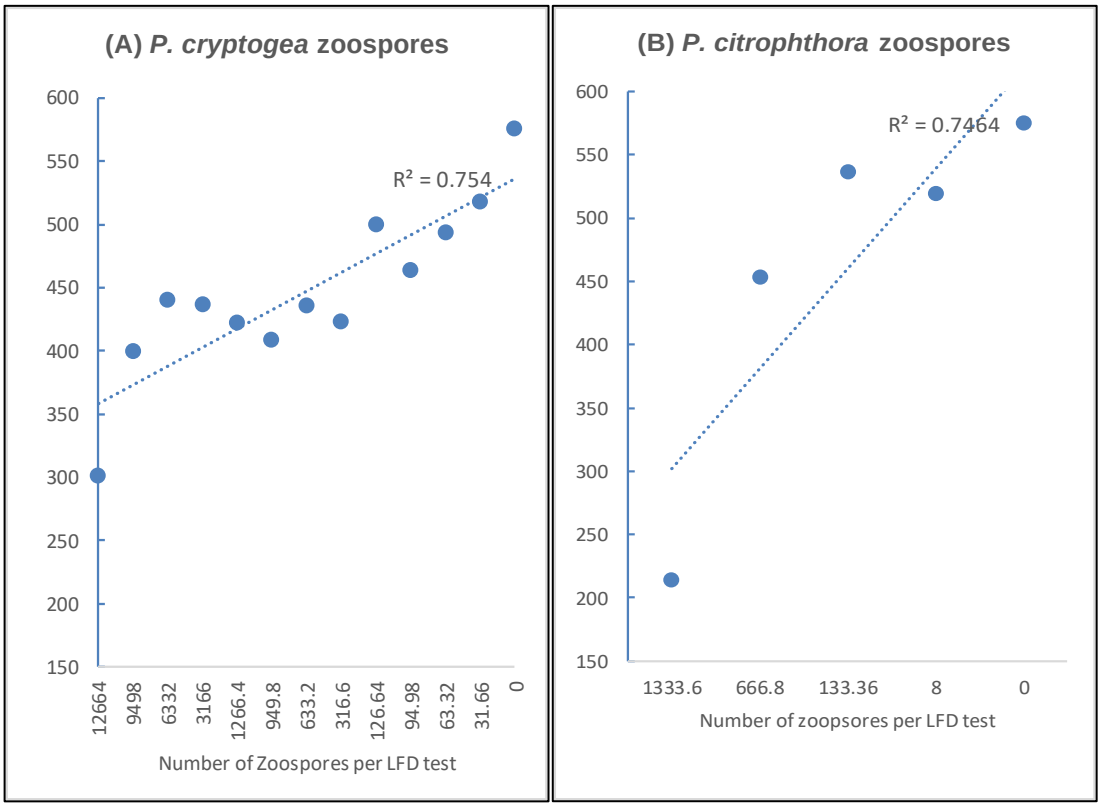
Figure 16. Detection of dilutions of *Pythium ultimum* var. *sporangiferum* mycelium, extracted in B2 buffer by 4B5 *Pythium* clades F/G/I-specific antibody in LFD tests



extracted mycelial antigen material) sprayed onto the lateral flow membrane (as described above) was either *P. rubi* or *P. cryptogea* in origin. These assays demonstrated that both 3H7 and 3C4 could readily detect both *P. cryptogea* and *P. rubi* down to a threshold of 50 ng or less of antigenic material (Figure 15). Antibody 4B5 has so far only been tested in LFD format against mycelial extracts and gives reasonably good sensitivity detecting down to between 500 and 50 ng of antigen per LFD (i.e 500-50 ng/100µl, Figure 16).

Assessing spiked field samples: The next phase of assessing the efficacy of the new LFD tests was to determine their ability to discern target pathogen material in non-sterile field samples. This assessment involved spiking relatively 'dirty' pond water with known concentrations of zoospores of either *Phytophthora* cryptogea or *P. citrophthora*, membrane filtering these samples, re-suspending the contents of half of each filter membrane in a small volume of B2 buffer and testing the suspensions with an LFD. With antibody 3H7 this process gave a detection limit of less than 30 spores per test for *P. cryptogea* and as low as 8 per test for *P. citrophthora* (Figure 17). An alternative variation was tried on the other half of each

Figure 17. Detection of dilutions of zoospores of (A) *Phytophthora cryptogea* and (B) *P. citrophthora* in spiked, non-sterile (and comparatively dirty) pond water, by LFD using 3H7 '*Phytophthora* general' antibody – increasing antibody reaction in the LFD test pad with increasing numbers of zoospores resulted in reductions in the intensity of the LFD test line (In these tests maximum line intensity was between 550 and 600 at zero zoospores = the 'zero control')

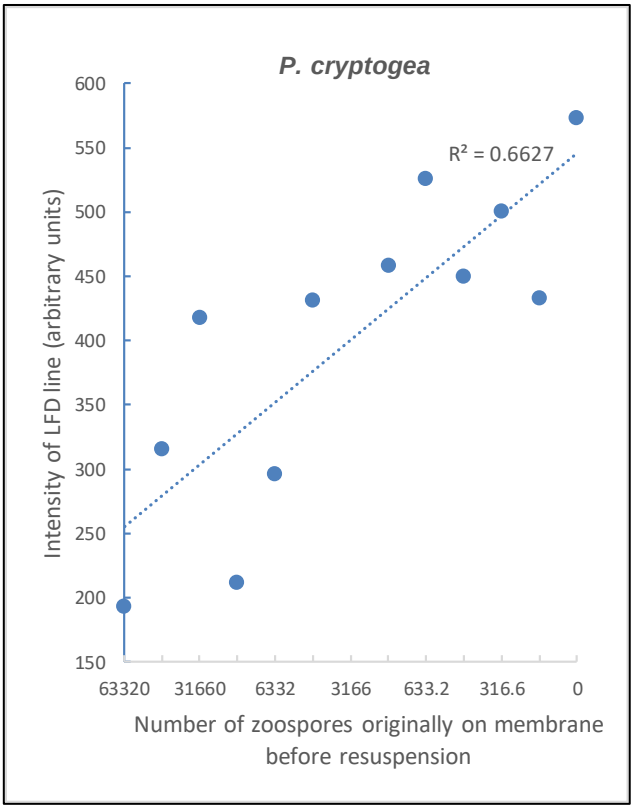


filter which was homogenised in 1.5 ml B2 buffer with glass beads and the centrifuged supernatant extract buffer was LFD-tested. This method gave more variable results (Figure 18) and possibly a higher detection limit of approximately 100-150 spores per test for *P. cryptogea* and such variable results for *P. citrophthora* (R^2=<0.4) they are not presented graphically. Assessments of antibody 3C4 with spiked field samples have been more limited, being restricted to the addition of zoospores of either *P. cryptogea* or *P. rubi* to 1 litre aliquots of selected field samples and comparing the LFD results from these with untreated aliquots

of the same samples. In these tests, 3C4 has consistently detected added spores down to concentrations of 10 spores per test. Antibody 4B5 has not yet been tested with zoospores of *Pythium ultimum*, although, as will been seen below, it is giving promising results in field tests in general.

