Simultaneous One-Tube Quantification of Host and Pathogen DNA with Real-Time Polymerase Chain Reaction

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Accepted for publication 3 May 2001.

ABSTRACT

Winton, L. M., Stone, J. K., Watrud, L. S., and Hansen, E. M. 2002. Simultaneous one-tube quantification of host and pathogen DNA with real-time polymerase chain reaction. Phytopathology 92:112-116.

Phaeocryptopus gaeumannii is a widespread foliar parasite of Douglas-fir. Although normally innocuous, the fungus also causes the defoliating disease Swiss needle cast in heavily infected needles. The extent of *P. gaeumannii* colonization in Douglas-fir foliage was estimated with real-time quantitative polymerase chain reaction (PCR) using TaqMan chemistry. In order to derive a normalized expression of colonization, both pathogen and host DNA were simultaneously amplified but individually detected by species-specific primers and TaqMan probes labeled with different fluorescent dyes. Detection of host DNA additionally provided an endogenous reference that served as both an internal positive control and adjusted for variation introduced by sample-to-sample differences in DNA extraction and PCR efficiencies. The genes employed for designing the TaqMan probes and primers were β-tubulin for the pathogen and a LEAFY/FLORICAULA-like gene involved in floral development for the tree host. Both probe/primer sets exhibited high precision and reproducibility over a linear range of 4 orders of magnitude. This eliminated the need to analyze samples in multiple dilutions when comparing lightly with heavily infected needles. Quantification of the fungus within needles was successful as early as 1 month after initial infection. Real-time PCR is the only method currently available to quantify *P. gaeumannii* colonization early in the first year of the colonization process.

Swiss needle cast has recently been implicated in a serious decline of Douglas-fir (*Pseudotsuga menziesii*) along the coastal region of Oregon (5). The disease is caused by internal needle colonization and subsequent stomatal blockage by ascomata of the fungus *Phaeocryptopus gaeumannii*. Normally, extensive colonization is found only in older (3 to 4 year) foliage, but severe disease symptoms are associated with extensive colonization of foliage less than 1 year old (5). Objective, quantitative measurement of foliage colonization by the pathogen in order to understand factors affecting its growth in young needles is a challenging aspect of research on this disease. Although abundance of ascomata (pseudothecia) on needle surfaces is well correlated with symptoms, quantification of pseudothecia by direct observation is labor intensive and unavailable during the first year of the disease cycle before fruiting bodies have developed.

Real-time polymerase chain reaction (PCR) is the most recent development in quantitative diagnostic methods and promises to be useful at very low levels of infection. The most frequently employed application of this technique utilizes TaqMan (Perkin-Elmer Applied Biosystems, Foster City, CA) chemistry (4,6,7) in conjunction with a sequence detection system (SDS) (7700; Perkin-Elmer). The fluorogenic TaqMan probe, labeled on opposite ends with a reporter dye and a quencher dye, anneals between the PCR primers. During the extension phase of the PCR, the 5′→3′ exonuclease activity of *Taq* DNA polymerase cleaves annealed probe molecules. Release of the reporter dye results in an intense fluorescent signal that is measured by the SDS during each cycle of the PCR process. TaqMan chemistry has contributed to the development of extremely specific, sensitive, and accurate assays to quantify pathogen infection in soybean seeds (15) and roots of both crop plants and forest trees (1). Unlike conventional end-point quantitative PCR, real-time PCR monitors PCR products as they accumulate in the exponential phase, before reaction components become limiting. In addition, because different reporter dyes can be attached to separate species-specific TaqMan probes, it is possible to simultaneously quantify both the host and pathogen DNA in infected tissues.

This report describes the development of a multiplexed TaqMan assay to simultaneously quantify both *P. gaeumannii* and Douglas-fir DNA in infected foliage to derive a normalized measurement of pathogen colonization.

