

Toxigenic Foliar Endophytes from the Acadian Forest



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Abstract This chapter describes the ecology of foliar endophytes of the Acadian Forest that dominates Canada's Maritime Provinces extending into Eastern Quebec and Maine. Recent evidence has illuminated the 'foraging ascomycete' life habit of fungi that can be endophytic in conifer needles. These fungi can occupy several eco-niches other than the needles including as saprophytes in aquatic or terrestrial environments or as endophytes of understory species. Structurally diverse secondary antifungal and antiinsectan metabolites appear to mediate the exchange between plant and fungus. The plant provides nutrients and shelter, the fungus increases plant fitness by contributing to tolerance to herbivorous insects or needle pathogens. This work is enabled by the advent of affordable sequencing capability, a dedication to fieldwork and alpha taxonomy, and directed investigations of the metabolites produced by these interesting fungi.

1 Introduction

Endophytes are an artificial assemblage of phylogenetically diverse fungi that can asymptotically infect and inhabit healthy plant tissue. Interest in fungal endophytes of plants was propelled by the discovery of the clavicipitaceous (Clavicipitaceae, Hypocreales) endophytes of cool-season grasses, which produce toxic alkaloids associated with livestock toxicoses and resistance to insect pests (Clay 1988; Siegel et al. 1990). These fungi systemically infect their hosts and may increase growth and seed production and confer tolerance to the host from a variety of abiotic and biotic stresses such as drought, heat, waterlogging, heavy metal

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toxicity, herbivory and fungal pathogens (Kuldau and Bacon 2008; Monnet et al. 2001; Rice et al. 1990; Song et al. 2015; Xia et al. 2015). Because of the economic importance of the impact on livestock and their potential use as inoculants of turfgrass against insects, clavicipitaceous endophytes received much attention and consequently shaped the endophyte research paradigm.

However, the clavicipitaceous endophyte model is not applicable to foliar endophytes of woody plants (Rodriguez et al. 2009). Instead of a phylogenetically-coherent group of taxa which display host-specific, systemic, and frequently vertically-transmitted life strategies, foliar endophytes of woody plants are phylogenetically very diverse, frequently exhibit broad host preferences, can be euryoecious, and are horizontally-transmitted. A significant knowledge gap in the general biology and taxonomy of woody plant endophytes exists. This is exacerbated by the immense phylogenetic diversity and lack of research investigating endophyte life histories.

Collectively, endophytes interact with their hosts along the endosymbiont-pathogen continuum, depending on a balance of antagonisms involving the immediate phenotype status of the fungus and host (Schulz and Boyle 2005). In trees, these host-endophyte interactions are often studied in the context of mutualism, for example investigating possible benefits bestowed upon the host by endophyte infection, such as decreased herbivory, disease resistance, drought tolerance, and plant growth promotion (e.g.: Arnold et al. 2003; Hanada et al. 2010; Khan et al. 2016; Rocha et al. 2011). Many of these mutualistic associations are explained by the production of endophytic natural products in planta. Endophytes are widely considered as an unexplored treasure trove of new biologically active compounds (Aly et al. 2010; Strobel and Daisy 2003). During the portion of their lifecycle associated with a host, there is constant metabolic interaction between the endophyte and plant. The endophyte-plant metabolic equilibrium must balance interactions between the fungal species, host, epiphytes, and plant pathogens. This type of constant interaction fosters an environment selective for endophyte genotypes capable of producing extensive secondary metabolite mixtures fulfilling an ecological function. The lack of knowledge regarding the taxonomy of foliar endophytes of woody plants provides a rare opportunity to prospect for new chemistries in understudied taxa.

Foliar endophyte communities of the Acadian forest and their natural product diversity have been the subject of extensive investigation over the past three decades. Last reviewed by Sumarah and Miller (2009) and Miller (2011), we update here the progress made studying the natural products, ecology and taxonomy of some abundant, phylogenetically diverse conifer endophytes. Consideration is given to the reintroduction of selected endophyte genotypes into seedlings to increase forest resiliency during reforestation efforts as part of integrated forest pest management strategies.

2 Challenges Detecting and Identifying Endophytes

Culture-dependent endophyte studies involve rigorous sterilization of plant tissue surfaces, for example by serial passage through ethanol and sodium hypochlorite, to kill non-endophytic fungi (e.g. epiphytes or viable propagules on the leaf surface) and other organisms. The surface-sterilized plant tissue is then cut into segments, placed on suitable agar media and incubated. Mycelia emerging from plant tissue segments are excised and subcultured to generate axenic cultures. Capturing more biodiversity from plant tissue can involve the addition of other methods such as enzymatic digestion of plant tissue, use of non-standard media, or higher throughput protocols (Greenfield et al. 2015; Prior et al. 2014). Culture-based studies generate viable strains, which enable further work such as multilocus phylogenetic analyses, omics studies, secondary metabolite profiling, inoculation experiments, and the ability to describe novel species represented by acceptable holotypes and ex-type living strains.

Traditional endophyte studies rely upon morphological characters to distinguish, group, and identify endophyte strains. Identification to genus and species is feasible when fungi produce taxonomically-informative morphological characters such as conidiomata or ascomata in vitro. Inducing sporulation in sterile cultures can be a laborious process with varying success. This requires prolonged incubation under various culture conditions, with treatments including different artificial and natural media, light regimes, incubation temperatures, gradual desiccation, mechanical stimulation, flotation in still or aerated water, and the addition of natural substrates such as sterilized plant leaves (Knapp et al. 2015; Sokolski et al. 2006a; Tanney et al. 2016a). Recalcitrant sterile cultures are generally unidentifiable because hyphae typically lack morphological characters allowing for precise taxonomic designation. Hence unidentified endophytes are often designated simply by morphotype descriptions (e.g.: “sterile white” and “sterile pigmented” in Johnson and Whitney 1989). The absence of identified strains, especially for strains not accessioned in culture collections, can result in the inability to make meaningful conclusions or comparisons between past and modern studies. Identification relying solely on morphology and other phenotypic characters can be problematic due to cryptic speciation, convergent evolution, and hybridization (Raja et al. 2017).

Previous attempts to identify particular sterile endophytes included, for example, the use of a specific absorbed immunoserum with on-section immunogold labeling to identify *Lophodermium piceae* hyphae in symptomless *Picea abies* needles by immunoelectron microscopy (Suske and Acker 1989). However, the most important development in the categorization and identification of sterile endophyte strains was the use of molecular DNA tools for fingerprinting and barcoding (Haemmerli et al. 1992; Leuchtmann and Clay 1993; Wilson et al. 1994). Most contemporary endophyte studies use the nuclear ribosomal internal transcribed spacer (ITS) barcode to identify or group endophyte strains. ITS, the official fungal barcode, is readily amplified using standard primers, well-represented in public DNA sequence databases, such as GenBank, and provides good species resolution,

although insufficient interspecific variation is known among some taxa, requiring the further use of secondary barcodes (Badotti et al. 2017; Samson et al. 2014; Schoch et al. 2012; Stielow et al. 2015). The more conserved large-subunit rRNA (LSU) gene is frequently used in conjunction with ITS. Similar to ITS, LSU is also efficiently amplified using standard primers (e.g. LR0R and LR5; Vilgalys and Hester 1990) and is well-represented by abundant reference sequences (Stielow et al. 2015). LSU provides good generic or higher rank taxonomic classification and may be readily aligned across distantly related taxa, which is useful for estimating phylogenies of diverse communities, placing new fungal lineages, and analyzing basal lineages (Liu et al. 2012; Porter and Golding 2012). A good description of a typical workflow for identifying cultures using molecular tools and morphological characters is presented by Raja et al. (2017).

Next generation sequencing (NGS) technology now enables the amplification of endophyte fungal DNA directly from plant tissue, bypassing the labor and selective biases associated with culture-dependent methods and capturing a large number of reads at a relatively low cost. However, NGS studies may also have shortfalls such as amplification biases and failure to discriminate between DNA from living or dead cells (Tedesoo and Lindahl 2016; van Dijk et al. 2014).

Despite these advances, an analogous problem that plagued pre-molecular culture-based studies still confronts both culture-dependent and culture-independent studies relying on DNA sequence-based identification: the precise identification of endophyte strains or sequences lacking taxonomically-informative data. When a fungus belongs to a species, genus, or even family that is unrepresented by accurately identified reference sequences, for example in an accessible DNA sequence repository such as NCBI GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) or UNITE (Abarenkov et al. 2010), its identification using DNA sequences may be irresolvable. The inability to identify endophyte sequences yields results similar to that of traditional studies, however instead of “sterile white” and “sterile pigmented” there are “fungal endophyte sp.,” “Ascomycota sp.,” and “Leotiomycetes sp.”. Accordingly, many endophyte studies report endophyte taxa that are often unidentified to species or genus (Arnold and Lutzoni 2007; Huang et al. 2016; U’Ren et al. 2012).

However, unidentified endophyte DNA sequences accessioned in GenBank and other sequence repositories can be a valuable resource. Comparison of unidentified sequences connected with biogeographic data can provide insight into endophyte ecology despite the absence of a binomial name. These sequences can also be clustered into molecular operational taxonomic units (MOTU) and presented as putative species hypotheses (Abarenkov et al. 2010). The ability to cluster MOTUs into species hypotheses with associated aggregate data has led some mycologists to advocate for a sequence-based taxonomic system allowing for the description of fungi based solely on ENAS data (De Beer et al. 2016; Hawksworth et al. 2016; Hibbett and Taylor 2013; Hibbett et al. 2016). This movement is partly justified by the growing number of unidentified sequences that certainly represent a portion of the currently unknown fungal biodiversity. Names provide consistent identifiers that facilitate the communication of taxonomic concepts; for example, the name

Aspergillus fumigatus provides a wealth of associated information compared with a unique digital identifier from GenBank. Taxonomic instability can also present issues in consistent identification of sequences over time as the quality of sequences databases changes, e.g. correction of misidentifications and identification of previously unidentified sequences (Arnold and Lutzoni 2007).

Although promising, the premature adoption of a taxonomic system accepting ENAS-based species descriptions is likely to create a legacy fraught with doubtful or redundant names. For example, named-but-unsequenced species will be re-described solely because of their absence from sequence databases. MOTU-delineation thresholds used in automated bioinformatics pipelines are variable across studies and dependent on the taxa being investigated. Sequence similarity thresholds used for MOTU delineation (e.g. 95–97%) attempt to compensate for intraspecific sequence variation and possible sequencing errors (Jumpponen and Jones 2009; Lindner et al. 2013; Vrålstad 2011). The result can be the inflation or underestimation of species diversity based on ITS MOTUs, especially when generic similarity thresholds values are applied to a phylogenetically diverse range of taxa with different expressions of ITS variation. In a culture-based study using multilocus phylogenetic analyses, Gazis et al. (2011) provided a demonstration of the shortfalls of species delineation using only ITS sequences for species complexes and advocated the use of more than one gene for species delineation or, if not feasible, the use of a higher ITS similarity threshold (99–100%) for a more accurate diversity estimate. Conversely, intra-isolate and even intrasporal variation of ITS and LSU can be high in Glomeromycota species, thus more stringent delineation thresholds can result in an overestimation of species diversity (Thiéry et al. 2012, 2016). The inability of ITS to discriminate species complexes or distinct species sharing similar ITS sequences is established for some well-studied genera such as *Diaporthe* and *Penicillium*, leading to the use of secondary markers (Udayanga et al. 2014; Visagie et al. 2014). A prudent approach mindful of the taxa being studied, rather than an automated taxonomic pipeline, should be adopted when describing novel taxa, including endophytes, from ENAS.

Endophyte species identification is therefore not constrained by the ability to generate strains or sequences, but by: (1) the lack of reference sequences; (2) the current approach to this taxonomic problem; and (3) a dearth of taxonomists. The growing dependence on sequence-based identification of fungal specimens and cultures and the shift towards biodiversity studies relying solely on ENAS highlights the need for accurately-identified reference sequences connected with voucher specimens and, when feasible, strains accessioned in public culture collections. If sterile endophyte strains or endophyte sequences cannot be identified by ITS barcodes and other gene sequences because of a lack of reference data, then the clear solution is to expand upon the available reference sequences—and knowing where to look.

3 Beyond the Leaf: The Foraging Ascomycete Hypothesis

Xylariaeous (Xylariaceae, Xylariales) endophytes are detected in varying abundance in most endophyte studies involving woody plants, regardless of geographic location or host (e.g. Davis et al. 2003; Dreyfuss and Petrini 1984; Okane et al. 2008, 2013; Petrini and Petrini 1985; Petrini et al. 1995). This ubiquity and apparent host-neutrality has led to much interest and speculation into the role of endophytism in fungal ecology. In this context, Carroll (1999) posited the foraging ascomycete hypothesis, suggesting that for some saprotrophs, endophytism is a secondary life-history strategy facilitating persistence and dispersal in the absence of substrates suitable for reproduction (i.e., a primary host). This latent saprotroph strategy may involve a fungus persisting as a quiescent thallus for some time until the host tissue senesces or dies, at which point the expansive colonization of the fungus into the host substrate ensues. The fungus may cycle through foliage as an endophyte for some time until it comes into contact with a suitable primary host and sexual reproduction or another facet of its life history is permitted. A fungus capable of endophytically infecting host foliage is afforded a refuge buffering it from UV radiation, drought, starvation, competition, mycophagy, and other stresses, while being provided with a source of nutrition and a vehicle for persistence and dispersal. *Picea* trees, for example, retain needles for several years to over a decade depending on environmental conditions (Hom and Oechel 1983; Okane et al. 2008; Osono et al. 2013; Reich et al. 1996), presenting a refuge amenable for long-term persistence by endophytes. Thomas et al. (2016) demonstrated the direct transmission of endophytes from leaves to woody substrates by means of physical contact. Thus the shedding of foliage may act as a dispersal mechanism for saprotrophic endophytes capable of growing on substrates present in the forest floor. Thomas et al. (2016) also isolated Xylariaceae endophytes from 38 tree species in a tropical cloud forest and connected all five *Xylaria* endophytes with saprotrophic (stromatal) life stages. *Hypoxylon pulvicidum*, a pantropical endophyte producing antiinsectan nodulisporic acids, was detected in several independent studies and eventually described following the collection of stromata on decaying wood (Bills et al. 2012). U'Ren et al. (2016) and Okane et al. (2008) also provided evidence connecting saprotrophic and endophytic Xylariaceae species.

Undoubtedly, life histories of foliar endophytes of conifer trees can be entirely restricted within the host foliage, for example *Lophodermium piceae* (Rhytismataceae, Rhytismatales). *Lophodermium piceae* is a ubiquitous *Picea* endophyte that can persist as an endophyte throughout the life of the needle, producing conidiomata (spermatia) and ascomata following needle senescence (Barklund 1987; Livsey and Barklund 1992). Ascospore discharge coincides with vernal shoot and needle flush, when the ascospores are forcibly ejected, adhere to needles by means of a gelatinous or mucoid sheath, and consequently germinate and infect the newly emerging needles (Osorio and Stephan 1991). The fungus then persists as an endophyte until needle senescence either occurs naturally or accelerated by stress.

Converging evidence indicates the foraging ascomycete hypothesis is also applicable to phylogenetically-diverse endophytes outside Xylariales, for example diverse families within Helotiales (Leotiomycetes). Conifer endophytes may produce reproductive structures on specific non-foliar tissues of the same host species or on other hosts such as hardwood trees. For example, *Phialocephala scopiformis* (Mollisiaceae, Helotiales) is a common foliar endophyte of *Picea*, also occurring as branch endophyte (Kowalski and Kehr 1995). Tanney et al. (2016a) discovered that the apothecial sexual state of *P. scopiformis* commonly occurred on decaying *Picea* wood and fallen branches, and suggested the fungus might systemically infect hosts. Conversely, *Phialocephala piceae*, also a foliar endophyte of *Picea*, instead produces its sexual state on decaying hardwood, indicating a broader host range and complex life history (Grünig et al. 2009; Menkis et al. 2004; Tanney et al. 2016a). Tanney et al. (2016a) described the first collection of *Phialocephala piceae* apothecia in nature, occurring erumpent from decaying *Acer saccharum* (sugar maple) branches, and hypothesized its presence as a cambium endophyte in the hardwood host. More sampling is required to determine the endophyte host range of *P. piceae*; i.e.: is it restricted to *Picea* or capable of infecting diverse hosts?

Some fungi are able to infect a broad range of hosts as endophytes but reproduce on a narrow range of alternate hosts with some narrow preference observed, such as the griseofulvin-producing *Xylaria* sp. discussed below (Fig. 1), *Nemania* (Xylariaceae, Xylariales), *Pezicula*, and wood-decaying basidiomycetes such as *Peniophora* and *Trametes* (Chen et al. 2015; Lee et al. 2016a; Martin et al. 2015; Yuan et al. 2011). Many studies have observed the presence of sporulating endophytes outside of their plant host (e.g. Chaverri and Gazis 2011; Koukol et al. 2012; Yuan and Verkley 2015; Okane et al. 2008; Tanney et al. 2016a; Thomas et al. 2016; U'Ren et al. 2016), indicating many endophytes are also capable of functioning as saprotrophs.

Emerging evidence of alternating saprotrophic-endophytic life histories of woody plant endophytes supports the idea that, in woody plants, endophytism should also be considered a facultative life history strategy that may occupy all or only part of the fungus' life, rather than endophytism being strictly an obligate mode of trophism. The foraging ascomycete hypothesis might explain why endophytism is common throughout Dikarya and may also provide a strategy to identify unknown endophytes, one that involves considering the broader ecology of endophytes. The strategy encouraged by Tanney (2016) is straightforward: culturable endophytes that are unidentifiable based on current sequence data or an absence of taxonomically-informative characters are identified by connecting them to identifiable specimens using DNA barcodes (Fig. 2). Specimens of named-but-unsequenced or undescribed species containing taxonomically-informative characters are inferred to represent saprotrophic reproductive states of unidentified endophytes based on hypotheses derived from phylogenetic or taxonomic evidence (Fig. 3). The resulting DNA barcodes generated from identifiable herbarium specimens, ex-type cultures, new field collections, and sporulating cultures are used to connect unidentified

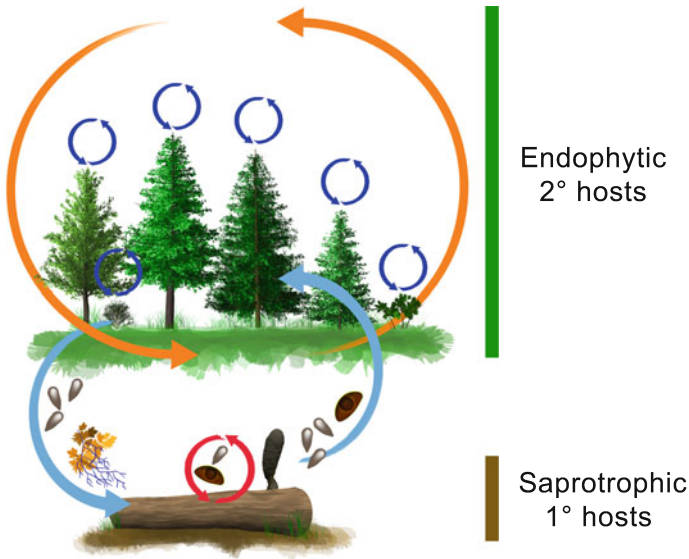


Fig. 1 Hypothetical life history of a *Xylaria* sp. exhibiting an endophytic-saprotrophic life cycle with host-switching. The fungus is capable of endophytically infecting a broad range of overstory and understorey plants, persisting and cycling within and among host canopies indefinitely. Endophytic infections are transmitted to primary substrates (e.g.: decaying *Acer saccharum* wood) when host leaves senesce and abscise by means of conidia disseminated by insects, rainsplash, and air currents, or by direct contact of leaves with the primary substrate. Ascomata and conidiomata are produced on the primary substrate and, via airborne ascospores or disseminated conidia, enable saprotrophic colonization of new primary substrates or endophytic infection of host foliage

endophytes with names. This chapter will provide some examples of both this approach, as inspired by Carroll's (1999) foraging ascomycete theory, and the synergy of combining chemistry and taxonomy to more effectively target endophytes that produce biologically active natural products.