Field tests of LFDs in comparison with conventional diagnostic methods:

Figure 18. Detection by 3H7 '*Phytophthora* general' antibody of dilutions of *Phytophthora cryptogea* zoospores recovered from spiked, non-sterile pond water by membrane filtration followed by Fast-Prep homogenisation – LFD tests being carried out on the supernatant post centrifugation. (In this test the maximum line intensity was 573 at zero zoospores = the 'zero control')



A total of 647 samples were collected from a wide range of horticultural nurseries and production facilities and the results of tests from these have been grouped into five categories: protected edibles, protected ornamentals, hardy nursery-stock and trees (HNS), field salads and vegetables and soft fruit (Table 5a-e). Of the three antibodies deployed in LFD format, 3H7 ('*Phytophthora* general') was the first to be developed and was applied to all samples. The second antibody to be developed, 3C4 (*Phytophthora* clades 1/7/8) was applied to 307 of the samples, whereas the most recently developed, 4B5 (*Pythium* clades F/G/I) has so far been applied to 67 samples. A total of 332 samples tested positive for *Phytophthora* sp. with antibody 3H7 and all except six of these have been confirmed as containing *Phytophthora* sp. by conventional isolation and plating techniques and observing morphological features,

especially sporangium formation. All six 3H7-positive samples where *Phytophthora* could not be confirmed by culturing techniques were water samples from sources that had been chlorinated with sodium hypochlorite and contained no detectable viable oomycete CFU. Of the 307 samples tested with antibody 3C4, 177 proved positive for *Phytophthora* clade 1/7/8 and the majority (132) of these positive tests were also confirmed by conventional tests supported by selective use of ITS sequencing. The remaining 45 positive tests could not be confirmed by conventional methods within reasonable time limits, although the six chlorinated samples mentioned above also tested positive with 3C4, reducing the number of unverified tests for 3C4 down to 39. *Pythium* tests using antibody 4B5 have been applied to the 67 most recently-collected samples and of these, 33 have given positive reactions and all have been confirmed to contain *Pythium* sp. by conventional plating methods, although only 20 of these have so far been confirmed as clade F, G or I.

Conventional plating methods have also focussed on the samples that did not test positive in LFD tests in order to verify these 'negative' results. Importantly (perhaps remarkably), no *Phytophthora* isolates have so far been obtained from the 315 samples that tested negative with 3H7. With the *Pythium* tests using 4B5, 18 of the 34 samples that tested 'negative' have been found to contain *Pythium* sp., although none of these have been confirmed as *Pythium* clades F, G or I yet.

The samples data has been presented grouped by 'sector' and sample type (Table 5a-e) for ease of handling, and to demonstrate the distribution of sampling both across sectors and broad sample category. Depending on how bait tests are considered, either 31% of samples were water tests, 22% plant tissues, 10% growing media, 9% swab tests and 28% mycelium, or if bait tests are considered plant tissue samples (which physically they are), then the percentages of water and plant tissue tests change to 15% and 38% respectively . Some caution is necessary in interpreting the data as presented because the pattern of sampling was not consistent, this study being primarily aimed at assessing the robustness of LFD tests developed and not intended or designed to address epidemiological patterns. Nevertheless, there are some patterns of interest. The overall proportions of all samples from the field salads and vegetables and the protected edibles categories testing positive for *Phytophthora* with 3H7 were lower, at 20% and 32% respectively, than the other categories of soft fruit (56%), HNS (60%) and protected ornamentals (61%). This result may in part be influenced by the fact that a large proportion of samples from both of these categories were testing complete 'unknowns' that turned out to be other pathogens such as *Fusarium* spp. (e.g. *Fusarium*-infected asparagus, which in the early stages can resemble *Phytophthora* rot) – nevertheless, very useful 'negative' tests. On the other hand, samples collected from HNS, protected ornamentals and soft fruit nurseries have associated with either known, or strongly

suspected oomycete (*Phytophthora* or *Pythium*) disease problems. With the water tests there were some biases, and a large proportion of samples collected on HNS and protected ornamentals nurseries were collected from sources such as runoff ditches, gutters and outdoor reservoirs with a known potentially high risk of contamination with pathogenic *Phytophthora* species. This selectivity is reflected in both the relatively high proportion of samples testing positive for *Phytophthora* with 3H7 (56% in protected ornamentals and 79% in HNS) and the very high proportion of these giving positive results when tested for pathogenic clades 1/7/8 with 3C4 (78% in protected ornamentals and 83% in HNS). In contrast, the majority of water tests carried out on soft fruit nurseries were on river water and whilst the proportion of these containing *Phytophthora* propagules was still quite high at 50%, a much lower proportion (9%) of these tested positive for *Phytophthora* clades 1/7/8. These tests were confirmed by high incidences of clade 6 species *Phytophthora gonapodyides, P. chlamydospora* and occasional *P. lacustris* (all species known to be common in river and pond water (Nowak *et al*., 2015; Scibetta *et al*., 2011; Reeser *et al*., 2011) and not normally considered a threat to horticultural crops although occasionally reported as being associated with declines of certain tree species under appropriate conditions (Akilli *et al*., 2013; Kanoun-Boulé *et al*., 2016; Milenković *et al*., 2012; Nechwatal *et al*., 2012)) seen in conventional agar plates and confirmed by ITS sequencing, plus small numbers of clade 1 *P. cactorum* isolated in all 3C4 positive samples. The lower incidence of *Phytophthora* positive tests in water samples from protected edibles nurseries reflects the fact that these were mostly samples taken from systems without any obvious disease symptoms – the single positive 3C4 test was confirmed by morphology and ITS as *Phytophthora cryptogea*, whilst this and the other two 3H7 *Phytophthora* positive samples all contained *P. gonapodyides*. Another general observation with water tests was that generally membrane filtration gave more *Phytophthora* positive tests than using baits, except on soft fruit nurseries. This result that may be linked to the fact that strawberry leaf pieces were a component of the 'generic' baits used to test most samples and these may have resulted in higher bait 'catches' on soft fruit nurseries and a higher proportion of 3C4 positive tests with baits compared to membrane filters in these samples.

