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Detection of airborne inoculum of *Leptosphaeria maculans* and *Pyrenopeziza brassicae* in oilseed rape crops by polymerase chain reaction (PCR) assays

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The potential use of DNA-based methods for detecting airborne inoculum of *Leptosphaeria maculans* and *Pyrenopeziza brassicae*, both damaging pathogens of oilseed rape, was investigated. A method for purifying DNA from spores collected using Hirst-type spore samplers and detecting it using polymerase chain reaction (PCR) assays is described. For both pathogens, the sensitivities of the DNA assays were similar for spore-trap samples and pure spore suspensions. As few as 10 spores of *L. maculans* or *P. brassicae* could be detected by PCR and spores of both species could be detected against a background of spores of six other species. The method successfully detected spores of *P. brassicae* collected using spore traps in oilseed rape crops that were infected with *P. brassicae*. *Leptosphaeria maculans* spores were detected using spore traps on open ground close to *L. maculans*-infected oilseed rape stems. The potential use of PCR detection of airborne inoculum in forecasting the diseases caused by these pathogens is discussed.

Keywords: *Leptosphaeria maculans*, light leaf spot, PCR, phoma leaf spot, *Pyrenopeziza brassicae*, spore trapping

Introduction

Leptosphaeria maculans and *Pyrenopeziza brassicae* are both damaging pathogens of oilseed brassicas, particularly winter oilseed rape (*Brassica napus* ssp. *oleifera*), where they cause major yield losses. *Leptosphaeria maculans* causes phoma stem canker, which can be economically damaging in many areas of the world, particularly in Europe, Australia and Canada (West *et al*., 2001). Yield losses attributable to stem canker are usually less than 10%, but losses of between 30 and 50% have been reported (West *et al*., 2001). *Pyrenopeziza brassicae* causes light leaf spot, one of the most damaging diseases of winter oilseed rape crops in the UK, causing estimated yield losses of up to 22% (Fitt *et al*., 1997). The disease can also be severe on winter oilseed rape crops in other northern European countries (Paul & Rawlinson, 1992). As no cultivars are available that are fully resistant to either pathogen, disease control relies heavily on the use of fungicides (West *et al*., 1999a,b; Gilles *et al*., 2000). However, disease severity varies from season to season for both pathogens (West *et al*., 1999a; Gilles *et al*., 2000) and consequently fungicide treatments are often applied inappropriately. Thus, to make efficient use of fungicide control, there is a need for forecasting systems to predict the risk of severe epidemics in different regions and seasons.

There are similarities in the epidemiology of the two diseases on oilseed rape in the UK. Both pathogens can be dispersed by airborne ascospores (McCartney & Lacey, 1990; Hall, 1992), which are probably responsible for the initial infection of winter oilseed rape crops in the autumn (Gilles *et al*., 2000; West *et al*., 2001). *Leptosphaeria maculans* ascospores infect leaves of young crops causing leaf spotting (phoma leaf spot). The fungus grows systemically from the leaf spots down the petiole to infect the stem, causing stem cankers (Hammond *et al*., 1985). It is the canker phase of the disease cycle that causes most yield loss. However, fungicides do not control the pathogen once it has reached the stem and it is therefore crucial to treat the crop when the pathogen is only in the leaves (Gladders *et al*., 1998). Similarly, ascospores of *P. brassicae* are thought to initiate epidemics of light leaf spot in autumn-sown oilseed rape crops (Gilles *et al*., 2000). After initial infection, the disease can be further spread

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by predominantly splash-dispersed asexual conidia, although secondary spread by ascospores may also take place in the spring and summer (McCartney & Lacey, 1990). Autumn sprays are often needed to control light leaf spot in the UK (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 of forecasting the availability of airborne inoculum in the autumn, when newly emerged crops are susceptible to infection.

Conventional methods of monitoring airborne fungal spores use either microscopy or cultural methods. Both approaches are time consuming, require experienced personnel and may be unreliable. Ascospores and conidia of *P. brassicae* are almost identical and are nondescript hyaline spores easily confused with spores of other fungi, e.g. the saprophyte *Unguicularia* cfr. *raripila* (Inman *et al*., 1992), making identification by microscopy difficult. Culturing requires a suitable selective medium, which is not available for these pathogens, and *P. brassicae*, in particular, is a slow-growing fungus that can often be overgrown by contaminant fungi and bacteria. Thus, for inoculum monitoring to become a practical tool in the assessment of risk for light leaf spot and stem canker on oilseed rape, alternative methods need to be developed.

