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Methods for Integrated Air Sampling and DNA Analysis for Detection of Airborne Fungal Spores

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Integrated air sampling and PCR-based methods for detecting airborne fungal spores, using *Penicillium roqueforti* as a model fungus, are described. *P. roqueforti* spores were collected directly into Eppendorf tubes using a miniature cyclone-type air sampler. They were then suspended in 0.1% Nonidet P-40, and counted using microscopy. Serial dilutions of the spores were made. Three methods were used to produce DNA for PCR tests: adding untreated spores to PCRs, disrupting spores (fracturing of spore walls to release the contents) using Ballotini beads, and disrupting spores followed by DNA purification. Three *P. roqueforti*-specific assays were tested: single-step PCR, nested PCR, and PCR followed by Southern blotting and probing. Disrupting the spores was found to be essential for achieving maximum sensitivity of the assay. Adding untreated spores to the PCR did allow the detection of *P. roqueforti*, but this was never achieved when fewer than 1,000 spores were added to the PCR. By disrupting the spores, with or without subsequent DNA purification, it was possible to detect DNA from a single spore. When known quantities of *P. roqueforti* spores were added to air samples consisting of high concentrations of unidentified fungal spores, pollen, and dust, detection sensitivity was reduced. *P. roqueforti* DNA could not be detected using untreated or disrupted spore suspensions added to the PCRs. However, using purified DNA, it was possible to detect 10 *P. roqueforti* spores in a background of 4,500 other spores. For all DNA extraction methods, nested PCR was more sensitive than single-step PCR or PCR followed by Southern blotting.

Conventional methods for identifying and enumerating airborne fungi and other microorganisms rely on microscopic or cultural techniques and, as a consequence, are time-consuming and laborious. Additionally, microscopy is unreliable for detection of the small, nondescript spores produced by many fungi, while cultural techniques are unsuitable for detection of spores that are slow growing, or nonculturable in vitro and the choice of medium may influence which species can grow. These difficulties have restricted the use of routine air sampling in the study of plant, animal, and human diseases.

Recently, however, molecular methods have been used in the development of diagnostic tests for a variety of fungi involved in plant diseases (8, 25, 26, 28, 30). While the potential of these techniques for detection of airborne spores has been recognized for some time (12, 14), there have been few reports on progress in this area. The use of immunoassay in the detection of airborne plant pathogenic fungi has been investigated (10, 20, 21). However, the application of these methods is restricted by the difficulties of developing antibodies showing the required specificity. DNA-based detection methods offer greater potential for sensitive and specific detection, and some progress has been made in the detection of airborne bacteria using these techniques (1, 2, 9, 15, 17). Progress with fungi has been slower, although detection of *Pneumocystis carinii* (recently reclassified in the fungi [formerly in the protozoa]) in aerosols has been achieved by PCR (16, 24), and the detection of *Stachybotrys chartarum* spores in air samples by PCR methods has been recently reported (7, 23). The earlier paper (7) described the development of a real-time PCR assay for quantification of *S. chartarum* inocula, including the testing of five air samples. The later paper (23) described a case study in which PCR-based detection of *S. chartarum* was used as one method of monitoring the success of measures taken to control airborne fungal spore concentrations. In both investigations, PCR-based monitoring of airborne fungal spores formed only a small part of the studies. The aims of our work were to develop, compare, and optimize DNA extraction techniques and PCR-based methods suitable for the detection of airborne fungal spores; to determine the levels of detection that could be achieved; and to study the effect of airborne inhibitors on the techniques.

As a model for these studies, we chose *Penicillium roqueforti* because it produces spores typical in size of many of those found in air samples; it can be easily identified by light microscopy; it is readily cultured and sporulates freely, producing large numbers of spores; it is nonpathogenic to humans; and a suitable PCR-based detection assay was already available (19).

MATERIALS AND METHODS

Collection and enumeration of *P. roqueforti* spores. *P. roqueforti* isolate C2709, which was isolated from wheat grain at Rothamsted, United Kingdom, was used for the work. It was cultured on absorbent cotton wicks soaked in potato dextrose agar (Oxoid Ltd., Basingstoke, United Kingdom) and incubated at 25°C. The cultures sporulated in about 1 week, and the wicks containing sporulating colonies could be stored for at least 10 weeks before use. *P. roqueforti* spores released into the air from the wicks were collected into 1.5-ml Eppendorf tubes using a miniature cyclone air sampler (Burkard Manufacturing Co. Ltd., Rickmansworth, United Kingdom) operated close by. The air sampler was of a new design and collected airborne particles directly into Eppendorf sampling tubes by using the cyclone principle to remove the particles from the air. We used this sampler because it collects a dry sample directly into a vessel suitable for use with PCR

