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A polymerase chain reaction (PCR) assay for the detection of inoculum of *Sclerotinia sclerotiorum*

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Abstract

The development of a polymerase chain reaction (PCR) assay for the detection of inoculum of the plant pathogenic fungus *Sclerotinia sclerotiorum* is described. The PCR primers were designed using nuclear ribosomal DNA internal transcribed spacer sequences. Specific detection of DNA from *S. sclerotiorum* was possible even in the presence of a 40-fold excess of DNA from the closely related fungus *Botrytis cinerea*. PCR products were obtained from suspensions of untreated *S. sclerotiorum* ascospores alone, but DNA purification was required for detection in the presence of large numbers of *B. cinerea* conidiospores. Specific detection of inoculum of *S. sclerotiorum* was possible in field-based air-samples, using a Burkard spore trap, and from inoculated oilseed rape petals. The assay has potential for incorporation into a risk management system for *S. sclerotiorum* in oilseed rape crops.

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a phytopathogenic fungus with a wide host range and geographical distribution, as a result of which, it is one of the most non-specific, omnivorous and successful of plant pathogens (Purdy, 1979). *S. sclerotiorum* survives in the soil as sclerotia, which are produced in infected tissue. Ascospores produced by carpogenic germination of sclerotia are the major source of inoculum for infection by *S. sclerotiorum* (Willets and Wong, 1980). Infection occurs when ascospores germinate on non-living or senescent plant parts, and then infect healthy plant tissue (Purdy, 1979; McCartney and Lacey, 1991). The impact of *S. sclerotiorum* depends on the weather conditions, the host plant and the timing of ascospore release, and thus varies enormously from year to year. From the early 1970s, most of the emphasis for controlling plant disease has centred on fungicide treatments (Kharbanda and Tewari, 1996). However, routine spraying to protect against infection by *S. sclerotiorum* is not desirable because infection occurs only when the time of ascospore release and weather conditions are favourable (Fitt et al., 1992). It is likely that the use of fungicides, at least on sunflower and oilseed rape, will continue to provide cost-effective disease control in high risk situations. Therefore, current emphasis is aimed at development of disease control strategies based on environmental monitoring and disease risk assessment (Sweet et al., 1992).

Monitoring of airborne ascospores offers a direct measure of the risk of crop infection. For example, airborne ascospore concentrations have been related to subsequent disease development in both sunflower and oilseed rape crops (McCartney and Lacey, 1991; 1992; 1999; McCartney et al., 1999). Traditional methods of detection of airborne inoculum are time-consuming, labour intensive and subjective since they rely on identification of spores by microscopy or culture-based techniques. Ascospores of *S. sclerotiorum* are small,

non-descript and similar to spores of a number of other fungal species and therefore are extremely difficult to identify morphologically with certainty. Although the pathogen can be cultured, it is slow growing and it can take several days to obtain results. Thus, for monitoring of airborne inoculum to be used in disease risk assessment, new, accurate and easy to use methods are needed. Recently, the potential of molecular methods for the detection of airborne micro-organisms has been recognized (MacNeil et al., 1995; McCartney et al., 1997). DNA-based methods have been used to detect several species of airborne bacteria (Alvarez et al., 1994; 1995; Junhui et al., 1997; Mukoda et al., 1994; Palmer et al., 1995). At present, detection of airborne inoculum using DNA-based methods has been reported for only three fungal species: *Pneumocystis carinii* (Olsson et al., 1996; Wakefield, 1996), *Stachybotrys chartarum* (Haugland et al., 1999; Vesper et al., 2000) and *Penicillium roqueforti* (Williams et al., 2001; Calderon et al., 2002).

The main aim of the research reported here was to develop and test a PCR-based assay for the detection of airborne inoculum of *S. sclerotiorum*. In oilseed rape crops, *Sclerotinia* infection is nearly always caused by ascospore-infected petals sticking to leaves, allowing the pathogen to penetrate the petiole, leading to infection of the stem. In Canada, measurement of the percentage of canola (oilseed rape) petals infested with *S. sclerotiorum* was identified as a potential indicator of future disease risk (Turkington et al., 1991). Therefore, the specific PCR assay was also tested on oilseed rape petals infected with *S. sclerotiorum* ascospores.

