Fungal Ecology 31 (2018) 1–8

Contents lists available at ScienceDirect

Fungal Ecology

journal homepage: www.elsevier.com/locate/funeco

Detection of foliar endophytes and their metabolites in *Picea* and *Pinus* seedling needles

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ARTICLE INFO

Article history:
Received 6 March 2017
Received in revised form
11 September 2017
Accepted 14 September 2017
Available online xxx

Corresponding Editor: Barbara Joan Schulz

Keywords:
Conifer endophytes
Choristoneura fumiferana
Cronartium ribicola
Griseofulvin
Pyrenophorol
Vermiculine
Rugulosin
Forestry

ABSTRACT

The needles of *Picea glauca* (white spruce) and *Pinus strobus* (white pine) trees infected with toxigenic fungal endophytes contain varying concentrations of their secondary metabolites that are toxic to either insect pests or needle pathogens. In the present study, liquid chromatography-mass spectrometric methods to determine needle concentrations of metabolites of four endophyte species were developed. The endophytes considered were a *Phialocephala* sp. (vermiculine) and *Phialocephala scopiformis* (rugulosin) from white spruce, as well as a *Xylaria* sp. (griseofulvin) and *Lophodermium nitens* (pyrenophorol) from white pine needles. To ensure that needles were infected with the associated fungal endophyte, suitable qPCR-based methods were also developed. There was a high degree of concordance between the qPCR analysis of the fungal mycelium and the LC-MS/MS quantification of the associated metabolites. Concentrations of the antifungal compounds griseofulvin and pyrenophorol were present in amounts that affect conifer needle diseases including white pine blister rust caused by *Cronartium ribicola*. Similarly, concentrations of the antiinsectan compounds vermiculine and rugulosin were in the range known to reduce the growth of *Choristoneura fumiferana* and mitigate foliage damage.

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1. Introduction

In describing the continuum of interactions between fungi and woody plants, Keener (1950) noted, “Between these extremes, cases of facultative parasitism and facultative saprophytism … much has been revealed concerning what some investigators imply are ‘borderline’ associations involving bacteria and fungi in more or less mutual relationships with their host.” During the early 1970s, extensive studies of fungi present in surface-sterilized conifer needles were conducted in the Pacific Northwest of the USA. These studies revealed that the foliage of all conifer species examined were colonized by fungi that did not damage the needles (Bernstein and Carroll, 1977; Carroll and Carroll, 1978). The latter authors speculated on the nature of this interaction, “Possible benefits to the host trees might include antagonism towards pathogenic needle parasites and surface saprophytes, delay of needle senescence, or a decrease in needle palatability for grazing insects.” It was subsequently demonstrated that the endophyte *Rhabdocline parkeri* appeared to reduce Douglas fir (*Pseudotsuga menziesii*) damage caused by *Contarinia* gall midges (Carroll, 1986). The culture filtrate extracts of a strain of this fungus reduced the growth rate and increased mortality of *Choristoneura fumiferana* (eastern spruce budworm) larvae and were toxic to HeLa cells (Miller, 1986).

Similarly, early observations suggested that needle endophytes could reduce the impact of needle cast diseases. For example, Darker (1967) reported that *Hendersonia pinicola* introduced secondary to an infection by *Lophodermella concolor* (= *Hypodermella concolor*) “was so abundant that the black masses of spores hung in festoons on the needles of jack pine (*Pinus banksiana*) and blackened the green leaves of vegetation below.” During the following year, no evidence of either primary nor secondary species was found at the same site. Apparently, the *L. concolor* infestation in this small area was completely wiped out. It was speculated that the endophyte reduced the amount of available nutrients thus preventing the pathogen from fruiting, thereby acting “as natural biological control agents” (Darker, 1967). Recently, we reported the isolation of needle endophytes from eastern white pine (*Pinus strobus*) in southern New Brunswick. Provisionally identified within

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http://dx.doi.org/10.1016/j.funeco.2017.09.003
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the Massarinaceae, two strains were subsequently identified as *H. pinicola*. These strains produced potently antifungal culture filtrate extracts and several new metabolites (Richardson et al., 2015), perhaps offering some additional insight to the observations made previously by Darker. Another similar, early observation suggested that *Lophodermium conigenum* appeared to exclude the needle cast pathogen *Lophodermium seditiosum* from Scots pine (*Pinus sylvestris*; Minter, 1981).

A systematic investigation of conifer endophytes from the Acadian forests of New Brunswick and Maine began in 1983–84 with stands near Fredericton, NB (Miller et al., 1985; Johnson and Whitney, 1989). Extracts of culture filtrate for some of these fungal strains were also toxic to spruce budworm larvae and cells (Miller, 1986). Later collections of needle endophytes isolated from white spruce (*Picea glauca*), red spruce (*Picea. rubens*) and black spruce (*Picea. mariana*) in the Acadian forest revealed that 5–10% of recovered strains produced antiinsectan metabolites (Clark et al., 1989; Calhoun et al., 1992; Findlay et al., 1995, 2003; Sumarah et al., 2008b, 2010; Sumarah and Miller, 2009; Tanney et al., 2016b). Inoculated as seedlings with the endophyte *Phialocephala scopiformis*, its dominant insect toxin rugulosin is produced within the needles at effective concentrations ($\geq$0.1 μg g^{-1} dry weight; Sumarah et al., 2005, 2008a), reducing the growth rate of spruce budworm larvae (Miller et al., 2008; Miller, 2011). The measured concentration of rugulosin in conifer needles explained most of the variance associated with the observed effect on *C. fumiferana* growth (Miller et al., 2008). Further, the fungi persist in the mature tree, producing effective rugulosin concentrations in the needles more than a decade after inoculation (Frasz et al., 2014).