Molecular techniques, based on DNA analysis, have been recognized as potential alternative methods for detecting airborne fungal spores (MacNeil *et al*., 1995; McCartney *et al*., 1997) but, until recently, little progress had been made in their use. Polymerase chain reaction (PCR) techniques have been used to detect a few species of fungi in air samples (Olsson *et al*., 1996; Wakefield, 1996; Haugland *et al*., 1999; Vesper *et al*., 2000; Calderon *et al*., 2002; Williams *et al*., 2001). Wakefield (1996) detected *Pneumocytis carinii* DNA, using PCR-based assays, in three out of five samples collected outdoors using a Hirst-type spore trap (Burkard trap; Hirst, 1952, 1967) and in one sample taken in an animal house. More recently, Calderon *et al*. (2002) demonstrated that DNA of *Penicillium roqueforti* could be purified successfully from Hirst-type and rotating-arm air samplers and detected using a PCR assay. This shows that PCR-based assays used in conjunction with conventional spore traps have potential as a method for detecting airborne fungal spores, providing suitable primers are available. However, these methods appear not to have been used to detect plant pathogenic fungi.

Specific PCR primers have been developed for *P. brassicae* (Foster *et al*., 1999; Foster, 2000). *Leptosphaeria maculans* isolates can be divided into two types (A or B) and primers are available for the more aggressive A type (Taylor, 1993; Williams & Fitt, 1999). Thus, there is potential for use of these PCR assays in conjunction with conventional air samplers for field monitoring of airborne inoculum of these two pathogens. This paper reports a series of experiments to determine the potential of combining air sampling using Hirst-type samplers (Burkard 7-day recording spore trap, Burkard Manufacturing Co., Rickmansworth, UK) with inoculum detection using PCR assays.

Materials and methods

Collection and enumeration of spores

Pyrenopeziza brassicae conidiospores and *L. maculans* ascospores were collected as described by Gilles *et al*. (2001) and Toscano-Underwood *et al*. (2001), respectively, from infected oilseed rape leaves and stems. Spore suspensions of each species were prepared using 0·1% Nonidet P40 (Sigma, St. Louis, MO, USA) and their concentrations were estimated microscopically using a particle counting chamber. The *P. brassicae* suspensions were adjusted to 2×10^4 conidia μL^{-1} and serial dilutions were then made for use in DNA-extraction experiments (see below). The concentrations of the *L. maculans* ascospore suspensions were generally lower than those for *P. brassicae* conidia, therefore, in some cases, the undiluted suspensions were used in the experiments. Spore suspensions were also pipetted evenly across $9{\cdot}5 \times 24$ mm pieces of Melinex tape (Burkard Manufacturing Co. Ltd, Rickmansworth, UK) covered with a thin layer of a 5 : 1 mixture of petroleum jelly (Vaseline) and paraffin wax (British Aerobiology Federation, 1995). The tape and coating were the same as those used in the spore traps used in this study (see below). The tape was allowed to dry and used in DNA-extraction and PCR-assay experiments.

Suspensions containing *P. brassicae* conidia and *L. maculans* ascospores against a background of *Botrytis cinerea*, *Cladosporium cladosporioides*, *Erysiphe cruciferarum*, *Penicillium roqueforti* and *Lycopodium clavatum* spores were prepared in 0·1% Nonidet P40. *Botrytis cinerea*, *P. roqueforti* and *C. cladosporioides* were taken from culture collections maintained within the Plant-Pathogen Interactions Division, IACR-Rothamsted, UK. *Erysiphe cruciferarum* was obtained from R. Kennedy, Horticultural Research International, Wellesbourne, UK and *L. clavatum* spores were obtained, dry, from BDH Laboratory Chemicals Ltd, Poole, UK. These suspensions were used to test the DNA-extraction/PCR-assay protocol.