Table 5: Total numbers of LFD tests carried out on field samples, showing numbers of tests carried out on different sample types and the numbers of positive tests found with each LFD test type, and categorised by horticultural sector: a) Protected Edibles; b) Protected Ornamentals; c) Hardy Nursery-stock (HNS); d) Field Salads and Vegetables; e) Soft Fruit.

(*In each table, figures followed by the same superscript letter were for tests that were positive with 3H7 alone or with both 3H7 and 3C4 – no tests were positive for 3C4 without also giving a positive with 3H7*).

5a) Protected Edibles

Sample/Test type		**3H7** (*Phytophthora* 'general')		**3C4** (*Phytophthora* clades 1/7/8)		**4B5** (*Pythium* clades F/G/I)	
		No. Tests	No. +ve Tests	No Tests	No. +ve Tests	No Tests	No. +ve Tests
Water tests	**Membrane**	14	3[a]	10	1[a]	5	3
	Bait	10	0	10	0	4	1
Plant tissues		18	13[b]	18	11[b]	1	0
Media samples		1	0	1	0	0	0
Swab tests		10	0	10	0	4	2
Mycelium/CFU		8	4[c]	8	4[c]	2	1

5b) Protected ornamentals

Sample/Test type		**3H7** (*Phytophthora* 'general')		**3C4** (*Phytophthora* clades 1/7/8)		**4B5** (*Pythium* clades F/G/I)	
		No. Tests	No. +ve Tests	No Tests	No. +ve Tests	No Tests	No. +ve Tests
Water tests	**Membrane**	32	18[a]	24	14[a]	2	0
	Bait	37	16[b]	17	13[b]	3	2
Plant tissues		33	25[c]	9	9[c]	5	3
Media samples		34	23[d]	9	9[d]	0	0
Swab tests		18	3[e]	6	2[e]	6	4
Mycelium/CFU		66	43[f]	18	11[f]	11	5

5c) Hardy Nursery-stock (HNS)

Sample/Test type		3H7 (*Phytophthora* 'general')		3C4 (*Phytophthora* clades 1/7/8)		4B5 (*Pythium* clades F/G/I)	
		No. Tests	No. +ve Tests	No Tests	No. +ve Tests	No Tests	No. +ve Tests
Water tests	**Membrane**	29	23[a]	28	19[a]	3	3
	Bait	44	24[b]	23	16[b]	3	3
Plant tissues		18	14[c]	5	5[c]	2	2
Media samples		7	6[d]	7	5[d]	0	0
Swab tests		22	5[e]	4	1[e]	0	0
Mycelium/CFU		74	38[f]	47	26[f]	10	4

5d) Field Salads & Vegetables

Sample/Test type		3H7 (*Phytophthora* 'general')		3C4 (*Phytophthora* clades 1/7/8)		4B5 (*Pythium* clades F/G/I)	
		No. Tests	No. +ve Tests	No Tests	No. +ve Tests	No Tests	No. +ve Tests
Water tests	**Membrane**	0	0	0	0	0	0
	Bait	0	0	0	0	0	0
Plant tissues		32	4[a]	6	2[a]	6	0
Media samples		12	4	0	0	0	0
Swab tests		0	0	0	0	0	0
Mycelium/CFU		10	3	0	0	0	0

5e) Soft Fruit

Sample/Test type		3H7 (*Phytophthora* 'general')		3C4 (*Phytophthora* clades 1/7/8)		4B5 (*Pythium* clades F/G/I)	
		No. Tests	No. +ve Tests	No Tests	No. +ve Tests	No Tests	No. +ve Tests
Water tests	**Membrane**	22	11[a]	2	1[a]	0	0
	Bait	12	8[b]	2	2[b]	0	0
Plant tissues		42	19[c]	15	12[c]	0	0
Media samples		10	8[d]	10	5[d]	0	0
Swab tests		10	2[e]	10	2[e]	0	0
Mycelium/CFU		22	15[f]	8	7[f]	0	0

Discussion

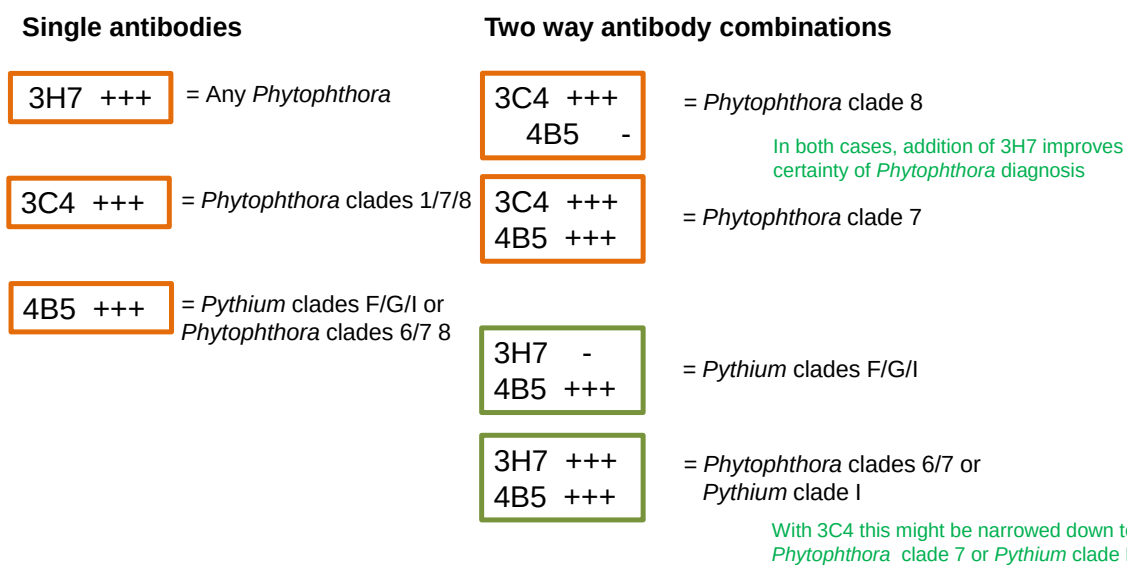
In the final year of this project the last 21 fusions were completed, leading to the production of 18 antibodies suitable for cross-reactivity testing and bringing the total number of fusions in the project to 51 and the number of testable antibodies to 43. By necessity, the cross-reactivity testing of antibodies raised in this project has been far more stringent than is often the reported norm. This level of stringency – testing new antibodies against a panel of 45 'target' and 'non-target' oomycete species plus key non-oomycete species plus further tests for selected promising antibodies – was necessary as a large majority of the situations where field tests using oomycete LFDs would be deployed are likely to contain at least one and probably several of the non-target organisms selected for the panel. This fact was established/confirmed in the work within this project to develop a culture collection of representative isolates, where the many non-target organisms isolates isolated from diseased plant, contaminated water and media samples and their frequency were recorded. Often non-target organisms were closely related taxonomically to pathogen species, for example the non-plant pathogenic oomycete species *Pythium lutarium, Phytophthora gonapodyides* and *Saprolegnia ferax* were all frequently seen, and cross-reactions with such related non-targets has previously caused problems with antibody tests by resulting in all-too-frequent false positive tests. Despite such stringent cross-reactivity screening, at least six of the monoclonal antibodies raised in this project have shown some potential, and three of these gave excellent results and were applied to the LFD format for further efficacy assessments and testing on field samples.

