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Development of a qPCR Assay for the Detection and Quantification of the Fungal Pathogen *Calonectria canadiana* on Conifers

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ABSTRACT

A real-time PCR TaqMan assay was developed for the detection of *Calonectria canadiana*, a fungal pathogen responsible for damping off, root rot and seedling blight in conifer forest nurseries in central and eastern North America. While highly significant in Quebec Forest nurseries, coniferous seedling mortality decreased significantly when nurseries transitioned from bare root to container seedling production. However, over the past few years, this pathogen has re-emerged as a threat and millions of container white spruce seedlings were culled in two nurseries in eastern Quebec. A sensitive detection and quantification assay for *C. canadiana* was essential to investigate the biological and environmental factors driving this new epidemic. We designed primers and a TaqMan probe targeting the internal transcribed spacer (ITS) of *C. canadiana*. The resulting Ccan TaqMan assay successfully differentiated *C. canadiana* from other soil-borne pathogens of the Nectriaceae encountered in Quebec Forest nurseries. The limit of detection of the assay was established at eight copies of *C. canadiana* ITS. The Ccan TaqMan assay quickly identified the presence of the pathogen in both symptomatic and asymptomatic white spruce (*Picea glauca*) seedlings. Furthermore, we demonstrated that the pathogen was more easily detected when DNA was extracted from necrotic needles at the base of the stem rather than from necrotic roots. This molecular tool will greatly aid in understanding the biology and epidemiology of *C. canadiana*.

1 | Introduction

Calonectria canadiana (syn. *Cylindrocladium canadense*) (Crous) is a fungal pathogen that affects conifers, causing root rot, damping off and seedling blight in spruce and pines (Cox 1954). This disease has had a significant impact on the production of coniferous seedlings in North America. In the late 1950s and early 1960s, mortality rates of 60%–90% were observed among conifer transplants in three Wisconsin nurseries (Thies and Patton 1970). Within a single year, the fungal pathogen destroyed 80% of the black spruce (*Picea mariana*) seedlings in a Minnesota nursery, resulting in its closure (Menge and French 1976). Juzwik, Honhart, and Chong (1988) conducted a survey of five forest nurseries in Ontario and found that up to 40% of spruce seedlings were infected by *C. canadiana* (previously attributed to *Cylindrocladium floridanum*). Prior to the mid-1990s, millions of coniferous seedlings were being killed annually by root rot pathogens, with *C. canadiana* being the primary culprit, in Quebec nurseries (Canada 1995). The significant impact of the pathogen reported up until the 1990s was associated with the bare-root production of coniferous seedlings. However, losses due to Cylindrocladium root rot became minimal when the majority of nurseries transitioned to container production by the

mid-1990s (Bonneau et al. 1997). It was hypothesised that microsclerotia were the main infectious propagules, allowing the fungal pathogen to overwinter and persist from one crop to the next (Thies and Patton 1970). The switch to container production, using fresh peat moss substrate free of *C. canadiana* in each production cycle, was identified as the primary factor contributing to the reduction of Cylindrocladium root rot in forest nurseries (Sutherland 1991).

Beginning in the late 2010s, there was a resurgence of Cylindrocladium root rot in two forest nurseries located in Gaspesia, eastern Québec. Although both nurseries produce seedlings exclusively in containers, millions of white spruce seedlings have been lost to *C. canadiana*. The epidemiology of these current epidemics remains unclear. Affected seedlings with chlorotic foliage have been observed mid-season, with surrounding seedlings later developing the same symptoms. The infection foci expand radially, and by the end of the growing season, they contain dead, chlorotic and cankered seedlings. While some containers are recycled, it appears unlikely that microsclerotia, present in necrotic root fragments contaminating recycled containers, and carried over from one production cycle to the next, can fully account for the progression of symptoms observed in the production fields of the Gaspesian nurseries. Therefore, monitoring the distribution of *C. canadiana* inoculum in different tissues of seedlings exhibiting varying symptoms, as well as in samples collected from different dispersal pathways (such as air and water), is crucial for elucidating the epidemiology of the ongoing epidemic in the Gaspésie region. Currently, there are no protocols available to detect the presence of *C. canadiana* in environmental samples other than through conventional morphology-based methods involving isolation in pure culture and microscopic observations, which are resource intensive. Quantitative PCR (qPCR) is a specific and sensitive tool for detecting and quantifying microbial propagules in various environmental matrices. Thus, the objective of this study was to develop a highly sensitive and specific TaqMan assay for the detection of *C. canadiana*, subject it to a rigorous set of validation tests and assess its effectiveness for detecting the pathogen in different environmental samples.

