CHARACTERIZATION OF SECONDARY METABOLITES PRODUCED BY FOLIAR
ENDOPHYTES OF RED AND BLACK SPRUCE

by

Blake Donald Green

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Carleton University
Ottawa, Ontario

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Blake Donald Green

Abstract

Eastern spruce budworm (*Choristoneura fumiferana*) is a cyclic pest responsible for widespread defoliation of conifer trees in the Acadian forest. During epidemics, affected forestry areas are vast, making insecticidal protection impractical. Research has shown endophytes produce toxins *in planta* at effective concentrations that deter spruce budworm herbivory. Red and black spruce endophytes were studied here. Five isocoumarins (**1-5**) were isolated from *Phaeocryptopus cf. gaeumannii*, where compound (**1**) is a new structure. Two new pyrones (**6, 7**), two phthalides (**8, 9**), two isocoumarins (**10, 11**) and tyrosol (**12**) were isolated from an undescribed Rhytismatales species. Phomopsolides A-C (**13-15**) were isolated from a newly described fungal endophyte, *Diaporthe maritima*. Cryptosporiopsin (**16**), 5-hydroxy cryptosporiopsin (**17**), cryptosporiopsinol (**18**) and mellein (**19**) were produced by *Pezicula sporulosa*. A majority of these compounds possessed antibacterial (*Bacillus subtilis*, *Escherichia coli*) and/or antifungal activity (*Saccharomyces cerevisiae*, *Microbotryum violaceum*).

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1. Introduction

Fungi

The fungal kingdom is comprised of a highly diverse group of unicellular and multicellular eukaryotic organisms. Fungi are heterotrophic and present in all inhabitable environmental niches worldwide as they can metabolize many forms of organic matter (Ferreira et al., 2006). Some fungi are saprophytes that live on decaying organic matter and in doing so provide the essential ecological function of nutrient recycling. Other fungi are parasitic or pathogenic and are responsible for many diseases observed in plants and animals. The last group of fungi are known as endophytes as they colonize the inside of plants without causing apparent damage (Figure 1.1). Endophytes are not limited to fungi as plants also associate with algae and bacteria, but the term endophyte used hereafter will refer to fungal endophytes. Endophytes have been found within all plants studied to date, but their existence and ecological importance have largely been overlooked until recently (Petrini, 1991; Arnold & Lutzoni, 2007). To date, only *ca.* 35,000 endophytes have been studied and an estimated 465,000 remain undescribed (Schmit & Mueller, 2007; Sieber, 2007).

The identification of fungi is often difficult meaning multiple approaches are necessary. Taxonomists typically combine the use of traditional and molecular methods. Traditional methods involve varying culturing conditions (e.g. pH, water activity, nutrient sources) to elicit distinguishable morphological features (Schmidt & Lodge, 2004). With molecular methods, the internal transcribed spacer (ITS) region between the small and large ribosomal RNA subunit genes is sequenced and compared to available databases. In fungi, this ITS sequence is commonly

referred to as the universal barcode and customary for fungal identifications at the species level (Scholch et al., 2012). The main limitation with molecular methods is the need for alpha taxonomy and a reliable reference sequences. With the vast number of undescribed endophytes, it is often difficult to find meaningful matches in currently available databases. Another interesting approach that can be a helpful addition to traditional and molecular methods is to compare and contrast the metabolite and lipid profiles of fungi (Boysen et al., 2000; Dörge et al., 2000). This approach is most useful when trying to distinguish between similar fungi as the presence of diverse metabolites or lipids profiles corresponds to different genes or gene expressions.

Figure 1.1: Image of an endophyte growing out of a surface-sterilized needle.

Endophytes

There have been two major driving forces behind the extensive research into these cryptic fungal species. Since the discovery of penicillin, fungi have been extensively investigated as a source of bioactive compounds as their diverse nature translates to the production of metabolites with an array of chemical complexity and diversity. For example, a review article from 2005 illustrated the antibacterial, antifungal or antitumor activity of 1,500 compounds that have been isolated

between 1993 and 2001 (Pelaez, 2005).

The other important driver is understanding plant-endophyte interactions and the influence of this interaction on ecosystems. Endophytes are classified into three groups: mycorrhizal endophytes associated with plant roots, clavicipitaceous endophytes that colonize grasses and non-clavicipitaceous endophytes. The current plant-endophyte interaction model is based on grass endophytes since they have been the most extensively studied due to their agricultural impact (Rodriguez et al., 2009). Multiple studies have shown grass endophytes conferring various benefits to their grass host in exchange for protection and dispersion. Observations of decreased food intake, weight loss and gangrenous extremities in cattle grazing on cool season fescues have been linked to neurotoxic ergot alkaloids and indole diterpenes produced by grass endophytes (Bacon et al., 1977; Mortimer et al., 1982). Reduced insect herbivory in agronomically-relevant fescues have been linked to grass endophytes producing pyrrolopyrazine and pyrroizidine alkaloids (Bush et al., 1997; Clay & Schardl, 2002). Clay (1997) demonstrated that grass endophytes produce metabolites that stimulate germination and increase biomass production of field fescues by 30%. The seaweed endophyte, *Mycosphaerella ascophylli*, has been shown to increase the drought tolerance of the intertidal species *Ascophyllum nodosum* (Garbary & London, 1995; Garbary, 2009). Overall, grass endophytes have been widely shown to increase host tolerance to abiotic and biotic stresses. In return, grass endophytes infect all above-ground tissues of their host to propagate the next generation via the host seed (vertical transmission). The production of fungal metabolites that deter herbivory, promote growth and increase host tolerance of the plant host, in exchange for dispersal represents a plant-endophyte mutualistic relationship.

The relationship (mutualism, commensalism or parasitism) of endophytes in non-clavicipitaceous plants is not as clearly defined. For instance, a meta-analysis of 157 primary publications was able to statistically show the defensive mutualistic role grass endophytes play in deterring herbivory, but was unable to show this association with non-claviceptaceous endophytes (Saikkonen et al., 2010). In the late Carboniferous period 300 million years ago, angiosperms came into existence by diverging from gymnosperms (Sieber, 2007). This divergence coincided with the divergence of the Helotialean and Diaporthalean taxonomic orders of fungi. Presently, the leaf tissues of angiosperms and gymnosperms is dominantly colonized by Diaporthalean and Helotialean endophyte species, respectively. This suggests that plant-endophyte interactions co-evolved over millions of years, in which the lack of associated disease suggests this relationship to be either mutualism, commensalism or low-virulence parasitism (Sieber, 2007). However, the number of scientific studies is still too limited to clearly detail the symbiosis between endophytes and non-clavicipitaceous plants or why the relationship started.

The current theory is centralized around the lifespan of plants being longer compared to common pests and pathogens that attack them (Miller, 1986). This concept is highlighted in woody plants, such as conifers, as their long lifespans result in slower gene mutations/evolutionary adaptations. Additionally, woody plants are attacked by a variety of animal and insect herbivores as well as fungal pathogens due to their long lifespan. As such, woody plants tend to produce a high abundance of generalized secondary metabolites to ward off a variety of pests and pathogens (Miller, 1986). As expected, this results in weaknesses in their natural defenses. For instance, balsam fir (*Abies balsamea*) produce an array of terpene metabolites predominately in their needles, in which the abundance of these terpenes increases with age (Hunt & Rudloff, 1977).

These terpenes have been shown to mitigate fungal pathogens but are ineffective towards insects such as the Eastern spruce budworm (*Choristoneura fumiferana*), which prefer feeding on young needles (Miller, 1986). It is believed that non-clavicipitaceous plants have overcome this evolutionary limitation by developing mechanisms that allow endophytes to live within their foliage while preventing endophyte biomass from increasing. As the life span of endophytes is much shorter than woody trees, they are able to mutate at a much faster rate. This means faster evolution to selective pressures, such as insects that have been shown to have fungal endophytes in their digestive systems. Taken together, endophytes will develop the genes to produce secondary metabolites targeting their natural predators faster than plants and trees take advantage of this feature by allowing endophytes to live within their foliage (Miller, 1986).

Conifer Endophytes

Research into the foliar endophytes of coniferous trees was initially pursued following the suggestion by Carroll & Carroll (1978) that the needle endophyte *Rhabdocline parkeri* was improving the tolerance of Douglas fir (*Pseudotsuga menziesii*) towards the gall forming needle pest called gall midges (family *Cecidomyiidae*). Douglas-fir trees showing infection of the needle endophyte *R. parkeri* were able to increase the mortality of gall midge larvae (Sherwood-Pike et al., 1986; Carroll, 1986). Miller (1986) showed that fungal extracts of *R. parkeri* slowed the growth rate and increased the mortality rate of spruce budworm larvae when introduced into a synthetic diet. This discovery lead to the postulation that conifer endophytes improve the fitness of their host by producing an array of anti-feedants to deter insect herbivory. This possibility was highlighted in the monograph published on the population dynamics of spruce budworm in New Brunswick (NB) between 1945 and 1983 (Royama, 1984). Eastern spruce budworm is a cyclical

pest responsible for large scale mortalities in the Acadian forests of Eastern Canada and Maine. While modeling the population dynamics of the spruce budworm, Royama described five mortality factors: parasitism, predation, food shortage, weather and an unknown fifth agent. His prediction of an unknown factor arose from unclear population oscillations occurring in late stage larvae that could not be completely explained by parasitism. Extensive research has been performed under the hypothesis that toxigenic endophytes are the fifth agent.

Over the past 25 years, endophytes have been collected from various conifers in the Acadian forest in order to better define the number of toxigenic endophyte genotypes present in the region (Miller, 2011). During the initial sampling of balsam fir (*Abies balsamea*), red spruce (*Picea rubens*), white spruce (*P. glauca*) and black spruce (*P. mariana*), it was observed that the proportion of endophytes with toxigenic properties was extremely low (~5%; Johnson and Whitney, 1989; Wilson et al., 1994; Miller et al., 2002). Another observation was that approximately 20% of the toxigenic strains originating from NB in the first collections produced rugulosin (Figure 1.2), which was known to have broad spectrum biological activity (Miller et al., 2002). Growth chamber tests showed spruce budworm larvae feeding on detached white spruce needles colonized by the rugulosin producing endophyte *Phialocephala scopiformis* had significantly reduced growth in comparison to the un-inoculated control needles. In growth chambers and in field tests white spruce saplings inoculated with *P. scopiformis* continued to produce rugulosin above the effective toxin level of $0.5 \mu\text{g g}^{-1}$, which was shown by the reduction in spruce budworm growth (Miller et al., 2002, 2008).

Figure 1.2: Chemical structure of rugulosin produced by *P. scopiformis*.

During these studies, nursery seedlings intended for reforestation were observed to lack diverse endophyte communities (Miller et al., 2002; Sumarah et al., 2008a). In a field test, un-inoculated woody plants (balsam fir and red, black and white spruce) were placed two meters around a white spruce tree that had been inoculated with the endophyte *P. scopiformis*. After 3 years, *P. scopiformis* was detectable by enzyme linked immunosorbent assay (ELISA) in only the white spruce saplings that surrounded the test tree (Miller et al., 2009). Contrasting to grass endophytes, this discovery contributed to the sparse data suggesting non-clavicipitaceous endophytes transmit horizontally rather than vertically (Miller et al., 2009; Miller, 2011). It also demonstrated that *P. scopiformis* had host specificity towards white spruce (Miller et al., 2009). This discovery of horizontal transmission helped explain the reduced endophyte diversity observed in tree stands that have been reforested/afforested artificially or naturally following a deforestation event (fire, forestry; Petrini, 1991; Miller, 2011). This work emphasizes the importance of understanding endophyte diversity and ecological function in relation to maintaining endophyte diversity during reforestation efforts and improving the fitness of tree plantations to common pests and pathogens.