The most rigorously tested of these antibodies was 3H7, which had excellent genus specificity for *Phytophthora* with very limited cross-reactivity, most notably with the pathogen species *Pythium ultimum*, but therefore possibly other *Pythium* clade I species although this does not seem to have been problematic so far in fields tests carried out with LFDs using this antibody. Either used alone, or in combination with 3H7, the second antibody to be tested in LFD format, 3C4, has given very promising results. Initially this antibody gave strong signals for pathogenic species in *Phytophthora* clades 7 and 8 (most notably *Phytophthora rubi* {7}, *P. cinnamomi* {7}, *P. fragariae* {7}, and *P. cryptogea* {8}), with a weak signal for the clade 1 species *P. cactorum*. However, in field tests 3C4 was found to give medium to strong signals for *P. cactorum* and later *P. infestans* (also clade 1) as well. Importantly, this antibody did not detect non-pathogenic members of *Phytophthora* clade 6, which makes it particularly useful for tests of irrigation water from open reservoirs or rivers or of samples irrigated with such water where normally non-pathogenic species such as *Phytophthora gonapodyides* and *P. chlamydospora* are commonly encountered in quantity (Scibetta *et al*., 2011, Reeser *et al*., 2011). The third antibody to be adapted to the LFD format, 4B5, which detects *Pythium*

clades F, G, and I, is in the early stages of field testing. Whilst detecting mainly soft rot and seedling blight pathogens such as *Pythium ultimum*, 4B5 is also potentially useful in improving the resolution of tests with 3H7 and 3C4.

Whereas 3H7 can be effectively used alone to give diagnoses not dissimilar to those currently possible with the Adgen and Pocket Diagnostics *Phytophthora* LFD test kits, when the LFDs developed in this project were run concurrently on samples, more refined diagnoses were possible by a process of matching and elimination using the different antibodies' ranges of specificity. For example, if using 3C4 alone a positive test would indicate a high likelihood of *Phytophthora* species from clades 1, 7 or 8, whereas when used in conjunction with 4B5, a positive 3C4 test and a negative 4B5 would indicate *Phytophthora* clade 8, whilst a 3C4 positive and a 4B5 positive would indicate clade 7 and if these tests were accompanied by a positive result with 3H7 this would greatly increase the confidence in the overall *Phytophthora* diagnosis (see Figure 19). Some of the other promising antibodies raised in this project but not taken to LFD may be of use in these kinds of combinations, for example 6A1 which in combination with 4B5 and 3C4 could give better resolution of *Pythium* species especially *P. dissotocum* and its allies. In field tests of the LFD tests it was sometimes possible to assess the possibilities of such combined use. For example, similar symptoms of collar/crown rot in anemones occurred on several nurseries and were investigated at two sites using 3H7, 3C4 and 4B5 in combination. On one nursery, the results were consistently 3H7 positive, 3C4 positive and 4B5 negative indicating a *Phytophthora* clade 8 infection and the pathogen was later confirmed by plating and ITS sequencing to be the clade 8 pathogen *Phytophthora cryptogea*. On the second nursery a more complex situation arose, in this case different symptomatic samples gave differing responses in tests; one group of six plants gave 4B5 positive tests only, whilst a group of the same variety on a separate bed 4 out of 6 tests gave 3H7 and 3C4 positives with 4B5 negative but the remaining two plant tests gave 3H7, 3C4 and 4B5 positive. In this case, ITS sequencing has not been carried out, but a *Phytophthora* species morphologically consistent with *P. cryptogea* was isolated from the second group but not the first whilst *Pythium* HS and *Pythium irregulare* have been isolated from plants in the first group. On this last nursery *Pythium* sp. had been identified by a clinic as the cause of the crown rot, although clearly the cause of the problem on the wider nursery was more complex.

Figure 19. Specificities of the three monoclonal antibodies used in LFD format and examples of diagnosis refinements possible when 2 tests are run concurrently.