2 | Materials and Methods

2.1 | Description of Fungal Material and Culture Methods

The fungal isolates used in this study are listed in Table 1. Most of these fungal strains were isolated from diseased conifer seedlings in Quebec Forest nurseries, and stored in the cryo-preserved mycological culture collection at the Laurentian Forestry Centre. Mycelial plugs from the liquid nitrogen storage were cultured on Oxoid 2% malt extract agar (Thermo Fisher). Identity of the isolates was confirmed by Sanger sequencing of the rDNA amplicon obtained using ITS1F and ITS4 primers (Gardes and Buns 1993). Sequences of the *tub2* barcoding gene were obtained to confirm the identity of the *C. canadiana* isolates recently collected in Gaspésie (Figure S1). To obtain *C. canadiana* conidia, the CFL5878 isolate was grown on synthetic nutrient-poor agar (SNA) (Nirenberg 1981) supplemented with 0.2% lactose instead of glucose.

2.2 | Design of the qPCR Assay

The ribosomal RNA region, including the internal transcribed spacers ITS1 and ITS2 and the 5.8S ribosomal RNA region, was chosen as the target gene for our detection assay as it is the most commonly used fungal barcoding sequence (Schoch et al. 2012). ITS sequences from the target and sister species were downloaded from GenBank (Table 1) and aligned with the BioEDIT software (Hall 1999) using ClustalW (Thompson, Higgins, and Gibson 1994). The alignment was manually examined to select primers and probe sites. Oligonucleotides were ordered from Integrated DNA Technologies (IDT Inc., Coralville, IA, USA), and experimentally assessed on a panel of DNA extracted from the isolates listed in Table 1.

2.3 | DNA Extraction

For DNA extraction from pure cultures, the fungal isolates were grown on malt extract agar (MEA) overlayed with a sterile cellophane sheet. The mycelium was scraped with a sterile scalpel blade, transferred to a 2-mL Eppendorf safe-lock microtube and homogenised twice for 1 min 30 s at 30 Hz with a 3-mm tungsten bead. DNA from these samples was extracted using the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Following tissue homogenisation, as described above, DNA from conidia and plant tissues was extracted with a QIAcube using the QIAamp DNA micro kit (Qiagen), with an overnight incubation at 56°C in the lysis buffer supplemented with 1 μL Reagent DX (Qiagen), and according to the manufacturer's instructions.

2.4 | Real-Time qPCR Conditions

The qPCR reactions were prepared in a MicroAmp Fast Optical 96-Well Reaction Plate sealed with MicroAmp Optical Adhesive Film. All qPCR reactions were run in a 7500 Fast qPCR instrument (Thermo Fisher Scientific, Waltham, MA, USA). TaqMan reactions of 10 μL contained 1× SensiFAST Probe No-ROX mix (Meridian Bioscience, Cincinnati, OH, USA), 500 nM of CcanF and CcanR primers and 100 nM CcanProbe (Table 2). TaqMan cycling conditions were as follows: 5 min at 95°C followed by 40 cycles of 15 s at 95°C, and 45 s at 60°C.

2.5 | Standard Curve on Serial Dilutions of a gBlock Fragment From the *C. canadiana* ITS

A gBlock including the *C. canadiana* ITS-targeted sequence was ordered from IDT Inc. (Figure S2). Absolute quantification of the gBlock solution diluted at 1 ng/μL in nuclease-free water was performed by SYBR Green qPCR. Reactions were performed in a final volume of 10 μL and contained 1X QuantiTect SYBR Green PCR Master Mix (Qiagen), 0.5 μM of each of the Ccan forward and reverse primers (Table 2) and 1 μL of template DNA. Thermocycling conditions were set at 95°C for 15 min, followed by 50 cycles at 95°C for 15 s, 51°C for 30 s and 65°C for 90 s. Fluorescence was read at the end of the extension step. Absolute

TABLE 1 | List of fungal isolates used in this study[a].