Naturally released ascospores of *P. brassicae* and *L. maculans* were collected using Burkard 7-day recording spore traps. The traps collected airborne particles on wax-coated Melinex tape attached to a slowly rotating drum, so that particle concentration could be recorded over a 7-day period. One trap was operated on open ground and surrounded by pieces of oilseed rape stems showing symptoms of stem canker and actively releasing *L. maculans* ascospores, placed on the ground. This trap was run from February to March 2000. A second trap was operated from May to June 2000 in a winter oilseed rape crop (cv. Apex) that showed symptoms of light leaf spot (*P. brassicae*). The spore trap tapes were replaced every 7 days. After exposure, each tape was cut into 24-mm sections, representing 12 h exposure periods. Each section was cut

in half along its centreline in the direction of rotation. One half was permanently mounted on a microscope slide using Gelvatol containing Trypan blue stain (British Aerobiology Federation, 1995). This slide was examined under a light microscope to determine whether or not *L. maculans* or *P. brassicae* spores were present. The number of *P. brassicae* spores deposited on selected slides was also estimated by counting using a light microscope. The other half of each section was placed in a 0·5 mL microtube for DNA extraction and PCR analysis (see below). The number of *L. maculans* ascospores deposited on selected Burkard spore trap samples collected in an oilseed rape field by J. West, IACR, Rothamsted, UK, were also counted and the samples subjected to PCR analysis in the same way.

Spore disruption and purification of DNA

Spores were disrupted by shaking 200 μL of spore suspension together with 0·2 g of acid-washed Ballotini beads (8·5 grade, 400–455 μm diameter) in a FastPrep® machine (Savant Instruments, Holbrook, New York, USA). Previous tests showed that shaking at 5 m s^{-1} for two periods of 40 s, with 2 min cooling on ice between them, efficiently disrupted these spores. DNA was purified from 50 μL of the disrupted spore suspensions using a modified version of the method described by Lee & Taylor (1990). The samples were mixed with a lysis buffer (containing Tris, EDTA, SDS and β-mercaptoethanol), incubated at 65°C for 1 h, extracted with phenol:chloroform and precipitated with isopropanol. At the isopropanol precipitation step, 20 μg of glycogen (Roche Diagnostics Ltd, Lewes, UK) were added to act as a carrier for the DNA during centrifugation (Tracy, 1981; Williams *et al*., 2001). The DNA pellet was dissolved in 50 μL of molecular biology grade water.

A similar method was used to extract DNA from spores deposited on Melinex tape samples. Tests were done to determine the best settings for the FastPrep® machine to remove and disrupt deposited spores. Pieces of coated tape containing *P. brassicae* conidia or *L. maculans* ascospores were placed into microtubes containing 0·2 g acid-washed Ballotini beads (8·5 grade, 400–455 μm diameter) and processed using different FastPrep® velocity settings (4–6 m s^{-1}) and different volumes of 0·1% Nonidet P40 (200–250 μL). Tape samples were examined, using a light microscope, before and after processing. The best conditions were a FastPrep® velocity setting of 6 m s^{-1} and processing for two periods of 40 s, with 2 min cooling on ice between them, using 220 μL of 0·1% Nonidet P40. Under these conditions the processing not only removed the wax deposit and its contents from the tape, but also disrupted approximately 90% of the spores contained in the deposit. This procedure was used in all experiments with Melinex tape samples from laboratory tests and from Burkard spore traps. DNA was purified from the resulting suspensions.

All PCR-assay experiments (laboratory and field samples) included negative (reagent only) controls consisting of spore-free Nonidet P40 solution or spore-free Melinex tape samples treated in the same way as the samples containing spores. Tests using *P. brassicae*- and *L. maculans*-specific primers and fungal consensus primers (see below) showed that both the Melinex tape and the coating did not contain detectable fungal DNA.

PCR detection of *P. brassicae* and *L. maculans* spores

Ribosomal DNA (rDNA) from all samples was amplified using universal fungal ribosomal primers ITS4 (5′-TCC TCC GCT TAT TGA TAT GC) and ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G) (White *et al*., 1990). These primers amplify a region of DNA stretching from the 3′ end of the 18S-like gene to the 5′ end of the 28S-like gene, including the 5·8S gene and the two internal transcribed spacer (ITS) regions. Each 25-μL reaction mixture contained 20 mM Tris HCl (pH 8·4), 50 mM KCl, 1·5 mM $MgCl_2$, 0·2 mM deoxyribonucleoside triphosphates (dNTPs), 25 pmol of each primer, 0·5 units of Platinum *Taq* (Invitrogen Corporation, Carlsbad, CA, USA) and 5 μL of purified DNA sample solution. Cycling conditions were 95°C for 10 min, then 30 cycles of 94°C for 30 s, 42°C for 2 min and 72°C for 2 min.

