Evaluation of Resistance to *Rhabdocline* Needlecast in Douglas Fir Variety Shuswap, with Quantitative Polymerase Chain Reaction

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Accepted for publication 15 December 2009.

ABSTRACT

Catal, M., Adams, G. C., and Fulbright, D. W. 2010. Evaluation of resistance to *Rhabdocline* needlecast in Douglas fir variety Shuswap, with quantitative polymerase chain reaction. *Phytopathology* 100:337-344.

A quantitative polymerase chain reaction assay was developed that could detect DNA of *Rhabdocline pseudotsugae* and *R. oblonga* among DNA of Douglas fir needles to a limit as low as three copies of target DNA. Differential infection rates of two varieties (seed sources) of Douglas fir interplanted in a field were studied in relation to staggered

bud breaks. Infection of Douglas fir var. San Isabel corresponded to ascospore release times for *Rhabdocline* spp., whereas infection of var. Shuswap Lake did not occur throughout the spore release period during 2 years of study, despite abundant inoculum and adequate moisture during bud break. *Rhabdocline* spp. DNA was never detected in Shuswap Lake and disease symptoms were not observed in any year. We provide evidence that Shuswap Lake is resistant and probably immune to *Rhabdocline* spp. infection and *Rhabdocline* needlecast under Michigan conditions.

Michigan is a major producer of plantation-grown Christmas trees in the United States and Douglas fir, *Pseudotsuga menziesii* (Mirb.) Franco, currently accounts for >15% of Christmas tree plantings in the state (1,14). There are two recognized subspecies of Douglas fir, coastal (*P. menziesii* subsp. *menziesii*) and intermountain (*P. menziesii* subsp. *glauca* (Beissn.) E. Murray); only the latter are grown in Michigan (22,25). Populations of intermountain Douglas fir are susceptible to needlecasting diseases and Cooley gall adelgid (*Adelges cooleyi* Gillette). Shuswap Lake (*P. menziesii* subsp. *glauca* var. *caesia* (Schwer.) Fitschen) is a seed source (provenance) from British Columbia resistant to Cooley gall adelgid (13). Furthermore, past evaluations have rated Shuswap Lake as resistant to *Rhabdocline* needlecast (13,31). In Michigan, Shuswap Lake breaks bud $\approx$ 2 weeks after other Douglas fir seed sources, which may help prevent late spring frost damage. Delayed bud break corresponding with an observed resistance to *Rhabdocline* needlecast in Shuswap Lake has led to speculation that bud break may be occurring when the preponderance of inoculum is exhausted.

Rhabdocline needlecast is one of the most important diseases of Douglas fir in Michigan and can cause serious damage in Christmas tree plantations if not properly managed (10,22–24). The disease causes chlorosis, premature needle loss, and reduced growth that decreases the quality and market value of Christmas trees. *Rhabdocline* needlecast is caused by five species of *Rhabdocline*; however, *Rhabdocline pseudotsugae* Syd. and *R. oblonga* (A. K. Parker & J. Reid) J. K. Stone & Gernandt are the only species found in Michigan (3,22,25). In addition, *R. parkeri* Sherwood, J. K. Stone & G. C. Carroll is known as an endophyte of Douglas fir needles (28,29) and sometimes is present in Michigan trees (3).

The *Rhabdocline* needlecast cycle begins in spring when ascospores are released from fruiting bodies maturing on the previous year's infected needles. *Rhabdocline* ascospores infect newly emerging needles shortly after bud break in the spring (4,5,18) and may continue into the summer. Monitoring the time of ascospore release, quantifying the inoculum, and utilizing resistant seed sources should contribute to better management of the disease, allowing growers to reduce or eliminate chemical treatments.

Douglas fir seed sources that appear resistant to *Rhabdocline* needlecast, such as Shuswap Lake, or individual trees of naturalized populations, are selected by visual disease rating under natural disease pressure (4,13,19) by astute seed collectors and growers. However, visual assessment has not been studied in context with the amount of infection or colonization of needle tissue by the pathogens or by the mode of resistance involved. Therefore, methods involving a more accurate assessment with quantification would be of value when screening Douglas fir for resistance to *Rhabdocline* needlecast.

Quantitative polymerase chain reaction (qPCR or real-time PCR) offers rapid and reliable DNA detection with high specificity and sensitivity. qPCR increasingly has been used to detect and quantify fungal plant pathogens, including Swiss needlecast in infected Douglas fir tissue (2,16,17,27,32). qPCR has also been used to monitor fungal colonization and screen plants for disease resistance where it proved to be better than traditional techniques (11). Furthermore, qPCR has been reported to be useful in detecting and quantifying airborne spores of *Fusarium circinatum* captured in spore traps (28).