Species	Isolate ID	Host	Isolation date	Source	Ccan assay detection
Calonectria canadiana	CFL 852	*Picea glauca*	1966	LFC[b]	+
	CFL 5846	*P. glauca*	1996	LFC	+
	CFL 5847	*P. glauca*	1996	LFC	+
	CFL 5848	*P. glauca*	1995	LFC	+
	CFL 5854	*Picea mariana*	1992	LFC	+
	CFL 5855	*P. mariana*	1992	LFC	+
	CFL 5870	*P. mariana*	1995	LFC	+
	CFL 5871	*P. mariana*	1992	LFC	+
	CFL 5872	*Juglans nigra*	1993	LFC	+
	CFL 5878	*P. glauca*	2021	LFC	+
	CFL 5879	*P. glauca*	2021	LFC	+
	CFL 5880	*P. glauca*	2021	LFC	+
	CFL 6283	*Pinus resinosa*	1996	LFC	+
	CFL 6291	*P. mariana*	1996	LFC	+
	CFL 6346	*P. strobus*	1995	LFC	+
	CFL 6351	*Juglans cinerea*	1995	LFC	+
	HSP4	*Pinus sylvestris*	2016	RJ[c]	+
	HSP60	*P. sylvestris*	2016	RJ	+
	HSP64	*P. sylvestris*	2016	RJ	+
	HSP65	*Picea abies*	2016	RJ	+
Corinectria fuckeliana	CFL 4771	*Abies balsamea*	1959	LFC	–
	CFL 879	*Picea abies*	1968	LFC	–
Fusarium acuminatum	CFL 5849	*P. mariana*	1995	LFC	–
	CFL 5850	*P. glauca*	1995	LFC	–
	CFL 5858	*P. strobus*	1995	LFC	–
Fusarium avenaceum	CFL 5862	*P. resinosa*	1995	LFC	–
	CFL 5863	*P. resinosa*	1995	LFC	–
Fusarium graminearum	CFL 5859	*P. resinosa*	1995	LFC	–
	CFL 5860	Unknown	1995	LFC	–
Fusarium oxysporum	CFL 5867	*P. glauca*	1995	LFC	–
	CFL 5868	*P. glauca*	1995	LFC	–
Fusarium solani	CFL 5191	*Ulmus americana*	1986	LFC	–
Fusarium sp. 1	CFL 5861	*P. strobus*	1995	LFC	–
Fusarium sp. 2	CFL 5843	*P. mariana*	1995	LFC	–
Fusarium sporotrichioides	CFL 5851	Unknown	1995	LFC	–
	CFL 5864	Unknown	1995	LFC	–
Ilyonectria crassa	CFL 5865	*Picea rubens*	1995	LFC	–
	CFL 5869	*A. balsamea*	1995	LFC	–

(Continues)

TABLE 1 | (Continued)

Species	Isolate ID	Host	Isolation date	Source	Ccan assay detection
Ilyonectria cyclaminicola	CFL 828	Soil	1965	LFC	–
Ilyonectria pseudodestructans	CFL 5857	*P. rubens*	1995	LFC	–
Nectria cinnabarina	CFL 1017	*Acer saccharum*	1987	LFC	–
	CFL 5856	*A. saccharum*		LFC	–
Nectria coccinea	CFL 1034	*Fagus grandifolia*	1967	LFC	–
Neonectria ditissima	CFL 3014	*F. grandifolia*		LFC	–
Neonectria neomacrospora	CFL 961	*A. balsamea*	1967	LFC	–
	CFL 963	*A. balsamea*	1967	LFC	–
	CFL 964	*A. balsamea*	1967	LFC	–
Neonectria sp. 1	CFL 5844	*P. glauca*	1995	LFC	–
	CFL 5845	*P. mariana*	1995	LFC	–
Thelonectria sp. 1	CFL 5842	*P. mariana*	1995	LFC	–
	CFL 5853	Unknown	1995	LFC	–
	CFL 5866	*P. rubens*	1995	LFC	–
Thyronectria balsamea	CFL 3067	*A. balsamea*	1961	LFC	–

[a]Species names were updated to reflect the currently accepted nomenclature, www.indexfungorum.org (accessed on 17 January 2024).
[b]Mycological culture collection of the Laurentian Forestry Centre, Quebec City, Quebec, Canada.
[c]DNA samples provided by Robert Jankowiak, University of Agriculture in Kraków, Kraków, Poland.

TABLE 2 | Details of the oligonucleotides used for the Ccan TaqMan assay.

Oligonucleotides	Target gene	Position	Tm	Primer length	Amplicon length	Sequence (5′ → 3′)[a]
Cyl canF	ITS	317–35	54.6	19	143	TCATTTCAACCCTCAAGCA
Cyl canR		445–60	51.3	16		CACCGAGACTCCAGAG
Cyl canLNA		337–49	60.05	13		6FAM-CAAGCTC^{+}C^{+}C^{+}GAAG-ZEN

[a]Nucleotides marked with a «+» are locked nucleic acids (LNA).

quantification of the number of ITS copies was obtained using a Java program based on linear regression of efficiency (Rutledge 2011). Serial dilutions ranging from 5,838,323,613 to 0.1 ITS copies per µL were prepared and 1 µL was used as template DNA to run at least three replicate reactions of Ccan TaqMan qPCR per dilution. Ct values were modelled by linear regression, using ITS copies as predictor variable. The efficiency of the amplification was calculated as:

$$\text{Efficiency}\,(\%) = 1 - 10^{(-1/\text{slope})} \times 100$$

2.6 | Limit of Detection

The limit of detection ($LOD_{95\%}$) of the *C. canadiana* assay was determined theoretically and empirically. For the theoretical determination, we used the data obtained from standard curve experiment. A successful amplification was defined as any amplification with a Ct value under 40. Positive and negative qPCR results were modelled by binary logistic regression (probit model), using ITS copies as predictor variable. Following this process, the $LOD_{95\%}$ value was experimentally tested on 20 replicates of a solution containing eight copies of the *C. canadiana* ITS.

