Distribution of the foliar fungal endophyte *Phialocephala scopiformis* and its toxin in the crown of a mature white spruce tree as revealed by chemical and qPCR analyses.

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Abstract: *Phialocephala scopiformis* is a foliar fungal endophyte of white spruce that produces the anti-insect compound rugulosin and other compounds in lower amounts. Seedlings inoculated with this and other toxigenic endophytes have increased tolerance to the spruce budworm, *Choristoneura fumiferana*. The presence of rugulosin in the diet and in needles infected by *P. scopiformis* reduces the growth rate of the insect. One of 300 white spruce trees inoculated as a seedling in 2001 was chosen to investigate the distribution of *P. scopiformis* and its principal toxin rugulosin throughout the crown. To facilitate the detection of the fungus in small samples, a qPCR assay was developed based on the ITS region of fungal ribosomal DNA targeting a genetic polymorphism unique to *P. scopiformis*. The assay was specific, with a method limit of detection 100 ng mycelium/g needle sample with high reproducibility and accuracy. We found that 11 years after inoculation, *P. scopiformis* DAOM 229536 and its toxin were detectable in needle samples distributed throughout the crown. Of the 109 samples tested, 100% of the rugulosin-positive samples also tested positive for *P. scopiformis* DAOM 229536 DNA in the qPCR assay. The mean and median needle rugulosin concentrations were > 2 times that required to reduce the growth of spruce budworm.
Introduction

Endophytes are fungi that colonize the internal tissues of plants without presenting any symptoms of disease (Carroll 1988). Most is known about endophytes of cool season fescues. Infection by species of *Epichloë/Neotyphodium* in various grass species results in the accumulation of potent toxins in leaf tissue. These toxins limit herbivory by invertebrate pests and grazing animals (Kuldaau and Bacon 2008). Carroll and Carroll (1978) suggested that foliar endophytes of conifers may have similar effects on herbivorous insects. They suggested decreased palatability for grazing insects as a possible benefit for the trees. This group subsequently investigated the association between Douglas fir (*Pseudotsuga menziesii*) and the needle endophyte *Rhabdocline parkerii* (Sherwood-Pike et al. 1986). An extract of culture filtrate of this endophyte resulted in reduced growth rates and mortalities when incorporated into synthetic diets of *Choristoneura fumiferana* (spruce budworm) at 10 µg/g (Miller 1986). This and the suggestion by Royama (1984) that there was an unknown agent (“fifth agent”) that affected spruce budworm populations in New Brunswick prompted surveys of foliar endophytes of conifers in the Acadian forest (Miller 2011). Over the past 30 years, we have demonstrated populations of white and red spruce (*Picea glauca, P. rubens*) foliar endophytes produce mixtures of different compounds toxic to insects (Sumarah and Miller 2009; Sumarah et al. 2010).

The white spruce foliar fungal endophyte *Phialocephala scopiformis* DAOM 229536 (Helotiales, Ascomycota) produces rugulosin as the major insect toxin along with emodin and skyrin (Calhoun et al. 1992). When incorporated in insect diets, rugulosin is toxic to spruce budworm (*Choristoneura fumiferana*), hemlock looper (*Lambdina fiscellaria*) and spruce budmoth.
(Zeiraphera canadensis) at a concentration of 10 mM (Sumarah et al. 2008). This corresponds to ~0.5 µg/g needle (Miller 2011). When white spruce trees are infected with this endophyte, the growth rate of spruce budworm (Choristoneura fumiferana) is significantly reduced (Miller et al. 2002; Miller et al. 2008; Miller 2011; Quiring, unpublished data).

