New Methods for Detecting and Enumerating Fungal Spores of Plant Pathogens

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Abstract

Information on the presence or absence of airborne spores or other particles would be useful in an increasing number of areas including agriculture. Traditional methods used for detecting and enumerating of airborne spores of fungal plant pathogens are time consuming and require specialist knowledge. Some spore types (e.g. ascospores) are difficult to differentiate using these methods. To facilitate this, new methods, which can be used to accurately differentiate fungal spore types, are required. A Burkard 7-day volumetric spore trap used in combination with an immunofluorescence test has been developed to detect and quantify field-trapped ascosporic inoculum of *Mycosphaerella brassicicola* (the ringspot pathogen of brassicas). This test has also been found useful in the validation of more rapid user-friendly immunoassay based trapping procedures. A microtiter immunospore trapping device, which uses a suction system to directly trap air-particulates by impaction into microtiter wells, has been used successfully for the rapid detection and quantification of ascosporic inoculum of *M. brassicicola*. The system shows potential for the rapid field-detection of airborne ascosporic inoculum of the ringspot pathogen.

Keywords: *Mycosphaerella brassicicola*; ringspot; ascospores; detection

INTRODUCTION

Microscopic examination of tapes or slides on which the microflora has been impacted has been widely used to quantify air-borne microflora. Alternatively selective agars and impingement into liquids can be used to determine the presence or absence of target species (BURGE & SOLOMON 1987). However these methods are of limited value in the detection and quantification of the air-borne spores of obligate fungal plant pathogens. These techniques also require considerable amounts of time and expertise if accurate counts are to be obtained. Many fungal spores can also contain allergenic compounds for example; allergic rhinitis and asthma can be caused by airborne allergens. Other fungal spores (*Alternaria* spp. and *Cladiosporium* spp.) have been associated with allergenic reactions (TOVEY *et al*. 2000). Airborne fungal spores are the main mechanism of dispersal of many plant pathogenic and saprophytic fungi and thus are found in a very wide range of environments. Detecting airborne fungal plant pathogens would be useful in crop protection if this could be done rapidly and accurately. For example it has been reported that one or two peaks in sporangial concentration in the air of the potato blight pathogen *Phytophthora infestans* preceded the first observed symptoms of the disease in the field (BUGIANI *et al*. 1998). Recently the potential for using serological methods and molecular methods to detect and quantify airborne bioparticles has been recognized (BURGE & SOLOMON 1987; DECOSEMO *et al*. 1992; MCCARTNEY *et al*. 2001).

Immunological methods can be used to provide rapid and cost effective estimates of target spores in the air-borne microflora provided that there are proper sampling systems available. This paper describes a system developed for the immunoquantification of airborne spores of *Mycosphaerella brassicicola*, the

ringspot pathogen of brassicas. A new microtiter immunospore trapping device (MTIST) that uses a suction system to directly trap air spora by impaction into microtiter wells has been developed (KENNEDY *et al*. 2000) and used in these studies in comparison with more conventional techniques.

MATERIALS AND METHODS

Antibody production and cross reactivity tests

A polyclonal antiserum (PAb) was raised in a female New Zealand white rabbit to an ascospore suspension of *M. brassicicola* as described by KENNEDY *et al*. (1999). The PAb was coded 96/10. A monoclonal antiserum (MAb) was raised (using a co-immunogen Mab EMA 185) to an ascospore suspension of *M. brassicicola* as described by WAKEHAM *et al*. (2000). The MAb was coded EMA 187. Antibodies were screened for cross-reactivity against a range of fungal airborne spores using immunofluorescence (If) as described WAKEHAM *et al*. (2000).

Detection and quantification of ascospores of *M. brassicicola* using conventional Burkard spore trapping and immuno uorescence (IF)

Sporulating cultures of *M. brassicicola*, were produced as described in KENNEDY *et al*. (1999). A Melinex tape was incubated for 2 h at room temperature in 5% (w/v) bovine serum albumin (BSA) (Sigma A-7030) PbsTinc (0.05% anti-bacterial tincture of merthiolate in phosphate buffered saline, PBS) before positioning inside a Burkard volumetric spore trap (Burkard Scientific, Uxbridge, UK). The Burkard spore trap was operated under controlled environmental conditions where it was exposed to sporulating cultures of *M. brassicicola*. The tape was processed for IF by attaching tape pieces to a glass microscope slide before incubating with polyclonal antibody (PAb 96/10), diluted 1:100 in blocking buffer (PbsTinc 0.05% Tween 20, 0.1% casein). After incubation at room temperature for 60 min the slides were carefully rinsed with distilled water. Anti-rabbit IgG FITC conjugate (Sigma F0382) diluted 1:100 in blocking buffer containing the counterstains Evan's blue (Sigma E-0133) and eriochrome black (Sigma E-2377) (at 0.05%) were added to the tape. The counterstains were used to block the autofluorescence of air-spora, bacteria and pollen. Following incubation in darkness at room temperature for 30 min the tape was carefully rinsed with distilled water and air-dried in darkness. After which the slides were mounted with DAKO fluorescent mounting medium (DAKO 53023). The tapes were viewed under UV episcopic-fluorescence microscopy.

