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
Project leader:	Tim Pettitt and Alison Wakeham Institute of Science and Environment University of Worcester, Henwick Grove, Worcester WR2 6AJ Tel: 01905 842643 Fax: 01905 855234 Email: t.pettitt@worc.ac.uk
Report:	Annual report, June 2017
Previous report:	June 2016
Key staff:	Tim Pettitt (Oomycete lead) Gary Keane, Simon John Emma Edwards
Location of project:	University of Worcester
Industry Representative:	Name: Gary Taylor
Date project commenced:	01/06/2015
Expected completion date:	31/06/2018

© Agriculture and Horticulture Development Board 2017. All rights reserved

DISCLAIMER

AHDB, operating through its AHDB Horticulture division seeks to ensure that the information contained within this document is accurate at the time of printing. No warranty is given in respect thereof and, to the maximum extent permitted by law the Agriculture and Horticulture Development Board accepts no liability for loss, damage or injury howsoever caused (including that caused by negligence) or suffered directly or indirectly in relation to information and opinions contained in or omitted from this document.

Copyright, Agriculture and Horticulture Development Board 2017. All rights reserved.

No part of this publication may be reproduced in any material form (including by photocopy or storage in any medium by electronic means) or any copy or adaptation stored, published or distributed (by physical, electronic or other means) without the prior permission in writing of the Agriculture and Horticulture Development Board, other than by reproduction in an unmodified form for the sole purpose of use as an information resource when the Agriculture and Horticulture Development Board is clearly acknowledged as the source, or in accordance with the provisions of the Copyright, Designs and Patents Act 1988. All rights reserved.

AHDB (logo) is a registered trademark of the Agriculture and Horticulture Development Board. All other trademarks, logos and brand names contained in this publication are the trademarks of their respective holders. No rights are granted without the prior written permission of the relevant owners.

[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]
[Position]
[Organisation]
Signature .... Date ....

Report authorised by:
[Name]
[Position]
[Organisation]
Signature .... Date ....

© Agriculture and Horticulture Development Board 2017. All rights reserved

CONTENTS

GROWER'S SUMMARY 5

Headline 5

Background 5

Summary 7

Financial Benefits 8

SCIENCE SECTION 10

Introduction 10

Materials and Methods 11

Results 14

Discussion and Conclusions 35

References 39

© Agriculture and Horticulture Development Board 2017. All rights reserved

GROWER SUMMARY

Headline

- A representative cross-sector collection of identified isolates of *Phytophthora* and *Pythium* species from plant roots, collars and crowns as well as compost and water samples has been assembled. More than 30 isolates of commonly seen non-target species have also been identified and collected. These collections are important for two reasons: firstly to make sure antibodies are raised to isolates/species that represent current disease threats to the UK industry and secondly for testing the specificity and efficacy of the antibody tests developed in this project. Although a good collection has been assembled it will continue to be expanded with ongoing nursery visits and incoming clinic samples.
- Progress with antibody development is very promising with antibodies already assessed with potential to discern +/- oomycete, +/- *Phytophthora,* and combinations that can discern *Phytophthora* clade 7 (includes *P. fragariae* and *P. rubi*) and clade 8 (includes *P. cryptogea* and *P. ramorum*). A further two *Phytophthora* antibodies are still to be assessed plus 10 raised to *Pythium* species. Four *Phytophthora* antibodies have been selected for the first series of field tests using the LFD format.

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 seed rot diseases. Often occurring just after planting, as young seedlings emerge. *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 range of crop types. Often associated with above ground plant parts i.e. shoot apex, leaf, stem and fruit they are also responsible for root and crown 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,

© Agriculture and Horticulture Development Board 2017. All rights reserved

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 however are commonly used only 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 at least four species are even mycophagous, some with the capacity to elicit disease resistance mechanisms in plants (Vallance *et al*., 2009) and therefore even 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'.

This AHDB-funded project (CP 136), now in its final year, 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 kits 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 kits, this project has 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. More details about the types of test being developed are provided in the year 1 (2016) Grower Summary for this project.

© Agriculture and Horticulture Development Board 2017. All rights reserved

Summary

***Nursery visits and continued isolate sourcing, identification and collection across each of the sectors*:**

Regular site visits have continued throughout the second year of the project and, together with isolates taken from plant clinic samples sent to Worcester, and isolates kindly supplied by James Townsend at Stockbridge Technology Centre, isolates collected from visits have been assembled into a representative culture collection containing more than 110 cleaned and identified isolates of *Pythium, Phytophthora*, and a wide range of background non-pathogen species.

Although at a slower pace, collections continue and isolates are being identified based on their morphology, with key isolates having their identifications confirmed by PCR and ITS DNA sequencing. The culture collection is important for its use in the extensive cross-reactivity testing needed to make sure that the antibodies raised in the project are 'doing what it says on the tin' and detecting specific pathogens and NOT common background species of oomycetes and fungi.

***Continued antibody and molecular probe development and testing*:**

The second year of this project has seen the major raising, purifying and testing of antibodies for the later development of detection tests. The bulk of the antibodies raised to Phytophthora species have passed through their first level of tests, although there are still some important fusions to complete and cell lines to test.

Cross-reactivity testing so far has revealed that we have a good general 'oomycete-specific' polyclonal antibody. This will be useful for the development of water tests where, in combination with a viability marker, it could form the basis for an effective test for the efficacy of irrigation water treatments. Key to this test will be a suitable viability marker, and for this a series of monoclonal antibodies have been raised to live zoospore preparations as well as a polyclonal antibody to a specific glycoprotein (CBEL) that is involved in the zoospore germination process. These antibodies will be tested against live (infectious) zoospore cysts and cysts killed by different sterilisation treatments (UV, heat, and chlorination - each of which can have quite different effects of the cysts' structures and contents), in the hope that they will be able to clearly discern the 'live' from 'killed' cysts.

In addition to these antibodies, a monoclonal antibody (3H7 H3) has been found to be nearly genus-specific – detecting only *Phytophthora* species plus the pathogenic species *Pythium ultimum*. Several other monoclonal antibodies have shown promising levels of specificity, detecting *Phytophthora cryptogea* only or a combination of *P. cinnamomi, P. rubi*, and *P.*

© Agriculture and Horticulture Development Board 2017. All rights reserved

cryptogea. We are now exploring whether it will be possible to deploy a combination of these antibodies in a test to identify, (a) *Phytophthora* sp., (b) *P. cryptogea* (*Phytophthora* clade 8), and (c) *P. rubi/P. cinnamomi* (*Phytophthora* clade 7). The first experimental lateral flow test strips are due to be ready for laboratory evaluations with field samples in August 2017.

Two more antibodies raised to *Phytophthora* are still to be tested for cross-reactivity and further final batch will be ready by the end of September 2017.

With the Pythium antibodies, the first batch of 10 antibodies are ready for cross-reactivity testing in July 2017, but there are also still fusions being set up with the potential to raise a further 16 or so antibody cell lines for testing later in year 3.

An important part of this project has focussed on developing a multiplexing platform for rapid clinic-based testing of field samples. This would allow the rapid, simultaneous testing of samples for a number of different possible pathogen species in a single sample at the same time. In year 1 of this project, some promising results were obtained following an oligonucleotide (DNA-based) approach. This involved attaching oligonucleotide probes to coloured magnetic beads that allowed capture and quantitation of species-specific amplified pathogen DNA via a MAGPIX Luminex detection system (this system is described in more detail in the year 1 annual report {Wakeham *et al.,* 2016}). However, despite successful amplifications of pathogen DNA and successful coupling of capture oligonucleotides to magnetic beads, it has proved impossible to adapt the oligonucleotide array already developed by other researchers (Tambong *et al*., 2006) for use on a magnetic bead array. Nevertheless, this does not mean that a multiplex system is not attainable within this project and work is currently under way to determine whether it is possible to run multiple antibody tests concurrently using the same MAGPIX testing platform and the effective antibodies raised in this project.

Financial Benefits

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 spread through inadvertent introduction is increased. 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.

© Agriculture and Horticulture Development Board 2017. All rights reserved

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. Targeted application of control measures will help delay the onset of pathogen resistance to fungicides, thus prolong 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.

© Agriculture and Horticulture Development Board 2017. All rights reserved

SCIENCE SECTION

Introduction

Background:

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 worldwide. A significant proportion 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 condition. 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. The use of molecular and immunoassay based techniques for improved pathogen detection have been described in a crop setting. The programme of work below describes the development of disease management tools with an aim to ascribe risk of waterborne oomycete species, to measure *Pythium* and *Phytophthora* presence and, more specifically, identify the key oomycete plant pathogens involved or generic oomycete propagule viability.

The overall aims of this project are reviewed in detail in the year 1 annual report (Wakeham *et al.*, 2016, pp. 8-12). The aims of year 2 of the project were:

1) to continue with nursery visits and processing clinic materials in the assembly of a representative culture collection (pathogenic and non-pathogenic oomycete species and key 'background' species), for cross-reactivity testing of antibodies and prototype test kits.

2) raise, purify and test antibodies as a) viability markers, b) generic oomycete markers, c) genus (*Phytophthora* or *Pythium*) markers and d) clade/species markers. And finally

3) continue the development of a multiplexing clinic-based pathogen detection, diagnosis and quantitation system using an oligonucleotide array-based approach.

