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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.

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GROWER SUMMARY

Headline

- Oomycete species have been isolated from environmental samples taken at different times during the UK horticultural production season, from a range of affected commercial sectors – hardy nursery stock (HNS), protected ornamentals, protected edibles and soft fruit. A comprehensive DNA typed Oomycete culture collection is in development to represent key sectors of horticultural production.

Background

Oomycetes and crop disease. Oomycete diseases cause significant losses across a range of agricultural and horticultural commodities worldwide. 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 different crops. Often associated with above ground plant parts i.e. shoot apex, leaf, stem and fruit, they are also responsible for root and crown rots.

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 the widespread dissemination of *Pythium* species across UK nurseries via danish trolleys reported by White (PC 097). 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 development of symptoms can highlight where and when treatments are needed, thereby reducing disease epidemics significantly. However, methods for the isolation and identification of Oomycete crop pathogens 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.

Best practice diagnostic tests: Conventional plating of plant tissue, water filtrate or soil suspensions onto semi-selective agars containing antibiotics is a simple and useful procedure for isolating and identifying *Pythium* and *Phytophthora* (oomycetes) species. Unfortunately, these methods often tend only to be used after disease symptoms have been observed. Whilst useful and relatively simple to carry out, their accurate interpretation requires much experience and skill and they can give variable results, especially with plant tissues, or where pathogen propagules have entered dormancy. Direct measurement of Oomycetes can also be achieved from soil by dilution plating from water by membrane filtration-resuspension plating and from plant tissues by comminution followed by plating dilutions onto selective agar plates and counting the resulting colonies. Baiting techniques have been used since the 1960s for both *Phytophthora* and *Pythium* detection in water and in soils and can be very effective, although of variable sensitivity, as they are dependent on the quality and physiological state of the plant tissues being used as baits. The main drawback of these 'best practice' techniques is the time required to generate information i.e. often too slow to assist with making on-site disease management decisions. This has led to a situation of routine, often prophylactic deployment of fungicides/oomyceticides. With ineffective targeting and overuse, a build-up of widespread fungicide resistance has been reported, with lost efficacy resulting from enhanced fungicide degradation. With a considerable pressure to move away from routine pesticide application to targeted crop treatments (pesticides and biological) greater depth of knowledge by producers and their staff is required to identify problems quickly.

On-site diagnosis Rapid (under 10 minute) point of care assays (POCs), originally developed for medical applications (e.g. Unilever clear blue home pregnancy testing kit) have successfully been adapted to achieve reasonably accurate diagnoses of some plant diseases. An early example of this was reported by Agri-Diagnostics associates who developed flow through tests for detection of *Phytophthora*, *Pythium* and *Rhizoctonia* species on infected root, stem and leaf samples. Commercially-available kits have since been made available and used by the UK horticultural industry. However, whilst useful for confirmation of disease in plants showing symptoms, the value of these tests has not yet been demonstrated for some environmental samples (eg. growing substrates) or for the pre-symptomatic infection of plant material. Their use in conjunction with plant tissue baits has been shown with some promise in irrigation water tests. However, these tests as they stand, fail to distinguish between live and dead pathogen propagules, negating their value in assessment of pathogen kill when assessing the efficacy of control treatments. Moreover, these tests are not able to

differentiate different *Phytophthora and Pythium* species. For *Pythium* this is particularly important given close to 300 species have been proposed. Many of these are saprophytic, frequently found in cultivation and a significant number are not pathogenic to crops. The inability of these tests to distinguish pathogenic from non-pathogenic species (or even bio-control i.e. *Pythium oligandrum*, *P. nunn*, *P. perioplocum* and *P. acanthicum*), and the inability to separate viable from non-viable propagules is problematic for reliable diagnosis. Nevertheless, on-site diagnosis can be effective, as recently seen in the UK for diagnosis of Oomycete pathogens causing sudden oak death. Here, a lateral flow device (on-site device) has been used in the UK by Fera Plant Health and Seed Inspectorate to monitor the spread of *Phytophthora ramorum* and *P. kernoviae*. The lateral flow device is used as a first screen of suspected infections, with confirmation of positive tests later made by molecular PCR (polymerase chain reaction).

New approaches to disease diagnosis. In order to quickly and accurately diagnose disease potential, new test systems are required. Innovative work continues to be carried out in the medical and defence industries to provide early warning of infectious agents and these technologies have the potential to provide useful tools for the management and control of diseases in plant cropping systems. However, it is important to understand from the outset the economies of scale associated with crop production and the sampling processes required to allow appropriate test coverage. For on-site testing by growers and agronomists, ease of use and test reliability are important, but ultimately adoption in agricultural systems will be driven by costs. The programme of work described in this project will attempt to address these issues using both molecular (DNA) and immunological (antibody) tests. If successful, these tests could deliver robust, economically viable systems to provide timely information directly to the front line to allow informed disease management decisions to be taken. The approaches that will be taken in this project for diagnosis of Oomycete species are outlined below:

Oomycete diagnostic assay development – A project overview: This programme of work seeks to develop a set of Oomycete disease management tools that can be used both by growers and crop clinics. Two test formats will be developed for use by the UK horticultural industry. For on-site testing the objective will be to develop a multiplex antibody-based lateral flow to measure the presence/absence of *Pythium* and *Phytophthora* species and, more specifically, identify key Oomycete plant pathogen(s), and if possible a generic Oomycete test will be produced for propagule viability.

Lateral flow tests consist of a carrier material containing dry reagents that are activated by applying a liquid sample. Movement of this liquid allows passage across various zones where molecules have been attached that exert specific interactions with target analytes. Results are generated within 5 - 10 minutes, with the formation of a control and test line(s) as appropriate to the sample and the test type (Figure 1, lateral flow qualitative test). They are designed for single use and are available commercially for a wide range of applications.

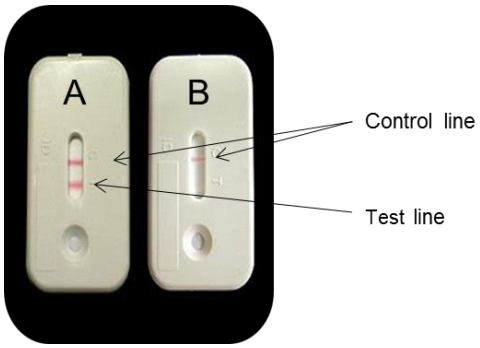

Figure 1. Visual assessment (by eye) of a qualitative double antibody sandwich lateral flow assay for disease risk. A - Control and test line development indicates risk of pathogen presence; B - Control line but no test line development – low or no risk.

Development of these type of tests require diagnostic probes which selectively recognise target Oomycete molecules. Hybridoma technology provides the capability to generate highly specific monoclonal antibodies (MAbs) which can be expressed from maintained cell lines to discriminate at the genus, species and at different stages of an organism's life cycle.

The second approach that will be investigated is the use of published molecular probes (DNA based) to detect and identify *Pythium* and *Phytophthora* species associated with cankers, stem and root rots of a wide range of horticultural crops. The detection of beneficial Oomycetes will also be considered and incorporated into a molecular test array format. For the purpose of this project the test will be aimed at crop clinic usage. The probes used may prove transferable to field based assays, but this will not be developed or evaluated within the remit of this project.

To facilitate this work it is critical to isolate and identify Oomycete species present in environmental samples at different times within UK horticultural production season, and to ensure the representation of key sectors affected by Oomycete root and collar rot pathogens: HNS, protected ornamentals, protected edibles and soft fruit. For this purpose site visits will be made to selected UK nurseries. Using traditional best practice techniques, isolations will be made for Oomycetes and where possible identification made by DNA sequence analysis.

Summary

Isolate oomycete species from plant and environmental samples across each of the sectors: Site visits have been made during project Year 1 to selected UK commercial propagators (HNS, protected ornamentals, protected edibles and soft fruit). Using traditional best practice techniques, isolations for Oomycete pathogens have been made from a range of environmental samples to include water, growing and plant material. Additionally, environmental samples sent by AHDB levy members have been processed for Oomycete infestation.

Develop a comprehensive horticulture-based *Pythium* and *Phytophthora* isolate collection: A significant element of the project in Year 1 has involved the development of an Oomycete culture collection with species verifications made by DNA sequencing methods. From environmental samples aggressive *Pythium* root rot species with broad host range have been identified. These include *P. aphanidermatum, P. dissotocum, P. hyphal swelling group, P. intermedium*, *P. irregulare*, *P. intermedium* and *P. ultimum*. Also, *P. violae* (cavity spot on carrot), *P. kasmirense* (isolated from choisy root), *P. lutarium* (spinach roots), *P. pectinolyticum* and *P*. *utonaiense* (isolated from water associated with strawberry production) have been identified. Highly destructive *Phytophthora* species with wide host range were also found. These include *P. cactorum* (strawberry), *P. cinnamomi* (*Chamaecyparis* roots), *P. citrophthora* (*Buxus* roots), *P. cryptogea* (geranium roots). Also, *Phytophthora gonapodyides* (pathogen on Oak) from nursery drain-water, P*. mississippiae* and *P. syringae* (pathogen associated with citrus, apple and pear) from river water.

For this project, a current Oomycete collection is fundamental to the development of relevant diagnostic probes. Identification of the DNA typed species isolated from commercial horticultural production may however also prove useful to AHDB projects outside the scope of this study. As mentioned previously, reliable and affordable detection and diagnosis are key to effective Oomycete disease management. Knowing which *Phytophthora* and *Pythium* species are of economic importance to UK horticulture production will assist in the development of meaningful diagnostic tests. For example, the ability to discriminate presence of saprophytic species frequently found in cultivations and not pathogenic to crops, and potential biocontrol agents (*Pythium oligandrum*, *P. nunn*, *P. perioplocum* and *P. acanthicum*) which can aggressively attack other Oomycete species.