Detection and quantification of ascospores of *M. brassicicola* using ELISA under controlled conditions

Spores of *M. brassicicola* and *E. cruciferarum* were trapped in microtitre wells using an MTIST spore trap as described in previous studies (KENNEDY *et al*. 2000). Using the MTIST, air-spora within a controlled environment cabinet (Sanyo Gallenkamp, Loughborough, Leicestershire, UK; Cat No. SGC970/C/RO-HFL operating at 94% r.h. with continuous light and intermittent wetting of 0.3 min/60 min period) was sampled over a 24 h period (20 l air/min) with sampling time periods of 30 min, 1, 2, 4, and 12 h. 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*. From each of four sampling periods two wells were examined for the spatial distribution of the impacted spores. Microstrips were stored at –20°C until processed through PTA-ELISA as described in previous studies (KENNEDY *et al*. 2000). Spore numbers in microtitre wells were also counted.

Immunomonitoring field-trapped airborne inoculum of *M. brassicicola*

In field studies a Burkard 7-day volumetric spore trap and a field modified MTIST spore trap were positioned adjacent to each other and 6 m outside a 24 m × 12 m ringspot inoculated plot. Over a period of 1 month airborne levels of *M. brassicicola* ascospores were monitored and infection levels recorded on exposed Brassica trap plants. The B-7-day spore trap was operated continuously over a 1 month period (Melinex tape changed at 7-day intervals). A Delta T data logger (Delta T Devices Ltd) was used to activate the MTIST when relative humidities (r.h.) of > 93% were recorded. The MTIST deactivated when the r.h. fell below 80%. Microtiter well strips were processed by PTA-ELISA (WAKEHAM *et al*. 1999) employing both PAb 96/10 and an anti-rabbit IgG SEEKit, and MAb EMA 187 and an anti-mouse IgG SEEKit (Harlan Sera Lab Ltd, UK). For each of these sampling periods, four 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 sampling period 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.

RESULTS

Specificity of antisera

Employing immunofluorescence (IF) PAb 96/10 cross-reacted with each of the ascosporic stages of the fungi tested (Table 1) and to the conidial stage of *Mycosphaerella pinodes* and *Pyrenopeziza brassicae*. Of the hybridoma cell lines identified from the MAb screen (mice co-immunized with MAb EMA 185 + ascospore preparation) only one cell line was identified (MAb EMA 187) which was positive to the ascospore wall of *M. brassicicola* in IF. Of the fungi tested using IF, cross-reactivity of MAb EMA 187 was limited to components of the ascospore wall of *Pyrenopeziza brassicae* (Table 1).

Table 1. Reactivity of PAb 96/10 and MAb EMA 187 to a range of fungi employing immuno uorescence (IF)

	PAb 96/10	MAb EMA 187
Ascospore		
Mycosphaerella brassicicola	+	+
Mycosphaerella pinodes	+	Not tested
Mycosphaerella cryptica	+	–
Mycsophaerella nubilosa	+	–
Sclerotinia sclerotiorum	+	–
Pyrenopeziza brassicae	+	+
Gaeumannomyces graminis var. *tritici*	+	–
Conidia		
Mycosphaerella pinodes	+	–
Pyrenopeziza brassicae	+	–
Ascochyta fabae	–	–
Ascochyta rabiei	–	–
Ascochyta lentis	–	–
Ascochyta allii	–	–
Botrytis cinerea	–	–
Botrytis squamosa	–	–
Alternaria brassicae	–	–
Alternaria brassicicola	–	–
Aspergillus avus	–	–

Immunomonitoring airborne inoculum of *M. brassicicola* in controlled environment

Ascospores of *M. brassicicola* and conidia of *E. cruciferarum* were distributed throughout the base of each microtitre well. With increasing conidial concentration (> 100 per microtiter well) aggregation of impacted conidia was observed. However at levels > 1000 conidia per microtiter ascospores of *M. brassicicola* were obscured. Following PTA-ELISA a correlation of $r^2 = 0.9655$ was observed with PAb 96/10, between number of ascospores per microtiter well and the absorbance figures recorded (Figure 1). There was no correlation between number of conidia of

Table 2. Comparison of PTA-ELISA using PAb 96/10 and MAb EMA 187 to MTIST samples collected in the field with ringspot lesions on trap plants and immuno uorescence (IF) detection

Date	Ringspot lesions	PAb 96/10	MAb EMA 187	IF test
14–15/09/1999	0	0.347	0.015	9
22–23/09/1999	26	0.396	0.396	31
23–24/09/1999	29	0.316	0.010	86
24–27/09/1999	204	1.040	0.129	207
15–18/10/1999	1	0.550	0.033	34
18–19/10/1999	0	0.105	0.003	2
19–20/10/1999	0	0.072	0.007	0
20–22/10/1999	26	0.574	0.072	19
22–25/10/1999	826	1.046	0.200	280