© Agriculture and Horticulture Development Board 2017. All rights reserved

Materials and Methods

Isolation, clean-up, identification and collection of representative isolates of Pythium and Phytophthora and relevant 'background' species

Nursery visits and reception of diseased plant materials for the purposes of collecting representative isolates of oomycete pathogens for testing antibody probes as well potential 'background' species for cross-reactivity tests, continued throughout year 2 of the project. 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).

Investigate potential to develop diagnostic probes to molecules associated with oomycete viability

Following successful treatment of irrigation water to remove plant pathogens, killed and non-viable pathogen cellular material often remains in treated water and can give positive immunological tests (Wedgwood, 2014). The aim of this section of the project is to develop a diagnostic test that can identify a molecule present in living pathogen material and discriminate viable from non-viable material and thereby provide a reliable measure of water treatment efficacy.

Two approaches to the objective of developing a 'viability marker' have been followed: a) the direct production of a panel of monoclonal antibodies against freshly encysted zoospores, and b) raising a polyclonal antibody against the CBEL (cellulose-binding elicitor lectin) glycoprotein which is expressed in the early stages of zoospore encystment and germination. The rationale behind these two approaches is explained in detail in the year 1 annual report (Wakeham *et al.*, 2016. pp. 16-19).

The production of the monoclonal cell lines was as described in the year 1 report (pp. 17-18). For the polyclonal antibody, purified recombinant CBEL glycoprotein (29kDa) was produced by BioServ UK. This was a *Phytophthora parasitica* CBEL corresponding to GenBank ID X97205 (nucleotide) and CAA65843 (protein) gene, which were sub-cloned into a bacterial expression vector to provide expression of His-tagged protein via a T7 promoter. Expression in *E. coli* (BBL21(DE3)) was according to the method of Gaulin *et al* (2006). Presence of expressed CBEL protein was determined by NuPAGE and Western blot (anti-His). A single rabbit was immunised five times at Bioserve UK, following a 77 day immunisation schedule, with 300µg protein per 500µl boost, at 4 different sites, between 12/12/16 and 06/02/17. Following final exsanguination, a protein A purification of the polyclonal antibody was carried out before passing to Worcester for testing.

© Agriculture and Horticulture Development Board 2017. All rights reserved

Development of antibody and molecular probes to assist diagnosis of Pythium and Phytophthora in environmental and plant tissue samples

Antibody test format

Oomycete Phylum/Class-specific test: A polyclonal antiserum was raised to a mixed antigen preparation consisting of a combined soluble fraction of isolates of *Phytophthora rubi, Ph. cactorum, Ph. cinnamomi*, *Pythium: oligandrum, P. irregulare, P. dissotocum, P. ultimum* var. *sporangiiferum*, *Saprolegnia ferax and S. parasitica*. The antiserum was prepared according to the methods described by Wakeham *et al*. (1997) and following the immunisation protocol of Wakeham and White (1996). Full details of the procedures, the isolates used and the production of an IgG-purified antiserum are presented in the year 1 annual report (Wakeham *et al*., 2016, p 19).

Phytophthora *and* Pythium *genus-specific tests:* Mice were immunised as described in the year 1 annual report (Wakeham *et al*., 2016, pp. 19-20) with a mixture of fast-prepped mycelial extracts (<30 kDa fraction only) from cultures of *Phytophthora cactorum, P. rubi* and *P. cryptogea*.

A similar preparation of mycelial extracts from cultures *of Pythium aphanidermatum, P. sylvaticum* and *P. ultimum* var. *sporangiiferum* has been used to immunise mice in June 2017, with fusions anticipated in September.

Phytophthora *and* Pythium *'clade-specific' monoclonal antibodies:* In an attempt to raise antibodies with some degree of clade specificity mice were immunised with single-species preparations of *Phytophthora cactorum* (Clade 1), *P. rubi* (Clade 7) and *P. cryptogea* (Clade 8), following the same overall procedure as for the *Phytophthora* genus-specific monoclonal antibodies above.

Similarly, mice have been immunised with single-species preparations of *Pythium aphanidermatum* (Clade A), *Pythium sylvaticum* (Clade F) and *Pythium ultimum* var *sporangiiferum*.

Cross-reactivity tests: Cross-reactivity tests were carried out against mycelial extracts prepared from cultures of a representative range of target and non-target oomycete, and non-oomycete species. This range is not exhaustive and further isolates/species are still being added to tests alongside positive controls. Mycelial extracts were prepared for tests by growing mycelium in clarified V8 broth (Johnston & Booth, 1983), mycelium was homogenised in 0.01M phosphate-buffered saline (PBS, pH 7.4) using a FastPrep®-24, with glass beads at a speed of 6.0 for 40 seconds, and the protein concentration was determined by NanoDrop™

© Agriculture and Horticulture Development Board 2017. All rights reserved

following centrifugation, and adjusted to 0.04 mg ml^{-1} in PBS. Antibodies were used at appropriate dilutions determined by titration against 0.04 mg ml^{-1} of their relevant antigen to give absorbance values in the range 0.5-1.0. Assessments of reactivity were by ELISA using TMB (3,3′,5,5′-Tetramethylbenzidine, substrate for ELISA, read at 450nm after addition of 1.5M Sulphuric Acid).

Oligonucleotide test format

The background to developing a versatile multiplexing platform capable of both qualitative and quantitative detection of target oomycete species, theoretically in a single reaction volume, using oligonucleotides with sequence heterogeneity as probes for individual species in a MAGPIX array is explored in the year 1 annual report (Wakeham et al., 2016, pp. 22-24)

DNA was extracted from mycelium of the selected target species (*Pythium ultimum var sporangiiferum, Pythium sylvaticum, Pythium irregulare, Pythium nunn* and *Phytophthora cactorum* Table 1) using the Fast DNA spin kit (MP Biomedical) according to the manufacturer's instructions. Extracted DNA was diluted to 1/10 in molecular grade water. PCR amplification of the ITS region was achieved using protocols and primers developed by Cooke et al and Tambong *et al.* (2006), using Biomix Red based amplification. Both methods were used in order to determine the most robust amplification for use in the bead array. Amplification from the PCR was confirmed by performing gel electrophoresis using 1% agarose gel and GelRed (Biotium). Amplified DNA was purified using QIAquick PCR purification kit (Qiagen). DNA was then used in the Magpix assay system(Luminex). The procedures followed are fully described in the year 1 annual report (Wakeham *et al*., 2016, pp.23-24.).

Table 1. Oligonucleotide sequences of the capture probes used to detect species-specific DNA, and of the biotinylated probes used to confirm bead coupling in the MAGPIX array.

Species	Probe type	Sequence (5'-3')
Pythium ultimum var. *sporangiiferum*	Capture probe	(AmC12)ACCGAAGTCGCCAAAA
Pythium sylvaticum	Capture probe	(AmC12)TTGGTATATTTGTTTATGCACA
Pythium irregulare	Capture probe	(AmC12)TGTTGCATGCGCGGCT
Phytophthora cactorum	Capture probe	(AmC12)ATAGCTCAGTTGCTTG
Pythium nunn	Capture probe	(AmC12)TTGTGCCGTTGCTGTTGTC
Pythium ultimum var. *sporangiiferum*	Biotinylated probe	(Btn)TTTTGGCGACTTCGGT

© Agriculture and Horticulture Development Board 2017. All rights reserved

Pythium sylvaticum	Biotinylated probe	(Btn)TGTGCATAAACAAATATACCAA
Pythium irregulare	Biotinylated probe	(Btn)AGCCGCGCATGCAACA
Phytophthora cactorum	Biotinylated probe	(Btn)CAAGCAACTGAGCTAT
Pythium nunn	Biotinylated probe	(Btn)GACAACAGCAACGGCACAA

Results

Isolation, clean-up, identification and collection of representative isolates of Pythium and Phytophthora and relevant 'background' species

With continued nursery visits and sampling through year 2 of the project a representative collection of over 110 described isolates has been assembled (Table 2). Although more cultures are still be collected and processed, the numbers of fresh isolates being added to the collection has now slowed and isolates are being selected and used in sensitivity and cross-reactivity testing of the antibodies being raised in the main part of this study. So far 39% of the accessions in the collection originate from water samples, 45% from plant tissue samples, 4% from swabs, 2% from compost and media (this is slightly less than the true representation, as some collections from 'water' actually consist of media runoff/wash-through samples) and 10% un-provenanced sources.