The Acadian Forest

The Acadian forest region covers the majority of Maine and the three Maritime Provinces as well as parts of eastern Quebec and New England. This forest region is uniquely high in biological diversity as it is located where the warm and humid Southern Gulf Stream hits the cold Labrador Current (Loo & Ives, 2003). The forest region is comprised primarily of red, black and white spruce, balsam fir, yellow birch and sugar maple but also contains red pine, Eastern white pine, eastern hemlock and beech (Miller, 2011). Analyses of the forest structure over long time scales have identified small changes due to tree diseases and forest fires but attributes most of the alterations to early European settlers and the cyclical pest *C. fumiferana* (Figure 1.3; Loo & Ives, 2003). In the 1600s, early settler's extensively harvested pine to build establishments to the point where pine stands today are estimated to span half the area they previously covered (Lorimer, 2008).

Spruce budworm outbreaks have been document since as early as 8240 before present (BP) through macrofossil analysis of spruce tree cores obtained from an 8,600 year old bog in Eastern Quebec (Miller, 2011). Since 1760, there has been six documented outbreaks of spruce budworm in New Brunswick (Forbes et al., 1998). Since the 1900s, these outbreaks have increased in intensity and frequency (Loo & Ives, 2003). For context, it is believed that prior to the 1900s spruce budworm outbreaks occurred every 42 to 72 years, yet the documented outbreaks since the 1900s have occurred every 19 to 34 years (Blais, 1983). At the peak of the last outbreak, 50 million hectares of coniferous forests were defoliated (Natural Resources Canada, 2016). To mitigate this widespread damage, chemical pesticides were sprayed over 4 million hectares, totaling a cost of *ca.* 200 million dollars (Ireland, 1980; Sumarah & Miller, 2009). Since then, these chemical

pesticides have been deregistered due to environmental and safety concerns (MacLean et al., 2002).

Figure 1.3: Spruce budworm (*C. fumiferana*) larvae defoliating conifer needles.

Through this extensive damage, spruce budworm has changed the populations of coniferous tree species of the Acadian forest. A study by Hennigar et al. (2008) analyzed the differences in spruce budworm defoliation in balsam fir and red, white and black spruce. Between 1984 and 1992, they measured defoliation in 136 tree stands that possessed a range of the following variables: species composition, stand maturity, foliage protection and net defoliation change from the previous year's measurement. Doing so ensured their results accounted for external factors. For instance, insecticides used for foliage protection work more efficiently on balsam fir than on spruce trees (Lysyk, 1990). This is because spruce foliage is denser, which prevents the insecticide droplets from penetrating into the inner foliage layers (Lysyk, 1990). As such, protected stands will overstate the amount of spruce defoliation in comparison to balsam fir defoliation. At the end of this long term study Hennigar et al. (2008) found that balsam fir was most susceptible to budworm defoliation and that white, red and black spruce had *ca.* 70, 40 and 30% as much defoliation,

respectively, in comparison. They attributed these differences to when these tree types begin to grow new leaves (budburst) and how this coincides with budworm larvae development (Hennigar et al., 2008). In red and black spruce, budburst occurs 13 days later compared to balsam fir. This is too late for spruce budworm larvae that are in early instar stages and looking for foliage to consume. This results in higher larval mortality and reduced defoliation of red and black spruce. In white spruce, budburst occurs only 4 days after balsam fir budburst, which is not long enough to cause extensive larvae mortality. Instead the difference in defoliation were attributed to white spruce ability to grow larger shoots at a faster rate and having more overall foliage per unit area than balsam fir (Hennigar et al., 2008). This translates to there being less defoliation on white spruce as they are combating the damage by growing faster than balsam fir. These findings are crucial to mitigating future spruce budworm deforestation events as they highlight that the damage will be more severe in stands with higher percentages of balsam fir and white spruce. This will permit more directed insecticidal foliage protection approaches.

In an effort to combat the extensive budworm deforestation, over 900 million conifers have been planted over the past 60 years (Miller, 2011). However, analyses have shown no major change in the percentage of hardwood in the Acadian forest during this period (Etheridge et al., 2005, 2006; Amos-Binks et al., 2010). The cyclical damage caused by spruce budworm indicates there is a pressing need to improve the fitness of our coniferous forests as the affected area is too expansive to utilize pesticides and pruning techniques. As exemplified by the antifeedant properties of the rugulosin producing endophyte (*P. scopiformis*) of white spruce, endophytes may be a viable option in mitigating forest damage. However, inoculating trees intended for reforestation with only one toxigenic endophyte is insufficient as it limits forest endophyte diversity, and spruce budworm

could develop tolerance. This project is an example of a continuing effort to discover more endophytes producing biologically active secondary metabolites that serve an important ecological function.

Metabolism

Primary metabolism is essential for the survival and reproduction of all organisms involving the breakdown of nutrients from the environment into energy. Organisms use primary metabolic machinery and pathways (glycolysis, Krebs cycle, fatty and nucleic acid metabolism) to convert the stored potential energy present in complex macromolecules into useable chemical potential energy in the form of adenosine triphosphate (ATP). Organisms subsequently utilize ATP to fuel unfavorable cellular processes that are necessary for growth and proliferation. The intermediary and end products generated during these catabolic processes are known as primary metabolites. Although there is an extensive chemical diversity of secondary metabolites, most originate from the primary metabolites acetyl-CoA, mevalonate and shikimic acid (Figure 1.4; Manitto, 1981). Acetyl-CoA is the starting material for oxidative respiration via the Krebs cycle. Due to its importance it can be generated by multiple resource pathways. For instance, simple sugars are catabolized into pyruvate by glycolysis, which can be oxidatively decarboxylated into acetyl-CoA. Acetyl-CoA can also be generated by the beta-hydroxylation of fatty acids or through the deamination and oxidation of amino acids.

In secondary metabolism, acetyl-CoA is utilized by the acetate pathway to generate a diverse family of secondary metabolites known as polyketides (Manitto, 1981). Alternatively, three acetyl-CoAs can be condensed together and subsequently reduced to generate mevalonate. Mevalonate

is used by the mevalonate pathway to produce a diverse family of secondary metabolites known as terpenes (Manitto, 1981). The last major primary metabolite used in secondary metabolism is shikimic acid. It is formed from combining the phosphoenolpyruvate intermediate of glycolysis with the erythrose-4-phosphate intermediate of the pentose phosphate cycle (Haslam, 1974). Shikimic acid is then used in the shikimate pathway to generate the aromatic amino acids (tyrosine, tryptophan and phenylalanine). Subsequent modifications then convert these aromatic amino acids into various families of secondary metabolites such as alkaloids, lignans and cinnamic acid derivatives (Haslam, 1974; Dewick, 1997).

Figure 1.4: The primary metabolite origins of the secondary metabolite classes (adapted from Dewick, 1997).

Secondary metabolism is not essential for the growth or survival of the Ascomycete fungi previously discussed here since it yields no usable energy. Producing secondary metabolites is energetically demanding. When essential nutrients are abundant, organisms produce minimal amounts of secondary metabolites (Wicklow, 1981). Instead, all available resources are used for growth and proliferation in order to colonize nutrient sources. This is considered a type of competition as colonizing the available space prevents other organism from getting a foothold. A classic example of this is shown in barnacles in the intertidal regions of Scotland. With *Chthamalus* barnacles growing up on in the rocky areas and *Balanus* barnacles down by the shore. Connell (1961) removed the *Balanus* barnacles and observed *Chthamalus* barnacles were able to grow down by the shore. Yet, when Connell (1961) reintroduced the *Balanus* barnacles, the *Chthamalus* barnacles were overcrowded and eventually receded back up to the rocks where *Balanus* barnacles could not grow due to desiccation.

Similar to excluding an organism by occupying the available space, secondary metabolites are believed to ward off competition as many have been found to be biologically potent (e.g. antibacterial, antifungal, antiinsectan; Pelaez, 2005). Organisms capable of producing secondary metabolites increase their production when essential nutrients such as carbon, nitrogen and phosphorous become scarce in the environment (Wicklow, 1981). It is believed that by forgoing some energy to create secondary metabolites, organisms are able to monopolize the remaining nutrient source by warding off competitors.

Although secondary metabolites arise from few primary metabolites, there is enormous chemical diversity due to the range of modifications that are performed. As these modifications are catalyzed

by numerous enzymes, organisms will produce unique profiles of secondary metabolites as they possess different sets of biosynthetic genes, often organized into clusters (Hoffmeister & Keller, 2007). This is why genetically diverse tree-endophytes are a potential untapped trove of novel, biologically active secondary metabolites (Miller, 1986).

Polyketides are a major class of secondary metabolites that originate from the acetate pathway mentioned above. This pathway has a high degree of similarity to fatty acid biosynthesis as both operate by sequential additions of C_2 units (Manitto, 1981). However, rather than adding CH_2 subunits, the acetate pathway sequentially adds acetyl subunits ($CO-CH_2$) to form a polyketide chain (Figure 1.5). In fungi, this addition is limited to one module as fungi elongate the polyketide chain using type I polyketide synthases (PKSs; Manitto, 1981). These PKSs elongate the polyketide chain by exchanging the chain between two thiol groups. Starting out, PKSs will bind acetyl-CoA to one of the thiol groups. Then, sequential malonyl-CoA groups are bound to the other thiol group as they are used to elongate this bound acetyl group. This is done by a Claisen condensation, which results in an acetyl group addition to the growing chain as well as the evolution of carbon dioxide (Keller et al., 2005). After a variable number of elongation steps the PKSs will release the polyketide chain by hydrolysis or cyclization (Hoffmeister & Keller, 2007). The resulting polyketide core is then modified (e.g. oxidations, reductions, methylations) to form an array of polyketide structures.

Figure 1.5: Acetate pathway illustrating polyketide biosynthesis (adapted from Deacon, 2005).

In conifers, two potent polyketide metabolites isolated from foliar endophytes are pyrenophorol and vermiculine (Figure 1.6). Both natural products are 16 membered homomeric macrolides. Pyrenophorol was produced with other macrolides and sesquiterpenoids from several *Lophodermium nitens* strains originating from Eastern white pine (*Pinus strobus*; Sumarah et al., 2011). Pyrenophorol inhibited the growth of the biotrophic pathogen *Microbotryum violaceum*, which serves as a proxy for *Cronartium ribicola*, the fungus that causes the disease white pine blister at concentrations as low as 5 μM (Sumarah et al., 2011). Vermiculine was obtained from a white spruce endophyte and showed toxicity to spruce budworm cells at 2 $\mu\text{g mL}^{-1}$ (Findlay et al., 2003).

Figure 1.6: Chemical structures of the macrolides pyrenophorol and vermiculine.

