THE DEVELOPMENT AND APPLICATION OF IMMUNOLOGICAL TESTS WITHIN HORTICULTURAL CROP DISEASE MANAGEMENT SYSTEMS.

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1 A CRITICAL OVERVIEW IN SUPPORT OF THE PORTFOLIO OF EVIDENCE FOR THE AWARD OF PhD

1.1 BACKGROUND TO THE PORTFOLIO OF EVIDENCE

Plant pathogens, crop loss and disease diagnosis. Worldwide and on an annual basis diseases of agriculture and horticulture cropping systems impact significantly on crop yield and quality. These losses can be quantitative and or qualitative. Quantitative losses result from reduced productivity, leading to a smaller yield per unit area. Qualitative losses may result from the reduction of an essential substance, a change in taste, cosmetic appearance or contamination of the harvested product by mycotoxin producers. It has been estimated that an average of 20 – 30% of crop yield is lost annually from the field, even in crops where pesticides and cultivars with improved genetic resistance to pests and diseases are used (Oerke, 2006) . Among crops, the total global potential loss due to pests and diseases varied from 50% in wheat to more than 80% in cotton production (Oerke, 2006). The perpetrators of these losses have been classified by their impacts into groups: reducers (pathogens), leaf senescence accelerators (pathogens), assimilate sappers (nematodes, sucking arthropods, pathogens), light stealers (weeds, some pathogens) and tissue consumers (chewing animals, necrotrophic pathogens) (Boot *et al.*, 1983).

A major problem for producers is that diseases are moving targets that evolve in response to agricultural practices and environmental change. Despite a clear increase in pesticide use, crop losses have not significantly decreased during the last 40 years (Oerke, 2006). Pesticide use has however enabled farmers to modify production systems and to increase crop productivity. With increasing globalization, travel and the international trade in plants the risk of disease through inadvertent introduction of pathogens is however exacerbated (Brasier, 2008). The emergence and spread of new diseases, or more aggressive or pesticide-resistant biotypes is an inevitable part of the evolutionary process chain. To tackle these issues effectively and, with the increasing restrictions placed by the 'green environment', requires the development and adoption of disease control systems that are sustainable with the use of lower-input farming systems (Beddington, 2010; Godfray *et al.*, 2010). With over five million tonnes of pesticide applied world-wide annually (Scharma *et al.*, 2013) this poses producers with significant challenges to:

- by 2050 increase yield production by 70 -100% to meet the global food security requirement (Godfray *et al.*, 2010);
- reduce crop protection inputs, energy and greenhouse gas emissions;

- react to the emergence of new disease threats resulting from the effects of climate change.

Most horticultural crops in the UK are currently produced using a variety of pesticides. In relation to the Arable market, the horticultural sector provides a limited market and accounts for only 3.7% of non-grassed cropped land. Nevertheless with more than 300 crops and with a combined home-produced value of around £2.23 billion (farm gate value), it provides significant economic value to the UK economy (Agriculture and Horticulture Development Board, 2013). However as a result of EU legislation there is reduction in broad spectrum fungicides available to the Horticulture Industry. Despite the development of the ‘off-label’ system for minor uses the number of pesticides that are currently approved for use on horticultural crops is under ever increasing pressure. To utilise these efficiently and with optimal disease control performance there is a move away from routine pesticide application to targeted crop treatments (pesticides and biological) based on regular monitoring. This requires a greater depth of knowledge by producers and their staff to identify problems quickly. Yet the ability to quickly diagnose many disease pathogens still does not exist. Simple, accurate, diagnostic tools for growers are required. With increased restrictive legislative pressure on the use of protectants within the cropping season, the diagnostic test should not necessarily be limited to a single disease but provide a more holistic approach to plant / disease pressure.

Disease diagnosis and pathogen detection are central to the ability to protect crops and natural plant communities from invasive agents (Miller *et al.,* 2009). Early diagnosis can provide the grower with useful information on optimal crop rotation patterns, varietal selections, control measures, harvest date and post-harvest handling. Pathogen detection prior to infection can reduce disease epidemics. Classical methods for the isolation and identification of crop pathogens are commonly used only after disease symptoms are observed. Such processes are often time consuming, with a reliance of the organism to be cultured and availability of expert taxonomic training for accurate identification. These processes frequently delay the application of control measures at potentially important periods in crop production. As a result blanket pesticide application has been common practice. The timely detection and identification of economically important diseases in a commercial cropping environment will provide 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 then incorporated within an integrated crop disease management system (ICDMS).

Once a disease is identified the pathogen concentration coupled to the associated environmental parameters is required to determine accurate disease thresholds at which damage may occur (Scherm and van Bruggen, 1995). This information needs

to be translated in a timely and accessible way to growers for targeted and cost effective control measures to be taken to enable disease containment or eradication. For this purpose extensive ecological studies need to be conducted, studying the responses of a pathogen in relation to both biotic (microbial, plant) and abiotic factors (light, temperature, humidity etc.) of its environment (Lievens and Thomma, 2005). 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 (Wakeham and Carisse *et al.*, 2005; Kennedy, 2010). In successful delivery of ICDMS, sampling procedures and sample size will prove critical to account for spatial variation of pathogen incidence within a cropping system. With the development of new diagnostic technologies often the material required for analysis reduces as test sensitivity increases. This has the potential to be particularly problematic in cases where disease potential ahead of infection or at a sub-clinical level of infection is a requirement. Equally in plant health quarantine, seed and certification of transplant stock, the sample size and sampling strategy is critical to identifying and determining an accurate disease potential. However this issue may be overcome by the isolation and concentration of the target pathogen(s) from the sample medium ahead of testing. Nevertheless the sampling plan should be performed in a manner that ensures a statistically representative sample (Ranjard *et al.* 2003).

The increasing concern about pesticides in the environment, the long term usage for effective control of disease and rising production costs have provided the platform for scientists to focus their attention towards the development of more rapid and accurate disease diagnostic systems. The pioneering work developed in the medical field during the latter decades of the previous century provides a plethora of techniques. Many of these have been developed and deployed commercially at a global scale, to provide diagnostic point of care systems to a wide audience range (clinical diagnostics, drugs of abuse testing, fertility, allergy). Equally, as a result of recent global activity, the defence industry has had to respond to the development of rapid, highly sensitive and accurate systems to deliver on-line responses to the threat of biological warfare activity (RAZOR® EX Anthrax Air Detection System). These emerging technologies increasingly move the emphasis towards nanobiotechnology (Jeong-Yeol and Bumsang, 2012). There is a real expectation that the mobile phone in the future is likely to provide a global laboratory platform for many of these techniques. The challenge for the plant scientist however remains the ability to identify, select and modify these systems to provide a diagnostic tool that is able to deliver useful information to the end user, appropriate to the delivery point *i.e.* in a lab or on-site and mindful of the economies of scale in an agricultural or horticultural setting. Suitable validation of the test and robustness in use will markedly affect the deployment and successful uptake by industry, laboratories and or inspection agencies.

With this in mind, immunoassays have been investigated for their use to provide simple, inexpensive and robust diagnostic tools to monitor disease epidemics. Following the work of Yalow and 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 (diagnostic probe) and antigen (target analyte / disease propagule) the interaction can be made quantitative or qualitative. The immunoassay system provides a biochemical process that is highly transferrable from a commercial centralised laboratory offering a test with high throughput, specificity and sensitivity to a simple point of care test system (POC) operated by a non-specialist. These POC tests are designed to be used at or near the site where the problem is located, do not require a permanent dedicated space and can provide results quickly (within minutes). Originally these types of tests were devised for a clinical setting and used in doctors' offices, hospitals, and in patients' homes. POC diagnostic devices are used to provide quick feedback in many sorts of investigations, *i.e*. enzyme analysis, drugs of abuse, infectious agents, toxic compounds, metabolic disorders, allergens, ovulation and pregnancy testing (Posthuma-Trumpie *et al*., 2009). Existing laboratory assays such as the widely used enzyme-linked immunosorbent assay (ELISA) often translate to the simplified POC format whilst retaining the tests original performance characteristics. The results can be qualitative (yes/no) or made quantitative by using a digital reader. The Clearblue Advanced Pregnancy POC system combines two tests within one system and an inbuilt digital reader to report to the end user a written display of "Pregnant" or "Not Pregnant". A quantitative reading of 1-2, 2-3 or 3+ is displayed to indicate by how many weeks. This integrated technology is currently limited to the pharmaceutical industry where a strong global market can support the financial investment required in test development and delivery. However with the surge in POC development to ever expanding markets more companies are coming on line to deliver generic POC readers which can be tailored to specific product lines. Examples of this are the ESE-Quant Lateral Flow System which can be purchased from QIAGEN (www.quiagen.com) and Skannex (http://www.skannex.com) offers the SkanSmart hand held system for multiplex POC specific test formats. However in horticulture, where the financial return can be limited, quantitative measurement may prove too expensive (portable reader circa £1300).

In 2005, over 6 million POC tests were performed in USA hospitals and by 2011 this was forecast to exceed 1.2 billion (Esposito, 2008). Workers outside the biomedical industry were at first slow to realize the potential of these powerful, robust and easy to use systems. However by the late 1970s to the early 1980s there was an increase in applications to which immunoassays were applied, including veterinary, medicine, agriculture, the food industry, environmental health and forensic science (Pal, 1978; Wardley and Crowther, 1982; Foulkes and Morris, 1985; Aoki, 1987). This was in

part due to the advent of monoclonal antibody production (Köhler and Milstein, 1975) which revolutionized the field of immunology. This technique using hybridoma technology and a myeloma cell line provided the capability to produce and select monoclonal antibody cell lines with a specificity to a particular epitope of an antigen (target analyte). This process had two important advantages: first, because of the method of selection it was possible to choose only those antibody-producing clones which had the desired specificity; second, the cell lines could be stored and the antibodies produced in large quantities when required. The successful development and commercialisation of Unipath's Clearview home pregnancy test launched in 1988, using a homogeneous format (a system which is able to measure bound label without the need to separate bound and free label), provided evidence to a global audience of the speed, simplicity of use, specificity, sensitivity, robustness and low cost that an immunological based diagnostic assay test could provide. Bangs (www.bangslabs.com; Technote 303) report a production cost of $0.35 per POC test and a shelf life of 12 to 24 months.

In agriculture, immunoassays using polyclonal antisera (antibodies isolated from blood serum of immunised animals) were first reported for the detection of viruses and bacterial plant pathogens in infected plant tissues (Voller *et al*., 1976; Clark and Adams, 1977; Nome *et al*., 1980). The potential of this approach for fungi was demonstrated by Casper and Mendgen in 1979. Later, Johnson (1982) reported the diagnosis of *Epichloe typhina* colonization in tall fescue (causing toxicity syndrome in cattle) using a polyclonal antiserum. However discrimination of the pathogen was limited to genus level. Where the use of this technology was being successfully applied worldwide for screening plant material for viruses (Raju and Olson, 1985; Burger and Wechmar, 1988), the poor specificity achieved to the structurally more complex fungal plant pathogens (Drouhet, 1986) hampered the development of immunologically accurate diagnostic probes for commercial applications (Mendgen, 1986, Barker and Pitt, 1988). Fungi share conserved glycoprotein antigens that are highly immunogenic (Notermans and Soentoro, 1986) and can result in the production of multiple antibodies which may bind selectively to both related and non-related species (Mohan, 1989 a,b). It has been reported that the glycoprotein fraction of the fungal immunogen predominantly elicits an immunodominant response from the host (Priestley and Dewey, 1993; Viudes *et al*., 2001; Da Silva Bahian *et al*., 2003). With the advent of hybridoma technology however, those researchers who had access to the process were able to pursue the development of monoclonal antibody probes to achieve levels of specificity at the species level (Dewey *et al*., 1990; Priestley and Dewey, 1993) isolate level (Keen and Legrand, 1980; Hardham *et al.,* 1986) and to discrimate epitopes present at stages within a pathogens life cycle (Estrada-Garcia *et al*., 1990). For fungi, where within the life cycle structurally different stages can exist, probe specificity has however the potential to be problematic. For this reason the organism and the application of the test should be well understood. The combination of both antibody types (monoclonal and

polyclonal) has sometimes been found beneficial to achieve an appropriate test specificity and / or sensitivity. Where non-specific binding to host tissue is observed the use of antibody combination types for capture and labelling of the target antigen (target disease component) has also been found useful (Priestley *et al.,*1993).

At this time, the candidate was working in an organisation which had capability to produce polyclonal antisera. Through an internal collaborative study with Dr N. Lyons (at that time a visiting medical and plant technologist), the candidate was introduced to the use of polyclonal antibody based systems and, their ability to discriminate closely related plant pathogen species. This culminated in a refereed paper (White *et al.*, 1994). The candidate also worked with Dr Lyons towards developing a system to detect slow-growing *Pythium* species in plant and soil (Lyons *et al.*, 1990). A problem with classical taxonomy for the identification of slow growing fungal species from environmental samples (combination of selective medium and microscopic analysis) is that they can be masked by faster growing species. However by employing a period of biological amplification, either baiting with host tissue or by enrichment of soils with semi-selective media, pathogen presence and viability can then be assessed using immunoassay technology (Klopmeyer *et al.*, 1988; Yuen *et al.*, 1993; Thornton *et al.*, 1999). However these tests are likely to remain as qualitative tests and, can prove as timely as conventional media based isolation processes. Where considerable advances were at this time being made was in the development and use of simple immunodiagnostic assays to confirm disease symptoms in infected plant tissues (Rittenberg *et al.*, 1988). A commercial company (Agri Diagnostics, Cinnaminson, USA), using a simple immunoassay flow through system (POC test format), marketed devices for plant-side testing of turf (i.e. golf courses) for infestation of *Rhizoctonia*, *Sclerotinia* and *Pythium* species. The tests could be used by non-scientists and provided results within the hour. In soils however the efficient and simple extraction of fungal antigens was limiting progress. Issues of sensitivity, specificity, non-specific binding of the probe and assay inhibitors within soil components were reported (Dewey *et al.*, 1997; Otten *et al.*, 1997). This had necessitated the pre-treatment of soils (drying, grinding, centrifugation and floatation processes) to recover pathogen resting structures prior to the assay. As a result, the methodologies developed were laborious, lacked an economy of scale and often required extensive laboratory space prior to analysis (Wallace *et al.*, 1995; Miller *et al.*, 1997). In a horticultural context, the ability to develop a highly specific, rapid and sensitive assay is somewhat irrelevant if the pre-extraction process is slow and attracts a cost in staff processing time with facility overheads.

In 1993, the candidate was provided with the opportunity to develop a diagnostic approach towards identifying concentrations of the plant pathogen *Plasmodiophora brassicae* in field soils. An early decision was required on whether to pursue antibody based systems or to adopt a nucleic acid based approach. From the late

1980s a rapid development of molecular techniques for pathogen identification had been observed. While the extraction of nucleic acid and comparison by restriction fragment length polymorphism (RFLP) was proving useful in the laboratory it was likely that the PCR based assay would offer greatest flexibility as a disease management tool. The increasing ability to sequence pathogen genomic content provided a capability to design specific and sensitive primer sets to amplify target pathogen DNA by PCR to detectable levels. The reported sensitivity and specificity of the PCR assay system was attractive. Mills et al., (1994) reported on *Colletotrichum* species specific primers, developed from the internal transcribed spacer regions of the ribosomal DNA repeat units, which were able by PCR to selectively amplify *C. gloeosporioides* from femtogram quantities of fungal DNA. It was estimated that 10 fg of DNA could represent as little as 100 picograms of mycelium, which provided the potential for sub-clinical detection in infected host plants.

Interestingly, the internal transcribed spacers (ITS) of ribosomal DNA have since been reported to be the most widely sequenced DNA region of fungi (Peay *et al*., 2008) and recommended as the universal fungal barcode sequence (Schoch *et al*., 2012). Consisting of alternating areas of high conservation and variability it has proved universal for the development of highly specific and sensitive primer sets for use in PCR based diagnostic tests to discriminate target fungal plant pathogenic species in complex environmental samples (Klemsedal *et al*., 2008; Lees *et al*., 2012). Target organism genomic sequences can readily be compared using DNA-similarity searches like BLAST (Altschul *et al*., 1997) and DNA and RNA sequence databases such as the International Nucleotide Sequence Database (INSD). However some caution is required, as it has been reported that less than 1% of the estimated 1.5 million viable fungal species have been sequenced for the ITS region. As many as 20% of all fungal sequences deposited in the INSD may be incorrectly annotated to species level (Bridge *et al*., 2003, Nilsson *et. al*., 2006). There are also concerns with the classification of species solely as a result of DNA region or gene analysis. Classical identification of plant pathogens has relied heavily on morphological and biological features (van der Plaats-Niterink, 1981). These relationships are not always conveyed when compared by genomic analysis. Kipling and Fabinoff (2004) reports on the myth of the DNA barcode for species classification and reasserts the requirement of morphological analysis in the identification and classification process.

In the field of medical mycology, the uptake of PCR as a diagnostic tool has been constrained by the lack of standardization (De Pauw *et al*., 2008). Nevertheless over the past two decades PCR based techniques have expanded to become some of the most widely used laboratory assays for the direct detection of low levels of pathogenic microbes in environmental samples (Theron *et al.,* 2010). In a

horticultural setting this approach could prove invaluable, in particular for disease quarantine and certification. However to develop a robust, inexpensive DNA based assay system to be delivered by crop clinic laboratories and or directly for use on-site by growers at this time looked challenging. The candidate determined that for successful delivery and uptake of a test to evaluate the pre-planting risk of clubroot disease in soils for the UK horticulture industry, would require a test format which considered the following characteristics :

- specificity to identify the target pathogen at species level;
- sensitivity which could translate a measureable disease risk;
- capability for the test to be used in tandem with other high throughput testing systems used routinely in crop clinics e.g. enzyme-linked immunosorbent assays (ELISA);
- sampling processes / sample volume;
- robustness, simplicity and speed of the test;
- capability of the test to be translated for on-site usage by growers;
- cost - profit margins and emotional attachment to crops are low.

The specificity and the sensitivity that could be achieved by PCR was persuasive in choice of test development. However with multiple races of clubroot identified worldwide and with mixed pathotypes routinely identified from infected hosts, ironically specificity could prove to be problematic. Equally test sensitivity would not provide an overriding factor in choice. The degree of infestation of soil by resting spores has been shown to correlate with clubroot infection on plants. Concentrations in excess of 1×10^5 spores g^{-1} soil are generally required for severe and uniform disease expression (Buczacki and Ockenden, 1978). A further consideration was the use of the test in crop clinic environments. Preceeding the cropping season a high throughput of soil samples would be expected. The molecular detection of fungal pathogens in plant and soil material requires the pre-extraction of DNA (Schaad, 2002). Measurement of the amplicon product following PCR analysis was at this time by gel electrophoresis. The number of samples which could be processed on a daily basis was time and rate limited. Equally an exceptionally high level of hygiene would be required to prevent contamination.

Where immunoassay formats reported challenges in the discrimination and measurement of plant pathogens in soil, similar problems were reported for PCR. For example, careful optimisation and evaluation of the PCR melting and annealing temperatures to prevent the formation of undesirable secondary structures such as primer dimers was required (Saiki *et al.*, 1988; Bej and Mahbubani,1992). Sample inhibitors such as humic and fulvic acids, pesticide residues and organic material were all reported to inhibit the DNA polymerase enzyme (Kong *et al.*, 2003). Equally,

colloidal matter which has a high affinity for DNA was reported to affect the measurement of DNA within samples (Wilson, 1997; Theron and Cloete, 2004). The presence of these in field samples has the potential to affect the amplification process and test sensitivity (Cai *et al*., 2006; Lombard *et al*., 2011; Stewart-Wade, 2011).

Having given consideration to the merits and shortcomings of the available assay systems, the candidate decided that for successful uptake by the industry, adopting an immunological approach provided greatest opportunity to achieve a diagnostic test for the measurement of clubroot in soil. However the limitation at this time to the use of polyclonal antisera, posed a significant challenge in developing a probe of suitable specificity. *Plasmodiophora brassicae*, an obligate plant pathogen, was at this time considered a fungus (recent molecular phylogenetic analyses now suggests that it belongs to the protist phylum Cercozoa in the kingdom Rhizaria (Niwa *et al*., 2011)). Using a resourceful approach in immunogen selection and purification of an immune serum could overcome this problem. With time, opportunity to access monoclonal antibody technology may arise to provide both serum types and achieve a test specificity with sensitivity, if required. Nevertheless, whether employing a molecular or immunological approach, the sample matrix (soil) and inhibitor(s) presence would likely prove problematic across the range of soil compositions which exist in the UK. Also composed of differently sized aggregates and, with microbial populations that are not evenly distributed, a small sample volume for test analysis required consideration when developing a sampling strategy to provide a suitable 'field' test coverage. Although at this time there were reports of successful isolation, concentration and detection of pathogenic bacteria from contaminated feedstuffs (Mansfield *et al*., 1993), faeces (Luk and Lindberg, 1991) aquatics (Bifulco and Schaefer, 1993) and soil (Mullins *et al*., 1995) by immunological approaches. These factors, linked with providing an inexpensive platform suitable for the end user and place of operation, necessitated in the mind of the candidate development of an immunological assay system.

1.2 RATIONALE OF THE PORTFOLIO OF EVIDENCE

Paper 1

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

(referenced in 43 peer reviewed literature citations)

Background

With the increasing concern of pesticide usage and potential harmful effects to the environment, an increased focus was provided to systems which could either optimise the efficient use of approved chemicals for disease control measures or provide other solutions. In 1992 a broad spectrum pesticide, methyl bromide, principally used as a fumigant, was identified as an ozone depleting substance. White (1984) had reported on the use of this chemical for the effective control of clubroot of brassicas by soil fumigation. By 1997, the UK along with other countries agreed a global scheme to eliminate its use and production. For the horticultural industry this posed a serious problem. The phase out of this chemical and others (thiabendazole/iodophor complex, benomyl, thiophanate methyl, carbendazim, calcium cyanamide, dazomet, manganese-zinc-iron-dithiocarbamate, a phenolic mixture, sodium tetraborate and thiophanate-methyl), which in the past have given partial control as soil incorporations (Buczacki, 1973; Buczacki *et al.*, 1976; Dixon and Wilson, 1983, 1984, 1985), now provided growers with few strategies to control the disease. With Brassica crops being of high economic importance in the UK new approaches were required to enable those control processes left (improving drainage, liming to raise soil pH, rotation of susceptible crops with non-cruciferous crops and the planting of resistant varieties) to be predictively deployed for maximum effect.

In the UK, growers of horticultural crops frequently rent land on a yearly basis. Often with limited knowledge of previous cropping histories, the capability to forecast clubroot disease risk would be beneficial prior to contractual agreements being made. The concentration of infestation of the soil by clubroot resting spores has been shown to directly affect the degree of clubroot infection. Resting spore concentrations in excess of $1x10^{5}$ g^{-1} soil are required for severe and uniform disease expression on test plants. Additional factors such as the conducive or suppressive nature of the soil may also influence the concentration required (Rouxel *et al.*, 1988). As the pathogen only grows within living tissues it is not possible to use standard dilution plating techniques to quantify numbers of pathogenic propagules within soil samples. Resting spores can be observed directly in soil

samples using microscopy however this can be very inaccurate, requires specialist knowledge and is highly labour intensive. Some studies have shown that stains can be used to identify resting spores from soil and to determine their viability (Takahashi and Yamagichi, 1989). However the accepted approach developed by Melville and Hawken (1967) relies on the observation of gall formation on bait plants exposed to standard quantities of test soil. This type of test has the disadvantage of being labour intensive, costly and slow (6 weeks).

Approach

As explained in Paper 1, researchers have attempted to develop methods to detect and quantify resting spores of *P. brassicae* using polyclonal antiserum (Arie *et al.*,1988; Lange *et al.*, 1989). The former did not provide an indication on whether the antiserum was of suitable specificity for use in soil. An immunofluorescence process with centrifugation of the soils was described as part of the assay system. Lange (1989) developed a dot immunobinding reaction to confirm infection in plant root tissues. A method to isolate the resting spores from infected host material by filtration and thereafter by suspension in a density gradient was provided. Hardham (1986) had reported on the increased specificity achieved when immunogen was prepared to zoospore cysts rather than the mycelial stage of the pathogen's life cycle. Lange (1989), suggested that the careful isolation of the resting spore structure as a source of immunogen provided an antiserum which was both sensitive and of a specificity that could be used in the soil environment. Equally, simple cell-free surface washings from agar culture have proved suitable as immunogen for antibody production (Dewey *et al.*, 1990) and more accessible during simple sample extraction processes. For the purpose of this work the candidate decided to examine the use of different immunogen preparations in the development of a polyclonal diagnostic probe and how these could be applied in test development for application in soil. It was important from the outset to identify the parameters of a test suitable for use by the horticultural industry and from this direct test development accordingly. The two previous works (Arie *et al.*, 1988; Lange *et al.*, 1989) had focussed on developing systems to identify the resting spore structure. Raising antibody probes to the soluble fraction of the pathogen provided an opportunity to increase flexibility and the potential to develop rapid, high throughput screening technologies (enzyme-linked immunosorbent assay (ELISA)) and or adapt to point of care technologies (POC) to provide 'in-field' testing kits. These two systems would, if developed, provide growers with two options for testing:

- collect soils for ELISA analysis by a commercial laboratory (3-5 days);
- analyse soil samples on site for immediate results.

Paper 1 assesses a range of immunoassays for measurement of the resting spores of *P. brassicae* in soil, explores the potential to improve probe specificity and the

adaptation of procedures to optimise test results. This early work generated a wealth of knowledge which was subsequently utilised in the development of other antibody based probes to diagnose target fungal plant pathogens in water and air. The paper provided the platform for the candidate to demonstrate the potential to utilise a range of antibody based systems for diagnosis of disease risk ahead of infection. This included test applications for use in the laboratory and the potential for on-site testing using soil dip-sticks. The candidate made oral presentations of this work at International conferences. She submitted this work in part for the award of MIBiol CBiol by thesis. The candidate was examined by Dr Molly Dewey (Plant Sciences, University of Oxford) for this award. From this association, Dr Dewey and the candidate collaborated on a number of research themes from which a research paper was later generated (Paper 3).

Paper 2

WAKEHAM, A.J., PETTITT, T.R. and WHITE J.G. (1997). A novel method for detection of viable zoospores of *Pythium* in irrigation water. Annals of Applied Biology 131, 427-435.

(referenced in 18 peer reviewed literature citations)

Background

The candidate was involved in works to identify inoculum and sources of *Pythium* species involved in seedling 'damping off' of ornamentals in UK nurseries. Over 150 species of *Pythium* have been described, occur on a wide and diverse host range (reported as parasitic on plants, animals (such as fish or crustaceans) and humans (causative agents of arteritis, keratitis, cutaneous or subcutaneous infections) and are of worldwide distribution (Kirk *et al*., 2008). Belonging to the family Pythiaceae and class Oomycetes, *Pythium* has a basic life cycle with resting, sexual oospores and asexual sporangia which in many cases are capable of releasing large numbers of infectious, motile zoospores. The propagation of bedding plants is a successful and vital component of the UK horticultural industry. Losses due to *Pythium* infestation can be high and are often triggered by specific environmental events. The propagation of monocultures of ornamentals in high pressure growing conditions provides a context for heavy losses to occur. 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 represents a real threat. Conventional plating of plant tissue or soil suspensions onto semi-selective agars containing antibiotics is a simple and useful procedure for isolating and identifying oomycetes. This process is commonplace in plant clinics but requires time and is skill dependent. Quantification

may be achieved by communition of the material followed by plating dilutions onto selective agar plates and counting the resulting colonies (Pettitt and Pegg, 1994). Baiting techniques have been used since the late 1960s for both *Phytophthora* and *Pythium* detection (Werres *et al.*, 2014). However, baits are of variable sensitivity, being dependent on the quality and physiological state of the plant tissue used and can provide confirmation of presence/absence with a limited capacity for quantification (Tsao, 1983). In the early 1990s, a commercially available qualitative flow-through immunoassay system was marketed for the plant side testing of *Pythium*, *Phytophthora* and *Rhizoctonia* species (Agri Diagnostic, USA). This test provided confirmation to the presence or absence of disease on plants expressing disease symptoms. However the sensitivity and specificity of these tests, along with their inability to differentiate between live and dead disease, prevented their use in pre-symptomatic plant health assessment. The value of these tests for environmental (substrates and water) sampling was unknown and, of genus specificity, might jeopardise indigenous biocontrol agents.

In test development consideration of the existence of fungal species that contain pathogenic and non-pathogenic or even beneficial strains should be considered. This is a known phenomenon for complex species such as *Fusarium oxysporum*, *F. solani*, *Rhizoctonia solani* (Recorbet *et a*l., 2003). *Pythium*, which is one of the most frequently described oomycete plant pathogens, can be a parasite of other fungi. This provides real potential in use as a biocontrol agent for horticulture and agriculture (Martin and Hancock, 1987; Paulitz *et al.*, 1990; White *et al.,* 1992). Others are primarily saprobes and found abundantly in the environment. In horticulture, many different *Pythium* species occur across a range of cropping systems. The challenge in developing a suitably specific and sensitive probe will rely on the capability to identify and detect the pathogens responsible for crop specific diseases. For this purpose, the candidate was involved in a study to identify those species which were highly aggressive on ornamental hosts. After which a polyclonal antiserum was to be developed for incorporation within an immunoassay format to provide the timely prediction of disease outbreak ahead of symptom development.