The objectives of this study were to (i) develop a qPCR assay for quantifying *Rhabdocline* needlecast in Michigan, (ii) monitor and quantify *R. pseudotsugae* and *R. oblonga* in needle tissue of Shuswap Lake and other seed sources, (iii) monitor and quantify ascospores captured in spore traps, and (iv) compare spore release time with bud break of susceptible and resistant seed sources.

MATERIALS AND METHODS

Fungal strains and plant materials. Fungal strains used in this study are listed in Table 1. Isolates were grown and main-

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doi:10.1094/PHYTO-100-4-0337

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tained on malt extract agar (Difco Laboratories, Detroit) when possible. Fruiting bodies of *Rhabdocline* spp. on needle samples were identified to species based on morphological characteristics as described by Parker and Reid (26) and Stone and Gernandt (30).

Samples of needles of Shuswap Lake and San Isabel were collected from a Douglas fir Christmas tree plantation in Lake City, MI (Missaukee County) during April through June during 2 years. For preliminary assays, needle samples from current and 1-year-old needles with disease symptoms were collected in the middle of June of a previous year and frozen for future use. During the first study year, collection and testing started 21 April when new buds began to emerge and ended 20 June. During the second study year, collection and testing started 23 April when new buds began to emerge and ended 19 June. Shuswap Lake and San Isabel trees grown as a mixed population in a plantation were labeled. Samples were collected twice a week until 26 May and once a week thereafter in the first year; in the second year, samples were collected once a week. Needle samples from uninfected Douglas fir and Fraser fir (*Abies fraseri*, a nonhost) trees were used as negative controls. Terminal buds were photographed each week.

DNA extraction. Fungal strains capable of growth in culture were grown on cellulose membrane-covered plates. Hyphae were scraped from the surface and lyophilized. DNA was extracted from these lyophilized cultures using Qiagen DNeasy Plant Mini Kits (Qiagen Inc., Valencia, CA). For *R. pseudotsugae* and *R. oblonga* (obligate pathogens), DNA was extracted directly from infected needles. Lyophilized hyphae (50 mg) or 100 mg of

needle tissue were soaked in 1 ml of hexadecyltrimethyl-ammonium bromide extraction buffer (8,9) and ground with an acid-treated, sterilized mortar and pestle. Following incubation at 65°C for 1 h, the homogenates were centrifuged at 13,000 rpm for 5 min and the supernatants were transferred to QIA shredder minicolumns (Qiagen Inc.). The flow-through fraction was precipitated with 95% cold ethanol before applying to DNeasy minicolumns. The quality and quantity of DNA extracts were determined by gel electrophoresis and measurements at 260-nm wavelengths with a GeneQuant II spectrophotometer (Pharmacia Biotech, Cambridge, UK).

Design and specificity of primers. Primers used in this study are listed in Table 2. Primer pair RPP1/RPP4 was designed for conventional PCR amplification of *R. pseudotsugae* in an earlier study (3). The primer pair RPPOBL-F/RPPOBL-R was designed from internal transcribed spacer (ITS) regions of ribosomal RNA to amplify both *R. pseudotsugae* and *R. oblonga* in qPCR (Fig. 1). The ITS sequences of *R. pseudotsugae* (AF462420-AF462421-AF462422, U92290, U92291), *R. oblonga* (AF260814), *R. obovata* (U92293), *R. weirii* (U92300), *R. epiphylla* (U92292), *R. parkeri* (AF260813, AF462423-27-29), and *Phaeocryptopus gaumannii* (AY826766) are available in the National Center for Biotechnology Information GenBank. Other needle-inhabiting fungi were sequenced by the author (3) or obtained from GenBank and included in the alignment for primer selection. The sequences were aligned using the programs MegAlign and SeqMan in the DNASTAR software package (DNASTAR Inc., Madison, WI). Primer Express software (Applied Biosystems, Foster City, CA) was used to design qPCR primers. Primers were

TABLE 1. Fungal strains used for determination of specificity of primers in real-time quantitative polymerase chain reaction assays