Table 2: Isolates of *Pythium, Phytophthora* and other oomycete species as well as representative 'background' species of filamentous fungi collected, cleaned and characterised in years 1 and 2. [*in the columns for verification by ITS and identification by morphology + = a positive identification - = no identification by this method and (+) = tentative or incomplete diagnosis by this method*]

Culture identifier	Taxonomic identification	Source	Verified by ITS sequences	Confirmed by morph-ology
	Oomycete species			
UW014	*Pythium aphanidermatum*	Tomato roots	+	+
SH CC332	*Pythium aphanidermatum*	Amaranthus	+	+
UW107	*Pythium coloratum*	Lettuce irrigation buffer tank	+	(+)

© Agriculture and Horticulture Development Board 2017. All rights reserved

Culture identifier	Taxonomic identification	Source	Verified by ITS sequences	Confirmed by morph-ology
UW120 & 123	*Pythium coloratum*	Strawberry runoff	+	(+)
UW023	*Pythium dissotocum*	*Brunnera* roots	+	-
UW053	*Pythium dissotocum*	Strawberry drainage water	+	(+)
UW055	*Pythium dissotocum*	Mixed ornamentals drainage water	+	-
UW058	*Pythium dissotocum*	Herbaceous ornamentals reservoir water	+	-
UW060	*Pythium dissotocum*	Irrigation water strawberry	+	-
UW82	*Pythium dissotocum*	Strawberry roots	+	(+)
UW100	*Pythium dissotocum*	Lettuce roots	+	+
UW121 &122	*Pythium dissotocum*	Strawberry runoff	+	(+)
C341	*Pythium* HS group	Strawberry roots	-	(+)
UW075	*Pythium intermedium*	Viola seedlings	+	(+)
UW086	*Pythium intermedium*	*Exacum* collar	+	-
UW056	*Pythium intermedium/ mamillatum*	Sweet William roots	+	-
UW068	*Pythium intermedium/ mamillatum*	Echinacea	+	-
UW013	*Pythium irregulare*	Unknown (SH CC298)	+	(+)
UW020	*Pythium irregulare*	MOPS/ADAS - unknown	+	+

© Agriculture and Horticulture Development Board 2017. All rights reserved

Culture identifier	Taxonomic identification	Source	Verified by ITS sequences	Confirmed by morph-ology
UW048	*Pythium irregulare*	CBS	+	-
SH CC345	*Pythium irregulare*	*Penstemon*	+	(+)
UW021	*Pythium kasmirense*	Choisya roots	(+)	(+)
UW017	*Pythium lutarium*	*Buxus* roots	+	(+)
UW018	*Pythium lutarium*	Spinach seedlings	+	(+)
UW062	*Pythium lutarium*	Herbaceous ornamentals irrigation water	+	(+)
UW114	*Pythium lutarium*	Roots and collar Anemone var. 'Wild Swan'	+	(+)
UW119	*Pythium lutarium*	Strawberry runoff	+	(+)
SH CC333	*Pythium myriotylum*	Hemp	+	+
UW049	*Pythium nunn*	CBS	+	-
UW044	*Pythium oligandrum*	CBS	+	+
UW047	*Pythium oligandrum*	CBS (*Pythium periplocum*)	(+)	-
UW079	*Pythium pectinolyticum*	Irrigation water strawberry	+	(+)
UW078	*Pythium pectinolyticum/ levoense*	Irrigation water (river)	(+)	(+)
UW051	*Pythium rostratum*	CBS	(+)	-
UW046	*Pythium sylvaticum*	CBS	+	-
UW084	*Pythium sylvaticum*	Dill roots	+	+
UW105	*Pythium sylvaticum*	Strawberry root rot (var Cambridge Favourite)	+	+

© Agriculture and Horticulture Development Board 2017. All rights reserved

Culture identifier	Taxonomic identification	Source	Verified by ITS sequences	Confirmed by morph-ology
UW050	*Pythium ultimum* var. *sporangiiferum*	CBS	+	(+)
C327	*Pythium ultimum*	Lettuce crown/root rot	-	(+)
C361	(*Pythium ultimum*)	Asparagus shoot rot	-	(+)
UW092	*Pythium ultimum* var. *ultimum*	Hemp roots (ex ADAS)	+	(+)
SH CC315	*Pythium ultimum* var. *ultimum*	Statice	+	(+)
UW061	*Pythium utonaiense*	Drainage water strawberry	+	(+)
C345	(*Pythium violae*)	Carrot	-	(+)
UW054	*Pythium* sp.	Drainage water strawberry	(+)	(+)
UW059	*Pythium* sp.	River water	(+)	(+)
UW072	*Pythium* sp.	Reservoir water	(+)	(+)
UW076	*Pythium* sp.	Irrigation line strawberry	(+)	(+)
SH CC315	*Pythium* sp.	Statice wilt	-	(+)
SH CC333	*Pythium* sp.	Hemp	-	(+)
C370	*Pythium* sp.	Swab test protected ornamentals benches	-	(+)
C388	*Pythium* sp.	Swab test herb production channels	-	(+)
C369	*Pythium* sp.	Isolate from SH – unknown provenance	-	(+)
C367	*Pythium* sp	Heuchera crown	-	(+)
UW108	*Pythium* sp.	Asparagus collar	(+)	(+)
UW131	*Pythium* sp.	Ornamentals reservoir (open)	(+)	(+)

© Agriculture and Horticulture Development Board 2017. All rights reserved

Culture identifier	Taxonomic identification	Source	Verified by ITS sequences	Confirmed by morph-ology
C291/1	*Phytophthora cactorum*	Strawberry crown (var Elsanta)	-	+
UW043	*Phytophthora cactorum*	CBS	+	+
UW103	*Phytophthora chlamydospora*	River water	+	(+)
UW015	*Phytophthora cinnamomi*	*Chamaecyparis* roots	+	+
UW025	*Phytophthora citrophthora*	Filter well bait	+	(+)
C290/2 Aii	(*Phytophthora citrophthora*)	*Buxus* roots	-	(+)
UW016	*Phytophthora cryptogea*	HNS roots	+	+
SH CC310	(*Phytophthora cryptogea*)	Geranium	-	(+)
C381	*Phytophthora cryptogea*	Tomato	+	+
SH CC350	*Phytophthora cryptogea*	Clematis	+	+
SH CC351	*Phytophthora cryptogea*	Clematis	+	+
UW073	*Phytophthora gonapodyides*	Bait – herbaceous ornamentals drain water	+	(+)
UW034	(*Phytophthora lacustris/ gonapodyides*)	Field header irrigation pipe – field veg.	(+)	(+)
UW098	*Phytophthora lacustris*	Filtered river water (Lugg – strawberries)	+	(+)
UW071	*Phytophthora missisippiae*	River water	+	(+)
SH CC349	*Phytophthora nicotianae*	Euphorbia	+	+
UW012	*Phytophthora palmivora*	Ivy – SH CC297	+	(+)

© Agriculture and Horticulture Development Board 2017. All rights reserved

Culture identifier	Taxonomic identification	Source	Verified by ITS sequences	Confirmed by morph-ology
UW042	*Phytophthora rubi*	CBS	+	(+)
UW064	*Phytophthora syringae*	River water	+	(+)
UW028	*Phytophthora* sp.	River water	(+)	-
UW066	*Phytophthora* sp.	Strawberry drain water	(+)	(+)
C344/1	*Phytophthora* sp.	*Taxus* roots & collar	-	(+)
C344/2	*Phytophthora* sp.	Poinsettia roots	-	(+)
SH CC312	*Phytophthora* sp.	Nemesia	-	(+)
UW045	(*Saprolegnia aenigmatica*)	CBS *Saprolegnia parasitica*	(+)	-
UW057	*Saprolegnia australis*	Reservoir water	+	(+)
UW029	*Saprolegnia ferax*	Greenhouse roof swab	+	(+)
UW035	*Saprolegnia ferax*	River water	+	(+)
UW067	*Saprolegnia ferax*	Drain water strawberry	+	(+)
UW080	*Saprolegnia ferax*	Irrigation line	+	(+)
UW030	(*Saprolegnia ferax*)	River bait	+	(+)
C377/B	(*Saprolegnia ferax*)	Irrigation water tank bait	(+)	(+)
	Non Oomycete species			
C435	*Botrytis cinerea*	‘Grey mould’ on ripe chilli pepper fruit	-	+
UW141	*Cordyceps confrugosa*	Strawberry fruit	+	(+)

© Agriculture and Horticulture Development Board 2017. All rights reserved

Culture identifier	Taxonomic identification	Source	Verified by ITS sequences	Confirmed by morph-ology
C271	*Fusarium* sp.	Untreated irrigation water	-	(+)
C272	*Fusarium oxysporum*	Echeveria glauca collar rot	-	+
C301	*Fusarium oxysporum*	Carnation root rot	-	(+)
UW106	*Fusarium oxysporum*	Strawberry roots (cv. Cambridge Favourite)	+	+
UW132	*Fusarium oxysporum*	Protected ornamentals runoff	+	+
UW142	*Fusarium oxysporum*	Protected ornamentals runoff	+	+
UW143	*Fusarium* sp.	Date Palm untreated runoff	-	+
UW144	*Fusarium oxysporum*	Banana crown	(+)	+
UW022	*Mortierella elongate*	Raspberry root	+	(+)
UW081	*Mortierella parvispora*	Irrigation water tank	+	-
UW011	*Mortierella zychae*	SH CC296	+	-
UW089	*Mortierella zychae*	Heuchera roots	+	(+)
UW027	*Mortierella* sp.	Irrigation tank	(+)	-
UW070	*Mortierella* sp.	Poinsettia drain water	(+)	-
UW125	*Mortierella* sp.	Rotted strawberry crown (cv. Elsanta)	(+)	(+)
UW019	*Mucor circinelloides*	Brunnera root	+	-
UW112	*Mucor hiemalis*	Strawberry crown rot (cv. Elsanta)	+	(+)
UW026	*Pholiota adiposa*	River water	+	-
UW031	*Pholiota* sp.	Irrigation holding tank bait	+	-