During time spent at UK industry holdings, examining the production process of bedding plants, it became apparent to the candidate that reservoirs and irrigation systems had the potential to provide a source of disease that could be quickly transferred across areas of the nursery. At one plant nursery visit, the candidate met with Dr Tim Pettitt, then a senior research scientist based at Efford, who was monitoring a chrysanthemum trial for *Phytophthora* infestation. Diagnosis of disease presence was measured by visual symptoms of the exposed plants, selective isolation from exposed roots on culture media and baiting of water samples. Following their meeting, the candidate determined that the development of an immunological approach to monitor disease in water systems had the potential to:

- sample increased volumes of water;
- be transferred to a crop clinic for use by technicians or by consultants with minimal laboratory processes required.
- discriminate viable from non-viable disease propagules.

The capability to differentiate between viable and non-viable organisms was important as in irrigation systems, soil and composting material treatment processes (heat, UV light, slow sand filtration) can lead to detectable organism presence (by immunological or molecular techniques) but which are dead or no longer present a disease risk.

Approach

Studies in Paper 1 had shown how antigenic material on the exterior of the disease propagule (spore) could be detected by the attachment of a specific probe (antibody) and visualised using a conjugated fluorescein marker. The candidate proposed that by isolating *Pythium* species from water on to a membrane, the trapped propagules (zoospores) could be concentrated and then quantified by the attachment of a visual marker. As in paper 1, the disease propagule would be labelled with a polyclonal antiserum. To remove the requirement of laboratory equipment (microscope) the antibody probe would be conjugated to an enzyme label to produce a red precipitating substrate which could be visualised using a magnifying glass. The candidate was keen to compare this process with an existing laboratory method routinely used to identify Pythiaceous contaminated nursery supplies. She collaborated with Dr Tim Pettitt and, with the inclusion of a pre-incubation step of the filtered membrane in a selective growth medium, devised a simple immunodiagnostic test which had the potential to be used by non-scientists and, was able to discriminate between live and dead disease propagules. The candidate published this work with collaborators Dr T. Pettitt and Dr G. White in 1997 followed by two further papers in 2002 and 2011.

The candidate continues to meet with Dr T. Pettitt to explore the potential for collaboration and to identify funding partners. The European Water Framework Directive (Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy) provides emphasis for closed and sustainable water systems in greenhouse areas by 2015. Sustainable solutions to deal with disease potential will become increasingly important if growers are required to recycle irrigation solution (van der Velt *et al.*, 2008). This provides the opportunity to further develop the assay system and adapt towards an on-line biosensor system.

Following this early work, the candidate was promoted to the position of higher scientific officer and shortly afterwards transferred to work within the foliar pathogen group to develop immunological approaches for the measurement of disease in air samples.

Paper 3

KENNEDY, R., WAKEHAM, A. J., BYRNE, K. G., MEYER, U. M. and DEWEY, F. M. (2000). A new method to monitor airborne inoculum of the fungal plant pathogens *Mycosphaerella brassicicola* and *Botrytis cinerea*. Applied and Environmental Microbiology p. 2996–3000.
(referenced in 42 peer reviewed literature citations)

Background
Prof. R. Kennedy had secured research funding on a DEFRA project (HH1795SFV) to study disease transmission of foliar fungal pathogens of vegetable brassicas for the prediction of disease risk. Discussions between the candidate and R. Kennedy had determined that existing sampling technology might be adapted to monitor airborne disease transmission events of fungal plant pathogens in the field. By raising a polyclonal antiserum, the candidate demonstrated that a target pathogen could be labelled, identified using an anti-antibody fluorescein conjugated probe and daily spore concentrations determined by selective visual discrimination once trapped on a collection surface. This methodology was published as part of a paper describing ascosporic inoculum production of the ringspot plant pathogen *Mycosphaerella brassicicola* (Kennedy *et al.,* 1999). This approach provided capability to identify a target disease in air samples ahead of symptom development on exposed plants and the opportunity to develop new more rapid methodologies. Prior to this, scientists had relied on identification of trapped spores on a Melinex tape by morphological analysis using bright field microscopy. This process relied on highly experienced scientific staff, was laborious, subjective and excessively time consuming. In a field environment, identification of Melinex tape trapped *M. brassicicola* ascosporic inoculum was deemed to be inaccurate based purely on light microscope observations. A later study reported that the reliability of data from Melinex tape bright field microscopy could result in sampling errors ranging from 7-55% of the mean value and miss 22 to 54% of the taxa present (Gottardini *et al.,* 2009).

During this period, the candidate remained in contact with Dr Dewey at Oxford. Dr Dewey was also working towards developing processes to monitor *Botrytis cinerea* in collected aerosols. Earlier studies to identify airborne disease transmission of *Botrytis cinerea* in cut flower production had focussed on the development of a petal test (Salinas and Schotts, 1994). Through discussion and collaborative work with an air sampling company (Burkard Manufacturing, Rickmansworth, UK) a new sampling prototype was devised. The new system (Microtitre Immunospore Trap (MTIST)) was based upon an agar personal air sampler and adapted to deliver fungal spores directly in to microtitre wells for quantification by PTA ELISA (plate trapped antigen enzyme-linked immunosorbent assay). By altering the trumpet delivery system an increase in spore coverage was achieved. The basis of the new system was to provide measurement of multiple bioaerosol targets and for the results to be generated within a few hours of sample receipt at the laboratory. The candidate and Dr Dewey confirmed the use of the new air sampler for collection of the spore types that they were currently involved with and the potential for a collaborative study.

Dr Dewey was established in the delivery of monoclonal antibody sera (MAbs) to label and measure fungal plant pathogens. This provided the candidate the opportunity first hand to work with MAbs and encouraged her to actively pursue this line for future studies. Due to the research groups she had previously been aligned to, the candidate had been limited to the production of polyclonal antisera. This had been a frustration although had allowed the candidate to examine the potential of antibody purification to improve serum specificity. However the introduction of a monoclonal antibody unit to the department at the candidate's place of work in the late 1990s now provided a potential for change. The candidate collaborated and wrote proposals on the development of MAbs to a number of plant pathogens. Two of these (*M. brassicicola* and *Albugo candida*) are described in Paper 5 and Report 1.

Approach

The rationale for paper 3 was based upon the development of a system to monitor aerosol concentration for multiple pathogen types with increased speed and accuracy. The enzyme-linked immunosorbent assay is probably the most widely used antibody-based test suitable for the quantitative measurement of large numbers of samples quickly (within hours), of high sensitivity and at relatively low cost. For this purpose it was proposed that aerosol delivery could be made across eight multi-wells of an ELISA strip. Each strip could be then be removed and trapped aerosol particulates selectively identified and concentrations measured by ELISA.

Clark and Adams first introduced the use of the ELISA for the quantification of plant viruses in host tissues in 1977. For viral and bacterial samples, many of the

commercial ELISA systems use a double antibody sandwich format (DAS ELISA). This can prove useful in capture and isolation of a target pathogen from a complex material and improve specificity with attachment of a second antigen specific labelled antibody. However, it is the plate trapped antigen (PTA ELISA) which is generally used in the diagnosis of fungal plant pathogens. As described earlier, the glycoprotein fraction of the fungal immunogen is reported to predominantly elicit an immunodominant response from the host. As a result, selected antibodies are often directed to this form of structure. In general, soluble glycoprotein structures do not lend functionally to the binding of two antibody types at one time. However glycoproteins often bind readily to the solid phase surface of an ELISA process and so do not require a capture antibody as in the DAS system. By sampling bioaerosols in to the microtitre wells a PTA ELISA could be carried out directly. For this purpose it was found necessary to improve the trumpet delivery design to provide increased spore coverage across the wells and with the spores remaining spatially separated. To provide multiplex capability the number of collection wells was increased to four eight well strips. When tested with *M. brassicicola* ascospores, there was little difference in mean distribution of impacted ascospores of *M. brassicicola* across the four strips. However distribution within each strip provided greater numbers of spores in the inner four wells. In a multiplex format design of the assay would require consideration of this. When the collected microtitire wells were processed by PTA ELISA, the quantitative measurements of each spore type correlated with the microscopic counts.

In support of this study, the candidate was interested to determine whether a relationship could be determined between pathogen aerosol concentration and disease development on susceptible host plants following optimal environmental conditions for infection to occur. This process was facilitated under controlled conditions where ascosporic inoculum from sporulating cultures of *M. brassicicola* was monitored by the microtitre immunospore trap (MTIST) and immunoquantified to provide an estimation of airborne biological threshold for ringspot disease expression on exposed plants. Later studies (paper 5) report on the translation of this technology to a field situation where disease transmission periods are identified. However, modification of the air sampler for this purpose, and the potential to operate remotely when optimal environmental parameters for spore release were met, are identified.

Paper 3 reports on the development of a new air sampling system which demonstrates the potential to monitor airborne disease transmission in a multiplex format and provide measurement of target diseases within a few hours of sample receipt. The collection efficiency is validated using a commercially available air sampling device, designed to sample airborne particles such as fungal spores and pollens, with performance akin to that described by Hirst in 1952.

Paper 4

WAKEHAM, A.J., KENNEDY, R. and MC. CARTNEY, H.A. (2004). The collection and retention of a range of common airborne spore types trapped directly into microtitre wells for enzyme-linked immunosorbent analysis. Journal of Aerosol Science. 35, 835-850.

(referenced in 8 peer reviewed literature citations)

Background

Bioaerosol particles include fungi, viruses, bacteria, pollen (as well as fragments and products of these organisms), plant or animal debris. They are ubiquitous in the ambient air and are characterized by a broad size spectrum (submicron values up to ~ 200 μm). They occur in a wide range of number concentrations which can be location and environment driven. Accurate field-compatible methodologies for the measurement of bioaerosols are essential. Bioaerosol samplers can be assigned into passive and active samplers:

- Passive deposition of bioaerosols on to collection surfaces is by gravitational pull and provides the simplest method for collection of bioaerosols. However, air turbulence can provide a significant factor in collection efficiency and is of little value for trapping particles <30 μm diameter. Passive deposition provides a qualitative measurement of airborne particles.
- The active collection of bioaerosols can broadly be characterised in to three groupings of impaction (separates particles from the air stream onto a solid collection surface), filtration (separates particles from the air stream by filtration through a membrane) and impingement (separation of particles from the air into a liquid).

High volumes of air can be sampled using active collection processes. The bioaerosol collection can be made into different fractions. Active collection processes, however, require a power source and can suffer from overloading of samples and are prone to loss of biological efficiency (measures the physical efficiency of collection but also the losses in viability of microorganisms during sampling that are caused by cell damage or stress, through impaction, dehydration and or hydration/osmosis). Particle size, wind velocity and the sampling device used will affect the collection efficiency of bioaerosol particulates (Ljungqvist and Reinmuller, 1998; Buttner *et al.*, 2002).

The microtitre immunospore trap (MTIST) samples at a flow rate of 57 L air per minute and provides a single impaction surface for the collection of bioaerosols. Air is drawn through a ‘trumpet’ and directed towards the base of a microtitre well. As the air turns away from the well surface particulates that cannot follow the flow are

impacted. The design concept of the MTIST was built around the capture of fungal spores involved in airborne disease transmission events of horticultural cropping systems. In application, a target spore size range of 1 to 30 μm was identified and of differing physical characteristics. The collection capability of the MTIST and, the effect of the ELISA process for quantitative measurement, had been reported on in Paper 3 but limited to two spore types. Reports previous have attributed to particles bouncing off dry impaction surfaces and the requirement of coating substrates to minimise this effect (Dzubay *et al*., 1976). Fungal bioaerosols constitute the major component of ambient airborne microorgansims (Hinds 1999; Yeo and Kim, 2002; Wu *et al*., 2004). For uptake of the technology characterisation of the MTIST for the collection of an extended range of fungal spore types would be required. The MTIST could prove useful across a range of disciplines to include plant, human and animal health.

Particles larger than 30 μm in aerodynamic diameter have low probability of entering the nasal passages and are thought to be less important in aspects of human health. Particles of 5-10 μm in diameter usually deposit in the passages of the nose and pharyngeal region. Smaller particles (1-5 μm) deposit in the tracheal bronchiolar region, whereas particles less than 1 μm in diameter are deposited on alveolar walls by diffusion (Owen *et al*., 1992). Studies have reported that the concentration of fungal bioaerosols is significant to the occurrence of human diseases and public health problems associated with acute toxic effects, allergies and asthma (Gravesen, 1979; Burge 2001; Bush and Portnoy, 2001). Fungal bioaerosols are of particular concern in healthcare facilities, where they can cause major infectious complications as opportunistic pathogens in patients with an immunodeficiency (Denning *et al*., 1997). The respirable size fraction of 1 to 10 μm is of primary concern as at this size fungal spores can become trapped within lung tissue and they are not easily expelled, which poses greater health risks. Airborne pollen is also of importance in human health with one of every five persons affected by allergic sensitisation. Pollen provides one of the most common triggers of respiratory allergy affecting lungs, nose and the eyes. Pollen grain counts in ambient air have traditionally been assessed to estimate airborne allergen exposure using Hirst style impaction samplers. For pollen and fungal spores, the Hirst spore trap is considered the reference method in aerobiology and with analysis by bright field microscopy (Hirst, 1952).

Approaches

Paper 4 sought to address the collection efficiency of the MTIST spore trap for a range of spore types of different shapes and sizes. Fungal spores are typically 1 μm to 30 μm in diameter (Gregory, 1973) and pollens have typical diameters of 17 to 58 μm. The behaviour of any particle in the atmosphere will depend upon its size, density and shape. Four fungal spore types were chosen to reflect their size and shape characteristics and importance in human health and or agriculture:

Penicillium: A number of *Penicillium* species have been identified as important mycotoxin producers. The predominant species found in human food and animal feeds was *Penicillium crustosum* (Frisvad and Thrane, 1995). *P. crustosum* is a very common fungus in food spoilage worldwide and several cases of animal intoxication associated with ingestion of *P. crustosum* contaminated feed have been reported (Arp and Richard, 1979; Hocking *et al.*, 1988; Naude`*et al.*, 2002). *Penicillium roqueforti*, a common saprotrophic fungus and of major industrial use in the production of blue cheeses, was used in this study as it was not considered a human pathogen but retained the size and aerodynamic characteristics of *P. crustosum* (smooth conidia, spherical, 2.8-4.2 × 2.6-3.5 μm).

Cladosporium species are not considered pathogenic for humans, except for immunocompromised patients. However, *Cladosporium* has the ability to trigger allergic reactions in sensitive individuals. In horticulture, the fungus is associated with rot on grape berries and can significantly reduce yield and wine quality at harvest (Briceño and Latorre, 2008). Recently *C. cladosporioides* has been identified as causative agent of sooty spot in post-harvest losses on satsumas (Tashiro et al., 2013). The airborne conidial stage is elliptical in shape with a smooth, verrucose or echinulate surface (3-7 x 2-4 μm).

Botrytis cinerea (causative agent of grey mould) infects over 200 cultivated plant species and causes significant economic damage to crops worldwide. Conidia are ellipsoid in shape (8-14 x 6-9 μm) with a rough surface but show no regular ornamentation. The conidia are reported to adhere to substrata immediately upon hydration although they are strongly inhibited by ionic or nonionic detergents (Doss *et al.*, 1993). The pathogen has been associated with human respiratory diseases including wine growers lung (Lee and Liao, 2014).

Erysiphe cruciferarum causes powdery mildew on Brassicas and can infect any above ground plant part of the vegetable brassica plant. Powdery mildew reduces plant growth and yield and in seed production a reduction in the quantity and quality is observed. Most horticultural brassicas are susceptible to infection by *Erysiphe cruciferarum* and these include Brussels sprouts, cabbage, Chinese cabbage, kohlrabi, broccoli, kale, mustard, collards, cauliflower, radish, and horse radish. Conidia of *E. cruciferarum* plays a vital airborne role in dissemination of the disease. They are relatively large fungal spores (30-40 x 11-15 μm), ellipsoid to cylindrical in shape, hyaline and with a surface characterised by net-like ridges (Cook *et al.*, 1997). *E. cruciferarum* is not considered a human pathogen.

Lycopodium clavatum, the common club moss, has large spores (35–45 μm) and is widespread in northern temperate, sub-arctic and alpine environments. The spore has a high concentration of fatty acids and forms a fine, emollient, hydrophobic

powder which has been utilised in theatrical makeup, as a dust to surgical gloves and in condom manufacture. However occupational exposure to *L. clavatum* has been reported to induce allergenic responses and since 1992 manufacturers in the UK have ceased to use the spore as a dusting agent (Cullinan *et al.*, 1993). As a result of size, commercial availability and the highly mono-dispersed state the spores are used often in particle bounce and impaction studies (Aylor and Ferrandino, 1985). It has been reported that the fraction of bounce is a strong function of particle size (Wang and John, 1987).

The MTIST air sampler allows the impaction of collected aerosols directly on to a flat polystyrene surface. Although not sampling at a high volume, compared to some commercially available particulate matter bioaerosol samplers (800 to 1100 litres air per minute), the potential for particle bounce is a cause for concern. Equally, the ELISA process has a requirement for the retention of target bioaerosol material throughout the immunoassay process to make quantitative measurement. The loss of spores at each stage could lead to an underestimation of the collected bioaerosol. To investigate this, a range of microtitire well coatings were evaluated for the improved collection and retention of spore types in bioaerosols:

- a mixture of petroleum jelly and paraffin wax has been used to coat the impaction surface of spore trapping systems (British Aerobiology Federation, 1995);
- gelatin, casein and bovine serum albumin are commonly used blocking buffers in the ELISA process, although usually used to coat ‘unbound’ sites post attachment of target material;
- poly-l-lysine which can be used in cell culture to promote cell adhesion to solid substrate.

The effect of wind speed on sampling efficiency was also investigated. In Paper 3, the MTIST device was used to sample bioaerosols at low wind speeds and there was no assessment of the behaviour of the device when sampling outdoors. A potential use of the air sampler was for studies in the field where variable wind speeds would occur. To allow adaption for this, the MTIST had been modified for outdoor sampling by adding an inlet manifold and mounting the sampler on a wind vane so that the inlet faces the air flow. To investigate the effect of windspeed on the collection efficiency of the MTIST for a range of spore types, the candidate contacted Dr Alistair McCartney at Rothamstead Research, UK to request a collaborative study using the wind tunnel facility that was sited there. The findings of this work were published in 2004 and have proved useful in later work funded by HDC for a range of fungal plant pathogens.

Paper 5

WAKEHAM, A.J. and KENNEDY, R. (2010). Risk Assessment Methods for the Ringspot Pathogen *Mycosphaerella brassicicola* in Vegetable Brassica Crops. Plant Disease 94, 851-859.

(referenced in 6 peer reviewed literature citations)

Background

During this period the DEFRA project (HH1795SFV) on which the candidate had been employed had come to a close. The work generated from this had demonstrated the potential to monitor field aerosols for inoculum. In association with the candidate, funding was secured by Prof. R. Kennedy from the Horticulture Development Council to further develop this approach and to translate this technology to commercial Brassica cropping systems. Early detection of disease can lead to an increase in production, an improvement of resource efficiency and make a substantial contribution to food security. In the airborne environment, many plant diseases are able to spread quickly between and within cropping systems. *Mycosphaerella brassicicola* is a major airborne fungal pathogen of vegetable brassicas in the UK and of widespread geographic distribution. In Brussels sprout production, 4 to 6 fungicide applications are routinely applied to control the disease and maintain the high quality of produce demanded by the market. With a 'field' latent period often of between two to three weeks, the disease can become established prior to symptom development. Additionally many of the diseases found on Brussels sprouts are difficult to diagnose correctly, appearing initially as small black spots. As disease symptoms progress, a major problem for growers and consultants is the ability to differentiate ringspot from dark leaf spot (caused by *Alternaria brassicae* or *A. brassicicola*). Similarly, older lesions of powdery mildew often resemble small black spots. This can result in a delay in application of suitable control measures at potentially important periods in crop production.

Monitoring environmental conditions necessary for infection can be used to determine risk of ringspot infection and other spot diseases of Brassicas (Kennedy and Graham, 1995). *Mycosphaerella brassicicola* requires periods of temperature and wetness to complete spore production within lesion structures. These requirements have been programmed into a computer based model to provide an infection risk (Brassica spot forecast: http://www.worcester.ac.uk/discover/6273.html). However, favourable environmental conditions often occur in the absence of disease inoculum. The ability to identify inoculum transmission periods of target spore bioaerosols at concentrations likely to initiate disease symptoms, could enable precision deployment of control measures at times of environmental disease risk. In Paper 3, the relationship between *M.*

brassicicola aerosol concentration and disease development on susceptible host plants had been evaluated. In Paper 5, the candidate looks at the potential of the MTIST air sampler to measure by PTA ELISA *M. brassicicola* spore concentrations in field bioaerosols. The use of this information to predict the onset of ringspot risk on Brussels sprouts in conjunction with an environmental forecast model is discussed.

Approach

The candidate included in the work proposal the production of a monoclonal antiserum (MAb) to the ascosporic stage of *Mycosphaerella brassicicola*. Initial purification of polyclonal antibody sera and the strategic use of immunogen preparation had prior to this been applied to good effect (Wakeham *et al.*, 2000). However the candidate considered that a MAb probe was a necessity in a commercial application. The candidate's experience in the selection of immunogen proved key in this work to provide a suitable cell line for the measurement of *M. brassicicola* ascospores. Having developed a suitable MAb, she focussed on validating the MTIST air sampler to monitor field aerosols. An earlier immunofluorescence air-sampling test developed by the candidate (Kennedy *et al.,* 1999) proved useful in this approach. Paper 5 reports in part on this and describes the use of the two air sampling systems to monitor disease transmission events of *Mycosphaerella brassicicola* over a distance and, within a field setting. Environmental factors of wind run (distance of the travelled wind over a period of time), rain and humidity are considered and their effects on the MTIST air sampler in measurement of spore concentrations. The paper considered also the development by Prof. R. Kennedy of an environmental disease forecast model. The study presents the potential to ascribe disease risk based upon two factors: inoculum availability and the environmental conditions during the period assessed. The MTIST system provides capability to quantify field inoculum concentration in a time frame that allows targeted control measures to be applied and prevent disease expression on the host plant. Offering a multi-test approach, this system is now used commercially in the UK for a number of horticultural plant pathogens.

Paper 5 proved pivotal in securing additional research funding from the now Horticulture Development Company (HDC) to develop and validate diagnostic assay systems to monitor airborne disease transmission events in commercial cropping systems of the following diseases of vegetables: dark leaf spot (*Alternaria brassicae A. brassicicola* complex, Light leaf spot disease (*Pyrenopeziza brassicae*), powdery mildew (*Erysiphe cruciferarum*), downy mildew (*Peronospora parasitica*) on onions and gummy stem blight (*Mycosphaerella melonis*) on cucurbits. For each of these pathogens, the MTIST air sampler has been evaluated for use in measuring bioaerosols for disease and more recently in the validation of more rapid 'in-field' POC diagnostic assay systems. These approaches are described in Report 1.

Report 1

WAKEHAM, A.J. and KEANE, G. (2011). Benchmarking predictive models, nutrients and irrigation for management of downy and powdery mildew and white blister. Horticulture Australia Ltd. HAL Final report VG07070, pp 28.

Background

This work was commissioned by Horticulture Australia Ltd and carried out in association with the Department of Primary Industries (DPI), Victoria, Australia. The work provided an extension to a DPI project which had focussed on the use of an environmental model to forecast disease epidemics of white blister (*Albugo candida*). The white blister risk model used had been developed for the UK industry to improve the efficiency of protectant fungicides in the control of the disease in vegetable brassicas (Kennedy, 2005). However the frequent occurrence of favourable environmental conditions provides opportunity to overestimate the real risk of disease establishment in crops.

At this time, the candidate was collaborating with Dr R. Faggian (DPI, Melbourne, Australia). Dr Faggian had developed a DNA molecular based approach to measure resting spores of *Plasmodiophora brassicae* in soil and water (Faggian *et al*., 1999). Through HDC funding, the candidate was investigating the ability to isolate, concentrate and quantify clubroot resting spores from infested soils using immunomagnetic separation. Used in conjunction with a 'lab on a stick' this approach (POC test) would provide the potential for the inexpensive 'in-field' testing of soils. This provided the opportunity for consultants and growers to make self-assessment of field soils for disease contamination and concentration. Dr Faggian was keen to explore the potential of this technology and the candidate was keen to utilise the molecular approach for comparative studies with the 'in field' immunological system. Dr Minchinton (DPI, Melbourne, Australia) became aware of this collaboration and of the 'in-field' diagnostic approach that was being applied by the candidate to both soil and airborne pathogens. From this, the candidate was approached by Dr Liz Minchinton to develop and submit a work package that would provide opportunity to develop an 'in field' test with capability to monitor bioaerosols for *A. candida*.

Approach

Based on host specificity more than 10 races of *A. candida* have been identified. Race 9 is identified as pathogenic on *Brassica oleracea* and is considered to have caused the recent outbreaks of white blister on Broccoli in Australia. Transmission of *A. candida* zoosporangia (spores) in bioaerosols has been observed using traps similar to those described by Hirst (1952). It is likely that they occur in high enough

concentrations to play an important role in transmitting the disease between vegetable Brassica crops in the field. To measure race 9 in field bioaerosols, monoclonal antibody technology was used in the development of a diagnostic probe. The candidate had previously used whole spores, mycelial washings and co-immunisation processes to achieve antibody probe specificity. However, given the requirement to selectively measure at the race level the candidate decided to approach the task by the isolation of molecular-weight fractions. MacDonald (1989) had reported that non-specific antibodies generally recognize antigen only in the high molecular weight range ($M_{r>}$150,000) whereas specific antibodies detected antigens in both the high and lower molecular weight range (M_r10,000). When mice were immunised with low molecular mass(<30 kDa) *Botrytis cinerea* and *Aspergillus flavus* culture washings a reduction in non-specific cell lines were observed (Bossi and Dewey, 1992; Priestley and Dewey, 1993). For the purpose of this study the candidate separated soluble washings from *A. candida* race 9 zoosporangia into molecular mass fractions of >30 kDa and <30 kDa for immunization. Gary Keane, as an animal licence holder, immunized the mice and carried out the monoclonal antibody hybridoma technology procedure.

The report provides the opportunity to draw upon the developments made in the portfolio of evidence and to demonstrate the step by step approach that is taken to develop a diagnostic test for application within a disease management system. The report documents the progression of the MTIST air sampler (laboratory test) and an 'in field' test (lab on a stick or point of care system (POC)) to provide near real-time monitoring of a plant pathogen in field bioaerosols. Detailed information is provided on the methodology for this and the field validation processes used. The purpose of this work was to develop a diagnostic system that could be used on-site, by the end user (grower) to make informed crop protection decisions. As in paper 5, this report provides an example of how information on inoculum availability can be used with an environmental disease forecast model as part of an integrated crop disease management system.

The in-field test adopted in this approach is often described as a lateral flow, lab on a stick, immunochromatographic test strip or POC and can provide qualitative and quantitative detection of target analytes. The test consists 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. Specific interactions can occur at these points with target analytes present in the sample. Results are usually generated with 5 – 10 minutes, with the formation of a control and test line as appropriate to the sample and the test type. They are designed for single use, can provide a multiplex test platform and are available commercially for a wide range of applications. The most well know of these is the Unilever Clear Blue Pregnancy Test Kit.

In-field tests developed by the candidate through HDC funding are available commercially and used in crop protection networks in parts of the UK (www.syngenta-crop.co.uk/brassica-alert). The tests are currently being trialled across Europe and Australia.

1.3 CONTRIBUTION OF OTHER PEOPLE TO THE PORTFOLIO OF EVIDENCE

Paper 1 – Dr J.G. White secured MAFF funding for the production of polyclonal antibodies to *Plasmodiophora brassicae* and development of a suitable assay to quantify resting spores in soil. The candidate was employed solely on the project and developed this area of work as her own. Dr J.G. White took no part in the writing of the paper but reviewed the text prior to journal submission. This work formed the basis of a submission for the award of MiBiol CBiol by thesis and it is certified that the work is a result of the candidate's (A. Wakeham) own investigation.

Paper 2 – This work was initiated and led by the candidate in collaboration with Dr T Pettitt. Dr Pettitt isolated and identified the fungal species from irrigation water by baiting and standard agar plate isolation with bright field microscopy. Dr Pettitt also produced the zoosporic material for tests carried out in the study. The candidate developed the zoospore trapping immunoassay and wrote the paper. Drs J.G. White and T. Pettit reviewed the work prior to journal submission.

Paper 3 – Drs Dewey and Meyer provided all works and protocols which related to studies with the plant pathogen *B. cinerea*. Prof. R. Kennedy provided research funding for the study through MAFF HH1795SFV and editorial support to the paper ahead of journal submission. The candidate was scientific lead for the study, wrote and liaised with the paper co-authors. She supervised a technician for the spore and disease enumeration work.

Paper 4 – Prof. R. Kennedy provided research funding for the study through MAFF HH1795SFV and editorial support to the paper prior to journal submission. Dr Mc Cartney contributed material, facilities and knowledge associated with the wind tunnel studies. The candidate developed the research area, provided the scientific lead for the study and wrote the paper liaising with the co-authors. She supervised two technicians for the enumeration of the collected spores.

Paper 5 – The paper is a culmination of work contributed to by both authors. Prof. R. Kennedy provided lead research on the controlled environmental studies, investigating ascospore release and the modelling of the data to include field wind run studies. The candidate provided lead research on all work associated with the development and monitoring of aerosols for the presence of ascosporic inoculum of

M. brassicicola. Both authors were involved in field experiment design, execution of the study, writing of the paper and journal submission.