Further examples of field sample testing quickly revealing interesting patterns were seen on strawberry nurseries and on a large tomato nursery. These examples were selected to demonstrate the possibilities revealed from deployment of rapid diagnostic tests in a field situation were questions thrown up by initial tests can be addressed and possibly answered. On three strawberry nurseries that kindly allowed sampling for tests, water for irrigation was abstracted from rivers and was tested for possible pathogens with 3H7 and 3C4. In all three cases river water frequently tested positive with 3H7, but only on two of these nurseries did the water also infrequently test positive with 3C4. In all these samples riparian clade 6 *Phytophthora* species were isolated and identified, whilst the samples testing positive appeared (suspected but not fully confirmed in all 3C4 positive samples) to also contain CFU of clade 1 potential strawberry pathogen *P. cactorum* – demonstrating that all 3H7 positives might be *Phytophthora* but that whilst a strong warning, not all '*Phytophthora* positives' constitute a direct disease threat. On the tomato nursery an entirely different question needed to be addressed. An unusual, slowly progressing but apparently non-lethal stem rot was present on some plants and was causing some yield decline in affected plants but did not appear to be spreading. The causal organism was identified as *Phytophthora infestans* and infections appeared to have been initiated at or near the time of grafting. The necrosis was slowly progressing up the stems of affected plants, but it was unclear whether the pathogen was present in all symptomatic plants and whether it was sporulating or able to spread within the greenhouse. Several different types of sample were taken: fallen leaf material, scrapes taken from browned and green sections of stem, water draining from blocks and swabs were taken of various horticultural plastic surfaces around the plants and from the stem surfaces.

Only 3H7 was used and this gave strong positive tests for all the tissue scrapes taken from browned stems but negative tests for all other samples including the stem surface swabs. Later, stem surface swabs were carried out on stems that had been maintained at 100% RH in the lab for 24 h and these all gave positive tests and showed visual signs of *Phytophthora* sporulation under the microscope. This assessment showed that at the time of sampling there did not appear to be a risk of disease spread and demonstrated the potential of using the LFDs to give a quick answer to an immediate question. Nevertheless, refinements are needed, not least to answer the question of viability.

Diagnosing the viability of pathogen inoculum in water samples is of key importance in monitoring disease management and determining disease risks. Immunodiagnostic assays do not discern between living and dead target cells and carry the risk of positively detecting dead pathogen inoculum, in some cases even when combined with baiting assays (Wedgwood, 2014). Part of this project attempted to develop probes for spore/inoculum viability. Whilst unable to identify probes capable of delivering an immediate answer on viability, when used with an incubation period of 3-5 h (or overnight) the ZTI procedure was successfully used with the oomycete general polyclonal antibody developed in this study, to differentiate live from dead spores by selectively staining spore germ tubes. Also, an incubation period combined with using killed controls for comparison, allowed the use of CBEL protein expression as detected using the CBEL antibody generated on the project to indicate viability. Of these assays, the ZTI is the closest to further development as a test, although the incubation period and current means of discerning germlings from dead material would necessitate use not in the field but in at least a basic technical facility.

During the collection and preparation of field samples to test the LFDs developed in this study, refinements of sampling and extraction protocols were tried, and whilst no formal comparisons were made, useful anecdotal observations were noted. For example, in water tests two conventional approaches were used; baiting and membrane filtration. Membrane filtration provided the means for immediate on-the-spot testing if completed using a hand pump (Figure 20) and was briefly recommended for early *Phytophthora* immunodiagnostic kits in the 1990s. Extraction of material from membranes by various homogenisation procedures was tried but the best method of preparing a membrane sample for LFD testing was to vigorously shake the membrane in a small volume of extraction buffer. This approach was also found to be an effective way to test growing media by flooding and washing through, collecting and testing the washings. Nevertheless, if time permits their use, baiting techniques should still be considered as they can provide a range of testing options depending on the system being assessed, questions being asked and potential baiting materials available (Werres *et al*., 2014). Swab tests were shown to be effective in

combination with LFDs, although in this case the best procedure for preparing them for LFD appeared to be homogenisation in B2 buffer containing glass beads and an 8 mm stainless steel ball, although vigorous shaking in buffer often sufficed. A number of different methods of extraction from plant tissues, mycelium and directly from growing media were used and the quickest, most straightforward of these was using BagPage® extraction bags in combination with a ball baring-based hand homogeniser (Bioreba AG, Figure 21). Other refinements and optimisations are always possible but tend not to be generic, depending more on the particular questions being asked of the diagnostic procedure, and sampling regime.

Future possibilities and applications for the antibodies developed:

Particular systems where there appears to be potential to further develop the LFDs produced in this study include a possible streamlining of current sampling for *Phytophthora ramorum* which in the UK has successfully used a *Phytophthora*-diagnostic LFD as a preliminary screen (Lane *et al*., 2007). The numbers of initial false-positive screens resulting from this procedure could potentially be greatly reduced by the deployment of 3C4 in initial screens. Other possibilities include deploying the potentially powerful combination of 3H7, 3C4 and 4B5 as rapid diagnostic tools discerning important pathogenic *Phytophthora* clades 7 (P. *fragariae, P. rubi* and *P. cinnamomi*) and 1 (*P. cactorum*) for the soft fruit sector – developing media and water tests and/or rapid probes for checking new planting stocks or even testing moisture swabs from cold-stored runners' packaging before planting to detect and avoid planting inoculum potentially emerging from contaminated batches (Pettitt and Pegg, 1994). The same combination of antibody tests might be assessed further in HNS sector crops, where the diversity of species and a high presence of clade 6 *Phytophthora* species makes the establishment of disease risks and the monitoring of disease management very difficult. Finally, key to any of these field-based studies and to making the LFD tests developed here more widely available would be the stability of the antigen on the line, as well as the antibodies attached to the gold beads on the test kit membrane pad (see Figure 3a & b for explanation of LFD structure). Monoclonal antibodies each exhibit different stability patterns and it is often necessary to add and optimise protein stabilisers to allow storage of batches of LFD strips for up to a year (or possibly even longer if needed) before use. In this study, the majority of LFD strips were used freshly-made, and although antibody 3H7 and 3C4 was demonstrated to be stable for well over one month, for any future programs of work involving 3H7, 3C4 or 4B5, developing the stability of these antibodies and their antigen stripes for longer periods would need to be studied and optimised.

There are three areas where progress is already being made. Firstly, the possibilities of using the Phytophthora specific LFDs to increase the speed of testing samples for commercial consultancy work were first realised when antibody 3H7 proved very reliable for 'colony

picking' (i.e. rapidly testing suspected *Phytophthora* colonies growing on agar isolation plates). A marked improvement over currently commercially-available kits, which tend to often give false positive tests with agar colony picks, this property allows 3H7 and 3C4 LFDs to be used to speed up accurate conventional diagnoses (e.g. in irrigation water tests). In addition the *Phytophthora*-specific LFD tests have been successfully used in combination with swab tests to verify the presence/absence of P. infestans inoculum in a recent outbreak of late blight in UK tomato nurseries (Pettitt *et al*., 2019). Secondly we are considering with APHA, the possibility of deploying experimental 3C4 LFDs with some inspectors to assess the possibility of increasing the specificity of their initial *in situ* screening for *P. ramorum.* And finally, the possibility of using 3H7 and 3C4 in raspberries as an economic selective screen prior to sequencing, to detect and identify *Phytophthora* infections in young canes is currently under consideration with the AHDB and UK soft fruit growers.