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analysis. There was therefore no need to remove the sample from a substrate, as would have been necessary with more conventional spore and pollen samplers. This equipment has been used to collect air samples for immunoassay analysis (6), but as far as we are aware, it has not been used for PCR analysis. Immediately prior to use, the dry spores thus collected were suspended in 0.5 ml of 0.1% Nonidet P-40 (Sigma, St. Louis, Mo.) in molecular-biology-grade water (Sigma). Microscopic examination showed that these samples consisted entirely of *P. roqueforti* spores with no visible fragments of mycelium or spores of other fungi. The spore concentration was estimated microscopically using a particle-counting chamber. The spore suspensions were adjusted to 2×10^4 spores/ml, and $\log_{10}$ serial dilutions were made for use in a series of DNA extraction tests. A fresh $\log_{10}$ serial dilution was prepared immediately prior to each experiment.

Preparation of spiked air samples to test the effect of background. The miniature cyclone air sampler was used to collect samples from ambient air into 1.5-ml Eppendorf tubes. Seven separate air samples were taken in four different greenhouses that house plants of a number of different species, including wheat, barley, and oilseed rape. Some of the plants were infected by fungal pathogens, particularly *Erysiphe* spp. For each sample, the miniature cyclone was operated for 10 min at a sampling rate of 20 liters min^{-1} and the sampler was moved around the greenhouse. The plants in the greenhouse were shaken during the sampling period to encourage spore release. Microscopic examination showed that airborne dust, pollen, and fungal spores, including those of *Erysiphe* spp. and *Peronospora* spp., were present in the samples. Air samples were stored dry at room temperature until use. The collected material was suspended in 1 ml of 0.1% Nonidet P-40 by vortexing for 2 min. The concentration of fungal spores of all types present was determined microscopically, and all air sample suspensions were diluted to provide 10^6 spores/ml prior to use. Log_{10} serial dilutions of *P. roqueforti* spores were then added to volumes of air sample suspensions. These spore suspensions, termed spiked air samples, were then used in DNA extraction tests. For each dilution of *P. roqueforti* spores, the concentration of air-sampled spores was maintained at 9×10^5/ml. Subsamples of spiked air sample suspensions were also examined microscopically for *P. roqueforti* spores. Accurate determination of *P. roqueforti* spores was not possible due to the presence of other indistinguishable spores in the samples.

Purification of DNA from spores and mycelium. Four methods of sample preparation were used for PCR analysis: (i) 5 μl of the spore suspension was added to the PCR tubes; (ii) 200 μl of the spore suspension was disrupted and 5 μl was added to the PCR tubes; (iii) DNA was extracted from 50 μl of the disrupted spore sample, the pellet was dissolved in 50 μl of molecular-biology-grade water, and 5 μl of the resulting DNA suspension was added to the PCR tubes; and (iv) 50 μl of the spore suspension was disrupted, the DNA was extracted and dissolved in 5 μl of molecular-biology-grade water, and the entire sample was added to the PCR tubes. Procedure iii is referred to as the large-scale DNA extraction, and procedure iv is referred to as the small-scale DNA extraction. Negative (reagent-only) controls, involving treating the spore-free Nonidet P-40 samples in the same way as the spore suspensions, were included in each preparation.

Disruption of spores (fracturing of spore walls to release the contents) was achieved by shaking spore suspensions with 0.2 g of acid-washed Ballotini beads (8.5 grade, 400 to 455 μm in diameter) for 8 min in a ball mill (Glen Creston, Stanmore, United Kingdom) or for two periods of 40 s in a FastPrep machine (Savant Instruments, Holbrook, N.Y.). Examination of samples of spore suspensions, by microscopy, before and after these treatments showed that they disrupted more than 90% of the *P. roqueforti* spores. DNA purification from disrupted spore suspensions was done using essentially the method of Lee and Taylor (11). This involves mixing the samples with a lysis buffer (containing Tris, EDTA, sodium dodecyl sulfate, and β-mercaptoethanol), incubating them at 65°C for 1 h, and then performing phenol-chloroform extraction and isopropanol precipitation. We modified the published protocol by adding 20 ng of glycogen (Roche Diagnostics Ltd., Lewes, United Kingdom) at the isopropanol precipitation step to act as a carrier for the DNA during centrifugation (22). The resulting pellet was dissolved in molecular-biology-grade water.

