REVIEW ARTICLE

Diagnostic tests and their application in the management of soil- and water-borne oomycete pathogen species

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Keywords: disease management, diagnosis, crop pathogens,

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Abstract

Oomycete diseases cause significant losses across a broad range of crop and aquaculture commodities worldwide. These losses can be greatly reduced by disease management practices steered by accurate and early diagnoses of pathogen presence. Determinations of disease potential can help guide optimal crop rotation regimes, varietal selections, targeted control measures, harvest timings and crop post-harvest handling. Pathogen detection prior to infection can also reduce the incidence of disease epidemics. Classical methods for the isolation of oomycete pathogens are normally deployed only after disease symptom appearance. These processes are often-time consuming, relying on culturing the putative pathogen(s) and the availability of expert taxonomic skills for accurate identification; a situation that frequently results in either delayed application, or routine 'blanket' over-application of control measures. Increasing concerns about pesticides in the environment and the food chain, removal or restriction of their usage combined with rising costs have focussed interest in the development and improvement of disease management systems. To be effective, these require timely, accurate and preferably quantitatve diagnoses. A wide range of rapid diagnostic tools, from point of care immunodiagnostic kits to next generation nucleotide sequencing have potential application in oomycete disease management. Here we review currently-available as well as promising new technologies in the context of commercial agricultural production systems, considering the impacts of specific biotic and abiotic and other

important factors such as speed and ease of access to information and cost effectiveness.

Introduction

The oomycetes are a large group of fungus-like microorganisms, with representatives in virtually every terrestrial, marine and freshwater habitat worldwide. A significant proportion of the oomycetes are parasitic, colonising and causing disease in a very diverse range of organisms from other protists to higher plants and animals (Beakes *et al*., 2012). Oomycetes cause significant losses across a broad range of agribusiness commodities worldwide, varying from the infamous and still highly destructive *Phytophthora* 'blight' of potatoes (Bourke, 1991), to *Saprolegnia* 'saprolegniosis' in farmed fish (Van West, 2006). In addition to these highly destructive diseases, many oomycete pathogens cause yield losses by attritional damage, for example in cereals where *Pythium* spp. causing lateral root necrosis have been dubbed the 'common cold of wheat' (Cook & Veseth, 1991), as well as storage rots (Cullen *et al.,* 2007), and declines in infected produce quality (Guehi *et al*., 2008).

Economic losses resulting from disease development can be reduced by accurate and early detection of pathogens. Accurate although not necessarily overly precise diagnosis (in many instances identification to genus is quite sufficient) is 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. Unfortunately, methods currently commonly adopted for the isolation and diagnosis of many pathogens are slow and normally, only deployed after disease symptoms have become apparent. In agricultural and horticultural production where profit margins are narrow and a policy of 'zero-tolerance' of disease expression in crop products has generally been applied by retailers, a culture of blanket fungicide applications has developed. With increasing global pressure to reduce pesticide inputs this approach will no longer be tolerated. In Europe, the recent introduction of the Sustainable Use Directive (SUD) (http://ec.europa.eu/food/plant/pesticides/sustainable_use_pesticides/index_en.htm) requires producers to demonstrate that they have taken alternative integrated pest and disease management (IPDM) measures to prevent pest & disease development before the use of spray applications of insecticides and/or fungicides. This situation will increase the pressure on producers and their staff to monitor and identify potential disease and pest problems quickly.

Pathogen detection prior to infection can reduce or even prevent disease epidemics by identifying when and where treatments and avoidance measures need to be applied. The timely detection and identification of economically important diseases in a commercial production environment provides the initial key to drive a successful and informed control strategy. It is however only part of the solution, the success of which will depend on how the information is evaluated and incorporated within integrated disease management systems (IDMS). Here we describe currently-

available and emerging techniques for the practical detection and diagnosis of oomycete pathogens and their potential role in the development of IDMS for soil and water-borne oomycetes.

Baiting, isolation and direct culturing of live propagules and identification by morphological characters

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*, *Phytophthora and Aphanomyces* species (Papavizas & Ayers, 1974; Ribeiro, 1978; Tsao, 1983; Hong *et al*., 2002; Pettitt *et al.*, 2002). Unfortunately, these methods often tend only to be used after disease symptoms have been observed and even then take valuable time to implement and interpret. The detection methods commonly used are those of baiting, culture plating, or a combination of both (Pittis & Colhoun, 1984). Whilst these procedures are 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 (Hüberli *et al*., 2000; Collins *et al*., 2012). Nevertheless, they do provide direct estimates of pathogen viability and allow the collection of representative cultures of live pathogen (Cooke *et al*., 2007) that can be used for determinations of pathogen 'fitness' by *in vitro* measurement of parameters such as sporulation, growth characteristics, pathogenicity, virulence and resistance to fungicides. Direct quantitation of pathogen propagules or inoculum can be achieved from soil by dilution plating (Tsao, 1983), from water by membrane filtration-resuspension plating (Pettitt *et al*., 2002; Büttner *et al*., 2014) and from plant tissues by comminution followed by plating dilutions onto selective agar plates and counting the resulting colonies (Pettitt & Pegg, 1991). Baiting techniques have been used since the 1960s for both *Phytophthora* and *Pythium* detection in water and in soils (Werres, *et al*., 2014), 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 (Themann *et al*., 2002; Hüberli *et al*., 2000). Baiting procedures are also likely to give a skewed picture of the potential pathogens present (Arcate *et al*., 2006) and are really best deployed for the detection of specific pathogen species using specific plant tissues. Nevertheless, they can provide confirmation of disease presence and have a limited capacity for quantitation *e.g*. by the most probable number (MPN) method (Tsao, 1960 & 1983). The main drawback of these 'conventional' techniques is the time required to generate information; measured in days rather than hours, which is 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 generally leading to ineffective targeting and overuse, and consequently resulting in the build-up of widespread fungicide resistance (White & Wakeham, 1987) and even lost efficacy resulting from enhanced fungicide degradation (Kenny *et al*., 2001). The current best practice 'conventional' diagnostic tests for root and stem rot oomycetes take upwards of 24 hrs with float tests (Ribeiro, 1978; Dhingra & Sinclair, 1995 – specific examples: 24h *Phytophthora*

in strawberry crowns, Pettitt & Pegg, 1994; overnight in HNS roots Pettitt *et al*., 1998) and between 3 and 10 days by conventional agar plating methods (Fox, 1993).

Molecular Approaches to Disease Diagnosis

Pioneering work in the medical field during the latter decades of the last century has provided an array of molecular-based techniques suitable for development of rapid diagnostic test procedures. Many of these have been commercialised to provide simple on-site diagnostic tests for medical use (Posthuma-Trumpie *et al.,* 2009). The defence industry has also provided invaluable input with, highly-sensitive and accurate systems for the detection and evaluation of biological warfare agents. For example the RAZOR® EX Anthrax Air Detection System. These emerging technologies are increasingly moving the emphasis towards nanobiotechnology (Jeong-Yeol & Bumsang, 2012). There is a real expectation that mobile phones will provide a global laboratory platform for many of these approaches (Ozcan, 2014). The molecular techniques considered here fall into two broad categories: immunologically-based assays and nucleotide-based assays, although there is overlap, with some procedures combining techniques from both categories.