Table 1. Specificity tests on the *S. sclerotiorum*-specific PCR assay. DNA purified from a number of *S. sclerotiorum* isolates and other fungi was tested in PCR assays with the consensus fungal primers ITS4/5 and the *S. sclerotiorum*-specific primers SSFWD/SSREV

Species (isolate)	Assay	
	ITS4/ITS5 PCR	SSFWD/SSREV PCR
S. sclerotiorum (PY1)	+	+
S. sclerotiorum (Great Harpenden)	+	+
S. sclerotiorum (31)	+	+
S. sclerotiorum (M23)	+	+
S. sclerotiorum (M1/44)	+	+
S. sclerotiorum (1992)	+	+
S. sclerotiorum (81)	+	+
S. sclerotiorum (M17)	+	+
S. sclerotiorum (S3)	+	+
S. sclerotiorum (R3)	+	+
S. sclerotiorum (R7)	+	+
Pyrenopeziza brassicae[1]	+	−
Plasmodiophora brassicae[2]	+	−
Leptosphaeria maculans[2]	+	−
Alternaria brassicae[2]	+	−
Alternaria brassicicola[1]	+	−
Alternaria alternata[2]	+	−
Alternaria infectoria[2]	+	−
Verticillium dahliae[2]	+	−
Peronospora parasitica[1]	+	−
Botrytis cinerea	+	−
Fusarium spp.	+	−
Cladosporium herbarum	+	−
Penicillium roqueforti[2]	+	−
Didymella spp.	+	−
Phytophthora parasitica[2]	+	−
Aspergillus nidulans[2]	+	−

[1]Supplied (as DNA) by S.J. Foster, IACR-Rothamsted (Foster et al., 1999).
[2]DNA prepared during earlier studies (Williams et al., 2001).
+ denotes detected and − denotes not detected.

Materials and methods

Culturing of fungi and spore collection

All fungi used in this study (Table 1) were obtained from the culture collection of the Plant Pathogen Interactions Division at Rothamsted Research. All fungi were maintained as mycelial cultures on potato dextrose agar (PDA) containing 0.005% chloramphenicol, 0.01% streptomycin sulphate and 0.005% neomycin. Isolates of *S. sclerotiorum* were stored as sclerotia at 4 °C. The sclerotia were produced by the wheat–perlite method (Sansford and Coley-Smith, 1992).

S. sclerotiorum ascospores were produced in modified Magenta vessels (Sigma Chemicals, Poole, UK) and harvested on cellulose filters (Millipore (UK) Ltd., Watford, UK). A 37 mm cellulose filter holder (Millipore (UK) Ltd., Watford, UK) was fixed to the lid of each Magenta vessel and a hole, which could be closed with a plug, drilled into the side wall. The whole assembly was sterilized with 95% ethanol in a laminar flow cabinet and left to dry before being assembled and the filters put in place. Sclerotia of *S. sclerotiorum* were removed from 4 °C storage, surface sterilized using 10% sodium hypochlorite, rinsed in sterile distilled water (SDW), and placed in the modified Magenta vessels containing sterilized coarse grade Perlite (Silverperl Products Ltd., Harrogate, UK). The cultures were kept at 15 °C in the dark until the

appearance of stipes (2–6 weeks) after which they were placed under near-UV radiation to encourage apothecia development (Mylchreest and Wheeler, 1987). Mature apothecia were formed after 3–4 weeks for isolates PY1 and Great Harpenden. Ascospores were harvested onto the cellulose filters by removing the plugs in the side wall and applying suction to the hole in the filter assembly using a vacuum pump. Ascospore-bearing filters were stored at −20 °C.

Cultures of *Botrytis cinerea* were grown on absorbent cotton wicks soaked in PDA. Conidiospores were harvested using a miniature cyclone air sampler (Burkard Manufacturing Co. Ltd., Rickmansworth, UK) that collects a dry air-sample directly into a 1.5 ml Eppendorf tube, and stored, dry, at −20 °C.

Preparation of spore suspensions

S. sclerotiorum ascospore-bearing cellulose filters were removed from −20 °C storage and ascospores suspended by vortexing the filters in 0.1% Nonidet P-40 for 60 s. Tubes containing *B. cinerea* conidiospores were removed from −20 °C storage and spores were resuspended by the addition of 0.1% Nonidet P-40 to the tubes and vortexing. Spore concentrations were calculated by counting ascospores in suspension in a particle counting chamber. The spore concentration was then adjusted to 2×10^6 ml^{-1} and $\log_{10}$ serial dilutions made for PCR experiments, or 1.3×10^5 ml^{-1} and $\log_5$ serial dilutions made for petal inoculation.