DNA was also extracted from mycelia of *P. roqueforti* and from 46 other fungal species (including 30 genera) for use as positive and negative controls in the detection assays (Table 1).

Detection of *P. roqueforti* DNA by PCR. Ribosomal DNA (rDNA) from all samples was initially amplified using the consensus fungal primers ITS4 (5′TC CTCCGCTTATTGATATGC) and ITS5 (5′AGTAAAAGTCGTAACAAGG) (29). 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 25 pmol of both primers, 0.5 U of Platinum *Taq* (Life Technologies, Ltd., Paisley, United Kingdom), buffer (20 mM Tris HCl [pH 8.4], 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM deoxyribonucleoside triphosphates, and DNA (various amounts from $\log_{10}$ dilutions of *P. roqueforti* spores and 5 to 50 ng from mycelium or spores of other fungi). Cycling conditions were 95°C for 10 min and then 30 cycles of 94°C for 30 s, 42°C for 2 min, and 72°C for 2 min.

Specific detection of *P. roqueforti* was done in two ways. (i) We used the primers ITS183 (5′CTGTCTGAAGAATGCAGTCTGAGAAC) and ITS401 (5′CCATACGCTCGAGGACCGGAC) (19) in a single-step PCR. These primers recognize sequences within the ITS regions of *P. roqueforti* and thus amplify a fragment of DNA within the region amplified by ITS4 and ITS5. The reaction mixture for amplification using ITS183-ITS401 was the same as that for ITS4-ITS5 except that the $MgCl_2$ concentration was 2.5 mM and 3.75 pmol of each

TABLE 1. Fungal species used in the testing of methods for the detection of *P. roqueforti*[a]

Species	Isolate	Source	DNA preparation method
Absidia coerulea	W32	GLB	1
Alternaria alternata	Aa6	BDLF	1
Alternaria brassicae	KDLB	BDLF	1
Alternaria infectoria	Ai7	BDLF	1
Alternaria linicola	A133	BDLF	1
Aspergillus nidulans	C2393	JL	1
Botrytis cinerea	C1650	JL	1
Cephalosporium maydis	IMI 107621	GLB	1
Cochliobolus sativus	GP0380	GLB	1
Epicoccum nigrum	W10	GLB	1
Fusarium chlamydosporum	F4	GLB	1
Fusarium culmorum	Fc81	GLB	1
Fusarium merismoides	W33	GLB	1
Gaeumannomyces caricis	167	GLB	1
Gaeumannomyces graminis var. *tritici*	88/10-1	GLB	1
Gaeumannomyces incrustans	1823	GLB	1
Helminthosporium solani	256	GLB	1
Idriella bolleyi	W6	GLB	1
Leptosphaeria maculans	95A18	BDLF	1
Ligniera sp.	F69	MJA	2
Magnaporthe grisea	2692	GLB	1
Microdochium nivale	W34	GLB	1
Olpidium brassicae	F61	MJA	3
Omphalina pyxidata	CBS 783.87	GLB	1
Penicillium aurantiogriseum	C2706	JL	1
Penicillium brevicompactum	C2704	JL	1
Penicillium chrysogenum	C699	JL	1
Penicillium expansum	C2636	JL	1
Penicillium italicum	C1035	JL	1
Penicillium roqueforti	C2709	JL	1
Phialophora malorum	1847	GLB	1
Phoma foveata	228	GLB	1
Phytophthora capsici	PCT12	GLB	1
Phytophthora citrophthora	PCT1	GLB	1
Phytophthora colocasiae	PT105	GLB	1
Phytophthora meadii	H2	GLB	1
Phytophthora palmivora	H41	GLB	1
Phytophthora parasitica	H57	GLB	1
Plasmodiophora brassicae	N	MJA	4
Polyscytalum polypustulans	Pp248	GLB	1
Pseudocercosporella herpotrichioides	R132	GLB	1
Pyrenopeziza brassicae	Pc19	BDLF	1
Rhizoctonia solani	Rs19	GLB	1
Sclerotinia sclerotiorum	M17	JL	1
Spongospora subterranea	H	MJA	4
Wojnowicia graminis	GP0412	GLB	1

[a] Isolates were obtained from the culture collections of M. J. Adams (MJA), B. D. L. Fitt (BDLF), G. L. Bateman (GLB), and J. Lacey (JL) (all from the Plant Pathology Department, IACR-Rothamsted, United Kingdom). DNA preparation methods were as follows: 1, DNA was prepared using freeze-dried, ground mycelium from liquid cultures, according to the method of Lee and Taylor (11) but with an additional RNase digestion, phenol extraction, and isopropanol precipitation to remove contaminating RNA; 2, DNA was prepared from infected roots using the protocol previously described (27); 3, DNA was prepared from zoospores using the protocol previously described (27); 4, DNA was prepared from resting spores using the protocol previously described (27).