© Agriculture and Horticulture Development Board 2017. All rights reserved

Culture identifier	Taxonomic identification	Source	Verified by ITS sequences	Confirmed by morph-ology
C366	*Trichoderma* sp.	Irrigation water	-	(+)
UW137	*Trichoderma* sp.	Choisya roots	(+)	(+)
UW138	*Trichoderma* sp.	River water	-	(+)
UW140	*Trichoderma* sp.	Slow sand filter sand	-	(+)

Investigate potential to develop diagnostic probes to molecules associated with oomycete viability

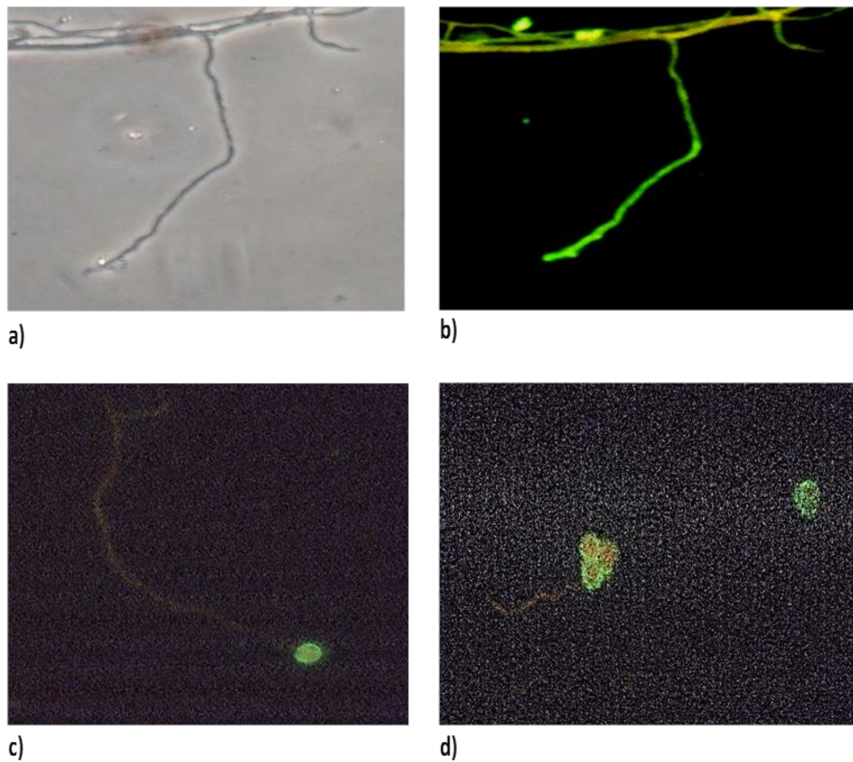
Following the procedures described in the year 1 annual report (Wakeham *et al*., 2016. pp. 17-18), two fusions were carried out and four monoclonal antibodies that bound to encysted zoospore preparations were identified. The cell lines established are detailed in Table 3 including their isotype and unique identification codes. Recognition of encysted zoospores and mycelial growth tips by these antibodies was confirmed by labelling with a fluorescence marker and viewing recognition sites by episcopic fluorescence microscopy at x400 magnification (see Figure 1 for results with UW 372 and UW 374). In addition to these tests, the antibodies raised in this section of the project were tested to see whether they might also be species or group specific and therefore potentially useful for deployment as additional Genus/Species/clade probes. This was achieved by testing them against extracts from a range of target and non-target species in cross-reactivity tests. These general cross-reactivity tests are described in the next section of this report.

Table 3. Details of cell lines raised as potential viability markers, raised to a preparation of mixed encysted zoospores of *Phytophthora cinnamomi, P. citrophthora* and *P. cryptogea*

Cell line	Isotype	Unique Code
3G9 D5 H5	IgM	UW 372
6A10 H1 A2	IgM	UW373
4E7 E1 B2	IgM	UW 374
3H7 H3	IgM	UW 375

© Agriculture and Horticulture Development Board 2017. All rights reserved

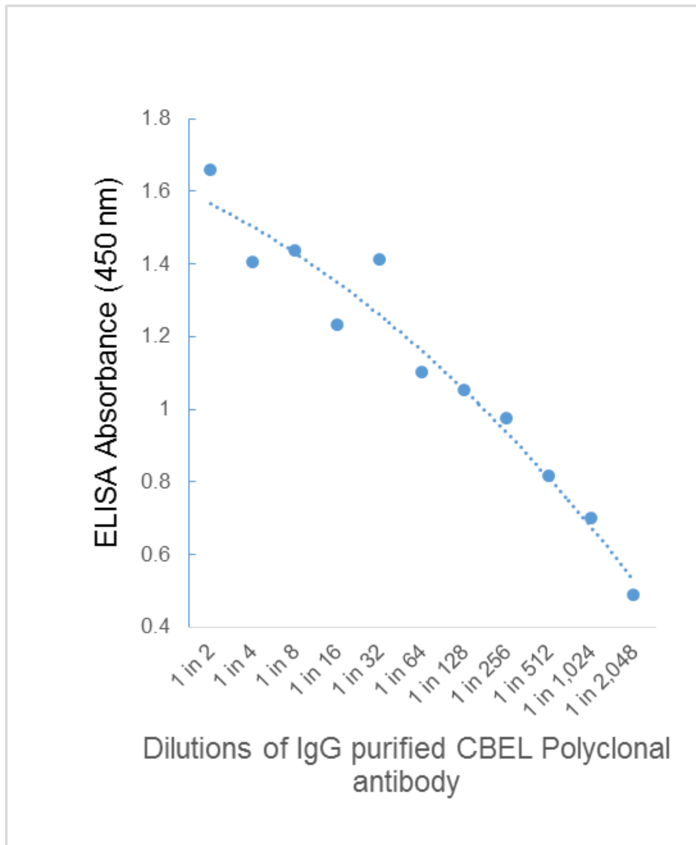
Figure 1. Monoclonal antibodies UW 374 recognising mycelia (a) & (b) and UW 372 recognising zoospore cysts (c) and (d) of *Phytophthora citrophthora,* visualised using fluorescence microscopy (x400)**.**



In sensitivity tests, the polyclonal antibody raised to the CBEL glycoprotein strongly recognises the purified protein (Figure 2). Furthermore, immunofluorescence microscopy using the polyclonal antibody appears to strongly indicate the localised distribution of the CBEL protein within encysted zoospores (possibly to adhesion sites Figure 3), as opposed to the more generalised fluorescence seen with the monoclonal antibodies raised to encysted zoospores (Figure 1). This indicates the specificity of the CBEL antibody although the practical implications of this localised staining will remain unclear until similar tests can be carried out on spores killed by water treatment techniques such as UV or chlorination to determine whether these may cause a disruption in, or eliminate the staining altogether. Such a disruption or elimination would indicate that the antibody has potential as a viability marker.

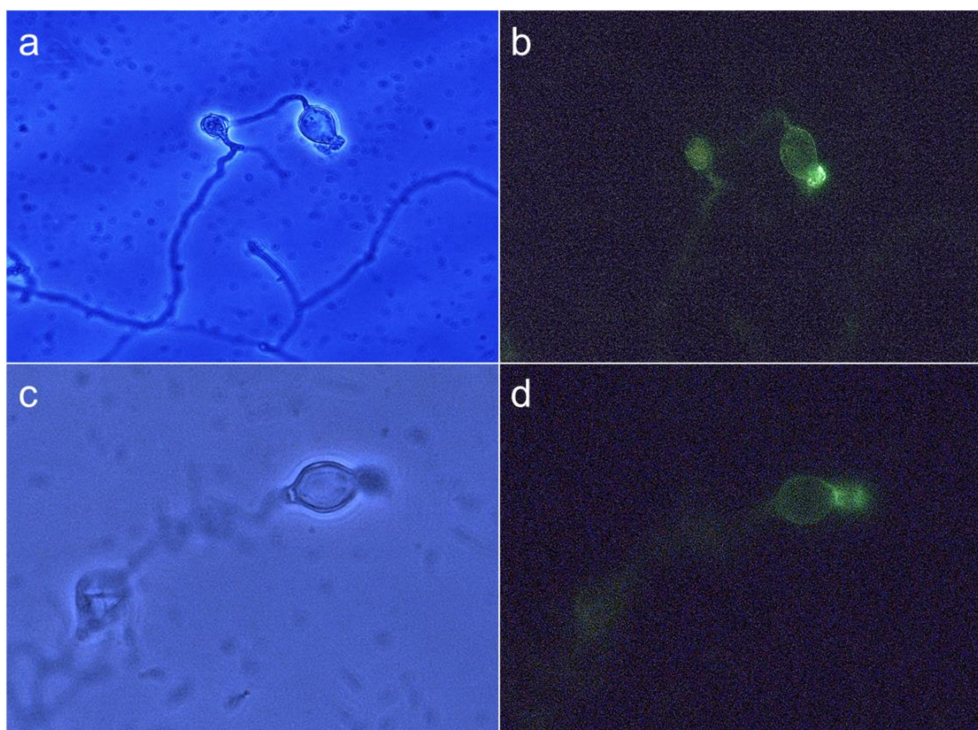
© Agriculture and Horticulture Development Board 2017. All rights reserved

Figure 2. Sensitivity of the polyclonal antibody (purified IgG) raised to CBEL, to the concentration of IgG-purified (CBEL) antigen as determined by ELISA (450 nm)



© Agriculture and Horticulture Development Board 2017. All rights reserved

Figure 3. Comparison between a light microscope images of germinating zoospore cysts (a & c) and recognition sites bound by the CBEL polyclonal antibody on the same spores labelled with a fluorescence marker and visualised by episcopic fluorescence (b & d).