MATERIALS AND METHODS

Fungal cultures and DNA extraction. The fungal isolates used in this study are listed in Figure 1. *P. gaeumannii* isolates were obtained by suspending needles bearing only *P. gaeumannii* pseudothecia over water agar and incubating in a moist chamber at 17°C. Individual needles were examined for the presence of pseudothecia, attached to petri dish lids, and suspended above the agar surface for 1 day. Individual ascospores were isolated from the agar surface with a fine needle and transferred to 2% potato dextrose agar (PDA) (Difco Laboratories, Detroit). Identical methods were used to obtain isolates of the epiphytic fungi *Rasutoria pseudotsugae* and an undescribed *Stomiopeltis* sp., both of which commonly occur with *P. gaeumannii* on Douglas-fir foliage. Endophytic fungi were isolated from surface-sterilized needles. Individual needles were immersed in 95% ethanol for 30 s, transferred to a solution of 50% commercial bleach (5% NaOCl) for 10 min, followed by 95% ethanol for 1 min. The needles were then cut aseptically into 2-mm segments and incubated on PDA at 17°C. Endophytic fungi growing from the cut ends were isolated on PDA and incubated for identification. Fungal cultures were

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Publication no. P-2001-1115-01R
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prepared for extraction by scraping approximately 30 mg of mycelium from the agar surface. Collected mycelium was placed in 2-ml microfuge tubes with 1 mm zirconia/silica beads (Biospec Products, Bartlesville, OK) and 1 ml of cetyltrimethylammonium bromide (CTAB) extraction buffer (2% CTAB, 100 mM Tris at pH 8.0, 20 mM EDTA at pH 8, 1.4 M NaCl, 1% polyvinylpolypyrrolidone, and 0.1% 2-mercaptoethanol) and shaken in a Mini-Beadbeater (Biospec Products) for 30 s at 5,000 rpm. After mixing, samples were incubated at 65°C for 2 h. The DNA was purified in 24:1 chloroform/isoamyl alcohol and further purified to reduce PCR inhibitors by passing the extract over QiaAmp Spin Columns (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions.

Douglas-fir needles (10 needles per sample) were placed in 2-ml microfuge tubes with two 5-mm glass beads, frozen in liquid nitrogen, and pulverized in a Mini-Beadbeater for 30 s at 4,200 rpm. After pulverization, samples were incubated in 1.5 ml of extraction buffer at 65°C for 2 h. The DNA was purified in 24:1 chloroform/isoamyl alcohol, precipitated from the aqueous phase by the addition of isopropanol, washed in 70% ethanol, and resuspended in 1 ml of Tris-EDTA (5 mM Tris at pH 8.0 and 0.5 mM EDTA).

Standards. Assay standards containing both *P. gaeumannii* and Douglas-fir DNA in proportions representative of naturally infected foliage were prepared from genomic DNA extracted from uninfected, greenhouse-grown Douglas-fir needles and *P. gaeumannii* grown in pure culture. DNA was extracted as described previously, except that ribonuclease A treatment (10) was included to facilitate accurate spectrophotometric measurement of DNA concentration. DNA concentrations in extracts from *P. gaeumannii* and Douglas-fir samples were determined by measuring optical density at 260 nm, diluted as needed, and combined so the ratio of Douglas-fir/*P. gaeumannii* in the DNA standards was comparable to that of infected foliage. Five 10-fold serial dilutions were prepared for use in calibration experiments and to serve as dual-species, one-tube quantification standards to be included in each assay. *P. gaeumannii* standards ranged from 0.1 to 1,000 pg, and Douglas-fir standards ranged from 0.1 to 1,000 ng.

Probe and primer design. The *P. gaeumannii* oligonucleotide probe/primer set was based upon β-tubulin sequences obtained from PCR amplicons of genomic DNA extracted from the isolates listed in Figure 1. PCR was performed in 50-µl reactions (1× enzyme buffer, 200 µM dNTP, 0.4 µM T1 and T22 [8], 2.5 units of Red*Taq* DNA polymerase [Sigma Chemical, St. Louis], and 10 to 100 ng of template DNA). Reaction conditions were 35 cycles of 60 s at 94°C denaturing, 60 s at 55°C annealing, and 60 s at 72°C extension. After amplification, PCR products were prepared for direct sequencing by isopropanol precipitation. Cycle sequencing in both 5′ to 3′ and 3′ to 5′ directions with primers T1 and T2 (8) were performed using dye-terminator chemistry on a fluorescent sequencer (ABI Model 377; Perkin-Elmer). Contigs were assembled and the overlapping sequences were edited using the

