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Detection and quantification of airborne inoculum of *Sclerotinia sclerotiorum* using quantitative PCR

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This paper reports the development of a new specific diagnostic technique to accurately quantify airborne inoculum of *Sclerotinia sclerotiorum* and discusses its potential use in disease-forecasting schemes, using examples of three contrasting epidemic seasons: 2007, when there was a severe epidemic of sclerotinia stem rot (SSR) in England and high numbers of airborne ascospores were trapped at Rothamsted, and, in contrast, 2003 and 2004, when the incidence of SSR in England was low and low numbers of airborne ascospores were trapped at Rothamsted. DNA was extracted from wax-coated plastic tapes, such as those used in Burkard (Hirst-type) spore traps and rotating-arm traps. A SYBR-green quantitative PCR (qPCR) method produced a linear relationship between ascospore numbers and *S. sclerotiorum* DNA (mean 0·35 pg DNA per spore) and was able to detect DNA representing as few as two ascospores. The technique was insensitive to DNA of the host plant, *Brassica napus*, and other plant pathogens, including *Sclerotinia minor*, *S. trifoliorum* and *Botrytis cinerea*, and common airborne fungal genera such as *Cladosporium* and *Penicillium*. There was no relationship between rainfall and numbers of airborne ascospores of *S. sclerotiorum* present at Rothamsted during the period of infection in the severe SSR season (2007).

Keywords: aerobiology, ascospore, molecular diagnostics, qPCR, sclerotinia stem rot, SYBR green

Introduction

Sclerotinia sclerotiorum is an ascomycete plant pathogen with a host range of over 400 plant species worldwide (Boland & Hall, 1994). It causes sclerotinia stem rot (SSR), a serious disease of oilseed rape (*Brassica napus*, canola) and also economically important diseases of sunflower, carrot, pea and lettuce. In western Europe, the severity of SSR epidemics differs from season to season and from region to region. Epidemics are initiated by airborne ascospores, released from fruiting bodies in the spring. The fungus survives crop-free periods as sclerotia in soil and crop debris, which, under suitable conditions, germinate carpogenicly to produce an apothecium, from which ascospores are released (Clarkson *et al*., 2007). Ascospores are not able to infect healthy tissues directly, but can infect senescent tissues such as petals, which provide a nutrient base enabling the production of oxalic acid and cell-wall-degrading enzymes that kill a zone of adjacent healthy plant tissue into which the pathogen can grow (Hegedus & Rimmer, 2007). Typically, petals either colonized by or coated with ascospores vector the pathogen onto lower leaves and branches to cause SSR. Control of SSR depends on the use of fungicides as there is no known race-specific host resistance (Hegedus & Rimmer, 2007). Numerous disease-forecasting schemes have been developed to aid SSR-related crop protection decisions in different countries, mostly based on weather conditions conducive to spore release coinciding with petal fall and adhesion of petals onto lower leaves (Turkington *et al*., 1991a,b; Nordin *et al*., 1992; Turkington & Morrall, 1993; Twengstrom *et al*., 1998). However, the pathogen population shows wide variation in responses to cool and warm periods needed to trigger sclerotial germination and spore release (Clarkson *et al*., 2007), which coupled with regional differences in climate and variations in microclimates within fields, leads to a wide variation in the timing of spore release. Consequently, inoculum-based forecasting schemes may be more accurate in predicting epidemic risk.

Air-sampling devices, such as the Burkard 7-day volumetric trap (Hirst-type), or rotating-arm sampler, impact particles present in air onto a thin layer of grease or wax coating a transparent plastic film attached to a supporting surface (Lacey & West, 2006). Traditionally, the wax-coated film is then mounted as a microscope slide and particles of interest identified and counted by