Figure 20. Membrane filtration of a water sample in the field using a hand-held vacuum pump and a membrane filter mounted in a bottle-top filter unit.

Figure 21. Bioreba hand homogeniser in use preparing a plant tissue extract in a BagPage® extraction bag. The bag contains a middle mesh layer that acts as a filter; the sample is placed on one side of the mesh and homogenised, then a clear sample is pipetted from the other side of the mesh.

Conclusions

- 17 fusions were completed and 51 cell lines raised against a range of selected *Pythium* and *Phytophthora* species and species mixtures, from which 43 antibodies were successfully produced and tested for cross-reactivity against a panel of 45 target and non-target species.
- Of the 43 monoclonal antibodies cross-reactivity tested, six showed useful levels of specificity. Of these, three were tested in the LFD format: 3H7 which gives very good *Phytophthora* genus specificity; 3C4 which is specific to *Phytophthora* clades 1, 7 and 8; and 4B5 which is specific to *Pythium* clades F, G and I. Of the remaining three antibodies, 6A1 recognises *Pythium* clade B and *Phytophthora* clade 8.
- Over 1000 LFD tests were carried out on 647 samples from the protected ornamentals, protected edibles, HNS, soft fruit and field crop sectors. Tests were backed up by conventional plating and morphology procedures as well as ITS sequencing of selected samples. Results from all three antibodies matched conventional assay results very well.

- In addition to the monoclonal antibodies, two polyclonal antibodies were raised: 1) an oomycete general antibody, raised for use in semi-specific viability assays to test water and growing media samples; and 2) a polyclonal antibody raised against CBEL protein as a potential zoospore viability marker.
- A lab-based viability assay was developed using the oomycete general polyclonal antibody in a zoospore trapping immunoassay (ZTI) that could discern between living and dead spores by selectively staining germ tubes after a germination-inducing incubation period.
- Combinations of 3H7, 3C4 and 4B5 tests were proposed, and have had limited testing, to improve the resolution of diagnoses possible to *Phytophthora* clade 7, *Phytophthora* clade 8 and *Pythium* clade I (plus other possibilities when the other 3 monoclonal antibodies are considered).
- Further development of the LFD tests and combinations assessed in this study is dependent upon the completion of stability optimisation studies for the antibodies in their LFD pads and their relevant antigen stripes. Stable batches of LFD strips could then be used to develop diagnostic applications for the HNS, soft fruit and other horticultural sectors.

Knowledge and Technology Transfer

Nursery visits – these were integral to the latter part of this project (overall, 55 nurseries and production sites visited on project).

Poster –Information poster for Hardy Nursery Stock Conference, Feb 2018 'Development of new detection devices for *Phytophthora* and *Pythium*'.

Workshop – Hands-on workshop on LFD devices at Ornamentals Conference Feb 2018.

Talks –

BPOA Conference Jan 2018 ' Tests for detection of oomycete root and stem rot pathogens'

Ornamentals Conference Feb 2018 ' Development and testing of diagnostic devices for rapid and precise early detection of oomycete root and collar rot pathogens'

TGA Conference September 2018 – Short talk on the recent *Phytophthora* infestans epidemic in UK tomatoes in which use of new LFDs will be described.

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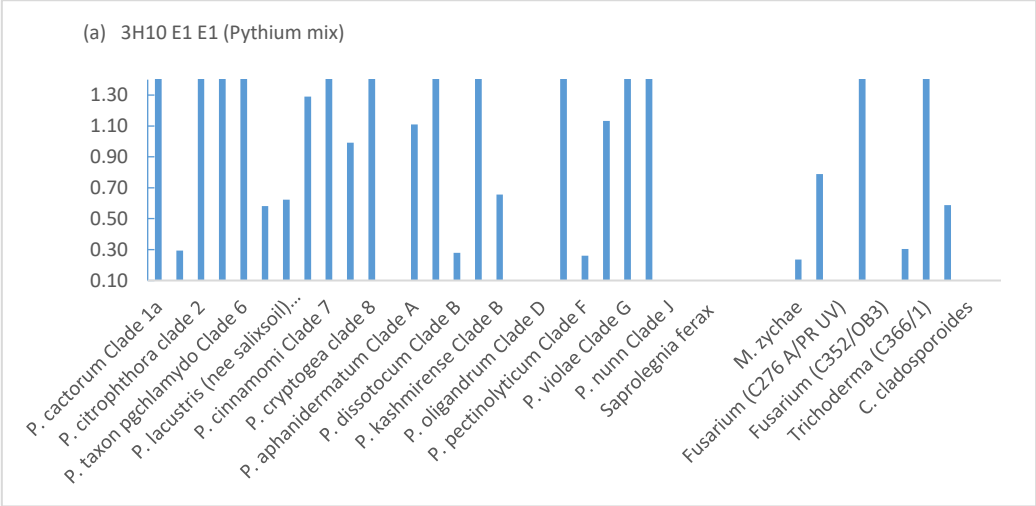
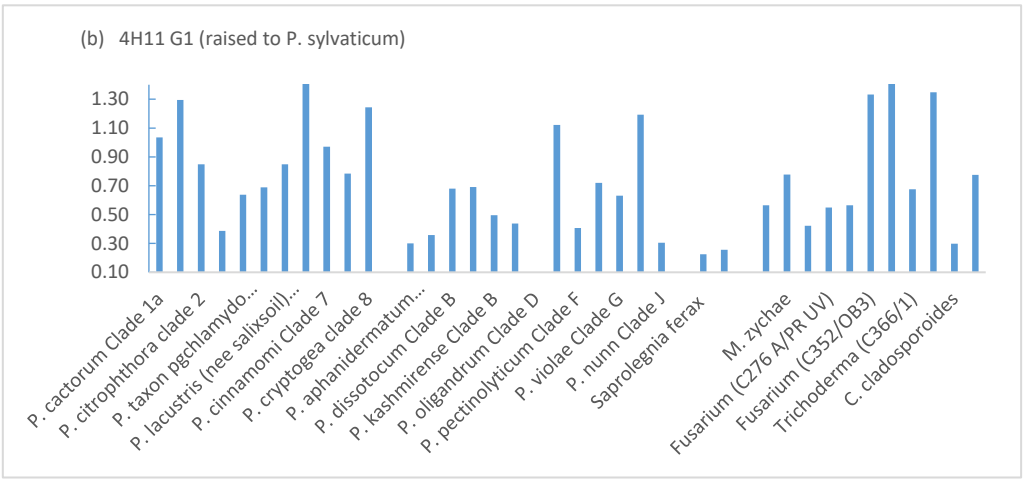
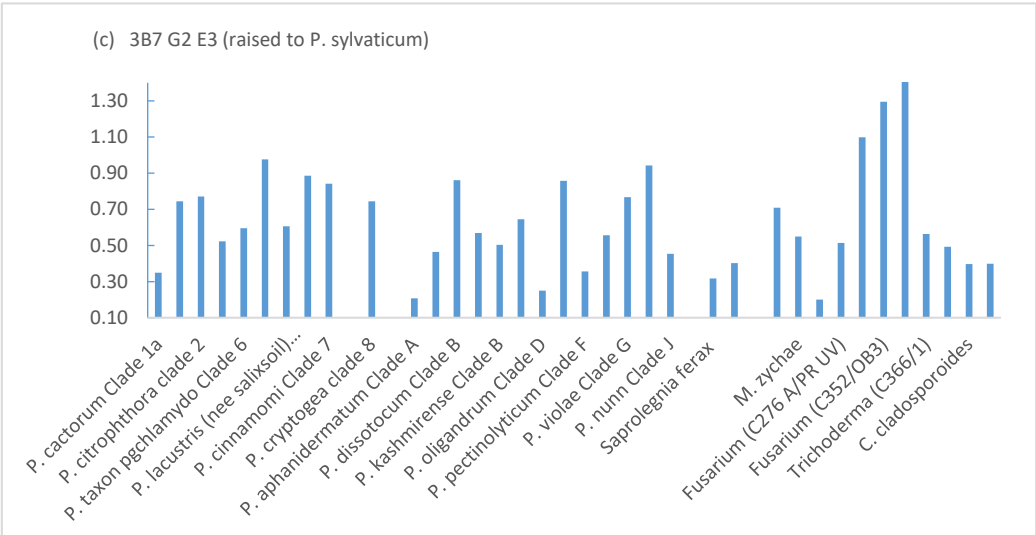
Appendices