Collection of air samples in the field

A Burkard seven-day recording spore trap (Burkard Manufacturing Co., Rickmansworth, UK) was operated, for 5 weeks, in an oilseed rape field in close proximity to 10 cm diameter plastic pots containing sclerotia of *S. sclerotiorum* bearing mature apothecia. The trap collected airborne spores on a clear tape (Melinex, Burkard Manufacturing Co., Rickmansworth, UK) coated with a thin film of a 18 : 100 (w/w) mixture of paraffin wax and petroleum jelly (Vaseline) (British Aerobiology Federation, 1995). Every seven days the exposed Melinex tape was removed from the trap and cut into sections representing 24 h periods. Half of each of these sections, cut along the direction of rotation, was mounted onto slides for microscopic examination and the other half was cut into sections representing 12 h periods and used for DNA purification.

Petal inoculation

Oilseed rape petals (cv. Bienvenue) were collected from glasshouse-grown plants approximately 1 h before use. Petals were stuck to the bottom of a Petri dish using a small amount of spore trap tape coating. A dilution series of ascospore suspension in 0.1% Nonidet P-40 containing 1.3×10^5, 2.6×10^4, 5.2×10^3, 10^3 or 0 ascospores ml^{-1} was made. Ten microlitres of each suspension was placed on each of the 10 petals (i.e. approximately 1300, 260, 52, 10 or 0 ascospores per petal). Five petals of each treatment were dried immediately by incubation at 60 °C for 10–15 min and then stored at −20 °C overnight. The remaining five petals were sealed inside the Petri dishes, with a piece of wet tissue to prevent the droplets evaporating, and incubated at room temperature overnight to encourage spore germination.

DNA purification from mycelium, spores, air-samples and oilseed rape petals

Mycelium from *S. sclerotiorum*, *B. cinerea*, *Cladosporium herbarum, Fusarium* spp., and *Didymella* spp. was grown on sterile cellophane discs (CPL Packaging, Avonmouth, Bristol, UK) on PDA plates at 25 °C in the dark (Foster et al., 1999). When mycelium covered the plates it was scraped off using the back of a scalpel blade and freeze-dried. Freeze-dried mycelium (30 mg) was ground in 2 ml screw-capped tubes (Alpha Laboratories Ltd., Eastleigh, UK) using metal rods shaped to fit the tubes. DNA was purified using a method based on that described by Lee and Taylor (1990). The samples were mixed with a lysis buffer (containing Tris, EDTA, SDS and β-mercaptoethanol) and incubated at 65 °C for 1 h; a phenol : chloroform extraction and an isopropanol precipitation were then performed. We modified the published protocol by adding an RNase digestion followed by a second phenol : chloroform extraction and isopropanol precipitation. The resulting pellet was dissolved in 50 µl molecular biology grade water. DNA of all other fungal species used had been prepared during earlier studies or were supplied by S.J. Foster, IACR-Rothamsted (Table 1).

Fungal spores were disrupted by violent agitation of 200 µl spore suspensions in 0.1% Nonidet P-40, in the presence of 0.2 g acid-washed, 400–600 µm diameter Ballotini beads (Jencons-PLS, Leighton Buzzard, UK). Agitation was done for 2 periods of 40 s, with

2 min on ice between each 40 s period, at 6 ms^{-1} in a FastPrep® machine (Savant Instruments, Holbrook, New York, USA). This method disrupted more than 99% of *S. sclerotiorum* ascospores as shown when samples were examined, before and after treatment, by microscopy. Disrupted spore suspension (50 µl) was transferred to a fresh tube and DNA was purified using the method of Lee and Taylor (1990) modified by the addition of 20 µg glycogen (Roche diagnostics Ltd., Lewes, UK) as a carrier for the DNA during isopropanol precipitation (Williams et al., 2001).

The same spore disruption and DNA purification process was used to prepare DNA from field-operated spore trap tapes, from oilseed rape petals and Melinex tape controls. Sections of Melinex tape (9.5 × 24 mm), corresponding to 12 h sampling periods, or individual petals were placed in 0.5 ml screw-capped tubes with 200 µl 0.1% Nonidet P-40 before being processed.

PCR sample preparation

Suspensions containing known numbers of spores were used in experiments to test the sensitivity of the PCR assays. Three different methods of preparing the spore suspension samples for PCR assay were compared: no treatment; disrupting spore suspensions using the FastPrep® machine; and purifying the DNA from disrupted spore suspensions. PCR assays used 5 µl of test sample. All experiments included positive controls (50 ng *S. sclerotiorum* DNA prepared from mycelium), and negative controls consisting of reagent only or spore-free Nonidet P-40 samples treated in the same way as spore suspensions.

