Grower Summary

CP136

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

Final report, August 2018

Project title:	Development and testing of single and multiplex diagnostic devices for rapid and precise early detection of oomycete root and collar rot pathogens for disease avoidance, management and control
Project number:	**CP 136**
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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.

GROWER SUMMARY

Headline

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

Background

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

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

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

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

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

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

Summary

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Financial Benefits

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

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

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