primer was used. Cycling conditions were 95°C for 10 min and then 30 cycles of 94°C for 30 s, 67°C for 1 min, and 72°C for 1 min, followed by 10 min at 72°C. (ii) We used a nested PCR. For these reactions, 1 μl of product from a previous ITS4-ITS5 PCR was diluted 1,000-fold, and rDNA was amplified in an ITS183-ITS401 PCR utilizing only 20 cycles of amplification but otherwise as described above. Preliminary experiments, using serial dilutions of ITS4-ITS5 PCR products and different numbers of cycles of the ITS183-ITS401 PCR, had shown these to be the optimum conditions for specific and sensitive detection (data not shown). In all cases, the PCR products were analyzed on agarose gels (1 or 2%) and the DNA was stained by ethidium bromide.

Southern blotting and probing. Product from ITS4-ITS5 PCRs was also used in Southern blotting and probing experiments for specific detection of *P. roqueforti* DNA. DNA from agarose gels was transferred to nylon membranes (Hybond NX; Amersham) by capillary blotting (13). For the oligonucleotide probe, the ITS183 primer developed for PCR by Pedersen et al. (19) was used. For labeling and detection, a nonradioactive probing method was used (AlkPhos direct; Amersham Pharmacia). The standard protocols recommended by the manufacturer were used but with increased amounts of DNA in the labeling (2 μg) and hybridization (16 ng/ml) reactions. Hybridization and washing conditions appropriate for the oligonucleotide based on its melting temperature (T_m) were used. The T_m was calculated to be 63°C using the Genetics Computer Group program MELT, and a hybridization temperature of 58°C was used (T_m − 5°C).

DNA sequencing and analysis. The sequences of ITS4-ITS5 PCR products were determined by purification from agarose gels using the Prepagene kit (Bio-Rad) and then sequencing using an ABI automated sequencer (PE Applied Biosystems, Foster City, Calif.) using cycle sequencing with the ABI Prism Dye terminator cycle sequencing ready reaction kit. Sequence editing and analysis were done using programs (SEQED and FASTA) in version 8 of the Wisconsin Package (program manual, Genetics Computer Group, Madison, Wis.).

Statistical analysis of comparisons. The results of the experiments to compare spore-processing methods and PCR detection methods were statistically analyzed to determine the best combination of spore processing and PCR assay. Most experiments were repeated between four and eight times, and the results of these replicates were used to estimate the probability of detecting spores (percentage of positive results) (μ) for different spore preparation treatments and PCR methods. A generalized linear model with binomial errors and logit link function (18) was used to model the logit probability of detecting a specific number of *P. roqueforti* spores(s) according to the following equation: $\log_{10}[\mu/(1-\mu)] = c + t + d + \tau \log_{10}(s)$, where c is a baseline intercept, t is an adjustment to the intercept that depends on the spore preparation method, d is an adjustment that depends on the PCR detection method, and τ is the slope of the line and depends only on the spore preparation method. The values of the fitted parameters allowed the methods to be compared.

Nucleotide sequence accession numbers. Sequences were deposited in the EMBL database with accession numbers as follows: *P. roqueforti* (isolate C2709); AJ270764; *Penicillium brevicompactum* (C2704), AJ270769; *Penicillium aurantiogriseum* (C2706), AJ270765; *Penicillium chrysogenum* (C699), AJ270768; *Penicillium italicum* (C1035), AJ270766; and *Penicillium expansum* (C2636), AJ270767.

RESULTS

Development of PCR methods for detection of *P. roqueforti*. Although PCR and probing methods based on rDNA sequences were available for *P. roqueforti* (4, 19), modifications were needed to detect small numbers of spores. The specificity of the oligonucleotides used for PCR and probing for the *Penicillium* isolates used in our study was first checked by sequencing the ITS4 and ITS5 regions of the isolates used. These sequences have been deposited in the EMBL database (see Materials and Methods). Sequences for other isolates of some of the following species had already been determined elsewhere (19), (GenBank and EMBL accession numbers are in parentheses): *P. roqueforti* (X82358), *P. aurantiogriseum* (AF033476), *P. expansum* (AF033479), and *P. chrysogenum* (AF033465 and AF034449). When sequences were available for comparison, isolates of the same species had almost identical sequences (three or fewer nucleotides different), and any differences occurred away from the ITS183 and ITS401 sequences used for detection. The ITS401 oligonucleotide is not specific for *P. roqueforti*, but BLAST and FASTA searches of the GenBank and EMBL databases confirmed that ITS183 should confer specificity for *P. roqueforti* (and the closely related *Penicillium carneum*) in PCR and probing assays as had already been suggested (4, 19).