Develop antibody and molecular probes to assist diagnosis of *Pythium* and *Phytophthora* in environmental samples: In Year 1 of the project, development of diagnostic probes has commenced with hybridoma production to a range of Oomycete targets isolated from UK nurseries in 2015 and 2016. Already, antibody cell lines have been identified which, in preliminary studies, discriminate between an Oomycete zoospore (motile stage in water) and the mycelial stage (supporting growth). These cell lines could prove useful in developing a test able to discriminate between the viable Oomycetes and dead material. This type of test would be a very powerful tool allowing growers to carry out rapid, meaningful, low-cost, routine *in-situ* testing of irrigation water treatment systems, as well as in other growing mediums where non-viable inoculum can give rise to false positive results.

Sample preparation has been undertaken as a first step towards the development of antibody-based probes to selectively discriminate *Pythium* and *Phytophthora* species and, more specifically, a test to identify key plant pathogen(s) involved. For example, an aim of the project will be to develop a general on-site *Phytophthora* lateral flow test and, if possible, differentiate between *P. cactorum*, *P. fragariae* and *P. cryptogea.* The rationale being that *Phytophthora cactorum* has a broad host range, but in the UK is important in fruit production causing fruit and crown rot of strawberries and collar rot of apples in addition to stem rot in a wide range of HNS species. *P. fragariae* has been identified as the causative agent of red core/red steele disease and is a significant disease risk in both strawberry and raspberry crops. And *P. cryptogea* is a very important pathogen across most sectors of UK horticulture with a broad host range and is aggressive, causing crop losses, particularly in HNS, protected ornamentals and tomatoes. Similar tests will be developed with an aim to identify *Pythium* species if present, and key species known to be aggressive on crop types.

A molecular (DNA) method, essentially based upon polymerase chain reaction (PCR) is also being assessed for the direct measurement of Oomycete inoculum in environmental samples. A selection of oligonucleotide array markers have been shown to bind to magnetic beads. Using a MAGPIX Luminex array system quantitative measurement of *P. sylvaticum* has been demonstrated. The assay system will now be tested using three different coloured bead sets bound with oligonucleotides specific for simultaneous measurement of *P. ultimum*, *P. irregulare* and *P. sylvaticum*. Other species will be added sequentially. This type of test would be laboratory based and essentially developed to provide an alternative to on-site tests. Diagnosis based on DNA arrays can be highly specific and sensitive allowing simultaneous detection of multiple pathogens present in a cropping system

Lateral flow tests are low cost, for use by growers on-site and should provide a useful first screen for Oomycete presence. DNA based tests offer the potential for enhanced sensitivity (detection of the pathogen when present at low level i.e. sub-clinical) and confirmation of the species involved.

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.

The predictive deployment of control measures will provide disease control systems that are sustainable, as an integral part of lower-input farming systems. The use of diagnostic tests will provide a significant step forward and help minimize the need for fungicide intervention.

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 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

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.

Development of an oomycete isolate collection:

In development of tests it has been important to consider which *Pythium* and *Phytophthora* species are of economic importance to UK horticulture production and their cross-sector relevance. Also, to identify saprophytic species frequently found in cultivations and not pathogenic to crops (Van der Plaats-Niterink, 1981). In addition, at least four species, *Pythium oligandrum*, *P. nunn*, *P. perioplocum* and *P. acanthicum*, are aggressively mycophagous and therefore potentially beneficial in disease control. For this purpose, site visits will be made during each of the three years of the project to selected UK commercial growers (HNS, Protected ornamentals, protected edibles and soft fruit). Using traditional best practice techniques, isolations for oomycete pathogens will be made from a range of environmental samples. These will include water and plant material. Additionally, environmental samples sent by AHDB levy payers will, where possible, be assessed for

oomycete infestation. A significant element of this project will be the development of an oomycete culture collection with species verifications made by DNA sequencing methods. The selection of *Phytophthora* and *Pythium* species for inclusion in detection array systems will aim to represent those most frequently encountered on UK nurseries (HDC PC97 and HNS 181), clinics and general observations or those which have the potential to be most economically damaging.

Development of detection array systems: The project will aim to develop several test formats to provide propagators with a choice of disease management tools. For on-site testing, lateral flow devices will be developed using antibody technology to identify oomycete targets. A multiplex format will be used and a series of tests aimed to measure oomycete presence. A generic oomycete test will aim to identify presence of the following oomycete types: *Pythium*, *Phytophthora*, *Saprolegnia, Achlya* and *Aphanomyces* species. Each of which are commonly present in water and growing media on nurseries. The *Saprolegnia*, *Achlya* and *Aphanomyces* species are not normally plant pathogens, but their spores and behaviour are similar to *Pythium* and *Phytophthora* so can be used as indicators for control treatment efficacy. For this reason, the potential to develop a diagnostic probe which can discriminate between the viable oomycete state and dead material will be investigated. If successful, the test would provide the capability to detect oomycete presence and determine viability. This would be very powerful tool allowing growers to carry out rapid, meaningful, low-cost, routine *in-situ* testing of irrigation water treatment systems. Also, in other growing medium where non-viable inoculum can give rise to false positive results. A generic viability probe should avoid this problem, providing an approach that will prove particularly useful in confirmatory tests of disinfestation treatments. Finally, it is hoped that probes can be developed to selectively discriminate *Pythium* and *Phytophthora* species (qualitative) and, more specifically, a test to identify key plant pathogen(s) involved.

This multiplex approach has been adopted as previous lateral flows developed have at best only been able to provide genus-specific detection. As reported previously, many different *Pythium* and *Phytophthora* species are present in horticultural cropping systems and their importance varies greatly with sector and crop. In addition to accurate discrimination between horticultural crop specific pathogens, an effective test should separate these from non-pathogenic and plant beneficial species. For example, a useful test would be able to discern between pathogens and indigenous oomycete biocontrol agents such as *Pythium oligandrum*. This ‘useful’ *Pythium* has been shown to be jeopardised by the application of inappropriate control measures. For example, the application of metalaxyl and mancozeb

based control products had a deleterious effect on soil populations of the biocontrol agent *Pythium oligandrum* (White and Wakeham, 1992).

The development of specific antibody probes to discriminate and measure *Phytophthora* and *Pythium* species will be directed by their economic importance to horticulture production and their cross-sector relevance. From past studies (HDC PC97 and HNS 181), clinics and general observations the species identified as important to the UK industry will be drawn into groupings (clades) and based on a number of criteria. We will consider not only the molecular internal transcribed spacers (1&2) phylogeny (Levesque & De Cock, 2004; Cooke et al., 2000; www.phytophthoradb.org) but serological and morphological characteristics (White et al., 1994; Plaats-Niterink, 1981). It is envisaged that any test developed could be used separately or simultaneously depending on the sample.

The aim of the *Pythium* on-site test would be to identify presence of *Pythium* species . If present, whether the species fall into specific clade groupings. This would be relevant from a disease control aspect. These would not be limited to but include: *P. aphanidermatum*, *P. deliense P. debaryanum*, *P. sylvaticum*, *P. irregulare*, *P. intermedium*, *P. ultimum*, *P. splendens, P. dissotocum and P. hyphal swelling group.* Similarly, a general *Phytophthora* test will be developed and, if possible, will aim amongst others to differentiate between *P. cactorum*, *P. fragariae* and *P. cryptogea.* The rationale being that *Phytophthora cactorum* has a broad host range but in the UK is important in fruit production causing fruit and crown rot of strawberries and collar rot of apples, also, stem rot in a wide range of HNS species. *P. fragariae* has been identified as the causative agent of red core/red steele disease and is a significant disease risk in both strawberry and raspberry crops. Whist *P. cryptogea* is a very important pathogen across most sectors of UK horticulture with a broad host range and is aggressive, causing losses, particularly in HNS, protected ornamentals and tomatoes.

Molecular (DNA based) probes. Methods, essentially based upon Polymerase Chain Reaction (PCR), have evolved from a complex specialised procedure to become an indispensable, routine tool used widely in the diagnosis of infectious diseases. Over the past two decades PCR and quantitative PCR (q PCR) techniques have expanded to become some of the most widely used laboratory assays for the direct measurement of low levels of pathogenic microbes in environmental samples (Theron *et al.,* 2010). The increasing ability to rapidly and economically sequence pathogen genomic content has enabled development of specific primer sets to selectively identify nucleotide sequences of fungal and oomycete species. Among the regions of the ribosomal DNA, the internal transcribed spacer (ITS) region has the highest probability of successful identification for the broadest range of fungi

(Schochl et al. 2012). Consisting of alternating areas of high conservation and variability the ITS region has proved popular for the development of highly specific and sensitive primer sets for use in PCR based diagnostic tests to discriminate target oomycete plant pathogenic species in complex environmental samples (Klemsdal *et al*., 2008; Lees *et al*., 2012). These processes have been successfully applied to develop molecular probes which are able to discriminate and measure many important pathogenic oomycete species (Beakes *et al*., 2012; Cooke *et al*., 2000; Lévesque & De Cock, 2004). Universal primers ITS4 and 6a have been found particularly useful for amplification of *Pythium* and *Phytophthora* species DNA (Cooke *et al* 2000). Other regions of the genome have also been described to reveal nucleotide base pair differences for the phylogenetic characterisation of *Phytophthora* and *Pythium* species. These include the mitochondrial cytochrome oxidase (cox 1 and cox 2) spacer regions and the nuclear translation elongation factor 1α- and β-tubulin gene (Kroon *et al*., 2004; Villa *et al*., 2006; Blair *et al*., 2008; Robideau *et al*., 2011). Databases also exist where DNA sequence data are stored and are available for species comparisons (www.phytophthoradb.org; www.phythophthora-id.org; www.q-bank.eu; www.boldsystems.org). In some cases these resources provide additional information such as diagnostic morphological features and aspects of biology.