PCR assays

Two primer pairs were used: the consensus fungal 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); and primers SSFWD (5′ GCT GCT CTT CGG GGC CTT GTA TGC) and SSREV (5′ TGA CAT GGA CTC AAT ACC AAG CTG), designed during this work, to detect *S. sclerotiorum*. Although this primer pair would also detect a few related *Sclerotinia* species (see below), for convenience, we will refer to them as '*S. sclerotiorum*-specific' in the rest of the paper. The ITS4/ITS5 primers amplify ribosomal DNA (rDNA) stretching from the 3′ end of the 18S-like gene to the 5′ end of the 28S-like gene and including the 5.8S gene and the two internal transcribed spacer (ITS) regions. The SSFWD/SSREV primers amplify a region within that amplified by ITS4/ITS5.

Each ITS4/ITS5 25 µl PCR reaction contained 25 pmol of both primers, 0.5 units of Platinum *Taq* (Life Technologies Ltd., Paisley, UK), buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM deoxyribonucleotide triphosphates and DNA (50 ng, except where stated otherwise, from mycelium or 5 µl from spore samples or oilseed rape petals). 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; followed by a final extension of 72 °C for 10 min.

For the *S. sclerotiorum*-specific PCR assay, each 25 µl PCR reaction mixture contained 5 pmol of both primers, 0.5 units of Platinum *Taq* (Life Technologies Ltd., Paisley, UK), buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl, 1 mM $MgCl_2$), 0.2 mM deoxyribonucleotide triphosphates, 1% formamide and DNA (50 ng–2 µg from mycelium, various amounts from spore samples, 5 µl from oilseed rape petals). A touchdown PCR was used with an annealing temperature range of 72–65 °C decreasing by 1 °C after each 2 cycles with 16 cycles at the minimum annealing temperature of 65 °C. Cycling conditions were: 95 °C for 10 min; then a total of 30 cycles of 94 °C for 30 s, annealing (as described above) for 1 min, 72 °C for 1 min; followed by a final extension of 72 °C for 10 min. PCR products from both assays were analysed on 2% agarose gels and the DNA stained by ethidium bromide.

Results

Design of PCR primers

The aim was to develop DNA-based assay systems that would allow specific detection of ascospores of *S. sclerotiorum* even in the presence of conidiospores of the related fungus *B. cinerea*. This pathogen is also air dispersed and is ubiquitous in many crops that are host to *S. sclerotiorum*, particularly oilseed rape and sunflowers. It was therefore essential that the PCR assay could distinguish between *S. sclerotiorum* and *B. cinerea*.

Ribosomal DNA sequences were sought for fungi closely related to *S. sclerotiorum* in the EMBL/Genbank databases. This was done using the SRS website (http://srs.ebi.ac.uk) and FASTA/BLAST searches using the previously published *S. sclerotiorum* sequence M96382. Sequences were aligned using the program PILEUP in the GCG package

(Genetics Computer Group, 1994). The accession numbers of the sequences used were: *S. sclerotiorum* M96382 (Z73799 and Z73800 are identical to this in the ITS4/5 region); *S. trifoliorum* U01218 (Z99676 is identical in the ITS4/5 region); *S. minor* Z99673; *S. borealis* AF067644; *B. cinerea* Z73765 (Z99664 is identical in the ITS4/5 region, Z73764, Z99663, Z99665 and Z99662 are identical in the regions illustrated in Figure 1); *Monilinia laxa* Z73785.

The potential *S. sclerotiorum*-specific primers SSFWD and SSREV were chosen using the sequence alignment (Figure 1) and analysed using the program Net Primer (http://www.premierbiosoft.com). This identifies regions of likely secondary structure and potential primer dimer formation and gives values for the T_ms. The specificities of the primers were checked using FASTA and BLAST searches of the EMBL/Genbank databases. This showed that they were identical to sequences of the very closely related species *S. trifoliorum* (accession numbers Z99676, U01218), *S. minor* (accession number Z99673), *S. glacialis* (accession number Z99669) and *Sclerotinia* sp. (accession numbers Z99679, Z99677). The assay reported here would therefore be likely to detect DNA from these species as well as *S. sclerotiorum*. However, the primers should not amplify DNA from any other fungi, including *B. cinerea*.