Terpenes are another major class of fungal secondary metabolites. They are assembled through sequential additions of C_5 subunits through the mevalonate pathway (Figure 1.7; Manitto, 1981). This pathway starts with two phosphorylations to convert mevalonate into 5-pyrophosphate. After a *trans* elimination reaction, the tertiary hydroxyl group and the carboxyl group are removed to generate isopentenyl pyrophosphate (IPP; Manitto, 1981). Isopentenyl pyrophosphate then isomerizes to form the reactive dimethylallyl pyrophosphate (DMAPP; Manitto, 1981). When an IPP condenses with DMAPP in a head-to-tail manner it isomerizes to conserve the reactive tail

(Manitto, 1981). This allows for continual C_5 additions, which is why terpenes are classified by the number of C_5 subunits added. For example, a C_5 structure is a hermiterene, C_{10} a monoterpene, C_{15} a sesquiterpene, C_{20} is a diterpene, etc. (Manitto, 1981).

Figure 1.7: Overview of acetyl-CoA being converted into terpene core by the mevalonate pathway (adapted from Keller et al., 2005).

These long terpene chains are then modified (e.g. cyclize, oxidize, reduce) by terpenoid synthases to increase their chemical diversity (Manitto, 1981). An example of sesquiterpenes isolated from endophytes are the heptelidic acid (HA), HA chlorohydrins and hydro-HA metabolites isolated from a needle endophyte of a balsam fir (Figure 1.8; Calhoun et al., 1992). When assayed against spruce budworm larvae, they found these terpenes to be toxic at concentrations as low as 5 to 15 μmol (Calhoun et al., 1992). Diterpenoids have also been isolated from balsam fir endophytes (Figure 1.8). These showed antifeedant properties to spruce budworm larvae at 6 μmol (Findlay et al., 1995a).

Figure 1.8: Chemical structures of heptelidic acid (HA), HA chlorohydrins and hydro-HA, and two diterpenoids toxic to spruce budworm larvae from balsam fir.

The shikimate pathway is the third major route to forming secondary metabolites. This pathway converts shikimic acid into the aromatic amino acids tryptophan, tyrosine and phenylalanine, and then further modifies them into aromatic secondary metabolites including alkaloids, lignans and cinnamic acid derivatives (Haslam, 1974; Dewick, 1997). Shikimic acid is biosynthesized by the condensation of erythrose-4-phosphate from the pentose phosphate pathway and

phosphoenolpyruvic acid (PEP) from glycolysis (Haslam, 1974). Initially, shikimic acid is coupled to a phosphate group and to another PEP to form 5-enolpyruvylshikimate-3-phosphate (Haslam, 1974). This intermediate is dephosphorylated to yield chorismate. At this point, the pathway branches to form the three aromatic amino acids. For example, the pathway towards tyrosine and phenylalanine begins with an isomerization of chorismate into prephenic acid (Haslam, 1974). Prephenic acid is oxidatively decarboxylated and then transaminated, using glutamate as a nitrogen source, to yield either tyrosine or phenylalanine, depending on whether the aromatic hydroxyl group is conserved (Haslam, 1974). Secondary metabolite enzymes perform a variety of modifications to these aromatic amino acids to form an array of secondary metabolites (Figure 1.9). For example, the grass endophytes that showed toxicity to mammals and insects produced potentially bioactive alkaloids and indole diterpenes such as ergonovine and peramine (Figure 1.10; Bush et al., 1997; Clay & Schardl, 2002).

Red and black spruce metabolites

As part of the ongoing effort to explore the endophyte ecology of the Acadian forest, 2000 red spruce endophytes were collected from nine areas across New Brunswick, Nova Scotia and Maine (Sumarah et al., 2010). Based on morphological characteristics and sequencing of a portion of the ITS, 150 strains were selected and incubated in liquid culture to study their secondary metabolites (Sumarah et al., 2010). The secondary metabolite extracts were tested for dietary toxicity to spruce budworm at 400 $\mu\text{g g}^{-1}$. Three toxic extracts were chosen for further study as they provided a good representation of geographic, taxonomic and natural product diversity (Sumarah et al., 2010). These further studies led to the characterization of 9 major metabolites (Figure 1.11), where none of these secondary metabolites were common to the three species (Sumarah et al., 2010).

Figure 1.9: Shikimic pathway showing biosynthesis of aromatic amino acids and subsequent secondary metabolite families (adapted from Herrmann, 1995).

Figure 1.10: Structures of ergonovine and peramine alkaloids produced by grass endophytes.

Although two of these endophytes could not be identified, they were deposited in the Centraalbureau voor Schimmelcultures (CBS) as CBS 121942 and CBS 121944. The last endophyte was identified as *Dwayaagam colodena* and was deposited in the Department of Agriculture, Ottawa, Mycology (DAOM) as DAOM 239833. Compound (A) was attributed for the toxicity of CBS 121942, compounds (D) and (E) for the toxicity of CBS 121944 and a variety of maleic anhydrides with structures similar to (G) for the toxicity of *Dwayaagam colodena* (Sumarah et al., 2010). Despite these metabolites possessing vastly different structures, all of them are unified in their toxicity to spruce budworm. This is important as toxin mixtures may delay the onset of spruce budworm tolerance. Additionally, inoculating nursery seedlings with a variety of representative endophytes rather than focusing on a single endophyte ensures forest endophyte ecology is maintained (Miller, 2011).

Figure 1.11: Structures secondary metabolites characterized from three red spruce endophyte strains of the Acadian forest.

There has also been a number of polyketide isocoumarins isolated from an endophyte of black spruce (Figure 1.12). Despite their structural similarities, only two of the compounds were found to be toxic to spruce budworm larvae (Findlay et al., 1995b). However, this is not likely a true representation of toxicity. A current theory is that fungi produce mixtures of toxins that combine to act synergistically (Calhoun et al., 1992). For instance, Dowd (1989) reported several non-toxic compounds from *Fusarium graminearum* that in turn had elevated toxicity and mortality to caterpillars when combined with the trichothecene deoxynivalenol. This phenomenon was also observed with *Penicillium* and *Aspergillus* species (Dowd, 1988, 1989). As such, it is possible that the array of isocoumarin metabolites from this endophyte increased in toxicity when acting together.

Figure 1.12: Chemical structures isocoumarins produced by a foliar endophyte of black spruce.

Project Aim

Research focused on conifer endophyte diversity of the Acadian forest over the past 25 years has resulted in many important discoveries. The first is that conifers grown in nurseries for reforestation purposes have low endophyte diversity as these non-clavicipitaceous endophytes spread through horizontal transmission. It has also been demonstrated that endophytes can be inoculated into conifer seedlings and that they can persist there for over 10 years despite natural needle shedding. After 10 years, the white spruce endophyte *P. scopiformis* continued to produce its dominant rugulosin toxin *in planta* at concentrations that effectively reduce spruce budworm growth and herbivory (Frasz et al. 2014). This thesis is part of a larger effort to explore the diversity of unknown toxigenic endophytes. The ideal end goal is that this will allow nursery trees intended for reforestation purposes to be inoculated with a variety of toxigenic endophytes.

Specifically, the work in this thesis entails screening endophytes for antifungal activity, choosing

toxigenic endophytes that have not been previously studied by the Miller group and characterizing the secondary metabolites that are responsible for this observed bioactivity. The aim of the project is to find novel secondary metabolites that are structurally distinct from the current set of metabolites known to be toxigenic to spruce budworm. If successful, future steps will involve *in vitro* testing of these novel metabolites against spruce budworm and potentially *in vivo* testing thereafter by inoculating the toxigenic endophyte into conifer seedlings.

2. Materials and Methods

Endophyte Collection

Branches of *Picea mariana* (black spruce) and *P. rubens* (red spruce) of various age classes were collected in New Brunswick, Canada and stored in plastic bags at 4 C. Endophytic fungi were isolated and subcultured from surface sterilized needles following the methods of Tanney et al. (2016a). Briefly, needles were surface sterilized by dipping each needle in 70% ethanol (EtOH) for one minute, rinsing with sterile ddH₂O, followed by a 7.5 minute immersion in a 10% bleach solution. After soaking in the bleach solution, needles were placed in 70% EtOH briefly, and rinsed with sterile ddH₂O again. Using sterilized forceps, each needle was carefully bisected cross-wise and plated on 2% malt extract agar (MEA). Needles were incubated at 25 C until sufficient growth was observed from the exposed needle tip. Isolates were subsequently subcultured, plated on fresh 2% MEA and incubated as described above. The collection of foliar endophytes studied here for natural products was maintained on 2% MEA and stored at 8 C, appendix II.

Fermentation and Extraction

Small scale fermentations were used to assess the natural product profiles and target fungi producing antimicrobial metabolites for each endophyte reported in appendix II. Under aseptic conditions, a portion of each endophytes 2% MEA plate was macerated in sterile ddH₂O. The resulting suspension was used to inoculate (5%, v/v) individual Glaxo bottles containing 1 L of autoclaved 2% malt extract (Bacto) broth. Cultures were incubated for 8 weeks without agitation at 25 C in darkness.

After the incubation period, the mycelia were separated from the culture filtrate by suction through Whatman #4 (Whatman GE Healthcare, UK) filter papers. The resulting volume and pH of the culture filtrates were recorded whereas the mycelia were lyophilized and stored at -20 C. Each culture filtrate was saturated with NaCl (20-30 g L⁻¹) and exhaustively extracted with ACS grade ethyl acetate (EtOAc). The aqueous culture filtrate was discarded and the organic layer was filtered through a Whatman #1 and anhydrous Na₂SO₄ prior to drying by rotary evaporation. The resulting crude extract was suspended in 3 mL of HPLC grade methanol (MeOH), passed through a 0.2µm PTFE syringe filter (Tisch Scientific, USA), dried under a gentle stream of nitrogen gas and weighed. Crude endophyte culture filtrate extracts were stored dry in amber vials at -20 C prior to natural product profiling and growth inhibition assays.

Based on LC-UV-MS chromatograms, bioassay result and phylogenetic analysis, from the 61 cultures grown in liquid culture, four were thoroughly investigated for natural products. Often this necessitates medium scale fermentations to isolate adequate amounts of metabolites for structural elucidations and bioassays. For medium scale incubations (15 L), a two-stage fermentation

approach was implemented. Fifteen 250 mL Erlenmeyer flasks containing 50 mL of 2% malt extract broth were inoculated as described above with the selected endophytes. Flasks were incubated on a rotary shaker (100 rpm) for 1 week in the dark at 25 C. Cultures were subsequently macerated, individually transferred to Glaxo bottles containing 1 L of the same liquid medium, and incubated without agitation as described above for 8 weeks. The resulting mycelium and culture filtrates were also treated as described above.

Figure 2.1: Flow chart illustrating the screening process used to select four endophyte species for secondary metabolites investigations.

Metabolite LC-UV-MS Analysis

Crude endophytes were analyzed by LC-UV-MS to access their natural product profiles. This was performed with a Waters 2795 separations module, Waters 996 diode array detector, and Waters MicroMass Quattro LC mass spectrometer (Waters Corporation, Milford, MA, USA). Metabolites were separated by a Kinetex C18 (100 x 4.6 mm, 2.6 mm) column (Phenomenex Inc., Torrance, CA, USA) with a mobile phase consisting of acetonitrile (ACN)-ddH₂O with formic acid (FA; 0.1 %, v/v). The linear solvent gradient was programmed from 5 to 100 % ACN-ddH₂O over 13 min with a flow of 1.0 mL min⁻¹. Column fractions generated from the normal phase chromatography of these extracts were also analyzed as described above. An aliquot of each crude extract (10 mg mL⁻¹) or column fraction (5 mg mL⁻¹) was dissolved in HPLC grade MeOH prior to LC-UV-MS analysis.