Report 1 – This work was commissioned by Horticulture Australia Ltd and in association with the Department of Primary Industries (DPI), Victoria, Australia. The work provided an extension to a DPI project which had focussed on the use of an environmental model to forecast disease epidemics of white blister (*Albugo candida*). The candidate developed and submitted the work package for approval to Dr Liz Minchinton (DPI researcher). Gary Keane, as licence holder immunized the mice and carried out the monoclonal antibody hybridoma technology procedure. The candidate executed all other work, and compiled and wrote the report.

1.4 EVALUATION OF THE PORTFOLIO OF EVIDENCE

In the 1970s, antibody-based immunoassays were reported for the detection of viruses and bacterial plant pathogens '*in planta*'. The detection and quantification of fungi proved more difficult but with the advent of monoclonal antibody technology, advances have been made. Nevertheless, immunoassay fungal test development has been slow and plant-side testing often restricted to disease identification only once symptom expression has been observed. This body of work reports on the development of antibody probes to ascribe disease potential ahead of symptom development and their use in commercial disease management strategies. The selected outputs show a progression of the technologies available for the study period and their adaptation for use to provide new sampling processes. A range of media are assessed and protocols devised for each to facilitate the measurement of crop pathogens in soil, water and air. To achieve the research objectives, the available methodology for the diagnosis of the selected disease targets is reviewed. The requirement and strategy for the development of new processes is identified and the developmental process described. Within the submitted material, examples of diagnostic test conception through to commercial uptake by industry are described. This work makes a significant contribution to monitoring specific disease epidemics and providing information on risk of crop pathogens in a range of environments. It should however not be overlooked that in the development and translation of these systems there are often considerable technical and biological challenges to overcome:

The first technical challenge concerned the development of the MTIST air sampler. This has led to successful uptake by industry, both in outdoor and protected crops (Brassicas, Alliums and more recently Cucumbers). Disease thresholds have been determined and the detection of pathogens in air samples can be measured using

either laboratory analysis (MTIST ELISA) or on-site testing for disease potential. The detection of pathogens in soil has proved more difficult. Issues of sensitivity, specificity, non-specific binding of the diagnostic probe and inhibitors are documented in environmental sample assay development.

Sample size with the efficient and simple extraction of pathogen material remains however probably one of the biggest hurdles in the development of a quick and sensitive test. With assay systems increasingly moving towards nanotechnology, smaller sample volumes are demanded. This presents difficulty in identifying a sampling strategy to provide suitable test coverage. This may lead to difficulties when transferred to a large cropping acreage. As documented earlier, soils can be particularly problematic, with a range of soil compositions, composed of differently sized aggregates and with microbial populations that are not evenly distributed. In air, the outdoor spatial sampling range or coverage is not well characterised. This could be problematic where regions differ in microclimates and / or geographical landscape, i.e. Cornwall and the Lincolnshire Fens.

For each of the media tested (air, soil and water) there should also be consideration of whether the test requires the capability to differentiate between viable and non-viable organisms. This may prove particularly critical in nursery irrigation systems or soil and composting materials where treatment processes can result in detectable pathogen but which no longer present a disease risk. Equally, test sensitivity should be at an appropriate level and of specificity that does not jeopardise indigenous bio-control agents.

Nevertheless, the capability now to multiplex for different pathogens affecting a cropping system, provides real potential to maximise the efficiency of available control treatments in an integrated disease management system (ICDMS). This is becoming increasingly important as crop protection actives are being withdrawn and or with a limited usage within a cropping season. Lievens et al., (2006) describes the development of quantitative PCR (q PCR) to measure the concentration of a number of economically important fungal pathogens of tomato in soils and plant material (*Fusarium solani, Rhizoctonia solani, Verticillium* species responsible for tomato wilt and *Pythium ultimum*). The MTIST sampler provides, by PTA ELISA, the discrimination in Brassica crops of powdery mildew, light leaf spot, ringspot, white blister and dark leaf spot inoculum. This approach can assist the cultivator to identify and optimise usage of control measures for disease complexes over a season rather than individual diseases as they occur on plants. Key to this, however, is the knowledge of pathogen concentration coupled to the associated environmental parameters. This information will provide accurate test disease thresholds at which

damage on crops may occur. However, some caution is required as disease progression may be influenced by the cultivar grown and the cropping conditions.

Once a suitable diagnostic prototype is available, it is essential that it is extensively validated against existing adopted systems (i.e. the isolation of pathogens by use of selective media, culture based morphological analysis, plant baiting, Koch's postulates) 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 with multiple 'non-skilled test' end users. A programme of work led by the candidate (HDC CP099A) to validate an on-site test for clubroot disease in field soils (Wakeham *et al.*, 2012) provided a series of workshops nationwide to evaluate grower usage of the test with artificially infested clubroot soils. From this, modifications were required to the test format to improve uniform sample delivery. Equally the necessity for an electronic reader was confirmed rather than a visual assessment by eye. Early collaboration with design engineers to make ergonomic improvements may support optimal test development and speed up commercialisation of the product. The system should be evaluated on a cost return basis and suitability of use. Growers are unlikely to invest in equipment that is expensive, requires an annual maintenance contract, staff to operate and the need to run the equivalent of a small laboratory.

During the period of this portfolio of evidence, on-site systems have continued to be developed as a first-line rapid defence screen for plants expressing visible disease symptoms. This is amply demonstrated in forestry disease management. A generic *Phytophthora* on-site lateral flow immunoassay device has been used in the UK by Fera Plant Health and Seed Inspectorate to monitor the spread of the oomycete pathogens *Phytophthora ramorum* and *Phytophthora kernoviae* on plants expressing disease symptoms. Initial positive diagnosis of *Phytophthora* presence has enabled the effective management of the disease by immediate quarantine and containment measures. Once a sample is identified as a potential risk from infestation confirmatory tests are undertaken in the diagnostic laboratory to fully characterize the strains involved using molecular q PCR (www.fera.defra.gov.uk/plants/publications/documents/factsheets/pramparks.pdf).
For the control of fire blight disease, a lateral flow device has been developed for the rapid identification and detection of *Erwinia amylovora* in host tissue (Braun-Kiewnick *et al.*, 2011). The 15 minute test was validated in-field and provides a valuable tool for plant inspectors and growers to improve phytosanitary management of fire blight disease. The test was compared to PCR and conventional plating techniques and reduced the provision of laboratory-generated results from 2 days to 15 min. The on-site test was reported to provide high-specificity and sensitivity with a lower detection limit (5.7 CFU/ml). These test formats can also be used as a quality control diagnostic tool to confirm on-site product suitability. Used commercially in the USA, a

lateral flow device has been developed to determine levels of stable *Botrytis* antigens in table and dessert wines (Dewey *et al.*, 2013).

The antibody probes used within each point of care test (POC) will ultimately prove central to whether the sensitivity and specificity can be attained at a level suitable for the application and commercialisation of the test. Approaches to combine nucleic acid molecular techniques in a lateral flow format (NALF) have been developed to provide highly specific and sensitive diagnostic assays for plant pathogens (Tomlinson *et al.*, 2010). However sample preparation (nucleic acid extraction and amplification) may for some plant pathogens prove a significant weakness for uptake of this technology for on-site usage.

1.5 DEVELOPMENT OF THE PORTFOLIO OF EVIDENCE IN FUTURE WORKS

The development of new technologies over the past few decades has been an exciting time for scientists involved in the development of methodologies to monitor disease epidemics. The work developed in the medical field during the late 20th Century has provided rapid, inexpensive, simple immunological assay systems that can be used on-site or at the point of care in a wide range of disciplines. In a laboratory environment, the simple 96 well ELISA format has been adapted to provide a high throughput multiplex array system (Luminex MAGPIX technology). Using a 96 well ELISA format there is the potential for 50 different fluorescence colour coded magnetic microsphere bead types to bind to homologous target analytes. By applying a magnetic field the bound target material and beads are retained free of sample and potential assay inhibitors. After this 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 quantification of target analytes in a sample. The microsphere imunnoassay is much shorter (1 h) than that of a standard ELISA system (4 h). 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 plant pathogen assay designed for use in seed screening to simultaneously detect four important plant pathogens: a fruit blotch bacterium (*Acidovorax avenae* sub sp. *Citrulli*), and three viruses (chilli vein-banding mottle virus, watermelon silver mottle virus and melon yellow spot virus) (Charlermroj *et al.*, 2013). This approach may prove useful for the isolation of multiple plant pathogens from complex samples such as soil, plant and water systems. Through grant applications, the candidate has recently acquired a MAGPIX system and aims to conjugate antigen-specific MAbs to the colour coded magnetic spheres for multiplex

analyte measurement in environmental samples. As a laboratory system, this will support the development of a crop clinic at Worcester for high throughput screening of samples. Equally, the National Pollen and Aerobiology Research Unit at Worcester may benefit from this technology in the development of improved systems to quickly monitor and discriminate airborne allergens in collected bioaerosols.

The University of Worcester has been identified by the Horticultural Development Company (HDC) as a centre of excellence for the development of crop diagnostic systems. The HDC Board has recently ratified funding of a five year strategic programme of work there (HDC CP099). The candidate has been responsible for the development of research grant submissions to this work area and designated by HDC as project lead on CP099. The candidate leads a team of five people in delivery of this work which will enable the development of new tests to target a range of diseases which commonly affect UK commercial horticulture cropping systems. The grant will also move to commercialise the existing HDC funded assay systems, as developed by the candidate over the past 10 years. One of the HDC research themes in CP099 aims to further develop and commercialise laboratory and ‘in field’ tests for the measurement of clubroot resting spores in soils. In paper 1, the candidate reported the potential of improving the assay system by direct capture of the resting spores from soil using specific antibody coated magnetic beads. Using immunomagnetic separation, the potential exists to isolate the resting spores to a ‘clean’ sample and concentrate in a small volume (100 µl) for test application. The current on-site test (immunochromatographic test strip (POC)) involves analysis of a 0.25 g field soil sample. The small sample size and the often patchy distribution of spores within soil provide concern about delivering a result that is representative of a field situation. The restriction on total volume of the sample that can be applied will limit test sensitivity. However by using magnetic spheres conjugated with *P. brassicae*-specific MAb fragments it has been possible to isolate and recover resting spores from 50 g soil lots and concentrate these in the volumes (<100 µl) required for the on-site test and with molecular methods (laboratory q PCR). This approach is currently under evaluation with a two year national soils testing programme (HDC CP099a).

The candidate has also been involved in an EU submission to develop a multiplex immunomagnetic diagnostic ‘in-field’ assay. If successful, this approach will provide the opportunity to selectively ‘fish’ magnetic antibody-coated beads in soil for three agricultural crop pathogens and associated viruses. One of these will include resting spores of *P. brassicae.* The project would involve collaborations with European research partners and a manufacturer to develop a portable field magnetic reader device. Additionally, the candidate is developing a UK consortium bid for Agri-Tech Catalyst funding to provide improved systems for sampling of fields for prediction of

disease infestation. This approach will utilise precision agriculture technologies (Global Positioning (GPS) and geographic information systems (GIS)) to investigate the potential of directed sampling to specific locations in the field conducive to disease development. In conjunction with Birmingham City University, the inclusion of smartphone software development to provide image analyse of the in-field test results will be included within this call to provide an inexpensive format to quantify test readings.

Monitoring bioaerosols for target pathogen and allergen concentration, however remains a research priority area. The new role at the University of Worcester provides the candidate with the opportunity to utilise her skills and knowledge base to complementary areas outside horticulture. A recent invitation to present and join a European workshop 'New technologies for airborne pollen/allergens monitoring' provides an opportunity, as part of the working group, to identify collaborative funding avenues to derive new technologies to monitor pollen and associated allergens. She is currently overseeing work at the NPARU to produce a pollen allergen immuno-monitoring system which could be rolled out alongside an existing UK horticultural fungal spore network. This is based on the MTIST air sampler (Paper 3) and a laboratory-based ELISA process. The potential to develop as an on-site format using daily cyclone collected aerosols with lateral flow technology (Report 1) forms part of an industry grant submission. The accurate and timely information on human exposure to airborne allergens of varying potency would be a useful development in forecasting respiratory conditions. Pollen monitoring networks currently operate worldwide to provide a continuous record of measurements and are an important source of exposure data (Hertel *et al.*, 2008). This practice has been followed for over 50 years and consists of volumetric air samplers (Hirst type traps) from which microscopic counts are made to determine pollen concentration and allergen exposure risk. However speed in analysis and accuracy provide significant limitations for this methodology. Moreover a recent study using a high volume cascade impactor with exposed membranes extracted for the immunoanalysis of allergen concentration has shown that pollen varies in its potency (Buters *et al.*, 2012). As the measurement of pollen concentration may not be a useful measure in terms of allergen exposure risk new approaches are required. As a result, the candidate has recently been involved in a NERC research grant submission bid to investigate allelic variation of Birch pollen in the production of Bet v 1 allergen. If successful it will afford the candidate the opportunity to investigate the loop-mediated isothermal amplification (LAMP) method (Notomi *et al.*, 2000) for on-site capability to measure Bet v 1 isoforms from DNA sequence information. Bet v 1 allergen is an important cause of hay fever in northern Europe. Bet v 1 isoforms from the European white birch (*Betula pendula*) have been investigated with non-allergenic, allergenic and hypoallergenic isoforms identified. Individual birch trees

have the genetic background to produce a mixture of Bet v 1 isoforms differing in terms of amino acid sequence and immunological impact (Schenk *et al*., 2006).

Interestingly LAMP, which is a novel nucleic acid amplification process under isothermal conditions (60 to 65°C), provides opportunities as a technique for horticultural and agricultural applications in monitoring disease epidemics. 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. This enables the visual assessment of amplification by the naked eye. It has been reported to be less affected by inhibitors (Francois *et al*., 2011) and as a result of its isothermal nature, has the potential to be deployed in the field. Because of its speed, robustness and simplicity, LAMP is gaining momentum in diagnostics in human medicine (Parida *et al*., 2008), and more recently in plant health (Kubota *et al*., 2008; Tomlinson *et al*., 2010; Buhlmann *et al*., 2013).

In the United States, a 'grower performed LAMP PCR' has been assessed for the detection-based management of spray programmes for grapevine powdery mildew in vineyards (Reiger, 2011). The LAMP process requires several steps to include heating and centrifugation. Although it could be carried out in a grower's office with desktop equipment, it was found that the participants were not consistently successful when interpreting the results. Equally, sensitivity of the assay system presented problems with the cross-contamination of spores from the collected bioaerosols. The spores were readily picked up and moved on peoples clothing and hands. Nevertheless this may be overcome by improved hygiene. The team is 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 protocol process to be performed on site and provides an application to an Android phone device for quantitative measurement of the target spores.

In complex environmental samples (plant and soil), nucleic acid–based tests in the field, however, remains a challenging goal. This is largely due to the reliance on pre-processing of samples (nucleic acid extraction), which is a rate and skill limited step due to the relatively complex nature of current nucleic acid extraction methods (King *et al*., 2008). If portable real-time PCR platforms are to be used successfully, they should ideally consist of completely closed systems capable of performing all steps of the assay. These steps include (1) nucleic acid extraction, (2) PCR set-up, (3) amplification and (4) unambiguous "calling" of results (Mikidache *et al*., 2012). A significant driver for use of these systems in the field will be ease of use and test reliability. It is likely that only those technologies that are cost-effective will be used in plant pathogen diagnostics. As reported earlier, this remains a particular

consideration to commercial agriculture and horticultural cropping systems where the profit margins and emotional attachment to crops are low.

Nevertheless molecular techniques look set to continue to provide a wealth of information that will be invaluable in disease epidemic studies. A recent proposal in which the candidate has been involved combines bioaerosol sampling, genomics, bioinformatics and modelling to evaluate infectious agents and allergens within the environment. A significant part of the work, if funded, will be carried out by a collaborating lead partner and is based around next generation sequencing (NGS). NGS is a high-throughput approach that generates thousands to millions of DNA sequences. However, obtaining and making sense of these sequences involves several complex stages, both at the lab bench and at the computer desk. With many 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 genomic sequences and interpreting this from voluminous, noisy, and often partial sequence data presents a serious challenge. Analysis requires considerable skill and understanding to avoid potential pitfalls and challenges in the process (Dewoody *et al*., 2013). NGS has the capability however to analyse complex environmental samples and from this identify uncultured known, unknown and novel pathogen variants (Breitbart *et al*., 2008; Adam *et al*., 2009; Bi *et al*., 2012; Harju *et al*., 2012) As a use in horticulture, plant virus identification currently reports at a cost of £1000 per sample analysis but this sum could reduce in the future Adams *et al*., (2009). NGS is thought likely to remain a sophisticated laboratory tool which will underpin fundamental genetic based studies to provide a new perspective to host-pathogen interactions and ecological studies. Nevertheless, Oxford Nanopore Technologies (ONT) MinION, a USB drive-sized real time sequencer, suggests real opportunities for point of care diagnostics particularly in areas of clinical health (https://www.nanoporetech.com/technology/the-minion-device-a-miniaturised-sensing-system).

In the area of bioaerosols and water, the candidate is keen to explore the use of microfluidics and nanotechnology to deliver inexpensive automated real-time monitoring systems. The environmental assay protocol processes, with which she has previously been involved, require input either in the collection process and or in processing of the test format. Fluidic cytometry provides a suitable environment for immunochemistry, microbial collection and transport. Workers have been able to differentiate fungal bioaerosols by flow cytometry using either intrinsic fluorescence or the staining with fluorochomes to nucleic material with light scatter (Day *et al*., 2002; Prigione *et al*., 2004). In water transport systems, fluidic cytometry has been demonstrated for use with dual staining to assess the viability of bacterial spores (Pianelli *et al*., 2005). With appropriate antiserum, the capability to discriminate target cells by immunofluorescence and measurement by cell flow cytometry is

documented (Vives-Rego *et al*., 2000; Stopa, 2000). More recently this approach has been miniaturized with a view to developing microfluidic systems and the use of biosensors for remote sensing capability (Alphonsus *et al*., 2010). By combining existing technologies (spore collection and diagnostics), the development of 'in line' automated sensing systems for horticultural crop pathogens within irrigation systems or glasshouse bioaerosols could be developed. To facilitate this aim, the candidate has organised working groups to consolidate expertise to support grant applications to Agri-Tech Catalyst. The Agri-Tech Catalyst is open for applications, to support businesses and academia in developing innovative solutions to challenges in the agri-tech sector. For both systems (air and water), the monitoring of disease propagules requires a synergistic approach with engineering solutions to concentrate and deliver collected sized propagules into a microfluidic channel suitable for biosensor analysis in an inexpensive disposable nanotechnology format. If this approach is unsuccessful, other funding streams that are to be explored include the Horizon 2020 framework where the EU 2015 water framework directive would lend impetus to developments in this area.

Bionsensor technology has already been applied to the monitoring of microorganisms in microfluidic systems to provide continuous data on target analyte(s) concentration (Deisingh and Thompson, 2004; Alphonsus *et al*., 2010). Biosensors provide capability for a target analyte to be selectively bound by a bio-receptor and with reaction measured by a transducer to provide a signal. The reaction may involve measurement of fluorescent bound molecules, the production of heat or the change in conductivity, mass or optical changes. Frantamico *et al*., (1988) provided early evidence of this technology with a surface plasmon resonance (SPR) biosensor that was able to detect 10 cells of *E. coli* in water and with capability of at least 50 separate analyses by regeneration. SPR is a surface-sensitive spectroscopic technique that can measure the interactions between biological molecules with surface-bound capture probes. A change in the measured refractive index is relative to the size of the particle binding to the sensor surface. The approach that is currently being considered is the use of fluorescein or magnetic bead specific antibody labelling to identify target species in the fluidic stream. Upstream of this reaction, the potential to integrate within the process a system to determine pathogen viability is under discussion. UV radiation, pressure systems, slow sand filtration, sonication, chemical biocides and surfactants may prove effective in removing the capability of the pathogen to survive or remain pathogenic; however its physical presence may remain for some time. Differentiating between the infectious and non-infectious state would be an important factor in delivering a useful test. As a prototype the system(s), if successful it would be assessed in a commercial environment with industry partners and a robust validation period to identify potential problem areas. The developed process would have applications in environmental monitoring to include public health.

A. Wakeham continues to build upon her international collaborative links and would like to attend and submit oral presentations to the International Aerobiology Congress in Australia in September 2014. This would allow her the opportunity to publicise the works described in this portfolio and to network with workers of other disciplines interested in the application of these systems. At the same time it would be useful to liaise directly with Dr L. Minchinton (recently transferred to the Department of Agriculture (Quarantine), Melbourne) and Dr R. Faggian (recently appointed Senior Research Fellow, Department of Agriculture and Food Systems, University of Melbourne) to progress for peer reviewed publication the works associated with the white blister integrated disease management system and to discuss the potential for new areas for collaborative research. The candidate is also keen to re-establish links with Prof. C. Mohammed (previous president of the Australasian Plant Pathology Society and Senior Research Fellow and Lecturer with the Tasmanian Institute of Agriculture, University of Tasmania) to revaluate collaborative potential for systems to monitor *Mycosphaerella* species (*Mycosphaerella cryptica* and. *M. nubilosa*) associated with Eucalyptus spot. The appointment of Dr C. Skjoth to the NPARU (atmospheric science) may prove useful in discussion on this topic with inclusion of his work associated with remote sensing using satellites or drones as funded under the SUPREME (Simple Unified Pollen and Spore Release Model) project.

1.6 DECLARATION OF WORKS PREVIOUSLY SUBMITTED FOR AN AWARD.

A. Wakeham submitted works associated with Paper 1 in part for the award of MIBiol CBiol by thesis. The candidate was examined by Dr Molly Dewey (Plant Sciences, University of Oxford) for this award. The thesis consisted of 162 pages and was entitled 'Serological detection of two major soil-borne plant pathogens'. For this purpose, polyclonal antisera were raised to two soil borne pathogens, (*Plasmodiophora brassicae* (clubroot disease of cruciferous hosts) and *Sclerotinia sclerotiorum* (white rot disease of Alliums), and diagnostic assay systems were developed and reported on. Paper 1 reports on work associated with *P. brassicae*.

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A New Method To Monitor Airborne Inoculum of the Fungal Plant Pathogens *Mycosphaerella brassicicola* and *Botrytis cinerea*

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We describe a new microtiter immunospore trapping device (MTIST device) that uses a suction system to directly trap air particulates by impaction in microtiter wells. This device can be used for rapid detection and immunoquantification of ascospores of *Mycosphaerella brassicicola* and conidia of *Botrytis cinerea* by an enzyme-linked immunosorbent assay (ELISA) under controlled environmental conditions. For ascospores of *M. brassicicola* correlation coefficients (r^2) of 0.943 and 0.9514 were observed for the number of MTIST device-impacted ascospores per microtiter well and the absorbance values determined by ELISA, respectively. These values were not affected when a mixed fungal spore population was used. There was a relationship between the number of MTIST device-trapped ascospores of *M. brassicicola* per liter of air sampled and the amount of disease expressed on exposed plants of *Brassica oleracea* (Brussels sprouts). Similarly, when the MTIST device was used to trap conidia of *B. cinerea*, a correlation coefficient of 0.8797 was obtained for the absorbance values generated by the ELISA and the observed number of conidia per microtiter well. The relative collection efficiency of the MTIST device in controlled plant growth chambers with limited airflow was 1.7 times greater than the relative collection efficiency of a Burkard 7-day volumetric spore trap for collection of *M. brassicicola* ascospores. The MTIST device can be used to rapidly differentiate, determine, and accurately quantify target organisms in a microflora. The MTIST device is a portable, robust, inexpensive system that can be used to perform multiple tests in a single sampling period, and it should be useful for monitoring airborne particulates and microorganisms in a range of environments.

Airborne spores of fungal plant pathogens have commonly been detected and enumerated by microscopic examination of surfaces on which spores have been impacted (2, 13). Sampling procedures may involve passive collection of spores by gravitational deposition (14) and/or sampling specific volumes of air with "active" spore-trapping devices (1, 9, 18). Such techniques require considerable amounts of time and expertise if accurate counts are to be obtained. In addition, sample identification is often not a realistic option, especially when there is no selective medium and when there are morphologically similar spores (such as the spores produced by ascosporic fungi).

However, technological advances in fungal diagnostics in which either antibody or nucleic acid probes are used (4, 6, 11) offer the potential for developing rapid systems for detecting and quantifying airborne spores of fungal plant pathogens. An immunoassay system developed by Spore View (Chaparral Diagnostics, Burlington, Vt.) utilizes passive deposition of ascospores of *Venturia inaequalis*, the causal agent of apple scab, on a membrane surface. Similarly, studies to develop an antibody-based immunoassay for early detection of *Sclerotinia sclerotiorum* (15), a major fungal pathogen of oilseed rape (*Brassica napus*), have relied solely on passive deposition of ascospores on rapeseed petals and subsequent mycelial growth. It is unlikely that these systems could detect pathogens at concentrations below a critical threshold level since only small volumes of air or small sample sizes can be assayed with passive sampling. Consequently, sampling methods, location, and the efficiency of sampling are crucial factors when high cropping acreages are examined with these systems. As a result, rapid assay formats in which large volumes of air are sampled are a prerequisite if accurate immunomonitoring of air spora is to be achieved. With vegetable production systems detection of small amounts of inocula is important because the presence of disease organisms at low levels can result in a loss of quality.

During development of a portable remotely operated fiber optic biosensor system for detecting aerosolized bacteria (19), research carried out by the U.S. Naval Research Laboratory revealed the potential of an immunoassay system used to sample relatively large volumes of air. However, a limitation of this system was the low limit of detection, 3,000 CFU of *Bacillus subtilis* subsp. *niger* per ml of air sampled. At present there are few systems that can accurately detect small amounts of inocula. Nevertheless, other workers (8), using a Burkard 7-day volumetric suction trap (B 7-day trap) as a trapping device, quantified airborne pollen allergens on polyvinylidene difluoride membranes by using immunoblotting and chemiluminescence techniques. Similarly, workers have developed an immunofluorescence test to detect and quantify airborne ascospores of *Mycosphaerella brassicicola*, the ringspot pathogen of brassicas. Trapped ascospores are labeled directly on B 7-day trap Melinex tape with polyclonal antibodies (PAbs) and anti-rabbit fluorescein isothiocyanate-conjugated antibodies (17). However, when these two methods are used as routine quantification methods, both tests are laborious, require expensive laboratory equipment for analysis, and include processes which require manual operation. Nevertheless, for foliar diseases in which the inoculum is a fundamental aspect of disease spread,

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immunoassays have the potential to produce novel quantitative data on the epidemiology of airborne pathogens.

In this paper we describe a new microtiter immunospore trapping device (MTIST device) which uses a suction system to directly trap air spora by impaction in microtiter wells, and this device can be used for rapid detection and quantification of ascospores of *M. brassicicola* and conidia of *Botrytis cinerea* by an enzyme-linked immunosorbent assay (ELISA). *M. brassicicola* is a foliar plant pathogen that has significant economic importance and causes ringspot of brassicas, and *B. cinerea* is a general pathogen that affects a wide range of crops, including brassicas, in which it causes grey mold in the field and post-harvest rot in stored white cabbage and other produce. Control of both diseases is problematic because of the nature of the airborne inoculum. A disease prediction model for *M. brassicicola* has been developed previously (16), but additional information concerning inoculum levels is required for effective disease control. Information derived from studies of environmental conditions and airborne *B. cinerea* inoculum levels should be important in disease prediction studies, rationalization of fungicide usage, scheduling harvest dates, and determining the storage conditions used for white cabbage and other brassica crops.

In this paper we describe using a MTIST device to immunomonitor airborne spores of two plant pathogens.

MATERIALS AND METHODS

MTIST device. The MTIST device which we used (Fig. 1 and 2) is a modified personal volumetric air sampler produced by Burkard Manufacturing Co. Ltd. (Rickmansworth, Hertfordshire, United Kingdom). This sampler is operated by a standard Burkard turbine suction unit, and air is drawn through the system at a constant rate of 20 liters per min (Fig. 1C). The volume of air sampled can be increased or decreased depending on the requirements of the test. Particulates in the airstream are channeled through delivery trumpet nozzles and directed across the base of each collection well of microtiter well strips [4 by 8 wells; catalog no. 9502 027; ThermoQuest (UK) Ltd., Basingstoke, Hampshire, United Kingdom]. The MTIST device is operated by rechargeable 6-V batteries which can provide 3 to 4 h of continuous operation. For longer periods or intermittent running conditions an electric mains model is available in 110- and 240-V versions. A sliding air control is located under the sampling chamber in order to allow rapid closure following sampling.

Immunoquantification of *M. brassicicola*. (i) Production of PAbs to *M. brassicicola*. A 100-ml ascospore suspension containing 2.5×10^4 ascospores that were collected in sterile distilled water (SDW) from cultures that were producing *M. brassicicola* pseudothecia (single-spore isolates CH195001, CH19500B, and C19500C) on senescent sprout leaf decoction agar (SLD agar) (17) was concentrated by freeze-drying (Modulyo 4K; Edwards, Crawley, United Kingdom). The sample was rehydrated in 15 ml of SDW and sonicated with a Soniprep apparatus (MSE, Crawley, United Kingdom) at a micron amplitude of 20 for a total of 15 min. The sonicated ascospore sample was freeze-dried as described above except that it was rehydrated in 5 ml of phosphate-buffered saline (PBS) (pH 7.2). The immunization protocol and serum collection method used have been described by Kennedy et al. (17). Using a DEAE Affi-Gel blue gel support (Bio-Rad, Hemel Hempstead, Herts., United Kingdom), we purified immune serum and collected the immunoglobulin G (IgG) fraction. The purified serum was designated polyclonal antiserum (PAb) 98/4/P, and the IgG concentration was 0.8 mg ml^{-1}. As a preservative, 0.05% tincture of merthiolate (Tinc) (1 mg of thimerosal per ml and 1 mg of pararosanoline per ml in ethanol) was added to the purified IgG serum before storage at −20°C in 100-μl aliquots.