Fig. 1. Comparison of the sequences and alignment of the β-tubulin gene region used for specific amplification and detection of *Phaeocryptopus gaeumannii* with other Douglas-fir needle fungi. Sequences for probes and primers in this study are shown in capital letters. Forward primer PGBT308F (upper arrow), probe PG336BTUB-FAM (solid line), and reverse primer PGBT429R (lower arrow).

TABLE 1. TaqMan probe/primer sets developed for quantification of *Phaeocryptopus gaeumannii* DNA in extracts of Douglas-fir foliage

Probe and primer	Sequence (5′→3′)	Target	Position	Fragment length (bp)
PGBT308F[a]	GGTACAATGGCACGTCTGATCTC	*P. gaeumannii*	308	…
PGBT429R[b]	GGACGCCTATATCGCAAGTCA	*P. gaeumannii*	429	122
PG336BTUBP-FAM[c]	CGAGCGCATGAACGTCTACTTCAACG	*P. gaeumannii*	336	…
LFY989F[a]	GGTCACAAACCAAGTATTTCGACA	Douglas-fir	989	…
LFY1102R[b]	TGTTCAACATCCAGGCAATGA	Douglas-fir	1102	114
LFY1015P-VIC[c]	TAACCGGCGCCTGAATGCTTCG	Douglas-fir	1015	…

[a] Forward primer.
[b] Reverse primer.
[c] TaqMan probes: the *P. gaeumannii* probe is labeled with the reporter dye FAM (6-carboxy-fluorescein; emission 518 nm) on the 5′ end, the reporter dye for the Douglas-fir probe is VIC, and both probes are labeled with TAMRA (6-carboxy-tetramethyl-rhodamine; 582 nm) on the 3′ end as a quencher.

Staden package (11). Because species-specific priming sites must be conserved within species to be useful, five *P. gaeumannii* isolates from different locations were sequenced. Sequence alignments were generated with ClustalX (13) and compared with regions unique to *P. gaeumannii* but invariant within the species. Candidate oligonucleotides were chosen such that at least one mismatched nucleotide was located at the 3′ end of each primer, whereas mismatches were placed near the middle of the TaqMan probe. These positions are the most destabilizing to non-target annealing (3,12).

The Douglas-fir probe/primer set was designed to be included in multiplex PCR as an endogenous reference and served as both an internal positive control (IPC) and as a normalizer. The IPC was used to distinguish uninfected needles from PCR inhibition. Normalization served to adjust *P. gaeumannii* estimates of colonization for differently sized needles, pipetting accuracy, and sample-to-sample variation in both DNA extraction and PCR amplification efficiencies. The Douglas-fir probe/primer set was based on homologs of the LEAFY/FLORICAULA genes, which control the transition from vegetative to floral development in *Arabidopsis* (14) and *Antirrhinum* (2). Sequences were obtained from three Douglas-fir cDNA clones (W. H. Rottmann and S. H. Strauss, *personal communication*) and *Pinus radiata* (GenBank Accession No. U76757), aligned with ClustalX, and compared with regions common to all sequences.

After identification of candidate oligonucleotides, analysis (percent G+C, theoretical melting temperature, and potential for unwanted oligonucleotide interactions) and final selection of TaqMan primer and probe sequences were performed using the ABI Primer Express program (Perkin-Elmer) according to the manufacturer's instructions. Both TaqMan probes used in this study were obtained from the Oligo Factory (Perkin-Elmer) and contained a TAMRA quencher dye conjugated to the 3′-terminal nucleotide. The *P. gaeumannii* probe contained the reporter dye FAM linked to the 5′-terminal nucleotide, whereas the Douglas-fir probe employed VIC as the reporter.