For each detection method used, the assay specificity was then checked experimentally using DNA extracted from 46 species of fungi (including 30 genera) in addition to *P. roqueforti* (Table 1 for the fungi tested and the DNA extraction protocols used). The only fungus to give a positive result in any of the tests was *P. roqueforti*. In single-step *P. roqueforti*-specific PCR assays, no PCR product was seen when testing fungi other than *P. roqueforti*. In the nested PCRs, a product was seen with other fungi after the initial ITS4-ITS5 PCR, but no band was seen when this product was subsequently tested using the *P. roqueforti*-specific primers. Also, no hybridizing bands were observed when ITS4-ITS5 PCR products from any fungus other than *P. roqueforti* were Southern blotted and hybridized with the *P. roqueforti*-specific probe, ITS183.

Comparison of methods for detecting *P. roqueforti* collected by air sampling. The four methods of sample preparation (see Materials and Methods) were tested on spores collected by air sampling in combination with the three methods of PCR detection described above. When untreated spore suspensions were added directly to the PCR mixture, *P. roqueforti* DNA was detected by the three detection assays. However, the sensitivity of detection was low (Table 2; Fig. 1). All the assays consistently detected 100,000 spores but never fewer than 1,000. Disrupting spore suspensions of *P. roqueforti* using Ballotini beads resulted in an improvement in sensitivity, with all detection assays consistently detecting the DNA from only 100 spores (Table 2; Fig. 1). In 86% of experiments using the nested PCR assay after spore disruption, the DNA from just one spore was detected. Results were similar when the large-scale DNA extraction was used in conjunction with disruption, although detection sensitivity using probing was reduced such that 1,000 spores were required for consistent detection (that is, detection in 100% of the experiments). Similarly, the small-scale DNA extraction demonstrated that in conjunction with nested PCR, the DNA from a sample of only 100 spores could be consistently detected (Table 2; Fig. 1). In some experiments, DNA from one spore was detected. Again, probing was found to be a less sensitive detection assay.

The generalized linear model fitted the experimental results well, and the model parameters are shown in Table 3. Comparison of the models for different PCR assays showed that for all spore treatments, the nested PCR was the most sensitive and reproducible and the ITS183-ITS401 PCR and PCR plus probing had similar sensitivities (Table 4). When the methods of preparing spores for PCR were compared, irrespective of detection method, the sensitivity using disrupted spores and large-scale DNA extraction were found to be similar but both were more efficient than the small-scale DNA extraction (Table 4). These three treatments had about the same rate of increase in detection probability with increase in spore numbers (τ in Table 3). Adding spores directly to the PCR was between 1 and 2 orders of magnitude less sensitive than using disrupted spores or large-scale DNA extraction (Table 4). The rate of response for untreated spores with increasing spore

TABLE 2. Detection of *P. roqueforti* DNA using different sample preparation methods and specific assays

Spore treatment	No. of spores processed	No. of spores added to PCR[a,b]	% of experiments with a positive result[c]		
			ITS183-ITS401 PCR	Nested PCR	Probing
Untreated spores	0	0	0 (0/6)	0 (0/6)	0 (0/6)
	1×10^2	1×10^2	0 (0/6)	0 (0/8)	0 (0/8)
	1×10^3	1×10^3	17 (1/6)	75 (6/8)	12.5 (1/8)
	1×10^4	1×10^4	100 (6/6)	100 (8/8)	62.5 (5/8)
	1×10^5	1×10^5	100 (4/4)	100 (8/8)	100 (8/8)
Spore disruption	0	0	0 (0/6)	0 (0/7)	0 (0/7)
	4×10^1	1×10^0	0 (0/6)	86 (6/7)	14 (1/7)
	4×10^2	1×10^1	83 (5/6)	100 (7/7)	57 (4/7)
	4×10^3	1×10^2	100 (6/6)	100 (7/7)	100 (7/7)
	4×10^4	1×10^3	100 (5/5)	100 (6/6)	100 (6/6)
Large-scale DNA extraction	0	0	0 (0/8)	0 (0/8)	0 (0/8)
	4×10^1	1×10^0	25 (2/8)	50 (4/8)	0 (0/8)
	4×10^2	1×10^1	43 (3/7)	71 (5/7)	0 (0/7)
	4×10^3	1×10^2	100 (8/8)	100 (8/8)	62.5 (5/8)
	4×10^4	1×10^3	100 (7/7)	100 (7/7)	100 (7/7)
Small-scale DNA extraction	0	0	Not tested	0 (0/4)	0 (0/4)
	1×10^0	1×10^0	Not tested	25 (1/4)	0 (0/4)
	1×10^1	1×10^1	Not tested	25 (1/4)	0 (0/4)
	1×10^2	1×10^2	Not tested	100 (4/4)	75 (3/4)
	1×10^3	1×10^3	Not tested	100 (3/3)	67 (2/3)