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microscopy. Airborne ascospores of *S. sclerotiorum* are difficult to identify by microscopy as they are similar to other ascospores and particularly those of closely related *Botrytis* species. Fortunately, it is now possible to integrate such traditional air-sampling formats with PCR-based molecular diagnostic techniques (Calderon *et al*., 2002; Fraaije *et al*., 2005; West *et al*., 2006). DNA replication during PCR can now be quantified by quantitative PCR (qPCR) to provide data on the abundance of the target, e.g. indicating the amount of DNA of a single species in an environmental sample, rather than simply its presence. Quantitative PCR was shown to be a valuable method to measure plant pathogen growth in plant tissues to evaluate host resistance (Fraaije *et al*., 2001), and to estimate abundance of fungal inoculum in environmental samples such as soil (Lievens *et al*., 2006; Chilvers *et al*., 2007). Freeman *et al*. (2002) reported a PCR assay for detecting spores of *S. sclerotiorum* that could be applied to air samples. This provided a qualitative indication of the presence (of at least 10 ascospores per reaction) or absence of inoculum, which has limited potential for forecasting and epidemiological studies compared to a quantitative diagnostic technique. However, the touch-down PCR method used by Freeman *et al*. (2002), which was required to avoid co-detection of *Botrytis cinerea*, was not suitable to be adapted for qPCR using the fluorescence-based SYBR green system, although other quantitative touch-down PCR methods have been developed (Larsen *et al*., 2004).

This paper reports the development of a new specific and quantitative diagnostic technique that can accurately quantify DNA of *S. sclerotiorum* in a background of DNA of other organisms (mainly plants and other fungi) and discusses its potential for use in disease-forecasting schemes using examples of three contrasting epidemic seasons: 2007, in which there was a severe epidemic of SSR in England, and, in contrast, 2003 and 2004, in which the incidence of SSR in England was low (Home Grown Cereals Authority, 2007).

Materials and methods

Primer design

Forward and reverse primers were designed from sequence data submitted to the GenEMBL database. The sequence (SSU07553) was for a *S. sclerotiorum* gene encoding for the mitochondrial small subunit rRNA intron and ORF1. The sequence was subjected to BLAST analysis (BLASTN) then aligned against related sequences. Forward and reverse sequences were selected over regions of specificity for *S. sclerotiorum* and subjected to BLAST analysis against the database to determine interactions with other sequences. Two primer sites were selected, mtSSFor (5′-AGGT AACAAGTCAGAAGATGATCGAAAGAGTT-3′) and mtSSRev (5′-GCATTAAGCCTGTCCCTAAAAACAAGG-3′) as having potentially good specificity towards *S. sclerotiorum* and no other sequences in the GenEMBL database.

Primer optimization

For determining the optimum annealing temperature for the primers, a PCR was set up on a gradient block PCR machine (MJ Research Inc.) with the gradient set to run between 45 and 55°C. PCR reaction volumes consisted of 20 μL containing 0·1 μM primers, 10 μL of 2× PCR reaction buffer and RedTaq (1·5 mMol Mg^{2+}; Sigma) and 1 μL DNA. PCR conditions were as follows: 95°C followed by 40 cycles of 94°C for 1 min, annealing temperature for 1 min and 72°C for 1 min, followed by a final extension step of 72°C for 5 min. The reaction was then cooled to 4°C until needed. PCR products were viewed on a 1·5% agarose gel in TBE buffer. Duplicate reactions were performed using *B. cinerea* as template DNA for comparison.

Optimization of qPCR and sensitivity testing

Quantitative PCR was performed in Bioplastic (EU) 96- × 0·2-mL PCR plates capped with Bioplastic EU optical, thin-wall eight-cap strip (Bioplastics) with the qPCR machine Mx3000P (Stratagene). SYBR-green mix (Sigma-Aldrich) was used for qPCR reactions. Primer concentrations were optimized according to the manufacturer's guidelines. Primers mtSSFor and mtSSRev were each included at a final concentration of 300 nM. Reference dye was added in accordance to the manufacturer's recommendations for use on the Mx3000P machine. The reaction mix was made up to 20 μL using ultrapure dH_2O.

PCR conditions were set up using the optimal annealing temperature determined in the gradient PCR (above). Conditions comprised 95°C for 2 min followed by 60 cycles of 95°C for 15 s, 50°C for 30 s and 72°C for 30 s. A dissociation curve produced after the first reaction demonstrated that the qPCR product had a sharp peak at 81°C. Therefore, an additional read step was added to the qPCR at 79°C to melt off any exogenous products, such as primer dimers, that could falsely contribute to the calculation of target DNA present. Reactions were performed in duplicate. The cycle threshold (CT) value for each qPCR was automatically calculated and analysed by the Stratagene MXPRO software (version 3·20).