Specific detection of *P. brassicae* DNA was done using nested PCR. The first round used specific primers Pb1 (5′-CAA CAT TGC CTG GTA TTG AGA AAC) and Pb2 (5′-ATC TGA TAC GCC TAC ACC GTC C) (Foster *et al*., 1999). Each 25-μL reaction volume contained 20 mM Tris-HCl (pH 8·4), 50 mM KCl, 2·5 mM $MgCl_2$, 0·2 mM dNTPs, 25 pmol of each primer, 0·5 units of Platinum *Taq* and 5 μL of purified DNA sample solution. Cycling conditions were 95°C for 10 min, then 30 cycles of 95°C for 1 min, 68°C for 1 min and 72°C for 1 min, followed by a final extension step of 72°C for 10 min. The nested PCR used specific primers PbN1 (5′-TGT AGA TGG AAC CCT ACC CGT ATT G) and PbN2 (5′-GTG ACC ACA ACG AAC CTT GTA TCA G) (Foster, 2000). The other components of the reaction mixture were as for PCR using primers Pb1 and Pb2. For these reactions, 1 μL of the PCR product obtained using Pb1 and Pb2 was added to the reaction mixture. Cycling conditions were 95°C for 10 min, then 20 cycles of 95°C for 1 min and 72°C for 1 min, followed by a final extension step of 72°C for 10 min.

Specific detection of *L. maculans* (A-type) DNA was achieved using PCR with specific primers D1 (5′-GCG TAA GAA GCG TGC CTT AGA GTC) and D2 (5′-TCC TGC TCC TAC TCC TTC TCT AGC) (Taylor, 1993). Although this primer pair will detect only A-type *L. maculans*, for convenience the primers and PCR are referred to here as '*L. maculans*-specific'. Each 25 μL reaction volume contained 20 mM Tris-HCl (pH 8·4), 50 mM KCl, 5 mM $MgCl_2$, 0·2 mM dNTPs, 0·165 μM of each primer, 27·5 μM tetramethylammonium chloride, 0·5 units of Platinum *Taq* and 5 μL DNA sample solution. Cycling conditions were 95°C for 10 min, then 35 cycles of 94°C for 30 s, 71°C for 1 min and 72°C for 1 min, followed by a final extension step of 72°C for 7 min.

Table 1 Detection of *Pyrenopeziza brassicae* conidia in spore suspensions or on Melinex tape using the *P. brassicae*-specific PCR. The replications were of the entire experiment (spore disruption, DNA extraction and PCR assay) not just the detection step

Suspensions			Melinex tape		
No. of conidia processed	No. of conidia in PCR[a]	Positive tests/ total number of tests	No. of conidia processed	No. of conidia in PCR[a]	Positive tests/ total number of tests
0	0	0/4	0	0	0/3
400	10	3/4	400	9	3/3
4×10^3	10^2	4/4	4×10^3	90	3/3
4×10^4	10^3	4/4	4×10^4	9×10^2	3/3
4×10^5	10^4	4/4	4×10^5	9×10^3	3/3
4×10^6	10^5	4/4	4×10^6	9×10^4	3/3

[a]Number of conidia processed (spore disruption and DNA extraction) multiplied by the fraction of the resulting DNA sample used in the PCR.

Table 2 Detection of *Leptosphaeria maculans* (A-type) ascospores in spore suspensions or on Melinex tape using the *L. maculans*-specific PCR. The replications were of the entire experiment (spore disruption, DNA extraction and PCR assay) not just the detection step

Suspensions			Melinex tape		
No. of ascospores processed	No. of ascospores in PCR[a]	Positive tests/ total number of tests	No. of ascospores processed	No. of ascospores in PCR[a]	Positive tests/ total number of tests
0	0	0/6	0	0	0/6
400	10	2/2	40	1	2/2
10^4	250	4/4	400	9	4/4
$1{\cdot}35 \times 10^4$	338	1/1	10^3	23	4/4
$2{\cdot}86 \times 10^4$	715	1/1	6×10^3	140	4/4
$3{\cdot}8 \times 10^4$	950	1/1	$3{\cdot}2 \times 10^4$	730	1/1
$4{\cdot}0 \times 10^4$	10^3	4/4	$7{\cdot}2 \times 10^4$	$1{\cdot}6 \times 10^3$	1/1
$5{\cdot}0 \times 10^4$	$1{\cdot}25 \times 10^3$	1/1			

[a]Number of ascospores processed (spore disruption and DNA extraction) multiplied by the fraction of the resulting DNA sample used in the PCR.