Where a laboratory/clinic environment is preferred, advances in molecular diagnostic test technology has provided the opportunity to couple PCR with high throughput pathogen detection multiplex arrays. PCR-based arrays generally consist of a high density of selected and synthesised immobilized nucleic acid sequences spotted onto a solid platform such as glass microslides, beads or nylon membranes (Epstein & Butow, 2000, Ishii et al., 2008). Published sequence sets exist for many *Pythium* and *Phytophthora* species and have been used to discriminate species in environmental samples using nylon membrane macro arrays systems (Lievens et al., 2003, Lievens et al., 2006, Lieven et al., 2012, Tambong et al., 2006, Chen et al., 2013). Following DNA extraction from an environmental sample, amplicons of a target DNA region are generated by PCR and bound with a fluorescent, biotinylated or enzyme label. Following a process of DNA hybridisation, amplicons which are able to bind selectively to immobilised target sequences of the array are visualised, either by direct fluorescence scanning or enzyme-mediated detection, to yield a semi-quantitative result (de Boer & Beurmer, 1999). In general, target amplification is based on the use of universal primers that recognize conserved sequences flanking variable domains in housekeeping genes, such as the ribosomal RNA gene. In this way, numerous targets can be amplified with a single primer pair, while target discrimination is performed afterwards on the array (Lievens *et al*., 2003 & 2011).

In this study, the development of nucleotide based multiplex array systems for target *Pythium* and *Phytophthora* species will be investigated both by membrane macro array and, with magnetic spheres using the Luminex MAGPIX technology. The MAGPIX system using colour-coded magnetic bead sets can provide a versatile multiplexing platform capable of performing qualitative and quantitative analysis of up to 50 target analytes in a single reaction volume and, in a variety of sample matrices. There have been several reports using this new technology to detect foodborne pathogens and toxins (Kim *et al*., 2010), three potato viruses in infected host tissues (Bergervoet *et al*., 2008) and a multiplex seed screening assay, simultaneously detecting four important plant pathogens: the blotch bacterium (*Acidovorax avenae* subsp. *citrulli*), and three viruses; chilli vein-banding mottle virus, watermelon silver mottle virus and melon yellow spot virus (Charlermroj *et al*., 2013).

Using these systems we hope to provide plant clinic capability to accurately identify a list of *Pythium* and *Phytophthora* species associated with cankers, stem and root rots of a wide range of UK horticultural crops. The detection of beneficial oomycete species will also be considered and incorporated within the array format. Initial candidates for this panel will include the following:

Phytophthora alni	*Phytophthora brassicae*	*Phytophthora cactorum*
Phytophthora cinnamomi	*Phytophthora citricola*	*Phytophthora citrophthora*
Phytophthora cryptogea	*Phytophthora fragariae*	*Phytophthora infestans*
Phytophthora nicotianae	*Phytophthora ramorum*	*Phytophthora humicola*
Phytophthora plurivora	*Phytophthora gonapodyides*	*Phytophthora austrocedrae,*
Phytophthora quercina	*Phytophthora rubi.*	*Phytophthora* generic

Pythium irregulare	*Pythium intermedium*	*Pythium sylvaticum*
Pythium ultimum var. ultimum	*Pythium ultimum sporangiferum*	*Pythium oligandrum*
Pythium nunn	*Pythium rostratum*	*Pythium torulosum*
Pythium paroecandrum	*Pythium sulcatum*	*Pythium polymastum*
Pythium violae	*Pythium debaryanum*	*Pythium aphanidermatum*
Pythium dissotocum	*Pythium vexans*	*Pythium attrantheridium*

Many of the *Pythium* species included in this panel have been found to be horticultural pathogens, whilst others are not often associated with plant diseases but are frequently found in water and compost samples and often require identification to be eliminated from clinic enquires e.g. *Pythium nunn* or *P. oligandrum* are potential important biological control agents

Materials and Methods

Isolate oomycete species from plant and environmental samples across each of the sectors

Background

To raise appropriate antibodies for relevant tests for current UK horticultural practice that do not give 'false positives', it is necessary to assemble and maintain a truly representative and up-to-date collection of oomycete pathogen species alongside non-pathogen species likely to be frequently encountered. This requires collections from all sectors of the industry, and in an ongoing series of nursery and production site visits samples of water, growing media, diseased and asymptomatic plants and swab-tests are collected for isolations and identification of the oomycetes present as well as representatives of more commonly seen fungus genera (e.g. *Penicillium* spp., *Trichoderma* spp., *Fusarium* spp. etc.).

Water samples

Water samples are collected in sterile 1 litre bottles and processed as soon as possible following the membrane filtration/colony plating procedure developed by Efford and Stockbridge House plant clinics and described by Büttner et al., (2014). A measured volume of water (normally 750-1000 ml) is filtered through a 47 mm diameter, 3 µm cellulose nitrate membrane filter using a Nalgene reusable bottle-top filter connected to a vacuum pump. Each membrane filter is then transferred to 5 ml of sterile resuspension medium (0.1% w/v aqueous agar solution) in a universal bottle and shaken vigorously for 3 minutes on a vortex mixer. To obtain isolates of filamentous fungi present in the sample, a single 0.5 ml aliquot of the resuspension medium is spreader-plated onto potato dextrose agar (PDA) in a 9 cm Petri dish. After this, selective antibiotic stock suspension (adapted BNPRA, Pettitt & Pegg, 1991) at 1:100 is added to the suspension and mixed prior to further 0.5 ml aliquots being taken and spreader-plated onto oomycete selective agar (adapted BNPRA) for oomycete isolations. All plates are incubated in the dark at 23^{o}C until colonies are observed, recorded and sub-cultured for collection, identification and potential collection/storage.

Growing medium/soil samples

Small samples of growing media or soil are collected in re-sealable polythene bags and processed (i) by baiting with appropriate plant tissue baits collected at the sampling site for this purpose (e.g. strawberry leaves for samples from a strawberry nursery), and (ii) by dilution and direct plating. Baiting of media is carried out following the procedure of Tsao (1960),

approximately 5g of medium is placed in a sterile glass Petri dish and flooded with sterile pond water (SPW) and submerged by approximately 2mm. Plant tissue baits are prepared by cutting into approximately 3mm squares and these are rinsed in 95% ethanol before being placed, 20 pieces per plate, in the SPW growing medium mix and incubated at room temperature on the bench top. After 24h, ten tissue pieces are removed from each petri dish, blotted on sterile tissue paper and plated onto oomycete-selective agar (adapted BNPRA). Direct plating follows the procedure of Pettitt et al. (2011), approximately 1g of growing medium or soil is added to 100 ml of sterile distilled water (SDW) and shaken vigorously for 5minutes. This suspension is diluted 1:10 and 1:100 in SDW and with the addition of BNPRA antibiotic stock solution at the same rate as added to agar plates, 0.5 ml aliquots of these suspensions are spread-plated onto BNPRA in 9cm Petri dishes and incubated in the dark at 23^{o}C.

Isolations from plant tissues and swab tests

Plant tissue pieces are cut from leading edges of necrosis or from edges 'water soaked' or discoloured areas with a flame-sterilised scalpel and either plated directly onto BNPRA oomycete-selective agar or surface-sterilised first by immersing in 3% sodium hypochlorite for 1 minute followed by 3 washes in SDW and blotting on sterile tissue paper. Root tissue pieces are generally only plated directly onto BNPRA; pieces for plating of 5-10 mm long are collected from areas showing root browning or randomly from the surface of the root ball when no obvious browning is seen, using flame-sterilised forceps. Swab tests follow an adaptation of the basic procedure used by White et al. (1998). Swabs of approximately 30 mm^3 volume are made from absorbent cotton wool and sterilised individually in 0.5 ml Eppendorf vials. To each vial 200 µl of 0.1% w/v sterile agar solution containing BNPRA stock at rate 1:100 is added. Sterile cocktail sticks or sterile forceps are used to hold swabs when used to collect material from surfaces such as footwear, bench tops, hose pipes etc. Once used the swab is returned to its appropriately-labelled Eppendorf vial and taken to the lab where it is either plated directly onto BNPRA or is mixed with 5 ml resuspension medium and taken through the plating procedure described above for membrane filters used for water tests.

Develop a comprehensive horticulture-based *Pythium* and *Phytophthora* isolate collection

Isolate clean-ups and identifications

Colonies of oomycetes and selected filamentous fungi isolated by the procedures outlined above are sub-cultured onto fresh plates of semi-selective agar (either BNPRA, cornmeal/pimaricin/rifamycin agar {pimaricin 100 mg l^{-1}; rifamycin 30 mg l^{-1}, Wakeham et al., 1997} or rifamycin agar). The majority of isolates have been purified by hyphal-tip culture with only a small proportion being the result of single spore isolations (although a large proportion of colonies from water samples can be assumed to originate from single spores). All new isolates are grown through vanTieghem rings (Cother & Priest, 2009), to remove hyphal-surface bacterial contaminants, before sub-culture for identifications and storage. For short-term maintenance cultures are maintained on quarter-strength PDA with rifamycin (30 mg l^{-1}), whilst for longer term storage they are grown on oatmeal agar slopes and refrigerated at 4^{o}C. Cultures are also grown on grass blades autoclaved in filtered pond water, on cornmeal agar and in clarified V8 broth to generate structures for morphologically-based identifications, primarily to genus level (van der Plaats-Niterink,1981; Dick, 1990; Ribiero; 1978; Erwin & Ribiero, 1996; Coker, 1923; Seymour, 1978 etc.), prior to selection for verifications using ITS sequencing.

Mycelium for rDNA extraction is grown on a 0.45 µm Supor membrane placed on the surface of 9 cm V8 agar plates. After 7 days of growth at 20^{o}C in the dark, membranes are lifted from the agar medium, initial inoculation agar plugs are removed and mycelium is released into sterile quarter-strength PBS solution by agitation with an L-shaped spreader. Mycelium is then separated from the PBS solution by centrifugation at 4000 rpm for 10 minutes and the pellets are stored at -20^{o}C prior to DNA extraction and PCR amplification. DNA is extracted from mycelium pellets using FastDNA Spin extraction kits (MP Biomedicals LLC) and amplified following the procedure of Cooke et al. (2000) using the universal primers ITS6^{a} and ITS4 (ITS6^{a} GAAGGTGAAGTCGTAACAAGG, and ITS4 TCCTCCGCTTATTGATATGC, White et al., 1990; Cooke et al., 2000). Amplified DNA is cleaned using QIAquick PCR purification kits (Qiagen Ltd), prior to sending to The Functional Genomics and Proteomics Laboratories at the University of Birmingham School of Biosciences for sequencing. Samples sent contained 5-10 ng of PCR product and 3.2 pmol μl^{-1} of one of the primers used for amplification (ITS6^{a}), made up to 10 µl with molecular grade water (Just Water, Microzone Ltd). Sequence data were processed using BLAST (Basic Local Alignment Search Tool)

analysis with the closest match being used to give identifications to be back-checked where possible by further morphological assessment.