2.7 | Standard Curves on DNA Extracted From Serial Dilutions of Conidia Supplemented With Different Matrices

Petri dishes containing SNA medium supplemented with 0.2% lactose were inoculated with 10^5 conidia of the strain CFL5878 incubated at 22°C for 7 days without Parafilm. The cultures were flooded with sterile distilled water supplemented with 0.01% Silwet (PhytoTech Labs, Lenexa, KS, USA). The conidia were gently dislodged with a sterile glass 'hockey stick'. The conidia suspension was filtered through a sterile 100 µm Corning Falcon Cell strainer (VWR, Mississauga, ON, Canada), and the conidia concentration was determined using a Neubauer haemocytometer. Dilutions of the stock solution

were made to obtain 50 μL aliquots containing 10, 100, 1000, 10,000, 100,000 and 1,000,000 conidia. These aliquots were flash frozen in liquid nitrogen and lyophilised overnight at −50°C in a FreeZone 2.5-L freeze-dryer (Labconco, Kansas City, MO, USA). For each lyophilised conidia density, either a silicone-coated rod or a glass fibre filter was added and processed as described in Tanguay et al. (2018). The DNA was extracted from three replicates of each conidia density, for each type of matrix, using the QIAamp DNA micro kit (Qiagen) as described previously, and eluted in 100 μL of EB buffer. One microlitre was used as template DNA to run triplicates of the Ccan TaqMan qPCR for each of the three biological replicates per conidia concentration. Ct values were modelled by linear regression, using the numbers of conidia from which the DNA was extracted as predictor variable.

2.8 | Case Study: qPCR Detection of *C. canadiana* From Different White Spruce Tissues and Seedlings Displaying Different Symptoms

In spring 2021, forest health technicians of the Quebec Ministry of Forest sampled 3-year-old white spruce seedlings in production at the SARGIM nursery highly impacted by the Cylindrocladium root rot. Comparable numbers of seedlings categorised as asymptomatic (62), necrotic (62), chlorotic (50) and showing collar damage (49) were sampled. The seedlings in the collar damage category were still green but displayed resin flow at the collar. Two subsamples of different tissues were collected on each seedling. One subsample contained five 1-cm-long pieces of necrotic roots and one piece (5 × 5 mm) of the main stem tissue collected at the soil–air interface. The second subsample contained five necrotic needles collected from the first 15 cm of the main stem. DNA was extracted from all subsamples using the QIAamp DNA micro kit (Qiagen), as described previously, and eluted in 200 μL of EB buffer. Technical triplicate qPCR reactions (total volume of 10 μL) of the Ccan TaqMan assay were run using 1 μL of a 1:10 dilution of each DNA sample.

3 | Results

The Ccan TaqMan assay developed here targets the region between positions 317 and 460 of the ITS1 sequence alignment for this taxon (not shown). Sequence alignments showed that the designed assay did not prevent amplification of sister species from *the C. kyotensis* species complex (Liu et al. 2020).

All 20 isolates of *C. canadiana* were successfully amplified with the designed assay (primers and probe presented in Table 2), whereas no amplification occurred with any of the other fungal species tested (Table 1).

We obtained a linear relationship between the log value of the target gene region copy number and Ct values for the *C. canadiana*–specific assay (Figure 1A). We ran the Ccan TaqMan assay on serial dilutions of gBlock, representing a portion of the *C. canadiana* ITS sequence, and used linear regression to model the obtained Ct values in function of the $\log_{10}$ ITS copies. There was a linear response of the Ct over quantities of the ITS copies. The ITS copies used as template for the assay had a significant effect