In all cases 5 μL of the PCR products were analysed on 2% agarose gels containing 0·5 μg/mL ethidium bromide.

Results

Spore suspensions

Experiments using serial dilutions of *P. brassicae* conidia and *L. maculans* ascospores collected from infected plants were carried out to determine the efficiency of the DNA purification methods and the sensitivity of detection. Results obtained using *P. brassicae*-specific primers are shown in Table 1 and those using *L. maculans*-specific primers in Table 2. Experiments were repeated up to four times. This replication was of the entire experiment (including DNA extractions), not just of the detection step. When PCR assays were repeated on particular samples, results were in good agreement (data not shown). For both fungi it was possible to detect the DNA equivalent of as few as 10 spores in the PCR using these methods.

Six experiments (milling, DNA extraction and PCR detection) were carried out using suspensions containing a mixture of equal proportions of spores of *L. maculans*, *P. brassicae* and five other spore types. Each PCR contained DNA from about 250 spores of each species. Both the species-specific PCR assays detected DNA in all six repeats of the tests. This showed that *L. maculans* and *P. brassicae* spores could be detected in the presence of large numbers of spores from other fungi.

DNA purification and detection of spores deposited on Melinex tape

The results of the experiments to detect *P. brassicae* conidia and *L. maculans* ascospores on Melinex tape are summarized in Table 1 (*P. brassicae*) and Table 2 (*L. maculans*). The sensitivity of detection, for both pathogens, was similar to that with experiments using spore suspensions. The *P. brassicae*-specific PCR amplified the expected product in all tests where *P. brassicae* conidia were present. In these tests the smallest number of spores detected was 10. Similarly the *L. maculans*-specific PCR amplified the expected product in all samples where *L. maculans* ascospores were present, including samples where DNA from only one spore was estimated to have been in the PCR.

Detection of *L. maculans* and *P. brassicae* spores in air samples

DNA was purified from 106 Melinex tape samples taken from the Burkard sampler that had been operated

