

Polymerase chain reaction (PCR)-based assays for the detection of inoculum of *Sclerotinia* spp

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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 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 from 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.

Key words: *Sclerotinia sclerotiorum*, stem rot, ascospores, spore trapping, *Botrytis cinerea*

INTRODUCTION

Airborne ascospores produced by carpogenic germination of sclerotia are the major source of inoculum for infection of crop plants by *Sclerotinia sclerotiorum*. Infection occurs when ascospores germinate on non-living or senescent plant parts, and then infect healthy plant tissue. For example, in oilseed rape crops ascospore-bearing petals stick to leaves causing initial infections. Monitoring of airborne or, for oilseed rape crops, petal-borne inoculum offers a direct measure of the risk of crop infection. Methods of detection of airborne inoculum based on microscopy or culturing are time-consuming, labour intensive and subjective. Methods based on DNA analysis have the potential to be used to detect airborne fungal spores. We report development and testing of a PCR-based assay for the detection of inoculum of *S. sclerotiorum* in air samples and on oilseed rape petals.

MATERIALS AND METHODS

Primer design: Ribosomal DNA (rDNA) 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 (Genetics Computer Group, 1994) searches using the previously published *S. sclerotiorum* sequence M96382. Sequences were aligned using the programme PILEUP in the GCG package. Potential *S. sclerotiorum*-specific primers were chosen using the sequence alignment and analysed using the program Net Primer (<http://www.premierbiosoft.com>). The specificities of the primers were checked using FASTA and BLAST searches of the EMBL/ Genbank databases.

PCR optimisation and specificity testing: A touchdown PCR was optimised for use with *S. sclerotiorum*-specific primers SSFWD and SSREV (see below), details of the protocol are given by Freeman *et al* (2002). The specificity of the PCR-assay was tested using DNA purified from a range of fungal species, including pathogens of oilseed rape (Freeman *et al*, 2002). DNA was purified from fungal mycelia using a method based on that described by Lee and Taylor (1990) (Freeman *et al*, 2002).

Sampling airborne ascospores. *S. sclerotiorum* ascospores were collected in an oilseed rape crop using a Burkard 7-day recording spore trap (Burkard Manufacturing Co., Rickmansworth, UK), operated close to 10cm diameter plastic pots containing *S. sclerotiorum* apothecia. The traps collected airborne particles on wax-coated Melinex tape attached to a slowly rotating drum. The spore trap tapes were cut into sections representing 12h exposure periods. Each section was cut in half along its centreline. One half was examined under a light microscope half was used for PCR analysis (see below).

Oilseed rape petal testing: Oilseed rape petals were inoculated with *S. sclerotiorum* ascospore suspension in to give approximately 1300, 260, 50, 10 or 0 ascospores per petal. DNA was purified from the petals and used in the *S. sclerotiorum* specific PCR assay (see below).

Purification of DNA from spores, air samples and petals: DNA for use in PCR assays, was purified by violently agitating the samples in 0.1% Nonidet P-40 solution containing acid-washed Ballotini beads, using a FastPrep® machine (Savant Instruments, Holbrook, New York, USA) (Freeman *et al*, 2002). This treatment disrupted more than 99% of the ascospores in each sample. DNA was purified from the disrupted samples as described by Williams *et al*, (2001).

RESULTS

Two regions, designated SSFWD and SSREV, were identified as potential specific primers (Fig. 1). The primers amplify a region within that amplified by the consensus fungal primers ITS4/ITS5 (White *et al*, 1990). FASTA searches of the EMBL/Genbank databases revealed that the primers were identical to sequences of the very closely related species *S. trifoliorum*, *S. minor*, and *S. glacialis*. The primers would therefore be likely to detect DNA from these species as well as *S. sclerotiorum*. PCR assays using SSFWD/SSREV were done using DNA from a range of different fungal species that are all either pathogens of oilseed rape, cereal pathogens or other air-dispersed fungi. The primers amplified DNA from *S. sclerotiorum* and not from any of the other species tested. They also amplified *S. sclerotiorum* DNA specifically, even in the presence of large amounts of DNA from the closely related species *Botrytis cinerea* (Fig. 2).

(a) Sequences around the forward primer site with primer SSFWD underlined

	1	40
<i>S. sclerotiorum</i>	GCTTTGGCGA <u>GCTGCTCTTC</u> GGGCCTTGT ATGCTCGCCA	
<i>B. cinerea</i> -..... -.....	