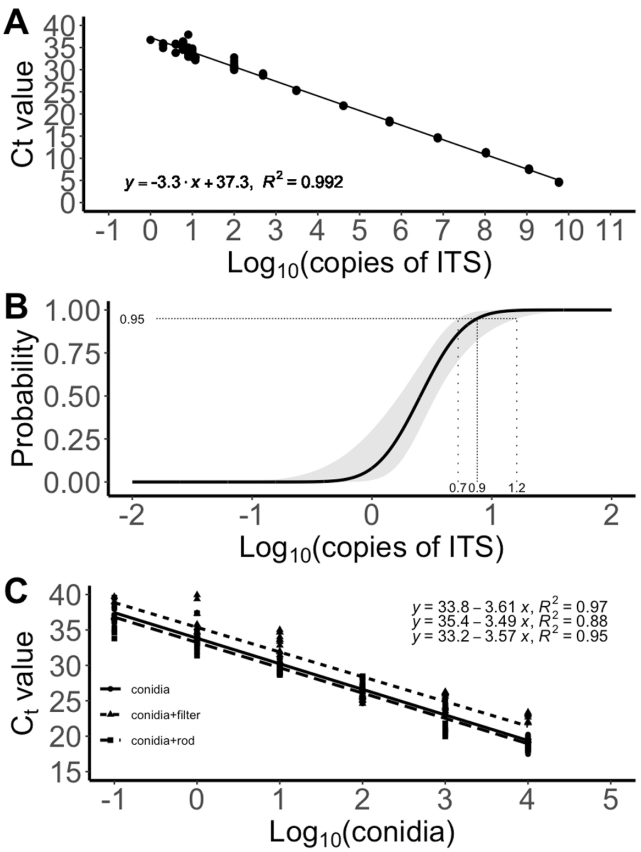

FIGURE 1 | Sensitivity of the *Calonectria canadiana*–specific TaqMan assay. (A) Standard curves of the Ccan TaqMan assay showing a linear response between the Ct values and Log_{10} of ITS copy numbers per reaction. (B) A binary logistic regression analysis estimating the $\text{LOD}_{95\%}$ of the Ccan qPCR assay at 7.56 ITS copies per reaction. (C) Multiple linear regressions of the Log_{10} of the conidia quantity present per reaction predicting the assay Ct values for DNA extracted from conidia alone, spiked with a plastic Rotorod or glass fibre filter paper used in field-trapping experiments.

on the Ct value obtained ($p < 0.01$) and the regression model explained 99.6% of the variance of the observed Ct values. Using the slope coefficient, we calculated a PCR efficiency of 101%.

The limit of detection ($\text{LOD}_{95\%}$), defined as the lowest amount of target DNA yielding positive results 95% of the time, was theoretically and experimentally determined for the Ccan TaqMan assay. The binary logistic regression analysis estimated the $\text{LOD}_{95\%}$ at 7.56 ITS copies per reaction, with 95% confidence intervals ranging from 5.23 to 16.18 ITS copies per reaction (Figure 1B). To provide an empirically validated $\text{LOD}_{95\%}$, we performed technical replicates of the assay using 8 ITS copies as template DNA. The assay returned a 95% positive detection rate with an average Ct of 34.63. Results with Ct over this cut-off would be defined as a negative response.

A linear relationship was observed between Ct values obtained with the Ccan TaqMan assay and DNA extracted from log number of *C. canadiana* conidia prepared in three different ways (i) conidia only, (ii) conidia combined with a silicone-coated trap rod (in the same tube) and (iii) conidia combined with a glass fibre filter. The latter two conidial preparations

were used, respectively, to mimic airborne spores sampled with Rotorod-like spore collectors and rain-borne spores collected passively with funnel traps. Standard curves were made by plotting the log number of conidia against the Ct value determined by the Ccan TaqMan assay (Figure 1C). DNA extracted from the three types of conidial preparations showed linearity across the complete range of dilutions assessed. ANCOVA was used to compare the coefficients of the linear regression curves. No significant difference was observed between the saturated model and the model that used conidia number and type of conidial preparation as predictors ($p = 0.75$), suggesting that the slopes of regression curves were parallel. The type of conidial preparation had a significant impact on the Ct values ($p < 0.001$). The emmeans package (Lenth 2020) was used to determine the effects of conidial preparations. At mean $\log_{10}$ conidia number (1.6502), the Ct values were significantly lower by 1.75 ($p < 0.01$, $CI_{95\%} = -2.35–1.14$) for the conidia alone, compared to the conidia plus the glass fibre filter. No significant difference was observed between the Ct values obtained from conidia alone and conidia plus silicone-coated rod ($p = 0.08$, $CI_{95\%} = -0.05–1.13$). Finally, the Ct values obtained from DNA extracted from conidia plus glass fibre filter were significantly higher by 2.29 ($p < 0.01$, $CI_{95\%} = 1.67–2.90$) when compared to Ct values from conidia plus silicone-coated rod. The number of conidia accounted for 96% of the variation in Ct values, while the type of conidial preparation accounted for an additional 1.5% of the variation in the Ct values.