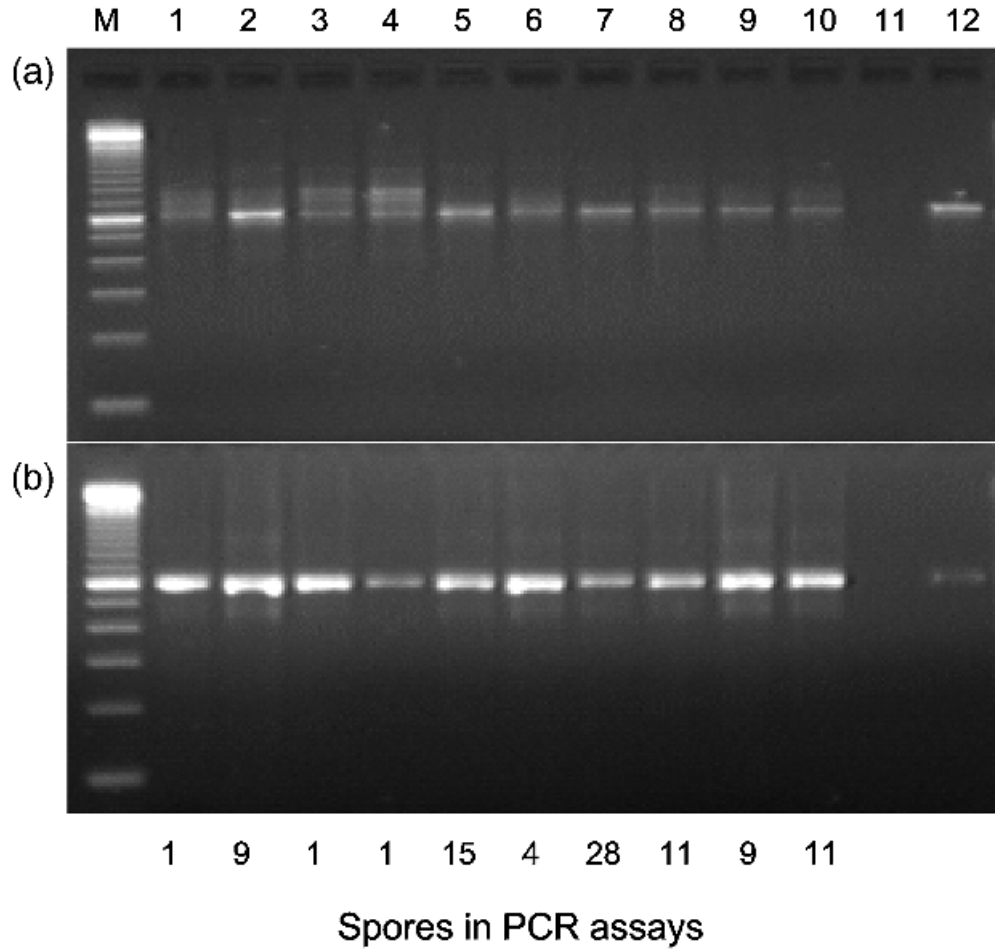

Figure 1 Detection of fungal DNA in air samples collected from oilseed rape crops using a Burkard spore trap. Each spore trap tape sample was divided into two equal pieces, one was examined by microscopy the other analysed using PCR. The numbers of *Leptosphaeria maculans* ascospores estimated to have been added to the PCR assays are given below each lane. (a) PCR products obtained using universal fungal rDNA primers and (b) PCR products obtained using *L. maculans*-specific primers. Lanes 1–10 are from 12 h samples collected on selected days (1 February 1999, 31 October 1999, 3 February 1999, 5 February 1999 and 27 February 1999, respectively). Lane 11 is a no-DNA control and lane 12 a positive control (50 ng DNA from *L. maculans* 95a15). M is the DNA size marker (100 bp ladder).

on open ground surrounded by pieces of oilseed rape stem infected with *L. maculans* and from 194 samples of tape taken from the Burkard sampler operated in an oilseed rape crop with symptoms of light leaf spot. Each tape sample corresponded to a 12 h air-sampling period. Paired samples were also examined under a light microscope (each 12 h air-sample was cut into two sections, one analysed by PCR the other examined by microscopy). DNA from all 300 samples was first amplified using the universal fungal rDNA primers ITS4/ITS5. Several bands were amplified in individual samples, indicating the presence of several different fungal species (Fig. 1). As expected, a large number of different fungal spore types were found when samples were examined under the microscope (especially in the case of samples from the oilseed rape crop), including spores of *Ganoderma*, *Coprinus*, *Agrocybe*, *Pleospora*, *Leptosphaerulina*, *Alternaria*, *Torula*, *Botrytis*, *Epicoccum*, *Curvularia* and *Helminthosporium*, as well as *Cladosporium*-like conidiospores and unidentified rusts, smuts, basidiospores and brown ascospores (aseptate or septate). There were also pollen grains, algae and dust amongst the other airborne particles on the tapes.

Fifty-four of the 106 samples collected over *L. maculans*-infected oilseed rape stems were found to contain *L. maculans*-like spores when examined under the microscope. In 41 of these samples (76%) *L. maculans* DNA was also detected in the *L. maculans*-specific PCR assay. In the remaining 52 samples, no *L. maculans*-like spores were detected by microscopy and no bands were seen in these samples using the *L. maculans*-specific PCR.

The numbers of *L. maculans*-like spores present on one half of each of ten 12 h tape samples (corresponding to 5 selected days), collected in an oilseed rape field, were also counted. The other half of each sample was subjected to PCR analysis. For each of the 10 tapes analysed, *L. maculans*-like spores were observed on one half of the tape and *L. maculans* DNA was detected by PCR on the