The effect of *B. cinerea* DNA on the sensitivity of the method was tested by comparing the quantification of a dilution series of *S. sclerotiorum* DNA (seven concentrations from 50 ng to 5×10^{-5} ng per reaction) alone and when each concentration was mixed with 5 or 0·5 ng of *B. cinerea* DNA. The sensitivity of the method was also compared with the sensitivity of the qualitative touch-down PCR method reported by Freeman *et al*. (2002) by testing the detection of a similar dilution series of *S. sclerotiorum* DNA (50 ng to 5×10^{-5} ng per reaction) alone or mixed with 5 or 0·5 ng DNA of *B. cinerea*, and visualizing the PCR products by gel electrophoresis.

DNA extraction for primer sensitivity testing

A new method was used to extract DNA from artificially ascospore-coated tape sections (production method

described later) in 2-mL screw-top tubes using 60 μL of a commercial detergent-based product, MicroLYSIS (Microzone) combined with an additional step in which 0·1 g acid-washed, 400- to 600-μm-diameter Ballotini Beads were added and shaken in a Fast Prep machine (FP120-Bio 101, Qbiogene) for 20 s at 4 m s^{-1}. The liquid was transferred to a 0·2-mL PCR tube and exposed to thermal cycling in a PCR block according to the manufacturer's protocol (65°C for 15 min, 96°C for 2 min, 65°C for 4 min, 96°C for 1 min, 65°C for 1 min, 96°C for 30 s, 20°C hold). Additionally, 2 mg polyvinylpyrrolidone (PVPP, Sigma-Aldrich) and 40 μL TE buffer (10 mM, pH 8·0) was added, vortexed and spun at 15 115 *g* for 15 min to remove polysaccharides, which can inhibit the PCR reaction. A 60-μL portion of the supernatant was removed to a new 0·2-mL tube to which 150 μL ethanol and 10 μL ammonium acetate (7·5 M, dissolved in water) was added, vortexed and spun at 15 115 *g* for 15 min. The supernatant was discarded and the remaining pellet air-dried and resuspended in 10 μL water. This was kept frozen at –20°C and 2·5 μL used per qPCR reaction.

DNA extraction for primer testing on archived air samples

DNA was previously extracted from daily outdoor Burkard trap samples from Rothamsted in 2003, 2004 and 2007 using a method based on that of Williams *et al*. (2001), but with numerous adaptations, hence the new method is reported in full here. Tape subsections 48 × 7 mm were cut into six equal-sized pieces and placed in a sterile 2-mL screw-topped tube. Acid-washed 400- to 600-μm-diameter Ballotini beads (50 μL), CTAB extraction buffer (500 μL, 2% CTAB, 100 mM Tris HCl, 1·4 mM NaCl, 20 mM EDTA) and β-mercaptoethanol (10 μL) were added to each tube. Samples were then subjected to two 40-s Fast Prep cycles before being incubated at 70°C for 60 min. Samples were then centrifuged for 15 min at 15 115 *g*. The supernatant was collected and extracted against 500 μL chloroform:isoamyl alcohol (24:1) and vortexed. Samples were centrifuged for a further 15 min and the upper aqueous phase (450 μL) was collected. To initiate DNA precipitation, 45 μL of 3 M sodium acetate and 900 μL ice-cold 100% ethanol were added and the samples were mixed by gentle inversion. Samples were then placed at –20°C for 1 h. DNA was pelleted by centrifugation at 15 115 *g*. for 10 min and the supernatant was discarded. DNA pellets were washed with ice-cold 70% ethanol. Pellets were allowed to dry at 37°C for 10–15 min and dissolved in 100 μL sterile dH_2O. The DNA was then archived at –20°C. Subsamples of 2·5 μL of the archived DNA were taken from alternate days in April and May in 2003 and 2004, and were tested by qPCR using the method above to quantify *S. sclerotiorum* DNA.