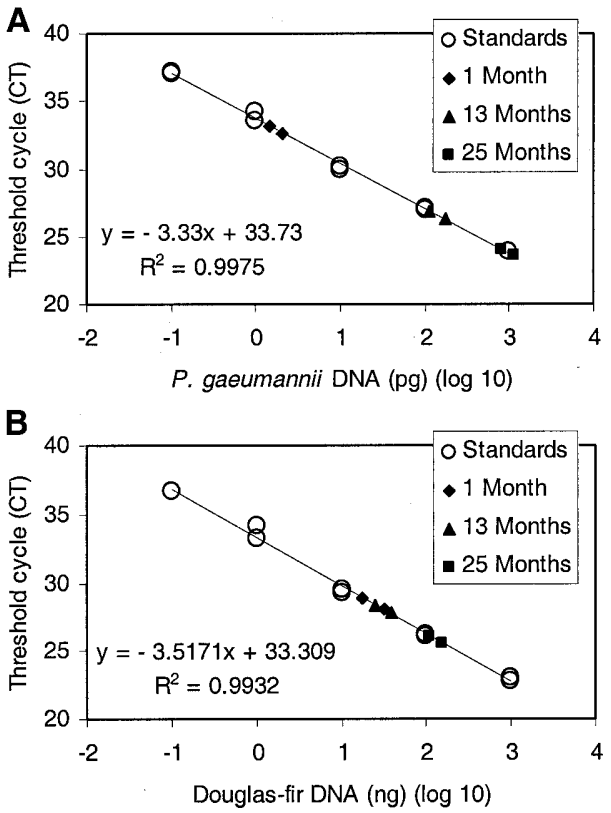

Fig. 2. Standard curves and unknown samples demonstrating the simultaneous quantification of **A,** *Phaeocryptopus gaeumannii* and **B,** Douglas-fir DNA present in foliage samples using TaqMan real-time polymerase chain reaction. Cycle thresholds (CT) were plotted against the log of genomic DNA standards of known concentrations and linear regression equations were calculated for the quantification of unknown samples by interpolation. Unknowns consisted of foliage samples collected from the most recent three needle cohorts at two highly diseased sites near Tillamook, OR. Individual cohorts had been initially infected at 1, 13, and 25 months prior to sample collection.

TaqMan PCR conditions and analysis. Reactions were performed in 15-µl aliquots with 1× TaqMan Universal Master Mix (Perkin-Elmer), 150 nM *P. gaeumannii* FAM-labeled probe, 150 nM Douglas-fir VIC-labeled probe, 60 nM forward and reverse primers for both organisms, and 5 µl of DNA template (various concentrations). Real-time quantitative PCR was performed by an automated ABI Prism 7700 SDS in MicroAmp optical 96-well plates or single tubes (Perkin-Elmer). Thermal cycling was completed in less than 2 h, and conditions consisted of 10 min at 50°C and 5 min at 95°C and 40 cycles of 95°C for 15 s and 60°C for 1 min. The SDS software collected data for both reporter dyes every 7 s from each well, generating a fluorescence profile for each amplification. The threshold cycle (CT) was recorded for each dye as the cycle at which fluorescent signal, associated with an exponential growth of PCR product, exceeded background fluorescence.

PCR controls in every assay included no template (negative) controls and the genomic DNA standards (positive) for both *P. gaeumannii* and Douglas-fir. Combined standards in 10-fold dilutions (described previously) were run in duplicate for each assay. Standard curves for both *P. gaeumannii* and Douglas-fir were generated by plotting the known DNA amounts against the CT calculated by the SDS software and calculating a regression equation. Unknown samples were quantitated from measured CT values by interpolation using the regression equation. Normalized estimates of *P. gaeumannii* colonization of Douglas-fir host foliage were obtained by dividing *P. gaeumannii* DNA estimates by Douglas-fir DNA estimates for individual samples.