(b) Sequences around the reverse primer site with primer SSREV (reverse complement) underlined.

	1	40
<i>S. sclerotiorum</i>	<u>AAGCTCAGCT</u> TGGTATTGAG <u>TCCATGTCAG</u> TAATGGCAGG	
<i>B. cinerea</i>T..... ..T.....	

Figure 1: Alignment of rDNA ITS sequences of *S. sclerotiorum* and *B. cinerea* in the regions used for *S. sclerotiorum*-specific primer design. Bases identical to *S. sclerotiorum* are shown as dots, gaps are shown as dashes. Bases 1-40 correspond to bases 86-125 (SSFWD) and 345-384 (SSREV) on *S. sclerotiorum* sequence M96382.

The PCR assays were tested using untreated ascospores, disrupted ascospores and DNA purified from ascospores. Similar ranges and sensitivities of detection ($1-10^4$ spores per PCR) were observed when using either untreated spores or purified DNA. The sensitivity of 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). However, when mixtures of suspensions of *S. sclerotiorum* and *B. cinerea* were used, the PCR assay detected about 10 spores in purified DNA samples, but did not even detect 10^4 spores when the other two treatments were used. DNA was purified from pieces of Burkard spore-trap tape (Burkard Manufacturing Co. Ltd., Rickmansworth, UK) containing known numbers of ascospores. The sensitivity of the PCR assay was the same whether the DNA was purified from the tape or from spore suspensions. DNA was also purified from the spore trap tapes collected in an oilseed rape field. For all samples, multiple bands were obtained in PCR assays using consensus fungal primers, corresponding to numerous fungal spores found on the tapes. DNA purified from some of the tapes, exposed at the end of May and at the beginning of June, gave positive results in the specific PCR. Small numbers of *S. sclerotiorum*-like ascospores were also found on these tapes.

The SSFWD/SSREV PCR assay was also used to detect ascospores on oilseed rape petals. The assay detected ascospores on petals containing about 50 or more ascospores, suggesting that the sensitivity of the assay was between about 50 and 200 ascospores per petal.

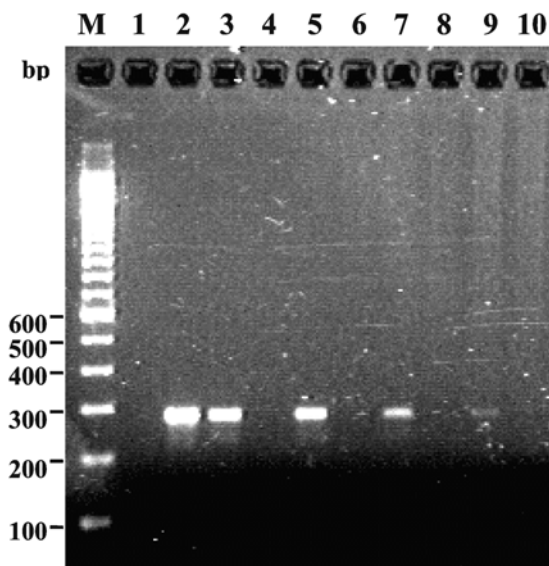


Figure 2: Specificity of PCR using SSFWD and SSREV primers. DNA was prepared from mycelium of *S. sclerotiorum* and *B. cinerea* and added, in different proportions, to the PCR. Lane M, 100bp DNA ladder (Life Technologies Ltd., Paisley, UK); lane 1, no DNA control. Ratios of *S. sclerotiorum*: *B. cinerea* 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 (where 1 = 50ng DNA).

DISCUSSION

The experiments reported here clearly demonstrate that it is feasible to detect ascospores of *S. sclerotiorum* in air samples and on oilseed rape petals, by purifying DNA and using specific PCR assays to detect it. The primers designed should also detect a few other *Sclerotinia* species, but not *B. cinerea*, a closely related fungus, common in the UK. In field crops cross reactivity with other *Sclerotinia* species may not be a problem as only the crop pathogen is likely to be present. 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 and, in the case of *S. sclerotiorum* on oilseed rape, by assessments of percentage petal infestation at early bloom. However, work is still needed to further assess the sensitivity under field conditions, to determine detection thresholds for epidemic development, and to assess the risk of cross contamination by non-target *Sclerotinia* species in the field.

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