***Immunoassays*:**

Background:

Following the work of Yalow & Berson (1959), using anti-insulin antibodies to measure hormone levels in blood plasma, immunological assay systems have provided an important contribution to analytical diagnostic test development. With an array of different labels and detection systems available, measurement of the antibody (immunological diagnostic probe) and antigen (target analyte/disease propagule) can be made quantitative or qualitative. This approach has been found to be highly transferrable, from commercial centralised laboratories offering tests with high throughput, specificity and sensitivity (for example the enzyme-linked immunosorbent assay (ELISA) Harlow & Lane, 1988) to simple point of care (POC) test systems operated by non-specialists. The latter being designed to be used at or near the site where a problem is located, do not require a permanent dedicated space and can provide results quickly (generally within minutes). They can provide quick feedback in many sorts of investigations, for example; enzyme analysis, drugs of abuse, infectious agents, toxic compounds, metabolic disorders, allergens, ovulation and pregnancy testing.

Polyclonal antibodies:

Using polyclonal antisera (antibodies isolated from blood serum of immunised animals), immunoassays 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; Nome *et al*., 1980). The potential of this approach for fungi was demonstrated by Casper & Mendgen in 1979. Later, Johnson *et al*. (1982) reported the diagnosis of *Epichloe typhina* colonization in tall fescue (causing

toxicity syndrome in cattle). Nevertheless, discrimination of the pathogen was limited to genus level in these early studies, and whist the techniques were being successfully applied worldwide for screening plant material for viruses (Raju & Olson, 1985; Burger & Von Wechmar, 1988), the poor specificity achieved to structurally more complex fungal and oomycete pathogens (Drouhet, 1986) hampered the early development of immunologically accurate diagnostic probes for commercial applications (Mendgen, 1986, Barker & Pitt, 1988).

As with fungi, the oomycetes share a complex array of antigenic sites that can induce a highly immunogenic and immuno-dominant response in the immunised animal. These include carbohydrate and protein complexes. For example, the *Phytophthora* cellulose binding elicitor lectin (CBEL-1), which plays an important role as a cell surface biomarker (pathogen associated molecular pattern (PAMP)) (Larroque *et al*., 2013). Mannose-containing heteroglycans such as galactomannans and rhamnomannans have also been identified as important derivatives of cell wall substances with importance towards immunogenic dominance. Enzymatic digestion and competitive inhibition tests demonstrate that galactosyl residues with β-linkages are immunodominant for *Aspergilus*, *Geotrichum* and *Cladosporium* antigens. Mannosyl residues with α-linkages provide immunodominance for *Mucor* antigens (Tsai & Cousin, 1993). The structure and complexity of these pathogens can thus lead to the production of antibodies able to bind selectively to both related and non-related species (Mohan 1989a & b; Notermans & Soentoro, 1986; Da Silva Bahia *et al*., 2003; Viudes, *et al*., 2001; Priestley & Dewey, 1993). This attribute can reduce specificity, consequentially new antibodies always need to be thoroughly screened against a range of target and non-target species when developing tests for specific pathogens/diseases.

Monoclonal antibodies:

With the advent of hybridoma technology (Köhler & Milstein 1975) there has been capability to generate highly specific monoclonal antibodies (MAbs, a single antibody type) which can bind selectively to complementary determining regions (CDRs) of pathogen targets. A similar approach has been adopted using antibody engineering (phage display technologies) to provide single-chain antibody variable fragments (scFvs) (Arap, 2005). Targeted to single epitope sites (CDRs), these diagnostic probes provide the opportunity to discriminate not only between groups of organisms, but also between different genera, species, isolates, and possibly life cycle stages of pathogenic fungi and oomycetes (Dewey *et al*., 1990; Priestley & Dewey, 1993; Keen & Legrand, 1980; Hardham *et al*., 1986, Arap, 2005).

For oomycetes, the ability to identify molecules at a specific stage in a pathogen's life cycle (e.g. zoospores or cysts) has been reported (Estrada-Garcia *et al*., 1990). Whilst this ability is desirable for detailed epidemiological research, such probe specificity does have the potential to be problematic in commercial test development. For example, where pathogens with multiple infective life cycle stages can co-exist.

For this reason the organism and the application of the test should be well understood. To overcome these issues the combination of antibody types (monoclonal and polyclonal) has been found beneficial to achieve an appropriate test specificity and/or sensitivity. Equally, where non-specific binding to host tissue is observed, the use of antibody combinations for capture and labelling of target antigens (target disease component) has also been found useful (Priestley and Dewey, 1993). These early successes have resulted in a rapid expansion of MAb-based immunoassay diagnostic procedures for the qualitative and quantitative measurement of fungal and oomycete pathogens (Dewey *et al*., 1993, Karpovich-Tate *et al*., 1998; Wakeham & Kennedy, 2010; Wakeham *et al*., 2012; Dewey *et al*., 2013 & Thornton & Wills, 2013). Availability of these probes from maintained cell lines may in the future prove a useful resource for fundamental host-pathogen interaction studies.

Enzyme-linked immunosorbent assay:

In test development, Clark and Adams (1977) introduced the use of the enzyme-linked immuno-sorbent assay (ELISA) for the quantitation of plant viruses in host tissues. This system is now used routinely in laboratories worldwide to provide high throughput, quantitative measurement of contamination/infection by viral, bacterial, fungal and oomycete plant pathogens in a range of environmental samples (Singh & Singh, 1995, Fang & Ramasamy, 2015). For viral and bacterial samples many of the commercial ELISA systems use a double antibody sandwich format (DAS ELISA). This format can prove useful in capture and isolation of target pathogens from complex materials and the attachment of a second antigen-specific labelled antibody, can provide improved specificity. A second type of ELISA is the plate trapped antigen (PTA ELISA). This assay is often reported for use in the diagnosis of fungal and oomycete plant pathogens (Dewey and Thornton, 1995, Wakeham *et al*., 2004). Antibodies raised to these targets are often directed to glycoprotein structures which bind readily to the solid phase surface of an ELISA process and so do not require a capture antibody (Kemeny, 1991; Nicolaisen & Justesen, 2007). Also, many soluble glycoprotein structures do not lend functionally to the binding of two antibody types at one time. Where these structures prove heat stable, this characteristic can be used in sample treatment to mitigate issues of antibody cross-reactivity (Dewey *et al*., 1997). The third type of ELISA system used routinely is the competitive ELISA (c ELISA). This format is used extensively in the detection of mycotoxins in food, pesticides in ground water and has been reported for the measurement of some soil-borne fungi and oomycetes in plants and soil. However, the development of such assay systems for the measurement of plant pathogens in environmental samples, especially soil, is particularly challenging. A good example of this problem is seen with carrot cavity spot caused in the UK by *Pythium violae* and *P. sulcatum* (Lyons & White, 1992; Hiltunen & White, 2002). A largely cosmetic disorder, cavity spot can rapidly render carrot crops unmarketable close to time of harvest, and since carrot crops are often grown in changing locations under contract and both species of *Pythium* have broad host ranges, a reliable test for the disease