The Ccan TaqMan assay was performed on DNA extracted from roots and collar and from necrotic needles sampled at the base of more than 200 seedlings binned in four classes of symptoms: asymptomatic, chlorotic, root collar damage and necrotic dead seedlings. The *C. canadiana* was more frequently detected when DNA was extracted from necrotic needles sampled at the base of the seedlings compared to DNA extracted from roots and collars. Among the different classes of symptoms, *C. canadiana* was more frequently detected in dead seedlings and seedlings showing collar damage (Figure 2A) than in other categories of symptoms.

A binary logistic regression was performed on the presence/absence detection data. The model showed that *C. canadiana* is likely to be detected 5.06×10^7 times ($CI_{95\%} = 17331 - 6.69 \times 10^{14}$) more often when the DNA is extracted from the necrotic needles rather than from the roots and root collar. In addition, when compared with DNA samples extracted from asymptomatic seedlings, the Ccan detection yielded positive results 8.18×10^4 ($CI_{95\%} = 209–8.99 \times 10^{10}$), 3.69×10^8 ($CI_{95\%} = 11293.78–2.08 \times 10^{19}$) and 1.92 ($CI_{95\%} = 1.31–4.44$) times more often from dead, root collar damage and chlorotic seedling classes, respectively (Figure 2B).

Using the linear regression equation obtained from the different numbers of conidia, and the obtained Ct values as predictor variable, the equivalent numbers of conidia detected in the DNA samples extracted from (1) the necrotic needles collected at the base of the seedlings and (2) the root and collar samples (Figure 2C) were determined. There were no strong relationships between the *C. canadiana* biomass detected in the necrotic needles, and in the root and collar samples ($R^2 = 0.17$), however, DNA samples extracted from necrotic needles contained at least 683 times ($10^{\text{intercept}[2.83]}$) more *C. canadiana* biomass than those extracted from roots and collar.

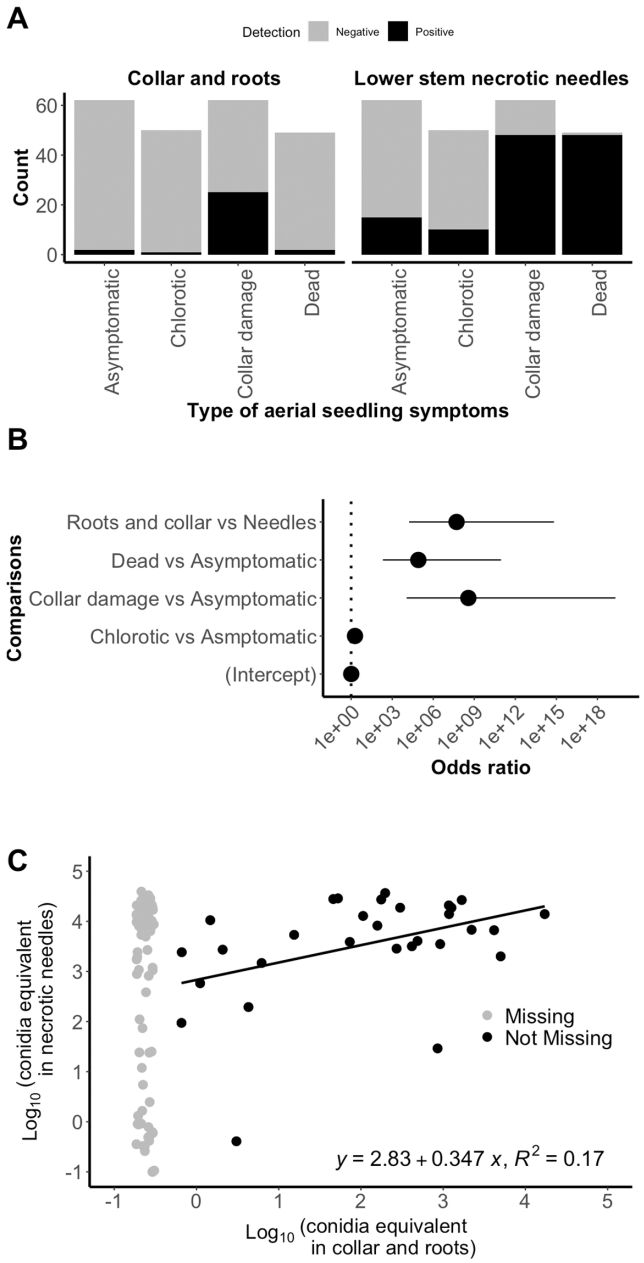

FIGURE 2 | Effect of symptoms displayed by sampled seedlings and tissues from which DNA was extracted for the detection of *Calonectria canadiana*. (A) Proportion of positive and negative detection of *C. canadiana* on seedlings showing different symptoms on DNA extracted from either root and collar or lower stem necrotic spruce needles. (B) Odds ratios for the detection of *C. canadiana* according to spruce tissue samples and class of disease symptoms. (C) Linear regression model showing the relationship between the biomass of *C. canadiana* quantified by qPCR in necrotic needles and root tissues of spruce seedlings.

