Grower Summary

CP 136

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

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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.

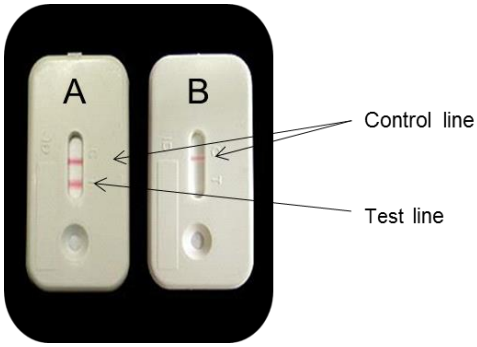

Figure 1. Visual assessment (by eye) of a qualitative double antibody sandwich lateral flow assay for disease risk. A - Control and test line development indicates risk of pathogen presence; B - Control line but no test line development – low or no risk.

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.