[a] Amount of DNA used in detection assay expressed as the equivalent number of spores.
[b] For probing experiments, these values relate to the number of spores added to the ITS4-ITS5 PCR done prior to blotting.
[c] Each set of values in parentheses is the number of experiments with a positive result per total number of experiments. For each concentration of spores, with each treatment the replication was of the entire experiment (including DNA extractions), not just the detection step.

numbers was also higher than that for the other two treatments (Table 3).

No positive results were observed with DNA from three other *Penicillium* species or from three other common airborne fungi (*Aspergillus nidulans, Botrytis cinerea*, and *Sclerotinia sclerotiorum*) in any of the *P. roqueforti*-specific assays used. Nevertheless, the ITS4-ITS5 PCR control assay confirmed that the DNA extracted from each of these species was amplifiable and that PCR inhibitors were not present (data not shown). *P. roqueforti* DNA was not detected by any of the specific assays in any of the control DNA extractions lacking added *P. roqueforti* spores (Table 2; Fig. 1).

Almost all of the experiments were repeated between four and eight times (actual values are given in Table 2), and this replication was not derived from simply repeating the detection step on serially diluted DNA from a few samples. Rather, it was derived from repeating the entire experiment, including the DNA extractions, which each time started from a range of

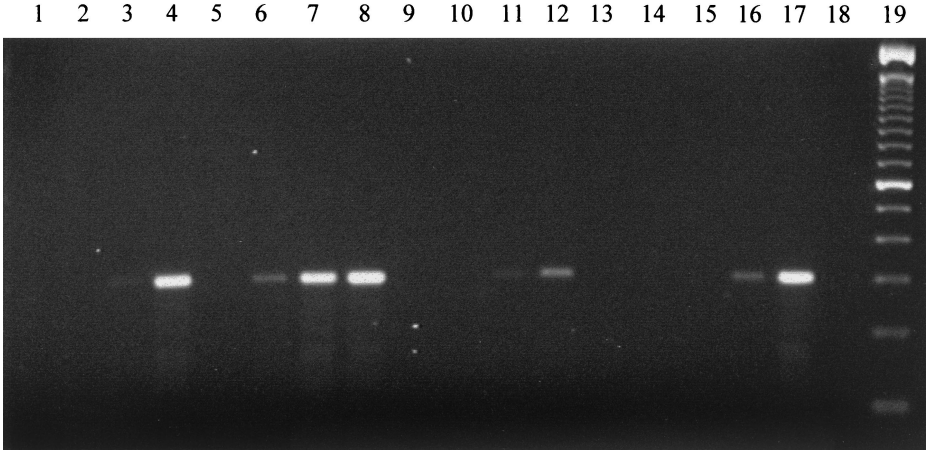

FIG. 1. Amplification of a 300-bp fragment of DNA from *P. roqueforti* spores by nested PCR. Lanes 1 to 4, 0, 100, 1,000, and 10,000 untreated spores added to the PCR; lanes 5 to 8, 0, 10, 100, and 1,000 disrupted spores added to the PCR; lanes 9 to 12, DNA from 0, 1, 10, and 100 spores purified by the large-scale extraction method; lanes 13 to 16, DNA from 0, 1, 10, and 100 spores purified by the small-scale extraction method; lane 17, *P. roqueforti* DNA extracted from mycelium (positive control); lane 18, water (negative control). Lane 19 is the DNA size marker (100-bp ladder; Gibco BRL).