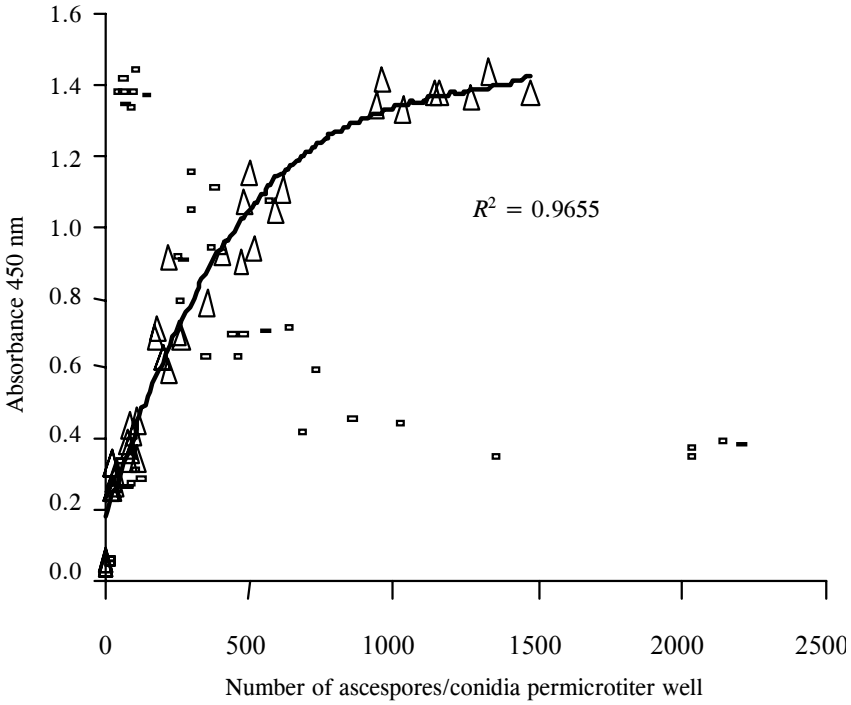

Figure 1. PTA-ELISA and the numbers of MTIST device – trapped ascospores of *M. brassicicola* (Δ) and *Erysiphe cruciferarum* conidia (▭)

E. ruciferarum per microtiter well and the absorbance values recorded (Figure 1).

Immunomonitoring field-trapped airborne inoculum of *M. brassicicola*

There was an incomplete data set for comparison of the results between the PTA-ELISA, the immunofluorescence test and the results of trap plant exposures (Table 2). The available results show a strong relationship between the PTA-ELISA using both PAb 96/10 and MAb EMA187 and the number of ringspot lesions on exposed trap plants. There was also a strong relationship between the results of the immunofluorescence test and the number of ringspot lesions however some variability in the results of the immunofluorescence test were observed associated with periods of heavy rainfall (20–22 October 1999).

DISCUSSION

Antibody based technology is frequently employed in plant pathogen immunodiagnostic systems (WERRES & STEFFENS 1994; DEWEY & THORNTON 1995). Using these techniques to detect and quantify target airborne spora rapidly will prove useful both as an immediate measure of spatial distribution of plant pathogenic fungi and as a research tool in quantatitive epidemiology. The B-7 day IF test has been particularly well suited in this respect and has been used in the development and validation of more rapid-based systems i.e the MTIST trapping system. As with any immunological based system the specificity of the antiserum used is critical to the success of the test. BARCLAY & SMITH (1986) established that co-immunization was a valuable method for raising MAbs specific to bacteria. Using this methodology it was possible to raise monoclonal antibodies which reacted positively to an area surrounding both non-germinated and germinated ascospores of *M. brassicicola*. These components are either released after impaction, or form part of the ascospore mucilage coating. The presence of mucilage on *M. brassicicola* ascospores ensures that they are able to attach to an impaction surface.

When the MTIST was used under controlled environmental conditions there was a close correlation between the number of ringspot MTIST trapped ascospores of *M. brassicicola* and absorbance after processing by PTA-ELISA. This was unaffected by the presence of larger *Erysiphe* spores. However the effect of germination by both spore types on the accuracy of detection was not investigated. This potentially could have influenced the results and requires further investigation. When the MTIST trap was used under field conditions little or no spore germination was observed within the microtitre wells. Although an incomplete data set was obtained (due to logger failure in turning the MTIST device on and off) however there was a very strong

relationship between the MTIST PTA-ELISA results (MAB EMA187) and the number of ringspot lesions on exposed trap plants (r^2 = 0.9947). The relationship between the MTIST PTA-ELISA results and the number of ascospores as detected using the IF test was also strong (r^2 = 0.8613). By optimizing trap position during periods of *M. brassicicola* ascospore release, it is likely that the trap can be used under field conditions to determine the likely transmission potential of ringspot at any crop location. Potentially the MTIST system will also enable the presence or absence and quantity of several target air-spora to be assessed at the same time either by immunoassay or by PCR using specific probes. As many plant diseases occur as a complex this aspect will be particularly useful. Advances in environmental data capture systems will allow operation of the MTIST only when conditions are favourable for target spore release and/or infection. This has great potential for use in epidemiological and disease management studies.

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