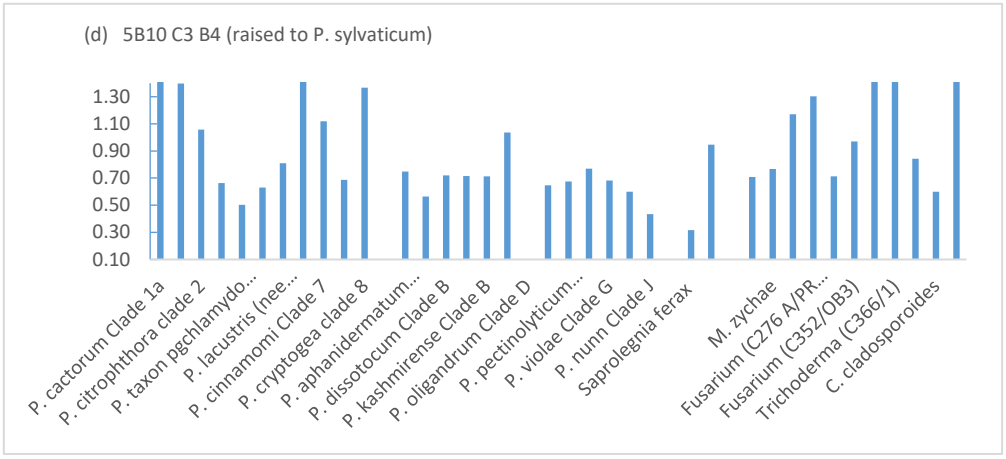
Appendix Figure 1a-j: Cross-reactivity results for antibodies from cell lines (a) 3H10 E1 E1, (b) 4H11 G1, (c) 3B7 G2 E3, (d) 5B10 C3 B4, (e) 1E4 A1 A1, (f) 4C11 B2 A2, (g) 2H2 H4 G4, (h) 6D11 D2 B3, (i) 3F4 G1 G1 and (j) 5C5 B4 D2.