risks of new fields is highly desirable. Based on the work of Lyons & White (1992), a laboratory diagnostic competitive ELISA test was developed to monitor pathogen oospore concentrations in soils (White *et al.* 1995, 1996, 1997). Although this procedure showed some promise, results for oospore detection did not always tie up well with subsequent observed disease. Such results might in part be explained by variation in environmental conditions during cropping as cavity spot disease incidence and severity can increase rapidly in wet conditions (Suffert & Montfort, 2007), but other factors may also have confounded test results. For example, the assay used polyclonal antiserum, and since many oomycete species are found naturally-occurring in soil, the antibodies used may have reacted with these or antigenically related species. Also, there is the potential of soil inhibitors; assay sensitivity and non-specific binding have been reported for other soil immunoassays (Kageyama *et al.* 2002, Otten *et al.* 1997). Finally, soil composition could have an impact, and with irregular distributions of pathogen propagules creating problems for accurate, representative sampling (Phelps *et al*., 1991).

Concentrating samples to assist detection:

To overcome these potential challenges to immunoassay efficacy, workers have attempted to develop simple and efficient extraction systems for isolation of target pathogens from soil. However this process has proven one of the biggest hurdles in the development of quick and sensitive plant pathogen diagnostic immunoassays (Dewey & Thornton, 1995). Many soil tests still resort to the use of a biological amplification stage (soil-baiting) to provide target analytes at readable concentrations (Yuen *et al.* 1993; Thornton *et al.* 2004). A beneficial consequence of this approach is that such tests are able to provide valuable information on viability of the target organism. However, they are also generally reduced to being qualitative or semi-quantitative and can end up proving as time-consuming as conventional media-based isolation tests.

Another approach has been the inclusion of a pre-treatment, for example drying, grinding, centrifugation or floatation processes, to recover pathogen resting structures. Unfortunately, these processes often prove laborious, lack economy of scale and, require considerable laboratory space prior to analysis (Wallace *et al*., 1995; Wakeham & White, 1996; Miller *et al*., 1997), and the development of a rapid, highly sensitive and inexpensive assay becomes somewhat irrelevant if the extraction process is lengthy, laborious and costly in time and labour. For this reason, simple and rapid processes to isolate and concentrate disease propagules from soil are still being actively sought. Separation of bacteria has been successfully achieved by immuno-magnetic capture, with isolation, concentration and detection reported from contaminated feedstuffs (Johne *et al*. 1989; Mansfield & Forsythe, 1993), faeces (Luk and Lindberg, 1991) aquatics (Bifulco & Schaefer, 1993) and soil (Mullins *et al*. 1995). Recently, this approach has been adopted to isolate and concentrate resting spores of the clubroot plant pathogen from infested UK

horticultural and agricultural soils (Kennedy & Wakeham, 2013). Monoclonal antibodies specific to *Plasmodiophpora brassicae* (causal agent of clubroot) and conjugated to super paramagnetic spheres have been used to 'fish' soil for *P. brassicae* resting spores. The labelled spores are isolated from the soil matrix and concentrated by exposure to a magnetic field. Quantitation of the isolated spores is determined either by quantitative polymerase chain reaction (qPCR) (Lewis, 2011) or by using an on-site ten minute lateral flow (immuno-chromatographic) test (Wakeham *et al*. 2012).

Prospects of multiplex testing of complex environmental samples:

The magnetic capture concept has been developed to provide immuno-array tests capable of multiplex testing for more than one pathogen. Test platforms like the magnetic microsphere capture immunoassay system (Luminex MAGPIX technology) can be used to detect multiple plant pathogens in complex environmental samples such as soil. This system can deploy 50 sets different fluorescence colour coded magnetic microspheres, each of which can be coated either with target analyte or a target pathogen-specific probe. Using a 96 well ELISA format, samples for testing are aliquoted (20-100µl per well). Within each well there is the potential to deploy all 50 bead types at once, with each seeking and binding to a specific homologous target pathogen analyte. By applying a magnetic field, the beads with bound target material can be withdrawn from the sample and retained and separated from potential assay inhibitors. After this step an ELISA process is carried out and the magnetic sphere bound target analyte is identified by linking with a fluorophore (R-phycoerythrin) conjugated detector antibody. The MAGPIX system is able to identify the colour-coded magnetic bead and measure the fluorescence of the detector antibody to provide quantitation of multiple target pathogens in a sample. This approach provides 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. The assay time of the microsphere immunoassay (1hr) is much shorter than for a standard ELISA system (approx. 4hr).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). This platform should prove highly versatile for epidemiological studies and crop clinic work assaying for, isolating, concentrating and quantifying multiple plant pathogens in potentially complex samples, such as soil, plants or water, at moderate cost.

On-site testing

Point of care assays (POCs), originally developed for medical applications have successfully been adapted to achieve reasonably accurate diagnoses of some plant diseases. An early example of this process was reported by Agri-Diagnostics

Associates who developed flow through tests for detection of *Phytophthora*, *Pythium* and *Rhizoctonia* species on root, stem and leaf samples (Miller et al. 1990; Ellis & Miller 1993). Variants, for example immuno-chromatographic test strips (Wong & Tse, 2009) which are also known as lateral flow devices (LFD), are available worldwide for on-site testing of a range of viral, bacterial, fungal and Oomycete plant pathogen infections (e.g. www.neogen.com; http://www.envirologix.com; www.pocketdiagnostic.com). One of the earliest LFD tests for a fungus was the monoclonal antibody test developed for *Botrytis* spp. (Dewey & Yohalem, 2007; Dewey et al., 2008 & 2016). LFD tests comprise 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 usually generated within 5-10 minutes with the formation of a control and test line as appropriate to the sample and the test type (Figure 1). They are designed for single use, can be quantitative in measurement with a suitable reading device and a calibration curve (Wong & Tse, 2009), and are capable of providing a limited multiplex test platform. In plant protection LFDs are increasingly used to provide a first line rapid defence screen. This application is amply demonstrated in forestry disease management where a *Phytophthora* genus-specific test device has been used in the UK by the Animal and Plant Health Agency's (APHA) Plant Health and Seed Inspectorate (PHSI) to detect and monitor the notifiable oomycete pathogens *Phytophthora kernoviae and P. ramorum,* the causal agent of sudden oak death and dieback/leaf blight in a range of tree, shrub, and herbaceous species. Initial positive diagnosis of the pathogen has enabled the effective management of the disease on horticultural nurseries by immediate quarantine and containment measures (Kox *et al*., 2007; Lane *et al.*, 2007). Once a sample is identified as a potential risk from infestation confirmatory tests are undertaken to fully characterize the strains involved using DNA-based molecular techniques (www.fera.defra.gov.uk/plants/publications/documents/factsheets/pramparks.pdf). LFD tests have also been successfully used as quality control diagnostic tools. For example, rapid on-site detection of *Fusarium* mycotoxins using rapid on-site monoclonal antibody immunoassays specific to fusarins, T-2 toxin, zearalenone (F-2 toxin) and DON (Barno-Vetro *et al*., 1994; Casale *et al*., 1988; De Saeger & Van Peteghem, 1996; Maragos *et al*., 2008), can quickly identify levels of contamination in cereal crops. Envirologix, under their QuickTox label (http://www.envirologix.com) supply a range of lateral flow tests to provide quantitative and traceable test results for mycotoxins in commodity grains. Within this product range a lateral flow device is also available to rapid determine levels of stable *Botrytis* antigens in table and dessert wines (Dewey *et al.,* 2013). Lateral flow assay systems have also been developed and used to track horticultural biocontrol agents. Using a monoclonal antibody probe, active propagules of *Trichoderma* species can be detected in soil samples within 15 min of antigen extraction. These devices have also been used to detect oomycete animal-, and human-pathogens (Thornton & Wills, 2013).