Species	Isolate	Host	Source ^a
<i>Hormonema dematioides</i>	HD-DF	<i>Pseudotsuga menziesii</i>	Michigan
<i>Hormonema</i> sp.	H.sp-DF	<i>P. menziesii</i>	Michigan
<i>Meria parkeri</i>	MP-ATCC	<i>P. menziesii</i>	ATCC 62704
<i>Phaeocryptopus gaumannii</i>	PG	<i>P. menziesii</i>	Michigan
<i>Rhabdocline parkeri</i>	RP-ATCC	<i>P. menziesii</i>	ATCC 201660
<i>Phoma-like endophyte</i>	P.sp-DF	<i>P. menziesii</i>	Washington
<i>Botryosphaera</i> sp.	B.sp.-DF	<i>P. menziesii</i>	Michigan
<i>Aureobasidium pullulans</i>	AP	<i>Pinus sylvestris</i>	Michigan
<i>Sclerophoma pithyophila</i>	SP	<i>P. sylvestris</i>	Michigan
<i>Cyclaneusma niveus</i>	CN	<i>P. sylvestris</i>	CBS 495.73
<i>Lophodermium seditiosum</i>	LS	<i>P. sylvestris</i>	ATCC 28345
<i>L. pinastri</i>	LP	<i>P. sylvestris</i>	ATCC 28347
<i>Mycosphaerella dearnessii</i>	SA-1	<i>P. sylvestris</i>	Wisconsin
<i>Dothistroma pini</i>	DP	<i>P. nigra</i>	Michigan
<i>Cyclaneusma minus</i>	CM	<i>P. torreyana</i>	California
<i>Sphaeropsis sapinea</i>	SSP	<i>P. banksiana</i>	Michigan
<i>Sirococcus conigenus</i>	SC	<i>P. resinosa</i>	Minnesota
<i>Rhizosphaera pini</i>	RPIN-1	<i>Abies fraseri</i>	Michigan
<i>R. kalkhoffii</i>	RKA-ATCC	<i>Picea pungens</i>	ATCC 26605
<i>L. juniperinum</i>	LJ	<i>Juniperus</i> sp.	Michigan
<i>Kabatina juniperi</i>	KJ	<i>Juniperus</i> sp.	N. Carolina

^a ATCC = American Type Culture Collection. CBS = Centraalbureau voor Schimmelcultures.

TABLE 2. Primers used for conventional and real-time quantitative polymerase chain (qPCR) reaction detection and quantification of the target organism

Targets and primer designations	Sequence	Tm ^a	Amplicon (bp)
<i>Rhabdocline pseudotsugae</i> / <i>R. oblonga</i>			
RPP1 ^b	5'-CAGATCTCCCACCCGTGTGT	52	340
RPP4	5'-CCACTAGTTTAGGGCCGA	...	...
RPPOBL-F ^c	5'-CCCTTACGCCTTGCGTAGTCT	60	71
RPPOBL-R	5'-GAGGGGACACCGCCACTA	...	...
<i>Pseudotsuga menziesii</i>			
LFY989F ^d	5'-GGTCACAAACCAAGTATTTCGACA	60	123
LFY1102R	5'-TGTTCAACATCCAGGCAATGA	...	...

^a Denotes specific annealing temperatures used in the amplifications.

^b Primer pairs were used for conventional end-point PCR.

^c Primer pairs were used for real-time qPCR.

^d Primers were used for amplification of Douglas fir DNA as an endogenous control to check the quality of DNA used in qPCR and the quantity of host DNA.

synthesized at the Macromolecular Structure and Sequencing Facility (Michigan State University, East Lansing) using a 3948 Oligonucleotide Synthesizer (Applied Biosystems). A Douglas fir primer pair was included as endogenous control to check the quality of DNA extracts and distinguish uninfected foliage from PCR inhibition. For Douglas fir DNA amplification, the primers LFY989F and LFY1102R designed by Winton et al. (32) from LEAFY/FLORICAULA genes were used.

The specificity of primer pair RPPOBL-F/RPPOBL-R was tested against isolates of other fungi representing various pathogenic and endophytic genera from different conifers (Table 1). qPCR amplifications for specificity were carried out using genomic DNA extracted from pure cultures of each isolate. Suitability of each DNA sample for the assays was verified by amplifying the extract from each isolate with universal primers ITS1F and ITS4 (7).

Spore trapping and counting. Ascospore release of *R. pseudotsugae* and *R. oblonga* was monitored 15 May through 18 June in the first year and 24 April through 19 June in the second year. A Burkhard volumetric spore trap (Burkhard Scientific, Richmansworth, Hertfordshire, UK) was placed in the center of an abandoned Douglas Fir plantation of unknown provenance showing severe levels of *Rhabdocline* needlecast. The abandoned stand was 400 m from the trees monitored in the Douglas fir plantation under study and was considered a main source of inoculum for *Rhabdocline* spp. infection in the plantation. The Burkhard trap was adjusted to sample 0.6 m³ of air per hour, 1 m above the ground. Burkhard collection tapes were replaced and processed weekly. In the lab, the tapes were cut into 48-mm sections (representing 1 day) and mounted on microscope slides. Tapes were further divided into 2-mm segments representing 1-h periods and examined under a microscope at ×200 for the presence of ascospores. Five randomly chosen 1-h segments were examined for each day by scanning each hourly segment along the two parallel lines. The ascospores of the *Rhabdocline* spp. were identified and differentiated from the co-trapped spores of other fungi based on the aseptate bone- or dumbbell-shaped morphology described by Parker and Reid (26) and the size range described by Stone and Gernandt (30).