(a)

```
                    1                                                  40
S. sclerotiorum     GCTTTGGCGA GCTGCTCTTC GGGGCCTTGT ATGCTCGCCA
S. trifoliorum      .......... .......... .......... ....G.....
S. minor            .......... .......... .......... ..........
S. borealis         .......... .....-.... -......... GC........
B. cinerea          .......... .....-.... -......... ..........
M. laxa             .......... .....-.... -......... ..........
```

(b)

```
                    1                                                  40
S. sclerotiorum     AAGCTCAGCT TGGTATTGAG TCCATGTCAG TAATGGCAGG
S. trifoliorum      .......... .......... .......... C.........
S. minor            .......... .......... .......... ..........
S. borealis         .......... .......... ........C. C.........
B. cinerea          .....T.... .......... ..T....... ..........
M. laxa             ....A..... .......... ..T....... C.........
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Figure 1. Alignment of rDNA ITS sequences of *S. sclerotiorum* and related fungi in the regions used for *S. sclerotiorum*-specific primer design. The accession numbers of the sequences used were: *S. sclerotiorum* M96382 (Z73799 and Z73800 are identical to this in the ITS4/5 region); *S. trifoliorum* U01218 (Z99676 is identical in the ITS4/5 region); *S. minor* Z99673; *S. borealis* AF067644; *B. cinerea* Z73765 (Z99664 is identical in the ITS4/5 region, Z73764, Z99663, Z99665 and Z99662 are identical in the regions illustrated); *M. laxa* Z73785. Bases identical to those in *S. sclerotiorum* are shown as dots, and gaps are indicated by dashes. (a) The sequence around the forward primer site, with primer SSFWD underlined. Bases 1–40 correspond to bases 86–125 on *S. sclerotiorum* sequence M96382. (b) The sequence around the reverse primer site SSREV. The reverse complement of primer SSREV is shown underlined. Bases 1–40 correspond to bases 345–384 on *S. sclerotiorum* sequence M96382.

Specificity tests of the PCR assay

PCR assays using consensus fungal primers (ITS4/ITS5) and *S. sclerotiorum*-specific primers (SSFWD/SSREV) were done using DNA from a range of different fungal species (Table 1) including pathogens of oilseed rape, and other air-dispersed fungi. DNA from all of the species tested was amplified using the consensus fungal primers, but primers SSFWD/SSREV only amplified DNA from *S. sclerotiorum* and not from any of the other species tested (Table 1). The size of the SSFWD/SSREV PCR products from the *S. sclerotiorum* isolates was around 278 base pairs, as predicted.

Increasing amounts of *B. cinerea* DNA (0–2 μg) were tested in the *S. sclerotiorum*-specific PCR assay in the presence and absence of 50 ng *S. sclerotiorum* DNA (Figure 2). Results demonstrated SSFWD/SSREV

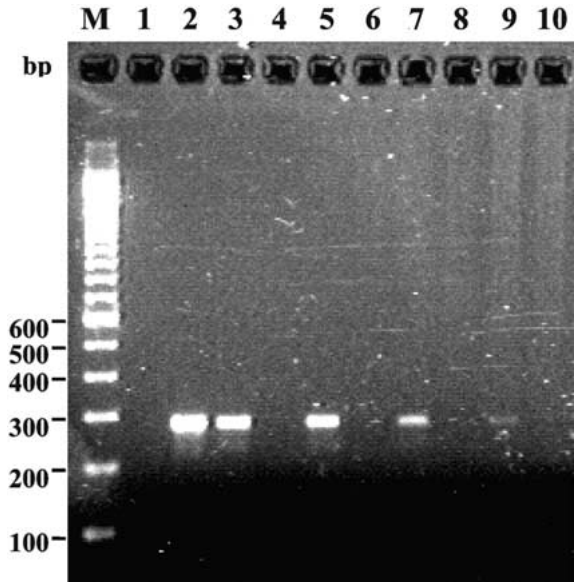

Figure 2. *S. sclerotiorum*-specific PCR using primers SSFWD and SSREV to amplify DNA prepared from mycelium of *S. sclerotiorum* and *B. cinerea*. Various quantities of *S. sclerotiorum* and *B. cinerea* DNA, in a total volume of 5 μl, were added to PCR reactions. Lane M, 100 bp DNA ladder (Life Technologies Ltd., Paisley, UK); lane 1, no DNA control. Ratios of *S. sclerotiorum*: *B. cinerea* DNA are (where 1 = 50 ng DNA): lane 2, 1 : 0; lane 3, 1 : 1; lane 4, 0 : 1; lane 5, 1 : 10; lane 6, 0 : 10; lane 7, 1 : 20; lane 8, 0 : 20; lane 9, 1 : 40; lane 10, 0 : 40.

amplified *S. sclerotiorum* DNA specifically, giving a single PCR product, even in the presence of large amounts of *B. cinerea* DNA.

Assays using untreated spores, disrupted spores and DNA purified from spores

Spore suspensions were prepared which contained 10-fold serial dilutions of *S. sclerotiorum* ascospores ranging from 0 to 2×10^6 spores ml^{-1}, in the presence/absence of *B. cinerea* conidiospores at a concentration of 2×10^6 spores ml^{-1}. PCR assays (primers ITS4/ITS5 and SSFWD/SSREV) were done using: (1) 5 µl of these suspensions directly (each assay was done once); (2) 5 µl of suspension immediately following spore disruption (the ITS4/5 PCRs were done twice and the SSFWD/SSREV PCRs were done once); (3) 5 µl DNA solution purified from a disrupted spore suspension (each assay was done three times). Each test was of the entire experiment, not just the detection step. Previous experience (Williams et al., 2001; Calderon et al., 2002) with *P. roqueforti* suggested that for samples other than pure spore suspensions DNA purification was necessary, therefore fewer tests were done with whole spores or disrupted spores than with purified DNA.