Potential limitations of POC immunoassays:

Although the single step POC immunoassay has its strengths there are potential weaknesses of the test format (Posthuma-Trumpie *et al.,* 2009). As with nucleotide based detection assays, the total sample volume that can be applied to this type of test is small (aprox. 100µl) potentially limiting sensitivity. As mentioned above, this problem may be addressed by pre-extraction treatments such as immuno-magnetic capture, although additional sample processing adds a level of complexity and detracts from the simplicity and speed of the single-step test approach. Application of the sample to the test is often drop-wise and this method has the potential to lead to a level of imprecision, especially if tests are being measured quantitatively using a reading device. In complex environmental samples, for example soils, food, or estuarine water, there is the capacity for the test strip to become blocked and inhibit the assay process. These problems can be reduced by incorporating pre-filtration materials into the lateral flow format, whilst sample volume issues can be addressed to some extent by deploying a range of sample pad sizes and formulations that allow increased volumes to be held prior to the immunoassay stage (www.millipore.com/diagnostics; www.whatman.com/DiagnosticComponents).

Dipstick and zoospore trapping immunoassays:

Although lateral flow tests (LFDs) have been found useful to quickly determine oomycete infections, available commercial tests are limited, by the antibodies used, in their ability to discriminate soil and water-borne oomycete pathogens at the species level. For Phytophthora, this situation is perhaps not so troublesome, as indicated above. However, it is highly problematic for *Pythium* species, a significant proportion of which are saprophytic, 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 (Ali-Shtayeh & Saleh, 1999; Martin & Hancock, 1987; Paulitz, *et al*., 1990: Vallance *et al*., 2009; White *et al*., 1992). The efficacy of these tests has also not yet been demonstrated for some environmental samples (e,g. growing substrates) or for the pre-symptomatic infection of plant material, although their use in conjunction with plant tissue baits has been used in irrigation water tests with some promise (Wedgwood, 2014). Importantly, these tests as they stand fail to distinguish between live and dead pathogen propagules, negating their value in assessing pathogen kill in measurements of the efficacy of pathogen control treatments. Cahill & Hardham (1994) overcame this shortcoming to some extent with *Phytophthora cinnamomi* by exploiting zoospore chemotaxis and developed a ‘dipstick’ test which could be carried out in water and on-site by unskilled operators. However, often only a limited proportion of the total number of zoospores present in a water sample is detected using this method (Pettitt *et al*., 2002). It may also be wise to include a step inducing cyst germination to prove viability as opposed to relying solely on chemotaxis (or apparent chemotaxis), since apple bait pieces were found to pick up non-viable pathogen material under comparable circumstances (Wedgwood, 2014). This

limitation could be very important in irrigation water supply where the number of zoospores per unit volume may be very low. Other workers have tried to overcome the problem by the development of a zoospore trapping immunoassay (ZTI – Wakeham *et al*., 1997). This process concentrates material from irrigation water by filtration onto a membrane. Following a short incubation with a selective medium, the viable zoospore-germlings, if present, can be visualised using a specific antibody probe conjugated to a coloured marker (see Figure 2). To date this assay is one of the most sensitive test procedures to have been successfully deployed in routine water assessments for the measurement of viable oomycete propagules (Pettitt *et al*., 2002; Bandte & Pettitt, 2014).

Improving LFD specificity using nucleic acid-based techniques:

Where specificity is problematic, LFD POC tests can be used in combination with nucleic acid molecular techniques (nucleic acid lateral flow (NALF)) to provide an on-site solution. This approach using loop-mediated isothermal amplification (LAMP, Notomi *et al*., 2000), has been applied for the detection of the oomycete pathogens *Phytophthora ramorum* and *P. kernoviae* from infected plant tissue (Tomlinson *et al*., 2010). After application to a chromatographic LFD test strip, which can also act as a preliminary genus-specific screen for *Phytophthora*, DNA is isolated and extracted from the membrane in <5 min with manual shaking in a small vial containing an extraction fluid. After extraction and applying LAMP, the target DNA is amplified using labelled specific primers. Detection of these labelled amplicon products is performed in a lateral flow test stip. Each of these steps (manual shaking to disrupt the sample before application onto the membrane, placing a section of the membrane into pre-prepared LAMP reaction mix and incubation in a heated block or water bath, and dilution of the LAMP reaction and application onto the chromatographic test strip) is deemed as sufficiently simple to potentially allow this method to be performed outside a conventional laboratory facility without extensive prior training. A result can be obtained in just over an hour. A LAMP assay for the detection of plant DNA (cytochrome oxidase gene) can be used in conjunction with pathogen-specific assays to confirm that the assay is working when a negative test results (Tomlinson *et al*., 2010). This technology is currently being used by trained operators, for example UK Plant Health and Seeds Inspectorate (PHSE). It will be interesting to see whether this type of test is taken up by industry for use at grower holdings to evaluate the risk of disease epidemics and the efficacy and timing of control measures.

Molecular Nucleotide Assays:

Background:

Molecular methods, essentially based upon Polymerase Chain Reaction (PCR), have evolved from a complex highly 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 provided a capability to design specific primer sets to selectively identify nucleotide sequences of fungal and oomycete species.