Air-sample processing and field observations

The qPCR method was initially tested on DNA extracted from *S. sclerotiorum* ascospores artificially deposited onto wax-coated plastic tapes as used in the Burkard spore sampler (Lacey & West, 2006). The ascospores were previously collected onto cellulose filters as reported in Freeman *et al*. (2002): cold-conditioned sclerotia were surface-sterilized and incubated in containers of moistened, sterile, coarse perlite in the dark at 15°C until stipes appeared, then placed under nUV light to encourage production of ascospores, which were harvested by suction onto the cellulose filters (Millipore Ltd). Once collected, ascospores were frozen at –20°C. An ascospore suspension was made in 10 mL distilled water by briefly vortexing quarter sections of the filters in sterile 50-mL plastic centrifuge tubes (Greiner Bio-One). Ascospore concentration was determined using a haemocytometer slide (*c.* 2×10^5 ascospores mL^{-1}) and a dilution series of 1/10 and 1/100 of the original concentration was made. The ascospore suspensions were sprayed onto wax-coated plastic tapes attached to the collection drum of a Burkard sampler, using a Chrom atomizer (CamLab), until tape sections were evenly coated with a fine deposit of spray droplets. These were allowed to dry in air before the tapes were removed from the collection drum and cut into 48-mm sections (the same length as for a 24-h Burkard trap sample). Unexposed wax-coated tapes were used to provide negative controls.

Each artificially ascospore-coated tape section was held at the edge with forceps and cut longitudinally along the centre line using scissors to give two subsections (each 7 × 48 mm). One subsection of tape was placed into a 2-mL screw-top tube (waxed surface facing the inside of the tube), labelled and stored at –20°C. The duplicate subsection was mounted on a microscope slide, stained with trypan blue in gelvotol (polyvinyl alcohol) and numbers of ascospores present were estimated by microscopy (Lacey & West, 2006). The total number of *S. sclerotiorum* ascospores was counted in two 200-μm-wide longitudinal traverses of the tape sections. The area of tape counted was, therefore, 5·7% of the total for the subsection, so the number of ascospores counted was then multiplied by 17·5 to provide an estimate of ascospore numbers per subsection of tape (the number per subsection of tape divided by 7·2 equates to the number of ascospores m^{-3} of air when derived from a Burkard trap operating at a flow rate of 10 L min^{-1}).

Air sampling and SSR incidence in oilseed rape crops

Air samples were taken from Burkard 7-day continuously recording spore samplers (Burkard Manufacturing Co. Ltd) operating outdoors according to standard methods reported in Lacey & West (2006). The outdoor air sampling site was at an area of grassland near to arable fields at Rothamsted in spring 2003, 2004 and 2007. Daily samples from the operational spore traps were treated in the same way as the artificially ascospore-coated tape sections, described above, to provide daily subsections 7 × 48 mm from which DNA was extracted and a duplicate subsection mounted as a microscope slide. A small subset of slides made with subsections of daily air

Table 1 Detection of DNA from different fungi and one plant species tested by PCR and qPCR using the new primer set

Species (isolate)	Detection by PCR	Detection by qPCR
Sclerotinia sclerotiorum (S3)	✓	✓
S. sclerotiorum (M24)	✓	✓
S. sclerotiorum (1999B)	✓	✓
S. sclerotiorum (Phy 1)	✓	✓
S. sclerotiorum (Great Harpenden)	✓	✓
S. sclerotiorum (31)	✓	✓
S. sclerotiorum (M23)	✓	✓
S. sclerotiorum (M1/44)	✓	✓
Botrytis cinerea (IMI 181038)	✓	x
B. cinerea (strawberry06a)	✓	x
B. cinerea (directly from grapes)	✓	x
B. cinerea (directly from *Pelagonium*)	✓	x
B. cinerea (directly from tomato leaf)	✓	x
S. minor (2317)	x	x
S. trifoliorum ('clover')	x	x
S. trifoliorum (R316)	x	x
Penicillium spp.	x	x
Cladosporium spp.(mixed 'rough' and 'smooth' spore types)	x	x
Leptosphaeria maculans	x	x
Pyrenopeziza brassicae	x	x
Brassica napus	x	x

x = not detected within 60 cycles of qPCR.

samples from Burkard spore traps were counted by microscope. Spores were only counted as ascospores of *S. sclerotiorum* if they were elliptical in shape within a size range of 7–10 μm by 3·3–4·6 μm and of similar pigmentation to reference slides. Confidence in the accuracy of count data from outdoor samples was low, however, because dense masses of pigmented spores and hyphal fragments may have masked the spores of *S. sclerotiorum* and because spores of other species might have looked identical to *S. sclerotiorum* ascospores. Ascospore numbers were therefore estimated from the mean DNA per ascospore found on artificially inoculated tapes.