Validation experiments. The specificity and sensitivity of the probe/primer sets were tested with genomic DNA from five isolates of *P. gaeumannii*, two to three isolates each of nine other Douglas-fir needle fungi (Fig. 1), uninfected, greenhouse-grown Douglas-fir foliage, and infected foliage both with and without visual signs of *P. gaeumannii*. Infected foliage was collected from the 1997, 1998, and 1999 needle cohorts from one moderately diseased tree from each of two diseased plantations (Juno Hill and North Fork) near Tillamook, OR (5). Initial infection dates for each cohort were estimated from phenology data recorded at each site.

According to the instruction manual for the SDS, the instrument is more sensitive to differences at lower concentrations of initial template amounts. Therefore, calibration experiments with spectrophotometrically measured amounts of Douglas-fir and *P. gaeumannii* DNA were performed on each probe/primer set, both separately and together, to determine their sensitivity and linear dynamic range. In addition, RNase-treated genomic DNA extracted from infected needles was compared with untreated DNA for differences in quantification and possible interference by RNA transcripts.

TABLE 2. Normalized estimates of *Phaeocryptopus gaeumannii* colonization (picogram of *P. gaeumannii* DNA per nanogram of Douglas-fir DNA) using interpolated data from Figure 2[a]

Time	North Fork	Juno Hill
1 month	0.05	0.12
13 months	4.56	4.65
25 months	7.28	7.43

[a] At both of the sites, estimates increased with the length of time following initial infection.

Because the TaqMan assay was intended to provide an indirect measure of *P. gaeumannii* biomass in foliage, the relationship between mycelium dry weight and estimated DNA content was examined for a single *P. gaeumannii* isolate. The isolate was grown for 3 months in 2% potato dextrose broth at 18°C. Harvested mycelium was then rinsed and dried overnight at 40°C. Prior to DNA extraction, dried mycelium pieces of various sizes were weighed.

Precision, or reproducibility, of the TaqMan assay on field samples was evaluated on Douglas-fir foliage from a single severely diseased tree from the Juno Hill site. One-year-old needles were stripped from branches, pooled, and separated into 12 subsamples of 10 needles randomly chosen from the pool. Prior to DNA extraction, all samples were stored at –20°C. Intra-assay precision, which could be affected by reaction-to-reaction differences in pipetting volumes and PCR efficiency and measurement among wells, was evaluated on 12 replicates of one of the DNA subsamples analyzed in a single assay. Inter-assay variability, which could additionally be affected by slight differences in reaction components, was evaluated on the same DNA subsample amplified over five separate assays. Inter-sample reproducibility, which could be affected by sample-to-sample differences in PCR efficiency, sample selection, and DNA extraction, was evaluated on separate extractions of 12 subsamples amplified in a single assay.

RESULTS

The sequences, locations, and amplicon sizes of the TaqMan probe/primer sets constructed for the quantification of *P. gaeumannii* and Douglas-fir in this study are listed in Table 1 and Figure 1. Neither of the TaqMan probe/primer sets amplified DNA extracted from any of the fungi cultured from Douglas-fir needles other than *P. gaeumannii*. The *P. gaeumannii* set did not amplify DNA extracted from uninfected needles, and the Douglas-fir set did not amplify DNA extracted from *P. gaeumannii* isolates. These results indicate no undesired cross-reactivity with nontarget genomic DNA present in the sample preparations. The *P. gaeumannii* set successfully amplified DNA extracted from all five *P. gaeumannii* isolates and infected needles, whether there were obvious signs of the fungus or not.