Similar ranges and sensitivities of detection (1 to 10^4 spores per PCR) were observed when using either untreated spores or purified DNA from *S. sclerotiorum* in the PCR assays. The PCR assay appeared to be inhibited when disrupted spore preparations were used; *S. sclerotiorum* was only detected at the highest spore concentration tested (10^4 spores in PCR). When mixtures of suspensions of *S. sclerotiorum* and *B. cinerea* were used, the PCR assay detected around 10 (2/3 tests) or more (all tests) spores in purified DNA samples, but there was no detection, at any of the concentrations tested when the other two treatments were used.

There was no amplification in any of the negative control PCRs containing reagent only or spore-free Nonidet P-40 samples treated in the same way as the spore suspensions. When only purified DNA from 10^4 *B. cinerea* spores was used, there was amplification in all tests using ITS4/5 PCR but no amplification was observed in any of the *S. sclerotiorum*-specific PCRs.

Detection of S. sclerotiorum *ascospores on Melinex tape*

Previous experiments had shown that PCR assays could amplify DNA purified from Melinex tape coated with the mixture used as a trapping surface in Burkard spore traps (Calderon et al., 2002). Spore suspensions were prepared which contained 10-fold serial dilutions of *S. sclerotiorum* ascospores ranging from 200 to 2×10^6 spores ml^{-1}. Aliquots (240 µl) of each spore suspension were deposited on pieces of coated Melinex tape and allowed to dry. DNA was purified from the Melinex tape and from 240 µl of spore suspension, after spore disruption. DNA (5 µl) purified from spore suspensions and from ascospores on Melinex tape was amplified using the *S. sclerotiorum*-specific PCR assay. The sensitivity of the PCR assay was the same whether the DNA was purified from the Melinex tape or from spore suspensions (Figure 3).

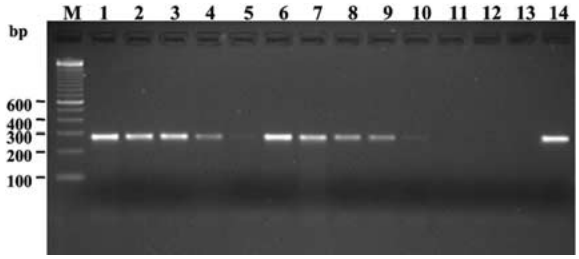

Figure 3. *S. sclerotiorum*-specific PCR using primers SSFWD and SSREV to amplify DNA prepared from *S. sclerotiorum* ascospore suspensions and from *S. sclerotiorum* ascospores deposited on Melinex tape. Lane M, 100 bp DNA ladder (Life Technologies Ltd., Paisley, UK); lanes 1–5 and 11, DNA purified from ascospore suspensions used in PCR; lanes 6–10 and 12, DNA purified from Melinex tape used in PCR; lane 13, no DNA control; lane 14, positive control using 50 ng DNA prepared from *S. sclerotiorum* mycelium; lanes 1 and 6, DNA equivalent of 1×10^4 ascospores added to PCR; lanes 2 and 7, DNA equivalent of 1×10^3 ascospores added to PCR; lanes 3 and 8, DNA equivalent of 1×10^2 ascospores added to PCR; lanes 4 and 9, DNA equivalent of 10 ascospores added to PCR; lanes 5 and 10, DNA equivalent of 1 ascospore added to PCR; lanes 11 and 12, ascospore-free negative controls.

Analysis of field air-samples

The Burkard spore trap was operated in the field for 5 weeks (3 May 2000 to 9 June 2000). Eight pots containing sclerotia and apothecia were placed round the trap on 8 May and a further eight pots on 12 May. DNA was purified from the exposed Melinex tape and amplified using the consensus fungal primers ITS4/ITS5 and the *S. sclerotiorum*-specific primers. Multiple bands were obtained in PCR assays of DNA purified from all 12 h samples during the sampling period using ITS4/ITS5, indicating the presence of DNA from a number of different fungal species.

Table 2. Detection of *S. sclerotiorum* in air samples, collected in a Burkard spore trap, by PCR amplification using SSFWD/SSREV. The trap was operated in an oilseed rape field from 3 May to 9 June 2000. Weeks one to six are consecutive seven-day periods from the beginning of sampling

Week	Day													
	1		2		3		4		5		6		7	
	am	pm	am	pm	am	pm	am	pm	am	pm	am	pm	am	pm
1	nd	−	−	−	−	−	−	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	−	−	−	−	−	−	−	−	−	+	−	+	+	+
4	−	+	−	nd	nd	+	+	−	−	+	+	+	+	+
5	−	+	+	+	−	−	−	+	+	+	+	+	−	+
6	+	+	+	+	−	nd	nd	nd	nd	nd	nd	nd	nd	nd

+ denotes detected, − denotes not detected and nd denotes not done.