TABLE 3. Parameters of the generalized linear model fitted to the percentage of positive results for each replicate set of experiments

Spore treatment or PCR type	Value (SE)[a]			
	c	*t*	τ	*d*
Spore treatment				
Untreated	−2.071 (0.566)	−11.54 (2.56)	3.985 (0.760)	
Disrupted	−2.071 (0.566)	1.225 (0.558)	2.349 (0.386)	
Large-scale DNA extraction	−2.071 (0.566)	0	2.149 (0.365)	
Small-scale DNA extraction	−2.071 (0.566)	−2.65 (1.21)	2.635 (0.695)	
PCR type				
ITS183-ITS401	−2.071 (0.566)			0
Nested	−2.071 (0.566)			2.592 (0.594)
Probing	−2.071 (0.566)			−1.169 (0.552)

[a] For explanation of the parameters, see Materials and Methods.

spore numbers. When PCR or probing assays were repeated on particular samples, results were in good agreement (data not shown).

Effect of other airborne particles on *P. roqueforti* detection. The effect of other airborne particles on *P. roqueforti* detection was tested by taking background air samples and spiking these with known numbers of *P. roqueforti* spores. Specific detection of *P. roqueforti* DNA was done using nested PCR. Experiments were repeated four or five times. The results (Table 5) showed that it was possible to detect *P. roqueforti* against a background of other airborne particles. However, the sensitivity was lower than when *P. roqueforti* spores were collected from the air and used without additional background (Table 2). Using the large-scale DNA extraction, with a background of 4,500 other spores, it was possible to detect the DNA from 10 *P. roqueforti* spores, but consistent results were obtained only using 1,000 spores. Using the small-scale DNA extraction, with a background of 45,000 other spores, the DNA from 100 *P. roqueforti* spores could be detected, but even the largest number of spores tested (100,000) was not consistently detected. *P. roqueforti* DNA was not detected using any of the DNA extraction and detection methods in any spore-free reagent controls or air samples lacking added *P. roqueforti* spores.

DISCUSSION

This is the first report of experiments to determine the minimum number of fungal spores, collected using an air sampler, from which DNA can be extracted and subsequently detected using PCR-based methods. Three spore treatments and three detection methods were tested, using *P. roqueforti* spores collected by air sampling, and the effects of background air-sampled material were also studied. Addition of untreated spores to the PCR was not a suitable option for detection of *P. roqueforti*. Detection was possible using untreated *P. roqueforti* spores collected from the air and used without additional background, but the sensitivity was poor, presumably because insufficient DNA was released. Although the PCR protocol used included an initial 10-min step at 95°C, microscopic examination showed that spores remained visibly intact after this treatment. However, preliminary experiments with the thin-walled conidia of *Leptosphaeria maculans* suggest that heating may be sufficient for lysis of some types of spores (data not shown). In spiked air samples, *P. roqueforti* DNA could not be detected using intact spores.

Disrupting *P. roqueforti* spores using Ballotini beads provided a simple protocol for improving detection sensitivity. When *P. roqueforti* spores were collected from the air and used without additional background, it was possible to detect 1 spore using this method and consistent detection was achieved with 10 spores. However, in spiked air samples, *P. roqueforti* DNA could not be detected using this method, presumably due to the effects of inhibitors, such as pollen and dust, in the samples (31).

The combination of extraction and purification of DNA was found to be the best method to produce a template for PCR from *P. roqueforti* spore suspensions or spiked air samples. In experiments where *P. roqueforti* spores were collected from the air and used without additional background, DNA from an initial sample of only 100 spores could be consistently extracted and detected by single-step or nested PCR, in some experiments, DNA from a single spore was detected. Using the large-scale DNA extraction to prepare template DNA from

TABLE 4. Number of spores required in a PCR for a 90% probability of detection estimated by the generalized linear model for the combinations of spore preparation treatment and PCR assay

Spore treatment[a]	Estimated no. of spores (mean and 95% confidence limits)								
	ITS183-ITS401 PCR			Nested PCR			Probing		
	Mean	Lower	Upper	Mean	Lower	Upper	Mean	Lower	Upper
A	9,268	3,972	21,627	2,070	1,091	3,926	18,197	7,211	45,920
B	20	6	67	2	1	4	62	16	237
C	97	24	384	6	2	18	339	71	1,614
D	421	60	2,938	44	13	148	1,169	139	9,863

[a] A, none; B, disruption; C, large-scale DNA extraction; D, small-scale DNA extraction.

TABLE 5. Detection of *P. roqueforti* DNA in spiked air samples using large- and small-scale DNA extractions and nested PCR

No. of *P. roqueforti* spores added to initial PCR[a,b]	% of experiments with a positive result[c]	
	Large-scale DNA extraction	Small-scale DNA extraction
0 (reagent control)	0 (0/4)	0 (0/5)
0 (air sample control)	0 (0/4)	0 (0/5)
10^0	0 (0/4)	Not tested
10^1	50 (2/4)	0 (0/5)
10^2	75 (3/4)	20 (1/5)
10^3	100 (4/4)	20 (1/5)
10^4	100 (4/4)	20 (1/5)
10^5	Not tested	80 (4/5)

[a] Amount of DNA used in detection assay expressed as the equivalent number of spores. For the large-scale DNA extractions, the number of spores processed was 40-fold larger than these values.