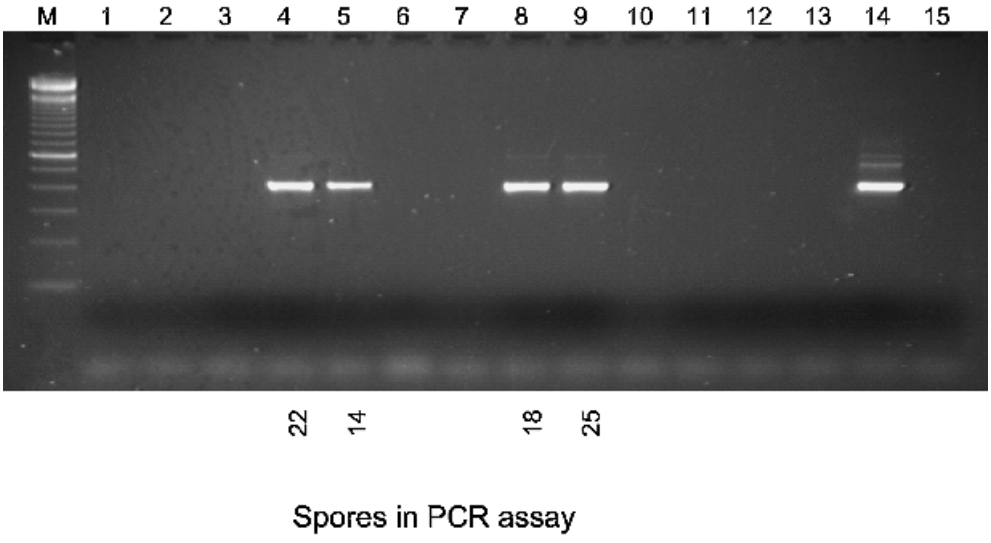

Figure 2 Detection of *Pyrenopeziza brassicae* DNA (by *P. brassicae*-specific nested PCR) in air samples collected using a Burkard spore trap in oilseed rape fields. Samples were collected over consecutive 12 h periods (23–29 March 2000). The numbers of spores estimated (by microscopy) to have been added to the PCR assays are given below each lane. In lanes 1–3, 6, 7 and 10–13 no *P. brassicae* spores were detected by microscopy. Lane 14 is a positive control (50 ng DNA from *P. brassicae*) and lane 15 is a no-DNA control. M is the DNA size marker (100 bp ladder).

other (Fig. 1). From the spore counts it was estimated that DNA from between 1 and 30 *L. maculans*-like spores was in the PCR, suggesting that the *L. maculans*-specific PCR assay may have detected DNA from as little as one spore in the PCR sample. The disparity between estimated spore numbers in the PCR and the intensity of the bands (Fig. 1) was probably due to a number of factors: (i) in conventional PCR the initial amount of target DNA is not necessarily reflected by the intensity of the final band; (ii) as the samples were from the field they could have contained differing amounts of PCR inhibitors; and (iii) the spore counts and PCR analysis were done using different pieces of tape.

Of the 194 samples collected by the spore trap in the oilseed rape crop, 124 were found to contain *P. brassicae*-like spores when examined under a microscope. The *P. brassicae*-specific PCR detected DNA in 54 of these (44%), but not in the 70 samples where no *P. brassicae*-like spores were found. The number of *P. brassicae*-like spores deposited on 13 consecutive 12 h tape samples (23–29 March 2001) was estimated. *Pyrenopeziza brassicae*-like spores were found on four of them and *P. brassicae* DNA was detected only from the corresponding four samples (Fig. 2). DNA from between 14 and 25 *P. brassicae*-like spores was estimated to have been in the PCRs. Because of the possibility of confusing *P. brassicae* ascospores with spores of other fungi (for example *U. raripila*), the number of *P. brassicae* ascospores on the tapes may have been overestimated. The difficulties in identifying *P. brassicae* ascospores may also help to explain why the PCR assay did not detect *P. brassicae* DNA in some of the samples where *P. brassicae*-like ascospores were observed. The PCR assay would not have detected DNA from other fungi such as *U.* cfr. *raripila*, but non-*P. brassicae* spores may have been counted as '*P. brassicae*-like'.

Although the total number of spores in the air samples was not estimated, the numbers of target spores (*L. maculans* or *P. brassicae*) were small compared to other spores. Thus, the specific PCR assays detected the presence of *L. maculans* or *P. brassicae* spores in air samples, even where there was a high background of other spores and particles.

Discussion

The results of both laboratory and field tests demonstrated that fungal DNA could be extracted from spores deposited on pieces of Burkard spore-trap tapes by violently agitating the sample in the presence of Ballotini beads and then purifying the DNA from the processed sample. The specific PCR assays detected DNA from the two target pathogens in the sample, even in the presence of DNA from a large number of nontarget spores and other biological material. Wakefield (1996) also demonstrated that DNA can be extracted from samples taken by a Burkard spore-trap and a cascade impactor and detected using PCR. In her experiments, a spore-disruption step (grinding with a mortar and pestle in liquid nitrogen) followed by a 16-h proteinase digestion step was used before DNA extraction. In the protocol described here the removal of the sample from the tape and spore disruption were done during the same step, simplifying sample processing.