Metabolite Chromatography

Medium-scale EtOAc culture filtrate extracts generated to obtain adequate metabolite amounts from selected endophytes were initially separated by flash column chromatography utilizing short silica gel columns. A step gradient elution system employing hexanes-EtOAc (0-100 %; v/v) in 10% increments followed by 5, 10, 20, and 50% EtOAc-MeOH (v/v) was typically utilized. Eluting metabolites were monitored by thin-layer chromatography (TLC) (0.2 mm silica gel 60 F₂₅₄ pre-coated) using 10 % (v/v) MeOH in chloroform (CHCl₃) as a mobile phase. TLC plates were visualized under both long (365 nm) and short wave (254 nm) light. Similar eluates were combined, dried by rotary evaporation, re-suspended in 5 mL of MeOH, filtered through a 0.2µm PTFE syringe filter and then dried again under a gentle stream of nitrogen gas. Column fractions were subsequently analyzed by LC-UV-MS as described above. Semi-preparative reverse phase

HPLC of fractions with interesting metabolite profiles or appreciable mass (>100 mg) were performed with an Agilent 1100 high performance liquid chromatography (HPLC) system (Agilent Technologies, Palo Alto, CA, U.S.A.) equipped with a diode array detector and Kinetex C18 column (250 x 10mm, 5 μ m; Phenomenex) using a mobile phase consisting of ACN and ddH₂O with 0.05% trifluoroacetic acid (TFA). HPLC elution gradients were metabolite specific; however, all had a flow rate of 4 mL min⁻¹. Column fractions were dissolved in HPLC grade MeOH to a concentration of 50 mg mL⁻¹ and chromatographed in 50 μ L aliquots.

***Phaeocryptopus cf. gaeumannii* (NB250-4H, DAOM 250863)**

Figure 2.2: *Phaeocryptopus cf. gaeumannii* (DAOM 250863) growing on a 2% MEA.

The crude extract mass (582.3 mg) of DAOM 250863 obtained from the 1 L screening fermentation was unusually high compared to most other foliar endophytes studied. LC-UV-MS analysis of the crude extracts revealed a relatively simple metabolite profile with six major peaks. As such, metabolite purifications for this endophyte were performed on the 1L extract rather than conducting a medium scale fermentation that is typically required. The normal phase column chromatography step was also not conducted due to the simplicity of the extract. Instead, the

extract was directly separated by semi-preparative HPLC. A linear gradient was programmed from 20 to 60% ACN-ddH₂O over 16 minutes to yield compound **1** (3.6 mg), **2** (2.9 mg), **3** (6.3 mg), **4** (3.0 mg) and **5** (14.5mg).

Figure 2.3: HPLC separation outlining major metabolites produced by DAOM 250863 (254nm).

Undescribed Rhytismatales species (NB236-1A)

Figure 2.4: Undescribed Rhytismatales species growing on a 2% MEA.

The crude EtOAc culture filtrate extract (3.0 g) of NB236-1A was fractionated by a short silica gel column that yield nine fractions. LC-UV-MS screening identified three chemically distinct fractions that were selected for subsequent metabolite purifications by semi-preparative HPLC. Fraction 1 (12.3 mg) eluted with 10% EtOAc in hexanes (v/v) and was further separated by HPLC using a linear gradient programmed from 55 to 85% ACN-ddH₂O over 23 minutes to afford compounds **10** (2.3 mg) and **11** (3.0 mg). Column fraction 4 (117.0 mg) eluted with 40% EtOAc in hexanes (v/v) and was separated using a gradient that increased linearly from 20 to 70% ACN-ddH₂O over 16 minutes that yielded compound **12** (5.4 mg) and **8** (4.0 mg). Compound **9** (4.7 mg) was purified from fraction 5 (373.2 mg) with an HPLC method that increased linearly from 20 to 90% ACN-ddH₂O over 21 minutes. When re-examining the LC-UV-MS chromatograms of the crude extract, two dominant peaks had yet to be isolated from the fractions investigated. Examination of each fractions UV and MS chromatograms indicated these metabolites were present within the polar 1:1 MeOH:EtOAc (v/v) fraction despite eluting in 50% ACN when performing metabolite screening by LC-UV-MS. Since polar fractions are typically difficult to separate by conventional reverse phase HPLC, 300 mg of fraction 9 (621.5 mg) was separated by PTLC (Partisil K6, 60A silica gel, 20x20cm, 250µm thickness, Whatman) with 10 % (v/v) MeOH in CHCl₃. Six bands were scrapped off the silica plate, filtered through a 0.2µm PTFE syringe filter and screened by LC-UV-MS. The two dominant metabolites present within the crude extract were detected in two of the six silica scrapings. These PTLC fractions were purified separately using an HPLC method that linearly increased from 40 to 80% ACN-ddH₂O over 17 minutes that afforded compounds **6** (9.9 mg) and **7** (9.3 mg).

Diaporthe maritima (NB365-71I, DAOM 628553)

Figure 2.5: *Diaporthe maritima* (DAOM 628553) growing on a red spruce twig (A), magnified to show pycnidia protruding through the plant tissue (B) and the conidiophores producing conidia (C).

Fractionation of the DAOM 628553 EtOAc culture filtrate extract (1.6 g) normal phase column chromatography yielded nine fractions. After screening each by LC-UV-MS, three fractions were selected for metabolite purifications by semi-preparative HPLC. Fraction 3 (131.3 mg) eluted with 20% EtOAc in hexanes (v/v) and was further separated using a linear gradient programmed from 15 to 100% ACN-ddH₂O over 15 min that yielded compound **13** (25.0 mg). Fraction 4 (504.0 mg) eluted with 30% EtOAc in hexanes (v/v) and was separated by a similar HPLC method programmed from 30 to 60% ACN-ddH₂O over 16 min, yielding compound **15** (4.6mg). Fraction 5 (227.9 mg) eluted with 40% EtOAc in hexanes (v/v) and was chromatographed with a linear method programmed from 25 to 100% ACN-ddH₂O over 18 min yielding compound **14** (15.3 mg).

Pezicula sporulosa (NB382-3D, DAOM 250862)

Figure 2.6: *Pezicula sporulosa* (DAOM 250862) growing on a 2% MEA.

The crude culture filtrate extract (1.65 g) of DAOM 250862 was separated into 11 fractions by normal phase flash column chromatography. LC-UV-MS analysis indicated major metabolites were predominantly in three fractions. Fraction 2 (117.3 mg) eluted with 20% EtOAc in hexanes (v/v) and semi-preparative HPLC employing a linear gradient increasing from 35 to 60% ACN-ddH₂O over 18 minutes afforded compound **16** (37.4 mg). Fraction 4 (116.1 mg) eluted with 40% EtOAc in hexanes (v/v) and a linear gradient programmed from 20 to 55% ACN-ddH₂O over 21 minutes yielded compound **17** (13.8 mg). Fraction 5 eluted with 50% EtOAc in hexanes (v/v) and was subsequently separated by a linear HPLC gradient increasing from 10 to 70% ACN-ddH₂O over 21 minutes. This fraction yielded 6.2mg of compound **18**. Compound **19** was detected in the crude culture filtrate of DAOM 250862 by data independent acquisition. Analysing the crude culture filtrate extract by HRMS allowed for comparison to a database of previously isolated secondary metabolites. By observing the correct mass at the appropriate retention time confirmed the presence of compound **19**.

Growth Inhibition Assays

The collection of foliar endophytes obtained from the Acadian forest was assessed for the production of antifungal metabolites using the Oxford disk diffusing assay (Vincent & Vincent, 1944). Crude extracts from the 1 L screening fermentations were dissolved in HPLC grade MeOH to prepare concentrations of 50 mg mL⁻¹. Ten µL aliquots were pipetted onto 10 mm sterile Whatman disks. Nystatin at a concentration of 1 mg mL⁻¹ and HPLC grade MeOH were the respective positive and negative controls. The MeOH was allowed to evaporate and disks were subsequently placed on 2% MEA plates that were previously smeared with 100 µL of a 0.10 optical density (OD; 600 nm) solution of either *Saccharomyces cerevisiae* or *Microbotryum violaceum*. Plates were stored in an incubator at 25 C in the dark and monitored every 24 hours for zones of inhibition. Each endophyte extract was tested in triplicate against both the representative yeast, *S. cerevisiae*, and the biotrophic pathogen *M. violaceum*, which serves as a proxy for *C. ribicola* (white pine blister rust).

Purified metabolites were tested for *in vitro* antimicrobial activity against *M. violaceum*, *S. cerevisiae*, *Bacillus subtilis* (ATCC 23857) and *Escherichia coli* (ATC 67878). *M. violaceum* was grown in 20 g L⁻¹ malt extract (Bacto), 2.5 g L⁻¹ peptone (Bacto), and 2.5 g L⁻¹ yeast extract (Sigma, St. Louis, MO, USA), whereas *S. cerevisiae* was inoculated and grown in 1 g L⁻¹ yeast extract supplemented with 10 g L⁻¹ glucose. Bacteria were inoculated and grown in 5 g L⁻¹ yeast extract, 10 g L⁻¹ peptone, and 10 g L⁻¹ NaCl. Nystatin was the positive control for antifungal assays and chloramphenicol was the antibiotic positive control. DMSO was the negative control for all assays. Metabolites were tested at 250, 25, and 2.5 µM in sterile 96-well microplates (Falcon 353072 Microtest-9, Franklin Lakes, NJ, U.S.A.). A 10 µL aliquot of each individual metabolite solution

dissolved in DMSO was added to 190 μ L microbial suspension. Assays were performed in triplicate and incubated at 28 C with a rotary table shaker providing gentle agitation (500 rpm). OD readings were taken every 2 hours for *S. cerevisiae*, *B. subtilis* and *E. coli* assays and every 12 hours for *M. violaceum* assays. OD measurements were made at 600 nm with a Molecular Devices Spectra Max 340PC reader (Sunnyvale, CA, U.S.A.). Antimicrobial OD data were analyzed by ANOVA followed by Tukey's test ($p < 0.05$) for significant differences (Systat V13.1; Systat Software Inc., Chicago, IL, U.S.A.) compared to the negative control (DMSO).

General experimental protocols

Metabolite NMR spectra were obtained on a Bruker Avance 400 Spectrometer (Bruker Instruments Inc., Milton, ON, Canada) at 400.13 (^1H) and 100 MHz (^{13}C) using a 5 mm auto-tuning broadband probe with a Z-gradient. Isolated natural products were dissolved in 600 μ L of CD_3OD (δH 3.30 and δC 49.0) (CDN Isotopes, Point Claire, QC, Canada) and referenced to the solvent peak. Homonuclear (^1H , ^{13}C , COSY, DEPT-135, and NOESY) and heteronuclear (HSQC and HMBC) NMR experiments were used to elucidate natural product structures. LC-HRMS data was acquired with a Thermo Q-Exactive Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA, U.S.A), coupled to an Agilent 1290 HPLC system. Crude extracts and pure metabolites were separated by an Agilent Zorbax Eclipse Plus RRHD C18 (2.1 $\times$ 50 mm, 1.8 μm) at a flow rate of 0.3 mL min^{-1} using mobile phases consisting of ddH_2O with 0.1% FA and ACN with 0.1% FA. The chromatographic method held 0% ACN for 30 seconds before increasing to 100% over 3 minutes. After holding at 100% ACN for 1 minute, the gradient returned to 0% ACN over 30 seconds. Two μL of each crude extract normalized to 1 mg mL^{-1} were analyzed by LC-HRMS. The LC-HRMS system also collected HRMS² data for every ionisable metabolite. Silica

gel (Silicycle; 40–60 μm) was utilized for all normal phase metabolite separations. UV spectra of all isolated metabolites were obtained using a Varian Cary 3 UV-visible spectrophotometer scanning from 190–800 nm. Optical rotations were determined with an Autopol IV polarimeter (Rudolph Analytical, Hackettstown, NJ, U.S.A.).