Culture collection

In addition to the collection of oomycetes and filamentous fungi collected from current horticultural businesses, isolates of key pathogen, potential biocontrol and some unidentified oomycete species have been obtained from Stockbridge Technology Centre, East Malling Research and the Centraal bureau voor Schimmelcultures (CBS). These isolates have all been taken through the clean-up, identification and long-term storage procedures described above.

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

Background

A wide range of water treatment techniques are used in UK horticulture to kill pathogens in the water. Killed and non-viable pathogen cellular material often remains in treated water and can give positive results in immunological tests (Wedgwood, 2014). An aim of this work is to develop a diagnostic test that can identify a molecule present in living pathogen material and discriminate non-viable material and therefore measure water treatment efficacy.

Zoospores are the key infective propagule of the water-borne oomycetes. During the early phases of germination zoospores encyst. Encystment can also be a survival reaction, as under certain conditions zoospore cysts can enter medium length periods of stasis and act as survival structures. Nevertheless, active encystment provides a very good indicator of spore viability. For successful infection it is important for newly-forming cysts to adhere to host surfaces before penetration (Hardham 2001;Tucker and Talbot 2001), and minutes after attachment to a solid surface an adhesive is secreted onto the ventral surface of encysting zoospores and the spores become sticky (Hardham and Gubler 1990). The adhesive process is part of the process of transformation of zoospores into cysts. Within 20–30 min the encysted spore is able to penetrate the underlying plant tissue. Adhesion is essential because a zoospore that is developing into a cyst must not be dislodged from a potential infection site, and because a mature cyst must be sufficiently glued to the host surface to provide leverage for successful penetration of the host tissue (Epstein and Nicholson, 2006). A number of molecule types have been reported in aspects of zoospore adhesion. Göernhardt et al. (2000) identified the car90 protein on the surface of *Phytophthora infestans* germlings. This protein is transiently expressed during cyst germination and appressorium formation. Gaulin et al.

(2002) identified the CBEL (cellulose-binding elicitor lectin) glycoprotein, which in transgenic strains lacking CBEL (DNA for transformation was prepared in *E. coli* strain), did not adhere to and develop on cellulose during *in vitro* studies. Evidence perhaps, that CBEL production could be used as a viability indicator. The zoospore trapping immunoassay (ZTI) developed by Wakeham et al (1997) utilises the collection of oomycete zoospores through a 5µm cellulose nitrate membrane filter. After 3-6h (or overnight) incubation, oomycete presence and viability is by immunodiagnostic analysis with specific attachment of a red pigment to attached zoospore germlings. Similarly, oomycete CBEL production has the potential to be monitored directly on cellulose filtrate membranes.

Two approaches have been investigated towards the development of an oomycete 'viability' marker and are described below:

Development of a monoclonal antibody to encysted zoospores

Immunogen (antigen) production: Isolates of *Phytophthora cactorum, P. cinnamomi* and *P. citrophthora* (Table 1) were grown on potato dextrose agar plates (PDA). According to the method of Pettitt et al (2002) young mycelial mats were produced by inoculating 15 ml aliquots of clarified V8 broth (Johnston & Booth, 1983) in 9 cm Petri dishes with plugs of mycelium taken from the edge of actively growing cultures on potato dextrose agar. After 5–7 days' incubation in the dark at 20°C, mycelial mats were transferred to starvation conditions by decanting the V8 broth and rinsing twice with sterile pond water (SPW). Rinsed mycelial mats were then replaced in their Petri dishes with 15 mL SPW and incubated at 20°C for a further 1–3 days to allow the development of sporangia. Synchronous zoospore release was achieved by chilling cultures containing sufficient sporangia at 4°C for 1 h. Suspensions of zoospores were collected in 50ml Falcon tubes after a further 2 h at room temperature on the laboratory bench. To induce encystment, a 15 min. settle period was followed by vortexing the tubes for 30 seconds. After centrifugation at 1300 x g for 10 mins, the liquid phase was removed and the encysted zoospores of each *Phytophthora* species were resuspended in PBS (phosphate buffered saline solution) and combined to provide a final zoospore concentration of $1.26x10^5$ ml^{-1}. The encysted zoospore suspension was aliquoted into 50 µl lots and stored at -20°C.

Immunization: Three female Balb C/Cj substrain mice were each immunised for induction of antibody secreting spleen cells (Kohler and Milstein, 1976) with 50µl of encysted zoospore antigen mixed with an equal volume of Titermax adjuvant (Sigma-Aldrich T-2684). The same mice were immunized on two further occasions at 28-day intervals with adjuvant. Collected tail bleeds (Kohler and Milstein, 1975) were titrated against their respective homologous

antigen preparation by plate trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) (Kemeny 1991) according to the method of Wakeham et al (2016). A mouse with a high tail bleed (end point> 2000) to each of the encysted *Phytophthora* zoospore preparations was selected. Following a final pre-fusion boost without adjuvant, the spleen was removed four days later. Spleen cell fusions were carried out according to a modified protocol (Kennett et al. 1978) with cell hybridomas topped up with fresh medium on day 7. By PTA-ELISA, the cell culture supernatants were screened 10 days after the cell fusion to the homologous antigen preparation (encysted zoospores of each *Phytophthora* species) as previously described. Cell lines which gave a positive result (>three times the negative control) were isolated for further study.

To determine antigen site expression, zoospores of *P. citrophthora* were applied in ¼ strength PBS solution on multi-well glass slides (model no. MIC3412, Scientific laboratory supplies, Nottingham, UK) and by immunofluorescence probed with each of the cell culture supernatants (Kennedy et al. 1999). Cell lines which produced antibodies that bound to the encysted zoospores were identified and cultivated for further testing.

Table 1. *Phytophthora* isolates used for production of encysted zoospores

Oomycete species	Host
Phytophthora citrophthora	Buxus roots
Phytophthora cinnamomi	Chamaecyparis roots
Phytophthora cryptogea	HNS roots

Viability adhesion marker

As described earlier, oomycetes express molecules that are able to interact with plant cell wall polysaccharides, such as cellulose. This interaction is thought to be mediated by **c**arbohydrate-**b**inding **m**odules that are classified into CBM family 1. The **c**ellulose-**b**inding **e**licitor **l**ectin (CBEL) represents the best described oomycetal CBM. An extracellular glycoprotein of 34-kDa it was first isolated from *Phytophthora parasitica*. This glycoprotein is widespread in the genus *Phytophthora* (Khatib et al., 2004) and in different oomycetes (Vilalba Mateos et al., 1998). Development of a recombinant protein for assessment as a potential viability marker for waterborne oomycetes is reported below.

P. parasitica CBEL corresponding to GenBank ID. X97205 (nucleotide) and CAA65843 (protein) gene 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).

Develop antibody and molecular probes to assist diagnosis of *Pythium* and *Phytophthora* in environmental samples.

Antibody diagnostic probe development

Polyclonal antiserum (Oomycetes genus test): In development of a diagnostic probe for selective discrimination of oomycetes in environmental samples (*Pythium, Phytophthora, Saprolegniaceae* species) an IgG purified polyclonal antiserum is in process. Preparation of the antigen was according to methods described by Wakeham et al (1997) and consisted of a combined soluble fraction of the isolates listed in Table 2. The immunization protocol commenced in May 2016 and according to a protocol described by Wakeham and White (1996).

Table 2. Species used in the production of a 'generic' oomycete polyclonal antiserum

Oomycete species	**Growth medium**	**Sample/Host**
*Phytophthora rubi**	V8 agar	Raspberry
Phytophthora cactorum	V8 agar	Strawberry
Phytophthora cinnamomi	V8 agar	Chamaecyparis roots
Pythium oligandrum	V8 agar	Soil
Pythium irregulae	V8 agar	Soil
Pythium ultimum var. sporangiiferum	V8 agar	Water
Pythium dissotocum	V8 agar	Strawberry
Saprolegnia ferax	V8 agar	Water
Saprolegnia parasitica	V8 agar	Water

**P. fragariae* cannot be used in this work as it is listed in Annex II of the Plant Health Directive 2000/29/EC and requires a licence to work on it irrespective of where the isolate originates. *Phytophthora rubi*, once classified as a variant of *P. fragariae* will be used as a substitute.

Production of monoclonal antisera

Phytophthora (genus) specific test: Monoclonal hybridoma technology was used in the development of a diagnostic probe for selective discrimination of *Phytophthora* species in

environmental samples. Antigen preparation was according to a method described by Wakeham et al (1997) and resulted in a combined soluble fraction of the isolates listed in Table 3. Using a Fast Prep device (Qbiogine FP120, Anachem Ltd, Luton, UK) the sample was mechanically disrupted according to the manufacturer's guidelines (3 x 15 seconds at a speed setting of 5). To prevent overheating and sample denaturation the sample was rested on ice for 5 min between each disruption phase. Thereafter, the sample antigen was transferred to a YM30 microcon centrifugal unit (www. millipore .com) and separated into two fractions of > 30 kDa and < 30 kDa according to manufacturer's guidelines. The <30 kDa fraction was retained and the protein concentration adjusted to 2 mg ml^{-1} prior to storage at -20°C in 50µl lots.

In May 2016, three 50µl sample lots were individually mixed with an equal volume of Titermax adjuvant (Sigma-Aldrich T-2684) and three female Balb C/Cj substrain mice were immunised for induction of antibody secreting spleen cells according to the method of Kohler and Milstein (1996). The same mice were immunized on two further occasions at 28-day intervals. Tail bleeds (Kohler and Milstein, 1975) will be collected in early July and titrated against their homologous antigen preparation by plate trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) (Kemeny 1991) according to the method of Wakeham et al (2016). Results of this and hybridoma production will be available in Year 2 of the study.