Our studies have demonstrated a reduced genetic diversity of needle endophytes in land previously disturbed by insect, fire and logging and reforested either by natural regeneration or tree planting. Grass endophytes are vertically transmitted (Kuldau & Bacon 2008) and clonal within a field (Arroyo García et al. 2002). In contrast, foliar endophytes of spruce in the Acadia forest are horizontally transmitted (e.g. Miller et al. 2009; Sumarah et al. 2008). Consistent with this observation, we have been unable to recover endophytes from surface sterilized needles of seedlings leaving nurseries (Sumarah et al. 2005; Miller et al. 2009). To address this, methods have been developed to inoculate nursery seedlings with representative endophytes from the region (Miller and Adams 2012). To facilitate this research both chemical and enzyme-linked immunosorbent assay (ELISA) based analytical methods have been used (Miller et al. 2008; 2009; Sumarah et al. 2008). ELISA is a robust approach to measuring fungal antigens in needles, quite sensitive and reasonably inexpensive. However, this ELISA analysis of needle samples is time-consuming and labour-intensive.

Sumarah et al. (2005) reported the results of chemical, ELISA and plating analysis of ~1200 white spruce seedlings inoculated in 2001 with P. scopiformis. In 2003, 300 P. scopiformis -positive and -negative trees from the same trial were planted in a test site near Sussex, NB. We have been following the persistence of the endophyte and its toxin in the trees and distribution in
the tree crown in the field since 2006. To date ca. 80 of the 300 *P. scopiformis*–positive trees have been sampled (Sumarah et al. 2008; Miller et al. 2009; Quiring & Miller, unpublished data).

The purpose of this paper is to describe the distribution of the fungus and its toxin throughout the crown of one of the inoculated white spruce trees planted in 2003, now ~5m tall. To facilitate the detection of the fungus in small samples, we developed a quantitative real-time PCR assay for the detection of *P. scopiformis* in white spruce needles. This was based on the internal transcribed spacer (ITS) region of ribosomal DNA, the accepted barcode marker for fungi (Schoch et al. 2012).

**Materials and Methods**

**Sampling**

In 2003, 300 trees successfully inoculated with *P. scopiformis* DAOM 229536 two years previous (described in Sumarah et al. 2005; 2008) were planted on the test site outside Sussex, NB. A similar number of trees from the same lot that were not successfully colonized were planted in a different section of the test plot. Control samples came from branches throughout the crown that were obtained from one of these uncolonized trees. A white spruce tree that had previously tested positive for rugulosin and *P. scopiformis* DAOM 229536 was selected from the endophyte field test. The mature tree was located at 65°26’5.067”W 45°57’55.909”N (near Havelock, NB) and was 4.6 m tall at the time of sample collection (October 2012).

Ten branch whorls around the tree were chosen starting at the bottom and ending at the top of the tree. For each whorl, four branches were selected. For each branch the first, second, and third year growth were collected. The needles (ca. 200 mg) and 6 samples of woody stem below the
bud were put in Falcon tubes on ice, transported to the laboratory on ice and stored at -30 °C until processing. Samples were freeze-dried, powdered and stored in vials at -20 °C until DNA extraction and rugulosin analysis. All freeze-dried, powdered needle samples (116) from the study tree were tested in triplicate.

For each branch the height from the base of the ground to where the branch started from the whorl was measured. The azimuth for each branch was determined using a compass facing out from the tree trunk. For each needle sample the distance from the trunk to the sample area was measured at a right angle. The distance from the needle sample area to the ground was also recorded.

**Rugulosin analysis**

Rugulosin analysis was performed on 116 needle samples after Miller et al. (2009) with modifications. An aliquot of 200 mg of freeze-dried ground needles was defatted using 20 mL petroleum ether for 50 min on ice. After filtration by suction, the petroleum ether extract was discarded and the suspension was extracted with chloroform (20 mL x 2, for 80 min each). The chloroform extract (10 mL) was washed with 10 mL of 5% NaHCO₃ solution and the organic layer was discarded. The aqueous layer was adjusted to pH 3 by the addition of 1 M HCl. The acidic solution was extracted with chloroform (10 mL x 2) and the organic layer was evaporated to dryness under a stream of nitrogen. The resulting extract was dissolved in 50 µL of MeOH and analyzed by HPLC.