The incidence of SSR in oilseed rape fields at Rothamsted (within 1 km of the air sampling site) was monitored each year. SSR was identified by the presence of white lesions girdling stems without any sporulating structures and often with a pattern of broad white and pale grey bands, produced as the lesion expanded. Meteorological data were collected by the Rothamsted synoptic meteorological station situated *c.* 1 km from the field experiments and air samplers in each season.

Results

Primer optimization and qPCR

A PCR fragment of 125 bp was generated from all eight UK isolates of *S. sclerotiorum* tested with primers mtSSfor and mtSSrev. A smaller PCR product (80 bp) was generated from DNA extracted from *B. cinerea*, but no PCR fragments were generated from any of the other fungal species tested, nor from *B. napus* DNA (Table 1). The primers were optimized for qPCR using the conditions outlined for use with the Stratagene qPCR machine. The optimum concentration for both primers was 300 nm per reaction and an additional read step (of 79°C for 30 s) was included in the qPCR reaction to melt away any exogenous PCR products that were not *S. sclerotiorum*. At this temperature no product was detected from samples containing *B. cinerea* DNA only, demonstrating that this primer set could be used for detection of *S. sclerotiorum* in mixed samples. The quantification of *S. sclerotiorum* DNA over a range from 50 ng to 5×10^{-5} ng was not affected by the presence of 0·5 ng *B. cinerea* DNA, compared to quantification of samples without *B. cinerea* DNA present (Fig. 1). However, 5 ng *B. cinerea*

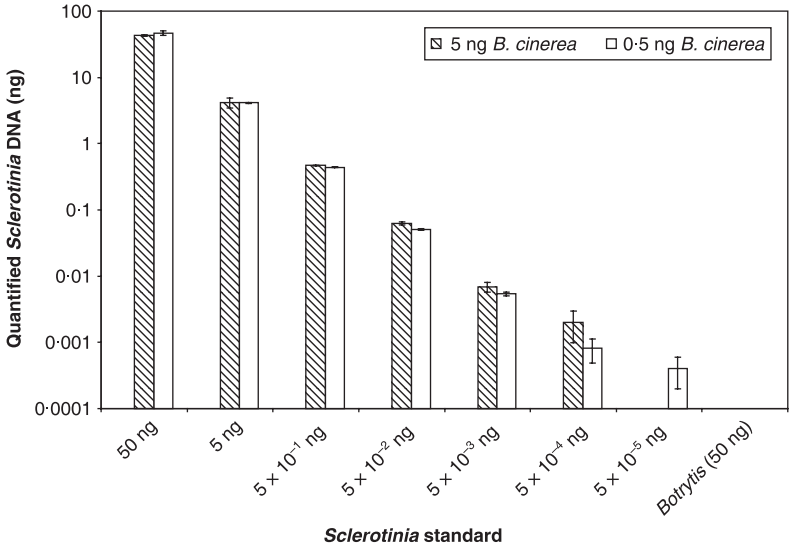

Figure 1 Quantification of a range of amounts of *Sclerotinia sclerotiorum* DNA ('*Sclerotinia* standard') in the presence of 5 or 0·5 ng *Botrytis cinerea* DNA. A sample of 50 ng *B. cinerea* DNA alone was also tested. Bars represent standard errors of means.

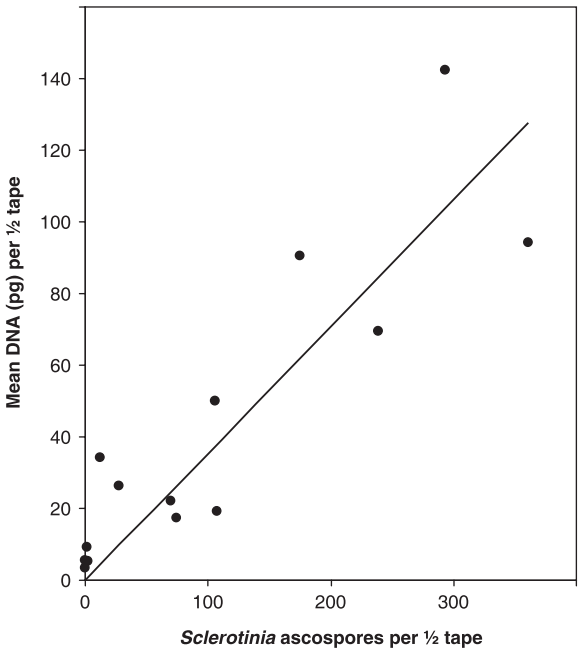

Figure 2 Relationship between numbers of ascospores estimated by microscopy (based on mean ascospore counts of two longitudinal traverses per half-tape section) on artificially-coated waxed tapes and amount of DNA of *Sclerotinia sclerotiorum* quantified by qPCR from the opposite half tape section (equation of fitted line is: y = 0·3538x; R^2 = 0·76, P < 0·001).