Nucleotide sequences and sequence databases:

The internal transcribed spacer (ITS) region of ribosomal DNA is reported to be the most widely sequenced DNA region of fungi (Peay *et al*., 2008). It has been recommended as the universal fungal barcode sequence (Schoch *et al.*, 2012), and as a consequence, has also been adopted for studies of oomycetes (Lévesque, 2011). Consisting of alternating areas of high conservation and variability ITS 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 in both crops and fish stocks (Beakes *et al*., 2012; Cooke *et al*., 2000; Lévesque & De Cock, 2004; Songe *et al*., 2015; Tuffs & Oidtmann, 2011). Other regions of the genome are also of use and have been sequenced 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 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), and in some cases these resources provide additional information such as diagnostic morphological features and aspects of biology. Target unidentified organism genomic sequences can readily be compared with database sequences for identification purposes, using DNA-similarity searches like BLAST (Basic Local Alignment Search Tool, Altschul *et al*., 1997) and DNA and RNA sequence databases, such as the International Nucleotide Sequence Database (INSD). However, caution is still needed in interpreting results of comparisons since for fungal species (bearing in mind that the renamed 'International Code of Nomenclature for algae, fungi and plants' (McNeill *et al*., 2011) still includes considerations on oomycete nomenclature under 'fungi' with a small 'f' (Schroeder *et al*., 2013)), it has been reported that less than 1% of the estimated 1.5 million extant species have been sequenced for the ITS region, and that as much as 20% of all fungal and oomycete sequences deposited in the INSD may be incorrectly annotated to species level (Bridge *et al*., 2003, Kang *et al*., 2010, Nilsson et. al., 2006). There are also concerns over the classification of species solely based on results of DNA region/gene analysis. Classical identification of oomycete pathogens for example has relied heavily on morphological and biological features (van der Plaats-Niterink, 1981). These relationships are not always conveyed when compared by genomic

analysis. Will & Rubinoff (2004) reported on the myth of the DNA barcode for species classification and reasserted the requirement for morphological analysis in the identification and classification process. Interestingly, in the field of medical mycology the uptake of PCR as a diagnostic tool has been constrained by the lack of standardization, such that PCR is not an accepted diagnostic criterion for the detection of human fungal diseases according to 2008 EORTC/MSG guidelines (De Pauw *et al.,* 2008). Nevertheless, sound oomycete phylogenies are now taking shape, taking morphological and physiological traits into consideration as well as sequence data from a range of regions in addition to ITS (e.g. *cox 1* Robideau *et al*., 2011; or β tubulin Villa *et al*., 2006) and attempts are being made to establish rigorous databases for this information (e.g. The *Phytophthora* Database: http://www.phytophthoradb.org and The Pythium Genome Database: http://pythium.plantbiology.msu.edu).

PCR *assay development*

As with immunoassay systems, consideration should be given to the test sample coverage and the environmental matrix assessed. Extraction and concentration processes may be required for the efficient and reliable amplification of low numbers of target genomic material. Melt and annealing temperatures of the PCR process should be optimised to prevent the formation of undesirable secondary structures such as primer dimers (Saiki *et al*., 1988; Steffan & Atlas, 1991). Environmental samples often contain potential assay inhibitors. These may include humic substances, pesticide residues and organic material (Kong *et al*., 2003). Colloidal matter which has a high affinity for DNA may also be present (Way *et al*., 1993; Wilson, 1997). The varied occurrence of these in field samples has the potential to disrupt the amplification process and affect test sensitivity (Lombard *et al*., 2011; Stewart-Wade, 2011).

qPCR:

qPCR provides a means of measuring the concentration target pathogen DNA, and thus estimating biomass in a sample, by monitoring DNA copy generation using conformational change of fluorescently-labelled probes with reference to a standard curve. This system is often referred to as real-time PCR as the fluorescently-labelled PCR products produced during each amplification cycle can be monitored as the reaction progresses. Where conventional PCR diagnostic testing can be confined by special room requirements to eliminate aerosol contamination (Regis *et al*., 2006) the 'closed' qPCR process can to some extent overcome this problem. It has also been shown to have advantages of speed, accuracy, and sensitivity over conventional PCR-based techniques (Schaad & Frederick, 2002). qPCR assays have been widely developed for oomycete pathogens (Cooke *et al*., 2007; Huang *et al*., 2010; Kernaghan *et al*., 2008; Lees *et al*., 2012; Li *et al*., 2014; Mulholland *et al*., 2013; Pavón *et al*., 2008; Strand *et al*., 2012; Tuffs & Oidtmann, 2011). Unfortunately, the costs of purchasing and installing a 'real time' laboratory operating system are high and with recurrent running costs, currently make this procedure an

unaffordable option for many laboratories. Nevertheless, where speed, specificity and sensitivity are priorities regardless of cost, analysis by qPCR can prove optimal for test delivery.

Quantitative PCR can also be performed outside of the conventional laboratory setting using a system originally developed for the US military to monitor bioterrorism-related outbreaks of anthrax. For this application the real time platform was supported by a portable battery and packaged in a large brief case to allow rapid field deployment in combat zones. The portable sampler has since been made commercially available (Cepheid Smartcycler Inc., Sunnyvale,California) and has been assessed for its quantitative capability on a small number of human pathogens as well as some 'notifiable' ('Quarantine', IPPC, 2015) plant pathogens (Bélanger *et al*., 2003; Tomlinson *et al*., 2005). These systems deploy fully automated sample preparation, using disposable cartridges that accept up to several millilitres of aqueous sample, completing preparation in less than five minutes, and provide limited multiplex detection on a single platform. Despite these developments, the molecular detection of microbial pathogens in plant material and other complex matrices, such as soil or infected animal tissues, is still limited by the need for the pre-extraction of DNA (Healey *et al.*, 2014). For this reason the on-site molecular testing of environmental samples has demanded not only a portable real-time PCR platform but also a simple and robust DNA extraction method. Recent success with field measurements of *P. ramorum* using the nitrocellulose membranes in LFD devices to extract sample DNA (Tomlinson *et al.,* 2010), indicate that perhaps for plant material at least, extraction is not as problematic as previously believed.

Loop-mediated isothermal amplification (LAMP)

Simpler, less expensive technologies have been sought to allow molecular based assays to be translated from the laboratory to the field. LAMP provides a novel nucleic acid amplification process under isothermal conditions (60 to 65°C) (Notomi *et al*., 2000). For this reason simple incubators, such as a water bath or a block heater, are sufficient for DNA amplification. As a by-product of the reaction a white precipitate of magnesium pyrophosphate is produced, which enables the visual judgment of amplification by 'naked eye'. LAMP has been reported to be less affected by inhibitors than other PCR procedures (Francois *et al*., 2011) and, because of its speed, robustness and simplicity is increasingly used for diagnostics in human medicine (Parida *et al*., 2008) and, more recently, in plant health (Kubota *et al*., 2008; Tomlinson *et al*., 2010; Bühlmann *et al*., 2013). In the United States the development of a 'grower performed LAMP PCR' has been assessed for the detection-based management of spray programmes for grapevine powdery mildew in vineyards (Mahaffee *et al*., 2011; Thiessen *et al*., 2015). Based on two years of results, a commercial company ran a feasibility trial to offer a grower-based test service. It was estimated that it would require US$2100 in capital equipment, US$60 in reagents and 25 minutes labour with a 1.5 hr process-time for 10 samples. This estimate did not however include the cost of an air sampler for collection of field

aerosols. The LAMP process consisted of several steps including extraction, heating, and centrifugation, and, although it could be operated in a grower's office with desktop equipment, it was found that participants were not consistently successful when interpreting the results. The company considered performing the LAMP service 'in-house' however opted to partner with a commercial laboratory to offer a laboratory quantitative PCR service (Reiger, 2013). As a result of the high sensitivity of the test, it was observed that one of the biggest concerns in the collection of samples for a commercial DNA-based testing service was the cross-contamination of samples. Spores could be easily picked up and moved on people's clothing and hands. For this reason they instituted clean practices whereby samplers wear gloves and protective clothing, which is changed between traps. Mahaffee and his team at the United States Department of Agriculture continue to work with growers to develop field tests that are more economical and easier to use. They are currently investigating the use of a hand-held, portable device called the Smart-DART (www.diagenetix.com/product-and-technology/smart-dart-platform) which allows the LAMP process to be performed on site and provides an application to an Android phone device for quantitative measurement of the assay process. If this approach is successful, a DNA extraction process will still need to be performed, but costs would be greatly reduced, with estimated complete system set-up costs of less than US $2000 in capital equipment and annual operating costs of US $400 (Reiger, 2013). This estimate does not include labour costs to operate the system, nevertheless, with appropriate economies of scale, this system could prove useful in a field situations where speed, sensitivity and specificity are key to a successful outcome.