The Douglas-fir probe/primer set was successful in detecting Douglas-fir DNA in both infected and uninfected needles. Therefore, this gene proved useful as both an IPC and normalizing gene. Samples were recorded as uninfected when the *P. gaeumannii* gene did not amplify and the Douglas-fir IPC gene was positive. Samples that resulted in negative reactions for the Douglas-fir gene were recorded as failed PCR reactions. The importance of the normalizing effect is evident in Figure 2, in which both host and pathogen DNA content were simultaneously estimated from total genomic DNA extracted from infected needle samples. Whereas *P. gaeumannii* DNA content in needles increased with needle age (Fig. 2A), the amount of Douglas-fir DNA was similar, but not identical, for all six samples (Fig. 2B). Because each sample consisted of 10 needles, the differences in Douglas-fir DNA estimates probably reflect differences in needle sizes and DNA extraction efficiencies. Normalized estimates of colonization are presented in Table 2, and indicate a trend of increased *P. gaeumannii* DNA over time, after adjusting for these potential sources of variation. Natural infection of foliage occurs between late May and late June (5). Additional evidence for normalizing performance was provided by the DNA standards of known concentrations. Despite being diluted over 4 orders of magnitude, the average ratio of CT values (*P. gaeumannii* CT/Douglas-fir CT) for all 10 multiplex reactions was 1.025 (±0.005 SE).

When run either separately or multiplexed, both probe/primer sets displayed high precision over a linear range of at least 4 orders of magnitude (Fig. 2). The correlations between CT and known DNA quantities were high for both *P. gaeumannii* (R = 0.998) and Douglas-fir (R = 0.997). *P. gaeumannii* was quantifiable between 0.1 and 1,000 pg (Fig. 2A), whereas Douglas-fir was quantifiable from 0.1 to 1,000 ng (Fig. 2B). These data were used to determine the appropriate dilution for DNA extracted from samples consisting of 10 needles and ensured that uninfected, lightly infected, and heavily infected foliage could all be quantified in a single assay. There was a linear relationship (R = 0.97) between *P. gaeumannii* DNA and biomass for the range that might be encountered in naturally infected needles (0 to 3 mg) (Fig. 3). Differences in estimated DNA quantities between RNase treated and untreated infected needle extracts were minor, therefore to save costs and the potential for variation introduced by additional steps, RNase treatment was not routinely performed in subsequent DNA extractions.

Average *P. gaeumannii* colonization estimates among the three experiments designed to assess the precision of the TaqMan system were not significantly different (P = 0.35 according to analysis of variance F test) (Fig. 4). However, there were differences in variability estimates among the experiments. For example, the standard error for the intra-assay experiment (±0.36 SE), in which 10 replications of a single DNA sample were amplified concurrently, was slightly but not significantly (P = 0.91; Levene's test [9] for unequal variance) less than that for the same sample amplified over five separate assays (±0.48 SE). Because each assay may have differed slightly in reaction components, due

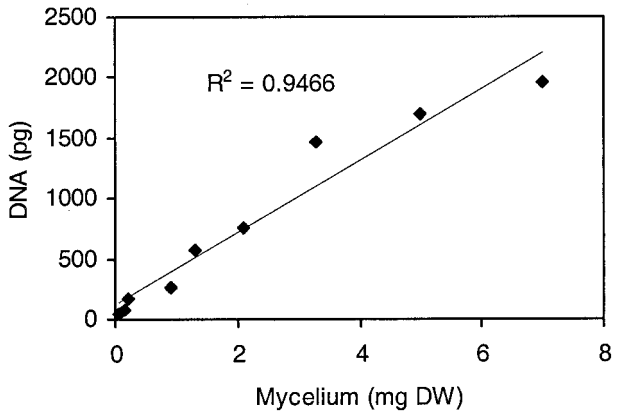

Fig. 3. Relationship between biomass estimates of dried *Phaeocryptopus gaeumannii* mycelium using real-time polymerase chain reaction (DNA) and dry weight (DW).

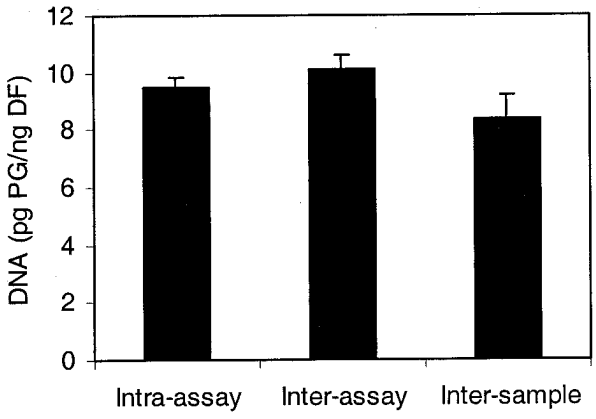

Fig. 4. Reproducibility of TaqMan estimates of *Phaeocryptopus gaeumannii* (PG) colonization of Douglas-fir (DF) foliage. Intra- and inter-assay reproducibility was evaluated on replicate reactions of DNA extracted from one foliage subsample amplified in single and multiple assays, respectively. Inter-sample reproducibility was evaluated in a single assay of 10 separate DNA extractions of different subsamples. Vertical bars represent standard errors.