Table 3. Species used in the production of a monoclonal serum for *Phytophthora* genus specificity test

Oomycete species	**Growth medium**	**Host**
Phytophthora rubi	V8 juice agar	Raspberry
Phytophthora cactorum	V8 juice agar	Strawberry
Phytophthora cryptogea	V8 juice agar	HNS roots

Phytophthora (species) specific: In preparation of monoclonal antibody diagnostic probes for the selective discrimination of *Phytophthora* species (possible clade groupings) isolates of *Phytophthora rubi* (clade 7)*, Phytophthora cactorum* (clade 1) and *Phytophthora cryptogea* (clade 8) were prepared as described above. However, prior to immunization the antigen for each isolate was kept distinct. The process for production of hybridoma cell lines was as described previously but mice were immunized with either fractions of *Phytophthora rubi*, *Phytophthora cactorum* or *Phytophthora cryptogea*.

Tail bleeds (Kohler and Milstein, 1975) will be collected and titrated against their homologous antigen preparation by plate trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) (Kemeny 1991) according to the method of Wakeham et al (2016) later in 2016. Results of this and hybridoma production will be available in Year 2 of the study.

Pythium (genus) specific test: As previously described, antigen preparation for the development of monoclonal antibody probes was according to a method described by Wakeham et al (1997) and resulted in a combined soluble fraction of *Pythium* isolates listed in Table 4. Using a Fast Prep device the sample was mechanically disrupted according to the manufacturer's guidelines (3 x 15 seconds at a speed setting of 5). As previously described, the sample was rested on ice for 5 min between each disruption phase.The sample antigen was thereafter transferred to a YM30 microcon centrifugal unit (www.millipore.com) and separated into two fractions of > 30 KDa and < 30 KDa. The <30 KDa fraction was retained and the protein concentration adjusted to 2 mg ml^{-1} prior to storage at -20°C in 50µl lots.

Immunization is scheduled for later in 2016 and will consist of three 50µl sample lots individually mixed with an equal volume of Titermax adjuvant (Sigma-Aldrich T-2684). Three female Balb C/Cj substrain mice will be immunised for induction of antibody secreting spleen cells according to the method of Kohler and Milstein (1996). The same mice will be immunized on two further occasions at 28-day intervals without adjuvant. Tail bleeds (Kohler and Milstein, 1975) will be collected and titrated against their homologous antigen preparation by plate trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) (Kemeny 1991) according to the method of Wakeham et al (2016). Results of this and hybridoma production will be available in Year 2 of the study.

Table 4. Species used in the production of a monoclonal serum for *Pythium* genus specificity.

Oomycete species	**Growth medium**	**Host**
Pythium aphanidermatum	V8 juice	Tomato roots
*Pythium sylvaticum**	V8 juice	Soil
Pythium irregulare	V8 juice	Sweet William
Pythium intermedium	V8 juice	Root material
Pythium ultimum	V8 juice	
*Pythium splendens**	V8 juice	
*Pythium deliense**	V8 juice	
*Pythium debaryanum**	V8 juice	

**environmental sample*

Pythium (species) specific test: In preparation of monoclonal antibody diagnostic probes for the selective discrimination of *Pythium* species (possible clade groupings) isolates of *Pythium aphanidermatum (*Clade A), *Pythium debaryanum* (Clade F) and *Pythium ultimum* var. *sporangiiferum* (Clade I) were prepared, as described previously. Once collected as a fraction of <30kDa each *Pythium* species was individually adjusted to 2mg ml^{-1} and separately stored at -20°C in 50µl aliquots.

On account of monoclonal antibody scheduling, the process for production of hybridoma cell lines is scheduled for early 2017. At this time three mice will be immunized with either fractions of *P. aphanidermatum, P. debaryanum or P. ultimum*. Tail bleeds (Kohler and Milstein, 1975) will be collected and titrated against their homologous antigen preparation by plate trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) (Kemeny 1991) according to the method of Wakeham et al (2016). Results of this and hybridoma production will be reported on in Year 2 of the study.

Molecular (DNA) diagnostic probe development

Background: Oligonucleotides with sequence heterogeneity for regions of the ITS have been described for *Pythium* (Tambong et al 2006) and *Phytophthora* species (Chen et al., 2013). The present study aims to utilise a selection of these oligonucleotide sequences to develop a MAGPIX array. This should provide a versatile multiplexing platform capable of performing qualitative and quantitative analysis of target oomycete species in a single reaction volume and in a variety of sample matrices.

For DNA application, the MAGPIX technology relies on coloured magnetic spheres coupled to oligonucleotides (capture probes) to capture complementary DNA sequences. To allow simultaneous detection of multiple species in the same reaction vessel a different bead colour set is used for each target species. There is potential to develop a 50 bead set assay, the principle of which is described below (Figure 2).

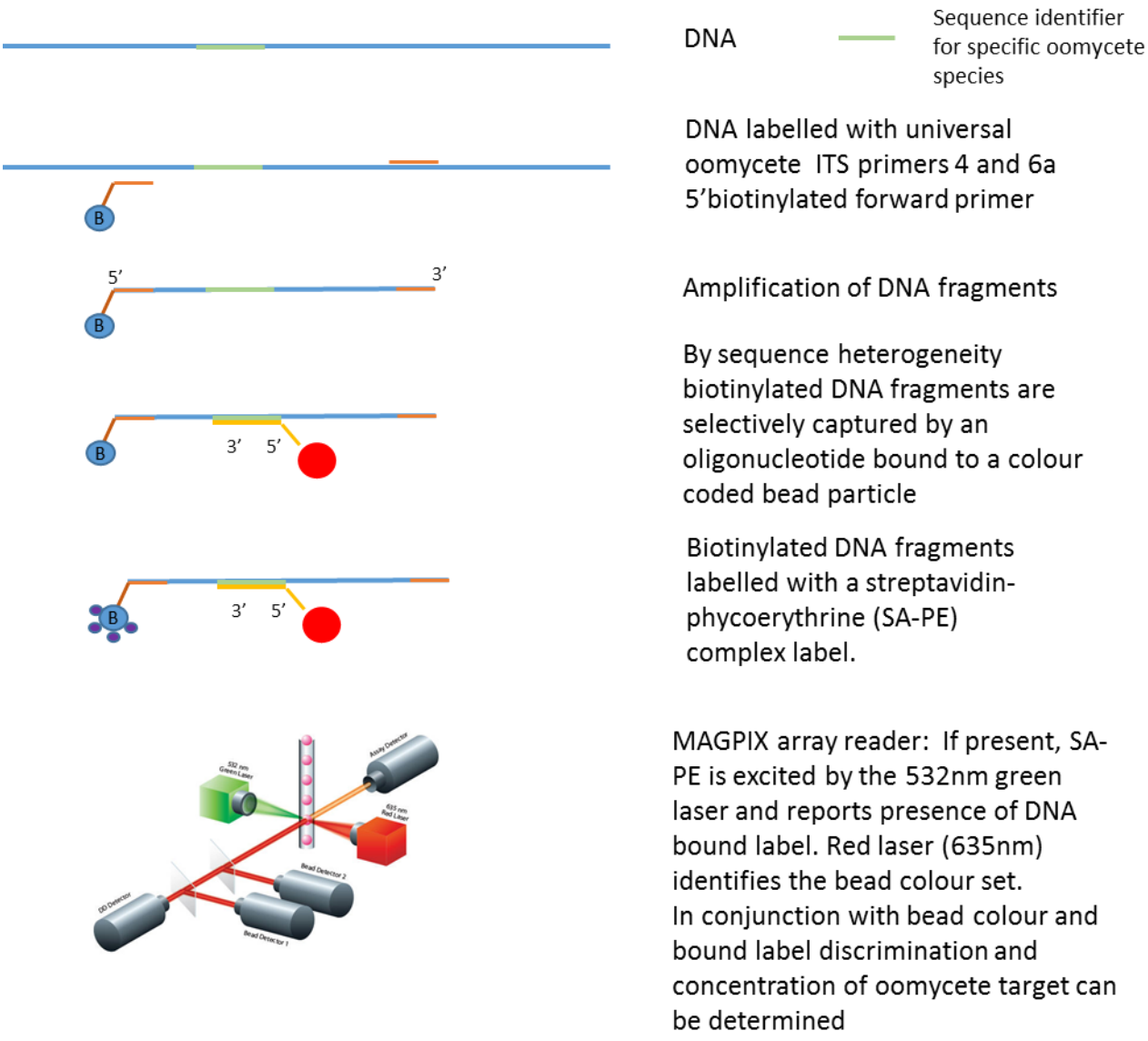

Figure 2. Schematic representation of DNA MAGPIX array process

MAGPIX Assay development: Oomycete species specific capture probes were created with amino-modified 3' terminus followed by 12-C spacers and species specific oligonucleotides (Sigma). Sequences are shown in Table 5. The sequence specific probes were coupled to MagPlex Microsperes (Luminex) using the direct hybridization protocol as suggested by the manufacturer (xMAP technology Cookbook). $5x10^6$ microspheres were re-suspended and sonicated, then pelleted using a magnet. Microspheres were then re-suspended in 0.1M 2-(N-Morpholino) ethanesulfonic acid (MES) buffer pH 4.5 and mixed with 0.2nM of capture oligonucleotide. 2.5µl of freshly made 10mg/ml 1-ethyl-3-(-3-dimethylamino) propyl carbodiimide hydrochloride (EDC) was added, vortexed, and incubated at room temperature for 30 minutes protected from light. Another 2.5µl of freshly made 10mg/ml EDC was added,

and, after vortexing, the tube incubated for 30 minutes at room temperature protected from light. Coupled beads were washed using 1ml of 0.02% Tween20 (Sigma) and suspended in 1ml of 0.1% sodium dodecyl sulfate (SDS). Finally, beads were pelleted using a magnet and suspended in 100µl Tris-EDTA (TE) pH 8.0. Beads were vortexed and sonicated after each wash.