Using the protocols described it was possible to detect about 10 *P. brassicae* spores (DNA equivalent) in the PCR assay; however, smaller spore numbers were not tested. For *L. maculans* the DNA equivalent of only one ascospore in the PCR assay was detected in some samples, including some field samples. For Burkard air samples, a sensitivity of 10 spores (DNA equivalent) detected in the

PCR translates to a 24 h average concentration of about 30 spores m^{-3}.

There is little information available on the ascospore concentrations needed to initiate disease epidemics in the field for either *P. brassicae* or *L. maculans*. Concentrations of ascospores of *P. brassicae* exceeding 1000 m^{-3} per 24 h have been measured in infected crops in the spring and early summer (McCartney & Lacey, 1990; Gilles *et al*., 2001). However, 24 h average ascospore concentrations in the autumn appear rarely to exceed about 50 ascospores m^{-3} (Gilles *et al*., 2001). There appear to have been few reports of measurements of *L. maculans* ascospore concentrations in field crops. Thürwächer *et al*. (1999) used Burkard traps to monitor airborne *L. maculans* ascospores over winter oilseed rape crops, but reported the results as spores per day not as concentrations. Their results suggest that if they had examined the whole collection tape for each day, ascospore concentrations would rarely have exceeded 10 ascospores m^{-3} (concentrations would have been higher if they had examined a smaller part of the tape). This suggests that during the initial infection phase airborne concentrations of *L. maculans* ascospores are small. However, 24 h average concentrations of *L. maculans* ascospores exceeding 50 ascospores m^{-3} were frequently measured from October to December over infected winter oilseed debris at IACR-Rothamsted (West *et al*., 1999b, 2000) and concentrations exceeding 1000 ascospores m^{-3} occurred occasionally. The protocol tested here is probably sensitive enough to detect ascospore release from crop debris but may need to be improved to detect the small concentrations that may be responsible for the initiation of light leaf spot and phoma leaf spot epidemics in the autumn. However, the above protocol used the DNA equivalent of only 1/44 of the original sample in the PCR detection assay. It may be possible to improve the detection of airborne spores present in low concentrations by sampling for longer periods or increasing the sample volume using an alternative sampler such as a rotating-arm sampler (McCartney *et al*., 1997). Alternatively, all of the tape could be processed rather than just part of it, or a larger proportion of the purified DNA could be used in the PCR assay.

Several methods have been proposed, or used, for forecasting the risk of the development of epidemics of light leaf spot (Gilles *et al*., 2000) and stem canker (West *et al*., 2001). However, the ability to monitor the presence of airborne inoculum (ascospores) of both pathogens would potentially give a direct measure of when crops were vulnerable to infections. Indeed, Gilles *et al*. (2000) suggested that *P. brassicae* ascospore release could be monitored, using spore traps, from 'depots' of developing apothecia as part of a risk-forecasting strategy. Similarly, measurement of pseudothecial maturation combined with monitoring of airborne ascospores, on a regional basis, has been suggested to improve regional forecasts for stem canker (West *et al*., 1999a,b). Unfortunately, the difficulty in monitoring airborne inoculum using conventional methods limits the practicality of such forecasting methods. The results presented here suggest that PCR-based assays have potential for routine monitoring of the presence of airborne inoculum of these two pathogens. However, more work is needed to further quantify, and improve if necessary, the field sensitivity of the PCR assays and to determine threshold ascospore concentrations needed for initial infection. The PCR assays used in this study 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, in recent years, the development of 'real-time' PCR equipment has allowed a direct estimate of the initial amount of target DNA in a sample to be made (Schaad *et al*., 1999). The protocols described here can successfully purify DNA from spore trap tapes and detect specific sequences using conventional PCR assays. In a recent preliminary experiment, these protocols were used to extract DNA of *Blumeria graminis* f.sp. *tritici* from spore trap tapes and detect and quantify it using a 'real-time' PCR assay (B. Fraaije, IACR-Rothamsted, UK, personal communication). Thus, spore trapping combined with 'real-time' PCR assays has the potential not only to detect airborne inoculum, but also to quantify it. Such methods would be suitable for assessing the risk of infection on a regional basis if a suitable network of spore traps were established and the results processed centrally.

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