4 | Discussion

In this comprehensive paper, we present the design, development and validation of a novel real-time PCR assay specifically crafted for the detection of *Calonectria canadiana*, a pathogen that historically had posed a significant threat to production of coniferous seedlings in North American forest nurseries. This assay is, to the best of our knowledge, an unprecedented tool tailored for the identification and study of this particular pathogen.

While designing the Ccan TaqMan assay, we had to make a trade-off between specificity and sensitivity. The assay was engineered to reliably distinguish *C. canadiana* from a range of other Nectriaceae, which are often found afflicting coniferous seedlings in the forest nurseries of Quebec. Despite these achievements, due to the intrinsic limitations posed by the scarcity of polymorphisms within the ITS ribosomal DNA sequences among closely related species, the specificity of the assay to *C. canadiana* was not absolute. We anticipate that multiple species within the *Calonectria kyotensis* complex may show a cross-reaction with our assay. However, the probability of detecting other species within the *C. kyotensis* complex in coniferous seedlings when sampled from the biogeographical regions of Central and Eastern North America is relatively low. This assertion is supported by Crous (2002) who determined that only *C. canadiana*, *C. kyotensis* (anamorph *Cy. floridanum*) and *C. morganii* (anamorph *Cy. scoparium*) have been isolated from diseased hosts of *Picea*, *Pinus* and *Abies* in eastern North America. Moreover, the taxonomic identification of *Calonectria* species isolated from coniferous seedlings in this region has historically been shrouded in confusion. Initial diagnoses of Cylindrocladium root rot and foliar blight were attributed to *C. morganii*, as reported by Cox (1954) and Anderson, French, and Taylor (1962). In a later study, Sobers and Seymour (1967) described a new species, *C. kyotensis* (syn. *C. floridana*, anamorph *Cy. floridanum*), identified as pathogenic to peach in Florida, which brought about a re-evaluation of the morphological features of the fungus impacting conifer seedlings in the Lake States area, ultimately leading to reclassification to *C. kyotensis* (Morrison and French 1969). Subsequent molecular analyses conducted by Kang, Crous, and Schoch (2001) revealed the existence of three distinct species within the *C. kyotensis* complex: *C. kyotensis*, *C. canadiana* and *C. pacifica*. These findings were bolstered by morphological examinations which revealed that the conidiophores of *C. canadiana* tend to exhibit less branching, and the conidia themselves are slightly longer than those of *C. kyotensis*. *Calonectria pacifica* was characterised by having the largest conidia among the trio of species within the complex. Although Kang, Crous, and Schoch (2001) identified isolates of both *C. canadiana* and *C. kyotensis* from diseased coniferous seedlings in Ontario, a study by Jeng et al. (1997) found ITS sequences that matched those of *C. canadiana* in strains from forest nurseries across Ontario, Wisconsin and Minnesota. This study further supported the presence of *C. canadiana*, as all historical and contemporary isolates from Quebec Forest nurseries were confirmed to be this species (refer to Table 1 for detailed data). Research studies conducted in the early 2000s identified *C. canadiana* as the only species responsible for Cylindrocladium root rot in the St-Modeste Forest nursery (Québec, Canada) (Vujanovic and St-Arnaud 2005; Vujanovic et al. 2007). The species *C. montana*, which has been reported to cause damping-off disease in European pine and spruce seedlings (Stępniewska et al. 2020), is now recognised as conspecific with *C. canadiana* (Liu et al. 2020). The role of *C. kyotensis* as a widespread pathogen affecting coniferous seedlings in the forests of Central and Eastern North America remains to be conclusively determined. Notably, additional species within the *C. kyotensis* complex have been recognised in recent taxonomical revisions (Liu et al. 2020). However, these species have not been reported as pathogens on coniferous seedlings, nor have they been detected in the Central and Eastern regions of North America.