Microscopic examination of the exposed Melinex tapes revealed that numerous spores, from a wide range of fungal species, were present as well as other biological and inorganic particles. Small numbers of *S. sclerotiorum*-like ascospores were also observed on some of the tapes. DNA purified from a number of samples collected from 21 May onwards, 12 days after the apothecia-containing pots were put out, gave a single PCR product, of the expected size for *S. sclerotiorum*, when used in SSFWD/SSREV PCR assays (Table 2). No positive results were obtained prior to this using the *S. sclerotiorum*-specific PCR assay. The intermittent pattern of positive results is typical of patterns of airborne *S. sclerotiorum* ascospore concentrations. The delay between placing apothecia in the field and observing bands in the *S. sclerotiorum*-specific PCR could have been caused by adverse weather conditions or the development of fresh apothecia.

Detection of S. sclerotiorum *ascospores on oilseed rape petals*

The aim of these experiments was to determine whether *S. sclerotiorum* ascospores could be detected using the *S. sclerotiorum*-specific PCR even in the presence of a high background of oilseed rape DNA. In the experiments DNA (5 µl) purified from uninoculated oilseed rape petals and petals carrying *S. sclerotiorum* ascospores was tested using the *S. sclerotiorum*-specific PCR assay. The sensitivity of the assay appeared to be slightly better when petals were not incubated at room temperature overnight (Table 3). Using this (no incubation) protocol, reliable detection was achieved when petals carried about 50 ascospores or more, and in one test (out of 5) a petal estimated to contain about 10 spores gave a positive result. Unfortunately, the main period of ascospore release from the artificial inoculations in the field occurred after flowering. Thus, it was not possible to test the PCR assay on field-collected infected petals.

Table 3. Detection of *S. sclerotiorum* ascospores on oilseed rape petals by PCR. Ascospores were placed on oilseed rape petals, then dried, and either stored overnight at −20 °C (0 h), or incubated overnight at room temperature to encourage spore germination (16 h). DNA was then prepared from the samples and tested using the *S. sclerotiorum*-specific PCR assay. For each concentration of ascospores, the replication was of the entire experiment (including the DNA extraction), not just the detection step. The number of positive tests/total number of tests for each ascospore number and incubation time are tabulated

Ascospores per petal	Positive number of tests/ total number of tests Incubation time (h)	
	0	16
0	0/5	0/4
10	1/5	1/4
52	5/5	3/5
260	5/5	4/5
1300	5/5	5/5

Discussion

The development of disease caused by *S. sclerotiorum* is related to concentrations of airborne inoculum (McCartney and Lacey, 1991; 1992; 1999; McCartney et al., 1999), therefore, monitoring of inoculum may aid in disease risk assessment. Semi-selective media have been used for the detection of airborne inoculum of *S. sclerotiorum* by exposing Petri dishes (Ben-Yephet and Bitton, 1985; Gutierrez and Shew, 1998) or using Andersen viable particle samplers (Venette and

Lamppa, 1998). These methods require incubation of large numbers of Petri dishes for several days before colonies can be counted and are therefore time-consuming and laborious. Concentrations of airborne ascospores of *S. sclerotiorum* have also been monitored using Burkard volumetric spore traps and rotating-arm spore samplers (McCartney and Lacey, 1992). These methods are also laborious and require considerable expertise to discriminate between spores of different fungal species, especially ascospores such as those of *S. sclerotiorum*. The experiments reported here demonstrate that it is possible to detect ascospores of *S. sclerotiorum*, even in the presence of a large amount of other biological material and inorganic particles, by purifying DNA from traditional spore traps and using specific PCR assays to detect it.