DNA reduced the quantification of *S. sclerotiorum* at the lowest amount tested, 5×10^{-5} ng, which was close to the limit for detection of pure *S. sclerotiorum* DNA. Quantification of *S. sclerotiorum* DNA over a range from 50 ng to 5×10^{-4} ng was not affected by 5 ng *B. cinerea* (Fig. 1).

The qualitative PCR method (Freeman *et al.*, 2002), was also found to be unaffected by the presence of *B. cinerea* DNA, but was less sensitive than the qPCR method reported here, with no amplification of PCR products visible for dilutions below a value of 0·05 ng (data not shown).

The specificity of the qPCR method was confirmed with DNA extracted from eight different UK isolates of *S. sclerotiorum*, two isolates of *B. cinerea*, putative *B. cinerea* conidia on a range of plant material, two other species of *Sclerotinia* (*S. minor* and *S. trifoliorum*), two other oilseed rape pathogens (*Leptosphaeria maculans* and *Pyrenopeziza brassicae*), two genera of fungi commonly occurring at high spore concentrations in air (*Cladosporium* and *Penicillium*) and *B. napus*, as listed in Table 1.

Artificially inoculated tape sections

Ascopore numbers estimated per tape subsection by microscope were directly related to *S. sclerotiorum* DNA quantities measured by qPCR in the ratio 0·35 pg DNA per spore (Fig. 2; R^2 = 0·76, P < 0·001).

Outdoor Burkard spore-trap samples, weather and SSR incidence

The pattern of airborne ascospores of *S. sclerotiorum* at Rothamsted showed low numbers of ascospores present in air during most of the flowering period in 2003 and 2004 (Fig. 3). A maximum number of four *S. sclerotiorum* ascospores m^{-3} day^{-1} was detected in air only at the start of flowering (mid-April) in 2003. Even fewer ascospores of *S. sclerotiorum* were detected over the sampling period in 2004. Oilseed rape experiments at Rothamsted in 2003 and 2004 had negligible reported incidences of SSR

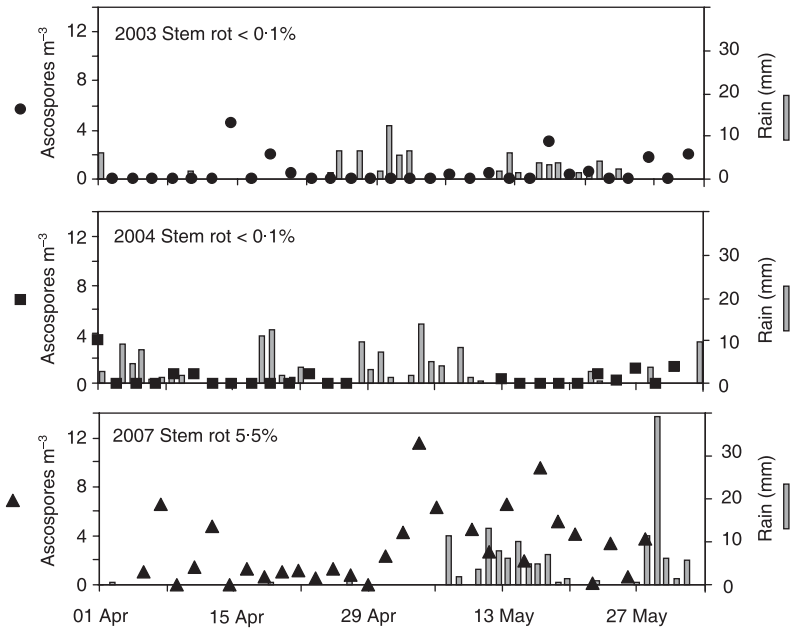

Figure 3 Changes in numbers of airborne ascospores of *Sclerotinia sclerotiorum*, rainfall and stem rot incidence in untreated oilseed rape at Rothamsted in 2003, 2004 and 2007. Ascospore numbers were estimated from the mean DNA per ascospore found on inoculated tapes (Fig. 2) and were not sampled in air directly within crops, but at a nearby site less than 1 km away.