Conventional PCR. Conventional PCR assays with primer pairs RPP1/RPP4 and ITS1F/ITS4 were performed in total volumes of 25 µl consisting of 12.5 µl of DNA template (1 to 2 ng/µl) and 12.5 µl of PCR mixtures. The mixtures contained 1× PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl, and 2.0 mM MgCl₂); 0.2 mM each dATP, dTTP, dGTP, and dCTP; 0.5 µM each primer; and 0.5 unit of Taq DNA polymerase (Invitrogen Life Technologies, CA). The amplifications were carried out on a DNA thermal cycler (Gene Amp PCR System 2400; Perkin-Elmer Cetus, Norwalk, CT). The cycling conditions consisted of an initial denaturation at 96°C for 3 min; followed by 35 cycles of 96°C for 1 min, 52°C for 1 min, and 72°C for 1 min; with a final

extension at 72°C for 10 min. PCR products were separated on 2% agarose (Gibco BRL, Grand Island, NY) in 1% Tris-acetate-EDTA buffer (100 mM Tris, 12.5 mM sodium acetate, and 1 mM EDTA, pH 8.0) by gel electrophoresis. Following electrophoresis for at least 1 h at 100 volts, the gels were stained with ethidium bromide, visualized by UV fluorescence, and photographed using AlphaImager (Alpha Innotech Corporation, San Leandro, CA).

qPCR. qPCR amplifications were performed in either Microamp optical 96-well reaction plates or single optical tubes with optical caps using the automated ABI Prism 7000 sequence detector system (Applied Biosystems). The reactions were carried out in a volume of 25 µl and contained 1× SYBR Green PCR Master mix (Applied Biosystems), 300 nM each primer, and 10 µl of DNA template dilutions. The thermal cycling protocol consisted of an initial incubation at 50°C for 2 min and denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Cycle threshold (Ct) values were calculated and analyzed using the ABI Prism 7000 SDS software, version 1.1. A melting curve analysis was performed at the end of each run to determine the presence or absence of the target sequences and to differentiate specific amplifications from nonspecific ones and primer-dimers.

qPCR was performed to determine the absolute quantities of *Rhabdocline* spp. in needle samples. DNA extracts from buds and newly emerged and 1-year-old needles were diluted 100-fold (≈20 ng per reaction) and amplified in qPCR assays for quantification. Three replications of test, negative (uninfected Douglas fir or Fraser fir needle), and nontemplate (PCR water) control samples were assayed in each replication.

In order to create standard curves, ITS genes of *Rhabdocline* spp. (*R. pseudotsugae* and *R. oblonga*) were amplified with the primers ITS1F and ITS4 from DNA extracted from fruiting bodies (excised from needles containing only the respective *Rhabdocline* sp.) (3). Resulting PCR products were purified (QIAquick PCR purification kit, Qiagen Inc.) and quantified using a spectrophotometer. The copies of target DNA molecules (purified ITS PCR product) were calculated. A subset of a 10-fold dilution series of fungal DNA ranging from 3.24 × 10⁷ to 3.24 × 10⁰ copies per reaction was amplified in each absolute qPCR assay in three replications to generate the standard curves.

Absolute qPCR assays were also performed to determine whether PCR inhibitors were present in Douglas fir DNA extracts. Each dilution series of fungal DNA used for standard curves was mixed with 20 ng of Douglas fir DNA from uninfected leaves which represented the proportions of naturally infected foliage to give the same final concentrations of 3.24 × 10⁷ to 3.24 copies per reaction. A standard curve was also constructed from amplification of these samples containing both fungus and plant DNA. This standard curve was used for the calculation of absolute quantities of *Rhabdocline* spp. DNA (copies) in infected Douglas fir tissues using the Ct values of test samples from the

Fig. 1. Diagram showing the locations of *Rhabdocline pseudotsugae* and *R. oblonga*-specific real-time polymerase chain reaction primers within the internal transcribed spacer region (ITS2) of ribosomal DNA. The sequences represent the conserved sequences of *Rhabdocline* needlecast pathogens: *R. pseudotsugae* (RPP), *R. oblonga* (ROB), *R. parkeri* (RPA), and *Phaeocryptopus gaumannii* (PGA) species available in the National Center for Biotechnology Information GenBank.

field. The absolute quantities of *Rhabdocline* total DNA were expressed as copies per milligram of fresh needle sample.

RESULTS

Primer specificity. In qPCR assays for specificity, the primer pair RPPOBL-F/RPPOBL-R amplified only DNA of target fungi *R. pseudotsugae* and *R. oblonga*. Primers consistently yielded Ct values <16 and highest fluorescence with DNA of both species. There was no amplification of DNA from any of 14 fungal strains in Table 1, including the Douglas fir endophyte *R. parkeri* and Swiss needlecast pathogen *P. gaumannii*, both colonizers of Douglas fir needles. Furthermore, melting curve profiles revealed two different peaks of 81.1 and 83.1 for the PCR products from *R. oblonga* and *R. pseudotsugae*, respectively, which allowed differentiating the two species (Fig. 2). The primers occasionally

Fig. 2. Melting curve analysis of quantitative polymerase chain reaction products of *Rhabdocline pseudotsugae* and *R. oblonga* amplified with primer pair RPPOBL-F/RPPOBL-R.

yielded Ct values of ≥ 36 with uninfected Fraser fir and Douglas fir DNA (negative controls) and nontemplate (water control) controls. However, melting curve analysis of those samples revealed temperature (T_m) peaks characteristic of nonspecific amplification and primer-dimer formations (Fig. 2).