Table 5. Sequences of oligonucleotides used to detect species specific DNA, and biotinylated probes used to check coupling efficacy

Species	**Probe type**	**Sequence**
Pythium ultimum var. *sporangiferum*	Capture probe	(AmC12)ACCGAAGTCGCCAAAA
Pythium sylvaticum	Capture probe	(AmC12)TTGGTATATTTGTTTATGCACA
Pythium irregulare	Capture probe	(AmC12)TGTTGCATGCGCGGCT
Pythium ultimum var. *sporangifarum*	Biotinylated probe	(Btn)TTTTGGCGACTTCGGT
Pythium sylvaticum	Biotinylated ITS 6a probe	(Btn)TGTGCATAAACAAATATACCAA
Pythium irregulare	Biotinylated ITS 6a probe	(Btn)AGCCGCGCATGCAACA

Coupling efficacy was tested using biotin tagged complementary oligonucleotides referred to elsewhere as biotin probes (Sigma). Biotin probes were reconstituted according to product insert, in molecular grade water (Microzone), then diluted to 1µM. The analysis method used was that suggested by the manufacturer. Coupled beads were sonicated, and diluted to 76 beads/µl. in TE, and 33µl added to reaction tubes. Tubes were made up to a total volume of 50µl using 200 fM of biotin probe in TE. Tubes were sealed, then heated to 95˚C for 5 minutes to denature the DNA, and allowed to hybridize at 56˚C for 20 minutes. Beads were then pelleted using a magnetic plate and the supernatant removed. Streptavadin coupled phycoerythrin (SAPE) reporter mix was diluted to 5µg/ml using TMAC (xMAP cookbook 2nd Edt.) and 75µl added to each tube. Tubes were returned to 56˚C for 5 minutes, and 50µl analysed using a Luminex MagPix. The same method was used to test biotinylated PCR product, creating a standard curve with a top point of 68pg of DNA, diluted in TE.

A non-specific, biotin labelled probe was used in a competition assay to determine whether it interfered with specific probe binding. Equal concentrations were added to the same tube (1µl of 5µg/ml), with 15µl TE and 33ul *P. sylvaticum* specific beads. The biotin labelled primer used for amplification was used for this purpose, as it should not bind amplified DNA.

Results

Isolate oomycete species from plant and environmental samples across each of the sectors and the development of a comprehensive Horticulture-based *Pythium* and *Phytophthora* isolate collection

During the first year of this project 14 nursery visits to collect representative isolates of oomycete and other species have been completed. In addition a further 16 samples of plant material and/or water samples have been sent by nurseries for isolations. From these activities several hundred isolations have been carried out from which a culture collection has been built up supplemented with isolates supplied by ADAS, Stockbridge Technology Centre, East Malling Research and CBS. So far 39 isolates of *Pythium* sp., 18 isolates of *Phytophthora* sp. and 7 isolates of *Saprolegnia* sp. have been assembled and the majority taken through morphological investigations and identifications by ITS sequencing (Table 6). In addition to oomycetes, a number of filamentous fungus species have also been collected. These consist either of species which can interfere with routine conventional isolation and identification processes (e.g. *Mortierella* and *Mucor* spp.), and species frequently encountered in horticultural production systems (e.g. *Fusarium* and *Trichoderma* spp.). Many more isolates in this group (especially isolates of *Fusarium* and *Trichoderma* spp.), are being held but have not yet been through the time-consuming identification process. Overall the collection currently contains more non-pathogen and 'semi-pathogenic' than pathogenic species. In addition, there are over 40 further unprocessed oomycete isolates from recent nursery visits and clinic samples awaiting identification.

Currently there is also a strong representation of water samples and under-representation of protected ornamentals nurseries although this is being addressed with more nursery visits planned. ITS sequencing is revealing some interesting (and useful) patterns. For example the expected high representation of *Saprolegnia* species in total oomycete counts from water tests has been confirmed and interestingly the majority of isolates so far investigated have turned out to be *Saprolegnia ferax*, a particularly useful non-phytopathogenic indicator species (Table 6). Other species of interest were *Phytophthora gonapodyides* and *P. mississipeae* in water samples. *Phytophthora gonapodyides* has been associated with *Phytophthora* decline in a number of tree species (Greslebin et al., 2005; Corcobado et al., 2010) whilst the pathogenicity of the recently described *P. mississippiae* still remain uncertain (Yang et al., 2013; Copes et al., 2015).

Table 6. Isolates of *Pythium, Phytophthora* and other oomycete species as well as selected filamentous fungi collected and identified so far.

Culture identifier	*Taxonomic identification*	*Source*	*Verified by ITS sequences*	*Confirmed by morph-ology*
	Oomycete species			
UW014	*Pythium aphanidermatum*	Tomato 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	+	-
C341	*Pythium HS group*	Strawberry roots	-	*(+)*
UW075	*Pythium intermedium*	Viola seedlings	+	*(+)*
UW056	*Pythium intermedium*	Sweet William roots	+	-
UW068	*Pythium irregulare*	Echinacea	+	-
UW013	*Pythium irregulare*	Unknown (SH CC298)	+	*(+)*
UW020	*Pythium irregulare*	MOPS/ADAS - unknown	+	+
UW048	*Pythium irregulare*	CBS	+	-
UW021	*Pythium kasmirense*	Choisya roots	*(+)*	*(+)*
UW017	*Pythium lutarium*	Buxus roots	+	*(+)*

Culture identifier	*Taxonomic identification*	*Source*	*Verified by ITS sequences*	*Confirmed by morph-ology*
UW018	*Pythium lutarium*	Spinach seedlings	+	*(+)*
UW062	*Pythium lutarium*	Herbaceous ornamentals irrigation water	+	*(+)*
UW049	*Pythium nunn*	CBS	+	-
UW044	*Pythium oligandrum*	CBS	+	+
UW047	*Pythium oligandrum*	CBS	*(+)*	-
UW079	*Pythium pectinolyticum*	Irrigation water strawberry	+	*(+)*
UW078	*Pythium dissotocum*	Irrigation water (river)	*(+)*	*(+)*
UW051	*Pythium rostratum*	CBS	*(+)*	-
UW046	*Pythium sylvaticum*	CBS	+	-
UW050	*Pythium ultimum var. sporaniferum*	*CBS*	+	*(+)*
C327	*Pythium ultimum*	Lettuce crown/root rot	-	*(+)*
C361	*(Pythium ultimum*	Asparagus shoot rot	-	*(+)*
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	*(+)*	*(+)*

Culture identifier	*Taxonomic identification*	*Source*	*Verified by ITS sequences*	*Confirmed by morph-ology*
UW076	*Pythium sp.*	Irrigation line strawberry	*(+)*	*(+)*
UW080	*Pythium sp.*	Reservoir water	*(+)*	*(+)*
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	-	*(+)*
C291/1	*Phytophthora cactorum*	Strawberry crown (var. *Elsanta*)	-	+
UW043	*Phytophthora cactorum*	CBS	+	+
UW015	*Phytophthora cinnamomi*	Chamaecyparis roots	+	+
UW025	*Phytophthora citrinum*	Filter well bait	+	*(+)*
C290/2 Aii	*(Phytophthora citrophthora)*	Buxus roots	-	*(+)*
UW016	*Phytophthora cryptogea*	HNS roots	+	+
SH CC310	*(Phytophthora cryptogea)*	Geranium	-	*(+)*
UW073	*Phytophthora gonapodyides*	Bait – herbaceous ornamentals drainwater	+	*(+)*

Culture identifier	*Taxonomic identification*	*Source*	*Verified by ITS sequences*	*Confirmed by morph-ology*
UW034	*(Phytophthora lacustris/ gonapodyides)*	Field header irrigation pipe – field veg.	*(+)*	*(+)*
UW071	*Phytophthora missisippiae*	River water	+	*(+)*
UW012	*Phytophthora palmivora*	Ivy – SH CC297	+	*(+)*
UW042	*Phytophthora rubi*	CBS	+	*(+)*
UW064	*Phytophthora syringae*	River water	+	*(+)*
UW028	*Phytophthora sp.*	River water	*(+)*	-
UW066	*Phytophthora sp.*	Strawberry drainwater	*(+)*	*(+)*
C344/1	*Phytophthora sp.*	Taxus roots & collar	-	*(+)*
C344/2	*Phytophthora sp.*	Poinsettia roots	-	*(+)*
SH CC312	*Phytophthora sp.*	Nemesia	-	*(+)*
UW044	*(Saprolegnia aenigmatica)*	CBS Saprolegnia parasitica	*(+)*	-
UW057	*Saprolegnia australis*	Reservoir water	+	*(+)*
UW029	*Saprolegnia ferax*	Greenhouse roof swab	+	*(+)*
UW035	*Saprolegnia ferax*	River water	+	*(+)*
UW067	*Saprolegnia ferax*	Drainwater strawberry	+	*(+)*
UW080	*Saprolegnia ferax*	Irrigation line	+	*(+)*

Culture identifier	*Taxonomic identification*	*Source*	*Verified by ITS sequences*	*Confirmed by morph-ology*
UW030	*(Saprolegnia ferax)*	River bait	+	*(+)*
	Non Oomycete species			
C271	*Fusarium sp.*	Untreated irrigation water	-	*(+)*
C272	*Fusarium oxysporum*	*Echeveria glauca* collar rot	-	+
C301	*Fusarium oxysporum*	Carnation root rot	-	*(+)*
UW027	*Mortierella elongata*	Brunnera root	+	*(+)*
UW081	*Mortierella parvispora*	Irrigation water tank	+	-
UW011	*Mortierella zychae*	SH CC296	+	-
UW027	*Mortierella sp.*	Irrigation tank	*(+)*	-
UW070	*Mortierella sp.*	Poinsettia drainwater	*(+)*	-
UW019	*Mucor circinelloides*	Brunnera root	+	-
UW026	*Pholiota adiposa*	River water	+	-
UW031	*Pholiota sp.*	Irrigation holding tank bait	+	-
C366	*Trichoderma sp.*	Irrigation water	-	*(+)*

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

Antibody diagnostic probe development (viability marker)

Each of the mice immunized with encysted zoosporic antigen reported an immune response when tested against homologous antigen by PTA ELISA (Figure 3). The mouse with the highest tail bleed titre (Mouse 1) was selected for the initial hybridoma fusion.