Over the past fifteen years, systematic surveys of endophytes from eastern white pine were conducted throughout New Brunswick. Echoing previous experiences, many of these strains produced potently antifungal compounds including several macrolides from *Lophodermium nitens* (Sumarah et al., 2011, 2015) and griseofulvin from a *Xylaria* sp. (Richardson et al., 2014). The macrolide pyrenophorol reduced the growth of the forest disease white pine blister rust (*Cronartium ribicola*) cultures at 5 μM (Sumarah et al., 2015). Strains of *L. nitens* produced mixtures of different antifungal compounds including racemic sesquiterpenoids and variable amounts of other macrolides including pyrenophorin (Sumarah et al., 2011; McMullin et al., 2015). There is extensive literature on the effect griseofulvin has towards plant pathogens including rusts. Depending on the fungus, griseofulvin inhibits growth in the 2–60 μM range (Richardson et al., 2014). Other antifungal compounds from white pine endophytes include chlorinated dihydrobenzofurans and xanthenes from the *H. pinicola* strains noted above (Richardson et al., 2015).

Research on the ecology and impact of foliar endophytes on spruce budworm populations has been critically dependent on methods to detect both the principal metabolites and fungi *in planta*. The purpose of this investigation is to report the detection of both *Picea* and *Pinus* endophytes and their dominant toxins (Figs. 1 and 2) from inoculated seedlings by qPCR and LC MS/MS, respectively, together with an analysis of the endophyte metabolites expected impact on the relevant insect pests or pathogens.

2. Materials and methods

2.1. Fungal material

qPCR assays were developed for the detection of four endophytes (Fig. 2; Table 1). This included *L. nitens* CBS 127939 and CBS 127941, and *Xylaria* sp. DAOMC 242774 and *Phialocephala* sp. DAOMC 229535. A previously reported qPCR method for the detection of *P. scopiformis* DAOMC 229536 (Frasz et al., 2014) was subject to further validation. Besides these fungi, several other foliar endophytes either phylogenetically related, or possible occupants of the same hosts, as well as phylloplane fungi common on needles (Miller et al., 1985) were used to assess specificity. No material cross reactivity was observed in any of the final assays (Supplementary Table S1).

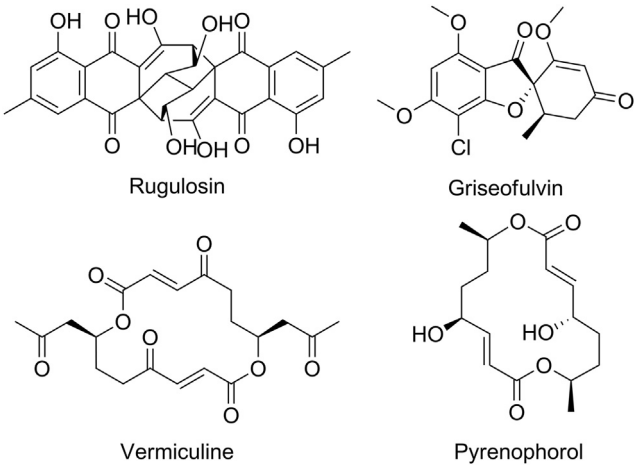

Fig. 1. Chemical structures of toxins produced by the endophytes studied. Rugulosin produced by *Phialocephala scopiformis*, vermiculine from *Phialocephala* sp., griseofulvin from a *Xylaria* sp., and pyrenophorol from *Lophodermium nitens*.

To produce mycelium for method development, all endophyte cultures were grown in Roux bottles containing 200 mL of 2% malt extract (Bacto, Becton, Dickinson and Company, Le Pont de Claix, France). Cultures were incubated at 25 °C in the dark for up to four weeks. Phylloplane fungi were grown on 5% malt extract agar for up to 2 weeks at 25 °C in the dark prior to DNA extractions.

2.2. DNA extraction

After incubation, mycelia were separated from their culture filtrates by suction through Whatman #4 filter papers (GE Healthcare Ltd., Buckinghamshire, U.K.) and lyophilized. For each strain (Supplementary Table S1), 10 mg of lyophilized mycelia were extracted with the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Catalog No. 12224-250) according to manufacturer's instructions. Minor changes included agitating samples after the addition of solution MD1 to release microbeads from the tubes and incubating tubes at 65 °C for 10 min to increase DNA yields. For needle phylloplane fungi, DNA was extracted as described above from aerial mycelium scraped from agar plates. DNA was quantified with the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Catalog No. P11496) according to the manufacturer's instructions. Fluorescence measurements were made in 96-well microplates by the FLUOstar OPTIMA (BMG LABTECH) multimode microplate reader (485/520 nm).

2.3. DNA extraction from needles

Initially, mycelia from the four endophytes considered were spiked onto appropriate control needles (pine or spruce depending on the endophyte host) to evaluate matrix effects. Both needles matrices would affect DNA extraction efficiencies, method limits of detection (LOD), and the co-extraction of PCR inhibitors. Mixtures of fungal mycelium and needles were lyophilized and subsequently powdered with a mortar and pestle in liquid nitrogen. An aliquot (300 μL) of each serial dilution (2 mg mycelium mL^{-1} to 2×10^{-11} mg mycelium mL^{-1}) were added to tube strips containing 20 mg of prepared conifer needles in duplicate.

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