3. Results

From the foliar conifer endophyte collection obtained from Acadian forest of Eastern Canada screened (Appendix II), four were selected for medium scale fermentations based on bioassay result, crude extract mass, LC-UV-MS chromatograms and novelty of the endophyte strain to the Miller group. Two endophytes were obtained from black spruce, *Phaeocryptopus* cf. *gaeumannii* DAOM 250863 and an undescribed Rhytismatales sp., and two from red spruce, *Diaporthe maritima* DAOM 628553 and *Pezicula sporulosa* DAOM 250862.

Metabolites of *Phaeocryptopus* cf. *gaeumannii* (NB250-4H, DAOM 250863)

Examination of the NMR (Table 3.1) and physiochemical (Table 3.2) data for compounds **1–5** isolated from DAOM 250863, indicated they were structurally similar isocoumarins. Compound **1** was isolated as a clear solid with the molecular formula $\text{C}_{10}\text{H}_{14}\text{O}_5$ determined by HRMS (m/z 215.0912 $[\text{M}+\text{H}]^+$). This formula indicates four units of unsaturation. The ^1H NMR spectrum displayed three oxygenated methines at δ 4.28 (m, H-6), 4.20 (dq, $J=9.3, 6.3$, H-3) and 3.11 (dd, $J=10.5, 9.3$, H-4), a methine at δ 2.71 (m, H-4a), two inequivalent methylenes at δ 2.29 (m, H-5), 1.25 (m, H-5), 2.64 (m, H-7) and 2.33 (dd, $J=19.0, 1.3$, H-7) and a methyl at δ 1.40 (d, $J=6.3$, H-9). Examination of the ^{13}C and DEPT-135 NMR spectra indicated three of the ten carbon signals

were quaternary. These signals were attributed to two sp^2 carbons at δ 175.0 (C-8) and 95.7 (C-8a) and a lactone at δ 172.3 (C-1). COSY cross peaks were observed from H-3 to H-9 and sequentially from H-3 to H-7. HMBC correlations from H-4a to C-8, C-8a and C-1 also support the presence of an isocoumarin core structure.

Findlay et al. (1995b) has previously reported a family of isocoumarins isolated from a black spruce endophyte. By comparison of their NMR data and optical rotations to previous reports they confirmed that two of their isocoumarins were (3*R*,4*aS*,6*R*)-6-hydroxyramulosin and (3*R*,4*S*)-4-hydroxymellein (Findlay et al., 1995b). The absolute stereochemistry of the remaining, structurally-similar isocoumarins was reported on the biological synthesis assumption that the *R* stereochemistry at C-3 is common to all (Findlay et al., 1995b). This assumption is continued with the isocoumarins reported here. For compound **1**, NOE correlations were observed from H-3 to H-4a and from H-4 to H-9. A lack of NOE correlation between H-4a and H-6 indicates the position 6 is of the *R* configuration, which is the same as an isocoumarin reported by Findlay et al. (1995b). Compound **1** is reported here as a new isocoumarin, (3*R*,4*S*,4*aR*,6*R*)-4,6,8-trihydroxy-3-methyl-3,4,4a,5,6,7-hexahydro-1H-isochromen-1-one, figure 3.1.

Figure 3.1: Structures of isocoumarins (**1-5**) isolated from *Phaeocryptopus* cf. *gaeumannii* (DAOM 250863).

Figure 3.2: Key HMBC and COSY correlations observed for compound **1**.

Compound **2** was isolated as a clear solid with the molecular formula $C_{10}H_{14}O_4$ determined by a HRMS $[M+H]^+$ peak at m/z 199.0963, indicating four units of unsaturation. The 1H and ^{13}C data for compounds **1** and **2** were very similar (Table 3.1). The 1H spectra of compound **2** revealed an additional methylene at δ 1.32 (m, H-4) and only two oxygenated methines at δ 4.56 (m, H-3) and 4.25 (m, H-6). COSY correlations were observed sequentially from H-3 to H-7, supporting an isocoumarin structure similar to compound **1** with a methylene at position 4 opposed to bearing a hydroxyl moiety. The experimentally obtained optical rotation ($[\alpha]^{25}_D$ 31.3 (c 0.06, $CHCl_3$)) closely matched the previously isolated natural product ($[\alpha]^{25}_D$ 40.8 (c 0.0009, $CHCl_3$) by Findlay et al. (1995b). As such, compound **2** is reported here as (3*R*,4*aS*,6*R*)-6,8-dihydroxy-3-methyl-3,4,4*a*,5,6,7-hexahydro-1*H*-isochromen-1-one.

Compound **3** was isolated as a white solid with the molecular formula $C_{10}H_{10}O_4$ determined by HRMS (m/z 195.0653 $[M+H]^+$), indicative of six units of unsaturation. The UV spectra showed absorption maxima at 220, 245 and 315 nm, suggesting the presence of an aromatic ring within the structure. The 1H NMR displayed three aromatic signals at δ 7.08 (dd, $J=7.5, 0.8$, H-5), 7.57 (dd, $J=8.4, 7.6$, H-6) and 6.94 (dd, $J=8.4, 0.8$, H-7), two oxygenated methines at δ 4.56 (o, H-3) and 4.55 (o, H-4) and a methyl group at δ 1.47 (d, $J=6.0$, H-9). Examination of the ^{13}C and DEPT-135 spectra showed four of the ten carbons were quaternary. These signals were ascribed to three sp^2 carbons at δ 144.1 (C-4a), 162.9 (C-8) and 108.0 (C-8a) and a lactone at δ 170.2 (C-1). COSY cross peaks were observed from H-5 through H-7 and from H-3 to H-4 and H-9 suggesting a similar isocoumarin structure as the previously elucidated metabolites **1-2**. The experimentally obtained OR $[\alpha]^{25}_D$ -13.8 (c 0.13, $CHCl_3$) was in agreement with the natural product (3*R*,4*S*)-4,8-dihydroxy-3-methylisochroman-1-one, previously reported with an OR of $[\alpha]^{25}_D$ -11 (c 0.0038, $CHCl_3$) by Findlay et al. (1995b).

Compound **4** was isolated as a clear solid with the molecular formula $C_{10}H_{12}O_4$ determined by an $[M+H]^+$ peak at m/z 197.0809, indicative of five units of unsaturation. The NMR signals were highly similar to the compounds described above. In comparison to **3**, compound **4** possessed a methine at δ 2.68 (o, H-4a), an inequivalent methylene group at δ 2.68 (o, H-5) and δ 1.98 (m, H-5) and two olefinic protons at δ 6.61 (m, H-6) and 6.07 (dd, $J=9.9, 3.3$, H-7). The reduction of the olefin group at C-4a to C-5 is supported a COSY correlation between H-4a and H-5 as observed for compounds **1** and **2**. Comparison of the experimentally obtained 1H and ^{13}C NMR was in accordance an isocoumarin reported by Findlay et al. (1995b). The experimental optical rotation ($[\alpha]^{25}_D$ 94.4 (c 0.03, $CHCl_3$)) had the same sign and similar magnitude to this isocoumarin ($[\alpha]^{25}_D$

164.0 (c 0.014, CHCl₃; Findlay et al., 1995b)). As such, compound **4** is reported here as (3*R*,4*S*,4*aR*)-4,8-dihydroxy-3-methyl-3,4,4*a*,5-tetrahydro-1*H*-isochromen-1-one, figure 3.1.

Compound **5** was also isolated as a white solid with the molecular formula C₁₀H₁₄O₄ determined by HRMS *m/z* 199.0963 [M+H]⁺, indicative of four units of unsaturation. The ¹H and ¹³C data for compound **5** were very similar to other biosynthetically related isocoumarins isolated from DAOM 250863 (Table 3.1). In comparison to **4**, compound **5** contrasted by the presence of an additional inequivalent methylene group at δ 1.99 (m, H-6) and 1.63 (m, H-6), a methylene group at δ 2.37 (o, H-7) and a lack of olefinic signals. Reduction of the C-6 to C-7 double bond in compound **4** is supported by the chemical shifts and COSY cross peaks observed sequentially from H-4*a* to H-7. Comparison of the experimentally obtained ¹H and ¹³C NMR was in accordance an isocoumarin reported by Findlay et al. (1995b). The experimental optical rotation of [α]²⁵_D 10.9 (c 0.5, CHCl₃) was in accordance that reported ([α]²⁵_D 16.7 (c 0.001, CHCl₃) by Findlay et al. (1995b). Compound **5** is reported here as (3*R*,4*S*,4*aR*)-4,8-dihydroxy-3-methyl-3,4,4*a*,5,6,7-hexahydro-1*H*-isochromen-1-one.

Table 3.1: ^1H and ^{13}C NMR data of isocoumarins **1-5** in CD_3OD .

	1		2		3		4		5	
Position	δ_{C} , type	δ_{H} (<i>J</i> , Hz)	δ_{C} , type	δ_{H} (<i>J</i> , Hz)	δ_{C} , type	δ_{H} (<i>J</i> , Hz)	δ_{C} , type	δ_{H} (<i>J</i> , Hz)	δ_{C} , type	δ_{H} (<i>J</i> , Hz)
1	172.3		173.3		170.2		172.7		172.5	
3	81, CH	4.2, dq (9.3, 6.3)	78.6, CH	4.56, m	81, CH	4.56, o	80.1, CH	4.15, dq (9.4, 6.3)	80.5, CH	4.16, dq (9.3, 6.2)
4	73.8, CH	3.11, dd (10.5, 9.3)	36.7, CH_2	1.32, m	69.5, CH	4.55, o	74.9, CH	3.32, o	73.9, CH	3.04, t (9.3)
4a	34.9, CH	2.71, m	27.7, CH	2.88, m	144.1		38.8, CH	2.68, o	40.9, CH	2.35, o
5	33.3, CH_2	2.29, m 1.25, m	37.9, CH_2	1.96, m	117.4, CH	7.08, dd (7.5, 0.8)	28.7, CH_2	2.68, o 1.98, m	27.1, CH_2	2.24, m 1.10, m
6	64.1, CH	4.28, m	64.3, CH	4.25, m	137.6, CH	7.57, dd (8.4, 7.6)	142.4, CH	6.61, m	21.4, CH_2	1.99, m 1.63, m
7	38, CH_2	2.64, m 2.33, dd (19.0, 1.3)	38, CH_2	2.65, dq (19.4, 2.6) 2.33, dq (19.4, 1.4)	117.5, CH	6.94, dd (8.4, 0.8)	124.8, CH	6.07, dd (9.9, 3.3)	29.7, CH_2	2.37, o
8	175.0		173.8		162.9		170.2		177.4	
8a	95.7		97.6		108		92.2		96.1	
9	18.9, CH_3	1.40, d (6.3)	22, CH_3	1.37, d (6.3)	17.8, CH_3	1.47, d (6.0)	18.3, CH_3	1.42, d (6.3)	18.7, CH_3	1.4, d (6.4)

Table 3.2: Physicochemical data for isocoumarin compounds **1-5**.