(< 0·1% plants affected). In contrast, the pattern of airborne DNA of *S. sclerotiorum* at Rothamsted in 2007 indicated that high numbers of ascospores were frequently present in air during the flowering period (early April to mid-May, peaking at 12 ascospores m^{-3} day^{-1}) and SSR incidence on untreated plants was unusually high for the region at 5·5% (Fig. 3). There was no substantial rain event during April 2007, when the large numbers of airborne ascospores were detected (recorded daily rainfall was < 0·2 mm, except for 0·4 mm on 2 April, 0·4 mm on 18 April and 0·8 mm on 26 April). Plots treated with fungicide (boscalid as Filan; BASF) on 27 March 2007 (growth stage 3·6–3·7; green-yellow bud; Sylvester-Bradley & Makepeace, 1985) had a lower incidence of SSR (2%) than untreated plots (5·5%) (based on samples of 20 plants in each of three treated or three untreated plots each of 20 different cultivars, i.e. 1200 plants each of treated or untreated plots; $P < 0{\cdot}001$). There was no cultivar difference in SSR.

Discussion

Quantitative PCR was found to be a rapid and accurate method to quantify inoculum of *S. sclerotiorum*. This paper reports the first successful primer set for quantifying the amount of *S. sclerotiorum* DNA from environmental samples (in this case collection tapes from spore traps) using qPCR. The method was also more sensitive than the PCR diagnostic method previously reported by Freeman *et al.* (2002) as it was able to detect DNA of *S. sclerotiorum* at amounts as low as 5×10^{-4} ng (0·5 pg; or representing 1·4 ascospores) in the presence of DNA of *B. cinerea*. The improved sensitivity combined with the ability to quantify inoculum by the qPCR method reported here is an important development because it allows very low amounts of inoculum to be detected and quantified. Low numbers of spores over a long period may be enough to cause a big SSR epidemic (i.e. the area under the spore curve is a key consideration), but low numbers of spores would not be detected using previously published methods. Furthermore, lower concentrations of spores are likely to occur in air at higher altitudes than immediately above the ground, where they are released, which means that air samplers deployed to sample air some distance from inoculum sources, such as on the roofs of tall buildings, may be used singly to provide a more general regional measurement of inoculum availability, rather than needing air samplers in numerous oilseed rape fields within a region. The primers used in the qPCR method reported here also produced a product with DNA from *B. cinerea* if used in end-point PCR. However, since the PCR products of the two species were different sizes and had different melting points, optimization of the qPCR conditions to include a heating step of 79°C per cycle prevented any quantification of *B. cinerea* DNA, along with any primer dimers that would otherwise produce fluorescence in the presence of the SYBR green dye. Hence, the qPCR method reported here was specific for quantification of DNA of *S. sclerotiorum*. The results from adding known amounts of *B. cinerea* DNA to different amounts of *S. sclerotiorum* DNA demonstrated that there was no reduction in sensitivity of *S. sclerotiorum* detection caused by the presence of DNA of this closely related species, except when extremely high amounts of *B. cinerea* (5 ng) were present with very low amounts of *S. sclerotiorum* (5×10^{-5} ng). It seems extremely unlikely that the amount of *B. cinerea* DNA necessary for this slight reduction in sensitivity of the technique would occur in air, particularly when sampled at 10 L min^{-1} above a crop. Furthermore, the reduction in sensitivity of *S. sclerotiorum* detection in the presence of relatively high amounts of *B. cinerea*, only occurred very close to the limit of detection of pure *S. sclerotiorum* DNA.