Multiplexing with PCR:

Where a laboratory/clinic environment is feasible, advances in molecular diagnostic test technology have provided the opportunity to couple PCR with high throughput pathogen detection multiplex arrays. These array systems were originally designed for gene expression profiling, gene discovery and single nucleotide polymorphism (SNP) analysis (Lockhart & Winzeler 2000; Mei *et al*., 2000). PCR-based multiplex 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 (Eptstein & Butow, 2000, Ishii *et al*., 2008). 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).

DNA arrays have been developed for the detection of plant pathogens in a range of environmental samples (Mumford *et al*., 2006; Boonham *et al*., 2007; Lievens *et al*., 2012). For *Pythium*, a DNA array containing 172 oligonucleotides complementary to specific diagnostic regions of the internal transcribed spacers (ITS) has been developed for the identification and detection of more than 100 species (Tambong *et al*., 2006). More recently a membrane-based oligonucleotide array has been developed to detect *Phytophthora* spp by using three DNA regions (ITS, cox1 and cox2-1 spacer). The array was validated with 143 pure cultures and 35 field samples, and showed promising sensitivity, being able to detect as few as 50 pg of PCR amplicon from pure laboratory cultures (Chen *et al*., 2013). Using a multiplex real-time PCR approach, other workers have reported a detection sensitivity ranging from 1 fg (gene with multiple copies) to 100 fg (single-copy genes) of target *Phytophthora* DNA (Schena *et al.,* 2006; Tooley *et al*., 2006). However each of these plant tissue assays was limited to the measurement of a few target species; *Phytophthora ramorum*, *P. kernoviae*, *P. citricola* and *P.quercina,* in symptomatic leaf samples in the former, and only *Phytophthora ramorum* and *Phytophthora pseudosyringae* in the latter test.

As a laboratory tool, the nucleotide-based array system can provide a highly specific and sensitive assay for the simultaneous detection of multiple pathogens present in a cropping system (Robideau *et al*., 2008) and has also been successfully used to identify species with fungicide resistance (Ishii *et al*., 2008). In general, macroarrays (immobilized nucleic acid sequences spotted onto reusable membranes) have been used for plant disease diagnosis as a result of cost, sensitivity and the more modest equipment requirements (Lievens *et al*., 2012). For commercial applications, Bio-art bvba (Belgium) have demonstrated the usefulness of this multiplex approach and report detection of a range of fungal, oomycete and bacterial plant pathogens (DNA MultiScan®, http://www.bio-art.org). Meanwhile, the ongoing, considerable advances in the areas of genomics and bioinformatics mean that ever more powerful molecular diagnostic methodologies continue to be developed. For the oomycetes, sequence data continue to generate and provide additional information for phylogenetic analysis and updating species classifications (Kamoun *et al*., 2014). Next generation sequencing (NGS) provides the capability to analyse and compare whole genomes of plant pathogens. The *Pythium ultimum* genome (42.8 Mb) is reported to encode for 15,290 genes of which extensive sequence similarity and synteny with the potato blight pathogen *Phytophthora infestans* is reported (Lévesque *et al*., 2010). More recently, analyses on the sequencing, assembly, and annotation of six *Pythium* genomes (*P. aphanidermatum*, *P. arrhenomanes*, *P. irregulare*, *P. ultimum var. sporangiiferum, P. vexans* and *P. iwayamai)* provides comparison with other plant pathogenic oomycetes including *Phytophthora* species*, Hyaloperonospora arabidopsidis*, and *Pythium ultimum* var. *ultimum* as well as related animal pathogens such as the important fish pathogen *Saprolegnia parasitica* (Bishwo *et al*., 2013).

Next generation sequencing:

Next generation sequencing offers a diagnostic tool that requires no previous knowledge of either a specific host or pathogen (Schuster, 2008). It is a high-throughput approach that generates thousands to millions of DNA sequences. However, as a diagnostic technique, obtaining and making sense of these sequences involves several complex stages, both at the lab bench and at the computer desk. With more and more organisms being sequenced, a flood of genetic data is being continually made available (Liu *et al*., 2012). Distilling meaningful information (bioinformatics) from the millions of new sequences from voluminous, noisy, and often partial sequence data, and interpreting it presents a serious challenge. Analysis requires considerable skill and understanding to avoid potential pitfalls and challenges in the process (Dewoody *et al*., 2013). Nevertheless, NGS does have the capability to analyse complex environmental samples and from these, identify uncultured known, unknown and novel pathogen variants (Adams *et al*., 2009, Harju *et al*., 2012, Bi *et al*., 2012, Breitbart *et al*., 2008) and is currently generating very interesting information on the diversity of oomycete species present in disease systems and natural ecosystems (Sapkota & Nicolaisen, 2015, Vannini *et al*., 2013). For plant virus identification Adams *et al*. (2009) reported a cost of £1000 per sample analysis but that this sum could reduce considerably in the future. However, for the time being NGS is likely to remain a sophisticated laboratory tool, underpinning fundamental genetically-based studies, to provide new perspectives on host-pathogen interactions and ecological studies. It will provide considerable support to the development of new diagnostic, molecular-based technologies.

Practical application of diagnostic tests*:*

Tests in the field:

Despite the many exciting developments in diagnostic technologies outlined here, application in the field is still often restricted by the need for (often complex) pre-processing of samples (e.g. nucleic acid extraction), which for environmental samples, especially soil, is still a rate- and skill-limited step (King *et al.,* 2008). Soil can be a particularly challenging environment to work with as texture, structure, pH, electroconductivity and moisture content can vary greatly within a sampling area and considerably influence pathogen distributions. The vertical and horizontal microbial composition will also have an association with the soil tillage, nutrient status and C:N ratio (Serrano *et al*., 2011; Coince *et al*., 2013; Lindhal *et al*., 2013).

For on-site testing, ease of use and test reliability are important, but ultimately adoption in agricultural systems will be driven by costs. This situation is a particular consideration for many plant cropping systems where the profit margins and emotional attachment to crops are low. A requirement for skilled staff and expensive equipment with reagents would not be easy to justify. However, as mentioned above, where legislative issues are a factor and potential of quarantine outbreaks a concern, the demand for specificity, sensitivity and speed may to some extent override the

costs. PCR-based technologies such as LAMP show great promise, and with immunodiagnostic POC tests and NALF technologies seem the most likely to have widespread field application in the short to medium term (Shan, 2011).