An Agilent 1100 HPLC system equipped with a diode array detector was used for quantification of the extract. A volume of 30 µL of the prepared extract solution was injected on a Phenomenex Synergi column (250 x 4.6 mm, 4 µm). The HPLC run was performed using a
linear gradient consisting of acetonitrile-water with TFA (0.1%, v/v) over 19 min with a flow rate of 1 mL min\(^{-1}\). Quantitation was performed by comparison of the peak area to that of an external rugulosin standard. The LOQ for rugulosin was 150 ng/g. Statistical analyses of rugulosin concentrations were performed using SYSTAT v13 (San Jose, CA).

**qPCR method development**

Fungal cultures used in the development of this assay (Table 2) were grown on 2% malt extract agar (MEA, Difco Laboratories) at 25 °C for 3 weeks prior to DNA extraction. For the purposes of primer and probe design, generation of standard curves and cross reactivity testing, DNA was extracted from 50 mg of mycelium from each fungal culture with an UltraClean Microbial DNA Isolation Kit (MoBio, Carlsbad, CA) according to the manufacturer’s instructions. DNA concentration was measured using a NanoDrop ND1000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE).

The complete ITS region of the extracted DNA was amplified by polymerase chain reaction (PCR) using primers ITS5: GGAAGTAAAAGTCGTAACAAGG (White et al. 1990) or ITS1F: CTTGGTCATTTAGAGGAAGTAA (Gardes and Bruns 1993) and ITS4: TCCTCCGCTTATTGATATGC (White et al. 1990) synthesized by Integrated DNA Technologies (Integrated DNA Technologies, Coralville, IA). PCR amplification was performed in a 20 µl volume using 12.2 µl ddH\(_2\)O, 0.2 µl of Taq DNA polymerase (invitrogen, Carlsbad, CA), 2.0 µl of PCR buffer (invitrogen, Carlsbad, CA), 1.2 µl of 50mM MgCl\(_2\), 0.4 µl (C) dNTPs, 1.0 µl of each primer and 2 µl of template DNA ranging in concentration from 7-10 ng/µl. PCR was conducted using a TECHNE TC-3000 thermocycler (Bibbly Scientific Ltd.) using the following parameters: initial denaturation at 94 °C for 10 min followed by 40 cycles of
denaturation at 94 °C for 15 sec, annealing at 53 °C for 30 sec, and extension at 72 °C for 90 sec. Final extension was at 72 °C for 10 min. The full length amplification product was sequenced in both directions by the Génome Québec Innovation Centre (Montreal, Canada). Sequences were edited using ClustalX software (Thompson et al. 1997). To generate the sequence alignment, the ITS sequence of *P. scopiformis* was aligned with the sequences of related *Phialocephala* species collected in NB and fungi associated with conifer needles including *Cladosporium cladosporiodes* (Table 2). In addition, ITS sequences of the following vouchered fungal isolates were obtained from NCBI GenBank and included in the alignment: *Phialocephala fortinii* FJ031031, *P. scopiformis* CBS 507.94 AF486126, *P. compacta* CBS 507.94 AF486125, *P. dimorphospora* CBS 300.12 AF486121 and *Aspergillus fumigatus* CBS 545.65 JN943566. All sequences were trimmed to begin with the ‘CATTA’ motif and sequences were aligned using Geneious R6 software (Biomatters Ltd., Auckland, NZ).

Primers and TaqMan probes were designed in Primer3 (Untergasser et al. 2012) and Geneious R6 (Biomatters Ltd.) using the alignment of relevant ITS sequences, targeting a 181 bp region in the ITS sequence containing sequence polymorphisms unique to *P. scopiformis*. Primers and probe were synthesized by Integrated DNA Technologies (Coralville, IA); forward primer 5’-GGTATACCCACCCGTGTCTI3’, reverse primer 5’-TAATCTCTCTGGCAGGCACATI3’ and probe 5’-TTGTGCTTTGGCAGGGCTGGCCTCCACTI3’. The probe was labeled at the 5’ end with the fluorescent reporter dye 6-carboxyfluorescein (FAM) and labelled with the quencher dye 6-carboxy-tetramethylrhodamine (TAMRA) at the 3’ end.