Our assay provides precise quantification of *C. canadiana*. The measured fluorescence signal was shown to be directly proportional to the number of ITS copies, demonstrating high sensitivity, as evidenced by the $LOD_{95\%}$ at 7.56 ITS copies. Additionally, the assay reliably quantified the number of conidia extracted in different matrices. Given that each nuclear genome has multiple copies of the ITS, we have demonstrated that the limit of quantification is below 0.1 conidia per reaction. This level of sensitivity is comparable to that of other qPCR assays targeting the ITS (Lamarche et al. 2017; Tanguay et al. 2018) and is predictably higher than assays based on single-copy genes, which are 10 to 100 times less sensitive (Abraham et al. 2018; Bergeron et al. 2019). Accurate quantification of pathogen biomass is crucial for epidemiological studies, enabling investigation into dispersion pathways (Gehesquière et al. 2013; Tanguay et al. 2018) and pathogen propagation in plant tissues and sporulation (Eshraghi et al. 2011; Langenhoven, Murray, and Crampton 2020).

Our comprehensive study sheds light on the detection patterns of *C. canadiana*, revealing that this pathogen is more easily detected in the necrotic needles located at the base of seedlings compared to within the root system. To optimise the detection of *C. canadiana*, it is recommended that DNA extraction be conducted from the necrotic needles at the base of the stem rather than from the roots. Previous research has demonstrated variations in the biomass of root pathogens depending on the specific plant parts sampled, as evidenced by studies conducted by Mercado-Blanco et al. (2003) and Saltos et al. (2021). Notably, Saltos et al. (2021) found that in susceptible *Capsicum* genotypes, the biomass of *Phytophthora capsici* was more prominent in the roots than in the hypocotyls. Despite these findings, the underlying biological mechanisms that drive these observations within the *C. canadiana*/spruce pathosystem remain unclear. While instances of seedling blight, attributed to species within the *C. kyotensis* complex, have been documented in conifers (Cox 1954; Bugbee and Anderson 1963; Cordell 1976; Barnard 1996), the most severe damage to spruce seedlings has been linked to root rot (Cox 1954; Juzwik, Honhart, and Chong 1988). The research of Anderson et al. highlighted that when black spruce seedlings exhibiting varying degrees of root rot were transferred and maintained under high humidity conditions in a mist chamber, they displayed blight symptoms on their needles and main stem, with sporulation occurring in recently deceased needles (Anderson, French, and Taylor 1962). This sequence of events and resulting symptoms observed align closely with conditions and findings from Gaspesian nurseries, where blight symptoms developed in seedlings subjected to ample overhead sprinkler irrigation. Consequently, it is plausible that in affected seedlings within the Gaspesian nurseries, *C. canadiana* initially infected the roots before progressing towards the crown. Our dataset indicated that despite the presumed root-based infection, the biomass of *C. canadiana* tends to be more concentrated in the needles at the base of the main stem than in the root system. This finding is corroborated by a prior study reporting the presence of a network of hyphae and conidiophores on needles of seedlings initially displaying symptoms of root rot, subsequently transferred to conditions of high moisture (Anderson, French, and Taylor 1962).

The chance of detecting *C. canadiana* varied with the visual symptoms displayed by the seedlings. While the fungal pathogen was readily detected in fully necrotic seedlings and seedlings showing collar lesions, the detection yield from chlorotic seedlings was comparable to that from asymptomatic seedlings. The low fungal biomass in tissues sampled from seedlings showing chlorosis was intriguing and deserves more investigation. Nitzan, Evans, and Johnson (2006) showed that in potato plants infected with *Colletotrichum coccodes*, the severity of chlorosis and necrosis on foliage was the outcome of natural plant senescence and was not caused by fungal colonisation. They also showed that fungal colonisation of plant tissues increased as the plant senescence progressed. This model fits our results and suggests that early spruce senescence, displayed by foliage chlorosis, is an inaccurate tool to diagnose the presence and amount of *C. canadiana* in spruce seedlings.

The reliability of the Ccan TaqMan assay to detect very low quantities of *C. canadiana* DNA, irrespective of the environmental conditions, highlights its diagnostic value on conifers. Given the limited specificity of the Ccan TaqMan assay, the species identity of samples yielding positive results can be confirmed by sequencing one or multiple barcodes discriminating *Calonectria* species (Liu et al. 2020). When applied in the field, this assay will enable studies to understand the factors involved in the epidemic currently affecting white spruce seedlings in Gaspesian forest nurseries. In addition, this qPCR assay will be of significant importance in evaluating the effectiveness of control treatments.

Author Contributions

P.T. and N.B. conceived the original idea and developed the theory. N.B. and A.P. contributed to sample preparation and performed the analytical methods. P.T. ran the statistical analyses and wrote the manuscript in consultation with L.B. and N.B. P.T. and L.B. supervised the project. All authors discussed the results, provided critical feedback and contributed to the final manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Peer Review

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/efp.12885.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.