Sample volume and sample strategy:

For agricultural industry use, the focus should be on how the test result will relate to the cropping system. Diagnostic platforms are evolving at a rapid rate and often the material required for analysis reduces as test sensitivity increases. For example, the use of nanotechnology provides potential for small, smart, inexpensive, real-time disposable diagnostic systems with field-portable electronic outputs. However, as the name suggests, nanotechnology will drive even smaller sample volumes. There is a real danger here that test accuracy will be compromised by the restrictive nature of the sample volumes used. This situation could be particularly problematic where disease is present at a sub-clinical level or patchy in large scale cropping systems or in the determination of 'disease-free' status in quarantine, assessments/certification of seed and transplant stock. For a sampling procedure to be effective, the potential spatial and temporal variation of target pathogen incidence within a cropping system need to be evaluated and accommodated by identifying appropriate levels of replication in time and space. Individual mycelia can extend to metres in composite length (Smith *et al*., 1982; Douhan *et al*., 2011) and microbial communities can display temporal variation in composition (Bush *et al*., 2003; Cacciola & Magnano di San Lio 2008; Courty *et al*., 2008; Pickles *et al*., 2010; Davey *et al*., 2012). This pattern may be short term in response to local weather events (Ristaino, 1991; Café-Filho *et al*., 1995), or to changes in cultural conditions (Kennedy & Pegg, 1990), or cyclic in relation to seasons and the phenology of host species (Weste & Vithanage, 1977; Khallil *et al*., 1993; Lindhal *et al*., 2013; Eyre & Garbelotto, 2015). Sample size and the sampling strategy will be critical factors of an accurate assessment of disease potential (Ranjard *et al*. 2003). If these hurdles can be overcome, the prospects for widespread uptake of molecular diagnostics within commercial disease management systems are considerable, although, ease of use and cost returns will prove key drivers in their uptake.

Viability of pathogen propagules:

In some areas of agriculture, the ability to reliably discriminate viable, from dead/inactivated oomycete propagules and the concentrations at which such discrimination might be possible is crucial. This situation is especially true in the testing of recycled water where, following treatments to kill pathogens, many dead pathogen cells and particles of debris can still be present. Differentiating between the infectious (viable) and non-infectious (non-viable) state remains a limitation of PCR (Stewart-Wade, 2011) as DNA persists for significant periods of time after the death of cells (Masters *et al*., 1994). Vettraino *et al*. (2010) and Chimento *et al*. (2012) approached this problem for the detection of *Phytophthora cambivora* and *P. ramorum* by targeting the mRNA of the cox genes for reverse transcription followed by PCR amplification. As an indicator of viability, mRNA is considered an

appropriate target since most mRNA species have a short half-life. In bacteria this time amounts to just a few minutes (Kushner 1996), whilst in fungi, the determination of mRNA half-lives for *Candida albicans*, suggest an enhanced period of between 4-168 min (Kebaara *et al*. 2006) and in protists, between 9.5 and 65 min in *Plasmodium falciparum*, depending on life-cycle stage (Shock *et al*., 2007). This potential length of half-life might be reasonable for tests indicating the viability of pathogen inoculum in soil, but is still potentially problematic for tests on the efficacy of water treatments applied to control oomycete pathogens where tests would be applied immediately post treatment and the risk of a high incidence of 'false positive' results could cause unnecessary and expensive disruption. To date, the only truly reliable measures of viable oomycete inoculum remain those that involve a germination step such as conventional agar plating (Büttner *et al*., 2014), adapted immunodiagnostic dipstick assays (Cahill & Hardham, 1994) and ZTI (Pettitt *et al*., 2002).

Uptake of molecular diagnostics within Integrated Disease Management Systems (IDMS):

In contrast to conventional single component control strategies, IDMS aim to combine biological, cultural, physical and chemical strategies in a holistic way (El Khoury & Makkouk, 2010). Early pathogen detection, diagnosis and quantitation are central to good crop protection, and successful IDMS depends upon the quality of diagnostic information, its evaluation, and incorporation within the system. For example, once a pathogen is identified, and inoculum concentration data are available, these can be assessed in relation to relevant associated environmental parameters to determine when disease-risk thresholds have been reached (Scherm & van Bruggen, 1995).

Mathematical plant disease prediction models have been developed and evaluated for a wide range of crop diseases, for example potato blight forecasts (Raposo et al., 1992) and MILIONCAST for *Peronospora destructor* downy mildew sporulation on onions (Gilles *et al*., 2004). These can predict optimum times for fungicide application based on meteorological measurements, improving efficacy and often reducing the number of sprays needed for good disease control in comparison to routine spray programmes. This optimisation could be greatly improved by the inclusion of real-time measurements of inoculum. Using slow and highly-skilled microscopic examination of spore trap tapes, measurements of concentrations of airborne sporangia of the potato blight pathogen *Phytophthora infestans*, demonstrated that peaks of airborne inoculum preceded the first observed symptoms of the disease in the field (Bugiani *et al*. 1998), whilst in onion downy mildew airborne inoculum must reach high concentrations before crop-to-crop transmission is possible (Gilles *et al*. 2004). The transmission of airborne pathogens from both within, and outside target crops can be modelled (Deardon *et al*. 2004; Deardon *et al*. 2006) and this approach provides scope for the development of regional disease forecasts. Early detection of incoming inoculum can be effectively achieved using

spore sampling network devices (Skelsey *et al*. 2009), and such measurements have been used to improve the precision of fungicide decision support systems for potato blight – guiding the timing, and also giving early and precise indications as to the efficacy, of disease management decisions (Fall *et al.,* 2015). More rapid and precise molecular techniques have allowed similar observations in potato blight and other disease systems (Skottrup *et al*., 2007; Kennedy & Wakeham 2008; Wakeham & Kennedy, 2010), readily providing sufficient time for the application of targeted protectant control measures (Wakeham 2014; West & Kimber, 2015; Thiessen *et al*. 2015).

As described above, many molecular procedures are best executed under controlled laboratory conditions, possibly providing intelligence to decision support networks. Klosterman (2014) described the coupling of field spore traps and quantitative PCR Assays for detection of the downy mildew pathogens of spinach (*Peronospora effusa)* and beet (*P. schachtii*). However, not requiring a pre-extraction or DNA amplification stage, the simplicity, relatively low cost and rapidity of immunological methods makes them an attractive proposition for in-field tests. In the UK, the Brassica Alert network of spore traps provides an inoculum based warning of airborne spore concentrations using lateral flow tests (http://www.syngenta-crop.co.uk/brassica-alert). Air samples are only tested when environmental conditions are suitable for *Mycospharella brassicicola* infection (Wakeham & Kennedy, 2010). In-field lateral flow tests have also been developed to monitor bio-aerosols for the oomycete pathogens *Peronospora destructor* (onion downy mildew) and *Albugo candida* (white blister on *brassica oleracea*) (Kennedy & Wakeham, 2008; Wakeham 2014). On the other hand, the specificity and sensitivity that can be achieved with molecular DNA-based methods is impressive and real advances have been made towards the deployment of DNA-based systems to the field and in the reduction of analysis cost (Thiessen *et al*. 2015).