The qPCR was performed in a Roche LightCycler 480 System (Roche Diagnostics, Basel CH) using LightCycler 480 Probes Master Mix (Roche Diagnostics). Each 20 µl reaction was
prepared with 10 µl 2X master mix, 6 µl ddH₂O, 0.25 µl of each primer (20 µM), 1.5 µl TaqMan probe (20 µM) and 2 µl DNA. Internal standard curve dilutions and non-template controls were run in each 96-well plate. Tenfold serial dilutions ranging from 8.6 ng-8.6 fg of *P. scopiformis* DNA were used to generate standard curves; each dilution was measured in triplicate. qPCR conditions were as follows: an initial denaturation step at 95 C for 10 min followed by 50 cycles at 95 C for 15 s, annealing at 60 C for 15 s and a final step at 40 C for 30 s to allow the 96-well plate to cool prior to handling. To verify the specificity of the probe, other conifer endophytes (*Lophodermium* and needle phylloplane fungi collected from New Brunswick) were analyzed using the qPCR assay (Table 2).

To explore the effect of the conifer needle matrix on fungal DNA extraction and the qPCR reaction, needles were spiked with *P. scopiformis* DAOM 229536 grown on 2% MEA. *P. scopiformis* mycelium was flash frozen in liquid nitrogen for 60 sec then ground to a powder with a sterilized mortar and pestle. The ground mycelium was diluted in deionized distilled water in tenfold serial dilutions in duplicate. One of each duplicate dilution of ground mycelium was added to 100 mg of freeze dried powdered white spruce needles. DNA extraction was then carried out as described above and qPCR was performed in triplicate following the parameters described earlier. To assess reproducibility, needle samples from 291 *P. glauca* seedlings inoculated with *P. scopiformis* DAOM 229536 were analyzed using our qPCR assay. Ten samples of *P. glauca* previously treated with an unidentified endophytic species of *Mycosphaerella* (DAOM 221611; Findlay et al. 2003) were extracted and analysed as the negative control. Sample DNA concentrations were calculated based on internal standard curve values generated within each run.
Freeze-dried powdered needle samples from the mature white spruce tree that were previously tested for the presence of rugulosin were weighed out into 75 mg aliquots. DNA was isolated with an UltraClean Microbial DNA Isolation Kit (Mobio) and qPCR was performed in 10 µl reactions in triplicate using the previously described qPCR method.

**Results and Discussion**

A qPCR assay for the detection of *P. scopiformis* DAOM 229536 in white spruce was developed based on the ITS gene region of fungal ribosomal DNA (Schoch et al. 2012). The assay was linear over four logs with an $r^2$ value of 0.99 (Figure 1). The method limit of detection was calculated to be $7.5^{-15} \text{g}$ of DNA by extinction of fluorescent signal (efficiency value 1.924). This translates to 1 ng of mycelium. The specificity of the assay was investigated using cultures of common conifer foliar endophytes and phylloplane fungi collected from the same region. No signal was detected from the three *Lophodermium* species needle endophytes or phylloplane fungi tested (Table 2). Three other *Phialocephala* species isolated as conifer needle endophytes from this region were not detected using our PCR probe. ITS gene sequencing identified them as *P. dimorphospora*, *P. cf. fortinii*, and *P. cf. glacialis*. A fourth *Phialocephala* isolate obtained from our study region produced a positive qPCR signal in our assay. ITS gene sequencing confirmed its identity as *P. scopiformis*.