Experiments with viable and killed zoospores of *Phytophthora cryptogea* and *Saprolegnia ferax* are currently in progress. This work is testing both the polyclonal anti-CBEL antibody and the monoclonal anti-cyst antibodies in both ELISA and immunofluorescence assays against dilutions of zoospores and cysts killed by different processes (heat, UV radiation, or oxidation by chlorine dioxide), to determine whether these treatments will make any difference to the binding capabilities of the various antibodies to the relevant proteins, and thereby determine the suitability of the antibodies as viability markers.

Development of antibody and molecular probes to assist diagnosis of *Pythium* and *Phytophthora* in environmental and plant tissue samples

Antibody test format

Oomycete Phylum/Class-specific test: Preparation of an IgG purified antiserum was completed in July 2016 and this was put through cross-reactivity tests (see below).

Phytophthora *and* Pythium *genus-specific tests:* Three fusions were carried out from immunised mice (13th October 2016, 28th November 2016 & 30th January 2017) and four new monoclonal cell lines were established as described in Table 4 below, including their isotype and unique identification codes. These antibodies were also put through cross-reactivity tests

© Agriculture and Horticulture Development Board 2017. All rights reserved

(see below). The first fusions from mice immunised with mixed *Pythium* mycelial extracts are planned for September 2017.

Table 4. Details of cell lines raised as potential *Phytophthora* genus-specific antibodies, raised to a preparation of mixed mycelial extracts from cultures of *Phytophthora cactorum, P. rubi* and *P. cryptogea*

Cell line	Isotype	Unique Code
5D3 C5	IgM	UW 386
3C4 A3	IgM	UW 387
2E6 A3	IgM	UW 398
2C8 C1	IgM	UW 399

Phytophthora *and* Pythium *'clade-specific' monoclonal antibodies:* One fusion has been completed with mice immunised with *Phytophthora rubi* (Clade 7) and ten new monoclonal cell lines were established as a result, these are described in Table 5 below, including their isotype and unique identification codes. Again these antibodies have been subjected to cross-reactivity tests (see below).

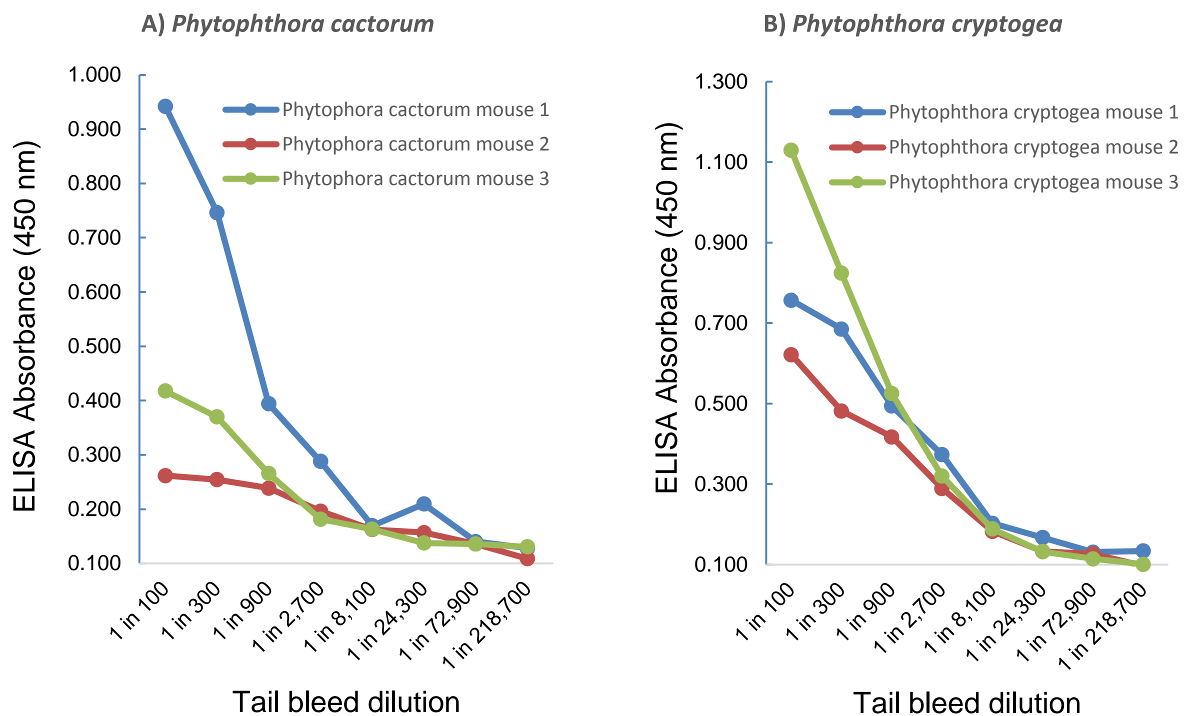
Table 5. Details of cell lines raised as potential *Phytophthora* clade (possibly species) - specific antibodies, raised to a preparation mycelial extracts from cultures of *Phytophthora rubi* (Clade 7).

Cell line	Isotype	Unique Code
3B6 A3 E1	IgM	UW 388
4F3 E2 G1	IgG3	UW 389
6C8 A1 H2	IgM	UW 390
1A11 G2 A10	IgM	UW 391
5F3 H1 G2	IgM	UW 392
5A9 H2 E1	IgM	UW 393
1A10 G3 C6	IgM	UW 394
2B5 G2 F3	IgM	UW 395
1H6 A3 H3	?	UW 396
4A2 G3 C31	IgG3	UW 397

© Agriculture and Horticulture Development Board 2017. All rights reserved

Good responses, showing high absorbance values for low dilutions and a clear decline with increasing dilution, were recorded from tail bleeds from mice immunised with single-species preparations of *Phytophthora cactorum* (Clade 1) and *P. cryptogea* (Clade 8) (Figure 4). and consequentially two fusions (one for each *Phytophthora* species) are currently under way.

Figure 4. Serum immune response of mouse tail bleeds to antigen preparations for A) *Phytophthora cactorum,* and B) *P. cryptogea,* determined by PTA-ELISA.



Two fusions have been carried out with mice immunised with *Pythium ultimum* var. *sporangiiferum* (Clade I) and to date 6 new monoclonal cell lines have been established. These cell lines are described in Table 6 and being prepared for the next set of cross-reactivity tests (see below). A further three fusions have been carried out with mice immunised with *Pythium aphanidermatum* (Clade A) and so far these have produced four new cell lines (UW 406-409, full details still to be confirmed). A third set of mice were immunised with a >30 kDa mycelial extract of *Pythium sylvaticum* in June 2017 and fusions from these mice are planned for September 2017.

© Agriculture and Horticulture Development Board 2017. All rights reserved

Table 6. Details of cell lines raised as potential *Pythium* clade (possibly species) -specific antibodies, raised to a preparation mycelial extracts from cultures of *Pythium ultimum* var. *sporangiiferum* (Clade I).

Cell line	Isotype	Unique Code
3C4 C6 A1	Not discerned yet	UW 400
4B5 F6 C1	Not discerned yet	UW 402
3E9 F4 B1	Not discerned yet	UW 401
2A8	Not discerned yet	UW 403
1A8	Not discerned yet	UW 404
1B6	Not discerned yet	UW 405

Cross-reactivity tests. Assessments of the cross-reactivity have been carried out using up to 45 defined individual defined isolates of oomycetes and non-oomycetes, on 16 monoclonal antibodies plus one polyclonal antibody, all raised to *Phytophthora* species. Many of these antibodies have shown very broad reactivity and are thus of not much further interest (Figure 5).

© Agriculture and Horticulture Development Board 2017. All rights reserved

Figure 5. Results of cross-reactivity tests showing results for antibodies with generally broad reactivities that probably preclude their use in further oomycete diagnostic test development.

© Agriculture and Horticulture Development Board 2017. All rights reserved

However, there were several antibodies that showed more specific reactivity that will be useful for identification purposes. Within this group of antibodies the degree of specificity varies, for example antibody 3H7 H3 reacts strongly with the majority of *Phytophthora* species assessed (Figure 6), and virtually no other species except to a much lesser extent with some *Pythium* species, especially the important pathogen species *P. ultimum* var *sporangiferum*. Used alone this antibody may potentially be useful for field diagnosis of *Phytophthora* species.