Assay sensitivity and DNA standard curves. In assays for construction of standard curves, the primer pair efficiently detected and amplified DNA of both *R. pseudotsugae* and *R. oblonga* through eight orders of magnitude of 10-fold dilutions starting with 3.24×10^7 initial amounts (copies) of template. Analysis of amplification profiles and melting curves indicated that assays with the primer produced reproducible and reliable Ct values up to 36 cycles in repeated assays. Detection was unreliable after 36 cycles because melting (T_m) peaks could not be distinguished from background. The detection limit of assays with the primer pair was as low as three copies of DNA per reaction. The 10-fold DNA concentrations lower than this yielded no or inconsistent Ct values and did not fit in a linear regression. A linear relationship was found when Ct values were plotted against the logs of the initial amounts of *R. pseudotsugae* and *R. oblonga* DNA. The correlation coefficients (R^2) for the standard curves were 0.9976 with *R. pseudotsugae* DNA and 0.9958 with *R. oblonga*.

In inhibition assays, the primer pair successfully amplified the same eight 10-fold serial dilutions of the target fungus DNA (*R. oblonga*) in the presence of Douglas fir DNA (Fig. 3A). Assays gave rise to reproducible and reliable Ct values of up to 36 cycles, as verified by amplification profile and melting curve analysis in two separate assays. Again, amplification after 36 cycles was not reliable because of absence of specific melting curve peaks. The standard curve produced by eight dilutions of purified fungal DNA (*R. oblonga*) mixed with Douglas fir DNA corresponded well to the standard curve obtained by the same dilutions of fungal DNA (*R. oblonga*) in water (Fig. 3B). The detection limit of assays in the mixed fungus and plant DNA was also three copies of target DNA per reaction. Amplification efficiency of the primers in the presence of Douglas fir DNA was nearly the same as that obtained from dilutions in water as indicated by slope values of standard equations. The linear relationship between the initial amounts of *R. oblonga* DNA in the presence of Douglas fir

Fig. 3. Inhibition assays of fungal DNA (*Rhabdocline oblonga*) mixed with plant (Douglas fir) using quantitative polymerase chain reaction (qPCR). **A**, Real-time amplification profile of 10-fold serial dilutions of *R. oblonga* DNA (purified internal transcribed spacer [ITS] PCR product) containing 20 ng of Douglas fir DNA in each reaction. Each amplification curve represents four replications. The starting ITS DNA copy numbers ranged from 3.24×10^7 to 3.24 copies. Delta Rn (ΔRn) values indicate the accumulation of fluorescent emission when normalized against the background emission. The figure shows the accumulation of fluorescence in the eight dilutions of fungal DNA over the 40-cycle-long PCR reaction. The horizontal line indicates the baseline. Cycle threshold (Ct) value shows specific cycle at which ΔRn passes the baseline. **B**, Standard curves were constructed from *R. oblonga* and Douglas fir (20 ng) DNA mixtures and used for conversion of Ct values to the fungal DNA amounts present in the field test samples from Douglas fir foliage. The log of the initial amounts (copies) of *R. oblonga* DNA + Douglas fir DNA was plotted against the corresponding Ct values.

DNA, and the Ct values, were similar to those observed in standard curves from only *R. oblonga* DNA in water. The correlation coefficient for the standard curve was 0.9944. These results confirmed that real-time PCR amplification assays with the primer pair were specific to target fungi and not inhibited by the presence of Douglas fir DNA.

Detection and quantification of *Rhabdocline* needlecast in Douglas fir seed sources. In initial assays with conventional PCR, *Rhabdocline* spp. were detected in current and 1-year-old needles from the seed source San Isabel collected in June in a previous year and stored frozen. The pathogens were not detected in current-year or 1-year-old needles from the seed source Shuswap Lake collected at the same time in repeated assays (data not shown). Likewise, current or 1-year-old needle samples of the variety Shuswap Lake collected in June in a previous year always tested negative for *Rhabdocline* spp. in preliminary qPCR assays (Table 3). Needles of San Isabel, regardless of needle year or age, tested positive in repeated assays. Infected current-year needles of San Isabel consistently yielded Ct values of 32 (average) whereas 1-year-old needles produced Ct values of ≤ 23 . An average of 4.34×10^3 *Rhabdocline* DNA copies per milligram of needle tissue was detected in the current-year needle samples whereas, in 1-year-old needles, the amount was 3.32×10^6 copies/mg.