to variation in pipetting accuracy, polymerase activity, and/or cycling temperatures, it is likely that the added variance was due to these factors. The greatest standard error was evident when different subsamples from a pool of needles were processed individually (±0.78 SE), and was significantly higher than both intra- and inter-assay variability ($P \leq 0.005$; Levene's test).

DISCUSSION

Accurate detection and quantification of pathogen colonization in host tissue is an important step in research on many plant diseases. These data are necessary for research on the ecology and epidemiology of plant pathogens and aid in evaluating potential resistant germ plasm. Traditionally, quantitative methodologies have relied either upon culturing the pathogen from infected tissues or visual identification and enumeration of pathogen propagules. While feasible for some pathogenic fungi at some stages of the disease cycle, many pathogens either lack distinctive characters at critical stages of the infection process or produce so many propagules that enumeration becomes unwieldy. For estimation of disease severity, Swiss needle cast research has depended on determining the proportion of stomata occluded with fruiting bodies, which first appear nearly 1 year following initial infection. Although this method has been useful for comparing severely diseased with infected, but apparently healthy plantations, real-time PCR has proved suitable for pathogen quantification early in the disease cycle, before fruiting bodies have developed.

To our knowledge, this is the first report on the application of real-time PCR technology to simultaneously quantify both pathogen and host DNA to derive a relative measurement of pathogen colonization of host tissue. Used in conjunction with the automated sequence detector, species-specific PCR primers and fluorogenic TaqMan probes enabled accumulating amplicons to be detected in real-time during the extension phase of the PCR reaction. In the early stages of developing this technique, we determined optimal probe and primer concentrations to prevent competitive interference between the two species in the multiplex PCR reaction (data not shown). This provided confidence that we scored uninfected needles accurately and enabled comparisons between needles of different sizes and developmental stages. In addition, multiplexing resulted in doubled assay output compared with separate tube amplifications.

Calibration experiments with known amounts of target DNA demonstrated that starting quantities that differed over 4 orders of magnitude were detected within the linear dynamic range of the TaqMan system. This enabled accurate quantification of *P. gaeumannii* colonization in severely diseased foliage and in apparently healthy foliage without the need to examine multiple dilutions of each sample. Precision and intra-assay reproducibility among replications were high for both the *P. gaeumannii* and Douglas-fir DNA standards as well as naturally infected foliage. Reproducibility decreased somewhat when replicates were compared between assays. Variance was greatest when subsamples from a single tree were examined. However, because the average colonization estimate for several subsamples was not significantly different from that of a single sample, one unreplicated sample per tree appears to be sufficient for routine stand-level quantification of *P. gaeumannii* colonization. A normal variance estimate for the entire subpopulation or treatment group should be sufficient for comparative analyses.

We are currently modifying the technique to detect and quantify *P. gaeumannii* ascospores deposited on grease-coated tape by an air-sampling spore trap. Initial tests are promising and have successfully detected ascospores on both laboratory- and field-inoculated tapes. Previous attempts to directly enumerate ascospores by microscopy have been confounded by the abundance of similar spores found in diseased forests. The application of real-time PCR will be critical for both observational and planned studies that address environmental and management parameters affecting inoculum potential in forest settings.

ACKNOWLEDGMENTS

Funding from the Swiss Needle Cast Cooperative of Oregon State University is gratefully acknowledged. We thank P. Reeser and W. Sutton for assistance with sample collection and DNA extraction and S. Strauss for sharing unpublished data.

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