A second PAb, designated PAb 96/10/4, which was previously raised by using whole ascospores of *M. brassicicola*, was also used in this study (17).

(ii) Determination of the optimal working dilutions of PAb 98/4/P and PAb 96/10/4 in a PTA-ELISA. Using a plate-trapped antigen ELISA (PTA-ELISA), we determined the optimal working dilution of each PAb for a whole-ascospore suspension of *M. brassicicola*, as described by Kennedy et al. (17).

(iii) Specificity of PAbs for fungal spores collected in SDW. PAbs 98/4/P and 96/10/4 were screened for cross-reactivity with a range of airborne fungal spores (Table 1). Fungal cultures were either grown on synthetic media or collected from infected plant material. Fungal spores were collected in SDW, and the concentration was adjusted to ~1,000 spores ml^{-1}. The spore suspensions were stored at −20°C until they were processed by the PTA-ELISA as described below.

For each fungal isolate 100 μl of a spore suspension was pipetted into each well of an 8-well microstrip. The microstrips were incubated overnight at 4°C, after which the unbound material was removed and the wells were washed twice

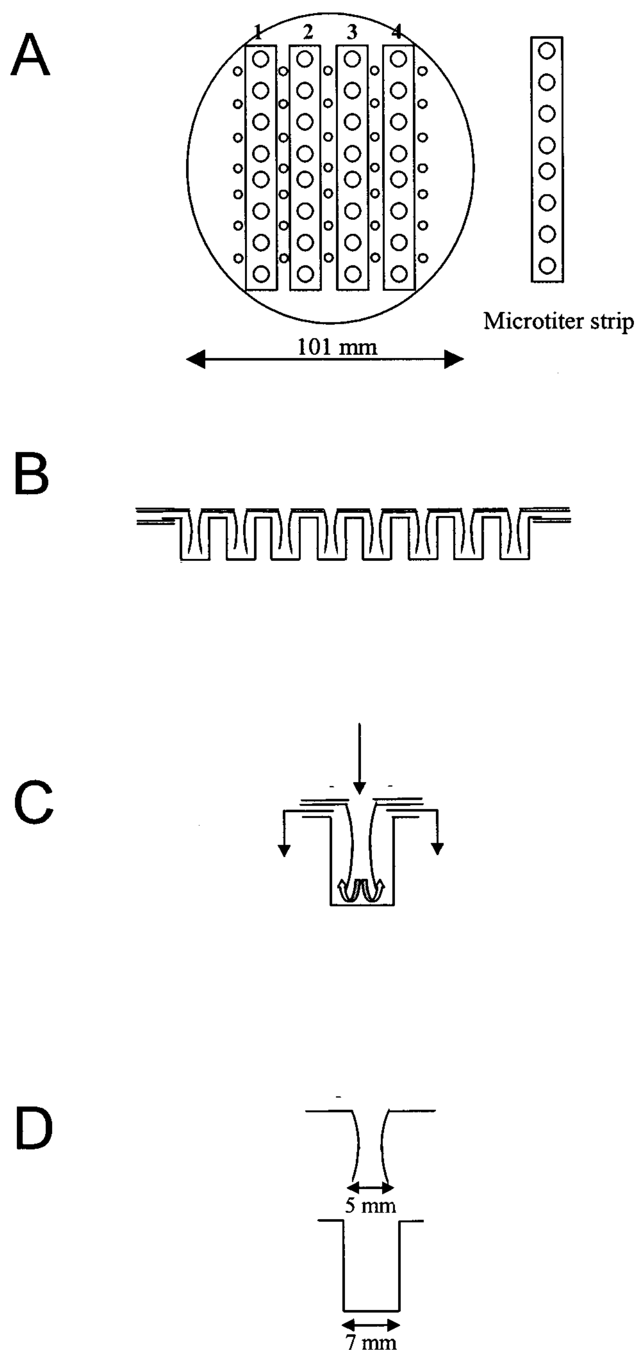

FIG. 1. Microtiter immunospore suction trap. (A) Overhead view of the MTIST device. (B) Cross-sectional view of the MTIST device. (C) Air movement during operation of the MTIST device. (D) Cross section of the MTIST device delivery trumpet and microtiter well.

(1 min each) with 200 μl of PBS-Tinc per well. The microtiter wells were blocked with 200 μl of 3% casein buffer (3% [wt/vol] casein in PBS) and incubated in a Wellwarm shaker incubator (Denley Instruments Ltd., Sussex, United Kingdom) at 37°C for 45 min. The residual blocking buffer was removed, and the wells were washed four times (1 min each) with 200 μl of PBS–Tinc–0.05% Tween 20 (PBS-Tinc-Tw). Two paired wells of each microstrip received 100 μl of PAb 98/4/P in PBS-Tinc-Tw (1:15) per well and 100 μl of PAb 96/10/4 in PBS-Tinc-Tw (1:50) per well. The remaining four wells of each microstrip received 100 μl of PBS-Tinc-Tw per well. Following incubation as described above, the wells were washed four times (1 min each) with 200 μl of PBS-Tinc-Tw. An anti-rabbit IgG SEEKit kit (catalog no. SSB-2003; Harlan Sera-Lab Ltd., Belton, Loughborough, United Kingdom) was used to amplify the signal generated by the bound

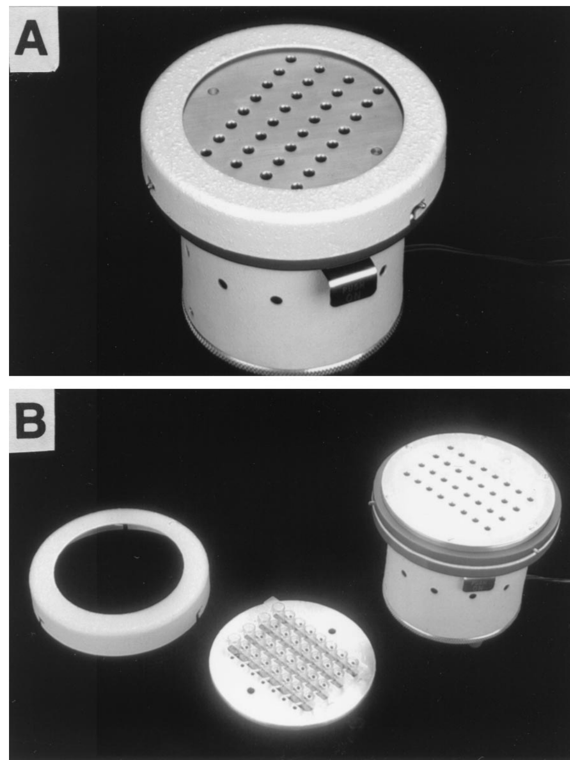

FIG. 2. (A) MTIST device. (B) Components of the MTIST device.

antibodies of PAbs 98/4/P and 96/10/4. As a negative control, a paired well of each microstrip, which had received no PAb during the previous incubation stage, was probed with the anti-rabbit IgG SEEKit kit. The optimal working dilution in PBS-Tinc-Tw for both the biotinylated antibody and the horseradish peroxidase-streptavidin complex was determined to be 1:10. The instructions of the manufacturer were followed. The incubation temperature and the time for each stage were the temperature and time described above; four 1-min washes with 200 μl of PBS-Tinc-Tw were performed after each of the two stages. To each of the eight wells of each microstrip 100 μl of 3,3′,5,5′-tetramethylbenzidene substrate (catalog no. T-3405 and P-4922; Sigma) was then added. The reaction was stopped by adding 25 μl of 2 M H_2SO_4. Absorbance at 450 nm was determined with a model HT11 ELISA plate reader (Anthos Labtech Instruments, Salzburg, Austria).

(iv) Specificity of PAbs for fungal spores trapped with the MTIST device. In an enclosed chamber, airborne spores of a number of fungal isolates (Table 1) were actively trapped in microtiter wells of microstrips (4 by 8 wells) by using the MTIST device. The number of spores trapped in each of the microtiter wells was determined by using a Nikon model TMS inverted binocular microscope. The microtiter strips were stored at −20°C until they were processed by PTA-ELISA. To determine the potential cross-reactivity of the two PAbs, only microstrips which contained approximately 1,000 spores per well were used in the PTA-ELISA. The PTA-ELISA was performed as described above, but no preincubation stage prior to blocking was included.

(v) Immunoquantification of trapped ascospores of *M. brassicicola* in controlled-environment cabinets with the MTIST device. Twelve sporulating culture plates containing *M. brassicicola* (CH195001, CH19500C, C19500D, C195001, C19500B, and DS1) were placed in a controlled-environment cabinet (catalog no. SGC970/C/RO-HFL; Sanyo Gallenkamp, Loughborough, Leicestershire, United Kingdom) operating at 94% relative humidity with continuous light and intermittent wetting for 0.3 min every 60 min. Discharged ascospores were collected by impaction in the microtiter wells of microstrips (4 by 8 wells) of the MTIST device, which was operated with a continuous airflow rate of 20 liters per min. Over a 96-h sampling period, the microstrips were changed at 30-min and 1-, 2-, 3-, 4-, 6-, 12-, and 36-h intervals. For each sampling period the total number of ascospores in each of the wells of each microstrip was determined with a Nikon model TMS inverted binocular microscope. Eight negative control microstrips, which had been removed from the MTIST device following operation for 2 h in a controlled environment with no sporulating cultures, and ascospore-containing microstrip wells were stored in 96-well multiframe holders [catalog no. 9503 060; ThermoQuest (UK)] at −20°C. The mean percent distribution of impacted ascospores for each sample in microtiter wells was determined by performing a single-factor analysis of variance. The ascospores in wells were immunoquantified by PTA-ELISA as described previously, but no preincubation stage prior to blocking was included. For each strip four wells received PAb 98/4/P, and the remaining four received PAb 96/10/4.

To determine the sampling efficiency of the MTIST device, a B 7-day trap was placed adjacent to the MTIST device and used as a reference trap. B 7-day traps have been used routinely to monitor fungal air spora (12, 13, 24, 26) and in a wide variety of air-sampling studies (3, 10). Ascospores on the Melinex spore tape were detected and quantified by immunofluorescence, as described by Kennedy et al. (17).

(vi) Immunoquantification of ascospores of *M. brassicicola* in a mixed fungal airborne population by using the MTIST device. Eight *Brassica oleracea* var. *gemmifera* plants (Brussels sprouts) which had been inoculated with *Erysiphe cruciferarum* (powdery mildew) and exhibited severe disease symptoms were placed in a controlled-environment cabinet operating as described above together with 12 sporulating culture plates containing *M. brassicicola*. Using the MTIST device as described above, the air spora in the cabinet was sampled over a 24-h period (20 liters of air min^{-1}) by using sampling periods of 30 min and 1, 2, 4, and 12 h. For two of the 4-h sampling periods and one 12-h sampling period, 40 additional but disease-free Brussels sprouts seedlings (*B. oleracea* var. *gemmifera* cv. Golfer) with three true leaves were placed in the controlled-environment cabinet at the following four positions: top left, top right, bottom left, and bottom right. Following each of these sampling periods the 40 *B. oleracea* plants were removed and placed into an environment with 100% humidity for 24 h. The plants then were removed, placed in a glasshouse, and grown at 15°C for 21 days. The plants were visually examined for expression of ringspot lesions. To confirm that ringspot (*M. brassicicola*) symptoms were present, infected leaf tissue was removed and surface sterilized for 1 min in aqueous sodium hypochlorite (4% [wt/vol] available chlorine), and organisms were isolated by using SLD agar (17).

For each MTIST device sampling period each well of each microstrip was examined with a Nikon model TMS inverted binocular microscope to determine the total number of impacted *M. brassicicola* and *E. cruciferarum* spores. For each of four sampling periods two wells were examined to determine the spatial distribution of the impacted spores. Microstrips were stored at −20°C until they were examined by using the PTA-ELISA as described above.

Immunoquantification of *B. cinerea*. (i) Production of a *Botrytis*-specific MAb. A hybridoma cell line secreting a *Botrytis*-specific monoclonal antibody (MAb), designated BC12.CA4, was raised by using splenocytes from a mouse which had been coimmunized with surface washings from a plate culture of *B. cinerea* (designated P-6 g) and supernatant from a hybridoma cell line that secreted the near genus-specific MAb BC-KH4 (5) at a 1:1 (vol/vol) ratio (22). Genus-specific MAb BC12.CA4 is an IgG1 antibody that has been shown to recognize a heat-stable epitope on an antigen expressed along the extracellular matrix of hyphae and on the surfaces of conidia of *B. cinerea* (22).

(ii) Immunoquantification of conidia of *B. cinerea* trapped with the MTIST device. A 3-mg conidial sample collected with a cyclone surface sampler (Burkard) in a sterile chamber by using six sporulating cultures of *B. cinerea* BC 404 grown on prune lactose yeast extract erythromycin agar (21) was added to a spore settling tower. With the MTIST device at the base of the spore settling tower and operating as described above, conidia of *B. cinerea* were actively trapped for 5, 10, and 15 s. Following each sampling period the microstrips (4 by 8 wells) were removed, and 100 μl of PDBG buffer (0.1% [wt/vol] potato dextrose broth [Difco], 1% [wt/vol] glucose in PBS) was added to each well. As a negative control, PDBG buffer was added to a microstrip which had not been exposed to *B. cinerea* conidia. The individual microstrips were incubated at room temperature for 16 h, after which the PDBG buffer was removed and the total number

TABLE 1. Fungal species used in cross-reactivity tests to determine PAb specificity

Fungal species	Growth medium or plant[a]
Aspergillus ruber	MSA
Paecilomyces variotii	MEA
Penicillium aurantiogriseum	MEA
Botrytis cinerea	PDA
Botrytis allii	PDA
Sclerotinia sclerotiorum	*Triticum*[b]
Alternaria brassicicola	PLY
Albugo candida	*B. oleracea*
Peronospora parasitica	*B. oleracea*
Erysiphe cruciferarum	*B. oleracea*
Puccinia allii	*Allium porrum*
Mycosphaerella brassicicola	SLD agar

[a] MEA, malt extract agar; PDA, potato dextrose agar; MSA, malt salt agar; PLY, prune lactose yeast extract agar.

[b] *S. sclerotiorum*-inoculated autoclaved wheat grain was placed 1 cm deep in John Innes no. 1 compost (23).

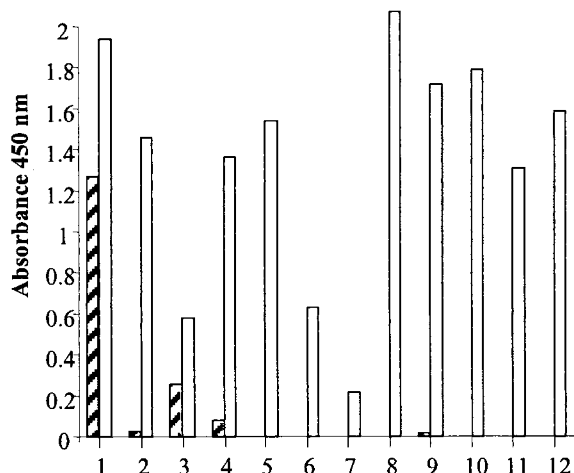

FIG. 3. Reactivity of PAb 96/10/4 with a range of airborne fungal spores, as shown by PTA-ELISA. 1, *Mycosphaerella brassicicola*; 2, *Peronospora parasitica*; 3, *Sclerotinia sclerotiorum*; 4, *Albugo candida*; 5, *Aspergillus ruber*; 6, *Botrytis allii*; 7, *Puccinia allii*; 8, *Botrytis cinerea*; 9, *Erysiphe cruciferarum*; 10, *Penicillium aurantiogriseum*; 11, *Paecilomyces variotti*; 12, *Alternaria brassicicola*. Cross-hatched bars, spores collected with the MTIST device; open bars, spores collected in water.

of germinated conidia in each well of each microstrip was determined by using a microscope. The microtiter wells then were washed with PBS containing 0.05% (vol/vol) Tween 20 (PBS-Tw) and blocked with 200 μl of 0.3% (wt/vol) casein in PBS containing 0.02% (wt/vol) NaN_3. Following incubation at room temperature for 10 min, the residual blocking buffer was removed, and the wells were sequentially incubated for 1 h at room temperature with 100-μl volumes of BC12.CA4 hybridoma supernatant and goat anti-mouse polyvalent (IgG, IgA, and IgM) peroxidase conjugate (catalog no. A-0412; Sigma) diluted 1:500 in PBS-Tw. After this the substrate 3,3′,5,5′-tetramethylbenzidene (catalog no. T-3405; Sigma) in acetate buffer (5) was added to each well, and the preparations were incubated for 10 min. The reaction was stopped by adding 50 μl of 3 M H_2SO_4. Absorbance at 450 nm was determined with a model MRX ELISA plate reader (Dynatech Laboratories Inc., Chantilly, Va.). The wells were washed between incubation steps four times with PBS-Tw.

RESULTS

Immunoquantification of *M. brassicicola*. (i) Specificity of PAbs 96/10/4 and 98/4/P in the PTA-ELISA. When fungal spores were collected in water and incubated in microtiter wells overnight, both antisera cross-reacted with each of the fungal spore suspensions tested. However, when fungal spores were actively trapped in microtiter wells by the MTIST device and were examined by PTA-ELISA without preincubation in water, a different pattern of recognition was observed. In the later tests the cross-reactivity with both antisera was limited to the ascospore stage of *S. sclerotiorum* (Fig. 3) (PAb 96/10/4).

(ii) Immunoquantification of MTIST device-trapped ascospores of *M. brassicicola*. Using a Nikon model TMS inverted binocular microscope at a magnification of ×200 with bright-field illumination, we confirmed that MTIST device-trapped ascospores of *M. brassicicola* were present in the base of each collection microtiter well, and the total number in each well was determined. We found that ascospores were distributed throughout the base of each well, and, as the ascospore concentration increased, the spores remained spatially separated. Employing a single-factor analysis of variance, we determined that significant variation in the mean percent ascospore distribution occurred in the microtiter well strips (Table 2); greater numbers of ascospores were collected in the inner four wells of each microstrip (Fig. 4). Except for wells 1 and 8, there was no significant difference between the strips (Table 2). Following PTA-ELISA, correlation coefficients (r^2) of 0.943 and 0.9514

TABLE 2. Mean distribution of MTIST device-trapped ascospores of *M. brassicicola* in microstrips (4 by 8 wells), expressed as percentages for the 96-h sampling period

Well no.	% of ascospores in:				
	Microstrip 1	Microstrip 2	Microstrip 3	Microstrip 4	SED[a]
1	2.94	2.56	2.37	2.33	0.23
2	2.87	2.48	2.57	2.73	0.26
3	3.44	3.04	3.43	3.11	0.24
4	3.69	3.26	3.56	3.53	0.26
5	3.74	3.53	3.83	3.44	0.24
6	3.69	3.48	3.62	3.50	0.21
7	2.88	3.09	3.13	3.03	0.22
8	2.38	3.11	3.01	2.59	0.21
SED[b]	0.25	0.26	0.199	0.229	

[a] SED, standard error of difference. The degrees of freedom was 36.
[b] The degrees of freedom was 72.

(polynomial) were obtained with PAbs 96/10/4 and 98/4/P, respectively, when we compared the number of ascospores per microtiter well and the absorbance values obtained from PTA-ELISA analyses (Fig. 5). The relative collection efficiency of the MTIST device in a controlled plant growth chamber with limited airflow was 1.7 times greater than the collection efficiency of the B 7-day trap.

(iii) Immunoquantification of ascospores of *M. brassicicola* in a mixed fungal airborne population by using the MTIST device. MTIST device-trapped ascospores of *M. brassicicola* and conidia of *E. cruciferarum* were identified in the bases of the collection microtiter wells (Fig. 6). The total number of ascospores of *M. brassicicola* and the total number of conidia of *E. cruciferarum* in each well were determined. Ascospores of *M. brassicicola* and conidia of *E. cruciferarum* were distributed throughout the base of each well, but the greatest numbers

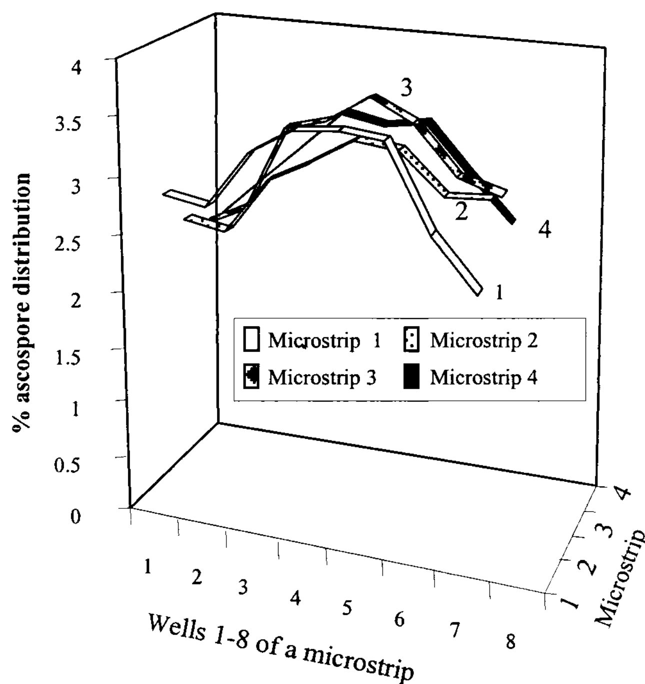

FIG. 4. Mean distribution of impacted ascospores of *M. brassicicola* for each of the microstrips (4 by 8 wells), expressed as percentages, for the 96-h sampling period.

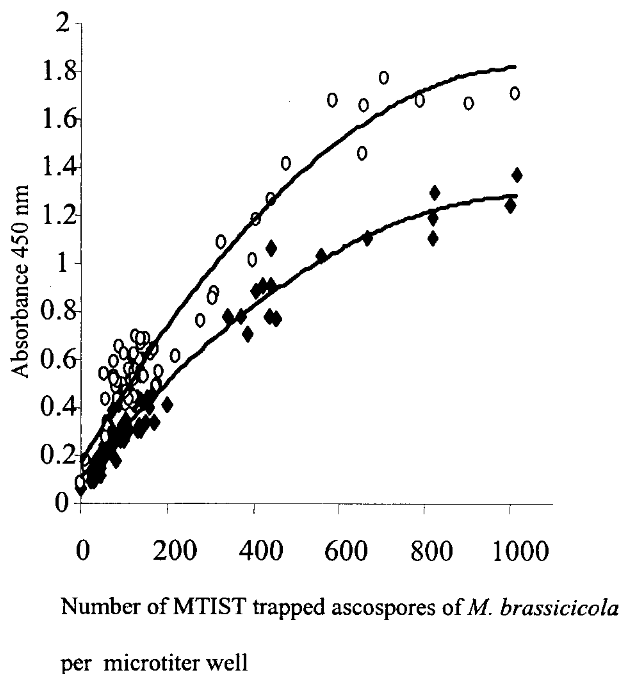

FIG. 5. Immunoquantification of MTIST device-trapped ascospores of *M. brassicicola* in a controlled-environment chamber, as determined with PAb 98/4/P (◆) and PAb 96/10/4 (○) by performing a PTA-ELISA.

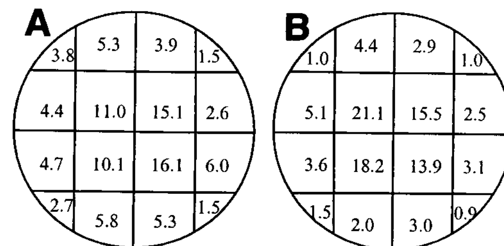

FIG. 7. Total numbers of MTIST device-trapped spores collected in eight wells, expressed as percentages in a single microtiter well. (A) *M. brassicicola*. (B) *E. cruciferarum*.

occurred in the central region of each microtiter well (Fig. 7). Low numbers of conidia of *E. cruciferarum* were observed on the sides of the microtiter collection vessels. As the conidial concentration increased (>100 conidia per microtiter well), aggregation of conidia was observed, and at levels of >1,000 conidia per microtiter well the ascospores of *M. brassicicola* were obscured. Following PTA-ELISA correlation coefficients of 0.8963 and 0.9655 (polynomial) were obtained with PAbs 96/10/4 and 98/4/P, respectively, when we compared the number of ascospores per microtiter well and the absorbance values (Fig. 8). There was not a correlation between the number of conidia of *E. cruciferarum* per microtiter well and the absorbance value when either PAb was used (Fig. 8).

Ringspot latent infection. All *B. oleracea* var. *gemmifera* cv. Golfer seedlings (Brussels sprouts) that had been exposed to the mixed fungal spore population had ringspot lesions (Fig. 9). Organisms isolated from the infected lesions on SLD agar were confirmed to be *M. brassicicola* (7). The position within the cabinet had little effect on the total number of ringspot lesions which developed on brassica seedlings exposed to spores. We observed that there was an association between the number of MTIST device-trapped ascospores of *M. brassicicola* per liter of air sampled and the total number of ringspot lesions that developed (Fig. 10). Leaf immaturity inhibited the development of *E. cruciferarum* lesions.

Immunoquantification of MTIST device-trapped *B. cinerea* conidia. As determined with the MTIST device for each sampling period (5, 10, and 15 s), *B. cinerea* conidia were present in the bases of the microtiter wells. To optimize the immunoassay, *B. cinerea* conidia were germinated by incubating them for 16 h in PBDG buffer prior to the PTA-ELISA. Recognition of ungerminated conidia by *Botrytis*-specific MAb BC12.CA4 is poor (22); this antibody predominantly binds to epitopes present in the extracellular mucilage of the conidial germ tube and mycelial wall. Using the absorbance values generated and the observed numbers of germinated conidia per microtiter

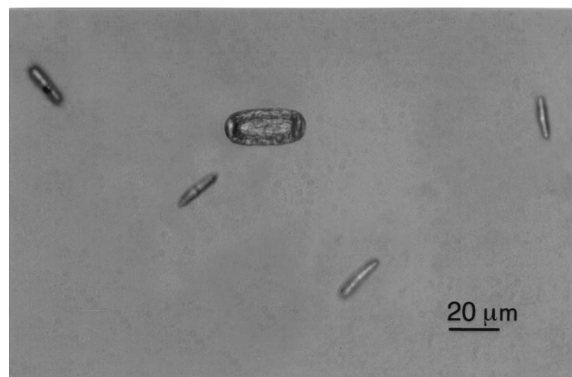

FIG. 6. MTIST device-trapped ascospores of *M. brassicicola* and a conidium of *E. cruciferarum* in the base of a microtiter well.

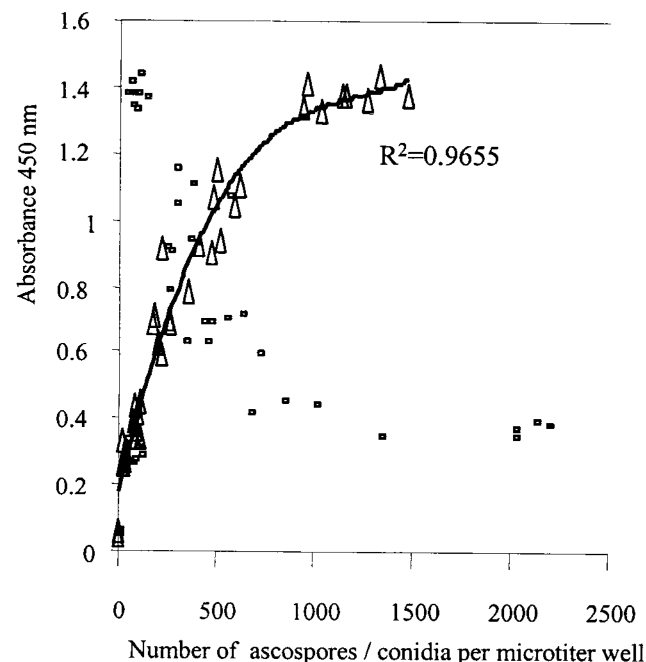

FIG. 8. Relationship of MTIST device-trapped ascospores of *M. brassicicola* (▲) and conidia of *E. cruciferarum* (□) in a PTA-ELISA analysis, as determined with PAb 98/4/P.

FIG. 9. Leaves of *B. oleracea* cv. Golfer exhibiting ringspot lesions.

well, we fitted a polynomial curve to the data to give a correlation coefficient of 0.8797 (Fig. 11).

DISCUSSION

In the past it has been impossible to quantify organisms in an airborne microflora accurately and quickly. A wide variety of methods have been used, which can be broadly divided on the basis of active or passive sampling techniques (1). With most of these techniques researchers depend on microscopic examination of impaction surfaces. Nonmicroscopic techniques have usually involved using selective agar media or trap plants. However, these methods have limitations since they rely on passive sampling and are not universally applicable. For example, problems can arise in detection of some slowly growing fungal species on agar, especially if the target organism occurs at low levels in the presence of large populations of other microfloral organisms. Additional problems are the incubation conditions used to express the target organism after it is trapped on agar and determining what might constitute a CFU. Using plants to express levels of target organisms in a microflora is equally problematic. For some diseases, symptoms are not expressed until weeks after infection has occurred. An additional problem is that not all organisms in an airborne microflora are pathogenic on plants. Therefore, it has been

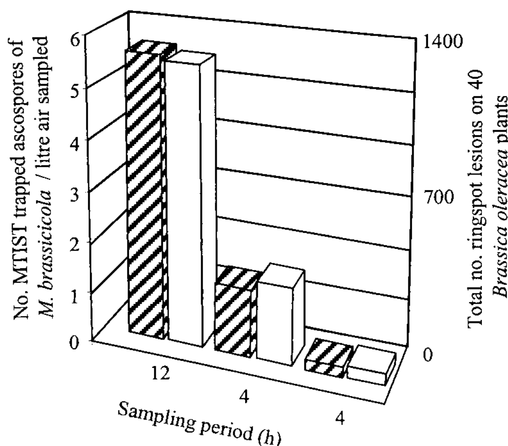

FIG. 10. Number of MTIST device-trapped ascospores of *M. brassicicola* per liter of air sampled and total number of ringspot lesions per sampling period on 40 exposed *B. oleracea* (Brussels sprouts) plants. Cross-hatched bars, ascospores trapped with MTIST device; open bars, ringspot lesions.

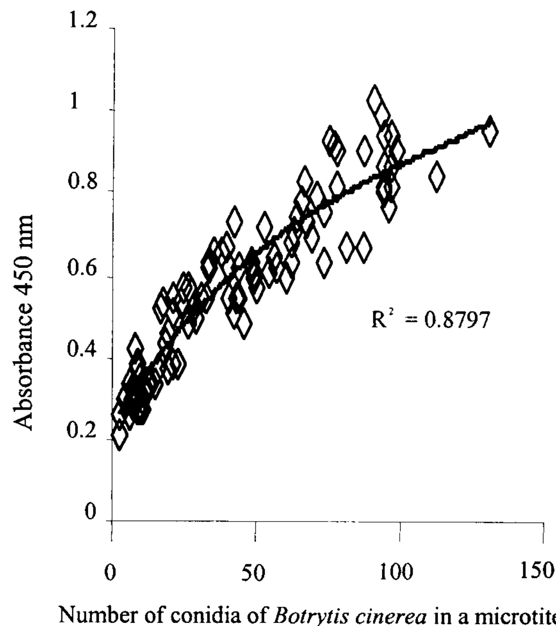

FIG. 11. Relationship between MTIST device-trapped germinated conidia of *B. cinerea* and corresponding PTA-ELISA absorbance values.

difficult in the past to measure the degree of airborne dispersal of fungal propagules, particularly when low levels have been present.

In the present study we developed new techniques and equipment which can be used to actively sample airborne microfloras. The equipment can be used to rapidly differentiate and accurately quantify target organisms in a microflora without microscopic examination. Additionally, target microflora can be quantified without long incubation periods after sampling. The values are also independent of the type and quantity of airborne spores in the air at the time of sampling. However, the specificity of the test depends on the antibodies used. The range of organisms used in cross-reactivity tests must reflect the range of organisms likely to be encountered in the field. In the cross-reactivity tests performed in the present study we used many pathogens and fungi likely to be found in horticultural brassica crops. Cross-reactivity with ascospores of other fungal species, including *Sclerotinia* species, might be problematic if the system described here was used to detect *M. brassicicola* in arable brassica crops. It is possible that *Sclerotinia* infection of arable brassica crops might interfere with counts in locations adjacent to horticultural brassica crops. However, this problem can be overcome by using more than one antibody in the system to accurately quantify the degree of cross-reactivity which may occur at any location. Additional information concerning the long-distance dispersal of the fungal pathogens would also be required.

It has been shown that long-distance dispersal of microorganisms does occur (25, 27). It has been demonstrated (20) that *Erysiphe graminis* spores can be transported 110 km year^{-1} in the main wind direction. The authors of these studies concluded that the probability that viable spores of plant-pathogenic species could cause disease was low but not zero. However, the importance of low numbers of propagules may be outweighed if, for example, they are of a different biotype than the resident population. Recent work has revealed the importance of transmission of a biotype of *Mycosphaerella graminicola* when sexual recombination with existing populations oc-

FIG. 12. Wind directional field-based MTIST device, operated by a data logger, for immunoquantification of field inoculum of *M. brassicicola*. This device is currently being evaluated.

curred at a significant level (26). In previous studies it was difficult to determine whether the levels of inocula were related to increases or decreases in disease occurrence. In previous studies of the dispersal and diurnal periodicity of airborne microflora the researchers used standard trapping techniques, such as B 7-day traps or rotorods. However, in controlled environmental tests in which the trapping efficiency of a B 7-day trap was compared to the trapping efficiency of the MTIST device, the latter trap was 1.7 times more efficient based on the number of spores trapped per cubic meter of air. However, this was not surprising given the characteristics of the B 7-day trap. The initial comparisons between the MTIST device and the B 7-day trap were carried out under controlled environmental conditions under which the airflow was low. For field operation some alteration in the design of the MTIST device will be required since at present collection occurs 90° to the direction of the airflow. A field-based wind directional MTIST device (Fig. 12) is currently under evaluation. Further studies will determine and optimize the trapping efficiency of the field-based MTIST device for different wind speeds and with variable airflow. Tests will be carried out to compare the trapping efficiency of the MTIST device and the trapping efficiencies of the B 7-day and rotorod traps and other samplers, notably the Anderson type of samplers, under variable-airflow conditions found in the field.

In the present study we found that with the MTIST device spores impact in the bases of microtiter wells, and observations in this study confirmed that ascospores of *M. brassicicola*, conidia of *E. cruciferarum*, and germinated conidia of *B. cinerea* adhere permanently to the microtiter wells. However, if adhesion to the plate does not occur (due to spore aggregation), there may be some loss of spores during the ELISA process (*B. cinerea* and *E. cruciferarum*). This may be overcome in the future by precoating microtiter wells with poly-L-lysine. Further investigations of such effects in which a range of propagule types from different airborne microfloras are used are needed in order to establish the general usefulness of this device. Aggregation, even at very high spore concentrations, was not observed for *M. brassicicola*.

Our results indicate that there is likely to be little interaction between large and small propagules provided that the antibodies used for one target pathogen do not cross-react with the other pathogen. However, when cross-reactivity occurs, it can be overcome by using appropriate pathogen-specific MAbs or even specific PCR probes. Also, since many propagules have a distinct diurnal periodicity and require specific conditions for release into the air, cross-reactivity might not be a significant problem in some applications. Interestingly, in this study the cross-reactivity observed with the PAbs was considerably reduced when airborne spores were collected with the MTIST device. This suggests that the nonsoluble ascospore wall antigens of *M. brassicicola* are specific.

The results obtained with the device under controlled environmental conditions indicated that there was a close correlation between the amount of ringspot disease, which appeared on trap plants placed inside the cabinet, and the number of MTIST device-trapped ascospores of *M. brassicicola* from source plates. The device could, therefore, prove to be useful in determining the potential risk of infection at specific locations. However, information on the numbers of target propagules trapped at any location could be interpreted easily only if additional information or models describing the effects of environmental variables on the biological responses are available. This approach would be particularly useful for plant-pathogenic fungi, with which the technology could be used to predict rapidly, with relatively little effort, the degree of disease transmission and the potential levels of disease.

For successful usage of the MTIST device further information is needed concerning the placement of traps in relation to sources of a target inoculum because of the vertical variation in the airborne concentration of propagules. The vertical variation in the aerial concentration of *V. inaequalis* ascospores in apple orchards has been determined by Aylor (2) by using rotorods. As determined by the *Venturia* studies the aerial concentration of ascospores 3 m above the ground was 94% less than the concentration 0.15 m above the ground. The vertical variation in the concentration of airborne spores is one factor which determines the likelihood of transport of microflora spatially.

Potentially, the MTIST device system could be used to assess the presence or absence and quantity of several target air spores at the same time either by immunoassay or by PCR in which specific probes are used. This is often important in studies in which organisms occur as complexes in particular ecological niches. Advances in environmental data capture systems should allow operation of the MTIST device only when conditions are favorable for target spore release and/or infection. This should result in greater accuracy in epidemiological disease studies. If the device is to be used to detect several pathogens simultaneously, a complete microstrip should be used for each target airborne inoculum type. This conclusion is based on the observation that the number of ascospores of *M. brassicicola* trapped in microtiter wells varied significantly within a microtiter strip but not between microtiter strips when the same sample was examined.

New techniques which can be used to rapidly and reliably differentiate airborne microflora should be useful in research programs. The information obtained could be used to predict spatial variation in plant-pathogenic propagules and the interaction between agricultural and natural ecosystems. However, the present system can operate only in a nonautomated format, and there is some delay between sampling and quantification of the microflora in a sample. Reducing this time interval will require further major advances in technology if sample processing is to be automated. However, by using immunochromatographic techniques it is already possible to process samples on site. The MTIST device is a portable, robust, and inexpensive system that can be used for multiple tests during a single sampling period, and it should be useful for monitoring airborne particulates and microorganisms in a range of environments.

ACKNOWLEDGMENTS

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Risk Assessment Methods for the Ringspot Pathogen *Mycosphaerella brassicicola* in Vegetable Brassica Crops

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ABSTRACT

Wakeham, A. J., and Kennedy, R. 2010. Risk assessment methods for the ringspot pathogen *Mycosphaerella brassicicola* in vegetable brassica crops. Plant Dis. 94:851-859.

Mycosphaerella brassicicola causes ringspot on Brussels sprouts, which can result in substantial yield loss in commercial production. Brussels sprout buttons are downgraded if this pathogen occurs on them. In this study, the effect of temperature and wetness duration was investigated on infection of Brussels sprouts using controlled environments (CE). The effect of temperature and wetness duration on inoculum production and ascospore discharge was also investigated. Infection by *M. brassicicola* was described using a mathematical model and was compared to estimates of ascospore availability obtained via a volumetric air sampler and immunofluorescence (IF). Infection of *M. brassicicola* was correlated ($r = 0.92$) with temperature during leaf wetness periods. The relationship between temperature and time to discharge of 5 and 50% of the cumulative total number of ascospores from ringspot lesions was $r = 0.99$ and 0.98, respectively ($P < 0.001$). In field experiments, an optimal wind run (the product of the average wind speed and the period over which that average speed was measured) of 250 to 500 km day^{-1} was required for the dissemination of ringspot inoculum to field bait plants. Quantification of *M. brassicicola* inoculum in collected field aerosols was possible using a monoclonal antibody in a plate-trapped antigen enzyme-linked immunosorbent assay. Precoating of the air sampler wells with sodium azide prevented trapped spores from germinating. Ringspot inoculum could be detected and quantified in air samples from commercial crops of Brussels sprouts in the United Kingdom. Low levels of ringspot inoculum measured within crops did not lead to disease development.

Accurate information about the presence of sufficient pathogen inoculum is required to predict plant disease occurrences in field settings (16). Traditionally, plant disease forecasting systems have relied upon environmental data singularly to predict disease occurrences in crops (14,16). Mathematical models describing the effect of temperature and wetness on pathogen infection have been developed for many types of plant pathogens. R. D. Magarey et al. (14) provide a comprehensive review of these systems. However, detecting and quantifying airborne spores could augment disease forecasting systems. By determining airborne spore numbers during a time period when environmental risk for a disease is high, protective disease control strategies could be implemented. Such an approach has been utilized successfully to control the potato late blight pathogen (*Phytophthora infestans* (Mont.) de Bary) where peaks of airborne spores have been detected many weeks before the observation of disease development in crops (3,15). A similar risk assessment method has been used for Botrytis blight (*Botrytis squamosa* Walker) on onion crops where thresholds of 15 to 20 conidia m^{-3} in the air were required for crop infection and, when detected, were used as a decision tool for making fungicide applications (4). A limiting factor of this approach is the reliance on a microscope for counts of field-trapped spores, which are difficult to accurately identify and quantify (11).

In European vegetable brassica production systems, airborne fungal diseases are a common problem (10,20). The most difficult disease to control is ringspot, caused by *Mycosphaerella brassicicola* (Duby) Lindau, which affects all vegetable brassica crop types. Brussels sprout and cauliflower crops are produced throughout the year in United Kingdom (UK) production systems. Ringspot is particularly problematic in the coastal areas of Lincolnshire, UK, where favorable environmental conditions occur frequently. Due to the cosmetic nature of damage by *M. brassicicola,* many opportunities exist for crop loss (20). Small amounts of disease on sprout buttons and cauliflower leaves can lead to downgrading the value of the product.

Inoculum thresholds for ringspot development on vegetable brassicas have been established (12). J. E. Cullington (6) demonstrated a relationship between ascospore inoculum availability, temperature and wetness on the number of ringspot lesions observed on trap plants if exposed to field conditions for more than 24 h. Seedborne inoculum is not significant in the epidemiology of *M. brassicicola* in the UK (6).

In this paper, we examine the environmental factors associated with ringspot inoculum availability and construct a simple ringspot forecasting method. Since there are currently few methods that can detect significant levels of fungal inoculum in air samples rapidly and accurately (12,13), we also report on using an innovative spore trapping system (microtiter immunospore trap [MTIST]) (12) allied to an immunological test (plate-trapped antigen enzyme-linked immunosorbent assay [PTA-ELISA]) to monitor airborne inoculum of *M. brassicicola.* In conjunction with the ringspot model, we designate the likely onset of disease occurrence in crops of Brussels sprout and cauliflower.

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MATERIALS AND METHODS

Temperature and ascospore development. Fully developed leaves of 6- to 7-week-old Brussels sprout plants (cv. Golfer) grown in a glasshouse were inoculated with a suspension of 2×10^4 *M. brassicicola* ascospores ml^{-1} (6). The inoculated plants were enclosed in polythene bags, which had been lightly sprayed inside with distilled water to provide humid conditions conducive to infection. After an incubation of 3 days in a growth room (15 to 18°C, 16-h day), the polythene bags were removed and plants were transferred to a glasshouse at a 12-h day:night temperature regime of 16:14°C. Plants were bottom-watered to ensure that inoculated leaves remained dry, preventing the onset of ascospore release on developing lesions. After 4 weeks, leaves bearing four to six ringspot lesions, approximately 10 mm diameter, were removed from the plants. Each leaf was placed adaxial side up on partially molten (approximately 30°C) distilled water agar (technical grade agar at 15 g liter^{-1} distilled water [DWA]) in a 14-cm-diameter petri plate. Leaf margins were submerged in the agar, anchoring the leaf to the medium. The agar was amended with the senescence inhibitor 6-benzylaminopurine (0.5 mg liter) following the method of Verma and Petrie (19). Petri plate lids were partially filled with DWA, providing a saturated internal environment conducive to pseudothecial development and ascospore discharge. After sealing the lids over the bases with Parafilm, plates were inverted and placed

in laboratory incubators maintaining temperatures of 5, 10, 15, 20, and 25°C. Two petri plates were incubated at each temperature, with lesions illuminated by low-intensity substage fluorescent lighting (12 h day). After 4 days incubation, glass coverslips were placed on DWA in the partially filled lids of all of the petri plates so that they were oriented directly beneath two lesions of similar diameter from the leaf above. Coverslips were replaced each day, and those from the previous 24-h period were mounted in lactophenol cotton blue to kill and stain discharged ascospores (6). Using bright field microscopy at a magnification ×400, counts were made of total ascospore numbers on each coverslip. Leaves incubated at each temperature were discarded and the counting discontinued when leaves reached an advanced state of decay. The experiment was repeated.

Temperature and wetness duration on ringspot development. Brussels sprout plants of the cultivar Golfer were raised in Hassy 308 modules (Erin Planter Systems Ltd., Herts., UK), cut into 12-cell units, in a mixture of 70:30 Levingtons Fisons F2 compost (Fisons, Ipswich, UK) and sand. Plants were then grown at a 12-h day:night temperature regime of 16:14°C until they had reached the third true leaf stage. Plants were placed uniformly within a glasshouse infection tent with wetted, sporulating ringspot infected crop material (6) for a period of four daylight hours to maximize ringspot inoculum exposure. The crop material placed in the tent, the source of ringspot inoculum, was evenly spread and prewetted using a humidifier to ensure 100% RH prior to introduction of the test plants. To assess airborne ringspot inoculum during this exposure period, a Burkard 24-h glass slide volumetric air sampler (Burkard Manufacturing Co., Rickmansworth, UK) was operated throughout the 4-h exposure period. Upon removal from the infection tent, inoculated plants were immediately placed within controlled-environment cabinets (Model no. SGC70/C/RO-HFL; Sanyo Gallenkamp, Leicestershire, UK), each operating with ambient lighting (100 $\mu mol \cdot s^{-1} \cdot m^{-2}$) at 8, 12, 16, 20, or 24°C. At each temperature, the cabinets were preprogrammed to mist the plants with distilled water for 0.3 min every 60-min period to maintain leaf wetness. Control plants were placed in a temperature cabinet without wetting at 12°C only. In each cabinet, there was a total of 288 plants in 24, 12-cell units. Using random numbers, two replicate 12-plant units were removed from each controlled-environment cabinet 1, 3, 7, 12, 18, 24, 30, 36, 42, and 48 h postinoculation. The plants were air-dried at 14°C and maintained in a glasshouse for a 4-week period with no overhead watering at a 12-h day:night temperature regime of 16:14°C. At 3 and 4 weeks postinfection, the total number of ringspot lesions observed on all leaves of each plant was recorded.

The *M. brassicicola* ascospores that were trapped on the exposed glass slides of the Burkard 24-h air sampler were labeled by immunofluorescence, as described by R. Kennedy (13), using a monoclonal antiserum (MAb), coded EMA 187 (21), bound to a fluorescein-isothiocyanate conjugate (F-0257, www.sigma-aldrich.com). The fluorescein-labeled *M. brassicicola* ascospores were then visually identified and enumerated by episcopic fluorescence (excitation and emission spectrum wavelength of 495 to 521 nm) using a Nikon Optiphot-2 microscope (×400) as described by R. Kennedy (13).

Modeling temperature and leaf wetness on ascospore production. The cumulative production of ascospores at each incubation temperature (5, 10, 15, 20, and 25°C) was calculated over time. The period of incubation required for production of 5% (T5) and 50% (T50) of the cumulative ascospore total was calculated as described by J. E. Cullington (6). A reciprocal transformation was performed on the data and the line of best fit produced by regression analysis. From ringspot developmental studies (above), the leaf wetness duration at which 50% of the plants (T_{50}) were infected by *M. brassicicola* at 8, 12, 16, 20, or 24°C was determined. The reciprocal of leaf wetness duration was used as an estimate of the rate per hour at which plants became infected at each temperature. Linear regression was used to describe the temperature rate relationship for periods of ringspot risk. This model was then used to validate field results.

Quantification of MTIST-trapped spores. Ten sporulating agar plate cultures of *M. brassicicola* (13) were placed in a controlled-environment cabinet (Model no. SGC70/C/RO-HFL; Sanyo Gallenkamp) at 94% RH with continuous light. The sporulating cultures were wetted intermittently for 0.3 min every hour as previously described. Airborne spores within the cabinet were collected by impaction in the wells of 4 × 8 well microstrips of a MTIST spore trap (Burkard Manufacturing Co.) over a 24-h period. The MTIST was operated at an air-flow rate of 57 liters min^{-1} (1.78 liters flow rate min^{-1} across each microstrip well) and with sampling time periods of 3, 4, and 12 h. Following each exposure period, the microstrips were removed, and using a Nikon TMS inverted binocular microscope (×400), each microtiter well was scanned so the trapped ascospores could be counted. The microstrips were then stored at –20°C until processed by PTA-ELISA. The PTA-ELISA was carried out as described by R. Kennedy (12) using at the primary antibody stage MAb EMA 187 (13) and then a Strept ABC Complex DAKO duet amplification system (Cat no. K0492; DAKO Ltd., Cambridge, UK). The blocking stage was reduced to 30 min, without shaking, and a 1% Casein buffer (1% wt/vol Casein in phosphate buffered saline [0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4; PBS]) was used. All wash stages were as described by R. Kennedy (12) but with the addition of 0.1% Casein.

Field detection of ringspot ascospores. Transplanted Brussels sprouts (cv. Golfer) and an overwintered cauliflower crop (cv. Jerome) were grown adjacent to each other and 5 m north of an established ringspot-inoculated Brussels sprout plot (source plot) in trials conducted at Warwick HRI, UK in 2000 (Fig. 1). No other brassica crops were present within a 5-mile radius of the experiment. All plot sizes were 5 × 5 m with a 50-cm spacing between each brassica plant row and a 50-cm within-row plant spacing. Seedlings were transplanted during the second week of May 2000. All plots were treated with 0.24.24 (NPK) fertilizer at 1,000 kg ha^{-1} prior to seedling transplant. Nitrogen at 140 kg ha^{-1} was applied at the time of transplanting the crop, with a further nitrogen application of 100 kg ha^{-1} applied 6 weeks after transplanting. Manual weeding was used to keep weed populations under control. At weekly intervals, the transplanted Brussels sprouts and the overwintered cauliflower plot were treated with the fungicide difenconazole (www.syngenta.crop.co.uk) according to the manufacturer's guidelines and at the recommended rate of 0.3 liter ha^{-1}. Twenty-one days prior to the start of the experiment, fungicide applications ceased. The ringspot source crop did not receive any fungicide applications. Air temperature, leaf wetness (LW), relative humidity (RH), and rainfall were recorded at 30-min intervals (Smartlog, Aardware Design, Walton on Thames, UK). Wind run, the product of the average wind speed and the period over which that average speed was measured, was calculated using an anemometer (Vector Instruments, Rhyl, Denbighshire, UK) of cup diameter 25 mm. This field trial was similar to a trial conducted in 1996 (6) and repeated in 2004; the transplanted cauliflower crop was omitted in the 1996 and 2004 trials, however.

Over a 1-month period (October to November), ringspot airborne spores were monitored continuously using a conventional Burkard 7-day spore trap (B7-day). The B7-day spore trap was placed at the center of the established ringspot source plot with the sampling inlet at the height of the crop canopy. An additional B7-day spore trap was sampled at a point 10 m north between the transplanted Brussels sprouts and overwintered cauliflower plot (Fig. 1). At 7-day intervals, the Melinex tape (Burkard Manufacturing Co.) for each spore trap was removed and ascospores of *M. brassicicola* were quantified by immunofluorescence (13). A field MTIST spore trap was also positioned within the

ringspot source plot and adjacent to the B7-day spore trap. A Delta T data logger (Delta T Devices Ltd., Cambridge, UK) was used to activate the MTIST (12,21,22) when RH was >80% and light intensity was >0.3 watts/m^2. MTIST operation ceased when either threshold was not fulfilled. Over a 4-week period, microstrips of the MTIST spore trap (4 × 8 wells) were removed after either 1, 2, or 3 days of field exposure and stored at –20°C. A PTA-ELISA was carried out as previously described. However, a positive control of eight coated ringspot antigen wells (1 × 8 well microstrip) and a negative control of eight uncoated/non-field-exposed wells was included within the assay. To determine additional background and/or nonspecific reactions, four wells of each of the microstrips did not receive MAb EMA 187 but PBSTwC (phosphate buffered saline, pH 7.2, 0.05% Tween 20, 0.1% Casein) alone. For each microtiter strip, the mean absorbance of the four wells that had not received EMA 187 was subtracted from the mean absorbance values generated from the four wells that received MAb EMA 187.

Pots of 10 disease-free Brussels sprout bait plants (cv. Golfer), each with 10 true leaves, were placed in the field for 24 h daily over the 1-month period to determine ringspot incidence. The plants were grown in the glasshouse as described above. Five plants were placed at the center of the source plot with a separation of 0.5 m between plants (arranged in two cross-secting diagonals) and five plants within each of the Brussels sprout and cauliflower plots. Following a 24-h field exposure period that covered full daylight periods when ringspot inoculum was produced, the plants were removed and placed in an environment of 100% humidity for 24 h. The plants were then removed, dried, and placed in a glasshouse at a 12-h day:night temperature regime of 16:14°C for 21 days. Plants were visually examined for typical ringspot lesions, and confirmatory isolations were made onto sprout leaf decoction agar since this medium was shown to improve the growth of *M. brassicicola* in vitro (13). Bait plant studies in 1996 (6) and 2004 were included in wind run studies where the same experimental procedures used in 2000 were adopted.

Ringspot risk in brassica production. At two sites (A and B) in Lincolnshire, in a coastal area of commercial brassica production, two freshly transplanted (disease-free at the time of planting) vegetable brassica crops were monitored for ringspot during June to October 2001. Site A coordinates were 53°6′29″ N, 0°14′11″ E, and site B coordinates were 52°58′29″ N, 0°2′52″ E (www.ordnancesurvey.co.uk). Within each crop, a B7-day and a field MTIST spore trap were sited and operated as previously described. The Melinex tapes of the B7-day traps were replaced every 7 days and processed by immunofluorescence as described previously. The microwell strips of the MTIST spore traps were changed at either 3-, 4-, or 5-day intervals and processed by PTA-ELISA. Air temperature, leaf wetness, relative humidity, and rainfall were recorded at 30-min intervals. The equipment used was powered on-site using a Rutland wind charger (model no. CA-12/08, CA-11/13; Marlec Engineering Co. Ltd., Northamptonshire, UK), and the parameters and procedures used were as previously described. Environmental data were downloaded daily and used in a mathematical model derived from the earlier linear regression study to describe the rate temperature relationship for periods of ringspot infection. During periods of ascribed risk, the crops were treated according to the manufacturer's guidelines with the fungicide difenconazole (www.syngenta.crop.co.uk) at the recommended rate

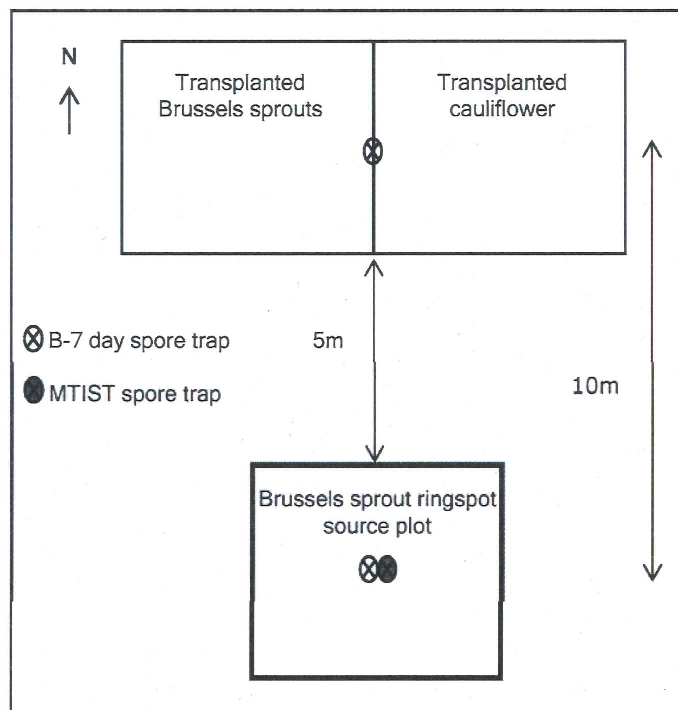

Fig. 1. Position of brassica crops and spore trapping equipment at Warwick HRI in 2000.

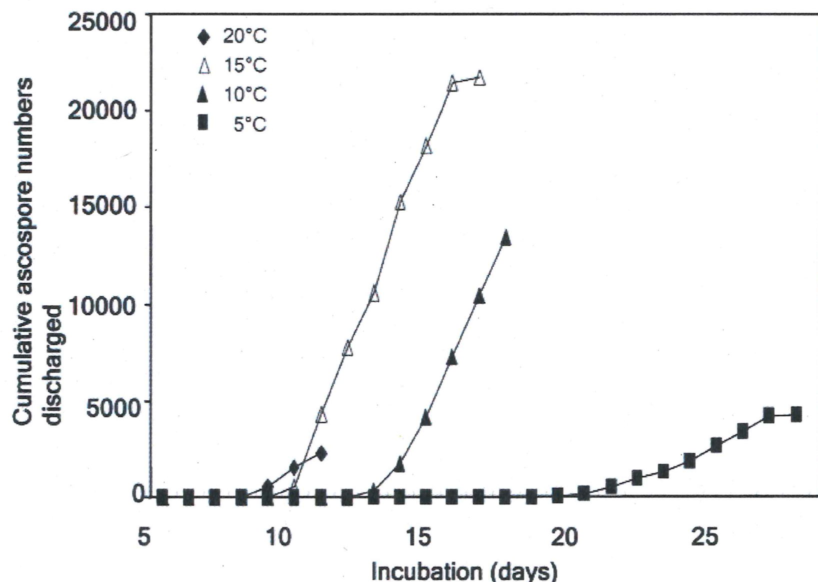

Fig. 2. Influence of temperature on *Mycosphaerella brassicicola* ascospore release from pseudothecia in laboratory petri dish assays.

(0.3 liter ha^{-1}). In each crop, a 5 × 5 m area, where no fungicide treatment was applied, was reserved for plant assessments. Five weekly assessments (July–August 2001) of ringspot lesion cumulative number were made on the four oldest leaves of nine tagged plants. The tenth leaf of each assessed plant was then marked with a plastic tag. This was necessary to calculate lesion number per plant due to leaf abscission. At each assessment, the leaves were referenced to the nearest tagged leaf. Isolations from leaf lesions onto V8 agar were as described previously to confirm the presence of *M. brassicicola*.

Inhibition of germination of MTIST-trapped spores. A B7-day and a field MTIST spore trap were positioned adjacent to each other within a heavily infected ringspot commercial Brussels sprout crop (site B; Fishtoft, Lincolnshire, UK) and operated as previously described. Prior to field exposure, MTIST microstrips received the following treatments using methods described by A. J. Wakeham et al. (22): 100 µl per well of distilled water was aliquoted to each of eight wells of a microstrip (A). The second strip contained 100 µl/well of a 0.5 mg ml^{-1} sodium azide (NaN_3; catalogue no. S-2002; www.sigma-aldrich.com) solution (in distilled water) (B); and to strips three (C) and four (D), a 1 mg and 1.5 mg ml^{-1} sodium azide solution was applied. The strips were air-dried at 50°C prior to field exposure. The pretreated MTIST microstrips were field exposed for either 3- or 4-day periods. Following each field sampling period, the four microstrips were collected, and to enhance conditions for trapped spore germination, four wells of each microstrip (A, B, C, and D) were separated and incubated at 15°C, 97% RH for 72 h before storage at –20°C. Those wells that did not receive the additional incubation step were stored at –20°C. All microstrips were processed by PTA-ELISA. However, two wells of each four-well microstrip segment did not receive the primary antibody stage of MAb EMA 187 but PBSTwC alone. All other stages were as previously described. The Melinex tape of the B7-day spore trap was processed by immunofluorescence and ascospore inoculum of *M. brassicicola* quantified.

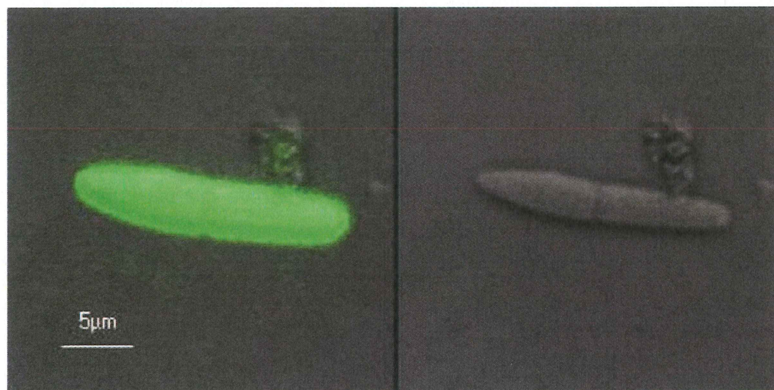

Fig. 3. Ascospore of *Mycosphaerella brassicicola* as viewed by confocal laser scanning microscopy: **A,** fluorescent image (green channel); **B,** grayscale (transmitted) image of the same area.

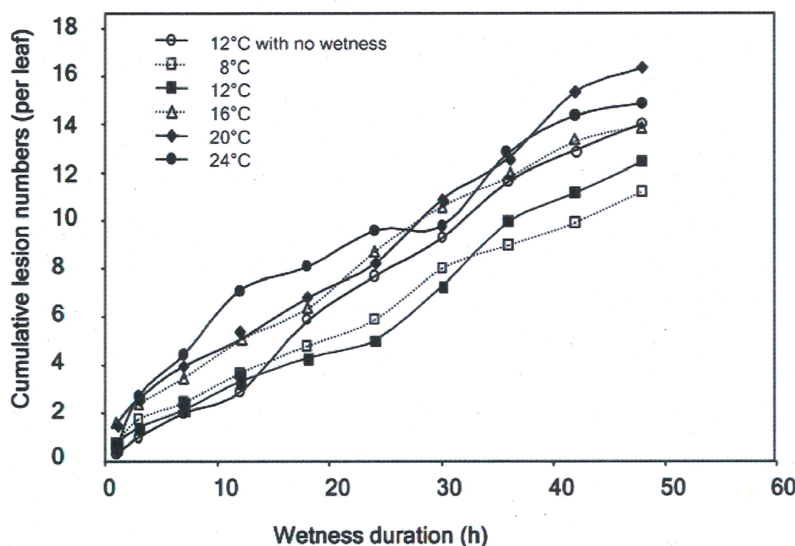

Fig. 4. Effect of leaf wetness duration on infection of vegetable brassica leaf tissue by *Mycosphaerella brassicicola* in a glasshouse infection tent study (lesion number = mean per plant based on 80 plants).

RESULTS

Temperature and ascospore development. Initial ascospore discharge occurred at 20°C after 8 days incubation (Fig. 2). At 15 and 10°C, ascospores were first seen after 9 and 12 days, respectively. At 5°C, lesions discharged ascospores after 18 days. Lesions incubated at 25°C showed sparse pseudothecial development, and no ascospores were collected. Lesions placed at 15 and 10°C discharged ascospores over a period of 7 and 5 days, respectively, after which leaves disintegrated and were discarded. At 5°C, lesions released ascospores over a period of 9 days, before the leaves disintegrated. At 20°C, leaves senesced rapidly and were discarded 3 days after the onset of ascospore release. Cumulative ascospore production by lesions was greatest at 10 and 15°C (experiment 1, Fig. 2). Similar results were observed in the repeated experiment, with a regression analysis of the relationship between the reciprocal of incubation period and temperature for each experiment being highly significant ($P < 0.001$).

Temperature and wetness duration on ringspot development. Ascospores of *M. brassicicola* were detected in the air 1 h after the Brussels sprout plants had been placed in the infection tent and exposed to the ringspot inoculated plant material. During this period, 232 *M. brassicicola* ascospores, which were collected from 2.4 m^{-3} of air by the B7-day air sampler, were identified by immunofluorescence (Fig. 3). The highest ascospore numbers were recorded after 2 h exposure. Ringspot infection was observed on all Brussels sprout plants incubated in the controlled environment cabinets. Lesion numbers increased steadily over time as wetness duration increased regardless of temperature (Fig. 4). For statistical analysis, the data were expressed as a rate (mean number of lesions per plant per hour). A graph (not shown) of this rate against time showed anomalous data (total 14 lesions) from 30 h at 24°C, and these data were omitted from subsequent analysis. The same graph showed that the control treatment (nonwetted plants) produced lesions at a steady rate over the whole time period but that the wetted plants took 3 h to settle to a constant rate of lesion development. A model describing ringspot ascospore infection used data collected from plants that received between 12 and 48 h of wetness over the temperature range 8 and 24°C. Regression analysis showed that the infection rate remained constant with time for each temperature regime. Rates were averaged over all times and regressed against temperature. Equation 1

shows the resulting linear relationship ($r = 0.92$):

$$r_i = 0.0056T + 0.1755 \quad (1)$$

where r_i is infection rate and T is temperature.

Modeling temperature and leaf wetness on ascospore production. Time to discharge of T5 and T50 of the cumulative total number of ascospores produced from lesions were estimated by linear interpolation. The times were inverted to produce rates (see below) and regressed against temperature. The relationships for both 5 and 50% were linear, with $r = 0.99$ and 0.98, respectively ($P < 0.001$):

$$r_5 = 0.0199 + 0.0058T \quad (2)$$

and

$$r_{50} = 0.0195 + 0.0046T \quad (3)$$

where r_5 and r_{50} are (1/T5) and (1/T50), respectively, and T is temperature (°C).

Quantification of MTIST-trapped spores. Ascospore concentrations were highest in the center of the base of exposed microstrip wells as previously reported by R. Kennedy (12). A correlation coefficient of $r = 0.91$ was obtained when MTIST-trapped *M. brassicicola* spore numbers per well were compared to the corresponding absorbance values generated in the PTA-ELISA (data not presented).

Field detection of ringspot ascospores. Average daily field temperatures ranged from 6 to 13°C, which according to equation 1 would produce between five and six lesions per plant per day on days when leaves were wet and subjected to the inoculum levels used in the controlled-environment experiments. During the 2008 crop season, leaf wetness was variable and almost all night periods had recorded wetness in the crop of 60 to 100% RH, which varied in duration depending on daylight conditions. Dry conditions were recorded for most of the daylight hours.

Lesion numbers were compared with wind run and wind direction. Results from bait studies from 1996 and 2004 were included in the analysis (Fig. 5). Lesion numbers per plant above 10 occurred only at wind runs greater than 200 km day^{-1} (Fig. 5). With the exception of bait plant lesion numbers recorded on 28 October 2000, lesion numbers on plants were related to the previous day's wind run. For example, 58 lesions per plant were observed on 17 October 2000, when a wind run of 380 km day^{-1} occurred on 16 October. Strong winds and heavy rain occurred on 28 October even though the previous day was exceptionally still, which may account for the discrepancy on this date. Wind runs of greater than 250 km day^{-1} occurred when the wind was from the south or southwest. There was an optimal wind run of 250 to 500 km day^{-1} for the spread of ringspot to bait plants (Fig. 5).

A correlation of $r = 0.7$ was estimated between the B7-day and the MTIST for airborne spores of *M. brassicicola* that were trapped (Fig. 6). For one sampling period (27 to 28 October 2000), however, the MTIST PTA-ELISA recorded a high absorbance value (0.37 nm) where the B7-day failed to trap corresponding levels of ringspot ascospores (Fig. 6). Nevertheless, for this sampling period, a high level of disease was recorded on the exposed bait plants (Fig. 7: 144 lesions per plant). For the next sampling period (28 to 29 October), both the MTIST and the B7-day failed to detect ascospores of *M. brassicicola* even though >60 lesions per plant was observed on the exposed bait plants. During this period, however, an exceptional level of rain (25 mm) fell over a short period of time, which may have compromised the trapping efficiency of each trap. With the exception of one 24-h period, infection of bait plants with >15 lesions/plant was limited to those exposed in the ringspot source plot. Nevertheless, in the Brussels sprout and cauliflower plots, sited 5 m from the source plot, a number of ringspot infection periods were observed

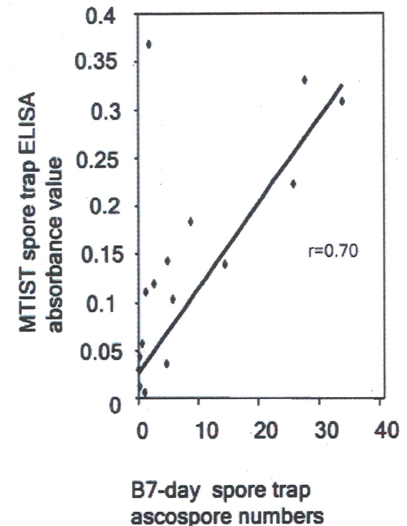

Fig. 6. Relationship of two air-sampling spore traps (corrected to a sample flow rate of 1.78 liters of air per min) for monitoring ascospores of *Mycosphaerella brassicicola* in the field.

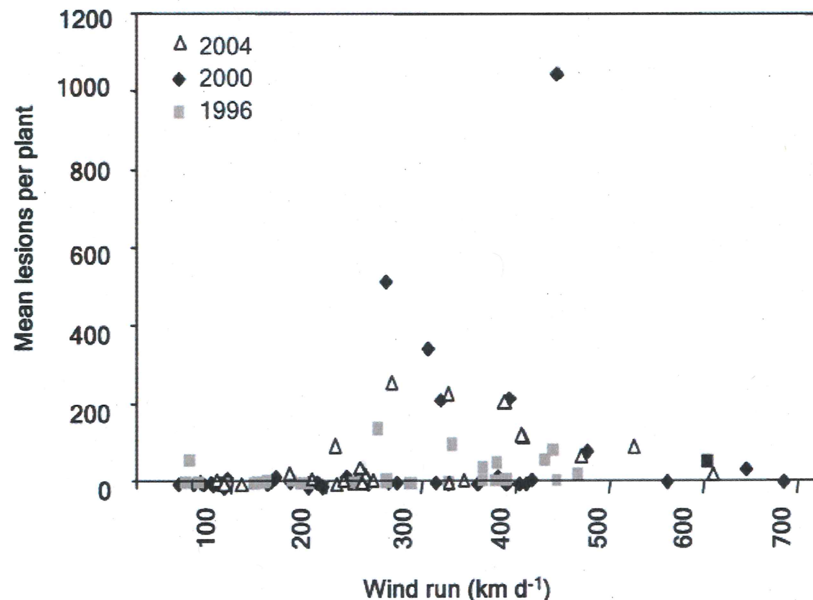

Fig. 5. Effect of wind run (dm^{-1}) on infection of trap plants by *Mycosphaerella brassicicola* in 1996, 2000, and 2004 field studies at Warwick, UK.

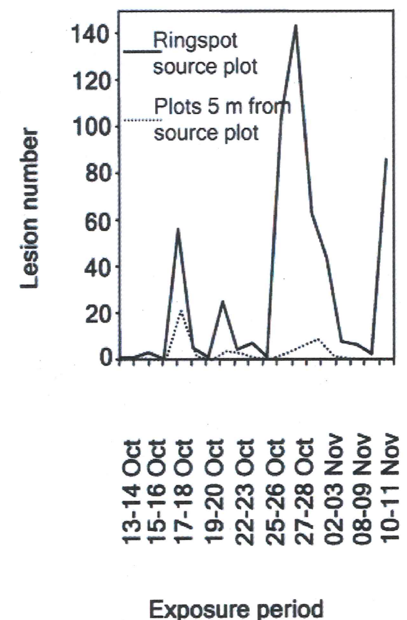

Fig. 7. Mean ringspot lesion numbers on trap plants in the field at Warwick, UK, exposed daily during fall 2000 to a ringspot source plot and an adjoining brassica plot 5 m away.

(Fig. 7). For these periods, a prevailing northerly wind was observed. On all other occasions, the prevailing wind was south, southwesterly, or southeast. Leaf isolations made on V8 agar medium confirmed the presence of *M. brassicicola.*

Ringspot risk in brassica production. There were many risk periods for *M. brassicicola* infection at both commercial sites in 2001. At site A, commercial ringspot ascospores were first detected between 6 and 10 July 2001 (B7-day = three ascospores per m^{-3} air sampled; MTIST PTA-ELISA absorbance value = 0.257 nm) (Fig. 8). For this period, the model (equation 1) predicted a high risk of infection. An antisporulant fungicide spray of difenconazole was applied in response on 9 July according to manufacturer's guidelines and at the label prescribed rate of 0.3 liter ha^{-1} (www.syngenta.crop.co.uk). Lesions of ringspot were observed on 13 July (Fig. 9) but only in the area of the crop (5 × m), which had not been sprayed with fungicide. During July and August, the model predicted six 24-ringspot infection periods. Fungicide sprays of difenconazole were applied on 29 July and 21 August.

During the 6-week trapping period, a linear relationship (r = 0.8675) was observed between MTIST PTA-ELISA absorbance values and corresponding number of B7-day trapped ascospores of *M. brassicicola* (Fig. 10, site A). At commercial site B, ascospores of *M. brassicicola* were first trapped on 30 June, but inoculum levels of *M. brassicicola* remained low (B7-day = <1 spore m^{-3} of air sampled; <0.1 nm MTIST PTA-ELISA) until 13 to 15 July. The model (equation 1) indicated a high risk of infection at the end of June, and a fungicide spray of difenconazole was applied to the crop on 10 July (due to prevailing weather conditions). Ringspot was not identified on nonsprayed plants until 20 July (Fig. 9); where fungicides were applied, the crop remained free of ringspot symptoms at both sites.

In contrast to site A, the two spore trapping systems used did not initially correlate well at site B (Fig. 10, site B). Upon examination of the 13 to 15 July trapping period, MTIST spore trap absorbance values (MTIST PTA-ELISA = 0.525 nm) assigned a much higher level of available inoculum than identified by the B7-day immunofluorescence spore trapping assay (3 spores m^{-3}). Further examination of the MTIST microstrip wells for this period revealed germination of the trapped spores. An additional two sampling periods, identified as having high levels of spore germination, were subsequently omitted from the study, and a linear relationship (r = 0.88) was then observed between the recorded MTIST PTA-ELISA values and B7-day trapped ascospores of *M. brassicicola* (Fig. 10, site B). At both sites during *M. brassicicola* infection periods, nonfluorescing spores of morphologically similar characteristics to *M. brassicicola* were found in high numbers when processed by immunofluorescence. Where these ascospores had germinated, binding of the MAb EMA 187 conjugated fluorescein was observed on the germ tube when viewed by UV fluorescence microscopy.

Data from each trial site indicated that trapped ascospore numbers related to the wind run on the day that they were trapped (Fig. 11). With the exception of three dates, the numbers of trapped ascospores above 10 were observed only at wind runs greater than 200 km day^{-1}.

Inhibition of germination of field MTIST-trapped spores. A linear relationship (r = 0.84) was observed between absorbance in wells that had not received a sodium azide coating and the number of B7-day IF trapped ascospores of *M. brassicicola.* Following a 72-h period at 93% RH, assay sensitivity increased but a nonlinear relationship was observed (r = 0.93) (Fig. 12A). Pretreatment of wells with 100 µl per well 0.5 mg ml^{-1} sodium azide resulted in a linear relationship while inhibiting germination of MTIST-trapped spores (Fig. 12B). However, when wells were pretreated with sodium azide at a concentration >0.5 mg ml^{-1}, assay sensitivity decreased (data not presented). At a concentration of 1.5 mg ml^{-1}, no correlation was observed between ascospores and the absorbance values of the MTIST PTA-ELISA (data not presented).

DISCUSSION

The objective of this study was to ascertain if measuring numbers of *M. brassicicola* ascospores in air samples could be used to predict the onset of ringspot risk within healthy crops of Brussels sprouts. Existing methods of forecasting ringspot

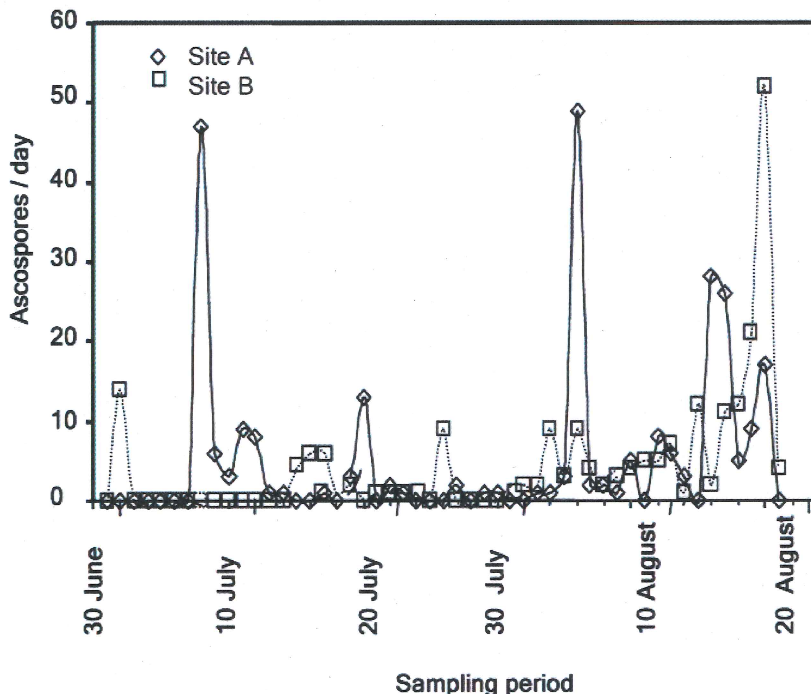

Fig. 8. Airborne ascospores of *Mycosphaerella brassicicola* in two commercial Brussels sprout crops in the United Kingdom in 2001 as determined by immunofluorescence.

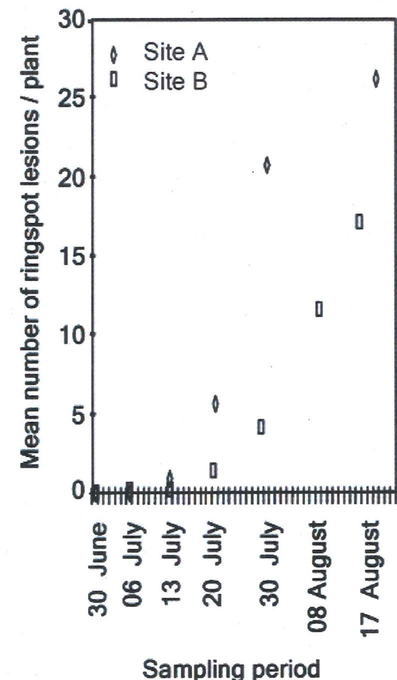

Fig. 9. Ringspot in two unsprayed commercial crops of Brussels sprouts in the United Kingdom in 2001.

depend solely on defining environmental field conditions necessary for infection (18). This approach to disease forecasting is standard in the control of many fungal diseases of horticulture and agricultural crops. However, we have shown that ringspot infection can occur over most environmental conditions typical of vegetable brassica crops in the UK. The time to development of ringspot inoculum and variability in the level of ringspot inoculum would appear to be the major components driving ringspot occurrence (Figs. 2 and 5). A highly significant relationship was observed between the results of the MTIST PTA-ELISA and the number of ringspot lesions that subsequently developed on exposed trap plants in the field at Warwick HRI, Wellesbourne. Monitoring epidemiologically significant levels of airborne field inoculum (MTIST PTA-ELISA) may prove a useful indicator of ringspot risk. One aspect which requires further investigation is the spatial variation in ascospore concentration in the field and its impact on risk assessment. Other studies within the UK on the vegetable brassica pathogen *Pyrenopeziza brassicae* Sutton & Rawl. (light leaf spot) have demonstrated that spore inoculum occurs at relatively high concentrations in the air before crop-to-crop transport is possible (9). Previous studies (12) have demonstrated the requirement for a threshold of ringspot spores to be reached before infections occur. It is unclear if this threshold would vary spatially among brassica vegetable production areas. Longer air sampling periods could be used to reduce the impact of variability.

This study demonstrates that air dispersal conditions (wind runs) have a major impact on inoculum availability. Discharged ascospores are likely to attain higher concentrations in the air on days with optimal wind runs. Very high wind runs appear to have the effect of dispersing the ascospores more widely, resulting in lower concentrations in the air. The results from trap plant exposure indicated that disease occurrence was significantly correlated with the wind speed on days prior to exposure and can be explained by the procedure used to expose trap plants. Plants were exposed at 6 P.M. during one day and collected at 6 P.M. the following day. This exposure pattern matches the pattern of inoculum availability where *M. brassicicola* ascospores are released from pseudothecia by light and no new inoculum is produced during dark conditions (6). Positioning of the spore traps above the level of the crop canopy would have enabled a better estimation of inoculum involved in long-range dissemination. Aylor (1) demonstrated the relationship between wind speeds and spatial variability over grass canopies with the apple scab pathogen *Venturia inaequalis*. Consequently, when monitoring the entry of primary inoculum, the positioning of the spore trap will prove critical in relation to the height of the crop. Other environmental conditions can also be associated with spore dissemination. Both spore traps failed to detect airborne spores of *M. brassicicola* on one occasion. However, disease was identified at a high level (>60 lesions per plant) on exposed trap plants. This situation appeared to be associated with heavy rainfall, and a similar effect was demonstrated for *Venturia inaequalis* by Aylor and Sutton (2).

The present study indicates epidemiological parameters have potential for integration with immunoassays measuring dispersal of fungal pathogens in crops. In the past, it has been impossible to quantify target organisms in the air accurately and quickly. However, the development of the MTIST device enables a portable, robust, and inexpensive spore trapping system that incorporates trapping technology alongside an existing well-established immunoassay (ELISA) (5,7,12). For successful adaptation, consideration of the characteristics of the target inoculum and use of the MTIST spore trapping device within the immunoassay format will require investigation (22). The numerous washing steps involved in ELISA mean that unless spores are properly attached to the impaction surface, they will be lost during the immunoassay stage. Ascospores of *M. brassicicola* have an exterior "sticky" mucilage coating which facilitate adhesion to the microstrip well throughout the ELISA process (12). Some studies (22) have demonstrated that spore attachment is not always a prerequisite of the assay. A correlation of $r = 0.93$ (nonlinear) was attained between the number of MTIST-trapped *Agaricus bisporus* spores and ELISA absorbances generated in spite of <1% of spores being retained post-ELISA. The necessity for retention of spores within the microtiter well will depend upon the antibody and target analyte. Processes in fungal spore attachment involve the production and release of extracellular adhesive to which antibody probes may be targeted. Where spore attachment can be reversible, the adhesive material may be retained (8,12).

Where spore retention is a requirement of the assay format, germination may facilitate attachment throughout the assay process. Studies involving the adhesion of germlings of *Botrytis cinerea* demonstrated that initial attachment involves relatively weak adhesive forces. Yet fol-

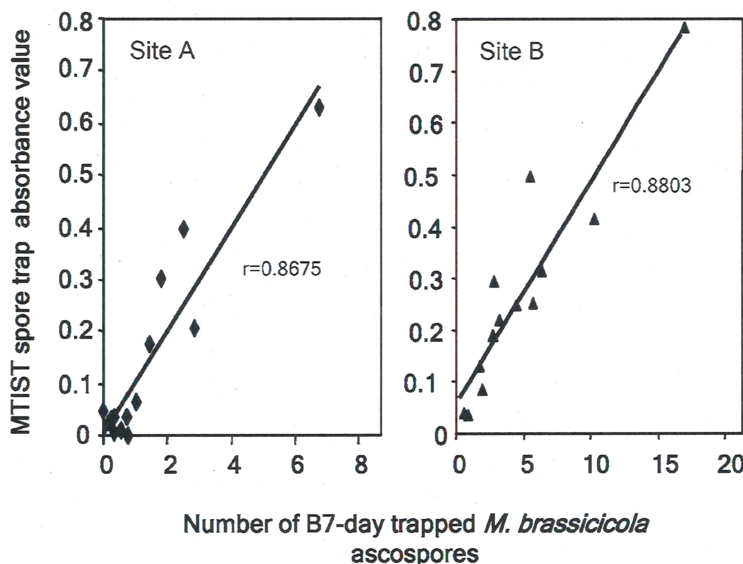

Fig. 10. Comparison of the microtiter immunospore trap (MTIST) immunoassay and the B7-day immunofluorescence test (B7-day sample flow rate corrected to 1.78 liters air/min) for ringspot transmission at two commercial brassica production sites in 2001.

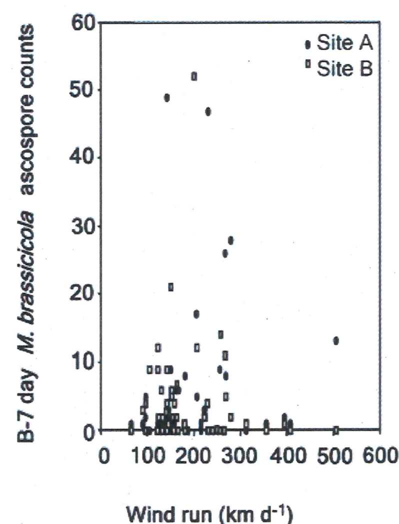

Fig. 11. Daily numbers of ascospores of *Mycosphaerella brassicicola* recorded at two commercial Brussels sprout sites in the United Kingdom in 2001.

lowing germination, conidia attach strongly to the impaction surface, proving resistant to removal by boiling or by treatment with a number of hydrolytic enzymes (8). Should germination be a prerequisite of the test, the specificity of the chosen antibody should be tested accordingly. The specificity of MAb EMA 187 to *M. brassicicola* is compromised on germination of fungal spores (21). At the two UK commercial field sites, where trapped spore germination occurred, MAb EMA 187 bound to the mycelial germ tube of spores similar in morphology to *M. brassicicola* but with no reaction observed to the spore wall. Whether this is reactivity to another spore type is not clear. The potential for variation or deletion of the *M. brassicicola* ascospore wall MAb-specific antigen determinant epitope site within a spore population may exist. Differential binding of species-specific MAbs to homologous spore wall antigen of *Agaricus bisporus* (basidiomycete mushroom spore) and *Bremia lactucae* (downy mildew on lettuce) has been observed and, with *Bremia lactucae*, antibody binding was related to spore maturation (A. J. Wakeham, *unpublished*). Future studies should consider the potential for reactivity of MAb EMA 187 to other spore types not previously tested (21) and whether differences in MAb-specific ascosporic wall antigen determinant epitope sites of *M. brassicicola* exist.

At commercial site B, the high summer temperatures (>20°C) and sea fogs gave rise to constant high humidity. Infection conditions as derived from this study were at times observed to be nonlimiting. An increased level of spore germination may have resulted in the overestimation and/or misidentification of *M. brassicicola* spore numbers, leading to misdiagnosis of ringspot risk. However, the pretreatment of microtiter wells with sodium azide (0.5 mg ml^{-1}) eliminated this problem. When using MAb EMA 187, it would be important for accurate estimation that ascospore germination did not occur. For commercial usage and in the absence of a B7-day spore trap to validate trapped spore numbers, the inclusion of a calibration curve would be a prerequisite within the assay format.

There is the potential to detect several target pathogens simultaneously using the MTIST spore trap in conjunction with specific antibody probes. Utilizing this information within environmental-based disease forecasting systems could prove important in disease prediction producing quantitative estimates of pathogen infection within crops. At present, many forecasting systems assume that inoculum is not limiting. However, the results from field trials in commercial and noncommercial situations indicate that this assumption is not valid. The results also point to the need for a quantity of inoculum to be present if significant crop infection is to occur and develop since low levels of ringspot inoculum did not lead to disease development. The amount of inoculum required for significant crop infection to occur could not be defined in this study. It is clear that this threshold may vary between pathogens and would be related to environmental factors and plant resistance. The MTIST spore trap can be used for multiple tests during a single sampling period so should prove useful for monitoring airborne particulates and microorganisms in a range of environments. In many disciplines, specific antibodies and optimized immunoassay systems are already in routine use and could be adapted for use with the MTIST spore trap.

The results also emphasize the need for rapid methods of detection if measures of target propagules within air samples are to be used in practical decision making for control of plant diseases. The ELISA is still a lab-based assay. However, the recent development and use of immunochromatographic test strips to rapidly detect and quantify fungal target inoculum *in situ* (11,17) exhibits considerable potential for monitoring airborne spores. Future work will investigate the potential for modifying the current spore trapping equipment and immunological test to improve both detection and quantification of airborne ascospores of *M. brassicicola* and other fungal pathogens of vegetable brassicas via a field-based rapid one-step assay system. The system described is used as an example where information on inoculum availability can be used directly with environmental disease forecast models to provide information for crop protection regimes.

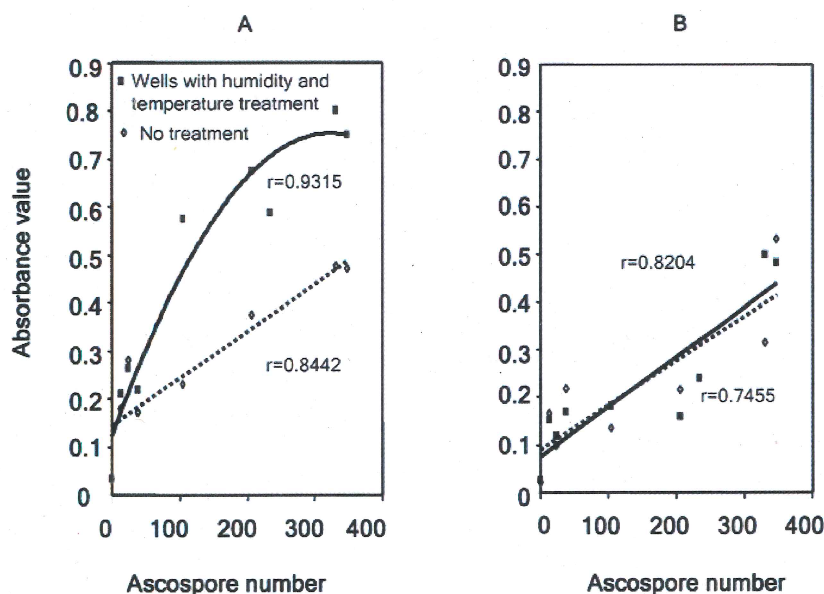

Fig. 12. Effect of humidity and temperature on field-exposed microtiter immunospore trap untreated microtiter wells (**A**) and 0.5 mg ml^{-1} sodium azide coated microtiter wells (**B**) as processed by plate-trapped antigen enzyme-linked immunosorbent assay.

ACKNOWLEDGMENTS

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Horticulture Australia Limited (HAL) – VG07070: Benchmarking predictive models, nutrients and irrigation for management of white blister.

Subcontract Final Report

National Pollen and Aerobiology Research Unit, University of Worcester,
Charles Darwin Building, Henwick Grove, Worcester WR2 6AJ

Project title:	Horticulture Australia Limited (HAL) – VG07070: Benchmarking predictive models, nutrients and irrigation for management of white blister.
Project leader:	A.Wakeham, National Pollen and Aerobiology Research Unit, University of Worcester, Henwick Grove, Worcester WR2 6AJ
Technical support	G. Keane
Report:	Final report
Previous report:	N/A
Location of project:	University of Worcester
Date project completed (or expected completion date):	[20th November, 2011]

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PROJECT BACKGROUND

White blister is caused by the oomycete pathogen *Albugo candida* and is a common disease of many economically important cruciferous vegetables and oilseed crops. Significant yield losses from this disease have been reported on the oilseeds *B. rapa* and *B. juncea* and, to a lesser extent, on susceptible lines of *B. napus.* Affected vegetables include broccoli, Brussels sprouts, cauliflower, radish, mustard, Chinese cabbage and turnip. The impact of disease in these crops is of a cosmetic nature and can render crops unmarketable. In Australia the disease has been commercially important in vegetable production since 2002

To date, more than ten distinct biological races of *A. candida* have been identified and classified based on host specificity. The recorded pathotypes in Australia are likely to be Races 1, 2, 3,4, 5, 6, 7, 9 and 10. Race 9 infects *B. oleracea* and is considered to have caused the recent outbreaks of white blister on broccoli in Australia. Current broccoli varieties tolerant to white blister are not available all year round. Disease management strategies to control the disease include routine spray programs alongside improved irrigation scheduling. In the UK, improved management of the disease and, reduced applications with effectiveness of the fungicides applied, has been achieved utilizing information from an environmental white blister disease risk forecast. This system is currently being trialed in Australia.

The present study aims to improve the environmental white blister disease risk forecast by including information on availability of *A. candida* airborne disease inoculum. Monoclonal antibodies, with recognition sites to *A. candida* Race 9 zoosporangia, will be produced and assessed within a PTA ELISA (plate-trapped antibody enzyme-linked immunosorbent laboratory test) for quantification of MTIST trapped field aerosols of *A. candida*. Preliminary studies will investigate the potential of an immunological chromatographic test strip (lateral flow) to provide ‘in field’ information on *A. candida* spores in collected field air samples.

YEAR 1

Production of Monoclonal Cell Lines

Introduction

The white blister pathogen *Albugo candida* is an obligate parasite which cannot be cultured on agar and will only grow and develop on host tissue. The pathogen can lie dormant by the formation of oospores on contaminated seed, in soil or on infected host material. During favorable periods oospore germination and sporangia production give rise to pustule formation on the host. Zoosporangia are dispersed by wind, rain, or insects to neighboring plants. The zoosporangial stage of the pathogens life cycle was identified for monoclonal antibody production (MAb).

Methods

Collection of zoosporangia. A hand held Burkard surface cyclone sampler (Burkard Manufacturing Co., Rickmansworth, Herts., UK) was used to collect zoosporangia of *A. candida* from the leaf surface of sporulating infected *B. oleracea* host material. One ml of chilled phosphate buffered saline ((PBS) 0.01M phosphate buffer, 0.0027M potassium chloride, 0.137M sodium chloride, pH 7.4) was added to the air sampler collection vessel. Using a spin mix (Gallenkamp Ltd, Cheshire) at high speed for five minutes the zoosporangia were suspended in to the aqueous phase. The spore suspension was filtered through a polyester Spectra mesh membrane (47µm pore size; NBS Biologicals Ltd, Huntingdon, UK) to remove any large contaminating material. The liquid phase was collected and, by using a membrane of 10µm pore size, bacteria and other small leaf contaminants were removed after filtration. The filtrate was collected and resuspended in 2ml chilled PBS and, presence of *A. candida* zoosporangia confirmed by bright field microscopy (x 400). A zoosporangial concentration of $1x10^7$ ml^{-1} was determined.

Immunogen preparation. Using a Fast Prep 120 instrument (www.qbiogene.com) at an operating speed of 5 for 25 seconds the *A. candida* zoosporangia were disrupted. The sample was rested on ice for 3 minutes and the process repeated twice. After microfugation in an MSE Microcentaur (www.mseuk.co.uk), operated at 10,000 r.p.m for five minutes, the soluble phase of the disrupted *A. candida* sample was retained and transferred to a YM30 microcon centrifugal unit (www. millipore .com). The sample was separated in to two fractions of > 30 KDa and < 30 KDa according to manufacturer's guidelines. The protein concentration of the >30 KDA fraction was adjusted to 2 mg ml^{-1} and stored at -20°C. Using a mini slide-a-lyzer dialysis cassette of molecular weight cut off 2 KDa (www.thermoscientific.com) the *A.candida* < 30 KDA fractions

was dialysed according to manufacturer guidelines in ½ strength chilled PBS buffer. The protein concentration of the recovered dialysed sample was determined, adjusted to 2mg ml^{-1} and stored at -20°C.

Immunisation. Three Balb C mice were immunized, by intraperitoneal injection, each with 50µl of > 30 KDa *A. candida* zoosporangia preparation mixed with an equal volume of Titermax adjuvant (Sigma T-2684). An additional three mice were immunized with the < 30 >2 KDa *A. candida* zoosporangia preparation. The process was repeated monthly over an 8 week period. To determine the immune response of each mouse to the homologous immunogen preparation, tail bleeds (50µl blood sample / mouse) were taken 7 days after the third immunization.

Tail bleeds. Using a standard plate-trapped antigen enzyme-linked ELISA (PTA-ELISA) polysorp microtitre wells (Catalogue No. 469957, Life Technologies Ltd, Paisley, Scotland) were each coated with 100µl of 10µg homologous antigen in PBS and incubated overnight at 4°C. The PTA-ELISA was carried as described by Wakeham (2004) but at the primary antibody stage doubling dilutions of each tail bleed were made in PBSTwC (PBS, 0.05% Tween 20 and 0.1% Casein [wt/vol]) to an end point of 1 in 128,000 and, each dilution probed against replicate homologous well-trapped antigen. A Strept ABC complex DAKO duet amplification system, (Cat.No. KO492; DAKO Ltd, Cambridge, UK) was used according to the manufacturers guidelines at the secondary Ab stage. Two weeks later a pre fusion immunogen boost was given, as described previously, to two mice. Mouse 1 received an immunogen preparation of *A. candida* <30KDa >2KDa and Mouse 6 >30 KDa. Four days later the two mice were killed.

Fusion. The spleens of two mice were removed and, for each, a fusion was carried out according to Warwick HRI standard protocol using a modified method of Kennet (1978). Antibody producing B cells isolated from the spleen were fused in vitro with lymphoid tumour cells (myeloma). The cell hybrids (hybridomas) were supported in Dulbecco's Modified Eagles's Medium (Code No. D5976, www.sigmaaldrich.com) containing 10% Foetal calf serum (DME). On day 6 100µl DME fresh medium was added to each hybridoma and, the medium changed on day 10. Cell tissue (TCS) culture supernatants were screened by PTA ELISA and immunofluorescence (6) 14 days after cell fusion for the presence of antibodies which recognized zoosporangial epitopes of *A. candida*. Cell lines which gave a positive results (> 3 times the negative control in PTA ELISA) were selected and twice cloned three times

Results

Tail bleeds. The two immunogen preparations (*A. candida* <30KDa >2KDa and, > 30 KDa *A. candida* zoosporangia soluble antigen) elicited an immune response in each of the mice, producing antibodies that reacted with either *A. candida* preparation as determined by PTA ELISA (Fig. 1.). Of the two types of immunogen preparation used, Mouse 1 and Mouse 6 were selected for fusion, as each proved optimal in the immune response to its homologous antigen.

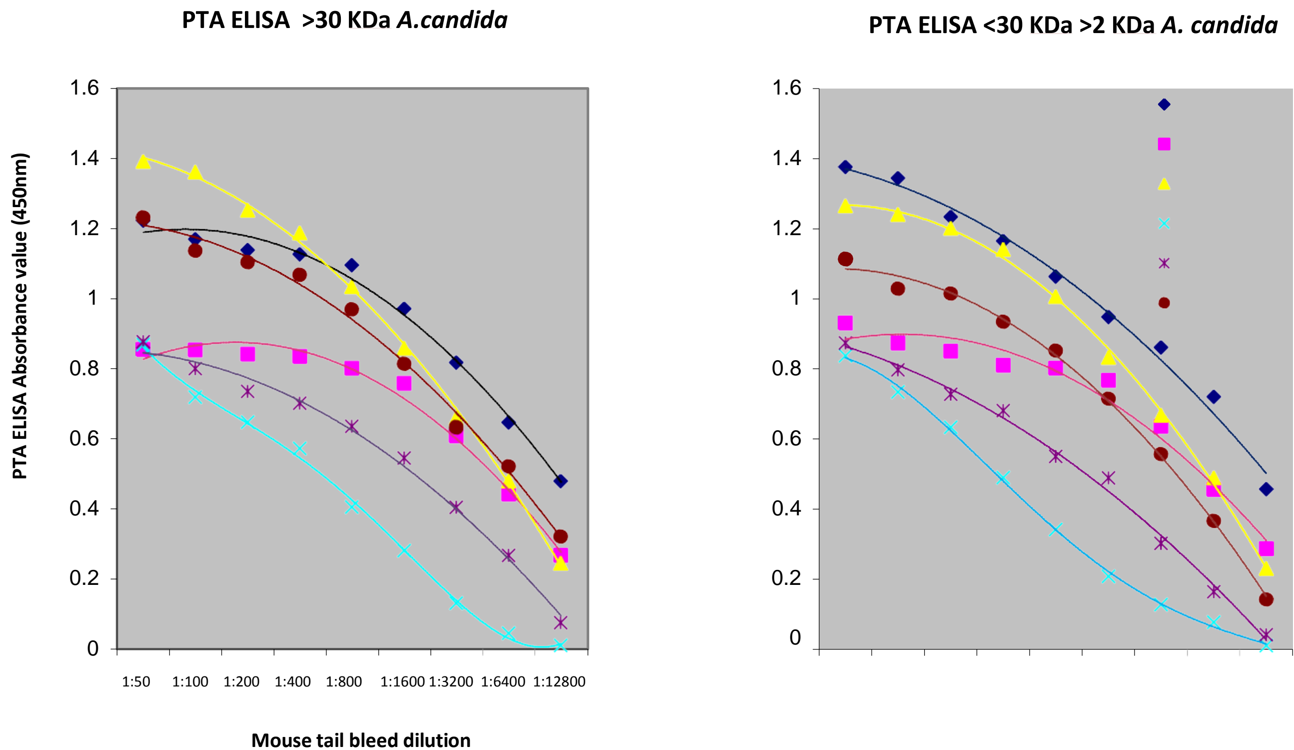


Figure 1. Immune response (polyclonal serum) of immunised mice to two *A. candida* zoosporangia preparations, as tested by PTA ELISA.

Fusion. Five cell lines which produced antibodies positive to *A. candida* by either PTA ELISA or IF were identified from Fusion 1 (Mouse 6 , immunized with >30KDa *A. candida*) and cloned. The cell lines were coded, the class and subclass determined (Table 1) and, for long term storage, retained in vials in liquid nitrogen at -185°C. Tissue culture supernatants (monoclonal antibodies) of each cell line were stored at -20°C in 250µl lots. Few positive cell hybridomas were identified from Fusion 2 (Mouse 1, immunized with <30 KDa > 2 KDa) and, each proved unstable during cloning.

Table 1. MAb cell lines raised to *Albugo candida*

Fusion	Cloned cell line	Class	Subclass	Code Name
1	4E1 D1 A2	IgM		EMA 256
1	5C5 E2 C4	IgG	1	EMA 257
1	3A10 D2	IgG	1	EMA 258
1	5F1 C10 A4	IgG	1	EMA 251
1	F1 F8 C4			EMA260

Discussion

From two fusions, five *A. candida* zoosporangia positive cell lines were identified and cloned. All of the cloned cell lines were from Fusion 1 (mouse immunized with >30 KDa *A. candida* soluble material). Tail bleeds (PTA ELISA tail bleeds) indicated a strong immune response to *A. candida* immunogen <30 KDa > 2 KD however post fusion few positive hybridoma cell lines were identified and, during cloning all proved unstable. Additional fusions would be required to determine whether this result was characteristic of the immunogen used.

Reactivity of selected Monoclonal antibody cell lines (MAbs)

Introduction

To determine specificity of the five selected MAb cell lines, the MAbs were screened by immunfluorescence (IF) against selected *Albugo candida* races and a range of fungal species (Table 2). Where the fungal species was cultivated on host material, spore collection was as described previously, using a hand held cyclone sampler and the spore sample made in to suspension with the addition of PBS. Spore numbers were adjusted to a final concentration of 1 x10^6 spore ml^{-1}. The remaining fungal species were inoculated on to a synthetic agar medium (Table 2), which had been pre-covered with PN60206 Supor 450 90 mm diameter membrane disc (www.pal.com). Fourteen days after inoculation the membranes were removed, and 5 ml PBS was added to each. Surface washings were taken by gently stroking the surface of the membrane with a sterile glass rod and, retained on ice whilst spore counts were made by bright field microscopy (x 400). Spore numbers were adjusted to a final concentration of 1 x10^6 spore ml^{-1}.

Table 2. Fungal species used to assess reactivity of the five MAb cell lines.

Fungal species	Code Name/Origin	Host / Growth Medium	Race
Albugo candida	UK	Cabbage	9
Albugo candida	AU03	Broccoli	9
Albugo candida	AU04	Broccoli	9
Albugo candida	AUO5	Broccoli	9
Albugo candida	AU06	Chinese Cabbage	7
Albugo candida	AU	Pak Choi	7
Albugo candida	AU	Soi Choi	7
Albugo candida	AU	Green stem Pak Choi c.v. Miyako	7
Albugo candida	AU	Radish	1
Albugo candida	AU	Radish c.v. radar	1
Albugo candida	AU15	Broccoli	9
Albugo candida	UK	Garden weed	
Albugo candida	UK	Shepherds purse	4
Albugo candida	AU	Shepherds purse	4
Albugo candida	AU	Rocket	
Albugo laibachii	Norway	Arabidopsis thaliana	
Ramularia	UK	Malt extract agar	
Fusarium culmorum	UK	V8 juice agar	
Puccinia allii	UK	Onion	
Oideum neolycopersici	UK	Tomato	
Erysiphe cruciferarum	UK	Brussels sprout	
Hyaloperenospora brassicae	UK	Lettuce	

Fungal species	Code Name/Origin	Host / Growth Medium	Race
Bremia lactucae	UK	Lettuce	
Pyrenopeziza brassicae	UK	Brussels sprout	
Botrytis cinerea	UK	Potato Dextrose Agar	
Stemphylium botryosum	AU	Agar	
Cladosporium cladosporioides	AU	Agar	
Alternaria alternata	AU	Agar	
Phoma lingam	AU	Agar	

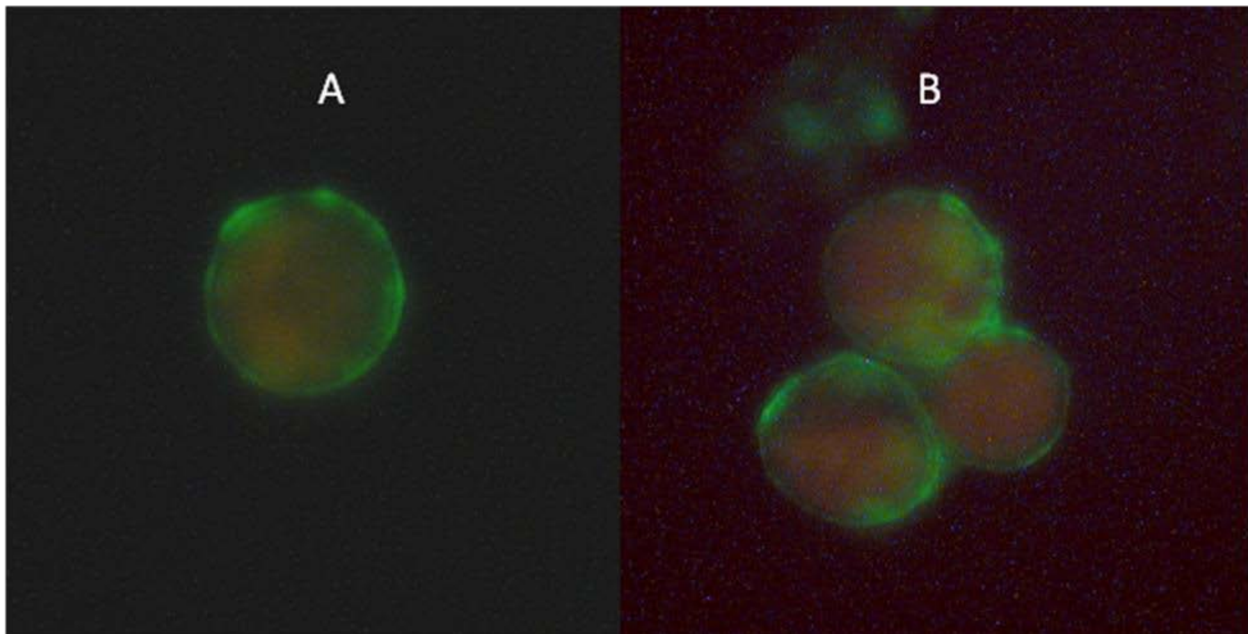
Methods

Microwells of a 29 Teflon coated 6 multiwell (8 mm diameter) glass slides (Code 63423-08, Electron Microscopy Services, Hatfield) were each coated with 30µl poly-l-lysine (Code No. Sigma P-1524 www.sigmaaldrich.com) in PBS (1mg ml^{-1}). Following a 10 minute incubation period at 18 to 20°C the slides were rinsed in distilled water and air-dried under a constant air-flow. To each well of a coated multiwell slide, 30µl of a fungal suspension was aliquoted and, retained in a humid environment for a 12 hr period. The glass slide was removed and allowed to air dry overnight. The slide was rinsed in PBS and air dried in a laminar flow cabinet. Tissue culture supernatants of each MAb cell line (Table 1) were diluted 1:2 in PBSTwc and, each applied at a rate of 30µl to a multiwell. The sixth well received PBSTwC alone. The slide was incubated for a period of 30 mins at 18 to 20°C in a moist chamber. The slide was gently rinsed with PBS and air-dried as described previously. 30µl of anti-mouse (whole molecule) fluorescein isothiocyanate conjugate (Cat. No. F-0527 www. sigmaaldrich.com) diluted 1:60 in PBSTwC Evans blue counterstain (6) was then aliquoted to each well and, incubated as previously described, in darkness. The slide was then rinsed, air dried in darkness and mounted in DAKO fluorescent mounting medium (Code no. S3023 www.dako.co.uk). This process was repeated for each of the fungal species listed in Table 2. The slides were viewed under U.V. with a fluorescent microscope using selective fluorescein excitation at wavelength 490 and a barrier filter at 520 wavelength.

Results

Each of the MAb cell lines bound to epitope sites present on the zoosporangial wall of *Albugo candida* race 9 (Plate 1a) and, to *A. candida* zoosporangia isolated from a garden weed (Plate 1b). Initial studies suggest that EMA 256 is *A. candida* species and is races specific, binding strongly to Races 9 and 1 and weakly to Race 4 and 7. All other fungal species and *A. candida* races tested (Table 2) were negative by IF. EMA 258 gave a similar profile but did not react with Race 4 or 7. Nevertheless, some recognition by EMA 258 was observed to the germ tube of *Erysiphe cruciferarum.* With the exception of EMA 256, each of the MAbs bound to epitope sites on the germ tube of *E. cruciferarum*. EMA 259 to the conidial stage. No reactivity to the other fungal species tested was observed (Table 2). Only EMA 257 reacted with *A. candida* zoosporangia isolated from Rocket (*Eruca sativa*).

Plate 1. *A. candida* zoosporangia labelled with EMA 256 conjugated to a fluorescein anti-mouse label as viewed by fluorescence microscopy (x 400)



Discussion

None of the cell lines selected proved *A. candida* race 9 specific. However MAb cell lines EMA 256 and 258 exhibited a level of specificity that may prove useful in the development of a rapid field based test for monitoring epidemiological significant levels of airborne inoculum of *A. candida,* in horticultural areas of Australia. Of the five MAbs, four bound to epitopes present on the germ tube of *Eryisphe cruciferarum* (Brassica powdery mildew), however no recognition to

Oidium neolycopersici (tomato powdery mildew) was observed. Additional studies could look to determine whether these MAb recognition sites relate to a protein / structure involved in the infection process / immune response of the host.

Competitive Lateral flow development

Introduction

The technical basis of the lateral flow immunoassay test (lfd) was derived from the latex agglutination assay (11). However establishment of the technology for the lateral flow test was not available until the late 1980's. Pioneering work in the development of a 'home test' for determination of human pregnancy assisted this technology to the wider market place, enabling complex laboratory processes to be carried out on-site by non-laboratory personnel. The simplicity of the design, requiring addition only of the sample and the compact and portable capability of the test, make it popular for development of a wide range of assay tests. The application of these tests has expanded beyond clinical diagnostics to areas as diverse as veterinary, agriculture, bio-warfare, food, environmental health and safety, industrial testing, as wells as newer areas such as molecular diagnostics and theranostics. Different configurations of the lateral flow assay exist however all require the basic elements of a solid membrane phase, a fluid transport and, a test specific labelled antibody.

For the purpose of this work, a competitive lateral flow assay format was used to develop a prototype field test for semi-quantification of trapped airborne inoculum of *A. candida*. A competitive lateral flow device (clfd), in the absence of a target sample (*A. candida* zoosporangia), will give rise to the formation of a test line. Rate of test line depletion will relate directly to target levels in the test sample. In the competitive format, the test line depletion is generally measured using a portable optical device.

Methods

Lateral flow construction. Competitive lateral flows (clfds) (Fig. 2), comprising a Millipore 180 HiFlow™ cellulose ester membrane direct cast onto 2ml Mylar backing (Millipore Corp, USA), an absorbent pad (www.whatman.com), sample pad (www.millipore.com) and a filter pad (www.whatman.com) were constructed for the detection of *A. candida* zoosporangia. A control line of an anti-mouse serum at 0.5 mg ml $^{-2}$ (www.sigmaaldrich.com) was sprayed directly on to the membrane surface using a flatbed air jet dispenser (Biodot Ltd,West Sussex, UK) at a constant rate of 50 m/s. A collected soluble fraction of an *A. candida* zoosporangial sample was adjusted to 0.5 mg ml^{-1} in ¼ strength PBS and applied as a test line to the membrane surface, as previously described. Membranes were air-dried overnight at 18 to 20° C and

sectioned in to 5mm strips before being individually housed in a plastic case (Advanced Microdevices, Ambala, India).

A 5µl gold anti-mouse IgM solution (Code BA GAMM 40, British Biocell International, Cardiff, UK) was pre-mixed with 30µl EMA 256 (Lot 1 diluted 1 in 50 conjugate buffer) before application to a pre-cut 5mm conjugate pad (www.millipore.com). The conjugate pad was laid horizontal and air-dried at 35°C for a period of 10 mins (or until dry). After which the conjugated antibody pads were stored individually in non-stick 0.5ml microfuge tubes.

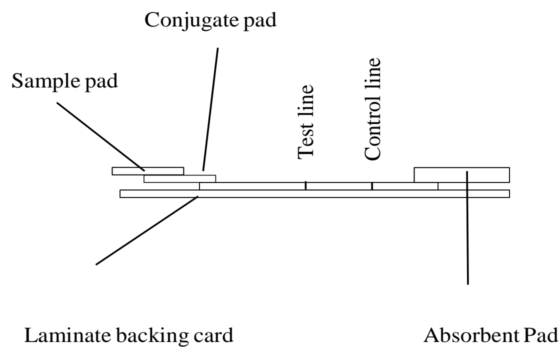


Figure 2. Lateral flow cross section (5mm strip)

Lateral flow test. Ten fold dilutions of $1x10^8$ to 100 *A. candida* zoosporangia (race 9) were prepared in Warwick HRI PVPC buffer. To 0.5ml non-stick microfuge tubes, each of which contained a pre prepared gold conjugate pad, 100µl aliquots of the zoosporangia dilution series were dispensed. Following a 1 minute incubation period to rehydrate the gold conjugated MAb, the contents of the microtube were mixed and transferred drop wise to a competitive lateral flow device (clfd). After 10 minutes the clfds were observed visually for the development of a control and test line. The test line area of each clfd was electronically measured using a Quadscan device (BioDot, Chichester, UK).

Results

With the exception of *A. candida* suspensions at $1x10^8$ and $1x10^7$ zoosporangia ml^{-1}, test and control lines were observed on each of the competitive lateral flow strips (clfd). Electronic readings of the test line area however showed a correlation (r^2=0.9777) (Fig.3) between the optical density of the test line area and the *A. candida* zoosporangia suspension applied to the clfd.

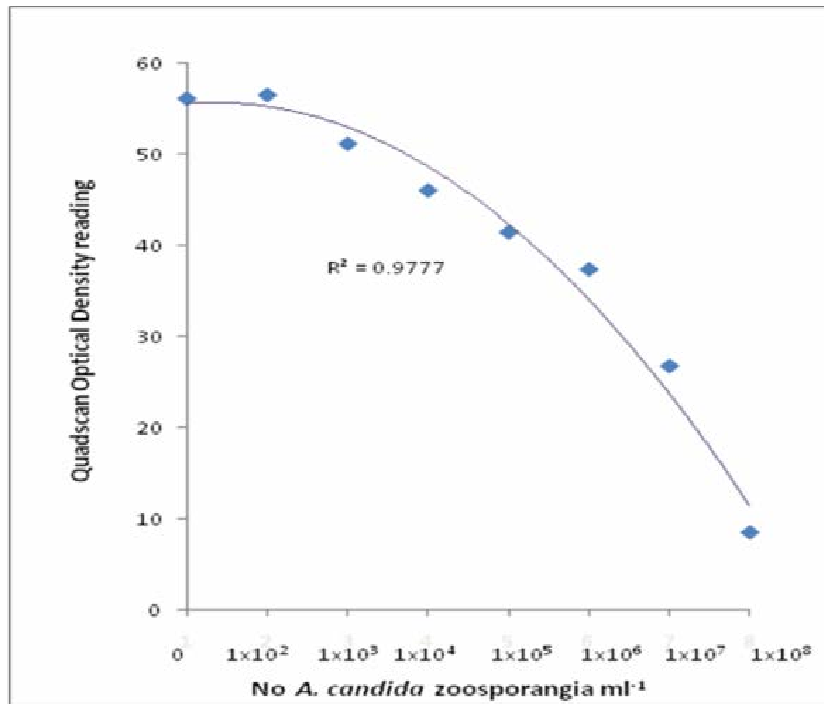


Figure 3. Change in lateral flow test line optical density with *A. candida* concentrations in test samples.

Discussion

The developed competitive lateral flow device enabled quantitative readings to be made of *A. candida* zoosporangia numbers when an electronic reader device was used. However test visual readings only enabled samples in excess of 1x 10^6 zoosporangia ml^{-1} to be identified. Further work will be carried out to optimize the assay format to increase visual test sensitivity whilst retaining good discrimination using an electronic reader. A competitive lateral assay format will be assessed in Year 2 using EMA 258 labelled to a gold carrier.