Other antibodies showed greater specificity and sometimes these specificities overlapped, indicating the potential to use two more antibodies with different reactivity together to identify clades or possibly even species, by inference. For example, antibody 3C4 A3 recognises *Phytophthora rubi* and *Phytophthora cinnamomi* (in the same clade = 7) in addition to *P. cryptogea* (clade 8; Figure 6), whereas antibody 1H6 A3 H3 gives such a strongly specific reaction to *P. cryptogea* (Figure 6), it may be possible to use these two antibodies together to discern between *P. cryptogea* (clade 8) and *P. cinnamomic/P. rubi* (clade 7).

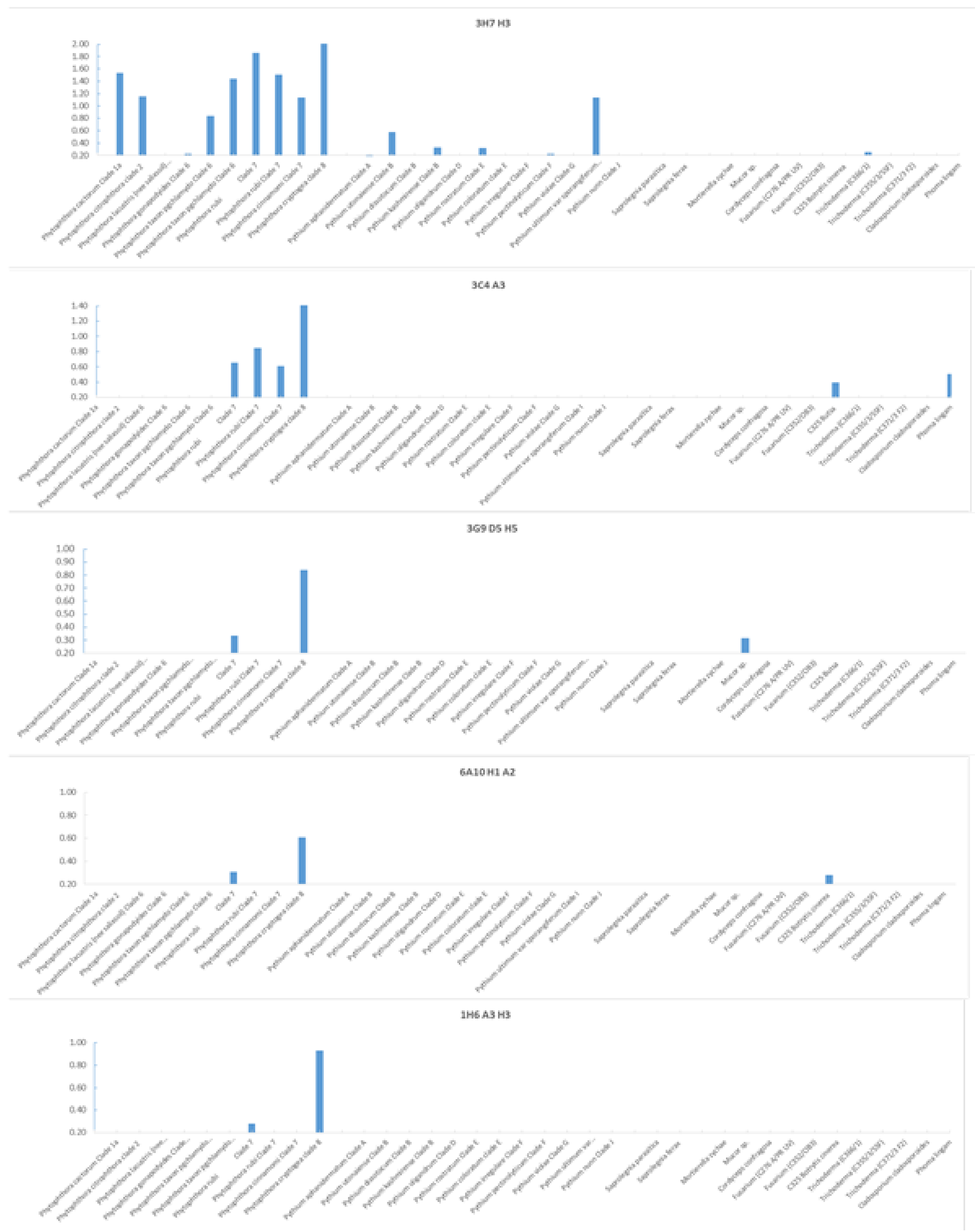
Whether the specificity demonstrated in Figure 6 is related to the individual species concerned or is clade specific will need to be explored further in planned tests with other species from clades 7 (e.g. *P. fragariae*) and 8 (e.g. *P. ramorum*).

The polyclonal antibody raised to a mixed antigen preparation (combined soluble fractions from mycelium of representative isolates of *Phytophthora, Pythium* and *Saprolegnia* spp.) has so far shown very promising cross-reactivity test results with strong reactions to key oomycete species (*Ph. citrophthora, Ph. cinnamomi, Ph. cryptogea, P. oligandrum, P. ultimum, P. rostratum, P. coloratum, S. ferax* and *S. parasitica*), with only very low reactions to non-oomycete species. The strong reactions to *P. coloratum, P. rostratum* and *S. ferax* indicate that this antibody will be particularly useful for water treatment efficacy testing, although assessments of its reactivity to the common water-inhabiting 'root nibbler' *Phytophthora* taxa, *P. lacustris, P. gonopodyides* and *P*. taxon. PG*chlamydo* are still to be completed.

Intensive cross-reactivity is ongoing, with a further set of 12 antibody cell lines ready for testing in July 2017 (10 anti-Pythium lines and 2 anti-Phytophthora {2 E6 A3 & 2C8 C1}), plus further tests anticipated for cell lines resulting from fusions later in the year (September/October).

© Agriculture and Horticulture Development Board 2017. All rights reserved

Figure 6. Results of cross-reactivity tests showing results for antibodies (3H7 {UW 375}, 3C4 {UW 387}, 3G9 {UW 372}, 6A10 {UW 373}, and 1H6 {UW 396}, all showing potentially useful specific reactions to *Phytophthora* species.



© Agriculture and Horticulture Development Board 2017. All rights reserved

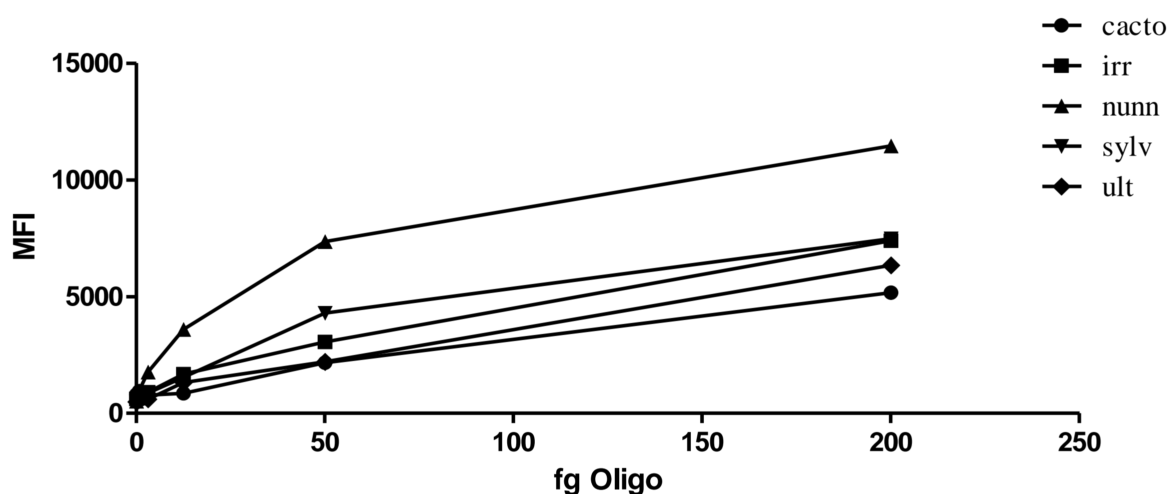
Oligonucleotide test format

Oligonucleotides are short DNA or RNA molecules that can be tailor-made in specific sequences and used as detection probes. In this study a specific section of DNA widely used in oomycete diagnostic tests (part of the ITS region), was amplified by PCR. This amplification results in lengths of sequence called amplicons that are specific to the particular species being amplified. Oligonucleotides were designed for each of five key species (*Phytophthora cactorum, Pythium sylvaticum, P. irregulare, P. ultimum* and *P. nunn*) that would specifically recognise only the amplicons of their target species (see Figure 8). Two oligonucleotides were used for detection of each amplicon and these were attached to beads for detection by the MAGPIX machine as illustrated in Figure 10. DNA extracted from the five target species was amplified successfully using the UNup and UNlo primer set (Tambong *et al*., 2006), and the ITS6 (Cooke *et al*., 2000) and ITS4 (White) primer set (Figure X1). ITS6 binds to a region overlapping the UNup (UN forward primer) annealing position. ITS4 primer binds to a region which overlaps the binding site of UNlo. DNA was amplified from *P. sylvaticum*, *P. ultimum*, *P. irregulare*, *P. cactorum* and *P. nunn*. Amplification using conditions recommended by Fessehaie *et al.* (2002), containing 2.0 mM $MgCL_2$ provided more robust amplification with both primer sets.