There was a 3-log decrease in the sensitivity of the *P. scopiformis* assay in the presence of the white spruce needle matrix (Table 1). The method limit of detection was 100 ng/g needle. This is comparable to the sensitivity of the ELISA method (Sumarah et al. 2005). From the 291 production samples tested to assess reproducibility, 165 samples tested positive (56.7%) for *P.
scopiformis. Based on triplicate analyses of 44 samples, the assay was 100% reproducible i.e. positive or negative. No qPCR signal was observed in the negative controls.

We found that the endophyte and its toxin were present throughout the crown of the white spruce tree studied. Approximately 90% of the samples taken from the crown were rugulosin-positive (Figures 2, 3). The mean and median of positives were 1.2 and 0.93 µg/g rugulosin, respectively. These values are > 2 times the low adverse effect concentration for spruce budworm growth on needles (Miller 2011). There was 100% concordance between the qPCR and the corresponding HPLC analysis for rugulosin a (Table 1 supplemental data). Four samples were positive by qPCR but below the limit of detection for rugulosin. Two of the 6 stem tissue samples contained rugulosin (0.3 µg/g each).

The ratio of toxin/unit fungal biomass is very well understood to be a fixed property (Miller 2011). Based on ELISA and chemical analysis, Sumarah et al. (2005) estimated that the mycelium to toxin ratio of P. scopiformis in the needles as ~2% w/w. The ratio estimated from the present data using the qPCR probe detection limit for mycelium was comparable. This provides additional evidence that the qPCR method employed here is providing an accurate estimate of fungal biomass in the needles. Many qPCR methods for fungi in the literature do not provide a linear relationship between fungal biomass and the PCR signal (Hospodsky et al. 2010), in plant material or other complex matrices (e.g. Dillon et al. 2007; Song et al. 2014).

Stefani & Bérubé (2006) examined endophytes from 28 trees in eastern Quebec and noted the majority of needles were colonized by one fungus but needles from the same branch were colonized by other endophytes. This is a similar result to that from surface sterilized needles.
from 2559 trees from two collections across the length and breadth of New Brunswick (see Sumarah & Miller 2009; Sumarah et al. 2010). However, surface sterilized needles of white spruce seedlings that were inoculated with various endophytes including *P. scopiformis* were colonized by one fungus based on plating and metabolite analysis (Miller et al. 2002). As noted, rugulosin and mycelium concentrations have been determined in ~25% of the trees from the 300 *P. scopiformis*-positive trees planted in 2003. Needle mycelium concentrations have been similar in this batch of seedlings since the trees were large enough to sample after inoculation, i.e. ca. 60-100 ng/g. The growth of the fungus diverts nutrients in the portion of the needle with a sufficient water activity for fungal growth (see Miller 2011). It appears then that when a foliar endophyte colonizes a tree as a seedling, competition for the resource limits colonization by other strains.

In summary, 11 years after inoculation, the fungus and its toxin rugulosin were distributed throughout the crown of a white spruce tree inoculated with *P. scopiformis* DAOM 229536 as a seedling (Figure 3). The distribution of concentrations of the toxin (Figure 2) was similar to earlier studies of the same inoculated seedling lot on the site (Miller et al. 2009; Sumarah et al. 2005; 2008). The mean and median concentrations were well above the concentration observed to reduce spruce budworm growth on the branches (Miller 2011; Quiring et al. unpublished data). There was 100% concordance between the chemical and qPCR assays. This represents a significant improvement over existing methods for rapid screening and detection of beneficial fungal endophytes in conifer needles.