4 Acadian Forest: A Case Study

The Acadian forest is the dominant forest region in the Canadian Maritime Provinces, into Eastern Quebec as well as Maine (USA) and including elements of the neighbouring Eastern Deciduous Forest and Boreal Forest Regions. Late-successional forest types are composed of the defining species *Picea rubens* (red spruce) and other long-lived, shade tolerant species including *Acer saccharum* (sugar maple), *Betula alleghaniensis* (yellow birch), *Fagus grandifolia* (American beech), *Pinus strobus* (Eastern white pine), and *Tsuga canadensis* (Eastern hemlock) (Mosseler et al. 2003a). Natural climax forest dynamics are generally driven by small gap-producing events, such as insect-, disease-, or wind-caused mortality,

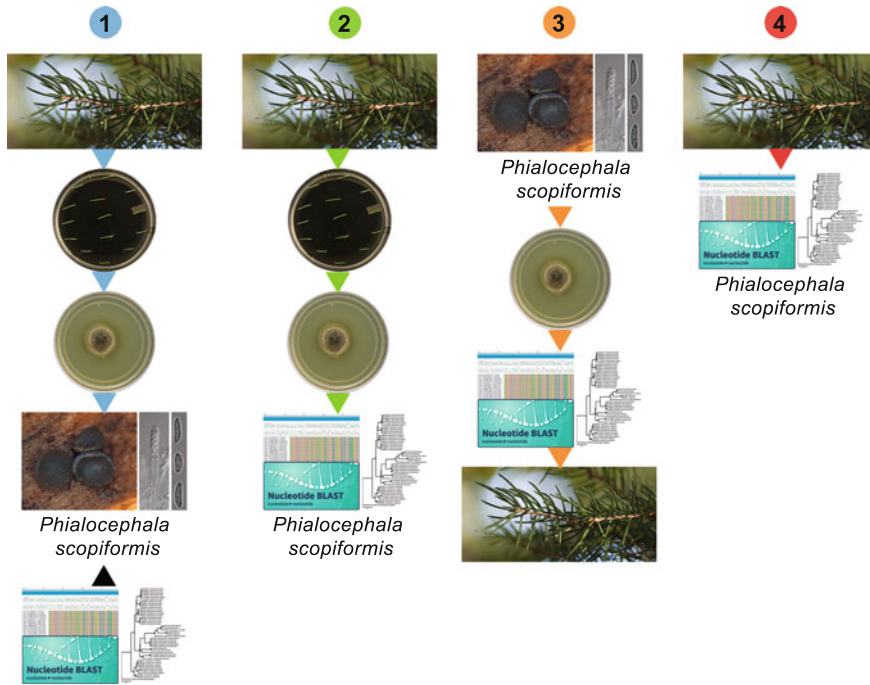


Fig. 2 General approaches to identifying endophytes, using *Phialocephala scopiformis* as an example. (1) Healthy, asymptomatic host leaves are surface sterilized, dissected, and incubated on an amenable media. Emerging mycelia is excised to yield an axenic endophyte culture. Sporulation is induced in vitro, enabling identification based on taxonomically-informative characters (e.g.: apothecia). Reference sequences (e.g. ITS and LSU) are generated for this species and accessioned in GenBank, allowing for its accurate identification in future studies. (2) The resulting axenic endophyte culture is sterile but rapidly identified by comparison or phylogenetic analysis with available reference sequences. (3) An identifiable, named-but-unsequenced field specimen, putatively representing the reproductive structure of an endophyte, is collected based on phylogenetically-informed inference. Sequences are generated directly from the fruiting body or from an axenic culture generated from ascospores, conidia, etc. Named sequences are accessioned into GenBank and can be used to identify this species when detected in future studies, for example from ENAS. (4) Fungal endophyte DNA is extracted and sequenced directly from host tissue. *Phialocephala scopiformis* is identified from the host by the availability of an authenticated reference sequence

with stand-replacing disturbances being rare (Lorimer 1977; Wein and Moore 1977). However, European settlement brought intensive deforestation activity and large-scale fire disturbances (Loo and Ives 2003). Additional disruptive human activities include persistent selective harvesting for *Picea* as well as conversion of mixed-wood forests to *Abies balsamea* (balsam fir)-dominated conifer forests after spruce budworm epidemics and fires (Blais 1983). In response to the spruce budworm epidemic peaking in 1975 forest managers planted white and red spruce. Over the past 70 years, the percentage of softwood in the Acadian forest has not materially changed (Amos-Binks et al. 2010; Etheridge et al. 2005, 2006).

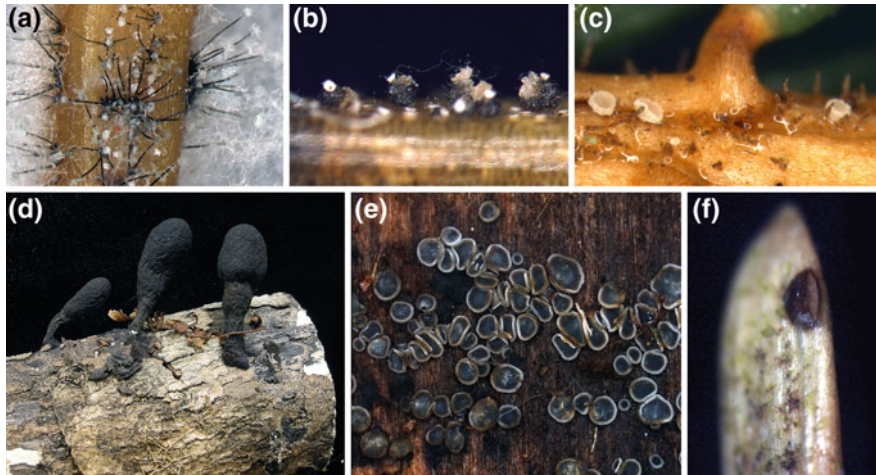


Fig. 3 Examples of diverse Ascomycota fruiting bodies connected with endophytic life histories. A. *Penicillium* section *Thysanophora* (Trichocomaceae, Eurotiales) conidiophores erumpent through *Picea rubens* needle stomata. B. *Rhizosphaera* sp. (Dothideaceae, Dothidiales) conidiomata erumpent through needle stomata, with stomatal wax plugs attached to conidiomata. C. *Hyphodiscus* sp. (Hyaloscyphaceae, Helotiales) apothecia occurring on living *Picea rubens* twigs. D. *Xylaria* sp. (Xylariaceae, Xylariales) stromata on decaying, partially buried *Acer saccharum* branch. E. *Phialocephala* sp. (Mollisiaceae, Helotiales) apothecia on decaying hardwood. F. *Micraspis acicola* (Tympanidaceae, Helotiales) conidioma on dead needle still attached to living twig

The Eastern spruce budworm (*Choristoneura fumiferana*) is one of the most destructive forest pests in Eastern Canada, capable of causing substantial decline in vigour and increased mortality in *Abies balsamea* and *Picea* species. The 1970s epidemic in Eastern North America resulted in substantial ecological disturbance and economic loss. Budworm outbreaks cause a significant loss of timber volume, an increase in forest fires, and substantial CO₂ emissions (Dymond et al. 2010; Fleming et al. 2002). Budworm outbreaks have occurred at periodic intervals of about 35 years (Royama 1984). Forty years after the peak of the last epidemic, in Quebec, almost 3.4 million hectares were moderately to severely defoliated by the budworm in 2014. This is roughly equal to the total number of hectares defoliated in the province from 1987–2007 (National Forestry Database 2017). Chang et al. (2012) estimate that from 2012–2041, uncontrolled moderate budworm outbreaks could cost the New Brunswick economy CDN\$3.3 billion and severe budworm outbreaks \$4.7 billion.

The driving factors initiating budworm outbreak cycles have not been fully elucidated, although epidemiological studies contradict the hypothesis of one epicenter spreading across the region and suggest that populations across the landscape cycle in unison (Royama 1984; Royama et al. 2005). Royama (1984) considered this basic cycle was determined by various density-dependent mortality factors during the third to sixth larval instars: parasitism, predation, food shortage, weather,

and the “fifth agent”. This last undefined variable was hypothesized to be a complex of cryptic causes to explain residual mortality. Royama deduced that population oscillation was ultimately driven by parasitism and the fifth agent.

Motivated by Royama’s intriguing “fifth agent” and the importance of the survival of feeding larvae in driving regional outbreaks, research was initiated under the hypothesis that this unknown variable consisted of toxigenic endophytes (Miller 1986, 2011). This has become a long-term collaborative research effort resulting in the discovery of antiinsectan metabolites produced by *Picea* endophytes, which may remediate the negative effects of Eastern spruce budworm through the inoculation and planting of endophyte-enhanced seedlings (Miller 2011). This research program is also screening *Pinus* endophytes for antifungal secondary metabolites to provide tolerance against white pine blister rust (*Chronartium ribicola*), an invasive disease which continues to decimate white pine in North America (Et-Touil et al. 1999; McMullin et al. 2015; Sumarah et al. 2015; Richardson et al. 2014, 2015).

Seedlings produced in greenhouses are typically void of endophytes (Miller et al. 2009). A primary objective of this program is to identify horizontally-transmitted toxigenic conifer endophyte genotypes to reintroduce stand diversity during reforestation efforts. An ongoing challenge remains developing knowledge regarding the biology and ecology of conifer endophyte collected from the Acadian forest (Tanney 2016). From a natural product discovery perspective, this type of information aids with the dereplication of isolates enabling new chemistries to be efficiently discovered. Akin to the challenges identifying endophytes described above, the natural product literature is full of errors with reports of metabolites produced by unidentified isolates, without deposited voucher specimens, lacking molecular data, with dubious identifications. Credible fungal natural product discovery is reliant upon proper taxonomic identification (Raja et al. 2017). Our approach of combining natural products chemistry with molecular methods, classical taxonomy, new field collections, and herbarium specimens is being implemented to provide more insight into the taxonomy and ecology of conifer endophytes. Examples of these endophytes producing ecologically important natural products collected from the Acadian forest are presented below, including relevant taxonomic, biological, and ecological discussions.

4.1 Toxigenic Endophytes of *Picea*

The identification of *Picea* needles harbouring endophytes capable of producing toxins affecting the growth of *C. fumiferana* pioneered subsequent surveys searching for diverse toxin-producing endophytes. Despite recent efforts to identify endophytic species of interest, some endophyte strains remain unidentified, although with improved taxonomic resolution, or represent undescribed species as part of ongoing taxonomic work. This was the case for two *Picea rubens* endophytes with culture filtrate extracts toxic to spruce budworm larvae in dietary bioassays. From undescribed *Phaeomoniella* sp. (Phaeomoniellaceae,

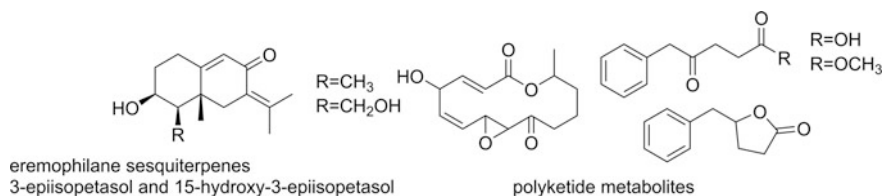


Fig. 4 Sesquiterpenes and polyketides metabolites characterized from the undescribed *Picea rubens* endophytes *Phaeomoniella* sp. CBS 121944 and unidentified strain CBS 121942

Phaeomoniellales) CBS 121944, the sesquiterpenes 3-epiisopetasol and 15-hydroxy-3-epiisopetasol were isolated as the major constituents of the anti-insectan extract (Fig. 4; Sumarah et al. 2010). These metabolites are structurally similar to eremophilane sesquiterpenes including PR-toxin, phomenone, and eremofortines produced by several *Penicillium* species (McMullin et al. 2014; Nielsen et al. 2006). Three additional polyketide metabolites were characterized from *Picea rubens* endophyte strain CBS 121942, which cannot be confidently placed in any family or order based on rDNA sequences (Fig. 4; Sumarah et al. 2010).