[b] The numbers of other spores (derived from air sampling) added per PCR were 4,500 for each of the large-scale extractions and 45,000 for each of the small-scale extractions.

[c] Each set of values in parentheses is the number of experiments with a positive result per total number, of experiments. For each concentration of spores, with each extraction method the replication was of the entire experiment (including DNA extractions), not just the detection step.

spiked air samples, 1,000 *P. roqueforti* spores were consistently detected against a background of DNA from 4,500 other spores collected by air sampling. In some experiments, as few as 10 spores could be detected in this background. Using the small-scale DNA extraction, 100 *P. roqueforti* spores could be detected against a background of 45,000 other spores, but consistent detection was not achieved at any of the spore concentrations tested. This may be because large amounts of PCR inhibitors are present in the DNA samples when processing so many spores and other air-sampled material.

In addition to enabling detection of small numbers of fungal spores in air samples containing pollen, dust, and large numbers of nontarget spores, using a DNA purification protocol also results in samples that should keep in better condition than crude extracts during long-term storage. When purifying DNA from very few spores, the addition of glycogen as a carrier for the DNA was found to be essential; without it, the DNA did not reliably precipitate and was easily lost (data not shown). Control reactions including glycogen indicated that it did not inhibit the PCR at the concentrations used in this study (data not shown).

When considering the sensitivity of the methods used, it is important to differentiate between the number of spores used in the extractions and the number used in the detection assays. Rather than extracting DNA from a large number of spores and then diluting it in order to assess detection efficiency, $\log_{10}$ serial dilutions of spores were used in the DNA extractions (Table 2). For example, it was possible to detect the DNA from one spore by nested PCR using the crude disrupted spore preparation, the small-scale DNA extraction, or the large-scale DNA extraction. However, to achieve this, the small-scale preparation required processing of only 1 spore, whereas the large-scale DNA extraction and the crude disrupted spore preparation required 40 spores to be processed.

In common with results of other studies, nested PCR was found to be a more sensitive detection assay than single-step PCR using *P. roqueforti*-specific primers (5). Both nested PCR and probing assays required an initial ITS4-ITS5 PCR to amplify any fungal rDNA present. As well as improving the sensitivity of detection, the probing and nested PCR approaches, using ITS4-ITS5 PCR as the first stage, have other advantages over single stage-specific PCRs. The initial ITS4-ITS5 PCR acts as a control to ensure that DNA has been extracted from the air sample and indicates whether PCR inhibition has occurred. For experiments where it is necessary to use the entire sample in the PCR, this approach allows more than one attempt at specific detection. This method would be particularly amenable to screening for multiple species in one sample. The ITS4-ITS5 PCR could be used to amplify DNA from all the fungi present in a sample, followed by a specific nested PCR or Southern blotting and probing for the individual fungal species being studied.

A number of positive and negative controls were included in the experiments. In addition to detection assay controls including samples without DNA and samples of DNA from nontarget species, negative controls for the DNA extraction process were included. These consisted of Nonidet P-40 samples lacking added *P. roqueforti* spores and were included for all spore treatments in all experiments. For experiments using spiked air samples, DNA was also extracted from air samples prior to addition of *P. roqueforti* spores. *P. roqueforti* DNA was not detected by any of the specific assays in any of these samples, demonstrating that the extraction reagents were all DNA free, that the air samples did not contain detectable indigenous *P. roqueforti*, and that cross contamination of samples did not occur. Similarly, by careful separation of equipment and areas used for DNA extraction, PCR preparation, and PCR product handling, problems associated with contaminating DNA or PCR products were avoided (3).

The results of this study suggest that this PCR technology has the potential for accurately detecting fungal spores of defined species in air samples. It is possible to detect some fungi by simply adding spores directly to the PCR without any processing. However, for sensitive detection, the spores may need to be disrupted to release DNA before it is amplified by PCR. In many cases, particularly when there is a large background of contaminating material or nontarget DNA, an additional DNA extraction step may be needed before PCR processing. The presence of nontarget biological particles and organic dust may inhibit the PCR process, thereby reducing the potential sensitivity of the assay. In addition, further research is required to quantify the effects of nontarget material in the sensitivities of PCR assays.

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