In comparison with airborne disease management, where decision support systems have been widely used for decades, IDMS for soil and water-borne pathogens is less well developed and for many key pathogens, distributions, rates of spread and especially, disease thresholds are still poorly understood. Nevertheless, useful diagnostic assay systems have been developed for a wide range of soil and water-borne pathogens. For example, the rapid and efficient detection of vascular wilt pathogens (*Fusarium solani*, *Rhizoctonia solani*, *Verticillium* species), and *Pythium ultimum* in plant tissues and irrigation water using DNA array technology (Lievens *et al*. 2006). In carrot soils, Klemsdal *et al*. (2008) designed PCR primers for ITS sequences to detect and identify the five most important *Pythium* species associated with cavity spot in Norway; *P. intermedium*, *P. sulcatum*, *P. sylvaticum*, *P. violae* and *P. vipa,* to provide the possibility of assessing disease risks before deciding whether to crop new fields. The assessment of these disease risks might be enhanced by the use of qPCR (Barbara, 2010), although carrot cavity spot is a complex disease system and research continues at the University of Warwick (UK) to improve assays

and their interpretation (Clarkson, J., 2016, AHDB project FV391a, personal communication). In commercial Brassica production, qPCR and immunoassay techniques have been compared for their value in IDMS for *Plasmodiophora brassicae* (clubroot) in soil (Wakeham *et al.,* 2015).

In river and water assessment, significant progress has also been made. Scibetta (2012) described the development and validation of two different rDNA primer sets for assessment of *Phythophora* species in environmental samples. Using an on-site knapsack system water borne propagules were collected directly from stream water prior to testing.

Monitoring specific oomycete species is more difficult in soil and water environments than in the air by the common occurrence of mixtures of fairly closely-related species; some pathogenic, many non-pathogenic, and some even mycophagous, with clear biological control potential. Reliable multiplex assays have yet to be developed that allow the monitoring representative *Pythium* species and their distributions in time and space in soils in relation to both disease occurrence and inter-specific competition/predation, although reasonably effective multiplex assays have been developed that can discern certain key pathogens in soil and water samples (Lievens *et al*., 2006; Abd-Elmagid *et al*., 2013). To be truly effective in future IDMS, such assay systems would also need to take the considerable progress made on monitoring biocontrol agents into consideration, for example hyperparasitic species of *Trichoderma* for which both immunodiagnostic and real-time PCR monitoring procedures have been developed (Thornton *et al*., 2002; Hagn *et al*., 2007; Savazzini *et al*., 2008).

Conclusion

The increasing concern about pesticides in the environment, removal or restriction of their usage and rising food production costs has focussed the attention towards the improvement of agricultural disease management systems. Early detection allied to key environmental parameters to control disease at the onset can lead to an increase in production, an improvement of resource efficiency and make a substantial contribution to food security. This approach has successfully been applied to measuring the transmission of crop pathogens in bio-aerosols and the timed application of control measures (Wakeham & Kennedy, 2010, Gent *et al*, 2013; Fall *et al*., 2015; West & Kimber, 2015). In water, growing media and soil progress is hampered by the environmental sample matrix, suitable sampling regimes to reflect the cropping area and target collection efficiency from the sample. Nevertheless, progress with molecular diagnostics of soil and water-borne oomycetes has been rapid over the last decade and there is an array of very promising technologies now beginning to be developed for field use.

The challenge for agricultural scientists however, remains the ability to identify, select and modify available systems to provide diagnostic tools that are able to deliver useful information to the end user. The development of these systems must

be appropriate to the delivery point, whether it is in a diagnostic laboratory or on-site, whilst remaining mindful of the economies of scale in agribusiness. Once developed, tests must be extensively validated and compared with existing adopted approaches (for example the isolation of pathogens by use of selective media, culture-based morphological analyses and baiting using plant tissues), and that this process is carried out across the range of environments in which the test will be used. If the test is to be carried out by non-scientists, the robustness of the system should be assessed in supported trials with multiple 'non-skilled test' end users. This process will require careful planning with a robust validation period.

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Figure Legends

Figure 1: Positive LFD test strips; on the left a strong positive for *Phytophthora*, showing clear control (C) and test (T) bands, and on the right a very weak positive for *Pythium*.

Figure 2: Developed ZTI membrane showing trapped and germinated *Phytophthora* zoospore cysts.

Figure 1:

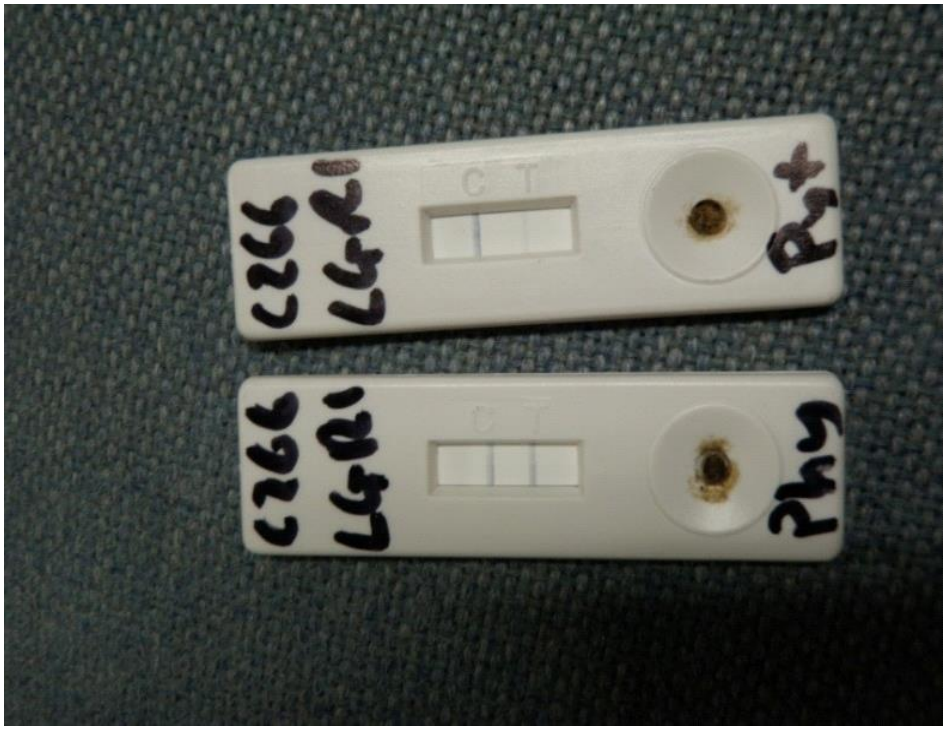

Figure 2: