SESSION 6A

DETECTION AND ENUMERATION OF PLANT PATHOGENIC INOCULUM

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Papers: 6A-1 to 6A-4

Recent advances in the detection of airborne inoculum of plant pathogens using molecular methods

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ASTRACT

Plant pathogen inoculum plays a vital role in the development of crop disease epidemics. Although the role of inoculum in the development of disease has long been recognised, information on inoculum availability has not been widely used in disease risk assessment or in disease forecasting. For many important crop pathogens inoculum is in the form of airborne spores. Conventional methods for assessing airborne plant pathogen inoculum rely on either microscopy or culturing techniques. Both methods are time consuming and, in the case of microscopy, require skilled personnel to identify the pathogen spores. The difficulties in measuring airborne inoculum probably contribute to the lack of its use in disease management. This paper considers the application of molecular methods, particularly those based on the polymerase chain reaction (PCR), in the development of novel methods for assessing airborne inoculum. A number of air sampling formats have been used in conjunction with PCR-assays for detecting airborne micro-organisms (mostly in the medical field) and their applicability for plant pathogen inoculum detection is considered. The limited available studies suggest that the sensitivity of detection of both bacterial and fungal spores is better when the spores are disrupted and DNA is extracted from the samples before PCR analysis. Current studies suggest that PCR-based spore detection is potentially very sensitive: some assays have detected fewer than 10 spores in field samples. Most examples of PCR-based inoculum detection, to date, have been non-quantitative. However, the use of "real time" PCR techniques appears to have the potential of making molecular analysis of airborne inoculum truly quantitative. The potential impact of the development of new molecular-based methods for airborne inoculum detection in plant pathology and disease management is briefly discussed.

INTRODUCTION

Forecasting the development of plant disease epidemics has been an important goal in plant pathology for many years. This goal has become more important in modern crop protection as the pressure to reduce the use of agro-chemicals increases, as an understanding of epidemic development can increase the efficiency of chemical disease control. Three conditions need to be satisfied to initiate a disease epidemic in a crop: inoculum of the pathogen must be present; the crop must be susceptible to the pathogen; and the environmental conditions must be appropriate for the pathogen to infect and develop within the crop. Ideally, the three elements of the so called "disease triangle" should be considered when trying to understand how specific disease epidemics develop in crops. Nevertheless, most disease forecasting

systems focus on the effects of environment on epidemic development, for example determining periods when the environmental conditions are favourable for infection (McCartney, 1997). However, even when environmental conditions are favourable, if inoculum is not present then disease will not develop.

For many fungal plant pathogens "inoculum" consists of airborne spores, either as a means of initiating the epidemic and/or as a means of secondary disease spread. For example, light leaf spot (*Pyrenopeziza brassicae*) and phoma stem canker (*Leptosphaeria maculans*), the two most important diseases of oilseed rape in the UK, are initiated in the autumn by airborne ascospores, although the pathogens do not cause economic damage until the following spring and summer (Gilles, *et al.*, 2000; West, *et al.*, 2001). For stem canker, fungicides do not control the pathogen once it has reached the stem and it is therefore crucial to treat the crop in the autumn when the pathogen is only in the leaves (Gladders, *et al.*, 1998). Similarly with light leaf spot, autumn sprays are often needed to prevent the development of damaging epidemics (Gilles, *et al.*, 2000), and generally need to be applied before symptoms are visible on the crop (Fitt, *et al.*, 1997). Thus, for efficient use of fungicides to control both diseases there is a need for effective methods to forecast the availability of airborne inoculum in the autumn, when newly emerged crops are susceptible to infection. Knowledge of the availability of airborne inoculum is likely to be important in forecasting epidemic development in other disease systems where the timing of infection is a limiting factor in the initiation of epidemics.

Although the importance of inoculum in the development of plant disease epidemics has long been recognised, there appears to have been surprisingly few studies of the relationship between disease development and inoculum availability. However, recently there has been interest in the relationships between airborne inoculum and weather aimed at developing inoculum forecasting systems for use in disease risk assessment. The apparent lack of use of information on airborne inoculum in disease forecasting systems probably stems from the difficulty in its measurement, hence the interest in inoculum forecasting. Conventional methods for identifying and enumerating airborne plant pathogen spores rely on microscopic or cultural techniques; both methods are time consuming and laborious. Microscopy can be unreliable, as the small, nondescript spores produced by many plant pathogens are often difficult to identify on morphological grounds. Cultural techniques are often unsuitable for detecting spores that are slow growing, or non-culturable *in vitro* (e.g. obligate pathogens) and the choice of medium may influence which species can grow.

The inclusion of information on pathogen inoculum has the potential to improve disease risk assessment for many pathogen systems. However, this is unlikely to happen until more reliable and faster methods are available to detect airborne plant pathogen inoculum. Recently two novel approaches have been considered: one based on immunoassay techniques; the other based on molecular methods. In this paper I will consider recent advances in the use of DNA-based methods for the detection of airborne spores of plant pathogenic fungi.

MOLECULAR TECHNIQUES

Over the last ten years molecular techniques based on the detection of specific DNA sequences have been increasingly used to detect and identify plant pathogens (Schots, *et al.*, 1994) but they are only now beginning to be applied to the detection of airborne inoculum. These methods are potentially highly specific; they can distinguish between different fungal

species, and within a single species (Ward & Adams, 1998). They are also potentially very sensitive: methods using the polymerase chain reaction (PCR) have detected DNA from a single fungal spore (Lee & Taylor, 1990). All DNA-based methods use specific nucleic acid sequences to identify the pathogen. The chosen sequences need to be unique to the target pathogen at the desired level of selectivity. Strategies for designing appropriate DNA target sequences have recently been discussed by McCartney, *et al.* (2002) and will not be further considered here.

PCR is probably the most useful molecular method for detecting plant pathogen spores. It is a method for synthesizing (amplifying) millions of copies of a DNA sequence. Briefly, in the first stage of PCR, DNA is separated its two complementary strands by heating to 95°C. Two primers, short pieces of single stranded DNA designed to match the ends of the region to be amplified, then bind only to regions of DNA with complementary base sequences. The primers define the sensitivity of the assay. This binding or annealing step is usually done at 40-65°C. In the third "elongation" step, a thermostable DNA polymerase extends the sequence from the primers using deoxyribonucleoside triphosphates (dNTPs) as building blocks. This completes the second strand of the two separated original strands. This third, step is usually done at 72°C. At the end of this first cycle of the PCR reaction, two copies of the target DNA exist where previously there was one. After about 25 such cycles about a million copies of the sequence have been produced. In practice the procedure is very simple. DNA, buffer, dNTPs, primers and DNA polymerase are mixed in a tube and placed in a computer-controlled heating block programmed to switch between the different temperatures required. The practical details of PCR, optimisation of conditions and applications of the technique are covered elsewhere (e.g. Dieffenbach & Dveksler, 1995; Edel, 1998).

Conventional PCR-based spore detection methods are not immediately quantitative. Although it is relatively easy to quantify the amount of a PCR product produced as a result of a successful PCR amplification, it is more difficult to relate this to the amount of target DNA, and hence spores initially present at the start of the reaction. This is because the reaction rate is exponential and consequently, slight variations in the amplification procedure can generate different amounts of final product from the same amount of starting material (Wang, *et al.*, 1989). As the PCR proceeds the concentrations of the reagents become limiting and a plateau phase is reached where the amount of product is no longer proportional to the original amount of template. However, with the advent of "real-time" PCR techniques in the last few years direct quantification of inoculum using PCR-based methods may now be possible. This technique measures, automatically, the accumulation of PCR products after each cycle in a closed-tube format, allowing the phases of the reaction to be monitored. PCR products can be monitored using either fluorescent DNA-intercalating dyes (e.g. ethidium bromide and SYBR Green I) or sequence-specific probe-based assays (Wittwer, *et al.*, 1997). The initial amount of target DNA in the reaction can be related to a "cycle threshold" (Ct), defined as that cycle number at which a statistically significant increase in fluorescence is detected. Target DNA can then be quantified by constructing a calibration curve that relates Ct to known amounts of template DNA. Because both specific and non-specific PCR products, as well as primer-dimer artefacts, can generate a signal when using DNA-intercalating dyes, highly specific primers are required. This method has only recently been used to quantify airborne fungal spores (see below).

DETECTION OF AIRBORNE SPORES

Although the potential of molecular methods for detecting airborne biological particles has been recognised, until recently little progress had been made in their use. Most of the effort has been in the field of medicine, and has included work on bacteria, mycoplasma, and fungi (McCartney, *et al.*, 2002). However, there have been a small number of reports on the use of PCR-based methods to detect airborne plant pathogen inoculum (Wiglesworth, *et al.*, 1994; Gonthier, *et al.*, 2001; Calderon, *et al.*, 2002a). Nevertheless it is clear that PCR-based detection combined with air sampling is potentially useful in the detection and quantification of airborne plant pathogen inoculum.

Several different types of air-samplers have been used in conjunction with PCR analysis of airborne pathogens. Gonthier, *et al.* (2001) used settle plates, the simplest type of air sampler, to collect spores of *Heterobasidion annosum*, a pathogen of conifers, which were cultured and PCR assays were used to identify the pathogen. Although the use of molecular diagnostics improved the accuracy of colony identification of the settle plate measurements, because the samples was still cultured the diagnostic did not increase the speed of spore identification. Settle plates containing water agar were used to collect sporangiospores of *Peronospora tabacina* (tobacco blue mould) (Wiglesworth, *et al.*, 1994), here the spores were washed off the plates and sonicated before being added to the PCR. Although settle plates are simple and easy to use their collection efficiency is generally low and they do not measure airborne spore concentrations.

More sophisticated samplers, volumetric air-samplers, are needed to measure airborne spore concentrations. One of the first types of volumetric air-samplers to be used with PCR assays were liquid impingers, which collect air samples direct into a liquid (Alvarez, *et al.*; 1994, Mukoda, *et al.*, 1994). There have been two recent reports of the use of PCR methods to detect spores of the fungus *Stachybotrys chartarum* in air samples collected using a SKC BioSampler® (SKC, Inc. Eighty-Four, PA, USA), a type of liquid impinger (Haugland, *et al.*, 1999b; Vesper, *et al.*, 2000). Liquid impingers sample direct into a liquid, which is an advantage for subsequent sample handling, however, they are often difficult to use in the field. Membrane filters have been used to collect fungi in medical studies (Olsson, *et al.*, 1998), but they appear not to have been used for plant pathogens. Filters are generally easier to use in the field than liquid impingers and are also available in pre-assembled filter holders (Olsson, *et al.*, 1998), which can reduce contamination problems in field use. Filter based air sampling formats may be very useful in PCR-based plant pathogen inoculum detection, provided that target DNA can be efficiently extracted from the filters. A miniature cyclone sampler, which collects a dry sample into 1.5ml Eppendorf sampling tubes, has recently been developed by Burkard Manufacturing Co., Rickmansworth, UK and has been used to collect airborne *Penicillium roqueforti* spores which were detected by PCR (Williams, *et al.*, 2001).

A number of types of air sampler use the small, but finite, inertia of the particles to separate them from a rapidly accelerating air-stream and collect them on a surface (inertial impaction) (McCartney, *et al.*, 1997). Such samplers are known as "impaction samplers" or "impactors", and a number of types have been used with PCR sample analysis. Conventional "impaction" samplers collect samples either on a culture medium or on a solid substrate such as a membrane filter or transparent film. Impaction samplers such as Hirst-type spore traps and rotating-arm samplers, which collect particles on to a transparent surface, are commonly used

in plant disease epidemiology (McCartney, *et al.*, 1997). The collected particles are analysed using optical microscopy. Wakefield (1996) used a PCR assays to detect the presence of a fungus (*Pneumocystis carinii*) in air samples collected using Hirst-type trap (Burkard spore trap, Burkard Manufacturing Co., Rickmansworth). The feasibility of using Burkard spore traps and rotating-arm samplers, which also collects particles on clear plastic tapes, to collect fungal spores for PCR analysis was studied the wind tunnel at IACR-Rothamsted using *P. roqueforti* as a model fungus (Calderon, *et al.*, 2002b). We have also used PCR analysis to detect inoculum of the oilseed rape pathogens *L. maculans*, *P. brassicae* in air samples collected in the field using Burkard spore traps (Calderon, *et al.*, 2002a). Wakefield (1996 removed the air sample from the spore trap tapes by washing with a detergent solution (Triton X-100) then ground the sample in liquid nitrogen with a mortar and pestle, before extracting the DNA after a 16h proteinase digestion step. In the method used by Calderon, *et al.*, (2002a, b) the deposit was removed from the spore trap tapes by violently agitating the sample in liquid in the presence of glass beads. This not only removed the sample from the tape but also disrupted the spores allowing DNA to be more efficiently purified from the sample. Thus, sample removal and spore disruption were done in a single step, simplifying sample processing. Tests showed that the spore-trapping medium did not significantly inhibit either the spore disruption or DNA purification. These studies show that "impaction" formats could easily form the basis for designing PCR-based plant pathogen inoculum detecting equipment.

The available studies suggest that the sensitivity of detection of both bacterial and fungal spores is best when the spores are disrupted before DNA is extracted from the samples. Several methods have been used to disrupt spores collected in air samples and include: grinding in liquid nitrogen (Wakefield, 1996), freeze thaw cycle (Alvarez, *et al.*, 1995; Pascual, *et al.*, 2001); violent agitation with glass beads "bead beating" (Haugland, *et al.*, 1999b; Vesper, *et al.*, 2000; Williams, *et al.*, 2001; Calderon, *et al.*, 2002a, b) and sonication (Wiglesworth, *et al.*, 1994; Mastorides, *et al.*, 1999). Haugland, *et al.* (1999a) and Zhou, *et al.* (2000) compared a number of methods for disrupting fungal spores and found that a form of "bead beating" was the most efficient of the methods compared. In the "bead beating" method used by Calderon, *et al.*, (2002a, b) to remove and disrupt fungal spores from spore-trap tapes, samples were placed in tubes containing sterile water, a surfactant and acid-washed Ballotini balls (400-455 µm diameter). The tubes were then shaken in a FastPrep® machine (Savant Instruments, Holbrook, New York, USA) for 2 periods of 40 seconds, with 2 min cooling on ice between. Williams, *et al.* (2001) found that this procedure disrupted more than 90% of *P. roqueforti* spores in spore suspensions. Although "bead beating" is an efficient method for disrupting spores there has been recent interest in the use of sonication for disrupting spores in preparation of PCR analysis (Belgrader, *et al.*, 2000). This approach has not yet been used to process air samples from filters or spore trap tapes. However, the method could prove to be an effective alternative to "bead beating" in the preparation of air samples for PCR analysis.

Integrated air-sampling/PCR analysis systems for detecting airborne plant pathogen inoculum are potentially very sensitive. The detection limit of the PCR used by Olsson, *et al.*, 1996 to detect *Pneumocystis carinii* was estimated to be 1 genome. There have been few systematic studies of the sensitivity of combined air sampling/PCR detection systems. A detection limit of about ten *P. roqueforti* spores per PCR for samples extracted from spore trap tapes was estimated from wind tunnel experiments where *P. roqueforti* and other non-target spores were released (Calderon, *et al.*, 2002b). However, Burkard spore trap samples taken in Mexico

City, where the concentration of non-biological particles was high, and analysed using fungal consensus primers suggested that for "fungal spores" the detection limit was between 50 and 200 spores per PCR (Calderon, *et al.*, 2002b). In contrast, there appeared to be little difference between the detection limit for "uncontaminated" spore tape samples and field-collected samples, for the oilseed rape pathogens *L. maculans* and *P. brassicae*, even though the field samples contained large numbers of non-target fungal spores and other biological and non-biological particles (Calderon, *et al.*, 2002a). In recent experiments at IACR a PCR assay detected about 10 ascospores of the plant pathogen *Sclerotinia sclerotiorum* in a background of 10^4 spores of the closely related fungus *Botrytis cinerea*, but only when the spores were disrupted and DNA extracted. The same PCR assay has also detected *S. sclerotiorum* ascospores on Burkard spore trap samples taken in an oilseed rape crop. The results of the limited number of "sensitivity studies" available suggest that "operational" sensitivity of air sampling/PCR detection systems may depend on the nature of the background non-target organisms in the sample, sample handling protocols and the PCR assay used. Thus, further work is needed to quantify and understand the field detection limits for detecting airborne plant pathogen inoculum by PCR assays. Indeed, it is likely that detection limits will differ for different target organisms, different sampling environments and different air sampling systems, and therefore will need to be quantified for different pathosystems.

Most of the PCR assays that have been used to detect airborne micro-organisms are not quantitative and can only be used to detect the presence of target spores (above a threshold) in air samples, not the number. However, "real-time" PCR techniques allowed a direct estimate of the initial amount of target DNA in a sample. Haugland, *et al.* (1999b) recently used a real-time PCR assay to quantify *S. chartarum* conidia and used it to test 5 air samples from contaminated houses with a detection threshold of about 2 conidia. In a recent preliminary experiment we have extracted DNA of *Blumeria graminis* f.sp. *tritici* from field exposed spore trap tapes and detected and quantified it using a 'real-time' PCR assay. Thus, spore trapping combined with "real-time" PCR assays has the potential not only to detect airborne inoculum, but also to quantify it.

FUTURE DEVELOPMENTS

It is clear that there is great potential for combining air-sampling methods with PCR-based diagnostics for detecting and possibly quantifying airborne inoculum of plant pathogens. However, there is much work still needed to identify and define spore trap design criteria optimised for use with PCR-detection methods. Furthermore, strategies for their use in disease management systems need to be developed. For many crop diseases basic biological information such as inoculum thresholds and an understanding of the role of inoculum in epidemic development are not available. Thus, PCR detection of airborne inoculum could play an important role in generating fundamental information on the basic biology of disease spread and development. PCR methods also have great potential in research on plant pathogen population structures. Combining such methods with air-sampling technologies could lead to insights into the spatial and temporal dynamics of plant pathogen populations.

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Quantification of airborne inoculum using antibody based systems

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ABSTRACT

Measuring airborne fungal spores has traditionally used methods, which are time consuming and involve specialist knowledge. However few attempts have been made to use more rapid techniques to quantify spore numbers in the air. Using immunological techniques provides a format for obtaining these estimates quickly and cheaply. The development of a new microtiter spore trapping system (MTIST) provides a spore trapping format where other immunological techniques such as ELISA (enzyme-linked immunoassay) can be used. However the system requires the use of specific antibodies for each target spore. The MTIST device is a portable, robust and relatively inexpensive system that can be used to perform multiple tests in a single sampling period. Field tests using the MTIST device in conjunction with ELISA has shown that the system is able to accurately trap and quantify ascospores of the ringspot pathogen of brassicas (*Mycosphaerella brassicicola*). Information on the transmission of inoculum can be obtained which can be used within crop protection programmes to rationalise pesticide applications. However the techniques can be readily adapted to monitor airborne particulates and micro-organisms in a range of environments.

INTRODUCTION

Information on airborne spore numbers has many potential users within medical, ecological or agricultural studies. Aerobiological studies have been used frequently in agricultural investigations. The classical methods used in aerobiological studies are however time consuming. There have been relatively few attempts to introduce newer methods in the detection and enumeration of airborne microflora. However if rapid methods could be used the resulting information could have enormous potential benefits within many areas of risk assessment. The intensive nature of agriculture and horticultural production in conjunction with climatic change and the growing need for sustainability means that this information could be used in crop protection regimes. Within horticultural production many opportunities exist for the transmission of fungal pathogens due to the sequential nature of production. In the past transmission of airborne fungal plant pathogens has been achieved using either the passive collection of spores by gravitational deposition (Aylor, 1998) and / or with specific volumes of air sampled by 'active' spore trapping devices (Aylor, 1993; Kohl *et al.*, 1994). Identification of the trapped inoculum has relied on either the microscopic examination of surfaces on which spores have been impacted on to and / or by agar plating. However using these systems sample identification requires considerable amounts of time and expertise if accurate counts are to be obtained. Where there are morphologically similar spore types (such

as ascosporic fungi) sample identification is often not a realistic option. To provide accurate information on inoculum availability (within a practical time interval to enable its usage) requires new systems which, can rapidly detect, differentiate and quantify target airborne inoculum.

Advances in fungal diagnostics, using either antibody or nucleic acid probes (Dewey & Thornton, 1995; Gray & Strachan, 1996), offer this potential. Immunoassays however are increasingly being exploited in the development of diagnostic kits as a result of their speed and relative simplicity. Test formats vary from the more traditional laboratory based enzyme-linked immunosorbent assay (ELISA) where multiple samples may be processed within hours (Clark & Adams, 1977) to kits developed for on site usage by 'non-scientific staff' (Danks & Barker 2001). All of these tests however rely on an antibody detection system and a linking visual identifier. The specificity of the antibody used for identification of the target fungal inoculum is fundamental to the reliability and success of the test.

Airborne ascospores of *Venturia ineaqualis* (apple scab) can be detected using the 'Spore View' immunoassay test, which is marketed by Chaparral Diagnostics (Chaparral Diagnostics, Burlington, Vermont, USA). Collection of ascosporic inoculum in this test is by passive deposition on to a membrane surface after which ascospore presence is determined by using a specific-labelled antibody probe. Similarly studies to develop an antibody-based immunoassay for the early detection of *Sclerotinia sclerotiorum* (Jamaux & Spire, 1994)) a major fungal pathogen on oilseed rape (*Brassica napus*), relies solely on the passive deposition of ascospores on to rapeseed petals and subsequent mycelial growth. It is unlikely however that these systems could detect pathogens below a critical threshold as with passive sampling only small volumes of air or small sample sizes can be assayed. Consequently, the importance of sampling, location of sampling and efficiency of sampling are crucial within high cropping acreages in these systems. As a result, rapid assay formats that sample large volumes of air will be a pre-requisite if accurate monitoring of fungal pathogens is to be achieved.

Currently there are relatively few systems that can detect accurately low amounts of inoculum. Nevertheless using a Burkard seven-day volumetric suction trap (B-7 day) as a trapping device (flow rate 10 L air per min) an immunofluorescence test has been evaluated for monitoring field inoculum of *Mycosphaerella brassicicola*, the ringspot pathogen of brassicas (Kennedy *et al.*, 1999). However, as a routine quantification method this test is protracted, requires expensive laboratory equipment for analysis, and employs processes which require manual operation. Nevertheless the potential exists to develop rapid assay formats that sample both large volumes of air and provide information of small amounts of inoculum which may be epidemiologically significant in disease spread. In vegetable production areas where cropping is intensive, transmission of inoculum is of great importance as, the presence of disease at low levels can result in quality loss. Ringspot on Brussels sprouts is caused by the fungal plant pathogen *M. brassicicola* and, under favourable environmental conditions, has the potential to cause major losses. Transmission of the disease is by windborne dispersal of ascosporic inoculum (Cullington, 1995). Control of the disease is problematical because of its airborne nature and the short periods of favourable environmental conditions required for release and infection. A disease prediction model for *M. brassicicola* has been developed (Cullington, 1995) but additional information on inoculum level is required for effective disease control. In this paper we discuss the potential using immunoassay for the detection and quantification of airborne inoculum of

Mycosphaerella brassicicola. The system is used as an example of how measurement of inoculum can be obtained in the field using immunological methods and how this might be used in crop protection regimes.

MATERIALS AND METHODS

Development of spore trapping equipment for integration within immunoassay format

The development of a new spore trap by Burkard Manufacturing Company (Rickmansworth, Hertsfordshire, United Kingdom) which combines with an enzyme-linked immunosorbent assay system (ELISA) (Kennedy *et al.*, 2000) offers the potential for rapid detection and quantification of target disease inoculum. The microtiter immunospore trap (MTIST) uses a suction system to directly trap air-particles by impaction into microtitre wells. Air is drawn through the MTIST device and particulates in the airstream are impacted on to the base of each collection well of 4 microstrips strips (Nunc Immunodiagnostics, F8 Polysorp 4 x 8 well microstrips, Catalogue No 469957). The volume of air drawn through the machine can be increased or decreased depending on the requirements of the test. Impacted target air particulates may be immunoquantified by ELISA using polyclonal or monoclonal antisera (Kennedy *et al.*, 2000).

Production of Antisera

A polyclonal antiserum was raised in a female White New Zealand rabbit to an ascospore suspension of *M. brassicicola* as described by Kennedy *et al.*, (2000) and coded 98/4/P. A monoclonal antiserum (MAb) was raised in a white female Balb mouse to an ascospore suspension of *M. brassicicola*. The MAb was coded EMA 187.

Selective quantification of airborne ascospores of *M. brassicicola*

Eight *Brassica oleracea* (Brussels sprouts) plants, which had been inoculated with *Erysiphe cruciferarum* (powdery mildew) and exhibited severe disease symptoms, were placed in a controlled environment cabinet together with twelve sporulating culture plates of *M. brassicicola*. The controlled environment cabinet was operated at a relative humidity of 94 % with continuous light and the plants and sporulating cultures wetted intermittently for 0.3 min / 60 min period. Air-spora within the cabinet were collected by impaction in to the microtiter wells of the 4 x 8 well microstrips of the MTIST. The MTIST was operated at an air-flow rate of 20 L per min and over a 24 hr period with sampling time periods of 30 min,1,2, 4, and 12 hr. For 2 of the 4 hr sampling periods and 1x 12 hr sampling period, forty additional but disease-free *B. oleracea* (Brussels sprouts) seedlings (cv. Golfer, 3 true leaves) were positioned in the controlled environment cabinet at 4 positions: top left, top right, bottom left and bottom right. Following each of these sampling periods the forty *B. oleracea* plants were removed, and placed into an environment of 100 % humidity for 24 hrs. The plants then were removed and retained in a glasshouse, at a temperature of 15 ° C for 21 days. Plants were visually examined for expression of ringspot lesions. To confirm ringspot symptoms infected leaf tissue was removed, surface sterilised for 1 minute in aqueous sodium hypochlorite (4 % w/v available chlorine) and isolations made on to sprout leaf decoction agar "SLD" (Kennedy *et al.*, 1999). For each sampling period of the MTIST, each well of each microstrip was viewed using a Nikon TMS inverted binocular microscope to determine the total number of impacted spores of *M. brassicicola* and *Erysiphe cruciferarum*. Microstrips were stored at –20 °C until processed through PTA-ELISA as above but this time employing PAb 98/4/P.

Monitoring field inoculum of *M. brassicicola*.
A B7 day volumetric spore trap and a field modified MTIST spore trap (Kennedy *et al.*, 2000) were positioned adjacent to each other and 6 m outside a 24m x 12m *B. oleracea* (Brussels sprouts) plot (c.v. Golfer). A 300 x 300 mm^2 Tygan gauze sachet sectioned in to 9 compartments, with each compartment filled with 10g of air-dried *M. brassicicola* infected leaf material, was positioned a further 3 m outside of the plot. The base of the Tygan gauze sachet was positioned 100 mm above ground level. The B-7day spore trap was operated continuously over a 3 week period and the Melinex tape changed at 7 day intervals. Detection and quantification of ascosporic inoculum of *M. brassicicola* on the exposed tapes was achieved by immunofluorescence (IF) as described by Kennedy *et al.*, (1999). Monoclonal antiserum EMA 187 and an anti-mouse FITC conjugate (Sigma F-0257) was used in these tests. A Delta T data logger (Delta T Devices Ltd) was used to activate the MTIST when relative humidities (r.h.) of > 80 % were recorded. The MTIST stopped sampling when the r.h. fell below 80 %. During a 3 week sampling period the microtiter strips in the MTIST (4 x 8 wells) were removed for processing after either 1 or 3 days field exposure. Microtiter well strips were processed by PTA-ELISA employing MAb EMA 187 and an anti-mouse IgG SEEKit (Harlan Sera Lab Ltd, U.K.). For each of these sampling periods, 4 seedlings of *B. oleracea* (Brussels sprouts c.v. Golfer, 3 true leaves) which had been grown in the absence of disease were positioned adjacent to the position of the two spore traps. Following each of the sampling periods the plants were removed and placed in an environment of 100 % humidity for 24 hrs. The plants were then removed dried and retained in a glasshouse, at a temperature of 12 - 14 °C for 21 days. Plants were visually examined for expression of ringspot lesions and confirmatory isolations made on to sprout leaf decoction agar (Kennedy *et al.*, 1999).

RESULTS

Antisera
Polyclonal antiserum raised to ascosporic inoculum of the ringspot pathogen *Mycosphaerella brassicicola* proved highly sensitive but lacked the specificity required for field detection studies. However improved specificity was obtained using MAb EMA 187 with reactivity (by immunofluorescence) limited to the ascosporic stage of *Pyrenopeziza brassicae* (Wakeham *et al.*, 2000).

Selective quantification of airborne ascospores of *M. brassicicola*
MTIST trapped conidia of *Erysiphe cruciferarum* (powdery mildew) and ascosporic inoculum of *M. brassicicola* were identified on the base of the collection microtiter wells. Spores of both fungal species were distributed throughout the base of each well, but the greatest numbers occurred in the central region of each microtiter well. A low number of conidia of *E. cruciferarum* were identified on the sides of the collection vessels. At conidial levels in excess of 1000 per microtiter well ascosporic inoculum of *M. brassicicola* was obscured. Following PTA ELISA a correlation coefficient of $r^2 = 0.9655$ was obtained when the number of ascospores per microtiter well and the corresponding absorbance values were compared (Figure 1).There was not a correlation between the number of conidia of *E. cruciferarum* per microtiter well and the observed ELISA values. An association between the number of MTIST device-trapped ascospores of *M. brassicicola* per cubic metre of air sampled and the total number of ringspot lesions that developed was observed. Leaf immaturity inhibited the development of *E. cruciferarum* lesions.

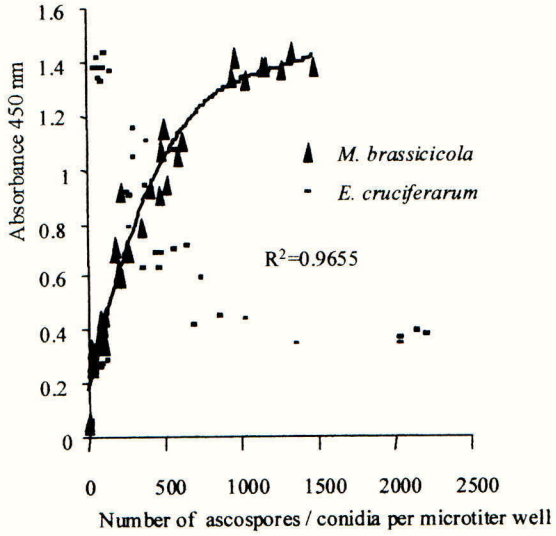

Figure 1. Relationship of MTIST trapped ascospores of *M.brassicicola* and conidia of *Erysiphe cruciferarum* in PTA ELISA;PAb 98/4/P

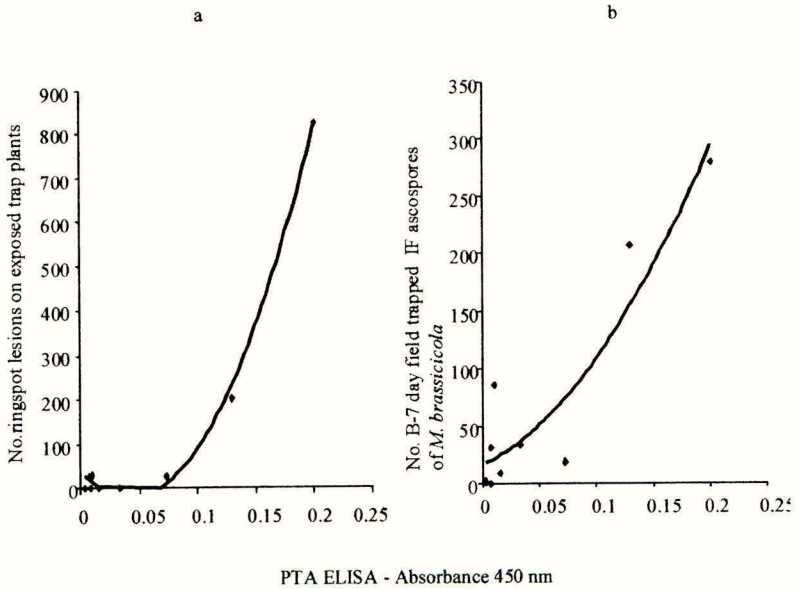

Figure 2. Relationship of MTIST PTA-ELISA (a) ringspot lesions on exposed *Brassica oleracea* trap plants and (b) the number of B-7 dayfield trapped ascospores of *M. brassicicola* by Immunofluorescence.

Monitoring field inoculum of *M. brassicicola*

Field MTIST absorbance values as derived from the PTA-ELISA and employing MAb EMA 187 show a high level of correlation between the level of ringspot observed on exposed trap plants (r^2=0.9947) (Figure 2a) and the numbers of ascospores trapped using the B-7day spore trap (r^2=0.8613) (Figure 2b). On some days over 800 lesions were recorded on the trap plants. This was equivalent to an absorbance value of ~0.2.

DISCUSSION

Immunoassays are increasingly being exploited in the development of fungal diagnostic assays (Werres & Steffens, 1994; Dewey & Thornton, 1995; Wakeham *et al.*, 1997) as a result of their speed, simplicity, relatively low cost and, the ability to perform on-site semi-quantitative assays (Miller *et al.*, 1988; Danks & Barker 2001). To incorporate immunoassays within spore trapping systems has the potential to revolutionise airborne monitoring systems providing a base for the rapid detection and quantification of target airborne particulates. 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 the latest trapping technology alongside an existing immunoassay technique (ELISA). Whilst the MTIST can be used for multiple tests during a single sampling period, it should prove useful for monitoring airborne particulates and micro-organisms in a range of environments.

In many disciplines specific antibodies and optimised immunoassay systems are in routine use. However consideration of the characteristics of the target inoculum and use of the MTIST spore trapping device with existing immunoassay systems will require investigation. A source of inaccuracy in using the MTIST for collection of particles is the retention of material after processing by ELISA. The numerous washing steps involved, means that unless spores are properly attached to the impaction surface they will be lost during the immunoassay stage. With an exterior 'sticky' mucilage coating, retention characteristics of the ascosporic inoculum of *Mycosphaerella brassicicola* proved optimal providing secure adhesion to the microtitre well throughout the ELISA process (Kennedy *et al.*, 2000). For other spore types germination will be required to 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 following germination conidia attach strongly to the impaction surface proving resistant to removal by boiling or by treatment with a number of hydrolytic enzymes (Doss *et al.*, 1995). Depending on the characteristics of the antibody probe, germination has not only the potential to provide adhesive properties to the spore but, also information of inoculum viability. A monoclonal antibody (coded:MAb EMA 212; HRI, Wellesbourne, Warwicks, UK) raised to conidia of *Alternaria brassicae* (dark leaf spot) has been determined as stage specific binding to an epitope present only at the point of germination. This epitope is not present at the conidial or mycelial stage.

Other studies have demonstrated that spore attachment is not always a pre-requisite of the assay. A correlation of r^2 = 0.9348 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 (Doss *et al.*, 1995).

Using the MTIST there was a good correlation between the number of ascospores of *M. brassicicola* trapped and the absorbance values generated by ELISA. In a mixed spore population, 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 react with other air particulates present. Tests in controlled environment conditions also demonstrated a close correlation between the amount of ringspot disease which developed on exposed plants place inside the cabinet and, the number of MTIST trapped ascospores of *M. brassiciciola*.

Field studies demonstrated that epidemiologically significant levels of *M. brassicicola* inoculum in the air can be detected both reliably and rapidly using the modified version of the MTIST spore trap. A highly significant relationship was observed between the results of the ELISA and the number of ringspot lesions that subsequently developed on exposed trap plants. With the exception of two sampling periods a good correlation was observed between the number of spores trapped using a conventional Burkard 7 day volumetric spore trap and that of the MTIST spore trap. The reason for variability in inoculum levels on these two dates is unclear. For usage of the MTIST spore trap further information is required on the spatial positioning of the traps and the effect of environmental conditions on their collection efficiency. Employing the MTIST spore trap in conjunction with specific antibody probes, there is the potential to detect several target pathogens simultaneously. Utilising this information within environmental based disease forecasting systems will prove important in disease prediction studies. At present many forecasting systems assume that inoculum is not limiting. However the results from field trials in heavily infected crops grown commercially and non-commercially indicate that this is not correct. The results also point to the need for a threshold of inoculum to be present if infection is to occur under optimal infection conditions. These results indicate that great improvements in the accuracy of forecasting systems are possible if immunological or other methods can be used to introduce estimates of inoculum within the forecast.

FUTURE WORK

Using the principle of immunochromatography many rapid, on-site disposable testing kits have been developed and used successfully within human medicine (Ketema *et al.*, 2001). The successful uptake and adaptation of this technology for the on-site detection of viral plant pathogens (Tanaka *et al.*, 1997; Danks & Barker, 2001) shows potential for the semi-quantitative detection of target fungal propagules. With optimisation and the utilisation of appropriate antisera this test format should enable the development of on-site testing kits which are both cheap and easy to use under field conditions. As a result it maybe possible to develop a range of in-field tests which could be used to monitor airborne inoculum levels in the field. This type of information, when used in conjunction with environmental disease forecast systems, would provide an accurate forecast based on actual disease risk.

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Use of flow cytometry in the detection of plant pathogenic spores

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ABSTRACT

A Partec PAS-III flow cytometer was used to differentiate sporangia of the late-blight pathogen *Phytophthora infestans* from other airborne particles. Using the PAS-III, light scatter and intrinsic fluorescence parameters could be used to differentiate sporangia from conidia of *Alternaria* or *Botrytis*, rust urediniospores and various pollens. Clear differentiation between *P. infestans* sporangia and *Blumeria* conidia was only possible using data analysis rules evolved using the methods of genetic programming, following staining with the fluorescent brightener Calcofluor white. Initial field data are presented and the potential application of these techniques to the prediction of late-blight epiphytotics in the field is discussed.

INTRODUCTION

Current methods for predicting the occurrence of late-blight of potato (*Phytophthora infestans*) rely on climatic modelling to identify conditions conducive to pathogen reproduction and thus to disease spread. Such methods can provide considerable savings to growers, as well as environmental benefits. In the UK, 'calendar spraying' is normal practice, with sprays commencing when the crop canopy has closed and continuing at 7-10 day intervals until crop desiccation / harvest. Use of forecasting models can reduce the number of required sprays by allowing a delay in the first fungicide application, and/or an extension of the interval between subsequent sprays (Hinds, 2000). However, such methods are not always reliable in all conditions. A multi-site evaluation of five forecasting systems from 1994 to 1997 indicated that their predictions differed from one another, and that the effectiveness of all the models varied from year to year. They were least effective in a 'low risk' year, when they recommended spraying even though no blight occurred (Bugiani *et al.*, 1998).

One important factor that is not taken into account by forecasting models is the amount of inoculum present in the immediate environment of the crop. Use of volumetric spore traps within potato fields, with identification and enumeration of sporangia carried out under the microscope, has given variable results. Schlenzig *et al.* (1998) detected inoculum in the air only after the first diseased plants were visible in the field, and concluded that sampling of the air was no more effective than a visual check of the crop in terms of identifying the start of the epidemic. In contrast, Bugiani *et al.* (1998) found that one or two 'peaks' in sporangia concentration in the air (10-20 sporangia m^{-3}) preceded the first observed symptoms, and used these data to confirm the predictions of climatic modelling. It is evident that the sensitivity of such methods would be greatly enhanced if an increased rate of sampling could be coupled with a means of identifying and enumerating the *P. infestans* sporangia automatically.

Flow cytometry is a well-proven technique that allows cells, spores and other particulates to be analysed individually (Davey & Kell, 1996; Shapiro, 1995). Particles are interrogated optically and, for each particle, measurements of several cellular parameters are recorded: these normally include forward light scatter, orthogonal ('side') light scatter, and one or more fluorescence parameters. Particles may show intrinsic 'autofluorescence' which may facilitates differentiation of

different species using flow cytometry (Vives-Rego *et al.*, 2000). In other cases, fluorescent dyes are needed to differentiate different microbes (Davey & Kell, 1996; Porter *et al.*, 1997). For example, forward and wide-angle light scatter coupled with DNA-binding fluorescent dyes have been used to differentiate spores of basidiomycete fungi (Allman, 1992).

Advances in computing and laser technology have recently led to the development of cheaper portable devices which can operate from a battery. Such devices offer the possibility of developing a field-based system, which if linked to a high-volume air sampler, could provide online measurement of airborne particles. The generic nature of the technology would allow the devices to be adapted to detect a wide range of plant pathogen propagules and thus provide advanced warning of impending epiphytotics. Here and in an earlier publication (Day *et al.*, 2002) we describe the use of flow cytometry linked to a high volume air sampler to provide sensitive and specific detection of *P. infestans* sporangia using a combination of staining with the fluorescent brightener Calcofluor white and genetic programming for data analysis.

MATERIALS AND METHODS

Source & preparation of fungal spores and pollen grains. An A1 mating type isolate of *P. infestans* (96.69) was maintained on rye A agar and sporangial suspension from plates were used to inoculate detached potato leaves. After 6-7 days incubation at 18°C, sporangia were washed from leaves with distilled water. Cultures of *Botrytis cinerea*, *Alternaria alternata* and *Penicillium chrysogenum* were maintained on potato dextrose agar and conidia were harvested by washing plates with distilled water. Conidia of *Blumeria graminis* var. *hordei* and rust urediniospores (*Puccinia coronata* f. sp. *avenae*, and, *P. recondita* f. sp. *tritici*) were harvested from infected plants by agitating leaves in 50 ml of distilled water containing a single drop of Tween-80. Grass, plantain and tree pollens were obtained locally. Spore / pollen suspensions were filtered through a single layer of muslin (average pore size ca. 0.5 mm) before being placed in the flow cytometer. For most analyses, particles were at a concentration of approximately 10^5 ml^{-1}. For some experiments, particles were fixed/killed by the addition of ethanol to the medium (50% final volume).

Staining of spores. A range of dyes were tested: $DiIC_1(5)$, $DiSC_3(5)$, TO-PRO, and SYTO (17, 59, 60, 61, 62, 63 and 64), Nile Blue A, FUN-1 and Calcofluor white M2R (Fluorescent Brightener 28; Tinopal UNPA-GX). Stock solutions were dissolved in dimethyl sulphoxide (DMSO) except for Nile Blue and Calcofluor white, which were made up in distilled water. After addition of dye, samples were routinely kept in the dark for 15 min at room temperature before being measured in the flow cytometer.

Flow cytometry. The Partec PAS-III Particle Analyzing System (Partec GmbH, Münster, Germany) was equipped with 488 nm argon-ion laser and 633 nm helium-neon lasers and a 100 W Hg arc lamp, and photomultiplier tube detectors for six optical parameters. For most experiments data were collected with respect to forward light scatter (FSC), side scatter (SSC), and four fluorescence (FL) parameters: FL1 (green; 515-560 nm), FL2 (orange; 575-605 nm), FL3 (red; >645 nm) and FL4 (red; 665-690 nm). For experiments using Calcofluor white, the PAS-III was operated with the 488 nm argon-ion laser and the mercury arc lamp as a UV light source. In this configuration, fluorescence from the UV excitation was recorded (at 450-460 nm) instead of SSC. Standard reference beads (6.0, 10.5, 41.5 and 66.0 μm diameter; AlignFlow Plus Flow Cytometry) were used for calibration. Data analysis was carried out using techniques of multiple gating (FCS Express, De Novo Software, Canada) and genetic programming (Gmax-Bio; Aber Genomic Computing,).

Air sampling. An XMX/2AL liquid impinger air sampler (Dycor Technologies Ltd., Edmonton, Canada) was customised to collect particles of 20 to 50 µm and coupled with a liquid impingement module to allow collection of particles into a small volume of water. A mesh screen over the inlet served to prevent very large particles from entering the system and particles smaller than 7 µm would be exhausted from the unit. A cowl and wind vane were added to permit air to be collected while facing into the prevailing wind. Air samples were collected by high-velocity impingement into 5 ml of distilled water in 50 ml disposable plastic centrifuge tubes with 1 mm nylon mesh, positioned above the liquid to reduce particle entrainment and loss of sample fluid by splashing. The flow rate on the XMX is variable and setting 5 (giving a measured flow rate of 600 $l.min^{-1}$) was found to be optimal, since some damaged sporangia were observed at higher settings.

RESULTS

Light scatter detection of *P. infestans* sporangia using the PAS-III. Peak channel numbers (PCN) for both FSC (forward scatter) and SSC (orthogonal or 'side' scatter) were plotted against particle width, particle length and particle volume, and against their logarithms. R^2 values >0.9 were obtained for FSC plotted against width, log(width) and log(volume) for the 'standard' beads but no such correlations were observed for the biological particles. Viable *P. infestans* sporangia and zoospores, *Blumeria* and *Botrytis* conidia and grass pollen showed relatively high FSC for their size (relative to calibration beads), whereas rust urediniospores, *Alternaria* conidia, killed *P. infestans* sporangia and *Plantago* pollen showed relatively low FSC. Correlation of side scatter with particle size was generally similar to, or better than, that of FSC (Day *et al.*, 2002). The ratio of SSC/FSC for killed sporangia and for grass / plantain pollens was relatively high compared to that of the opther fungal particles tested (fig. 1). It is also clear from fig. 1 that *Blumeria* conidia cannot be differentiated from sporangia of *P. infestans* using FSC and SSC alone.

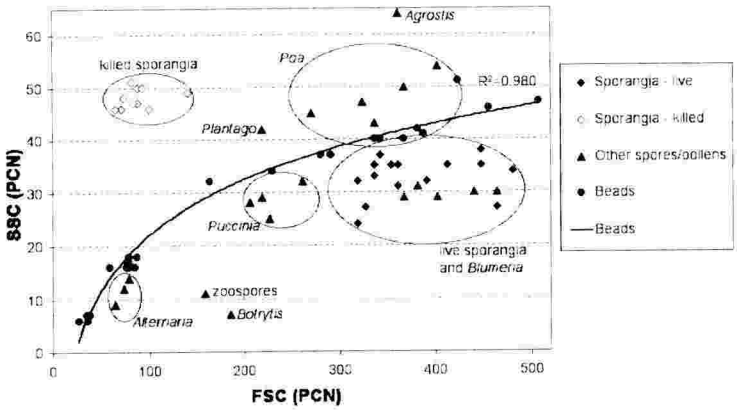

Figure 1. Correlation between SSC and FSC for standard beads, *P. infestans* sporangia and zoospores, other fungal spores and pollen. Logarithmic least squares best fit line calculated for beads only; R^2 calculated in MS Excel calculated using a transformed regression model.

Intrinsic fluorescence. Certain biological particles exhibited red 'autofluorescence', notably pollen, rust urediniospores, *Blumeria* conidia (particularly non-viable conidia), and, to a lesser degree, *P. infestans* sporangia. Both the fluorescence signals and the SSC signals are collected at an angle orthogonal both to the direction of the laser and to that of the sample fluid flow. Fluorescence signals

for each of the four wavelength 'windows' collected were plotted against SSC to determine which exhibited intrinsic fluorescence. Pollen, rust urediniospores, killed sporangia of *P. infestans*, and, to a lesser degree, viable *P. infestans* sporangia and *Blumeria* conidia showed varying degrees of orange and red FL (Fig. 2), which could be used as a further aid to differentiation.

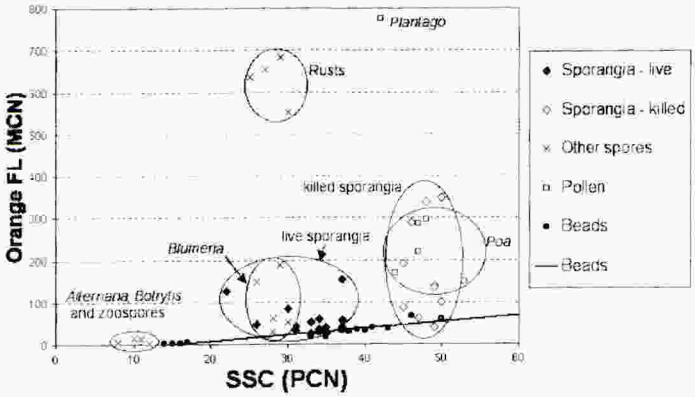

Figure 2. Correlation between orange autofluorescence (575-605 nm) and side scatter (SSC). *Agrostis* pollen also could not be shown on the same scale (Orange FL MCN > 3500). MCN = median channel number. Linear least squares best fit line for beads is shown.

Differentiation of *P. infestans* from other fungal spores using light scatter and autofluorescence. Using a combination of FSC, SSC and measurements of autofluorescence, *P. infestans* sporangia could readily be differentiated from all of the tested biological particles except for *Blumeria* conidia. Multiple gating was used to exclude particles that did not conform to the same FSC, SSC and FL parameters as the sporangia. For most of the spores (*Alternaria*, *Botrytis*, rusts) and pollens (*Agrostis*, *Plantago* and *Poa*) tested, <3% false positives were observed, though for *Blumeria* conidia, the number of false positives was extremely variable, ranging from 2% to 61% on different experimental days. Use of all six parameters for gating (i.e. inclusion of the two red FL parameters) did not consistently decrease the number of false positives, whilst always decreasing the number of true positives (i.e. the number of sporangia actually identified as sporangia).

Evaluation of fluorescent dyes. Sporangia and zoospores of *P. infestans* stained readily with nuclear dyes (TO-PRO-3®, SYTO® dyes), with the lipid-staining dye Nile Blue, with the membrane energization dyes $DiSC_3(5)$ and $DiI_1C(5)$ and with the fungal-specific stain FUN-1. These dyes were stained spores of *Penicillium*, *Alternaria* and *Botrytis* rather poorly, though they were less effective in differentiating sporangia from the various pollens tested. Differentiation of sporangia from pollen (but not from fungal spores) was more effective with SYTO-17 or TO-PRO-3, or with low concentrations (100 nM) of $DiSC_3(5)$. The multiple gating analysis was repeated for sporangia and other spores/pollens stained with these dyes, at concentrations of 1 µM or 10 µM. However, the number of false positives for pollens, *Blumeria* and rusts was either similar to or greater than the number that had been obtained in the absence of fluorescent dye. Thus, detection of *P. infestans* sporangia was not usefully improved by any of these red dyes.

The fluorescent brightener Calcofluor white stained sporangia and pollen effectively at a range of concentrations (10 µM, 100 µM and 1 mM), though sporangia were most effectively stained at 1mM. Staining of other fungal spores, including *Blumeria*, was much less effective. Multiple gating using FSC, UV and green and orange FL parameters indicated that Calcofluor

was effective for distinguishing sporangia from any of the other fungal spores tested (including *Blumeria*), and also from *Poa* pollen (Table 1).

Table.1. Percentage particles falling within gates defined for *P. infestans* sporangia, when stained with different concentrations of Calcofluor. Logical gating was used, where R1 and R2 were polygons drawn from plots of FSC vs green FL and UV vs orange FL respectively.

	Calcofluor concentration			
	0 µM	**10 µM**	**100 µM**	**1 mM**
P. infestans sporangia	58, 62, 67, 78, 87, 90, 90, 92, 92, 93, 96, 97, 97, 98, 98	69, 71, 77, 86, 86, 87, 89, 89, 90, 91, 92, 92, 94, 95	62, 77, 79, 81, 84, 85, 85, 85, 91	36, 55, 67, 72
Other fungal spores				
Alternaria	0	0	0	0
Blumeria	5, 31, 47, 65, 72	0, 0, 0, 0, 0	0, 1, 2	0
Brown rust	0, 0	0	0, 0	NT
Crown rust	0, 2	0	0, 0	NT
Pollens				
Anthoxanthum	0, 0	0, 0	0, 0	NT
Dactylis glomerata	1	0	NT	NT
Poa annua	0, 0, 1	0, 0, 3	0, 0, 1	0, 4, 5
Poa trivialis	10	0	NT	NT
Plantago	0, 0, 4	0, 0, 0	0	NT
Silver birch	1, 11	0, 1	NT	NT
Sycamore	0	0	0	NT

Use of Genetic Programming (GP) in Data Analysis. GP (Koza *et al.*, 1999) is a technique that allows the evolution of simple, interpretable rules from complex datasets. We have been applying GPs to a wide variety of problems in spectroscopy (Gilbert *et al.*, 1997; Johnson *et al.*, 2000). GPs proved to be a still more effective means of particle discrimination and allowed detection of 95% of true positives with a false positive rate of less than 1%. We note that the 'top' rule that was evolved contained a variety of non-linear operators, and that this type of approach is in contrast to the usual 2D or 3D displays where the separation of individual particles into classes via visual or computational clustering methods relies on them being linearly separable. This 'top rule' can be cast either as a small computer program or as the following equation:

$$Score = \frac{((x_{FSC} \times x_{UV}) - x_{FL3}^2)}{\sqrt{|\min(x_{FL4}, (x_{UV} - x_{FL2}))|}} \quad if \quad \sqrt{|\min(x_{FL4}, (x_{UV} - x_{FL2}))|} \neq 0$$

$$Score = 1 \quad if \quad \sqrt{|\min(x_{FL4}, (x_{UV} - x_{FL2}))|} = 0$$

$$x_{FSC} = FSC(488 \pm 5nm)$$
$$x_{UV} = UV(455nm \pm 5nm)$$
$$x_{FL2} = FL2(575 - 605nm)$$
$$x_{FL3} = FL3(> 645nm)$$
$$x_{FL4} = FL4(665 - 690nm)$$

$$Output = \frac{1}{1 + e^{(4.118731 - Score \times 3.591183 \times 10^{-5})}}$$

Detection of *P. infestans* in samples of air spora spiked with sporangia. Samples collected in aqueous suspension using the XMX air sampler (on the UWA campus) on various dates during early 2001 were spiked using sporangia at 50–1000 ml^{-1}. Samples were counted in the PAS-III and the resulting data were analysed either using multiple gating or the equation derived from the GP. Good correlation was found between observed and expected values for sporangial numbers when samples were analysed using four-parameter multiple gating and even better with the GP equation.

Detection of *P. infestans* sporangia under field conditions. Preliminary experiments at the ADAS experimental site at Llanilar in 1999 and 2000 showed that sporangia were detectable above potato fields and that these could be trapped using the XMX sampler. Ten minute sampling periods were found to be optimal. Restrictions imposed following the Foot and Mouth outbreak rendered it impossible to carry out field testing at Llanilar in 2001. However, a 0.5 ha field on UWA property was planted with potato cultivars Rocket and Premiere. Samples from the air above the crop were collected on a weekly basis using the XMX and were analysed in the PAS-III (using both GP and multiple gating), as well as being examined microscopically (haemocytometer). For the GP using the equation described above, sporangia were counted as those particles with a probability 'score' above 0.80). The crop was also examined weekly for signs of late-blight, which was first observed (as a result of natural infection) in two foci on the first of August. Given that it would take 5 to 7 days for symptoms to become visible, and that the foci observed each involved several plants, we would estimate that initial infection probably occurred two weeks earlier.

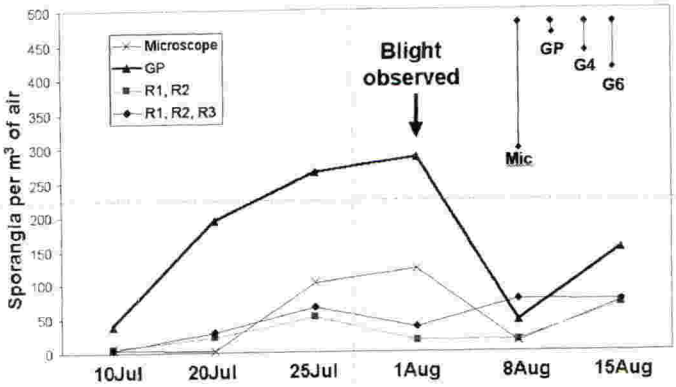

Figure 3. Estimated numbers of *P. infestans* sporangia m^{-3} of air, sampled above a potato crop planted at Penglais, Aberystwyth, in 2001 (mean of 3 samples per day). Vertical bars show LSD calculated by Duncan's Multiple Range Test of data from microscopic analysis (Mic), genetic programming (GP), and multiple gating using either four (G4) or six (G6) parameters. Significant differences in numbers of sporangia over the six sampling weeks were only indicated for data obtained by microscope ($P<0.001$) and GP ($P<0.05$).

Examination of the different methods of data analysis suggests that genetic programming (GP) was more effective than multiple gating (fig. 3). In each case, the variation between different samples taken on the same day was large, but in the case of the multiple gating data, such variation was so great that no significant difference was recorded between estimated sporangial numbers at the beginning, middle or end of the epidemic. Differences over time were observed, however, for the microscope data and the data analysed by GP. It is also apparent that the shapes of the graph are similar, for the microscope and the GP data, even though the estimated numbers of sporangia were lower for the microscope data. This may reflect the difficulty of counting sporangia in very dilute solutions under the microscope. The numbers of sporangia detected at different points in the epidemic (by GP) are higher than those reported from other studies, but not unreasonably so, e.g. over 60 m^{-3} from an infected crop (Gregory & Hirst, 1957). Bugiani *et al.* (1998) detected 'spikes' of around 20 sporangia m^{-3} prior to recording of disease occurrence, with 85 sporangia m^{-3} measured at the height of the disease progress curve. Numbers of pollen grains present in the samples were also estimated (up to 500 m^{-3}). Some attempts were made to differentiate pollen from different species,

DISCUSSION

A broadly linear relationship between log particle size and log FSC was observed for the 'standard' beads. This is in agreement with other experiments using either beads or physiologically-similar cells (Davey & Kell, 1996; Sharpless *et al.*, 1977)). As well as particle size, FSC is also influenced by refractive index (relative to the surrounding medium), by the presence in cells, or on their surface, of compounds that may absorb light at the illumination wavelength used, or by highly textured surface or internal structures which may also act to decrease the intensity of FSC (Sharpless *et al.*, 1977). These latter features have a greater influence on SSC than FSC, with SSC being less dependent upon particle size; typically, highest-intensity SSC signals are obtained from particles with the highest degree of cytoplasmic granularity (Davey & Kell, 1996). As well as the (fairly small) differences in FSC and SSC, differentiation of pollen and urediniospores from sporangia was facilitated by the intrinsic orange and red fluorescence of the former. Using a combination of FSC, SSC and FL parameters, sporangia could be distinguished from other fungal spores (except *Blumeria*) and pollen by multiple gating.

The ability of fluorescent dyes to enhance particle differentiation, in particular, to allow differentiation of *P. infestans* sporangia from *Blumeria* conidia, was assessed for a variety of different 'red' dyes. It was thought likely that fluorescent dyes would stain these two spore types differently, since *Blumeria* is a true fungus with chitin / glucan cell walls, while *P. infestans* is an oomycete classified in the Kingdom Chromista, with cellulose cell walls. However, for all the 'red' dyes tested, it appeared that the thickness or pigmentation of the wall influenced the effectiveness of staining more than did wall composition. The pattern of staining for *Blumeria* and pollen was similar to that for *P. infestans* sporangia. Moreover, the 'red' dyes tended to mask differences in intrinsic fluorescence, and so tended to make differentiation of sporangia from pollen and *Blumeria* less effective than for unstained particles.

Sporangia stained with Calcofluor could readily be differentiated using multiple gating from any of the other fungal spores tested, including *Blumeria*, with <1% false positives. Differentiation of sporangia from pollen grains using Calcofluor was less clear, since pollen stained in a similar manner to the sporangia. However, use of GPs allowed differentiation of sporangia from pollen, or from any of the other particles tested, again with <1% false positives at the level of the individual particle. Use of GP to analyse flow cytometry data could theoretically permit detection of a single sporangium within a complex sample. In practice, however, the detection threshold must be set higher in order to eliminate false positives. For example, if an 'early warning' system were set to trigger a warning for blight at, say, five sporangia per cubic metre of air, this would mean in theory that there could be up to 500 pollen grains present per cubic metre without triggering a warning (assuming <1% false positives for pollen).

Using GP, a clear increase in sporangial numbers was first apparent by 20th July, almost 2 weeks before symptoms were noticed in the crop. The system therefore appears promising, as a means of early blight detection. We recognise that more frequent sampling (including different years and at different sites) would be necessary for complete validation of the system. Differences in weather conditions are likely to affect the numbers of sporangia detected on different days. Indeed, even samples taken within minutes of each other demonstrated a high degree of variation in numbers of sporangia detected, suggesting that airborne spores travel in 'clouds', or, in the case of samples taken at the height of the epidemic, were liberated from the crop in irregular bursts, perhaps following gusts of wind. Sampling would certainly need to be carried out on a daily basis, with several samples taken per day.

ACKNOWLEDGEMENTS

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Sensors for early warning of post-harvest spoilage in potato tubers

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ABSTRACT

Post-harvest spoilage of potato tubers results in approximately £9 million annual loss in the UK alone. In this paper, we report work completed with funding from the British Potato Council to develop an electronic sensor system (e-nose) to enable early detection of spoilage diseases in stored potato tubers. The device is designed to detect the characteristic volatile organic compounds (VOCs) released during bacterial and fungal infections. Sensors were fabricated and tested for changes in their electrical resistance when exposed to the characteristic VOCs. For detecting bacterial soft rot caused by *Erwinia carotovora*, metal oxide sensors were selected for incorporation into the prototype device. This is portable and can be used without computer control after threshold values and settling criteria have been downloaded. One tuber with soft rot in 100 kg of sound tubers could be detected in a simulated storage crate at 18^0C. The device also detected an inoculated tuber within 10 kg of sound tubers, and followed the progression of disease in conditions typical of a commercial store (4^0C, 85% relative humidity).

INTRODUCTION

Annual losses of stored potato tubers in the UK amount to approximately £9 million (M. Storey, pers com.), with the largest single cause being the soft-rot bacterium *Erwinia carotovora*. This pathogen accounts for over 60% of total losses, with the remainder due to fungal pathogens, of which dry rot caused by *Fusarium coeruleum* and blight caused by *Phytophthora infestans* are the most significant.

Soft-rot is expected and tolerated in most commercial stores, but if conditions are favourable the disease spreads rapidly and losses approaching 100% occur. Processors report that in years when soft-rot is particularly prevalent, it is not unusual for up to 70% of the fresh weight of tubers to be washed away before the remainder is processed. This clearly has serious implications from both the quantity and quality aspects of production, and in relation to consumer concern. There is also potential for pollution when large amounts of carbohydrate enter water treatment plants and waterways.

Spoilage is detected presently by monitoring store temperature, visual inspection and the presence of characteristic odours. However, critical early stages of spoilage events are not detected by these methods, which leads to the problems experienced presently by the industry. Thus both store managers and processors require a system for the early detection of the causal microorganisms and the onset of spoilage events, to enable remedial action to be taken before soft-rotting becomes established.

Two approaches based on sensors within electronic nose devices (e-noses) to detect the characteristic volatile organic compounds emitted at the onset of soft-rot are possible. The first is to use relatively expensive, multi-purpose commercial e-noses that typically employ conducting polymer (eg polypyrrole) sensors. They have limited application in crops and their products because water vapour absorbs onto the polymer; this results in substantial baseline drift and unreliable detection of VOCs. The second approach is to develop relatively simple, cheap and task specific e-noses based on ceramic sensors that are far less sensitive to water. The development of such an e-nose, from identification of the characteristic VOCs, through fabrication and selection of suitable sensors, to their incorporation in a prototype device for laboratory evaluation and preliminary in-store testing, is reported here.

MATERIALS AND METHODS

Bacteria were isolated from uninfected and *E. carotovora* infected potato tubers (*Solanum tuberosum* cv. Maris Piper) and identified by fatty acid profile analysis as described previously (de Lacy Costello *et al.*, 1999). An *Arthrobacter* isolate and *Bacillus polymyxa* were present in both uninfected and *E. carotovora* infected tubers, and therefore the VOCs produced by all three bacteria following inoculation of healthy tubers were assessed by Gas Chromatography-Mass Spectrometry (GC-MS). The VOCs produced when *F. coeruleum* and *P. infestans* infected tubers were also determined using similar methods and by GC-FID analysis (de Lacy Costello, Evans *et al.*, 2001).

A number of ceramic (metal oxide) sensors were fabricated and tested for their electrical resistance changes to the key VOCs identified by GC-MS (de Lacy Costello *et al.*, 2000). On the basis of these tests, four sensors were selected for incorporation into a prototype e-nose. All the sensors were based on sintered alumina substrates (2.5 mm square), which had interdigitated electrodes coated with a conducting metal oxide layer (Figure 1). The undersides of the substrates had integral heating elements, as shown diagrammatically in Figure 2, and operated at 350°C. One sensor was fabricated with a thick film (> 1 μm) of a 50/50 w/w mixture of zinc oxide/tin dioxide, and the others were thin film sensors with 150, 500 and 940 nm thick coatings of tin dioxide. Negatively charged oxygen species absorbed by the tin dioxide are depleted at the surface following catalysis of VOC oxidation by the metal. This leads to a change in resistance of the sensor that is proportional to the concentration of the VOCs present.

The sensors were each housed in stainless steel chambers with volumes of either 10 or 20 cm^3 within a prototype e-nose (Figure 3). Air was drawn into the chambers through a sampling tube by a low voltage diaphragm pump at a rate that could be varied from 300-500 $cm^3 min^{-1}$. The e-nose could be powered either by a sealed lead-acid battery, making it a hand-held portable device, or by mains electricity. The resistance of each sensor was displayed on a screen on the front casing of the e-nose device, which could also be interfaced to a personal computer. This allowed pass/fail and system settling criteria to be set and downloaded, and for data to be collected and analysed at locations remote from the e-nose.

This e-nose was used for a number of laboratory trials, two of which are reported here. Firstly in a trial at room temperature (approx. 18°C), a square wooden crate (dimensions 1x1x1 m) was filled with 100 kg of sound tubers (approx. 900). The air was sampled for 60 s from each of 5 sampling points at the top of the crate (Figure 4), and the maximum change in resistance

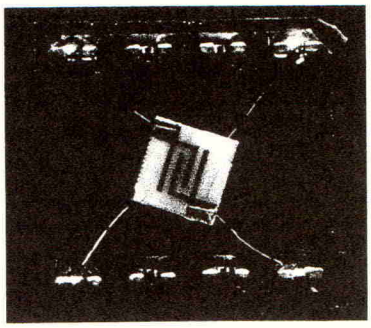

Figure 1. Ceramic sensor. Interdigitated electrodes on a sintered alumina substrate (2.5 mm square) are coated with a conducting metal oxide layer and a voltage is applied. Two other connections supply the heater (see Figure 2).

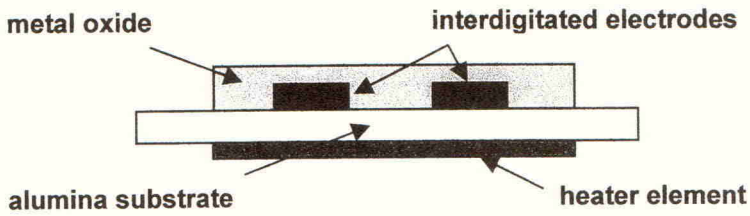

Figure 2. Diagrammatic cross-section of the ceramic sensor shown in Figure 1.

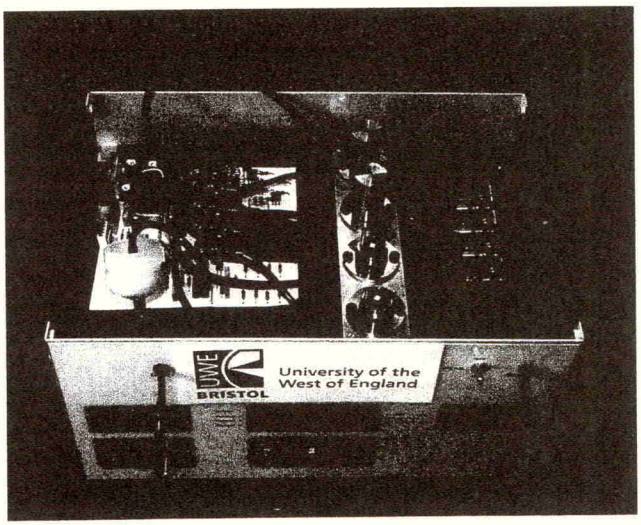

Figure 3. Prototype electronic nose, with upper casing removed to show the four stainless steel sensor chambers, with the front two opened to show the sensors.

calculated for each sensor. One *E. carotovora* infected tuber was then placed at the centre of the bottom of the crate, and the air sampled as before. In the second trial, air was sampled from a batch of 10 kg of sound tubers, and then from the same batch of 10 kg sound tubers but with one *E. carotovora* infected tuber added, over a period of 96 h in a cold-room at 4°C and 85% relative humidity. In both trials, the infected tubers had been inoculated 5-7 d earlier and incubated in the dark in a moist aerobic environment at 20°C, and showed moderate symptoms of soft-rot (< 25% of tuber affected).

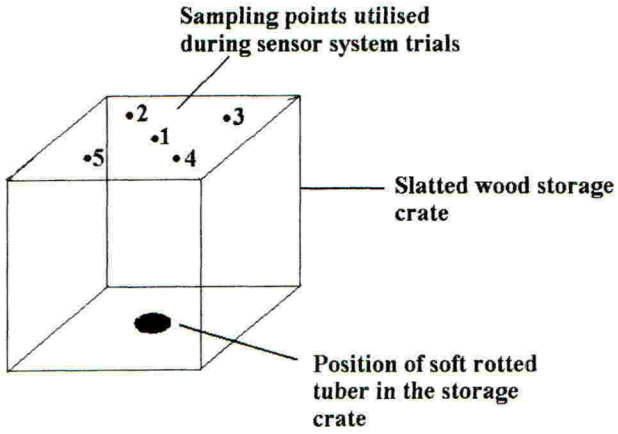

Figure 4. Sampling points in experiment with one *E. carotovora* infected tuber placed at the bottom of a crate filled with 100 kg of sound tubers.

RESULTS AND DISCUSSION

Bacteria identified by fatty acid profiling as *Arthrobacter* sp., *B. polymyxa, Bacillus pumilis, Bacillus cereus, Bacillus megaterium, Bacillus* sp., *Agrobacterium* sp. and *Cytophagus-Flavobacterium* complex were isolated from internal tissues of healthy surfaced sterilised potato tubers. These are therefore likely to be natural endobionts (endophytes) of the tubers (Gunson and Spencer-Phillips, 1994). Two of these, the *Arthrobacter* sp. and *B. polymyxa*, were also isolated from *E. carotovora* infected tubers, so they will contribute to the mixture of VOCs emitted from soft-rotted tubers. For this reason, it was important to determine their VOC profile so that the e-nose did not falsely identify the harmless activities of these endobionts.

GC-MS found 22 volatiles, comprising 10 alkanes, 4 alkenes, 2 aldehydes, and 1 each of a sulphide, ketone, alcohol, aromatic, acid and heterocyclic compound, unique to *E. carotovora* infection. *B. polymyxa* and the *Arthrobacter* sp. generated three and one unique compounds respectively. The specific identity of all the VOCs emitted is given elsewhere (de Lacy Costello *et al.*, 1999), but the data are presented here as metabolomic profiles by plotting all compounds by their retention time in the GC-MS column (Figure 5). This gives a bar code-like display and facilitates comparison of the organisms. It is clear that each produces a unique finger-print of VOCs when introduced to potato tubers, and future developments in

detection technology, perhaps linked to neural network processing, may be able to use the specific profiles to discriminate pathogens to species level. This would be particularly useful in a system that could detect non-indigenous statutory organisms, such as the brown rot pathogen *Ralstonia solanacearum* and the ring rot pathogen *Clavibacter michiganensis* ssp. *sepedonicus*, within bulked quantities of imported tubers.

Some 50 volatiles were identified in the headspace above tubers inoculated with *P. infestans* and *F. coeruleum* (de Lacy Costello, Evans *et al.,* 2001). The six most abundant VOCs, benzothiazole, 2-ethyl-1-hexanol, hexanal, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl) propyl propanoate, 2-methyl-3-hydroxy-2,4,4,-trimethyl-pentyl propanoate and phenol, were common to both pathogens. Butanal, 2-butenal, 3-methylbutanal, undecane and verbenone were specific to *P. infestans*, with 2-pentylfuran and copaene specific to *F. coeruleum*. Additionally, GC-FID analysis identified ethanol and 2-propanol in the liquid exudate and headspace from both infections. As for the bacteria, the VOC profiles from the two fungi are compared in Figure 6, which also shows relative abundance of the compounds. Thus future developments are likely to enable discrimination of fungal versus bacterial spoilage, and between different species of fungi.

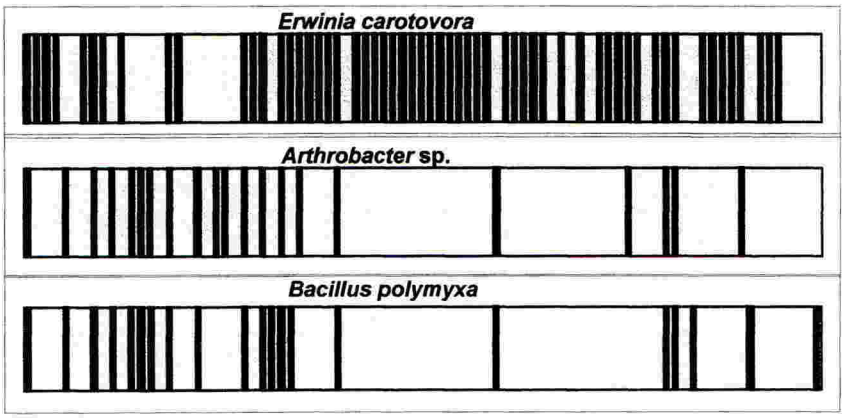

Figure 5. Metabolomic profiles from GC-MS analysis of volatile organic compounds emitted from potato tubers inoculated with *Erwinia carotovora*, *Arthrobacter* sp. and *Bacillus polymyxa*. Compounds are represented as bars against their relative retention time.

Full data for the sensor tests are published elsewhere (de Lacy Costello *et al.,* 2000; de Lacy Costello, Ewen *et al.,* 2001), but results from the two experiments described above are summarised here. In the trial with 100 kg of tubers in a wooden crate, the system was able to detect one *E. carotovora* infected tuber paced at the bottom of the crate when air was sampled from the top (Figure 7). The greatest response was seen at sampling point 1, which was in the centre directly above the infected tuber (Figure 4). Discrimination was also most pronounced with the thick film zinc oxide/tin dioxide sensor, which readily detected the presence of the single infected tuber at all sampling points. With the tin dioxide thin sensors

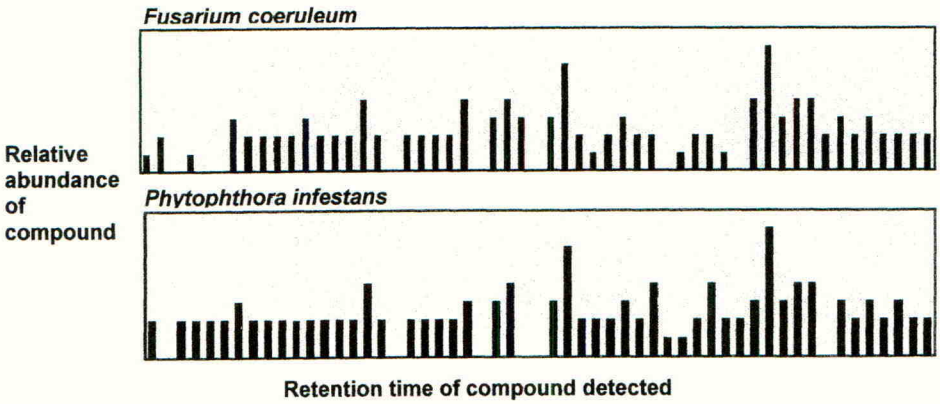

Figure 6. Metabolomic profiles from GC-MS analysis of volatile organic compounds emitted from potato tubers inoculated with *Fusarium coeruleum* and *Phytophthora infestans*. Compounds represented as in Figure 5, except that relative abundance also indicated.

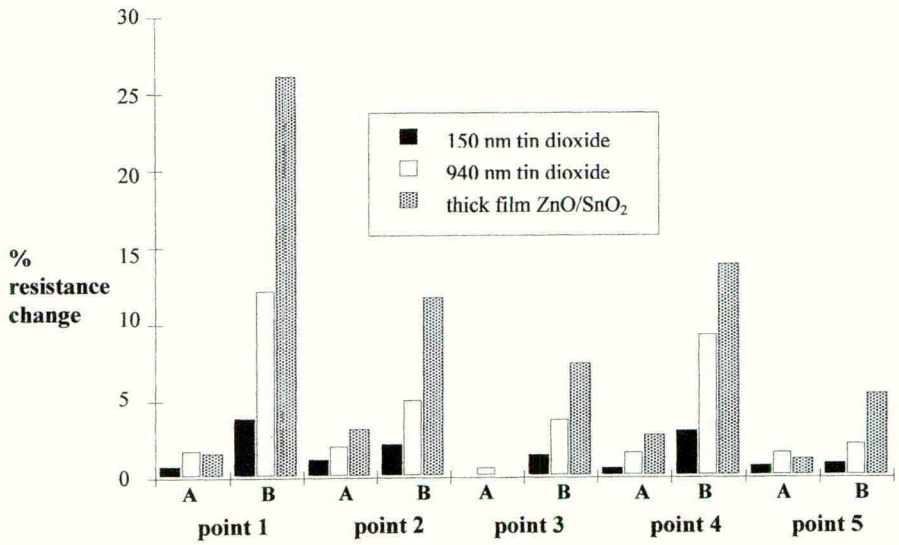

Figure 7. Response of individual sensors to 100 kg of sound tubers (**A**) and subsequently to 100 kg sound tubers with one *E. carotovora* infected tuber added (**B**), at five different sampling points in a wooden crate (see Figure 4). Reproduced from de Lacy Costello *et al.* (2000) with permission of The Institute of Physics.

tested, ability to discriminate was related to thickness of the film, with the 940 nm sensor giving consistently greater responses than the 150 nm sensor. The resistance of all four sensors changed to 95% of their maximum response in 30 s of exposure to VOCs, and returned to baseline resistance in less than 1 min purge with clean air.

To mimic more closely the conditions in commercial potato stores, the entire e-nose and tubers were subjected to 4°C and 85% relative humidity for the duration of the second trial. The change in resistance of the thick film zinc oxide/tin dioxide and a 500 nm thin film sensor were summed to give an overall system output for these two sensors (Figure 8). Good discrimination was obtained between the batches of tubers with and without an *E. carotovora* infected tuber present, with an overall efficiency of 90%.

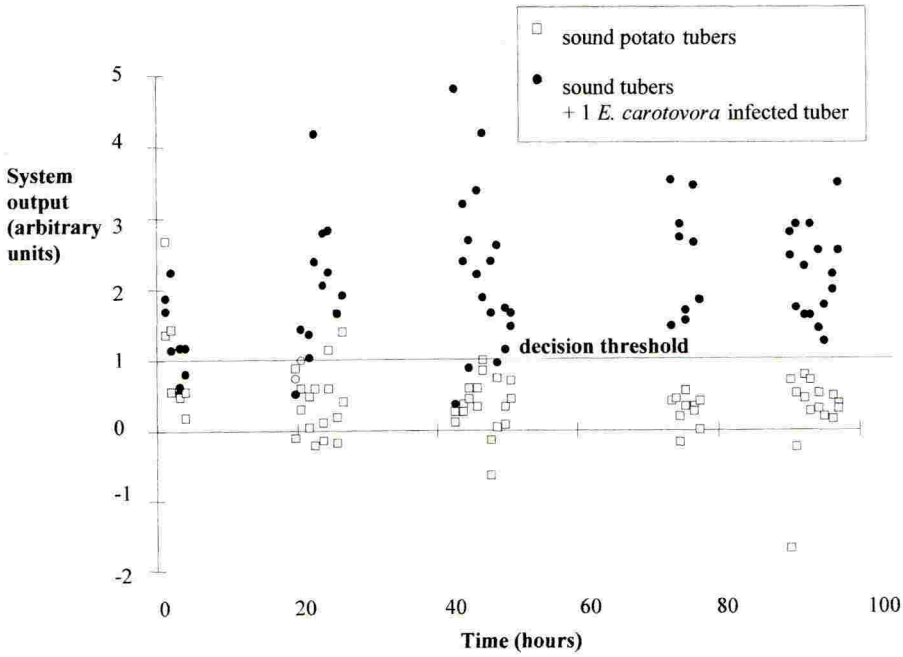

Figure 8. System output of the prototype electronic nose operating with two sensors over a period of 96 hours at 4°C and 85% relative humidity. Each data point represents the response to either 10 kg of sound tubers or the same 10 kg of sound tubers with one *E. carotovora* infected tuber added. Reproduced from de Lacy Costello *et al.* (2000) with permission of The Institute of Physics.

Although the sensitivity of the sensors was reduced at the lower temperature, baseline drift was also reduced allowing discrimination similar to that obtained at room temperature. The significant degree of overlap observed between the system output for sound and infected samples on the first day improved as the experiment progressed, so that by 96 h good separation of the data was observed. One probable reason for these observations is that the sensors were gradually equilibrating to the lower temperature and higher humidity. Additionally, the level of VOCs evolved was likely to have increased with progression of

infection, thus allowing better discrimination with time. This suggests that a period for equilibration will be required before data are collected when the e-nose is used in commercial stores.

Indeed the e-nose has now undergone trials in stores at the Sutton Bridge Experimental Unit of the British Potato Council. Although analysis of data is still underway, preliminary results indicate that the system can detect levels of infection well below what can be detected by conventional methods, and thus has the potential to provide early warning of spoilage events. This would enable store managers to undertake remedial action, for example by either increasing ventilation to dry out infected tubers or selecting for market potentially problematic batches, before soft rot reached a critical stage.

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