Biotinylated oligonucleotides were added at a range of concentrations to their relevant beads, before streptavidin/phycoerythrin was added and fluorescence intensity was measured to detect interaction The more successfully coupled beads, the higher the fluorescence intensity measured, and the amount of binding should increase with increasing oligonucleotide concentration Beads were successfully coupled for all five target species, as demonstrated by Mean Fluorescence Intensity (MFI) which correlated with biotin coupled probe concentration (Figure 7). However, the probes for each species showed different MFIs despite the use of the same concentration of oligonucleotide detection probe.

© Agriculture and Horticulture Development Board 2017. All rights reserved

Figure 7. Increasing binding of coupled beads to biotinylated complementary oligonucleotides as indicated by increasing MFI with increasing oligonucleotide concentration (fg).

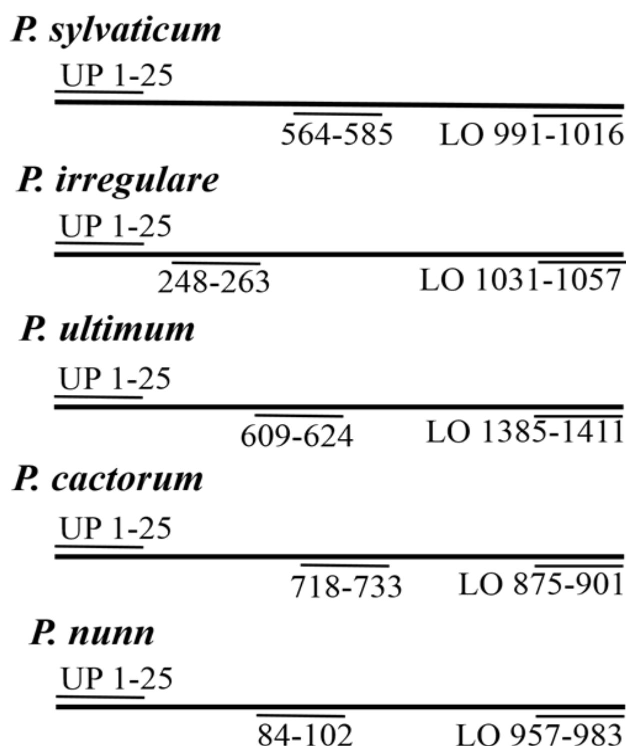


Unfortunately, after multiple attempts it was not possible to repeat the experiments which gave an apparent correlation between DNA concentration and reported fluorescence reported as an interim result last year (Wakeham *et al.*, 2017). Attempts were made with ITS6 and ITS4 primers (Cooke *et al.,* 2000), with fresh biotinylated primer stocks, with the forward and/or reverse primer biotinylated, but with no success. This indicates that the initial results were anomalous. As the maximum fluorescence achieved was an order of magnitude lower than that achieved with biotinylated probes, the earlier results appear to be unreliable. After consultation with the manufacturer, it was suggested that the MAGPIX instrument is likely only capable of detecting specific DNA when the probe binds within 100-300 bases of the bead (33-99nm). Therefore, the binding positions of each probe relative to the UN primer set (Tambong *et al.*, 2006) needed to be determined and the numbers of nucleotides between each probe and the amplicon chain's ends counted. Figure 8 shows the annealing positions for each species considered. ITS6 annealing position overlaps with UNup, ITS4 annealing position is one base different to that of UNlo, so for simplicity only UN primers are shown in Figure 8. As the probes for *P. nunn* and *P. irregulare* bind within the manufacturer's recommended region, all further attempts at generating standard curves focused on these species. The three other species investigated in this set of experiments were considered to be less likely to work, being outside of the specified distance. Other probes were specified by

© Agriculture and Horticulture Development Board 2017. All rights reserved

Tambong *et al,* (2006) but were generally considered less specific than the probes used in the study.

Figure 8. Simplified diagram showing the binding positions of selected probes in relation to UN primer set (Tambong *et al*, 2006). Numbers indicate nucleotides bound by primers and probes.



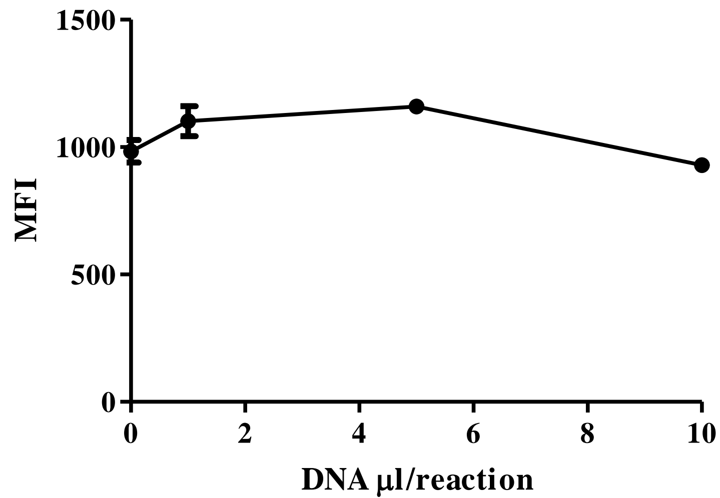
Lowering the hybridisation temperature was suggested by the manufacturer as a solution to the lack of detection. Assays were therefore performed at a range of hybridisation temperatures from 45ºC to 56ºC. No significant positivity was found at any of the temperatures tested, even with *Pythium nunn* DNA. The manufacturer only recommends testing down to 45ºC, any lower would lead to non-specific binding between DNA strands. Although some difference was seen between positive and negative samples for *Pythium nunn*, and *Pythium sylvaticum,* the signal achieved, with 5µl of amplified DNA was very weak. The manufacturer recommends between 1µl and 5µl per reaction. As the maximum difference between positive and negative is seen at lower temperatures, a hybridisation temperature of 46ºC was used to generate a standard curve using DNA amplified from *Pythium nunn*. Nevertheless, no increase in signal was found with increasing concentrations of DNA (Figure 9). Similar results were found for the other species tested (results not shown).

A further potential modification to the assay procedure was to increase the concentration of streptavidin/phycoerythrin (SAPE) used. However, increasing the concentration from 4 µg ml^{-}

© Agriculture and Horticulture Development Board 2017. All rights reserved

^{1} to 8 µg ml^{-1} (the maximum recommended rate) did not increase the MAGPIX signal relative to background levels.

Figure 9. Effect of *Pythium nunn* DNA concentration on the mean fluorescence intensity (MFI). Bars on means are standard errors calculated from results of duplicate runs.



© Agriculture and Horticulture Development Board 2017. All rights reserved

Discussion

A culture collection is never truly complete. Nevertheless, a comprehensive and reasonably representative culture collection has been assembled for this project. Key isolates have been cleaned and identified using both morphological characters and ITS sequencing. Isolates have been extensively used in both the raising of specific antibodies and more importantly in the ongoing cross-reactivity testing of new antibodies and test formats. Collections of isolates for the culture collection continue, although now at a much reduced rate as other parts of the project program take priority.

A wide range of water treatment techniques are used in UK horticulture to kill pathogens in the water. Unfortunately, killed pathogen cellular material often remains in treated water and can give positive immunological test results. So, there is real need for a quick and inexpensive test that can detect a molecule(s) that is only present in living pathogen material and therefore assist in diagnosis of water treatment efficacy. Such a viability test should not have great species specificity and ideally would detect viable material from all oomycete species. This type of test would allow more common non-pathogen species to be utilized as 'indicator' organisms for water treatment efficacy. Water treatments likely in UK protected cropping systems are : UV irradiation, sand slow filtration, oxidation (chlorine dioxide, hydrogen peroxide) and pasteurisation (80°C). A lateral flow test to reflect viable / non-viable propagules present in water system should differentiate protein markers of oomycete propagules as affected by these treatments. However, achievement of this is a highly ambitious goal.

Two approaches have been followed with the aim of developing a diagnostic probe to selectively differentiate viable from non-viable oomycete material. The first approach was to immunise mice directly with a 'mix' of native molecules associated with oomycete zoospore encystment from live zoospore cyst preparations to elicit an immune response and identify suitably 'viability-specific' probes by monoclonal antibody technology. Four antibodies have been directly produced in this way and are ready for testing. In addition to these, ten other antibodies that have been shown to have wide species reactivity in cross-reactivity tests may still be responding to proteins associated with spore viability and these are also ready for testing. As described above, tests for potential viability probes will be carried out with 'live' and 'killed' zoospore cyst preparations and are programmed to start in July 2017.