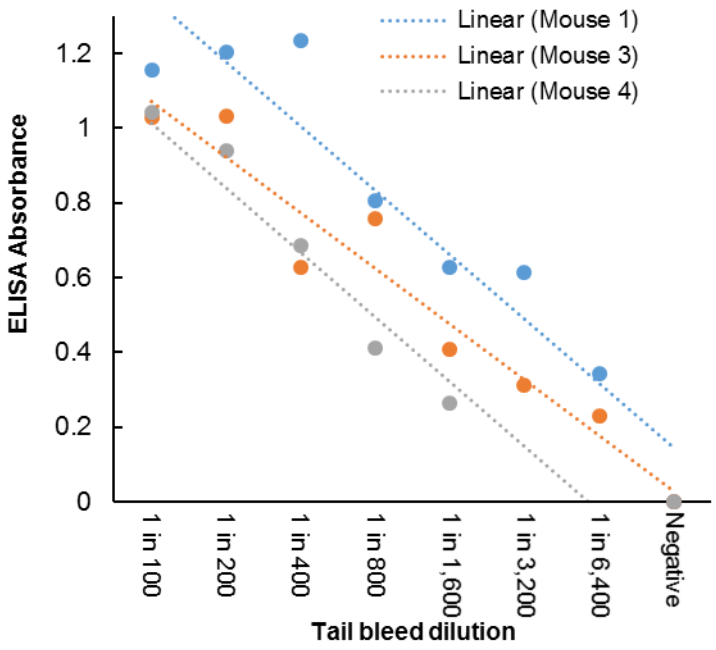

Figure 3. Serum immune response to homologous antigen of mice immunized with encysted zoosporic antigen as reported by PTA-ELISA.

At the time of writing this report (June 2016) 5 cell lines from fusion 1 have been identified by PTA ELISA as producing antibodies positive to the homologous antigen (oomycete zoosporic material). The cell lines are in the process of being cloned for stability and further study. By immunofluorescence, a number of the cell lines produced antibody types which were visualised as binding to epitopes present either on the encysted zoospore, soluble material exuded from the propagule or associated with the mycelial stage (Figure 4).

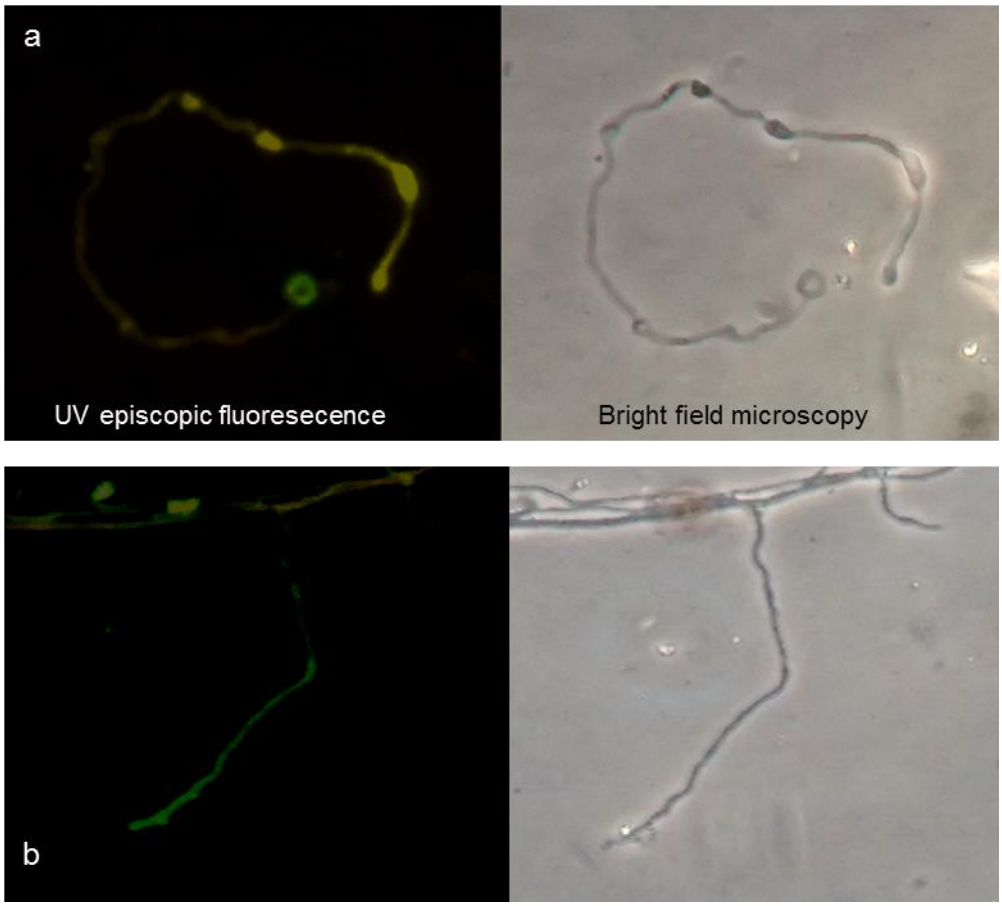

Figure 4. Encysted zoospore (a) and mycelial tip (b) recognition sites bound by tissue culture supernatant antibodies from hybridoma cell lines of Mouse 1, labelled a fluorescence marker and viewed by episcopic fluorescence (x400)

Viability adhesion marker

A DNA expression construct has been propagated in *E.coli* strain TOP10 to provide glycerol stocks and DNA purified for long-term archiving of the gene. An expression scale-up and purification of the recombinant CBEL is in process.

Develop antibody and molecular probes to assist diagnosis of *Pythium* and *Phytophthora* in environmental samples.

Antibody diagnostic probe development

Polyclonal antiserum (Oomycete genus test): The immunization schedule is in process and production of an IgG purified serum is scheduled for completion in July 2016.

Monoclonal antisera: For production of oomycete positive hybridoma cell lines, soluble antigen has been collected as fractions of <30kDa and stored at -20°C. An immunization schedule has been prepared and the first set of mice injected (*Phytophthora (genus) specific test)*. Hybridoma fusions for the remaining test formats (*Phytophthora (species / clade grouping) specific test, Pythium (genus) specific test, Pythium (species / clade grouping*) are due to take place throughout 2016 and into 2017.

Molecular (DNA) diagnostic probe development

MAGPIX Assay development: Dilution curves have been generated using biotin labelled complementary oligonucleotides, and DNA amplified from P. *sylvaticum* (Figures 5 and 6 respectively). The method used has good reproducibility with a mean CV of 6.975%. Both probe and DNA amplicon are detectable at similar concentrations, although probe gives higher fluorescence readings. Limit of quantification was estimated to be 14.8pg in 50µl reaction mix (22 fM). Non-specific biotin coupled probe does not interfere with signal produced by *P. sylvaticum* specific probe (Figure 7), there is no significant difference between the signals generated by *P. sylvaticum* specific probe alone, and *P. sylvaticum* probe with non-specific probe**.**

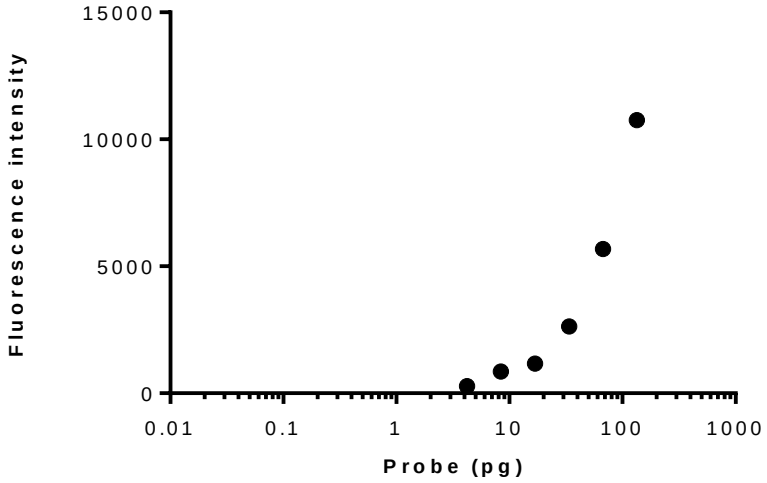

Figure 5. Fluorescence intensity produced by binding of *Pythium sylvaticum* specific beads to a biotin labelled complementary probe.

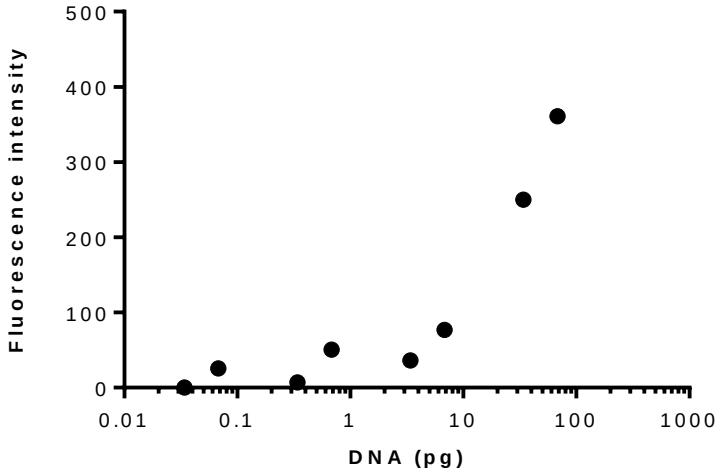

Figure 6. Amplified PCR product using DNA isolated and amplified from *Pythium sylvaticum*, shows pg per reaction