4.1.1 *Dwayaangam colodena*

Dwayaangam colodena is a common endophyte of *Picea mariana* and *P. rubens* in Eastern Canada, which motivated the investigation of its bioactive secondary metabolites (Sumarah et al. 2010). From a collection of *P. rubens* endophytes (ca. 2000 isolates) from New Brunswick, Nova Scotia, and Maine, 150 isolates were selected based on morphological characteristics and ITS sequence for further study (Sumarah et al. 2010). Sixteen of the selected isolates that grew well in vitro generated culture filtrate extracts toxic to *C. fumiferana* larvae when incorporated into a synthetic diet. The alkenoic acids cordyanhydrides A and B, and two structurally related precursors, were characterized from *D. colodena* DAOM 239833 (Fig. 5; Sumarah et al. 2010). These unusual natural products were initially isolated from the insect pathogenic fungus *Cordyceps pseudomilitaris* (Isaka et al. 2000). From a recent investigation of conifer endophytes of the Acadian forest, the same metabolites were detected from the antifungal extract of a *D. colodena* strain isolated from healthy *P. mariana* needles (Green 2016).

Dwayaangam colodena (incertae sedis, Helotiales) was discovered as both a *Picea* endophyte and an aquatic hyphomycete in boreal and mixed-wood forest streams in Eastern Canada (Sokolski et al. 2006a, b). The distinct stauroform conidia of *D. colodena* were collected from surface foam in running forest streams and also induced in vitro by placing cultures in a microcosm simulating river foam (aerated sterile distilled water). These observations evoke a complex life history involving endophytism and an aquatic saprotrophic and dispersal phase. Some root endophytes of riparian hosts are also aquatic hyphomycetes, a conceivable

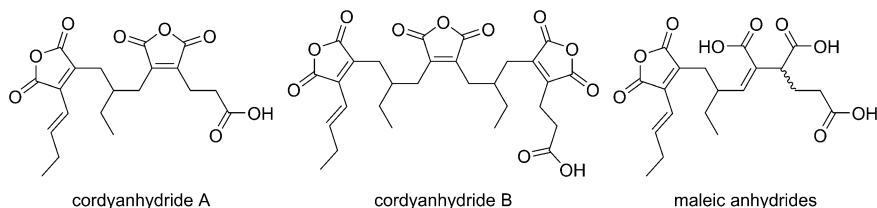


Fig. 5 Maleic anhydrides produced by *Dwayaangam colodena* strains collected as endophytes of *Picea mariana* and *P. rubens*

adaptation because riparian plants may extend part of their roots into streams and river (Selosse et al. 2008; Sati and Belwal 2005; Sridhar and Bärlocher 1992). Hypothetically, *D. colodena*-infected needles might naturally senesce, abscise, and enter nearby streams. The fungus would then initiate a more aggressive saprotrophic phase, colonizing the needle and producing water-dispersed conidia when exposed to aerated water. Sporulation might even be promoted during spring freshet events when water flow and aeration is increased, coinciding with bud break and needle extension of *Picea* hosts. The infection process of host foliage by *D. colodena* is unknown, but may be established by airborne ascospores from an as yet undescribed or unconnected sexual state.

Dwayaangam colodena conidia were also reported from rainwater collected from stands of *Picea abies*, *Pinus sylvestris*, and *Vaccinium myrtillus* in Europe, suggesting a wider geographic and host range and the production of conidia within the canopy (Révay and Gönczöl 2010; Sumarah et al. 2010). Conidia of so-called “terrestrial aquatic hyphomycetes” are frequently reported from arboreal environments, possibly arising from microhabitats such as bark fissures, lodged leaves and debris, phytohelma, or saprotrophic, parasitic, and epiphytic fungi sporulating ephemerally in surface condensation on leaves (Ando 1992; Chauvet et al. 2016; Révay and Gönczöl 2011; Stone et al. 1996). Thus the “terrestrial aquatic hyphomycete” conidia morphology might facilitate dispersal not only in aquatic habitats, but also through the canopy via throughfall and stemflow, along water films on plant surfaces, and by increasing trapping efficiency (Bandoni and Koske 1974; Gönczöl and Révay 2004; Sridhar and Karamchand 2009; Webster 1959).

4.1.2 Mollisiaceae

Mollisiaceae is a large, poorly sampled family comprising at least 1000 names from *Belonopsis*, *Mollisia*, *Phialocephala*, and *Tapesia* alone. Dark septate endophyte (DSE) *Phialocephala* species ubiquitous in roots are well-studied and recent evidence shows the prevalence of endophytism in *Mollisia*, a genus typically considered to be comprised of saprotrophic species (Tanney 2016). The *Picea* endophyte *Phialocephala scopiformis* DAOMC 229536 (Mollisiaceae, Helotiales) is the most extensively studied endophyte from the Acadian forest; its

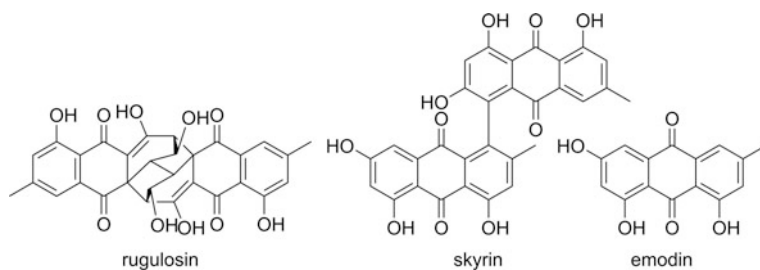


Fig. 6 Anthraquinones rugulosin, skyrin and emodin synthesized by the *Picea* endophyte *Phialocephala scopiformis*

anthraquinones rugulosin, skyrin, and emodin represent some of the first insect toxins characterized from conifer endophytes (Fig. 6; Calhoun et al. 1992). Incorporation of *P. scopiformis* culture filtrate extract or its dominant toxin rugulosin into a synthetic diet inhibited the growth of spruce budworm (*C. fumiferana*) larvae, hemlock looper (*Lambdina fiscellaria*), and spruce budmoth (*Zeiraphera canadensis*) in the low $\mu\text{g/g}$ range (Sumarah et al. 2008a). Greenhouse and nursery studies with three- and four-year-old inoculated seedlings were performed to determine the effect this toxigenic endophyte had on spruce budworm growth. When needle concentrations of rugulosin exceeded the low observed effect level of $0.5 \mu\text{g g}^{-1}$ for *C. fumiferana*, a dose response was observed (Miller et al. 2008). These experiments showed the reduction in growth was associated with effective endophyte toxin concentrations and provided incentive to further explore the biology of *P. scopiformis* and other Mollisiaceae species.

Phialocephala scopiformis, originally described as an endophyte from the periderm of living *Picea abies* branches, is a common foliar endophyte of *Picea* in Eastern Canada (Kowalski and Kehr 1995; Tanney et al. 2016a). As part of an ongoing investigation to identify unknown endophyte cultures by connecting them with sporulating field specimens, Tanney et al. (2016a) discovered the sexual state of *P. scopiformis* from fallen decaying *Picea* branches and wood. Its ascospores arise from typical mollisoid apothecia, which are grayish blue, 0.3–1 mm diam, and occur scattered to caespitose on the host substrate. The asexual state of *P. scopiformis* consists of dematiaceous penicillate conidiophores, which give rise to dimorphic conidia aggregating in slimy heads. Sumarah et al. (2005) successfully infected foliage of *Picea glauca* seedlings with *Phialocephala scopiformis* by wound inoculation of un lignified stems with a macerated mycelial suspension, suggesting *P. scopiformis* can cause systemic infections. Thus, it is also conceivable that colonization of cambium or inner bark by *P. scopiformis* (Barklund and Kowalski 1996) might arise from needle infections, and vice versa. Foliar infections are probably initiated by airborne ascospores and possibly by conidia vectored by insects or rainsplash, although the infection process remains to be studied in detail.

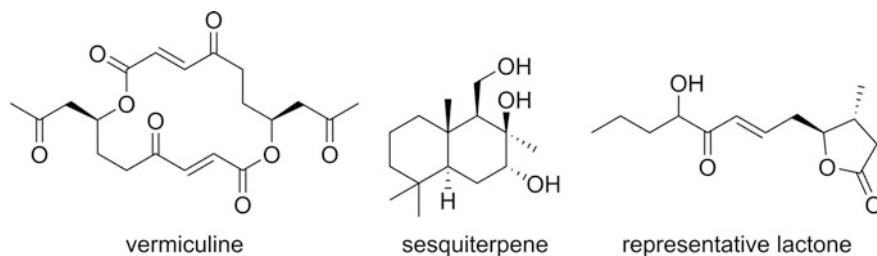


Fig. 7 Structures of the macrolide vermiculine, sesquiterpene and example γ -lactone produced by unidentified *Phialocephala* sp. DAOMC 229535

The culture filtrate extract of an unidentified *Phialocephala* sp. DAOMC 229535 endophyte isolated from *Picea glauca* produced the antibiotic macrolide vermiculine, a known sesquiterpene, and seven previously undocumented gamma-lactones (Fig. 7; Findlay et al. 2003). The sesquiterpene and four of the gamma-lactones at a concentration of $400 \mu\text{g g}^{-1}$ of synthetic diet were toxic to *C. fumiferana* larvae, whereas vermiculine was toxic to immortalized spruce budworm cells (Findlay et al. 2003). *Choristoneura fumiferana* mortality associated with exposure to this *Phialocephala* species' natural products prompted taxonomic investigation and inoculation studies analogous to those of *P. scopiformis*. Based on multigene phylogenetic analyses, *Phialocephala* sp. DAOMC 229535 is likely an undescribed species sister to *Phialocephala* s.s. (Tanney, unpublished data). Similar to *Phialocephala scopiformis* and *P. piceae*, we hypothesize that *Phialocephala* sp. produces mollisoid apothecia from decaying wood, probably *Picea* and/or unrelated hardwood species (Tanney et al. 2016a). Efforts to collect corresponding apothecia to facilitate identification, description, and isolation of new strains from ascospores are ongoing.

4.1.3 *Mycosphaerellaceae* sp.

An endophyte of *Picea mariana* and *P. rubens* that produces mixtures of ramulosin derivatives and isocoumarins toxic to spruce budworm has been the subject of investigation for some time. An isolate originally designated as *Conoplea elegantula* was reported from *P. mariana*. Its crude extract and several purified pentaketides were toxic to spruce budworm larvae and cells (Findlay et al. 1995). The crude extracts of two undescribed *P. rubens* strains (CBS 121943 and DAOMC 239830) were later shown to be toxic to spruce budworm in dietary feeding assays (Sumarah and Miller 2009). Based on ITS sequences, a subsequently studied strain from *P. mariana* (DAOMC 250863) was 100% identical to the *P. rubens* endophytes CBS 121943 and DAOMC 239830. However, the isolate investigated by Findlay et al. (1995) was not deposited, preventing a direct comparison (McMullin et al. 2017a).

McMullin et al. (2017a) reported that all conspecific strains were sterile, despite attempts to induce sporulation over a two-year period involving several media and growth conditions. Eight strains isolated as *Picea mariana* and *P. glauca* endophytes were included in a phylogenetic analysis using the ITS barcode, which placed them as a sister species to *Nothophaeocryptopus gaeumannii*. *Nothophaeocryptopus gaeumannii* (Mycosphaerellaceae, Capnodiales) is a common foliar endophyte and biotrophic pathogen of Douglas-fir (*Pseudotsuga menziesii*), and can cause substantial losses in productivity as the causal agent of Swiss needle cast disease. The disease is marked by chlorosis and low needle retention caused by reduced gas exchange due to the occlusion of stomata by erumpent ascomata (pseudothecia) (Manter et al. 2000). The endophytic Mycosphaerellaceae species from spruce and *Nothophaeocryptopus gaeumannii* are most closely related to *Pallidocercospora* and *Pseudocercospora* species reported as endophytes or associated with leaf spots from diverse hosts such as *Acacia mangium*, *Calophyllum longifolium*, *Eucalyptus* species, and *Ventilago denticulata* (McMullin et al. 2017a; Winton et al. 2007). Despite being relatively common, comprising 11% of total *Picea mariana* endophytes in Quebec (Stefani and Bérubé 2006), this species is probably undescribed. Sampling *Picea* needles in Eastern Canada for mycosphaerella-like pseudothecia or conidiomata will undoubtedly result in the connection of this unknown endophyte with identifiable field specimens.

4.1.4 *Diaporthe maritima*

Diaporthe maritima is a common *Picea mariana* and *P. rubens* endophyte described from the Acadian forest (Tanney et al. 2016b). Field collections connecting *D. maritima* with conidiomata erupting through twig surface and petioles on dead or dying twigs suggest it also occurs as an endophyte in healthy twigs and could potentially be a weakly opportunistic pathogen. *Diaporthe maritima* is a member of the *Diaporthe eres* species complex (DESC), which comprises at least 15 species that are morphologically highly variable and occur on diverse hosts (Gao et al. 2016; Udayanga et al. 2014; Yang et al. 2017). Species identifications within the DESC are difficult due to variability and overlap of morphology and ambiguous clades with short branches and moderate statistical support. However, recent epitypification efforts and the use of more informative genes provides more robust species concepts (Udayanga et al. 2014; Gao et al. 2016). ITS can be insufficient for differentiating closely related *Diaporthe* species and may result in discordant phylogenies, necessitating the use of additional secondary markers (e.g. EF1- α , Apn2, and HIS) (Udayanga et al. 2014). Consequently, endophyte surveys using only ITS or LSU will likely underestimate *Diaporthe* diversity.

Species within the genus *Diaporthe* represent multi-host endophytes and/or tree-associated fungi that may be observed on unrelated trees from different geographical locations (Pirttilä and Frank 2011; Rehner and Uecker 1994; Webber and Gibbs 1984). Phomopsolides A–C as well as a stable pyrone were characterized from two *D. maritima* strains (DAOMC 628553 and DAOMC 250334) isolated

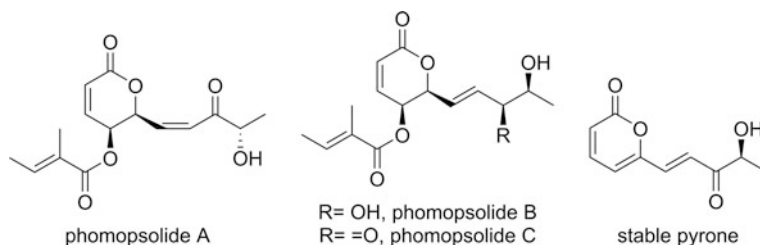


Fig. 8 Dihydropyrone phomopsolides and stable pyrone structures characterized from the *Picea* endophyte *Diaporthe maritima*

from healthy *P. rubens* needles (Fig. 8; Tanney et al. 2016b). This dihydropyrone class of compound was originally reported from *Diaporthe eres* (as *Phomopsis oblonga*) strains associated with the outer bark of healthy *Ulmus* trees (Grove 1985). DESC strains are known to invade the phloem tissue of *Ulmus* trees infected with *Ophiostoma ulmi*, the causal agent of Dutch elm disease (Webber 1981). Phomopsolides possess anti-feedant and anti-boring activity against adult female large elm bark beetles (*Scolytus scolytus*), an insect vector for *O. ulmi* (Claydon et al. 1985). Because of these biological activities, DESC species producing these metabolites were hypothesized to naturally mitigate Dutch elm disease by disrupting the breeding of the pathogen's insect vector (Webber 1981; Webber and Gibbs 1984).

Endophytic *Diaporthe* species are a large source of natural product diversity, with several of their natural products directly implicated in host tree-pest chemical ecologies. From these studies, phomopsolide B appears to be the most common metabolite reported (Claydon et al. 1985; Grove 1985; Horn et al. 1996; Tanney et al. 2016b). For example, a large proportion of North American and European *Silax* endophytes from the genus *Diaporthe* produced phomopsolide B (Horn et al. 1996). Besides phomopsolides, *Ulmus*-derived *Diaporthe* species synthesize pyrones, orsellinic acids, aromatics, and isocoumarins including (*R*)-5-methylmellein, tyrosol, and mellein. While these latter secondary metabolites are commonly produced by phylogenetically diverse fungi, interestingly, they are synthesized by *P. glauca* Rhytismataceae endophytes from the Acadian forest (Sumarah et al. 2008b; McMullin et al. 2017a). When incorporated into the diet of *C. fumiferana* larvae, the culture filtrate extracts of these *P. glauca* endophytes significantly reduced both their weight and head capsule size (Sumarah et al. 2008b). Together with the phomopsolides, (*R*)-5-methylmellein, tyrosol, and mellein all displayed in vitro antiinsectan activities, where phomopsolide A was the most active, towards the bark beetle *S. scolytus* (Claydon et al. 1985). Of particular interest is the identification of the same biologically active natural products synthesized by different tree-associated fungi of different geographic origin having potentially very important ecological roles.

4.1.5 Rhytismataceae

Rhytismataceae (Rhytismatales) contains 55 genera and ca. 730 species of endophytic, parasitic, and saprotrophic plant associates, many of which are exclusively associated with conifers (Kirk et al. 2008; Lantz et al. 2011). The general morphology of Rhytismataceae consists of an ascoma immersed in host tissue with a pigmented shield-like clypeus. This is closed in dry conditions and open in humid conditions to reveal a hymenium consisting of paraphyses that are simple or branched, filiform, free or embedded in a gelatinous epithecium, often connected by hyphal bridges near the bases, with cylindrical to clavate or circinate apices, and asci with undifferentiated thin-walled inamyloid apices (Fig. 9). The ascus forcibly ejects ascospores, which are typically fusiform to filiform and surrounded in gelatinous sheaths (Darker 1967; Lantz et al. 2011; Sherwood 1980). Many Rhytismataceae genera are known from their ascomata produced from needles (e.g. *Bifusella*, *Elythroderma*, *Isthmiella*, *Lirula*, *Lophodermiella*, and *Lophodermium*), branches and bark (e.g.: *Coccomyces*, *Colpoma*, *Therrya*, and *Trybliopsis*).

Rhytismataceae species can have both narrow host and substrate preferences, such as *Lophodermium nitens* and *L. piceae*, while evidence suggests some species may have narrow host preferences but broader, specialized substrate preferences. For example, *Trybliopsis pinastri* is specific to *Picea* and occurs as both a cambium and foliar endophyte, but produces ascomata and conidiomata only on dead, usually self-pruned, branches and never foliage (Barklund and Kowalski 1996; Livsey and Minter 1994; Tanney 2016). *Trybliopsis pinastri* is probably a latent pioneer saprotroph associated with self-pruning of branches, existing as a cambium endophyte for prolonged periods prior to the death of its harboring branch, after which it rapidly colonizes the substrate and eventually produces ascomata and conidiomata (Stone et al. 1996). The detection of *T. pinastri* as a foliar endophyte does not necessarily indicate its ability to persist in foliage, rather foliar infections might be transient and a dead end (i.e.: accidental endophytes), a probability resulting from high numbers of adhesive ascospores contacting foliage. However, the infection biology of *T. pinastri* is unknown, and it is conceivable that twig and branch colonization originates from both wounds and expansion from needle infections.

An unidentified Rhytismataceae endophyte (DAOMC 251461) collected from healthy *Picea mariana* needles produced several metabolite classes including dihydropyrones, phthalides, isocoumarins, and amino acid-derived compounds (Fig. 10; McMullin et al. 2017a). The two dihydropyrones, rhytismatone A and B, were reported as new metabolites, structurally similar to the potently antifungal compound alternaric acid. A phylogenetic analysis of Rhytismataceae endophytes collected from the Acadian forest determined that Rhytismataceae sp. DAOMC 251461 was distinct from three endophyte strains previously isolated from *Picea glauca* (CBS 120379–CBS 120381) with culture filtrate extracts toxic to spruce budworm in dietary bioassays (McMullin et al. 2017a; Sumarah et al. 2008b). These *P. glauca* strains produced mixtures of polyketide structures; however, similarities to the metabolites of Rhytismataceae sp. DAOMC 251461 were

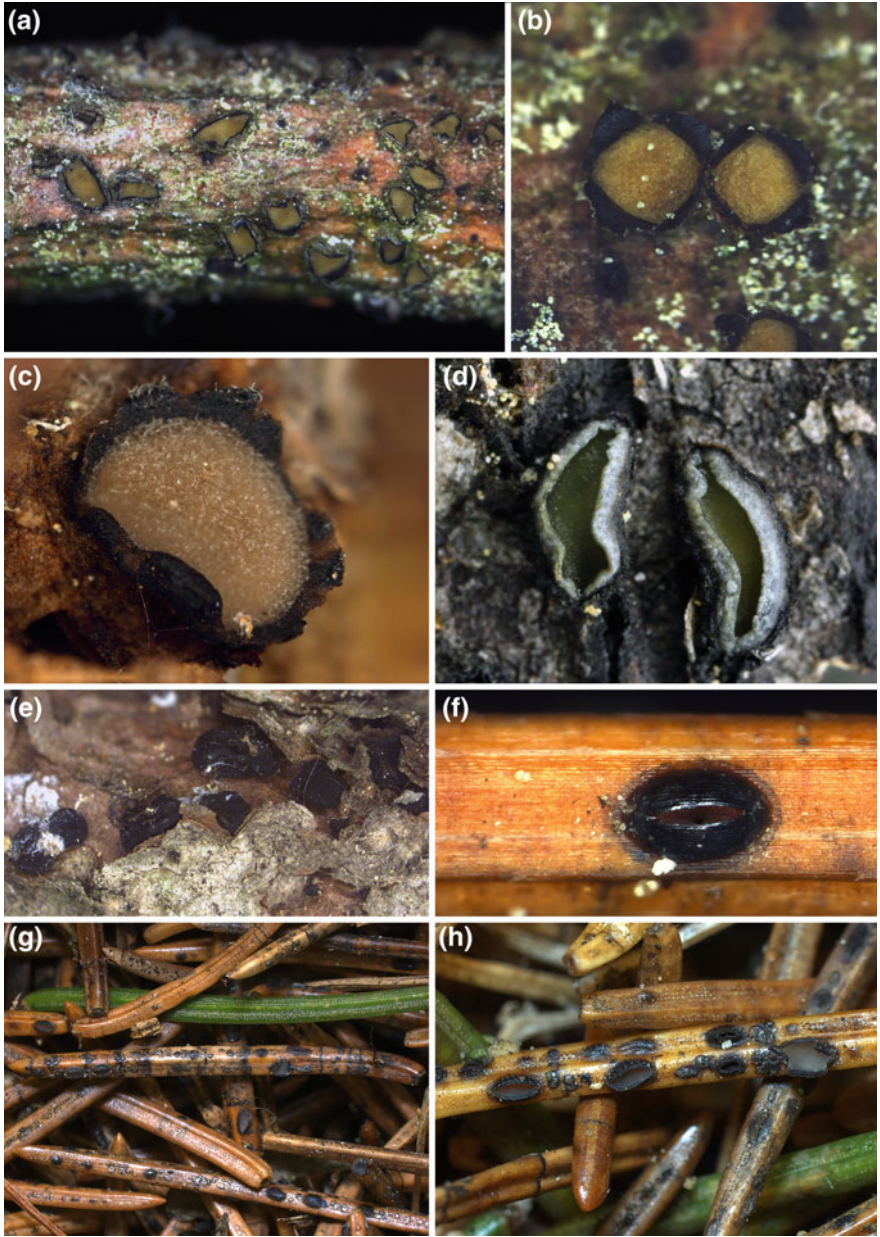
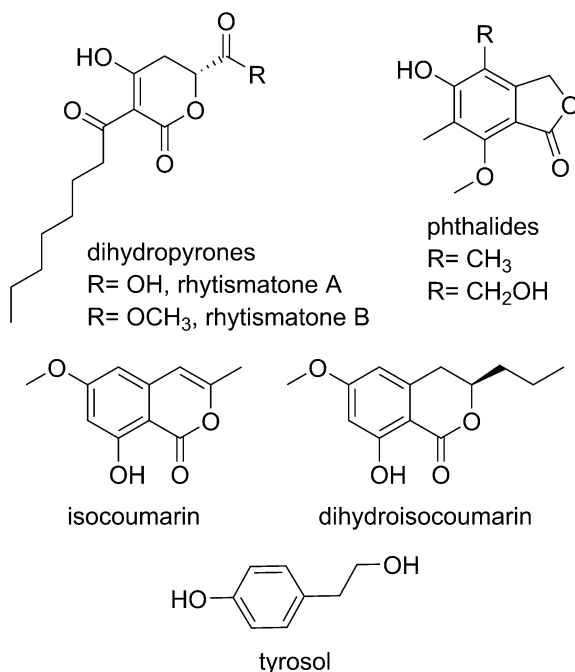


Fig. 9 Apothecia of some Rhytismataceae species detected as conifer endophytes. A–B. *Coccomyces strobil* on dead self-pruned *Pinus strobus* branches. C. *Coccomyces irretitus* on living *Picea rubens* bark. D. *Colpoma crispum* on fallen *Picea mariana* branches. E. *Trybliidiopsis* sp. On dead self-pruned *Picea glauca* branches. F. *Lophodermium nitens* on fallen *Pinus strobus* needles. G–H. *Lophodermium piceae* ascomata and conidiomata on fallen *Picea abies* needles

Fig. 10 Structures of dihydropyrones, phthalides, isocoumarins, and tyrosol produced by *Picea mariana* Rhytismataceae endophyte DAOMC 251461



observed. The amino acid-derived compound tyrosol was produced by all four conifer endophytes and both Rhytismataceae sp. DAOMC 251461 and *Tryblidiopsis* sp. CBS 120380 synthesised similar isocoumarins and phthalides (McMullin et al. 2017a; Sumarah et al. 2008b).

Rhytismataceae species comprise an important family of conifer associates commonly detected as endophytes. However, Rhytismataceae taxonomy is largely neglected by contemporary mycologists. Reference sequences are lacking, hindering sequence-based identification. Identification of unknown Rhytismataceae endophytes is dependent on generating reference sequences for named-but-unsequenced species from identifiable field specimens (ascmata) and actively describing novel species when encountered. Rhytismataceae sp. DAOMC 251461 is unidentifiable based on currently available reference sequences. However, an ITS phylogeny places it in a polytomous clade including genera consisting largely of branch-associated fungi, such as *Coccomyces*, *Colpoma*, and *Tryblidiopsis*, as well as *Lophodermium* species associated with foliage (McMullin et al. 2017a). Increased field sampling of conifer-associated Rhytismataceae ascomata will eventually connect this unknown species with identifiable material. For example, the *Picea glauca* endophyte CBS 120380 is an undescribed *Tryblidiopsis* species occurring on self-pruned *Picea* branches in Eastern Canada (McMullin et al. 2017a). Foliar endophytism should therefore be investigated in bark or branch-inhabiting Rhytismataceae with similar life histories as *Tryblidiopsis*, such as *Coccomyces*, *Colpoma*, and *Therrya* species. For example, *Therrya pinastri*

(misidentified as *Tryblidiopsis pinastri*) was isolated as an endophyte from *Pinus halepensis* needles and is possibly a branch endophyte (Botella and Diez 2011; Solheim et al. 2013). The characterization of new and ecologically important metabolites should encourage more attention on this understudied family (McMullin et al. 2015, 2017a; Reignoux et al. 2014; Sokolski et al. 2004; Sumarah et al. 2008b, 2011, 2015; Tanney and Seifert 2017; Wang et al. 2014).

4.1.6 *Pezicula sporulosa*

Three chlorinated cyclopentenones and (*R*)-mellein were characterized from the culture filtrate extract of an endophytic strain of *Pezicula sporulosa* (Dermateaceae, Helotiales) collected from *Picea rubens* in a mixed-wood stand dominated by *Betula alleghaniensis* and *Acer rubrum* (red maple) (Fig. 11; McMullin et al. 2017a). The characterization of the antifungal metabolite (+)-cryptosporiopsin from this endophytic *P. sporulosa* strain was not unexpected. This compound was originally reported from a *Pezicula* species (as *Cryptosporiopsis*) collected from decaying yellow birch wood (Stillwell et al. 1969; Strunz et al. 1969). A survey of endophytic *Pezicula* species from asymptomatic deciduous and coniferous trees in Northern Germany revealed that all eighty-five strains produced (*R*)-mellein, whereas only six strains produced (+)-cryptosporiopsin, suggesting it is taxonomically significant (Schulz et al. 1995). From this survey, other *Pezicula* species-specific biologically active compounds included 4-epi-ethiosolide, 2-methoxy-4-hydroxy-6-methoxymethyl-benzaldehyde, and (-)-mycorrhizin (Schulz et al. 1995). Interestingly, strains of *Lachnum papyraceum* associated with wood from Europe and North America produced mycorrhizin secondary metabolites with antiinsectan and nematicidal activities together with other chemical classes (Anke et al. 1995; McMullin et al. 2017a). (*R*)-mellein is also produced by a strain of an endophytic *Tryblidiopsis* species (CBS 120381) isolated from *Picea glauca* (Sumarah et al. 2008b). Other *Pezicula* species collected as endophytes of *Fagus sylvatica*, *Pinus sylvestris*, and *Tripterygium wilfordii* are a source of cyclic lipopeptides with potent antimicrobial activities (Noble et al. 1991; Strobel et al. 1999). Future efforts should focus on the identification of conifer endophytes producing cyclic peptides, because members of this chemical class including the enniatins are toxic to spruce budworm (Strongman et al. 1988; Sy-Cordero et al. 2012).

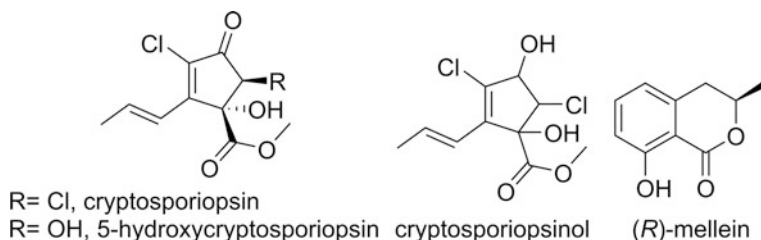


Fig. 11 Structures of the chlorinated cyclopentenones cryptosporiopsin, 5-hydroxycryptosporiopsin and cryptosporiopsinol and (*R*)-mellein produced by *Pezicula sporulosa*

Pezicula sporulosa is reported from a diverse range of hosts including conifers such as *Abies*, *Cupressus*, *Larix*, and *Pseudotsuga*, and hardwoods such as *Fagus*, *Pterocarya*, *Quercus*, and *Tilia* (Farr and Rossman 2016; Chen et al. 2015). In general, *Pezicula* species are usually detected as saprotrophs and sometimes weak pathogens on recently dead branches or stems, where they may produce ascomata (apothecia) and a sporodochial asexual state (= *Cryptosporiopsis*). *Pezicula* species are also commonly isolated as branch endophytes, which, combined with the habit of ascomata and conidiomata erupting through the bark, suggests they share a similar ecology with *Tryblidiopsis pinastri* and may be involved in the self-pruning of branches (Butin and Kowalski 1990; Kehr 1992; Kowalski and Kehr 1992). Endophytic *Pezicula* strains are known from roots, shoots, and leaves, and some studies described species occurring as both foliar and branch endophytes within the same host species (Barengo et al. 2000; Chen et al. 2015; Sieber 2002; Sieber et al. 1991; Sigler et al. 2005; Verkley 1999). For example, *Pezicula neosporulosa*, a common needle and branch endophyte described from *Abies* species in Europe and China, produces ascomata on necrotic bark of the same hosts (Yuan and Verkley 2015). Taken together, these observations indicate a general pattern for *Pezicula* species: the ability to endophytically infect a potentially broad range of hosts while exhibiting more narrow substrate preferences for the production of reproductive structures. Thus, endophytism in *Pezicula* might represent a secondary life history sensu the foraging ascomycete hypothesis.

4.2 Toxigenic Endophytes of *Pinus strobus*

Eastern white pine (*Pinus strobus*) is an economically important conifer for lumber and finished wood products with several potential value-added commercial opportunities. During the North American colonial period, pine was extensively harvested for ship masts and much of the Acadian forest was cleared for agriculture to support new inhabitants (Miller 2011). Natural populations of *Pinus* species are severely susceptible to the disease white pine blister rust (WPBR), caused by the obligate biotrophic parasite *Cronartium ribicola* (Fig. 12). This invasive species, native to Asia, was independently introduced to the west coast and east coast of North America during the early 1900s (Allen and Humble 2002). Since then, it has virtually eliminated *P. monticola* (Western white pine) as a viable commercial species in British Columbia and as of 2010, ~70% of *P. strobus* (Eastern white pine) populations in Quebec were afflicted with the disease (Natural Resources Canada 2012). *Cronartium ribicola* infects North American five-needle pines during all growth stages, with younger trees being the most susceptible, and is one of most important forest diseases. White pine blister rust is more pervasive in cooler, humid environments, making Northern latitudes particularly susceptible to the disease. The rust propagates by alternating between *Pinus* and *Ribes* (currant/gooseberry) species. During the spring, *C. ribicola* spores infect *Ribes* species, which subsequently sporulate in the autumn to infect *Pinus* tree needles.



Fig. 12 *Pinus strobus* branch infected with the forest disease white pine blister rust

Left untreated, the disease spreads sequentially from the needles, branches, and trunk, potentially killing the tree within a few years (Et-Touil et al. 1999). Silviculture disease management strategies have included pruning diseased branches and eradicating alternative hosts, or the use of fungicides (Maloy 1997). Despite this, *C. ribicola* remains a threat to Eastern *Pinus* species that are extensively utilized for reforestation purposes due to their rapid growth. Recognition and planting of *Pinus* trees tolerant to the disease remains an important mechanism of maintaining healthy stands.

As with other conifers, *Pinus* species harbour a diverse community of foliar endophytes (e.g. Oono et al. 2015; Prihatini et al. 2016; Qadri et al. 2014). Echoing our previous experiences with *Picea* endophytes, foliar endophytes of Eastern white pine producing antifungal natural products were targeted in an effort to identify genotypes capable of mitigating the effects of *C. ribicola*. A collection comprising more than 600 *P. strobus* endophyte isolates was assembled and isolations are still ongoing. Incorporating the taxonomy of the isolates facilitated natural product dereplication efforts to identify new chemistries from undescribed or unexplored species. From these collections, approximately 175 isolates were screened for antifungal activity using a modified Oxford assay, where approximately 25% showed antifungal activity. This approach resulted in identifying a greater percentage of biologically active extracts compared to previous collections screened where approximately 5% showed appreciable activity (Sumarah et al. 2015). An initial phylogenetic analysis of these isolates revealed that a large proportion of these strains were *Lophodermium nitens* (Rhytismataceae, Rhytismatales). This abundant *Pinus* endophyte, together with two other species, are discussed below in the context of their taxonomy, biologically active compounds, and ecological roles within the Acadian forest.

4.2.1 *Lophodermium nitens*

Lophodermium nitens is a ubiquitous endophyte of *Pinus strobus* in Eastern Canada, where its conspicuous ascomata are commonly encountered on fallen

P. strobus needles (Fig. 9f). Ascomata and conidiomata develop on fallen senesced secondary needles and mature in the spring and early summer following overwintering. Infection of new foliage is initiated by ascospores, which are released coinciding with bud break and needle extension. *Lophodermium nitens* endophyte infections may be prolonged but are not necessarily quiescent, rather they show signs of continual slow growth intercellularly between the epidermis and hypodermis, and later exhibit increasingly aggressive intracellular hyphal proliferation when needle senescence begins (Deckert et al. 2001). Endophytic *Lophodermium nitens* strains can be rapidly identified by comparing ITS sequences with available reference sequences derived from ascospore isolates.

From the culture filtrate extracts of *Lophodermium nitens* strains collected as white pine endophytes, the antifungal macrolides pyrenophorol, pyrenophorin, and dihydropyrenophorin were characterized together with phenolic bisabolane-type sesquiterpenoids (Fig. 13; Sumarah et al. 2011, 2015). Many of the sesquiterpenoids including sydonol were produced as natural racemic mixtures (McMullin et al. 2015). From in vitro assays, the macrolide chemical class was identified as the more antifungal constituent of these *Lophodermium* extracts where pyrenophorol and pyrenophorin both inhibited the growth of the biotrophic pathogen *Microbotryum violaceum* at 5 μ M (McMullin et al. 2015; Sumarah et al. 2011). The same concentration of pyrenophorol was shown to significantly reduce the dry cell mass of *Cronartium ribicola*, the causal agent of white pine blister rust (Sumarah et al. 2015). These early data indicated needle endophytes of *P. strobus* produce potentially antifungal metabolites that may inhibit the growth of forest diseases.

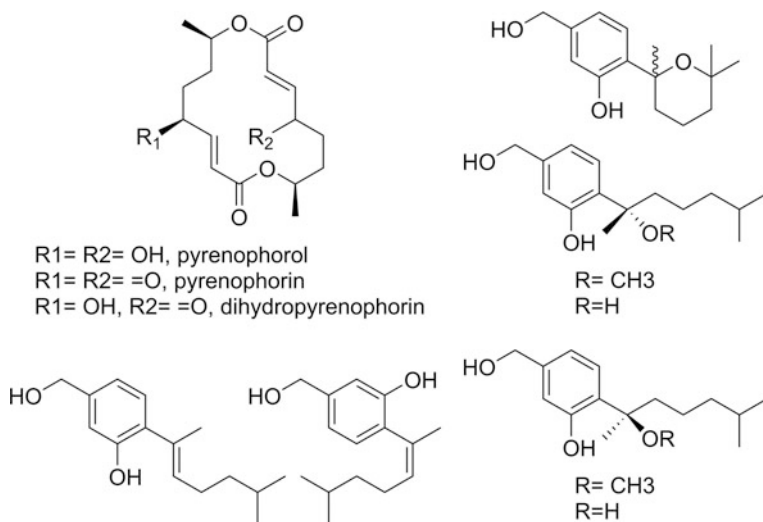


Fig. 13 Structures of the macrolides pyrenophorol, pyrenophorin and dihydropyrenophorin, and phenolic bisabolane-type sesquiterpenoids produced by *Lophodermium nitens*

4.2.2 *Hendersonia pinicola*

When assessing the *Pinus strobus* endophyte collection for in vitro antifungal activity, extracts of two strains (DAOMC 242779 and DAOMC 242780) representing an unidentified species provided the largest response. From their culture filtrate extracts, four dihydrobenzofurans and two xanthenes were characterized (Fig. 14; Richardson et al. 2015). The xanthenes and two chlorinated dihydrobenzofurans were new structures; the remaining dihydrobenzofurans were new natural products, previously identified as synthetic intermediates. Individually tested, each metabolite displayed in vitro bioactivity towards *M. violaceum* and the Gram-positive bacterium *Bacillus subtilis*; however, not as potent as the crude extract.

Based on ITS and LSU phylogenetic analyses, Richardson et al. (2015) suggested these toxicogenic strains represented a new genus and species within a novel lineage of Massarinaceae (Pleosporales). However, shortly after the publication of Richardson et al. (2015), Broders et al. (2015) isolated strains of *Hendersonia pinicola* from *Pinus strobus* needles, which share 100% similar ITS sequences with the unknown Massarinaceae sp. strains (Tanney, unpublished data). This exemplifies the importance of collecting and barcoding named-but-unsequenced forest fungi, as the reference sequences generated by Broders et al. (2015) facilitated the rapid identification of an unknown species previously unidentifiable to genus, despite repeated attempts to induce taxonomically-informative characters in vitro.

Hendersonia pinicola is a well-known secondary colonizer of pine needles, often found in association with *Lophodermella concolor* (= *Hypodermella concolor*), which causes a needle cast disease on two-needle pines. Darker (1967) wrote that these secondary fungi might be extremely important from an economic viewpoint by acting as natural biological control agents: “By robbing the primary fungus of its food supply they prevent the latter from fruiting and thus act as natural biological control agents. As an example, during the height of its development in 1961,

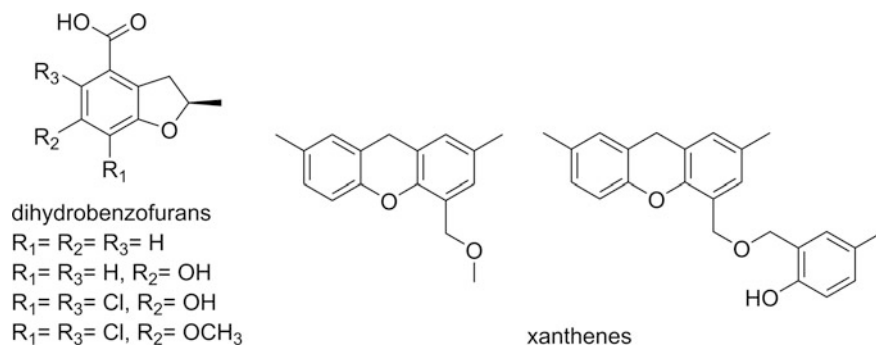


Fig. 14 Dihydrobenzofurans and xanthenes produced by the *Pinus strobus* endophyte *Hendersonia pinicola*

a collection (DAOM 88009) of *Hendersonia pinicola* Wehm., secondary after *Hypodermella concolor*, was so abundant that the black masses of spores hung in festoons on the needles of *Pinus banksiana* and blackened the green leaves of vegetation below. During the following year no evidence of either the primary or the secondary species could be found at the site of the earlier infection near North Onslow, Pontiac County, Quebec. Apparently the *H. concolor* infestation in this small area was completely wiped out.” Additional evidence suggests the potential biocontrol of species of *Lophodermella* through the reduction of inoculum availability by invading secondary *Hendersonia* species (Czabator et al. 1971; Jalkanen and Laakso 1986; Mitchell et al. 1976; Staley and Bynum 1972). The production of antifungal mixtures by *H. pinicola* could explain some of these earlier observations. *Hendersonia* is a large, neglected, and probably polyphyletic genus (Broders et al. 2015). However, earlier observations suggesting biocontrol potential and work by Richardson et al. (2015) should motivate future taxonomic work and natural product investigations.

4.2.3 *Xylaria* sp.

Recent studies have shown the ecologies of conifer endophytes from the Acadian forest are more complicated than anticipated. A currently undescribed *Xylaria* species was collected as a common endophyte of *Pinus strobus* and selected for further investigation, as Xylariaceae have been a rich source of diverse metabolites (Stadler 2011). The potently antifungal compound griseofulvin was isolated as a major constituent of its antifungal culture filtrate extract, together with dechlorogriseofulvin, a dihydrobenzofuran, and piliformic acid (Fig. 15; Richardson et al. 2014). The identification of piliformic acid (2-hexylidene-3-methylsuccinic acid) is chemotaxonomically important as it is produced characteristically by several temperate *Xylaria* species (Whalley and Edwards 1995; Richardson et al. 2014). Griseofulvin production has been reported from a narrower group of Xylariaceae species (Lee et al. 2016b; Sica et al. 2016; Park et al. 2005; Whalley and Edwards 1995). This antifungal metabolite is known to inhibit the growth of several plant

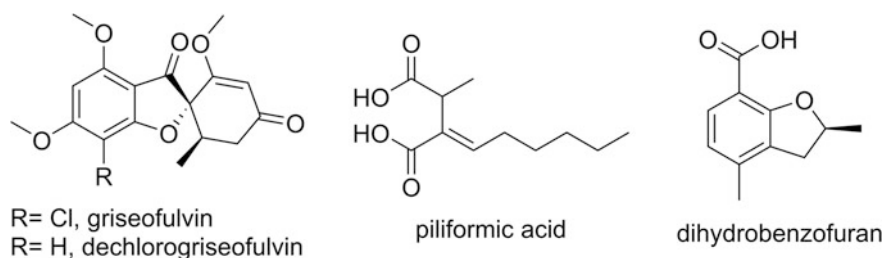


Fig. 15 Griseofulvin, dechlorogriseofulvin, piliformic acid and the dihydrobenzofuran produced by endophytic strains of the discussed undescribed *Xylaria* species

pathogens, including rusts, in the low μM range, and is not phytotoxic (Richardson et al. 2014). A corresponding study of fruit-bearing plants in Eastern Canada identified griseofulvin-producing *Xylaria* strains from lowbush blueberry stems (*Vaccinium angustifolium*). Phylogenetic analysis revealed these were the same undescribed *Xylaria* species and several strains isolated from both hosts produced griseofulvin in culture (Richardson et al. 2014). These observations have prompted seedling inoculation studies with this *Xylaria* sp. (described below) as well as further taxonomic and metabolomic investigations (Ibrahim et al., in preparation).

The phylogeny presented by Richardson et al. (2014) shows this *Xylaria* sp. was detected in other studies, including as an endophyte of the liverwort *Trichocolea tomentella* (Davis et al. 2003). *Xylaria* sp. is now known as an endophyte from *Picea glauca*, *P. mariana*, *P. rubens*, and has a diverse host range including other conifers and even mosses, bryophytes, and lichens (Ibrahim et al., in preparation). A hypothetical life history for *Xylaria* sp. illustrated in Fig. 1. is likely applicable to many endophytic *Xylaria* species.

5 Endophyte Inoculation Studies

The ecological roles of conifer endophytes within the Acadian forest have been the subject of considerable study over the past three decades. As described above, phylogenetically-diverse endophytes from the Acadian forest represent a prolific source of natural products with antifungal, antibiotic, and antiinsectan activities. Studies focused on the ecology and impact of foliar endophytes on spruce budworm or other forest pest and pathogen populations are critically dependent on methods to detect both the fungi and principal metabolites in planta. The development of polyclonal antibodies specific to endophyte mycelial proteins previously represented a suitable approach to monitor the infection and persistence of selected toxigenic strains in seedlings. Together with HPLC-UV analysis for rugulosin in needles, these analytical methods enabled earlier nursery and field studies with *Phialocephala scopiformis* (Sumarah et al. 2008a; Miller et al. 2008, 2009). While being adequately sensitive and specific, antibody-based approaches are very labour-intensive and require large sample amounts. A shift from ELISA to molecular diagnostic methods, such as qPCR, was made feasible by more reliable reference sequences and knowledge advancements in the taxonomy and phylogenetics of conifer endophytes, a prerequisite to developing more effective and reproducible molecular diagnostic methods.

The *Picea* foliar endophyte *Phialocephala scopiformis* was extensively studied over the past few decades because it synthesizes rugulosin, a toxin that negatively affects the growth of *C. fumiferana*. Previous efforts studying the colonization and persistence of *P. scopiformis* in *Picea glauca* trees relied upon ELISA methods together with HPLC-UV to monitor rugulosin concentrations (Miller et al. 2009;

Sumarah et al. 2005, 2008a). Using ELISA, Miller et al. (2009) showed that after three years, *P. scopiformis* was detected in 40% of the 250 *P. glauca* seedlings planted under the emerging canopies of 50 *P. glauca* trees previously inoculated with *P. scopiformis*. Armed with greater taxonomic insight for the endophyte strains producing insect toxins or antifungal compounds, qPCR methods were developed to monitor their persistence within conifers (Frasz et al. 2014; McMullin et al. 2017b). Using a *P. scopiformis*-specific qPCR probe targeting the ITS region, Frasz et al. (2014) tested 291 *P. glauca* trees eleven years after inoculation. They reported 56.7% of needle samples collected throughout the crown were colonized by *P. scopiformis*. Needle samples were also tested for rugulosin, where the mean ($1.2 \mu\text{g g}^{-1}$) and median ($0.93 \mu\text{g g}^{-1}$) rugulosin concentrations were approximately twice the in vitro minimum lowest observed effect level ($0.5 \mu\text{g g}^{-1}$). These results combined with those of Miller et al. (2009) demonstrated that *P. scopiformis* persists following inoculation, is horizontally-transmitted throughout the crown, and is capable of infecting proximal naturally regenerating *Picea* seedlings.

A follow-up study aimed to monitor three additional endophyte genotypes discussed above from inoculated *Picea glauca* and *Pinus strobus* seedlings by qPCR (McMullin et al. 2017b). Tandem mass spectrometric methods specific to each endophytes' principal toxin were also implemented to replace HPLC-UV analysis used previously to quantify rugulosin from needles. Seedlings were inoculated with a single endophyte species and their toxins were quantified to determine the antifungal and antiinsectan metabolites concentrations within host foliage. From inoculated *Pinus strobus* seedlings, endophytes and corresponding metabolites considered were *Xylaria* sp. DAOMC 242774 and *L. nitens* CBS 127939, which produce the antifungal compounds griseofulvin and pyrenophorol, respectively. From inoculated *Picea glauca* seedlings, the undescribed vermiculine-producing *Phialocephala* sp. DAOMC 229535 was evaluated together with the rugulosin-producing *Phialocephala scopiformis* DAOMC 229536. In all cases, there was a high concordance between the qPCR and LC-MS/MS results. All samples positive for the respective toxins were positive for the producing endophyte with the accompanying qPCR assay. The undescribed *Phialocephala* sp. was detected from 83% (25/30) of samples where the mean vermiculine concentration in needles was $0.5 \mu\text{g g}^{-1}$ from 82% (22/27) of samples (McMullin et al. 2017b). All samples ($n = 25$) tested for *P. scopiformis* DAOMC 229536 were positive for both the endophyte and its toxin. Akin to previous studies, the mean rugulosin concentration was in the range expected to affect the growth of spruce budworm larvae (> 1.0 ppm; see Frasz et al. 2014; Sumarah et al. 2005, 2008a).

Griseofulvin was detected from 78% (25/32) of *Pinus strobus* seedlings inoculated with *Xylaria* sp. DAOMC 242774, a high griseofulvin-producing strain in culture. Unlike the other endophyte metabolites quantified, mean and median griseofulvin concentrations were divided between two distinct groups. The mean griseofulvin concentration from the high toxin group equated to approximately $5 \mu\text{M}$ of the antifungal compound in needles (McMullin et al. 2017b). This concentration is in the range where griseofulvin is known to be toxic to several plant disease causal agents, including rusts (Napier et al. 1956). The mean concentration

of the macrolide pyrenophorol from white pine seedlings inoculated with *Lophodermium nitens* CBS 127939 was also in the low μM range (McMullin et al. 2017b). A 5 μM dose of pyrenophorol reduced the dry cell mass of a *Cronartium ribicola* strain, collected from afflicted *P. strobus* seedlings (Sumarah et al. 2015). As stated above, the macrolides pyrenophorol and pyrenophorin both inhibited the growth of the biotrophic pathogen *M. violaceum* at the same concentration (McMullin et al. 2015; Sumarah et al. 2011). In all cases, forest pests and pathogens would be exposed to a greater toxin concentration as a proportion of the biologically available water would be bound by needle structural components. These data further support the hypothesis that species-specific strains synthesize natural products that can increase host tolerance to pests and pathogens. These studies have been enabled by advances in modern analytical instrumentation and investments into the informative taxonomy of conifer endophytes from the Acadian forest.

6 Conclusions

Forests are critical to global ecological and economic integrity, yet relatively little is known concerning the composite forest mycobiota. Endophytes are omnipresent in forest ecosystems, where they represent a wealth of undescribed biodiversity and novel natural products, and likely play an important role modulating forest health. Improving our understanding of endophyte diversity and taxonomy is critical to developing a more holistic approach to forest health, including the development of integrative forest pest management tools. For example, finding higher rugulosin producers requires access to a large number of *Phialocephala scopiformis* strains. Previously, isolating *P. scopiformis* endophytes was a more-or-less chance occurrence; however, now countless single-ascospore *P. scopiformis* strains can be generated by collecting apothecia commonly occurring on decaying fallen *Picea rubens* branches. Other practical benefits of investing in taxonomic research of endophytes, and forest mycobiota in general, include improved surveillance and monitoring for invasive pathogens and more informed forest management practices.

The evidence marshalled in this chapter should provide strong support for field collecting efforts and the mining of invaluable resources such as herbaria and culture collections. Classical taxonomic knowledge is needed now more than ever and the synergistic marriage of both classical taxonomy and modern molecular methods leads to significant advances in mycology. Mounting evidence connecting endophytic and saprotrophic life histories cannot be ignored. Endophyte research programs should include efforts to connect unidentified endophytes with field specimens, which will increase the resolution of subsequent studies and provide invaluable reference sequences for studies using NGS. In general, funding agencies should be more supportive of “traditional” methods and fieldwork, because our lack of taxonomic knowledge cannot yet be circumvented by adopting new technology (see Truong et al. 2017). Further, the identification of novel or known bioactive secondary metabolites from conifer endophytes of the Acadian Forest Region is

possible only because of culturing endeavors, and screening is becoming incrementally more efficient because of taxonomy-guided chemistry. Future culture- or inoculation-based work should include connecting genomic data with phenotypic data to identify genetic signatures of endophytism and the mechanisms driving endophyte-saprotroph life histories and host-switching strategies.

This multi-disciplinary investigation of endophytes has already resulted in the discovery of novel species, new natural products, and assists the ongoing development of integrated forest pest management tools to combat forest pests and pathogens. Endemic endophytes are an overlooked source of forest biodiversity in the Northern hemisphere and may provide tools that protect managed forests from destructive native and alien species. One consequence of evidence garnered from endophyte research is that studies investigating resistance among forest tree populations must also consider the possible role of endophytes, and not just the host genotype, in expressed phenotypic variation.

Just as old growth *Picea rubens* forests are reservoirs of tree genetic diversity and possible superior seed sources (Mosseler et al. 2003b), these same forests also contain a great biodiversity of horizontally-transmitted endophytes and represent important sources of potentially commercially viable endophyte strains. Forest endophyte diversity is reduced by disturbances from insect pests and pathogens, fire, and commercial logging (Miller 2011). The high incidence of horizontally-transmitted endophytes detected from inoculated nursery seedlings suggests this approach provides a mechanism to reintroduce natural endophyte populations during reforestation efforts. Additionally, this knowledge also provides a pragmatic incentive to protect old growth forests and ascribes additional value to the retention of trees and coarse woody debris, which may serve as sources of endophyte inoculum for regenerating forests or for future discoveries.

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