Properties	1	2	3	4	5
Appearance	clear solid	clear solid	white solid	clear solid	white solid
Empirical formula	C ₁₀ H ₁₄ O ₅	C ₁₀ H ₁₄ O ₄	C ₁₀ H ₁₀ O ₄	C ₁₀ H ₁₂ O ₄	C ₁₀ H ₁₄ O ₄
HRMS [M+H] ⁺	215.0912	199.0963	195.0653	197.0809	199.0963
Calculated [M+H] ⁺	215.0914	199.0965	195.0652	197.0808	199.0965
OR [α] _D ²⁵ (CHCl ₃)	17.8 (c 0.18)*	31.3 (c 0.06)	-13.8 (c 0.13)	94.4 (c 0.03)	10.9 (c 0.5)
UV (MeOH) λ_{max} nm (log ϵ)	200 (3.12), 265 (3.50)	200 (3.10), 265 (3.60)	220 (3.32), 245 (3.23), 315 (3.06)	205 (3.41), 320 (3.36)	200 (2.21), 265 (3.89)

*-Optical rotation observed in methanol as insoluble in chloroform.

Metabolites of an Undescribed Rhytismatales species (NB236-1A)

The major metabolite from NB-236-1A was isolated as light yellow solid with the molecular formula C₁₄H₂₀O₆ determined by HRMS (m/z 285.1332 [M+H]⁺). The ¹H and ¹³C spectra revealed eighteen proton and fourteen carbon signals (Table 3.3), respectively. The UV spectrum displayed absorption maxima at 205, 230 and 270 nm. Examination of the mass spectrometry and spectroscopic data (Table 3.4) suggested compound **6** possessed a trisubstituted dihydropyrone-2-one core structure with five units of unsaturation attributed to one ring and four double bonds. The

^1H NMR spectrum displayed a single terminal methyl at δ 0.89 (t, J = 7.0), five aliphatic methylene signals between δ 1.28 and 1.64, an inequivalent methylene at δ 2.76 (dd, J = 17.2, 6.6) and 2.96 (dd, J = 17.2, 4.0), a methylene adjacent to a carbonyl at δ 2.85 (dt, J =7.1, 1.4) and an oxygenated methine at δ 4.90. Examination of the ^{13}C and DEPT spectra revealed five of the fourteen carbon signals were quaternary. These signals are attributed to a ketone at δ 195.3, a lactone at δ 174.1, a carboxylic acid at δ 172.6, and two sp^2 carbons at δ 99.6 and 197.5. COSY correlations were observed sequentially from the triplet methyl H-8' (δ 0.89) to H-2' (2.85) where five of the methylene signals (δ 0.89-1.65) appeared in the aliphatic region of the ^1H NMR spectrum. The chemical shift of H-2' and an HMBC correlation from H-2' to C1' (δ 195.3) place H-2' vicinal to a carbonyl supporting the presence of a 1-octanal moiety in compound **6**. Moreover, the dominant MS^2 fragment ion of compound **6** at m/z 127.1 $[\text{M}+\text{H}]^+$ suggested a C_8 side-chain bearing a single oxygen atom. The remaining COSY cross-peak was observed between H-5 (δ 2.76 and 2.96) and H-6 (δ 4.90). HMBC correlations were observed from both H-5 and H-6 to C-4 (δ 197.5) and C-7 (δ 172.6), where H-6 showed an additional correlation to C-2 (δ 174.1). These data established the presence of a trisubstituted 5,6-dihydropyrone-2-one core structure with a carboxylic acid functionality bound to C-6. The chemical shifts of C-3 and C-4, and the fact compound **6** undergoes a slow keto/enol tautomerization when left in CD_3OD indicated the 1-octanal moiety is attached at C-3. This type of chemical equilibrium was observed for podoblastin A, a metabolite with a similar 3-acyl-4-hydroxy-5,6-dihydropyrones core structure (Miyakado et al., 1982).

The absolute configuration of compound **6** at C-6 was determined to be *R* based on comparison of its experimentally determined optical rotation, $[\alpha]^{25}_{\text{D}} - 30$ (c 0.04, CHCl_3), to structurally similar compounds with a single chiral center. The absolute stereochemistry of podoblastin A at C-6 was

determined to be *R* by stereoselective synthesis which had an optical rotation of $[\alpha]_D -27.08$ (c 1.12, CHCl_3 ; Ichimoto et al., 1988; Miyakado et al., 1982). Dictyopyrone A shares the same core structure and is of the *S* configuration at C-6. The optical rotation of this metabolite as a natural product is reported as $[\alpha]^{25}_D 33.8$ (c 0.07, CHCl_3) and $[\alpha]^{25}_D 33.9$ (c 1.23, CHCl_3) when prepared synthetically using chiral reagents (Takaya et al., 2000). Since all of the structurally similar dihydropyrones have one chiral center at the same position, compound **6** is reported here as a new structure, (*R*)-4-hydroxy-5-octanoyl-6-oxo-3,6-dihydro-2H-pyran-2-carboxylic acid or rhytismatone A.

Figure 3.3: Structures of rhytismatones A (**6**) and B (**7**) isolated from the unidentified Rhytismatales sp.

Figure 3.4: Key HMBC and COSY correlations observed for compound **7**.

Compound **7** was isolated as a light yellow solid with the molecular formula $C_{15}H_{22}O_6$ determined by HRSM (m/z 299.1489 $[M+H]^+$). The increase in mass for compound **7** compared to **6** corresponds to the addition of a methyl group. Examination of the MS^2 spectra compound **7** revealed the same dominant m/z 127.1 $[M+H]^+$ fragment ion suggesting it also possessed a 1-octanal moiety. The 1H and ^{13}C NMR spectra (Table 3.3) of compounds **6** and **7** were very similar. However, the 1H and ^{13}C spectra of **7** displayed additional signals at δ 3.67 (s) and δ 52.5, respectively, indicative of a methyl ester functionality. An HMBC correlation from the singlet methyl H-8 (δ 3.67) to C-7 (δ 171.4) supported the presence of a methyl ester in compound **7**, compared to the carboxylic acid in compound **6**. The optical rotation of compound **7** $[\alpha]_D -67.7$ (c 0.4 $CHCl_3$) was also negative indicating an *R* configuration at C-6. Compound **7** is reported here as a new structure, (*R*)-methyl 4-hydroxy-5-octanoyl-6-oxo-3,6-dihydro-2H-pyran-2-carboxylate or rhytismatone B.

Table 3.3: ^1H and ^{13}C NMR data of compounds **6-7** in CD_3OD .

Position	6		7	
	δ_{C} , type	δ_{H} (J, Hz)	δ_{C} , type	δ_{H} (J, Hz)
2	174.1		174.1	
3	99.6		99.6	
4	197.5		197.6	
5	36.6, CH_2	2.96, dd (17.2, 4.0) 2.76, dd (17.2, 6.6)	36.5, CH_2	3.00, dd (17.1, 3.9) 2.85, o
6	79.1, CH	4.90, o*	78.9, CH	4.93, o*
7	172.6		171.4	
8			52.5, CH_3	3.67, s
1'	195.3		195.8	
2'	36.5, CH_2	2.85, dt (7.1, 1.4)	36.5, CH_2	2.85, o
3'	26.7, CH_2	1.65, p (7.4)	26.6, CH_2	1.65, p (7.4)
4'	30.1, CH_2	1.33, o	30.1, CH_2	1.33, o
5'	30.4, CH_2	1.33, o	30.4, CH_2	1.33, o
6'	32.8, CH_2	1.28, o	32.8, CH_2	1.28, o
7'	23.6, CH_2	1.31, o	23.6, CH_2	1.31, o
8'	14.4, CH_3	0.89, t (7.0)	14.4, CH_3	0.89, t (7.0)

*-Overlapping with CD_3OD solvent peak at 4.90 ppm.

Table 3.4: Physicochemical data for rhytismatones A and B (**6-7**).

Properties	6	7
Appearance	light yellow solid	light yellow solid
Molecular formula	C ₁₄ H ₂₀ O ₆	C ₁₅ H ₂₂ O ₆
HRMS [M+H] ⁺	285.1332	299.1489
Calculated [M+H] ⁺	285.1332	299.1489
OR [α] _D ²⁵ (CHCl ₃)	-30.0 (c 0.04)	-67.7 (c 0.4)
UV (MeOH) λ_{max} nm (log ϵ)	205 (3.72), 230 (3.70), 270 (3.90)	230 (4.02), 270 (4.21)

Compound **8** was isolated as a white solid with the molecular formula C₁₁H₁₂O₄ determined by HRMS (m/z of 209.0808 [M+H]⁺), indicating six units of unsaturation. The UV spectrum displayed absorption maxima at 235 and 265 nm suggesting an aromatic ring within the structure. The ¹H NMR spectrum displayed a methoxy functionality at δ 3.92 (s, H-10), an oxygenated methylene at δ 5.19 (s, H-3) and two methyl groups at δ 2.12 (s, H-8) and 2.15 (s, H-9). The ¹³C and DEPT-135 revealed that seven of the eleven carbon signals were quaternary. These signals were attributed to two oxygenated sp² carbons at δ 161.8 (C-5) and 157.3 (C-7), four sp² carbons at δ 148.2 (C-3a), 114.5 (C-4), 119.6 (C-6), 108.8 (C-7a) and a lactone at δ 172.0 (C-1). All signals from the ¹H spectrum appeared as singlets, thus, no COSY correlations were observed. HMBC correlations observed from H-8 to C-3a, C-4 and C-5, from H-9 to C-5, C-6 and C-7 as well as from H-10 to C-7 suggested the presence of a fully substituted aromatic ring. The observed HMBC

correlations indicate the methyl groups are *meta* arranged since they both couple to the hydroxylated C-5. Additional HMBC cross peaks were observed from the H-3 to C-1, C-3a and C-7a that indicate compound **8** is a phthalide. The ^1H NMR data (Table 3.5) for compound **8** is in accordance with the synthetic product 5-hydroxy-7-methoxy-4,6-dimethylisobenzofuran-1(3H)-one (Kato et al., 2008).

Figure 3.5: Structures of phthalides (**8-9**) isolated from the unidentified *Rhytismatales* sp.

Compound **9** was isolated as a white solid with the molecular formula $\text{C}_{11}\text{H}_{12}\text{O}_5$ determined by an $[\text{M}+\text{H}]^+$ ion at m/z 225.0757. The ^1H and ^{13}C NMR data (Table 3.5) for compounds **8** and **9** were very similar, indicating both structures were substituted phthalides. Compound **9** was 16 Da heavier compared to **8**, corresponding to an additional oxygen atom. The ^1H NMR spectrum displayed an additional hydroxylated methylene at δ 4.75 (s, H-8) that replaced a methyl functionality. HMBC correlations were observed from H-8 to C-3a, C-4 and C-5 demonstrated the methyl group of compound **8** at position 8 was replaced by a hydroxylated methylene. The ^1H data for **9** are in accordance with the synthetic intermediate 5-hydroxy-4-(hydroxymethyl)-7-methoxy-6-methylisobenzofuran-1(3H)-one reported by Cimmino et al. (2013).