**Acknowledgements**
This study was supported by the NSERC CRDPJ 421782-11 to JDM and K.A. Seifert and an IRAP (National Research Council) and Maritime Forest Research, Limited to JDM. Vara Kesnakurti Samba Siva, Miao Liu, and Julie Chapados (ECORC, AAFC) are thanked for assistance with development of qPCR assay. Joey Tanney is thanked for providing fungal cultures. Shona Millican (JD Irving) collected the needle samples and prepared the data for Fig. 2 which was produced by Wayne Handspiker (JD Irving). Sophia Dick (MFRL) assisted in the preparation of the samples for analysis. We thank anonymous reviewers for useful comments on the manuscript.
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fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and
Table 1. Amount of target fungal DNA in [g] extracted from pure cultures of *P. scopiformis* DAOM 229536 (column 2) and from white spruce needles spiked with pure cultures of *P. scopiformis* DAOM 229536 (column 3), as determined by qPCR. The amount of mycelium used for the DNA extractions is given in [mg] in column 1.

<table>
<thead>
<tr>
<th><em>P. scopiformis</em> mycelium (mg)</th>
<th><em>P. scopiformis</em> DNA (g)</th>
<th><em>P. scopiformis</em> DNA from mycelium + 100 mg needle</th>
</tr>
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<tbody>
<tr>
<td>100</td>
<td>1.10x10^{-9}</td>
<td>1.11x10^{-12}</td>
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<td>10</td>
<td>2.12x10^{-11}</td>
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<td>1.97x10^{-15}</td>
</tr>
<tr>
<td>0.1</td>
<td>8.94x10^{-13}</td>
<td>0</td>
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Table 2. Fungal strains used to test *Phialocephala scopiformis* DAOM 229536 ITS TaqMan probe.

<table>
<thead>
<tr>
<th>Fungal strain</th>
<th>Detection by <em>Phialocephala scopiformis</em> DAOM 229536 probe</th>
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<tbody>
<tr>
<td><em>Lophodermium nitens</em> CBS 127941</td>
<td>-</td>
</tr>
<tr>
<td><em>Lophodermium nitens</em> CBS 127939</td>
<td>-</td>
</tr>
<tr>
<td><em>Lophodermium nitens</em> CBS 127938</td>
<td>-</td>
</tr>
<tr>
<td><em>Lophodermium cf. piceae</em> CBS 127942</td>
<td>-</td>
</tr>
<tr>
<td><em>Phialocephala scopiformis</em> DAOM 229536</td>
<td>+</td>
</tr>
<tr>
<td><em>Phialocephala scopiformis</em> NB173</td>
<td>+</td>
</tr>
<tr>
<td><em>Phialocephala cf. glacialis</em> DAOM 229535</td>
<td>-</td>
</tr>
<tr>
<td><em>Phialocephala dimorphospora</em> NB157A</td>
<td>-</td>
</tr>
<tr>
<td><em>Phialocephala fortinii</em> NB285-2B</td>
<td>-</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em> CBS 545.65</td>
<td>-</td>
</tr>
<tr>
<td><em>Cladosporium cladosporioides</em> Paracel ID 1219013</td>
<td>-</td>
</tr>
<tr>
<td><em>Penicillium chrysogenum</em> DAOM 234056</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 1. Standard curve based on dilution of *Phialocephala scopiformis* DAOM 229536 DNA for TaqMan qPCR assay targeting internal transcribed spacer (ITS) region of fungal rDNA. Plot represents cycle threshold (Ct) versus the Log of DNA concentration for 9 experiments run in triplicate. *P. scopiformis* DNA was diluted from concentrations of 8.6 ng/uL-8.6 fg/uL in sterile water. $R^2 = 0.9996$, Ef = 1.924.
Figure 2. Rugulosin concentrations in the 116 samples from the tree studied as determined by HPLC; spruce; the length of each box shows where the central 50% of the values fall, with the edges at the first and third quartiles.

Figure 3. Range of rugulosin concentrations throughout the crown of the test white spruce tree as determined by high performance liquid chromatography (white sampling point < 0.02 µg/g; red 0.02 - 0.69 µg/g; green 0.7 - 1.39 µg/g; blue > 1.40 µg/g).
Fig. 2
needle rugulosin concentration

white < 0.02 ug/g
red  0.02 - 0.69 ug/g
green 0.7-1.39 ug/g
blue > 1.40 ug/g