The second approach towards achieving a viability probe was to raise a polyclonal antibody with selective reactivity to a purified recombinant glycoprotein molecule associated with the early stages of encystment and germination of zoospores. A well characterised glycoprotein associated with spore adhesion (CBEL) was selected and prepared and used to raise an anti-CBEL polyclonal antibody which has now been purified and put through some initial

© Agriculture and Horticulture Development Board 2017. All rights reserved

experiments using immunofluorescence microscopy, producing some dramatic images showing selective staining of the points of adhesion of zoospore cysts. The production and properties of the CBEL glycoprotein are discussed in more detail in the year 1 annual project report (Wakeham *et al.*, 2016, pp. 37-38). This antibody will be tested alongside the monoclonal antibodies as outlined above, in experiments planned for July 2017.

The second year of this project has seen the major raising, purifying and testing antibodies for the later development of detection tests. The bulk of the antibodies raised to *Phytophthora* species have passed through their first level of tests, although there are still some important fusions to complete and cell lines to test.

Cross-reactivity testing so far has revealed that we have a good general 'oomycete-specific' polyclonal antibody. This will be useful for the development of water tests where, in combination with a viability marker, it could form the basis for an effective test for the efficacy of irrigation water treatments. Key to this test will be a suitable viability marker, and for this a series of monoclonal antibodies have been raised to live zoospore preparations as well as a polyclonal antibody to a specific glycoprotein (CBEL) that is involved in the zoospore germination process. These antibodies will be tested against live (infectious) zoospore cysts and cysts killed by different sterilisation treatments (UV, heat, and chlorination - each of which can have quite different effects of the cysts' structures and contents), in the hope that they will be able to clearly discern the 'live' from 'killed' cysts.

In addition to these antibodies, a monoclonal antibody (3H7 H3) has been found to be nearly genus-specific – detecting only *Phytophthora* species plus the pathogenic species *Pythium ultimum*. Several other monoclonal antibodies have shown promising levels of specificity, detecting *Phytophthora cryptogea* only or a combination of *P. cinnamomi, P. rubi*, and *P. cryptogea*. We are now exploring whether it will be possible to deploy a combination of these antibodies in a test to identify, (a) *Phytophthora* sp., (b) *P. cryptogea* (*Phytophthora* clade 8), and (c) *P. rubi/P. cinnamomi* (*Phytophthora* clade 7). The first experimental lateral flow test strips are due to be ready for laboratory evaluations with field samples in August 2017.

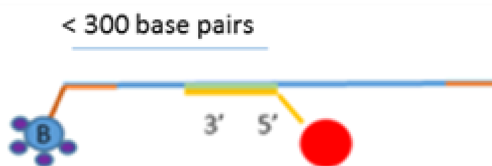
Two more antibodies raised to *Phytophthora* are still to be tested for cross-reactivity and further final batch will be ready by the end of September 2017.

With the *Pythium* antibodies, the first batch of 10 antibodies are ready for cross-reactivity testing in July 2017, but there are also still fusions being set up with the potential to raise a further 16 or so antibody cell lines for testing later in year 3.

© Agriculture and Horticulture Development Board 2017. All rights reserved

Despite successful amplification of DNA, and successful coupling of capture oligonucleotides to magnetic beads, it has not been possible to adapt the oligonucleotide array described by Tambong *et al.* (2006), for use on a bead array. It is possible that the amplicon length is interfering with the ability of amplicon to bind to the bead. Luminex specify the detection probe should bind within 300bp of the biotin/PE (Figure 10), which is the case for two of the species tested, but it was still not possible to detect binding. With longer amplicons there is an increased chance of secondary structure formation, which may block recognition of the bead-coupled oligonucleotide. To test this it would be possible to reduce the amplicon length after amplification by use of a restriction digest, or possibly a secondary, biotinylated probe coupled to biotin which could detect the specific amplicon. However, in practice this would add too many extra steps and make the assay too complex/cumbersome to be useful. It may also be possible to alter the primers used to amplify DNA. However, it has so far, not proven possible to identify primers which would produce shorter amplicons still capable of showing species differences, whilst also amplifying all the required species. Any primer set capable of amplifying the ITS region of all oomycetes, must be very long, as the primers must bind in conserved regions.

Figure 10. Diagram showing biotinylated DNA amplicon labelled with a streptavidin /phycoerythrine (SAPE) complex label and illustrating the recommended distance between the biotinylated end and the label.



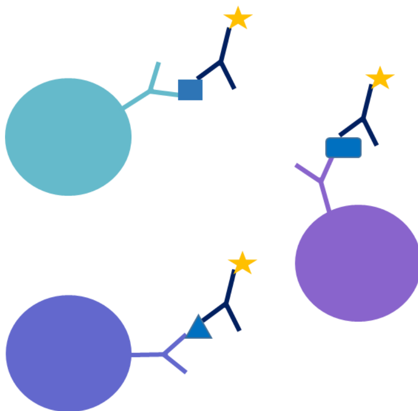
The initial study reported in year 1 annual report (Wakeham *et al.*, 2016, pp. 33-35) showed promising results with data generated from two runs each using DNA from a single amplification of *Pythium sylvaticum* ITS. However, as stated above, these results could not be repeated in year 2 of the project. This was initially considered to be the result of distances between the biotin label and the SAPE probe being too great, but subsequent assay work with *Pythium nunn,* with a length of approximately 100 bp and well within the recommended range, and with *Pythium sylvaticum*, just inside the range at 263 bp, has not given evidence of detection. It therefore seems wise to abandon the oligonucleotide approach to using the MAPIX multiplexing platform.

However, this does not mean that the concept of using the MAGPIX platform should be abandoned, as the system can be used to multiplex double-antibody sandwich (DAS) ELISA.

© Agriculture and Horticulture Development Board 2017. All rights reserved

This procedure would entail using monoclonal antibodies developed in this project – attaching them to them to MAGPIX beads. This procedure may work with just an antibody purification step, although since monoclonal antibodies are pentamers, their large size might be problematic within the MAGPIX assay system. Nevertheless, these large molecules can be split into 5 individual monomers by pepsin digestion. Monomers would then be attached to beads, and the bead colours would indicate identity of specific antigens, whilst antigen presence/absence could be marked by a second, antigen-specific antibody attached to a fluorescent label (Figure 11).

Figure 11. Diagram illustrating the concept of using species/clade-specific antibodies coupled to different colour MAGPIX beads in combination with an oomycete-specific secondary antibody labelled with a fluorescent dye for confirmation and quantitation of antigen presence.



There is a possibility that the oomycete general polyclonal antibody raised in this project would be suitable for this role – providing a second tier of identification by confirming +/- oomycete antigen. This work is now under way with the first stages being proof of concept (and antibody compatibility) by a series of DAS-ELISA assay with selected antibody combinations as well as the attachment of selected antibodies to MAGPIX beads.

© Agriculture and Horticulture Development Board 2017. All rights reserved

References

Cooke D.E.L., Drenth A., Duncan J.M., Wagels G., Brasier C.M. (2000). A molecular Phylogeny of *Phytophthora* and related oomycetes. *Fungal Genetics and Biology* **30**:17-32.

Fessehaie, A., De Boer, S.H., and Lévesque, C.A. (2003). An oligonucleotide array for the identification and differentiation of bacteria pathogenic on potato. *Phytopathology* **93**:262-269.

Gaulin E., Drame N., Lafitte C., Torto-Alalibo T., Martinez Y., Ameline-Torregrosa C., *et al* (2006) Cellulose binding domains of a *Phytophthora* cell wall protein are novel pathogen-associated molecular patterns. *Plant Cell* **18**:1766–1777. doi: 10.1105/tpc.105.038687.

Johnston J., Booth C., (1983) *Plant Pathologist's Pocketbook*, 2nd edition. Kew, UK: Commonwealth Mycological Institute.

Tambong J.T., De Cock A.W.A.M., Tinker N.A., Lévesque C.A. (2006). Oligonucleotide array for identification and detection of Pythium Species. *Applied and Environmental Microbiology* **72**:2691-2706.

Vallance, J., Le Floch, G., Déniel, F., Barbier, G., Lévesque, A., Rey, P. (2009). Influence of *Pythium oligandrum* biocontrol on fungal and oomycete population dynamics in the rhizosphere. *Applied and Environmental Microbiology* **75**:4790-4800.

Wakeham A., Pettitt T., Keane G., John S., Lewis M., Edwards E. (2016). *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.* Annual Report Year 1, Project CP136. AHDB, Stoneleigh Park, Warwickshire, UK.

Wakeham A.J., Pettitt T.R., White J.G. (1997). A novel method for detection of viable zoospores of Pythium in irrigation systems. *Annals of Applied Biology,* **131**:427-435.

Wakeham A.J., White J.G. (1996). Serological detection in soil of *Plasmodiophora brassicae* resting spores. *Physiological and Molecular Plant Pathology* **48**:289-303.

Wedgwood E. (2014). *Baiting and diagnostic techniques for monitoring* Phytophthora *spp. and* Pythium *spp. in irrigation water on ornamental nurseries.* Final Report on HDC Project HNS/PO188. Stoneleigh Park, Warwickshire, UK: AHDB Horticulture.

White, T.J., Bruns, T., Lee, S., Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for polygenetics. In: PCR Protocols: A guide to methods and

© Agriculture and Horticulture Development Board 2017. All rights reserved

applications. pp. 315-322. Eds. M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White. San Diego, USA: Academic Press.

© Agriculture and Horticulture Development Board 2017. All rights reserved