In real-time qPCR assays with buds or needle samples collected from 21 April through 20 June the first year, Shuswap Lake tested negative for *Rhabdocline* spp. in all the samples regardless of collection date and needle year (Table 4). *Rhabdocline* spp. also were not detected in buds or current-year needle samples of the seed source San Isabel until the third week of May. San Isabel was observed to break bud between 6 and 13 May (Fig. 4). The bud break period for the variety Shuswap was between 24 and 31

May. The current-year needles of San Isabel tested positive for *Rhabdocline* spp. starting 18 May. An average Ct value of 33 was obtained from 18 May through 12 June. The copies of *Rhabdocline* spp. DNA varied from 1.22×10^3 to 5.13×10^3 copies/mg needle tissue during the same period (Table 4). Noticeable increases in Ct value (29.5) and, therefore, the *Rhabdocline* DNA quantities (2.63×10^4 copies/mg) were observed in samples collected after 20 June. Melting curve analysis of these samples showed that needles on the same tree were found to be infected by either *R. oblonga* or *R. pseudotsugae* alone or often together. In dual infections, two distinctive melting-curve peaks of variable height were visible (Fig. 2). We did not observe peaks indicative of heteroduplexes of the different ITS fragments, although they were hypothesized to occur in such dual infections.

In the second year, in qPCR assays with bud or needle samples collected from 21 April through 20 June, the seed source Shuswap Lake again tested negative for *Rhabdocline* spp. in all samples regardless of collection time and needle age (Table 5). The needlecast fungi also were not detected in bud or current-year needle samples of the variety San Isabel until the fourth week of May. San Isabel was observed to break bud between 7 and 14 May (Fig. 4). The bud break period for Shuswap was between 23 and 30 May. The current-year needles of San Isabel tested positive for *Rhabdocline* spp. starting May 21. The Ct values obtained from 18 May through 19 June were 34.2 to 32.4. The quantities of *Rhabdocline* spp. varied from 9.73×10^2 to 4.05×10^3 copies/mg of needle tissue during the same period.

Spore trapping and quantification. The first-year data from spore traps indicated that *Rhabdocline* spp. had already initiated ascospore release when the spore trap was placed in the field (Fig. 5). Spore release was observed until the second week of June.

TABLE 3. Real-time quantitative polymerase chain reaction (qPCR) assays of needle samples from Douglas fir varieties Shuswap Lake and San Isabel collected in June during a prior year of epidemic disease

Variety	Collection date (prior year)	Needle age	Ct ^a	DNA copy/mg ^b
Shuswap Lake	15 June	1 year old	ND	0
Shuswap Lake	15 June	Current year	ND	0
San Isabel	15 June	1 year old	22.1	3.32×10^6
San Isabel	15 June	Current year	31.8	4.34×10^3

^a Cycle threshold (Ct) values in qPCR. ND = no detection after 40 cycles.

^b Absolute quantities (copies) of *Rhabdocline* spp. DNA (ITS PCR product) per milligram fresh needle tissue.

TABLE 4. Real-time quantitative polymerase chain reaction (qPCR) assays of needle samples from Douglas fir varieties Shuswap Lake and San Isabel collected from 21 April through 20 June in the first study year

Variety	Collection date	Needle age	Ct ^a	DNA copy/mg ^b
Prior year				
Shuswap Lake	15 June	1 year old	ND	0
Shuswap Lake	15 June	Current year	ND	0
San Isabel	15 June	1 year old	20.1	1.11×10^7
First study year				
San Isabel	21 April	Bud	ND	0
San Isabel	25 April	Bud	ND	0
San Isabel	28 April	Bud	ND	0
San Isabel	3 May	Bud	ND	0
San Isabel	6 May	Bud	ND	0
San Isabel	9 May	Bud	ND	0
San Isabel	12 May	Bud	ND	0
San Isabel	15 May	Bud	ND	0
San Isabel	18 May	Current year	33.3	1.91×10^3
San Isabel	23 May	Current year	33.9	1.22×10^3
San Isabel	26 May	Current year	33.9	1.24×10^3
San Isabel	31 May	Current year	33.9	1.28×10^3
San Isabel	2 June	Current year	33.2	2.13×10^3
San Isabel	5 June	Current year	32.7	4.44×10^3
San Isabel	12 June	Current year	32.6	5.13×10^3
San Isabel	20 June	Current year	29.5	2.63×10^3

^a Average cycle threshold (Ct) values obtained in qPCR from DNA extracts from three different needle samples (standard deviation [SD] ± 1.000) from three different buds (or newly emerging needles). Each sample was tested in triplicate (SD < 0.100 per replicate). ND = no detection after 40 cycles.