Table 3.5: ^1H and ^{13}C NMR data for phthalides **8** and **9** in CD_3OD .

	8		9	
Position	δ_{C} , type	δ_{H} (<i>J</i> , Hz)	δ_{C} , type	δ_{H} (<i>J</i> , Hz)
1	172.0		171.7	
3	69.8, CH_2	5.19, s	70.1, CH_2	5.30, s
3a	148.2		147.6	
4	114.5		117.4	
5	161.8		162	
6	119.6		120.1	
7	157.3		158.6	
7a	108.8		109.2	
8	11.3, CH_3	2.12, s	58.9, CH_2	4.75, s
9	9.1, CH_3	2.15, s	8.8, CH_3	2.15, s
10	62.4, CH_3	3.92, s	62.5, CH_3	3.95, s

Table 3.6: Physicochemical data for phthalide metabolites (**8-9**).

Properties	8	9
Appearance	white solid	white solid
Empirical formula	$\text{C}_{11}\text{H}_{12}\text{O}_4$	$\text{C}_{11}\text{H}_{12}\text{O}_5$
HRMS $[\text{M} + \text{H}]^+$	209.0808	225.0757
Calculated $[\text{M} + \text{H}]^+$	209.0808	225.0757
OR $[\alpha]^{25}_{\text{D}}$ (MeOH)		
UV (MeOH) λ_{max} nm (log ϵ)	230 (3.44), 265 (3.48)	230 (3.41), 265 (3.45)

Interpretation of the ^1H and ^{13}C NMR data (Table 3.7) for compounds **10** and **11** determined they were structurally similar isocoumarins. Their physiochemical properties are reported in Table 3.8. Compound **10** was isolated as a white solid with the molecular formula $\text{C}_{11}\text{H}_{10}\text{O}_4$ determined by HRMS (m/z 207.0651 $[\text{M}+\text{H}]^+$), indicative of seven units of unsaturation. The ^1H NMR spectrum displayed resonances at δ 3.86 (s, H-6-OMe) suggesting a methoxy functionality, two *meta*-coupled aromatic protons at δ 6.44 (d, $J=2.1$, H-5) and δ 6.45 (d, $J=2.1$, H-7), an olefinic proton δ 6.39 (q, $J=0.8$, H-4) and a methyl group at δ 2.24 (d, $J=0.8$, H-1'). Examination of the ^{13}C and DEPT-135 spectra determined six of the eleven carbon signals were quaternary. These signals represented three oxygenated carbons at δ 155.9 (C-3), 168.8 (C-6) and 164.8 (C-8), two conjugated sp^2 carbons at δ 142.1 (C-4a) and 100.6 (C-8a) and a carbonyl at δ 170.5 (C-1). COSY cross peaks from H-5 to H-7 and H-4 to H-9 as well as an HMBC correlation from H-5 to C-4 support a fully conjugated isocoumarin structure. The ^1H NMR data reported here are in accordance with the synthetic product 8-hydroxy-6-methoxy-3-methyl-isochromen-1-one reported by Nozawa et al. (1980).

Figure 3.6: Structures of isocoumarins (**10-11**) isolated from *Rhytismatales* sp.

Compound **11** was isolated as a light yellow solid with the molecular formula $\text{C}_{13}\text{H}_{16}\text{O}_4$ determined by HRMS (m/z of 237.1112 $[\text{M}+\text{H}]^+$), indicative of six units of unsaturation. Similar to **10**, the ^1H

NMR spectrum of compound **11** revealed a methoxy group at δ 3.82 (s, H-6-OMe) and two *meta*-coupled aromatic protons at δ 6.37 (d, J = 2.3, H-5) and δ 6.38 (d, J = 2.3, H-7). In contrast, three inequivalent methylene groups at δ 2.95 (dd, J = 16.3, 3.6 Hz, H-4), 2.87 (dd, J = 16.3, 11.2, H-4), δ 1.80 (m, H-9), 1.69 (m, H-9), and δ 1.59 (m, H-10), 1.50 (m, H-10), an oxygenated proton at δ 4.55 (m, H-3) and alkyl methyl moiety at δ 0.99 (t, 7.2, H-11) were observed. COSY cross peaks from H-3 to H-4 and H-9 and sequentially from H-9 to H-11 indicated a reduced isocoumarin structure with a longer aliphatic chain compared to compound **10**. The lack of olefin group creates a chiral center at position 3, which is supported by the alkyl chain protons having inequivalent methylene signals. The stereochemistry at C-3 was determined to be (*R*) based on comparisons of the experimental optical rotation ($[\alpha]^{25}_{\text{D}} - 8.0$ (c 0.15, CHCl₃)) to the published data for the synthetic (*R*) ($[\alpha]^{19}_{\text{D}} - 151$ (c 1.10, CHCl₃)) and (*S*) ($[\alpha]^{24}_{\text{D}} + 150$ (c 1.46, CHCl₃)) configurations (Uchida et al., 1998). Compound **11** was determined to be (*R*)-8-hydroxy-6-methoxy-3-propylisochroman-1-one. The structures of compounds **10** and **11** are shown in Figure 3.6 below.

Table 3.7: ^1H and ^{13}C NMR data for isocoumarins **10-11** in CD_3OD .

Position	10		11	
	δ_{C} , type	δ_{H} (<i>J</i> , Hz)	δ_{C} , type	δ_{H} (<i>J</i> , Hz)
1	170.5		171.6	
3	155.9		80.6, CH	4.55, m
4	105.5, CH	6.39, q (0.8)	34, CH_2	2.95, dd (16.3, 3.6) 2.87, dd (16.3, 11.2)
4a	142.1		143.3	
5	101.6, CH	6.45, d (2.1)	107.1, CH	6.38, d (2.3)
6	168.8		167.6	
7	101.4, CH	6.44, d (2.1)	100.4, CH	6.37, d (2.3)
8	164.8		165.6	
8a	100.6		102.7	
9	19, CH_3	2.24, d (0.8)	38, CH_2	1.80, m 1.69, m
10			19.3, CH_2	1.59, m 1.50, m
11			14.1, CH_3	0.99, t (7.2)
6-OMe	56.2, CH_3	3.86, s	56.1, CH_3	3.82, s

Table 3.8: Physicochemical properties of isocoumarins (**10-11**).

Properties	10	11
Appearance	white solid	light yellow solid
Molecular formula	C ₁₁ H ₁₀ O ₄	C ₁₃ H ₁₆ O ₄
HRMS [M+H] ⁺	207.0651	237.1112
Calculated [M+H] ⁺	207.0652	237.1121
OR [α] ²⁵ _D (CHCl ₃)		-8.0 (c 0.15)
UV (MeOH) λ_{max} nm (log ϵ)	240 (3.71), 277 (3.14), 327 (3.06)	222 (3.61), 267 (3.61), 302 (3.23)

Compound **12** was isolated as a brown solid with the molecular formula C₈H₁₀O₂ determined by an [M+H]⁺ peak in the HRMS spectrum at m/z 139.0764 (calculated [C₈H₁₁O₂]⁺ 139.0753). UV maxima (log ϵ) in MeOH were observed at 230 (3.12) and 278 (2.80) nm. The ¹H NMR data showed four aromatic methines, two at δ 7.03 (d, J = 8.3, H-3) and two at δ 6.70 (d, J = 8.3, H-2) as well as an oxygenated methylene at δ 3.68 (t, J = 7.2, H-6) and a methylene at δ 2.71 (t, J = 7.2, H-5). These NMR resonances are in accordance with the simple amino acid derived metabolite tyrosol (Figure 3.7; Takaya et al., 2007, Brenna et al., 2003).

Figure 3.7: Structure of tyrosol (**12**) isolated from an undescribed Rhytismatales sp.

Metabolites of *Diaporthe maritima* (NB365-71I, DAOM 628553)

Compound **13** was isolated as a yellow oil with the molecular formula $C_{15}H_{18}O_6$ determined by HRMS (m/z 295.1175 $[M+H]^+$), indicative of seven units of unsaturation. The 1H NMR spectrum revealed five olefinic protons at δ 6.26 (d, $J = 9.7$, H-3), 7.14 (dd, $J = 9.8, 5.9$, H-4), 6.39 (dd, $J = 11.8, 6.9$, H-1'), 6.71 (dd, $J = 11.8, 1.7$, H-2') and 6.87 (q, $J = 7.2$, H-3''), three oxygenated methines at δ 5.68 (dd, $J = 5.9, 2.9$, H-5), 6.01 (m, H-6) and 4.30 (q, $J = 7.0$, H-4'), and three methyl groups at δ 1.79 (s, H-2''-Me), 1.80 (d, $J = 6.3$, H-4'') and 1.32 (d, $J = 7.0$, H-5'). Examination of the ^{13}C and DEPT-135 showed four of the fifteen carbon signals were quaternary. These signals are attributed to three carbonyls, a lactone at δ 164.1 (C-2), a ketone at δ 204.3 (C-3'), an ester at δ 167.7 (C-1''), and a sp^2 carbon at δ 128.7 (C-2''). COSY cross peaks observed sequentially from H-3 to H-2', HMBC correlations from H-3 and H-6 to C-2 and the chemical shift of C-5 and C-6 indicate compound **13** possessed a dihydro-pyran-2-one core structure substituted at positions 5 and 6. The magnitude of the coupling constant, $J = 11.8$ Hz, between H-1' and H-2' indicated a *cis* olefinic group. The COSY cross peaks from H-2'' to H-4'', an HMBC correlation from H-5 to C-2'' and the chemical shift of C-5 suggested a tiglic ester is attached at C-5. An additional COSY cross peak was observed from H-4' to H-5' and HMBC correlations from H-4' and H-2' indicate C-3' is a ketone. The 1H and ^{13}C NMR data and molecular connectivity elucidated for compound **13** were in accordance with the polyketide metabolite phomopsolide A (Grove, 1985; Stierle et al.,

1997).

Figure 3.8: Structure of phomopsolides A-C (**13-15**) isolated from *D. maritima* DAOM 628553.

Compound **14** was also isolated as a yellow oil with the molecular formula $C_{15}H_{20}O_6$ determined by an $[M+H]^+$ peak at m/z of 297.1332, indicative of six units of unsaturation. The 1H and ^{13}C NMR spectra of compound **14** suggested a structure very similar to phomopsolide A (**13**) except for the reduction of the ketone moiety at C-3' that coincides with the loss of a unit of unsaturation. Instead, carbon and proton signals were observed at δ 76.9 (C-3') and δ 3.91 (m, H-3'), respectively, for comp **14**. The magnitude of the coupling constant ($J = 15.7$ Hz) between H-1' and H-2' indicated a *trans* olefinic group. Additionally, COSY cross peaks were observed sequentially from H-3 to H-4'. The 1H and ^{13}C data for compound **14** are in agreement with phomopsolide B (Grove, 1985; Stierle et al., 1997).