The *S. sclerotiorum*-specific primers were designed using the ITS region of rDNA. The ITS regions of nuclear rDNA evolve relatively rapidly and thus are highly variable showing differences between closely related species and sometimes within species (Ward and Adams, 1998; Ward and Bateman, 1999). This makes the ITS region a good candidate for developing specific PCR primers. In addition, rDNA is present in multiple copies in most fungal genomes, therefore, PCR assays that amplify rDNA should be more sensitive than those directed towards single-copy genes. Although the SSFWD and SSREV primers were designed specifically to detect *S. sclerotiorum*, they were also identical to sequences from a few other closely related *Sclerotina* species (*S. trifoliorum*, *S. minor* and *S. glacialis*) and should therefore also detect these fungi. However, the closely related fungus *B. cinerea*, airborne conidiospores of which are common in the UK, was not detected by the PCR assay, nor were a range of other oilseed rape pathogens and common airborne fungi. In oilseed rape crops the ability to detect other *Sclerotinia* species may not be a problem as other *Sclerotinia* species are typically not found on oilseed rape crops because of their lack of spores, their host range or both (Bom and Boland, 2000). The host range of field crops for *S. trifoliorum* appears to be restricted to forage legumes (Willetts and Wong, 1980). Although *S. minor* has been reported on oilseed rape in Australia (Davies, 1986), infection of its main hosts is generally via mycelial germination with little ascospore production (Willetts and Wong, 1980). The assay may also be of use in detecting these other *Sclerotinia* species in other situations.

The risk of stem-rot disease in canola (oilseed rape) has been related to the percentage petal infestation at early bloom (Turkington et al., 1991) and a commercial risk assessment system, based on culturing petals has been developed (Morrall and Thompson, 1991). Immunoassays using polyclonal antibodies have also been used to detect *S. sclerotiorum* ascospores on petals (Lefol and Morrall, 1996; Bom and Boland, 2000). Lefol and Morrall (1996) used immunofluorescence to detect ascospores, but this required microscopic examination of the petals which is time-consuming. Bom and Boland (2000) found little correlation between ELISA absorbance and either petal infestation or disease and attributed this, in part, to poor specificity of the polyclonal antibodies tested. *S. sclerotiorum* ascospores were successfully detected on oilseed rape petals using the *S. sclerotiorum*-specific PCR assay developed here. Reliable detection was achieved when petals contained 50 ascospores or more. Recent controlled environment studies of oilseed rape infection by ascospore-bearing petals suggest that between 50 and 100 spores per petal are needed to cause successful infections (McCartney et al., 2001). Thus, it appears that this PCR-based assay for oilseed rape petal infections by *S. sclerotiorum* has potential in assessing the risk of stem-rot development in oilseed rape crops. The method is faster than culturing and not subject to contamination by *B. cinerea*, which can be a problem with culture methods in the UK (P. Gladders, ADAS Boxworth, UK, pers. comm.). However, further work is needed to confirm the sensitivity in field situations.

This study demonstrates that it is possible to detect inoculum of *S. sclerotiorum* from both air-samples, collected using Burkard spore traps, and on oilseed rape petals. The spore removal and DNA purification methods should also work with other spore trapping formats that collect samples on surfaces such as tape or filters, provided that the collecting medium does not inhibit the PCR. The PCR-based assays, therefore have the potential to be incorporated into risk assessment systems for *Sclerotinia* diseases by enabling airborne inoculum to be relatively easily monitored (compared to conventional methods), and, in the case of *S. sclerotiorum* on oilseed rape, by assessments of percentage petal infestation at early bloom. However, further work is still needed to assess the sensitivity under field conditions, to determine detection thresholds for epidemic development, and to assess the risk of interference by non-target *Sclerotinia* species in the field.

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 quantity. However, it may be possible to adapt the

methods for use in real-time PCR assays that would allow inoculum to be quantified (Heid et al., 1996; Haugland et al., 1999).

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