^b Average absolute quantity (copies) of *Rhabdocline* spp. DNA in fresh needle tissues of three samples.

However, there was considerable day-to-day variation in the number of spores released. The second-year data indicated that *Rhabdocline* spp. did not release ascospores until the third week of May (Fig. 5). However, the number of observed ascospores was low when compared with similar dates in the prior year until 4 June, after which high numbers of spores were observed for the following 2 weeks.

DISCUSSION

Plant resistance is the most effective and environmentally sound method of controlling plant diseases. Selection and production of *Rhabdocline* needlecast-resistant seed sources of Douglas fir will play an important role in effective management of the disease. However, sensitive detection and accurate quantification of pathogens in host tissue are essential for evaluating seed sources for needlecast resistance. Traditional methods of detection and quantification of pathogens are dependent on culturing the pathogen from infected tissues. Such methods do not

succeed with *Rhabdocline* pathogens because they do not grow on laboratory media. In this study, we have developed a sensitive and effective real-time qPCR assay primarily to detect and quantify *R. pseudotsugae* and *R. oblonga* in Douglas fir foliage. However, the primer pair could also detect and amplify other pathogenic *Rhabdocline* spp. which occur in other regions of North America and, therefore, the qPCR assay could be used to quantify *R. obovata*, *R. epiphylla*, and *R. weirii* but not the epiphytic species *R. parkeri*. *R. pseudotsugae* and *R. oblonga* are the most common pathogenic species of *Rhabdocline* and cause epidemics in Douglas fir Christmas tree plantations in Michigan and elsewhere (4,18). The two species are routinely identified and differentiated indirectly, based on host symptomology and the pattern of epidermal dehiscence over the apothecium (21,22,30). The qPCR assay developed in this study allowed for direct and more accurate differentiation of the two pathogenic species, simultaneous detection, and quantification. The assay could be a reliable tool to measure the distribution and relative frequencies of the two species in epidemiological studies and, potentially, in comparing and quantifying disease severity. The technique for quantifying a fungus in field conditions needs to be specific so that native or other exotic fungal species can be excluded. In our test for specificity, we included common needle fungi that could be found in the plantations where many different Christmas tree species were grown together. The assay with designed primers did not amplify any of the fungi tested. The

Fig. 4. Developmental stages of Douglas fir seed sources Shuswap Lake and San Isabel needles during first *Rhabdocline* spp. infections detected by real-time quantitative polymerase chain reaction (qPCR) and PCR assays. Conditions of needles on A, 18 May and B, 26 May in the first year and C, 21 May and D, 28 May in the second year. Resistance of Shuswap Lake was intuitively believed due to the evident delayed bud break, whereas this study demonstrated that resistance was an inherent characteristic of the needles and that timing of bud break was not relevant.

Fig. 5. Enumeration of ascospores of *Rhabdocline* spp. captured with a Burkhard volumetric spore trap in the first and second years of study. First quantitative polymerase chain reaction positives for infected needle tissue in first and second study years were 18 and 21 May, respectively.

TABLE 5. Real-time quantitative polymerase chain reaction (qPCR) assays of needle samples from the Douglas fir varieties Shuswap Lake and San Isabel collected from 23 April through 19 June in the second study year

Variety	Date (second year)	Needle age	Ct ^a	DNA copy/mg ^b
Shuswap Lake	15 June	1 year old	ND	0
Shuswap Lake	15 June	Current year	ND	0
San Isabel	15 June	1 year old	21.6	4.0 × 10 ⁶
San Isabel	23 April	Bud	ND	0
San Isabel	30 April	Bud	ND	0
San Isabel	7 May	Bud	ND	0
San Isabel	14 May	Bud	ND	0
San Isabel	21 May	Current year	33.6	1.53 × 10 ³
San Isabel	28 May	Current year	34.2	9.73 × 10 ²
San Isabel	4 June	Current year	32.8	2.61 × 10 ³
San Isabel	11 June	Current year	32.6	2.82 × 10 ³
San Isabel	19 June	Current year	32.4	4.05 × 10 ³

^a Average cycle threshold (Ct) values obtained in qPCR from DNA extracts from three different needle samples (standard deviation [SD] ± 1.000) from three different buds (or newly emerging needles). Each sample was tested in triplicate (SD < 0.100 per replicate). ND = no detection after 40 cycles.
^b Average absolute quantity (copies) of *Rhabdocline* spp. DNA in fresh needle tissues of three samples.

high specificity of the assay was further shown when no amplification was observed in assays with the Douglas fir endophyte *R. parkeri*, the closest related species to the target fungi (29).