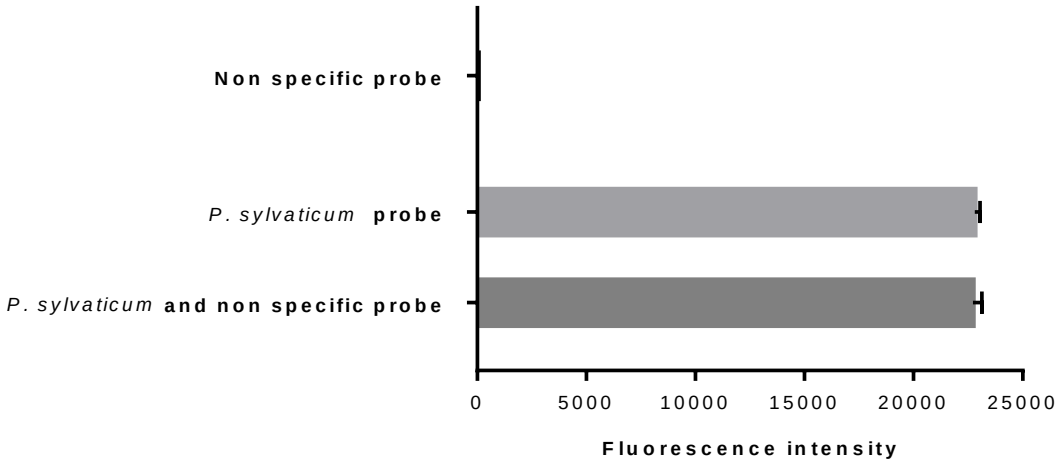

Figure 7. *Pythium sylvaticum* specific probe binding is not inhibited by competition with a nonspecific probe. Shows mean and standard error

Discussion

Current Oomycete culture collection: Of the 39 cultures of *Pythium* so far collected, 18 are known phytopathogens of varying virulence and host range. A further four species are relatively new and/or of unknown pathogenicity and another three are non-pathogenic (two of these are the mycophagous species *P. nunn* and *P. oligandrum*). Eleven Pythium isolates still remain to be identified to species level. Five of the 18 *Phytophthora* isolates collected have not yet been identified to species level, the remaining 13 species are pathogenic. These isolates are of importance in determining the sensitivity and specificity of detection assays. The isolation and identification activities involved in this study have served to verify that there are large numbers of propagules of non-pathogenic oomycetes present in most horticultural production systems. It is important to collect isolates from these groups as well as the pathogens to test for cross-reactivity of new detection assays and avoid the development of assays prone to 'false positive' tests. The most common non-phytopathogenic oomycetes found so far are members of the genus *Saprolegnia*, the most prevalent species is *Saprolegnia ferax*. This observation verifies previous assumptions based on morphological characterisations that *Saprolegnia* spp. and *S. ferax* in particular, predominate in irrigation water supplies and that this species provides an excellent indicator organism for testing water treatment efficacy (Pettitt, unpublished; Pettitt, 2016). The mycophagous *Pythium* species (*P. periplocum*, *P. oligandrum* and *P. nunn*), which have great potential for biocontrol, also have the capacity to confound pathogen detection assays.

Oomycete viability test. 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 tests. 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: 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 identified with an aim to develop a diagnostic probe to selectively differentiate viable from non-viable oomycete material. By immunizing mice directly with a 'mix' of native molecules associated with oomycete zoospore encystment there is potential to elicit an immune response and identify a suitably specific probe by monoclonal antibody technology. Screening hybridoma tissue culture supernatants by immunofluorescence has identified cell lines selective of molecules associated with *Phytophthora citrophthora* cyst and zoospore cycle stages. Additional hybridoma fusions will take place in 2016. Positive cell lines will be tested for reactivity towards other oomycete species (horticulture oomycete culture collection) and identification of other specific stages of the oomycetes life cycle i.e. cyst germ tube and soluble material associated with adhesion and germination.

The second approach towards achieving a probe with selective reactivity to a specific oomycete marker has been the development of a purified recombinant protein molecule. For use at the hybridoma screening stage and assessment as a potential immunogen. For this purpose, we have chosen a recombinant protein which is well characterised and associated with oomycete adhesion (CBEL). To achieve recombinant protein production, there are a number of expression systems available. These include *Escherichia coli*, baculovirus-mediated insect cell expression, yeast, and several mammalian based systems. Each has its advantages and disadvantage however only expression systems utilizing mammalian cells for recombinant proteins are able to introduce proper protein folding, post-translational modifications, and product assembly, which are important for complete biological activity. Disadvantages of mammalian cell protein expression are however expense, expression time and protein yield can be low. Also, the use of mammalian hosts for expression of correctly folded protein, presented to the animal, reside as small immunogenic fragments. Therefore, use of the expression systems will not guarantee the de novo generation of antibodies recognising native protein (personal communication Dr I Phillips). One of the organisms of choice for the production of recombinant proteins is *E. coli* and has become the most popular expression platform. *E. coli* is also reported as a suitable host for expressing stably folded, globular proteins from prokaryotes and eukaryotes (Rosano and Ceccarelli, 2014). Where membrane proteins and proteins with molecular weights above 60 kDa can be difficult to express, several reports have had success using this approach (Rosano et al., 2011). The CBEL protein is reported a molecular weight of 32 kDa and successfully used by Gaulin et al (2002) for production of *Phytophthora* transgenic strains lacking CBEL production. For this reason the method of Gaulin was followed and using the *E.coli* strain BL21(DE3) for gene expression.

Oomycete species immunoassay test development (diagnostic probes)

Polyclonal antisera (*selective on-site test for oomycete species*): Polyclonal antisera (antibodies isolated from blood serum of immunised animals) were first deployed in a phytopathological context for the detection of viruses and bacterial plant pathogens in infected plant tissues (Voller *et al*., 1976; Clark & Adams, 1977). The potential of this approach for the detection of fungal and oomycete pathogens has been tried. However, the poor specificity achieved to the structurally more complex fungal and oomycete pathogens (Drouhet, 1986) has hampered their use for commercial application. Like fungi, oomycetes share conserved glycoprotein antigens that can induce a highly immunogenic and immuno-dominant response in the immunised animal. These include carbohydrate and protein complexes. Of which the CBEL glycoprotein is an example of and plays an important role as a cell surface biomarker (pathogen associated molecular pattern (PAMP)) (Larroque *et al*., 2013). The potential for a diverse mix of antigens and immunodominant epitopes will therefore give rise to multiple antibodies against different epitopes of variable binding affinities and avidities.

In this study there is a requirement in test development for a diagnostic probe that can identify broadly at the oomycetes level so requires a probe that is not limited by specificity to a specific isolate(s). Polyclonal antisera offer multiple antibodies which can be directed against a range of targets so could provide recognition broadly across the oomycete genera. However, a polyclonal serum will consist of various antibody types which will differ in function and antigen responses. In test development this can be problematic in terms of specificity. Five major antibody types exist of which approximately 80% IgG, 15% IgA, 5% is IgM, 0.2% IgD, and a trace of IgE. In year 1 of this study, we have a developed a polyclonal antiserum which we hope to use selectively to identify oomycete species in environmental samples. However, with multiple antibody types, the potential for reactivity outside of the genus is high. By purification of the IgG fraction we anticipate that improved specificity can be gained. Part of the rationale for this is that the IgM fraction, which is associated with lower affinity and made predominantly during the early stages of exposure to foreign antigen, is associated with binding of glycoprotein structures. As reported earlier, the glycoprotein fraction of both fungal and oomycete immunogen is reported to predominantly elicit an immunodominant response in the host. Often, IgM isotypes are routinely selected in the production of monoclonal antibodies towards fungal receptors. Conversely, the IgG fraction, specifically IgG 2a, 2b and 3 are associated with recognition towards peptide regions of an epitope.

Once IgG purified, the oomycete positive polyclonal serum will be assessed in Year 2 for reactivity across the broad spectra of environmental isolates collected in this project. If reactivity is observed outside of the oomycete genera the combination of polyclonal and monoclonal (monospecific antibodies that are made by identical immune cells that are all clones of a unique parent cell) antisera can be assessed in a diagnostic format. For the detection of viral and bacterial samples this has been found to be beneficial in respect to test specificity and sensitivity. However, for fungi and oomycete assays this is often not the case. Probably a result of soluble glycoprotein structures which do not lend functionally to the binding of two antibody types at one time. For this reason, many of the diagnostic assays developed for fungal and oomycete crop pathogens rely on direct binding of the antigen to the solid phase surface e.g. plate trapped antigen ELISA and competitive lateral flow assay. Should this situation arise improved specificity may still be attained with the polyclonal antiserum by using a cross-absorption technique (Wakeham and White, 1995).

Monoclonal antisera *(oomycete species and clade specific on-site tests).* Where increased test specificity is required the use of monoclonal antibodies have been found useful to selectively discriminate oomycete epitopes at the species, isolate and stages of the life cycle ((*Phytophthora cryptogea zoospore)* Estradsa-Garcia et al., 1990). They can be used alone or in combination with either monoclonal or polyclonal serum. In this study, monoclonal cell lines have been developed (or in stages of production) to a range of *Pythium* and *Phytophthora* species. Given the requirement to selectively measure at both the genus and species level it was decided to approach the task of antigen production by the isolation of molecular-weight fractions. MacDonald (1989) reported that non-specific antibodies generally recognize antigen only in the high molecular weight range ($M_{r>}$150,000) whereas specific antibodies were directed against antigens in both the high and lower molecular weight range (M_r10,000). When mice were immunised with low molecular mass (<30 kDa) *Botrytis cinerea* and *Aspergillus flavus* culture washings a reduction in non-specific cell lines were observed (Bossi and Dewey, 1992; Priestley and Dewey, 1993). In this study, mice have been immunised with oomycete low molecular mass antigenic material (30kDa).

Molecular (DNA) diagnostic probe development

Published oligonucleotide array markers (Tambong et al., 2006) have been used towards the development of a MAGPIX Luminex array system for quantitative measurement of *Pythium* species. Preliminary results identify successful labelling of array beads with complementary oligonucleotides for *Pythium ultimum*, *P. irregulare* and *P. sylvaticum*. Detection and quantification of *P. sylvaticum* has been shown with a detection limit of 14.8pg DNA in a 50µl reaction mix. It has been estimated that for *Colletotrichum* species 10 fg of DNA could represent as little as 100 picograms of mycelium, and provide the potential for sub-clinical detection in infected host plants (Mills, 1994). Studies will now look to optimise and validate the standard curve for *P. sylvaticum* and check for cross-reactivity with other species at different concentrations. Using different coloured beads, oligonucleotides specific for *P. ultimum* and *P. irregulare* will now be tested to produce an assay capable of detecting the presence of all three simultaneously. From this point, other species will be added sequentially.

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