The qPCR method tested on artificially inoculated plastic-tape sections produced under laboratory conditions indicated that 0·35 pg DNA was quantified per *S. sclerotiorum* ascospore. The variability in the relationship shown ($R^2 = 0{\cdot}76$) can be explained by uneven distribution of the sprayed ascospores onto the tape surface, which could result in slight differences between numbers of ascospores counted by microscopy on one half of the tape and numbers detected by qPCR on the other half. The amount of DNA per ascospore was used to calculate ascospore numbers from DNA quantified from outdoor air, sampled using a Burkard spore trap, allowing as few as 0·1 ascospores m^{-3} air (1·4 ascospores per daily air sample) to be detected. In comparison, microscopic quantification of *S. sclerotiorum* ascospores in outdoor air samples containing other ascospores was less accurate than qPCR. This was partly because ascospore identification is difficult and also because counts made in two microscope traverses of the tape (typically 2–4 h of work) represented only 2·9% of the total trap surface counted [$(0{\cdot}4 \times 48$ mm$)/(14 \times 48$ mm$)$], hence it was usually not possible to detect fewer than 30–40 ascospores per air sample (i.e. representing 2–4 ascospores m^{-3} air sampled per day). *Brassica napus* DNA was included in sensitivity tests because large numbers of oilseed rape pollen grains would be expected in air at the same time as *S. sclerotiorum* inoculum, particularly if air samplers were located within an oilseed rape crop. The fact that *B. napus* DNA did not generate PCR product using the *S. sclerotiorum*-specific primer set, while large numbers of *S. sclerotiorum* ascospores could be quantified accurately on artificially inoculated tapes, suggests that the technique might be adapted to measure amounts of *S. sclerotiorum* DNA in host tissues to assess cultivar resistance in a similar way to that reported for other pathogens by Fraaije *et al.* (2001). The sensitivity of a PCR primer set for detecting DNA of airborne ascospores is also affected by the copy number of the target DNA sequence in the ascospore. In this specific case, it is not currently known how many copies of the target sequence are present per ascospore of *S. sclerotiorum*. Sensitivity is also affected by the method used to extract DNA from the sample. Both DNA extraction methods used in this study were found to be equally reliable (data not shown). There is potential for products present in a range of naturally occurring airborne particles to inhibit

the PCR reaction, resulting in a false negative result. The extraction methods and dilutions used in this study were not shown to produce any false negatives from air samples tested previously by the authors.

The results of tests of outdoor air samples show that this method has potential for use in inoculum-based forecasting schemes similar to those used in the USA for tobacco blue mould and soyabean rust (http://www.ces.ncsu.edu/depts/pp/bluemold/; http://www.ces.ncsu.edu/depts/pp/soybean-rust/howtoreadforecast.php). However, although some information is provided on the detection of inoculum of *S. sclerotiorum* in air as an example of an application of the new diagnostic method, other air-sampling formats than the Burkard (Hirst-type) spore trap may be integrated with DNA-based diagnostics. Formats such as wet cyclones and rotating-arm samplers typically sample much high volumes of air than the Burkard trap and so may provide increased sensitivity in terms of detection of low number of spores per m^3 air.

Many existing SSR forecasts include a component of rainfall in the decision process (Nordin *et al.*, 1992; Turkington & Morrall, 1993; Twengstrom *et al.*, 1998; Koch *et al.*, 2007). However, this study showed that in 2007, when there was an unusually severe and unpredicted SSR epidemic in the UK (Anonymous, 2007; Home Grown Cereals Authority, 2007), ascospores were present in air at Rothamsted during the flowering period of oilseed rape in high numbers, despite a lack of significant rainfall. This suggests that ascospores may be either transported in air from distant sources, or produced in large numbers from irrigated areas such as horticultural-production sites. The fact that in 2007 there was a widespread epidemic of SSR over most of England (HGCA-Defra CropMonitor data) and western Europe, suggests that airborne inoculum, while not necessarily from the same source, must have been present over whole regions. Further work is required to indicate how numbers of spores in air relate to the subsequent level of SSR infection (this depends on the proximity of the air sampler to the source of spores and the crop and in this case also the occurrence of conditions conducive to petals sticking to leaves and stems). The relationship between airborne inoculum and SSR disease is reported here only as an example application the new qPCR method. The observation that plots treated with fungicide in late March, 2007 (before flowering) showed reduced incidence of SSR can be explained by the fungicide protecting the leaves onto which infected petals subsequently fell, confirming the airborne infection pathway.

In addition to the example application tested in this study, the qPCR method reported here has potential for evaluating the presence and quantity of *S. sclerotiorum* in a wider range of environmental samples, such as soil, seeds or other plant tissues, such as petals or stems. In the case of airborne inoculum, further research is required to develop methods to rapidly apply the *S. sclerotiorum*-specific qPCR to air samples and to confirm that airborne inoculum is a reliable indicator of SSR risk by testing over a wider geographical range and a number of seasons.

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