Amplification efficiency and sensitivity is essential for detecting and monitoring fungal colonization at very early stages of infection. Assays for the standard curve showed that the primers amplified both *Rhabdocline* spp. with similar high efficiencies. Standard curves constructed from PCR products have often been used in qPCR assays (2,12,15,17). In this case, standard curves were created from PCR products amplified from the fruiting bodies of *R. pseudotsugae* and *R. oblonga* picked from infected needles because they cannot be cultured for DNA extraction. The observed sensitivity threshold of our assay was less than three copies and corresponding Ct values were ≤ 36 , which should allow for detection of low levels of *Rhabdocline* infection.

DNA extracts without PCR inhibitors are crucial for accurate quantification of fungal DNA in infected plants. PCR inhibitors can cause complete failure of PCR reaction or reduce assay sensitivity (6,24). In our inhibition assays, amplification of the target DNA had similar efficiency in the presence of Douglas fir DNA and in pure water, indicating that the DNA extracts do not contain PCR inhibitors and the amplification is not affected by Douglas fir DNA.

The amount of *Rhabdocline* DNA copies per milligram of needle tissue in relationship to dates of ascospore release and magnitude of airborne ascospores trapped was important to the interpretation of the epidemiology of the needlecast disease. Current-year needles became infected within 3 days of first detection of ascospores with the Burkhard spore trap (Fig. 5). This was true even in the second year of study, when the airborne spore load was near the lower limit of sensitivity for detection with the trap (Fig. 5). Further infection and colonization of the current-year needles apparently did not occur later, despite the many days of release of increasingly abundant numbers of airborne propagules, because DNA assays showed amounts of pathogen DNA copies per milligram remained steady at the initial level of 103 at the time ascospores were first detected. From the standpoint of selection pressure, one would see no role for the local release of increasing amounts of spores through the month, at least for the study years. The majority of spores which were released in later weeks (Fig. 5) could be important primarily for longer-distance dispersal, where dilution of spore numbers would be a factor. Our experiences in Michigan were that *Rhabdocline* needlecast disease usually occurred over large regions of the state in years of favorable climatic conditions and were not limited to local regions. Continuing this study over some years of weather unfavorable for infection, or with regard to hosts at a range of distances from the field releasing the ascospores, could improve understanding of the importance of amplitude and duration of ascospore release.

The colonization of needle tissue of San Isabel remained at 10^3 DNA copies/mg throughout the first season but 1-year-old needles had 10,000 to 1,000 $\times$ the copy numbers of pathogen DNA per milligram of tissue by 15 June (Tables 4 and 5). The low colonization level the first season could be interpreted as an initial endophytic and possibly biotrophic growth phase. The increase to 10^6 to 10^7 DNA copies/mg of tissue in the second season could correspond to the expected change to a virulent phase, perhaps including the launching of pathogenicity factors and acceleration of host colonization. An alternative scenario could be that year-old needles may be much less effective, physiologically, in resisting expansion of the pathogen in comparison with current-year needles. If the latter is the case, then perhaps resistance is evolving selectively in the current-year needles in Isabel but is not yet evident under the cultivation practices of plantation Christmas tree growing. Regardless, colonization of 10^6 to 10^7 DNA copies/mg of tissue appeared to be approximately the threshold extent resulting in needle desiccation, partial necrosis, and casting in San Isabel in Michigan.

Differential reactions of Shuswap Lake and San Isabel to *Rhabdocline* spp. as determined by qPCR assays were consistent with the status of the varieties as resistant and susceptible seed sources in previous studies (13,31). Shuswap Lake was not infected by *Rhabdocline* pathogens because fungal DNA was not detected in any needle samples tested, regardless of needle age or date (Tables 4 and 5). On the other hand, San Isabel was infected soon after bud break because significant levels of pathogen DNA were detected in newly emerged needles and older needles showed high levels of the pathogen. These results indicated that Shuswap Lake is completely resistant or immune whereas San Isabel is sensitive to *Rhabdocline* needle cast under Michigan field conditions. The observation that Shuswap Lake breaks bud 2 weeks later than San Isabel and, therefore, escapes the infection could not be the basis for disease resistance because *Rhabdocline* spp. continue releasing spores during and after bud break of Shuswap Lake (Fig. 5). Shuswap Lake was resistant to both species found in Michigan. It was not determined whether *R. parkeri* was present as an endophyte in this study because it was rarely encountered in Michigan when previously sought using DNA probes (3) and it was known to be not found in young needles (29).

The two seed sources in this study were chosen on the basis of our earlier observations and those of previous studies regarding their damage levels expressed during *Rhabdocline* needlecast epidemics. The qPCR assay we developed presents a rapid and reliable tool for monitoring *Rhabdocline* spp. infection and colonization in Douglas fir. The specificity, sensitivity, accuracy, and high-throughput nature of qPCR should allow efficient and dependable screening of large numbers of Douglas fir seed sources for resistance responses to *Rhabdocline* needlecast. The assay will especially be an invaluable asset for quantifying the fungal colonization in seed sources that become infected but fail to display symptoms or suffer from damage of the disease.

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