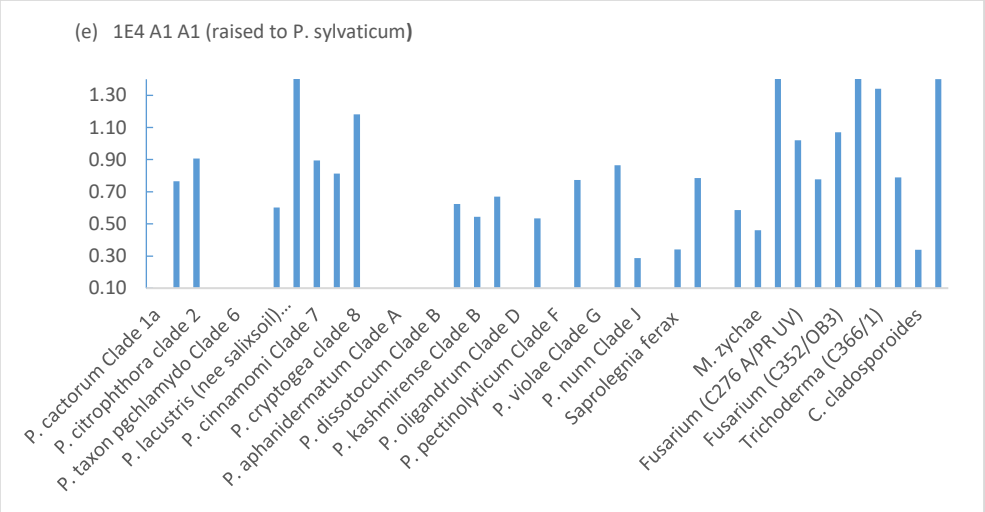


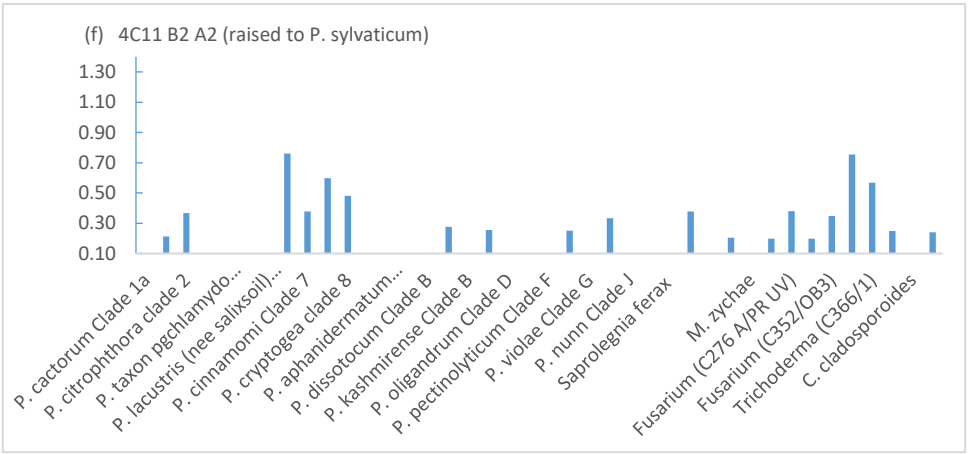




Appendix Figure 1a-j contd.







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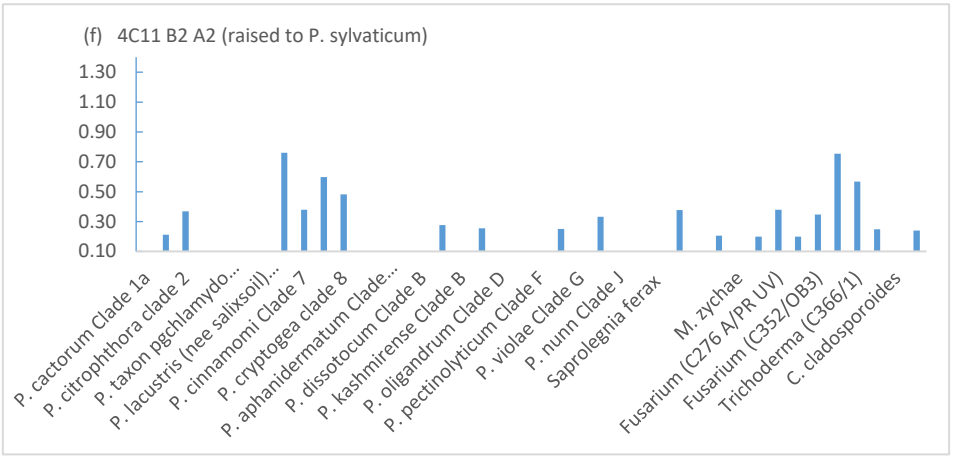

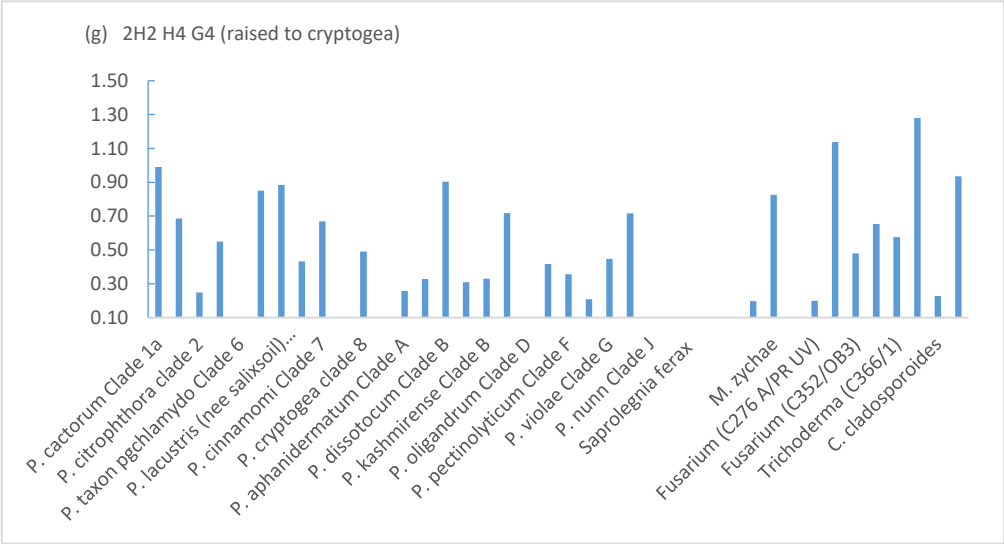

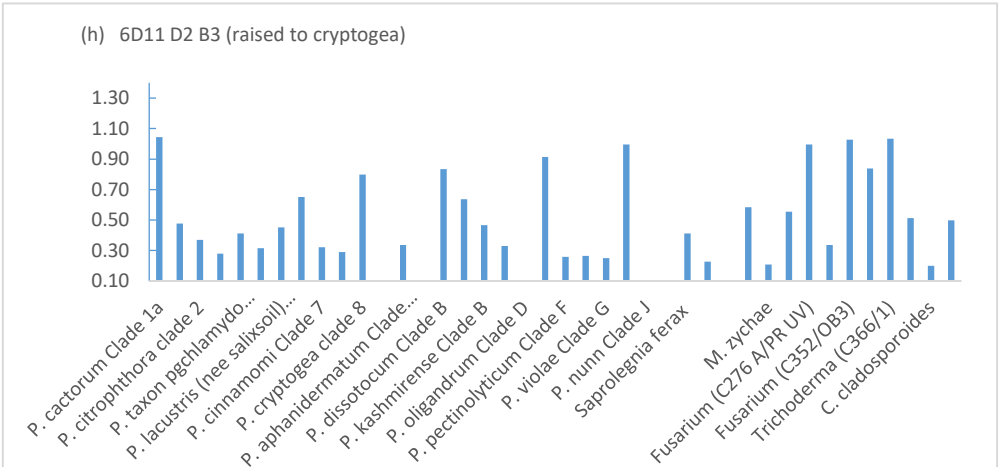

Appendix Figure 1a-j contd.