Summary

In Year one of the study five MAbs have been raised to zoosporangial material of *A. candida*. Reactivity tests have determined that two of the MAb cell lines may exhibit a level of sensitivity and specificity that could prove useful in a developed field test system. A competitive lateral flow prototype (clfd) has been developed using cell line EMA 256 and this will now be extended

to include EMA 258. However, the studies to date have demonstrated that in the present format for the test to be useful, quantitative readings would need to be made using a Quadscan device. In this format a calibration series of *A. candida* zoosporangia concentrations would be a requirement of the test and run concurrently with field samples to determine quantitative information on *A. candida* field trapped zoosporangia numbers. Future work will investigate the optimization of the assay format to enable visual clfd discrimination of trapped *A. candida* airborne inoculum that would give rise to disease development when under optimal environmental conditions. This may preclude the need for an electronic reader and an *A. candida* zoosporangial calibration test series.

YEAR 2

Monitoring airborne spores of *Albugo candida* in commercial Brassica crops in Australia using aerial spore traps.

Introduction

Accurate information about the presence of sufficient disease is required to predict plant disease occurrence in field settings (12). Traditionally, plant disease forecasting systems have relied upon environmental data singularly to predict disease occurrence in crops (9,12). Mathematical models describing the effect of temperature and wetness on pathogen infection have been developed for many types of plant pathogen. Magarey (2004) provides a comprehensive review of these systems. However, detecting and quantifying airborne spores could augment disease forecasting systems. By determining airborne spore numbers during a time period when environmental risk for a disease is high, and when protective disease control strategies could be implemented. This study reports on monitoring airborne inoculum of *Albugo candida* in commercial Brassica crops in Australia using air samplers placed within the crop canopy.

Commercial Broccoli crops *c.v.* Ironman and Steel, located on a property at Cunninghams Rd, Werribe, South Victoria were monitored for airborne disease transmission events of *Albugo candida* for two, two month periods between May 2010 and December 2010. Another commercial Broccoli crop c.v. Viper located on a property at O'Conners road, Werribee South Victoria was also monitored for airborne disease transmission events by *Albugo candida* for a further 2 month period in January-February 2011. Three air samplers: a Microtitre immunospore trap (MTIST), a volumetric 7 day sampler and a multi-vial cyclone air sampler, were operated within the cropping system. An environmental white blister disease forecast model was evaluated for use in determining periods of white blister risk.

Methods

Monitoring airborne disease transmission of *Albugo candida* in a commercial Broccoli cropping system using three air samplers

Microtitre Immunospore Trap (MTIST). A detailed description of the MTIST device, which is manufactured by Burkard Manufacturing Company (Rickmansworth, Herts, UK) can be found in Kennedy *et al.,* 2000 (7,15). In the outdoor version air is drawn thorough a manifold consisting of a plastic tube with a right angle bend placed over the sampler inlet. The manifold samples air through a 9cm diameter vertical circular inlet and directs it into the sampler body that is held horizontally. For field use, the sampler (including the manifold) is mounted on a wind

vane so that the manifold inlet faces into the wind. Within the sampler the airflow is channeled through 32 trumpet shaped nozzles each directed at the base of a microtitre well. The sampler contains four microtitre strips each containing 8 wells. The MTIST air sampler uses a suction system and particulates in the airstream are impacted on the base of each collection well of the four microtitre strips. The collected impacted target particulates may, if appropriate antibodies are available, be immunoquantified by plate trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA).

Monitoring *A. candida* spores in MTIST collected field aerosols . An MTIST spore trap was placed within a commercial field Broccoli crop. Held within the base plate of the machine were four coated eight well microtitre strips (0.1mg ml-1 Poly-L-Lysine (Sigma P-1524) in distilled water and sodium azide (Sigma S-2002). The MTIST spore trap was operated for 12H periods from 06:00 H to 18:00 daily. The coated microtitre strips were changed daily and prior to analysis stored at -20°C.

Enumeration of MTIST trapped *A. candida* spores. Visual examination of the base of field exposed microtitre wells determined that a high concentration of air particulates would prevent accurate enumeration of *A. candida* spores. The wells of two, eight well strips of each field sampling period were processed by PTA ELISA (7). Field trapped *A. candida* spores were identified and labelled using a monoclonal antiserum (MAb EMA 256). This process was amplified using an anti-mouse biotinylated conjugate linked to a streptavidin horseradish peroxidase system (Dako K-0492) and visualised by adding 100µl to each microtitre well of 3,3',5,5'-tetramethylbenzidene substrate (Sigma T-3405 and P-4922). The reaction was stopped by adding 25µl of a 20% 1M H_2SO_4 solution and the generated absorbance values were read at 450nm using a Biotek ELISA plate reader (EL800). This process was repeated using the remaining field exposed microtitre strips in conjunction with MAb EMA 258 (raised to *A. candida*) used in the assay format.

Burkard 24hr Volumetric glass slide air sampler A Burkard 24H volumetric air sampler which contained a glass slide coated with silicone (BC 380S, Basildon Chemical Co, Kimber road, Abingdon, Oxon, UK) operated at an air flow of 10 L of air per minute. Sampled air particulates impacted directly on to an area of the glass slide which corresponded to time intervals by movement of the glass slide over a 24 H period.

Monitoring *A. candida* spores in collected field air samples. The Burkard 24H glass slide volumetric spore trap was placed 2 m from and adjacent to the MTIST spore trap. The trap was loaded daily with a silicone coated glass slide and air particulates were impacted directly on to the surface. The glass slide was changed daily after 18:00 H and stored at -20°C.

Enumeration of trapped *A. candida* spores. Each glass slide was examined for the presence *A. candida* spores by bright field microscopy.

Burkard multi-vial cyclone air sampler. The characteristics of a cyclone air sampler are described by Ogawa & English (1995). Air is drawn through the sampler using a vacuum pump in the form of a cyclone. The height of the cyclone and air inlet, along with the width of the air inlet, air exhaust diameter and the diameter of the cyclone within the length of the exhaust pipe influence the relative efficiency of the trap. These characteristics have been drawn together and standardised within the Burkard cyclone sampler (Burkard Manufacturing Co.). The cyclone air sampler operates at an air flow rate of 10 to 15 L air / min.

Monitoring *A. candida* spores in collected field air cyclone samples. The multi- vial cyclone spore trap was placed 2 m from and adjacent to the MTIST spore trap in the commercial Broccoli field crop. The trap was loaded weekly with eight 1.5ml microfuge tubes (Sarstedt 2013/4). By an integrated automated mechanism each tube was exposed once for a 12 H period for collection of field air particulates. Each sampling exposure period was between 06:00 to 18:00 H daily. After each eight day period the field exposed tubes were collected and stored at -20^{0}C .

Enumeration of trapped *A. candida* spores. To each exposed microtitre tube 100µl of NPARU B2 buffer was added and agitated using a Gallenkamp Spin Mix for 5 seconds at high speed. A lateral flow device developed for field assessment risk of the white blister pathogen was identified to semi-quantify trapped airborne disease inoculum of *A. candida*. A 100ul aliquot of the spore suspension was applied to the sample pad of the lateral flow device (Plate 2A) and test line development was assessed 15 min. later using an ESE Quant reader (Plate 2B).

Plate 2. A lateral flow device for evaluation of field crop risk to white blister (A) and a hand held reader (B).

Results

Microtitre Immunospore Trap (MTIST). The PTA ELISA carried out on the field exposed wells for the sampling periods May - June 2010, November - December 2010 and January - February 2011 identified periods of when the Broccoli crops were at risk to infection by *A. candida* (Fig. 4). Low level disease potential by MTIST PTA-ELISA was determined within the May – June, 2010 sampling period. During November and December 2010 a high risk period was noted between 19th- 21st November 2010. A number of moderate risk periods were identified throughout the period (Fig. 4). For the third field sampling phase the Broccoli field crop was determined to be at low level risk *A. candida* until the end of January. A high risk period was then identified in the middle of February 2011.

Each field exposure period was monitored for white blister disease transmission using two different *A. candida* specific monoclonal antibody cell lines. Both exhibited a similar response pattern but with EMA 258 providing an enhanced signal. However this may be due to the activity used as EMA 258 was optimised at 1:2 whereas EMA 256 is used at a 1:200 dilution.

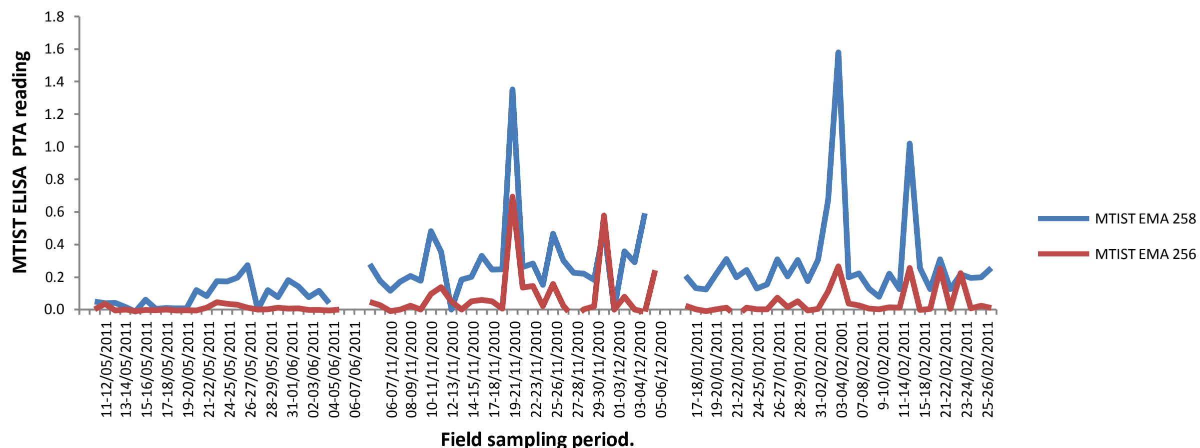


Figure 4. Monitoring in commercial field Broccoli cropping systems for white blister airborne disease transmission periods using an MTIST air sampler. Immunoquantification of *Albugo candida* by PTA ELISA.

24hr Volumetric glass slide air sampler. Enumeration of *Albugo candida* spores on the field exposed glass slides was carried out for the periods May-June 2010, November-December 2010 and January-February 2011. During May – June the airborne concentration of *A. candida* spores was low (Fig. 5). For the second field trapping period, inoculum was first identified at a low level in the airborne environment from the 7th - 13th November 2010. An increase in concentration was observed during the 21st - 22nd November (Fig. 5). *A. candida* exposure periods were then observed at the end of November

beginning of December, 2010 but at a reduced concentration. For the third sampling *A. candida* spores were observed at increasingly high spore concentrations and over several waves. For each of these three field monitoring periods an association was observed between the airborne concentration of *Albugo candida* spores trapped using the volumetric air sampler and the MTIST PTA ELISA (Fig. 6).

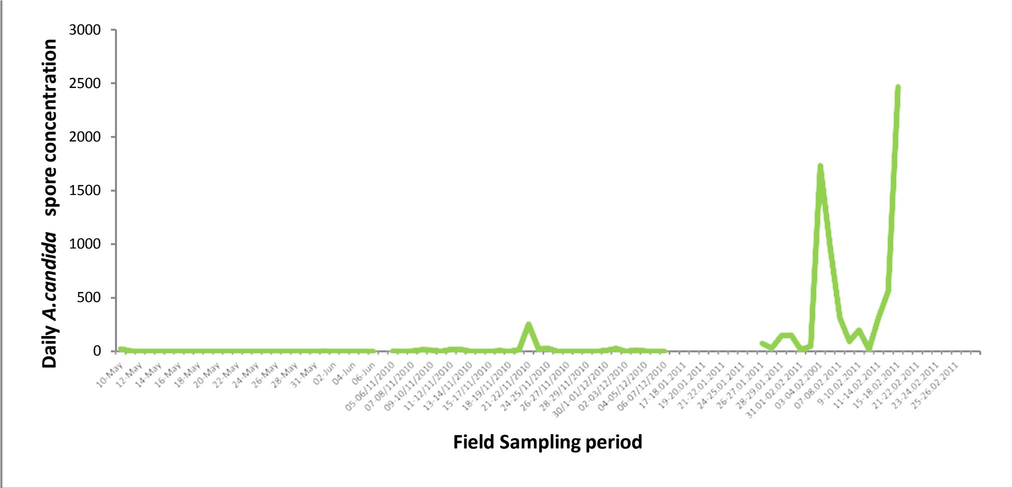


Figure 5. Measurement of airborne spore numbers of *Albugo candida,* collected daily using a volumetric air sampler onto glass slides and identified by bright field microscopy.

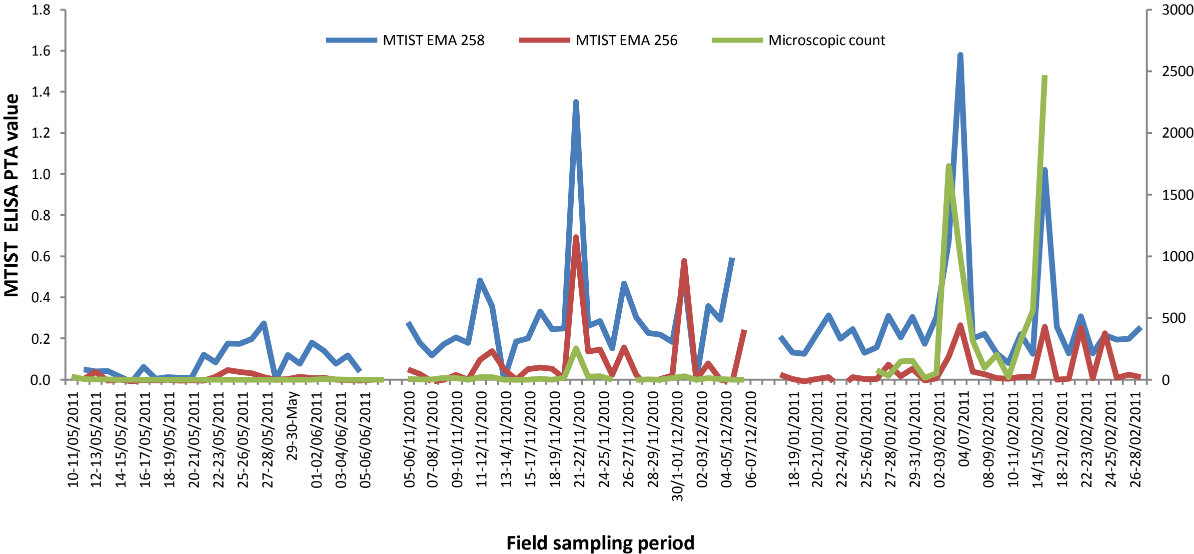


Figure 6. An overlay of daily field sampling periods in a commercial Broccoli crop of *Albugo candida* airborne spore concentrations as derived from a volumetric glass slide sampler and a MTIST PTA ELISA spore trap system.

Note. At the time of report submission no cyclone air samples had been received from the Australian collaborators. Results to follow on receipt.

Discussion

The MTIST ELISA shows potential for determining risk periods of *Albugo candida* spores in the field bioaerosols. Using bright field microscopy to determine the presence of *Albugo candida* spores impacted on to glass slides is extremely time consuming, requires expertise and results that are often available days after the event. Alternatively, the MTIST ELISA process enables results to be made available within two hours of the laboratory being in receipt of the field exposed microtitre wells and, by staff who only require basic training. The ability to produce results quickly provides information on *A. candida* inoculum availability ahead of symptom development. When this information is used in conjunction with an environmental based forecast for infection risk, growers can make an informed decision on an appropriate crop protection regime.

Development of a Lateral flow field test for quantification of *A. candida* in air samples.

Introduction

Year 1 of the study described the development of a competitive lateral flow prototype for semi-quantitative detection of *A. candida* spores. MAb EMA 256 was used as the specific antibody label. A conclusion of this work was to extend test development of a competitive lateral flow to include EMA 258 as a specific antibody label and make evaluation. Earlier studies had shown that the two MAb cell lines of EMA 256 and 258 each exhibited a level of specificity that may prove suitable for use in a field diagnostic test for *A. candida* (Race 9 (AC9) *Brassica oleracea*). From the tests carried out in this report it is proposed that EMA 256 is likely to be *A. candida* races specific (reacting strongly with Races 9, 1 and weakly with 4 and 7). EMA 258 was identified as *A. candida* races specific for 9 and 1 only. However by immunofluorescence EMA 258 was observed to bind to an epitope present on the germ tube of *Erysiphe cruciferarum* (Brassica powdery mildew).

This study describes comparative studies of two competitive lateral flow assay systems for quantitative detection of *A. candida* using either EMA 256 or 258 as a specific *A. candida* label.

Methods

Lateral flow test development. Competitive lateral flows were prepared as described in Year 1 of the study. In these experiments EMA 256 monoclonal antiserum (Lot 1) was pre mixed with gold anti-mouse at activities of 1:50, 1:100 or 1:500. EMA 258 (Lot 1) was at concentrate, 1:2 and 1:4 in conjugate buffer.

Lateral flow test line activity. To each of the prepared competitive lateral flow tests 100µl of buffer (no *A. candida* zoosporangia present) was applied to the conjugate pad (Plate 2A). Test line readings were taken after 15 minutes using an ESE Quant LFR reader (Qiagen UK) (Plate 2B).

Results

Lateral flow test line activity. As previously observed EMA 256 proved useful when incorporated in a competitive lateral flow test format for detection of *A. candida* zoosporangia (Fig. 7). However at a dilution of 1:500 (EMA 256, Lot 1) activity was reduced with little binding at the test line observed. Poor test line formation was observed when EMA 258 was employed in the competitive lateral flow assay format. An optical density reading could only be generated when the monoclonal antiserum was used undiluted (Fig. 7).

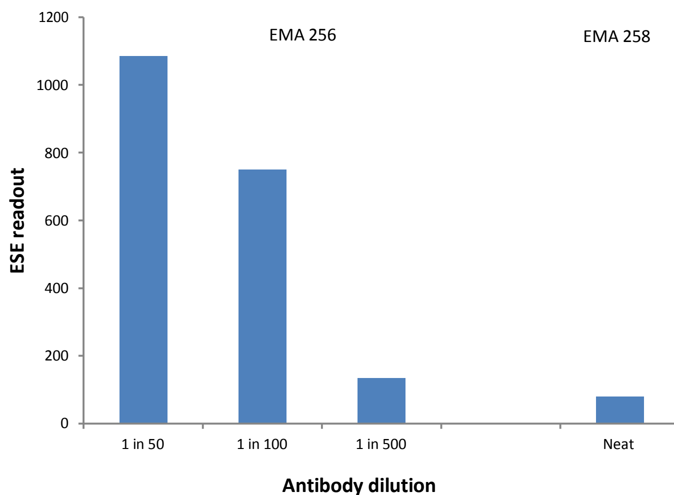


Figure 7. Competitive lateral flow test line values as generated using an ESE Quant portable reader.

Discussion

The competitive lateral flow format incorporating EMA 256 enabled production of a clear test line but MAb activity was limited at a dilution of 1 in 500. EMA 258 proved sensitive to *A. candida* zoosporangia when used in a PTA ELISA format (Fig. 4) but not when incorporated within a lateral flow assay. This contrasted with EMA 256, which demonstrated increased sensitivity to *A. candida* in a lateral flow test. If appropriate *i.e.* increased specificity of the lateral flow test is required, different label identifiers (latex, iron, carbon) could be investigated for labelling of EMA 258. The use of a double antibody system (combination of EMA 256 and 258) as a capture and identifier may also prove useful and provide increased specificity than the current competitive lateral flow assay format. However given the field MAb profiles generated in each of the ELISA assays there would appear to be little difference in specificity (Fig. 4). This may relate to the pre- coating of microtitre wells with sodium azide which has been shown to limit germination of trapped air spora (14). For the purpose of this study, a batch of competitive lateral flow devices was produced using EMA 256 as the label identifier for immunoquantification of *A. candida* inoculum in collected field aerosols.

Test predictions using the developed lateral flow kits for within crop and between crop spread of white blister inoculum in UK field trials

Introduction

In vegetable production systems airborne fungal diseases are a common problem (1,12,14). Brussels sprout and cauliflower crops are produced throughout the year in UK productions systems. Due to the cosmetic nature of damage by *A. candida* many opportunities exist for crop loss. Small amounts of disease on sprout buttons and cauliflower leaves can lead to downgrading the value of the product. Currently there are few methods that can detect significant levels of fungal inoculum in air samples rapidly and accurately (7,14,15). Using an innovative spore trapping system (Mircrotitre immunospore trap (MTIST)) (7) allied to an immunological test (plate-trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) we have reported on the potential to monitor airborne field inoculum of *A. candida*. In conjunction with a white blister environmental based forecast the likely onset of disease occurrence in crops can be determined.

The results demonstrate the need for rapid methods of detection if measures of target propagules within air samples are to be used in practical decision making for control of plant diseases. The ELISA test is still a laboratory based assay. However the recent development and use of immunochromatographic test strips (lateral flow) to rapidly detect and quantify fungal target

inoculum *in-situ* (5,13) exhibit considerable potential for monitoring airborne spores. The work reported below investigates the potential of using a multi-vial air sampler and a lateral flow test to detect and semi-quantify airborne field disease transmission events *in-situ* by non-scientific staff. The system described provides an example where information on inoculum availability can be used directly with environmental disease forecast models to provide information for crop protection regimes.

Methods

Monitoring airborne disease transmission events of *Albugo candida* in a commercial Brussels sprout crop. A Burkard multi-vial air cyclone and a MTIST air sampler were operated within a commercial UK Brussels sprout crop at Croppers Farm, Bickerstaff, Lancashire from August to October, 2011. Air temperature, leaf wetness, relative humidity and rainfall were recorded throughout this period using a Smartlog (Aardware Design, Walton on Thames, UK) and at 30 min. intervals the data was downloaded to provide daily disease risk periods of *A. candida* infection. Each air sampler was loaded weekly. Eight microfuge tubes of the multi-vial cyclone air sampler and 4x 8 well microtitre well strips of the MTIST sampler. Prior to field air sampling the microfuge tubes and microtitre wells were each pre coated with 100µl 0.1mg Poly L Lysine 0.05% sodium azide solution and air-dried. The field air samplers operated between 06:00 and 18:00 H daily. The automated mechanism of the cyclone air sampler provided each tube to a single daily exposure whilst the MTIST remained unchanged to provide a weekly reading. After each eight day period the field exposed tubes and the microtitre wells were collected and stored at -20^{0}C .

Enumeration of MTIST trapped *A. candida* spores. Visual examination of the base of MTIST trapped field exposed microtitre wells determined that a high concentration of air particulates would prevent accurate enumeration of trapped *A. candida* spores using bright field microscopy. The eight well strips of each field sampling period were then processed as described above by PTA ELISA for immunoquantification of *A. candida* inoculum.

Field exposed microfuge tubes of the multi-vial cyclone air sampler were assessed for *A. candida* inoculum by the addition of 110µl / tube of lateral flow extraction buffer. Each tube was agitated using a Gallenkamp Spin Mix for 5 seconds at high speed. An aliquot of 20ul was removed and if present *A. candida* spore numbers were determined by bright field microscopy (x 400). Using the remaining buffer, a lateral flow device for field assessment of the white blister pathogen, was used to semi-quantify *A. candida* inoculum of each field exposed microtube. A 100ul aliquot of each ‘field air sample’ suspension was applied to the sample pad (Plate 2A) of a lateral flow device and test line development was assessed 15 min. later using an ESE Quant reader.

Results

Monitoring airborne disease transmission events of *A. candida* in a commercial Brussels sprout crop. Weekly field air samples collected using the MTIST sampler and processed by PTA ELISA for *A. candida* inoculum determined that throughout the study the disease risk was low (14th to 21st September, 2011) or there was no risk of white blister infection (Fig. 8). In the earlier field study (Fig.4) the MTIST microtitre wells were changed daily (24h field exposure) whereas in this study a seven day cumulative air sampling process was recorded.

Microscopic counts of the collected cyclone field aerosols in liquid phase found no presence of *A. candida* inoculum. Each of the lateral flow devices used to assess white blister disease risk in the collected daily field aerosols provided a low / negative value when measured by an ESE reader (Fig. 9).

Throughout the sampling period of August to October 2011 the Brussels sprout crop was assessed visually for white blister. On each occasion no visible symptoms of white blister could be observed.

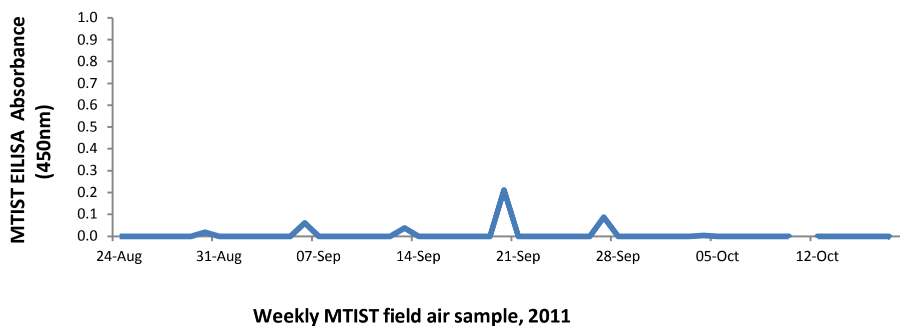


Figure 8. Weekly measurement of an MTIST field trapped bioaerosol for *Albugo candida* by PTA ELISA

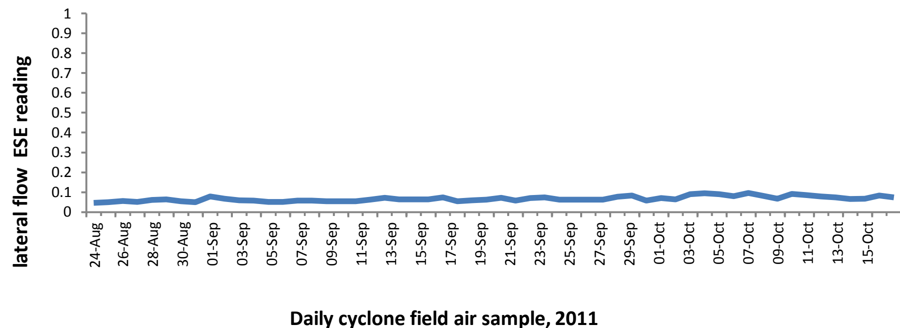


Figure 9: Daily bioaerosol measurements for *Albugo candida* inoculum using 'in field' lateral flow tests.

Discussion

In this study, the two immunoassay based sampling systems developed show potential to determine data on field airborne disease transmission events of *A. candida* in UK Brassica horticultural field cropping systems. The MTIST ELISA, which provided a weekly estimate of disease inoculum, identified low level *A. candida* spore concentrations during each of the sampling periods. Using daily aerosol samples the 'in field' lateral flow test provided negative results. Crop walking confirmed that no white blister disease development occurred within the exposed crop. Further work should now investigate whether daily or weekly sampling of airborne disease transmission events is required. The use of weekly estimates of disease inoculum in air samples has been reported for other diseases of field crops (14). In addition, to provide a robust field disease monitoring system it is necessary to determine the inoculum concentration required for symptom development to occur and, the effect of environmental parameters on this process. This could provide useful information in determining whether daily or cumulative field readings could then be made.

PROJECT SUMMARY

Airborne disease inoculum plays an important role in the development of disease epidemics on 'above ground' plant material of horticultural crops (1, 14). The two immunological air sampling systems developed for monitoring field aerosols of *A. candida* in commercial Brassica cropping systems demonstrate the potential for determining presence or absence of disease transmission events ahead of symptom development. In the past, air sampling processes have been limited to passive collection of disease inoculum by gravitational deposition and /or sampling specific volumes of air by suction air samplers. These techniques have been limited to laboratory analysis as they require considerable amounts of time and expertise if accurate counts are to be obtained. Nevertheless, the Burkard volumetric glass slide air sampler has in this study provided valuable information to assist in the validation of two new sampling processes. Both of which rely on monoclonal antibodies (MAbs) to selectively measure *A. candida* zoosporangia.

The development of the MTIST air sampling device enables a portable, robust and inexpensive spore trapping system that incorporates trapping technology alongside an existing well-established immunoassay (ELISA) (2,3,7). The production of two MAbs to zoosporangial material of *A. candida* have both proved equally useful in quantifying airborne disease inoculum of *A. candida* in field crops when used in conjunction with the MTIST PTA-ELISA format. With the addition of sodium-azide to the microtitre wells of the MTIST spore trap results indicate a test that is able to detect *A. candida* to race level. In an earlier study (14), field trapped

air-spora were inhibited from germinating when wells were pre-coated with sodium azide. The MTIST air sampler provides the potential to detect several target crop pathogens simultaneously providing suitable antibody probes are available. A limiting factor of the test system is however that although assay time is short (2 hours) a laboratory and specialised staff are still required to process the collected field samples.

The recent development and use of immunochromatographic test strips (lateral flow) to rapidly detect and quantify fungal target inoculum '*in-situ'* (5,13) exhibit considerable potential for monitoring airborne spores. In this study we have combined an existing air sampling system (cyclone air sampler) and developed a lateral flow assay test which can be used 'in situ' to determine crop exposure to *A. candida* disease inoculum. The test format provides a rapid assay platform with results provided within 15 minutes of the assay start time. The developed test also provides the potential for measurement of *A. candida* in collected field bioaerosols. This data can be recorded using a portable electronic reader. The current lateral flow assay system relies on a single MAb cell line (EMA 256). Results to date show promise for monitoring airborne disease transmission events of *A. candida*. However in this study no disease was observed for the duration of the field trial. Future studies should look to validate the system in cropping systems during periods of white blister infection. The integration of this information with the white blister model should be investigated.

The current study provides information of airborne disease transmission events of *A. candida* in commercial field crops using three different air sampling systems. The approach described is used as an example where information on inoculum availability can be used directly with environmental disease forecast models to provide information for optimal crop protection regimes.

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