Compound **15** was isolated as a yellow oil with the molecular formula $C_{15}H_{18}O_6$ determined by HRMS (m/z 295.1175 $[M+H]^+$), indicative of seven units of unsaturation. Compound **15** and phomopsolide A (**13**) had very similar 1H and ^{13}C NMR spectra, which suggested a geometric isomer. The magnitude of the coupling constant between H-1' and H-2' was 15.8 Hz, indicative of a *trans* double bond, opposed to the *cis* olefinic group of phomopsolide A (**13**). The NMR and

spectroscopic data for compound **15** was in accordance with phomopsolide C (Stierle et al. 1997). The structures of phomopsolides A-C (**13-15**) are displayed in Figure 3.8 and their accompanying NMR resonances (Table 3.9) and physicochemical data (Table 3.10) are reported below.

Table 3.9: ^1H and ^{13}C NMR data for phomopsolides A-C (**13-15**) in CD_3OD .

	13		14		15	
Position	δ_{C} , type	δ_{H} (<i>J</i> , Hz)	δ_{C} , type	δ_{H} (<i>J</i> , Hz)	δ_{C} , type	δ_{H} (<i>J</i> , Hz)
2	164.1		164.9		164.0	
3	125.3, CH	6.26, d (9.7)	125.0, CH	6.21, d (9.8)	125.3, CH	6.28, d (9.7)
4	142.4, CH	7.14, dd (9.8, 5.9)	143.1, CH	7.09, dd (9.9, 5.6)	142.6, CH	7.13, dd (9.8, 5.9)
5	64.7, CH	5.68, dd (5.9, 2.9)	65.3, CH	5.41, dd (5.6, 3.0)	64.2, CH	5.57, dd (5.9, 2.9)
6	78.4, CH	6.01, m	80.5, CH	5.21, dd (6.2, 2.9)	79.2, CH	5.47, m
1'	142.4, CH	6.39, dd (11.8, 6.9)	136.4, CH	5.98, ddd (15.7, 5.8, 1.1)	140.5, CH	6.88, dd (15.8, 3.5)
2'	126.1, CH	6.71, dd (11.8, 1.7)	125.7, CH	5.88, ddd (15.7, 6.3, 1.3)	127.5, CH	6.89, dd (15.8, 1.2)
3'	204.3		76.9, CH	3.91, m	202.5	
4'	74.2, CH	4.30, q (7.0)	71.5, CH	3.59, quint (7.0)	73.2, CH	4.39, q (7.0)
5'	19.6, CH_3	1.32, d (7.0)	18.7, CH_3	1.07, d (6.5)	19.9, CH_3	1.29, d (7.0)
1''	167.7, C		167.9		167.8	
2''	128.7, C		128.9		128.7	
3''	140.4, CH	6.87, q (7.2)	140.4, CH	6.88, q (7.2)	140.2	6.84, m
4''	14.5, CH_3	1.80, d (6.3)	14.5, CH_3	1.80, d (6.3)	14.5, CH_3	1.81, d (6.3)
2''-Me	12.1, CH_3	1.79, s	12.9, CH_3	1.82, s	12.0, CH_3	1.77, s

Table 3.10: Physicochemical properties of phomopsolides A-C (**13-15**).

Properties	13	14	15
Appearance	Yellow oil	Yellow oil	Yellow oil
Empirical formula	C ₁₅ H ₁₈ O ₆	C ₁₅ H ₂₀ O ₆	C ₁₅ H ₁₈ O ₆
HRMS [M+H] ⁺	295.1175	297.1332	295.1175
Calculated [M+H] ⁺	295.1176	297.1333	295.1176
OR [α] ²⁵ _D (MeOH)	+366 (c 0.3)	+136 (c 0.3)	+63 (c 0.2)
UV (MeOH) λ_{max} nm (log ϵ)	218 (4.41), 320 (3.12)	224 (3.62), 326 (2.10)	218 (3.53), 320 (2.68)

Metabolites of *Pezizula sporulosa* (NB382-3D, DAOM 250862)

Compound **16** was isolated as a yellow solid with the molecular formula C₁₀H₁₀Cl₂O₄ determined by an [M+H]⁺ peak in the HRMS spectra at m/z 265.0029, indicative of five units of unsaturation. The isotopic peak distribution of compound **16** which displayed an [M+H+2]⁺ peak with approximately 70% the abundance of the [M+H]⁺ ion, as well as an [M+H+4]⁺ peak, indicated the presence of two chlorine atoms. The ¹H NMR spectra displayed two olefinic signals at δ 6.96 (dq, J = 16.0, 7.0, H-2') and 6.56 (dq, J = 16.0, 1.7, H-1'), a deshielded methine at δ 4.79 (s, H-5), a methoxy moiety at δ 3.73 (s, H-2'') and methyl group at δ 1.96 (dd, 7.0, 1.7, H-3'). Examination of the ¹³C and DEPT-135 spectra revealed that five of the ten carbon signals are quaternary. These signals were a result of a ketone at δ 190.0 (C-4), an ester carbonyl at δ 171.0 (C-1''), two sp² carbons at δ 159.7 (C-2) and 130.1 (C-3) and a sp³ carbon at δ 84.5 (C-1). COSY cross peaks were

observed sequentially from H-1' to H-3' and HMBC correlations from H-1' and H-2' to C-2 connect a propanene moiety to C-2. The magnitude of the coupling constant, $J = 16.0$ Hz, between H-1' and H-2' indicated the double bond was *trans*. The chemical shift of H-5 and HMBC correlations from H-5 to C-1'', C-1 and C-4 suggested the presence of a chlorinated five membered ring. The ^1H and ^{13}C data for compound **16** are in agreement with (+) cryptosporiopsin (Figure 3.9), that is of the *S* configuration at both positions 1 and 5 (Strunz et al., 1969; Schulz et al., 1995). The optical rotation of cryptosporiopsin isolated in this study was $[\alpha]_{\text{D}} 114.6$ (c 1.71, CHCl_3) which was in agreement with reported optical rotation values, $[\alpha]_{\text{D}} 130$ (c 1.33, CHCl_3 , Schulz et al., 1995; $[\alpha]_{\text{D}} 129$ (c 1.35, CHCl_3 ; Strunz et al., 1969), for this metabolite.

Figure 3.9: Structure of cryptosporiopsin related metabolites (**16-18**) and (*R*)-mellein (**19**) isolated from DAOM 250862.

Compound **17** was isolated as a yellow oil with the molecular formula $\text{C}_{10}\text{H}_{11}\text{ClO}_5$ by an $[\text{M}+\text{H}]^+$ peak at m/z 247.0368. An approximate 3:1 isotopic peak distribution of $[\text{M}+\text{H}]^+ : [\text{M}+\text{H}+2]^+$ is indicative of a single chlorine atom within the structure. The ^1H and ^{13}C data for compound **17** were similar to compound **16** (Table 3.11). The carbon and proton chemical shift at position 5 increased from δ 67.0 to δ 83.1 and decreased from δ 4.79 to δ 4.37 in compound **17** compared to **16**, respectively. Additionally, the chemical shift of the adjacent carbonyl at C-4 increased from δ

190.0 to δ 196.5. The HRMS data, isotopic peak profile and increased chemical shift of C-5 indicated the chlorine at position 5 for **16** was exchanged with a hydroxyl group in compound **17**. This establishes the structure of **17** as the 1,5-dihydroxy, 3-chloro analog of cryptosporiopsin. Compound **17** was previously characterized from a *Cryptosporiopsis* species along with cryptosporiopsin (**16**) with 1*R* and 5*S* configurations (Strunz et al., 1974).

Compound **18** was isolated as a clear solid with the molecular formula $C_{10}H_{12}Cl_2O_4$ determined by an $[M+H]^+$ peak at m/z 267.0186. The same isotopic peak distribution observed for cryptosporiopsin (**16**) indicative of two chlorine atoms was observed for **18**. The NMR spectroscopic data for compound **18** and cryptosporiopsin (**16**) were similar; however, subtle differences were observed. A COSY cross peak between δ 4.44 (d, J = 6.7, H-4) and 4.17 (d, J = 6.7, H-5) not observed for compounds **16-17** and the chemical shift of C-4 (δ 75.9) were notable differences compared to the previously elucidated structures. These spectroscopic data and the HRMS data indicated compound **18** possessed a hydroxyl group at C-4 instead of a ketone functionality. Comparison of the 1H and ^{13}C data for compound **18** reported in Table 3.11 were in accordance with cryptosporiopsinol (Giles & Turner, 1969; Holker & Young, 1975). However, comparison of the experimentally determined optical rotation of **18**, $[\alpha]_D$ 23.6 (c 0.16, MeOH), had the opposite sign, $[\alpha]_D$ -90 (c 0.56, MeOH; Giles & Turner, 1969) compared to cryptosporiopsinol isolated from a *Periconia* species. The absolute stereochemistry of (-)-cryptosporiopsinol was determined to be 1*S*, 4*S* and 5*R* by X-ray crystallography (Giles & Turner 1969; Holker & Young, 1975). For compound **18**, an NOE between H-4 and H-5 indicated these protons were in the same plane. This observation was consistent with (-)-cryptosporiopsinol (Giles & Turner, 1969). The absolute

configuration of positions 1, 4 and 5 could not be determined for compound **18** and is reported here as (+)-cryptosporiopsinol.

Using a chemical standard of (-)-(*R*)-mellein (**19**) previously isolated from CBS 120379 (Sumarah et al., 2008b), the simple isocoumarin was detected from the crude culture filtrate extract of *P. sporulosa* DAOM 250862 by LC-HRMS. At the appropriate retention index, an $[M+H]^+$ ion was observed at m/z 179.0702, indicative of a metabolite with the molecular formula $C_{10}H_{10}O_3$.

Table 3.11: 1H and ^{13}C NMR data for cryptosporiopsin related metabolites **16-18** in CD_3OD .

	16		17		18	
Position	δ_C , type	δ_H (J, Hz)	δ_C , type	δ_H (J, Hz)	δ , type	δ_H (J, Hz)
1	84.5		84.4		88.6	
2	159.7		158.4		138.6	
3	130.1		130.0		133.9	
4	190.0		196.5		75.9, CH	4.44, d (6.7)
5	67.0, CH	4.79, s	83.1, CH	4.37, s	80.5, CH	4.17, d (6.7)
1'	122.4, CH	6.52, dq (16.0, 1.7)	122.6, CH	6.49, dq (16.0, 1.7)	122.3, CH	6.19, o
2'	145.5, CH	6.96, dq (16.0, 7.0)	144.2, CH	6.90, dq (16.0, 6.9)	134.3, CH	6.15, o
3'	20.2, CH ₃	1.96, dd (7.0, 1.7)	20.0, CH ₃	1.95, dd (6.9, 1.7)	19.3, CH ₃	1.8, d (5.3)
1''	171.0		171.9		174.3	
2''	53.8, CH ₃	3.73, s	53.6, CH ₃	3.70, s	53.3, CH ₃	3.76, s

Table 3.12: Physicochemical properties of compounds **16-18**.

Properties	16	17	18
Appearance	light yellow solid	yellow solid	clear solid
Empirical formula	C ₁₀ H ₁₀ Cl ₂ O ₄	C ₁₀ H ₁₁ ClO ₅	C ₁₀ H ₁₂ Cl ₂ O ₄
HRMS [M+H] ⁺	265.0029	247.0368	267.0186
Calculated [M+H] ⁺	265.0028	247.0367	267.0185
OR [α] ²⁵ _D (MeOH)	114.6 (c 1.71)	103.9 (c 0.64)	23.6 (c 0.16)
UV (MeOH) λ_{max} nm (log ϵ)	290 (4.06)	287 (4.06)	246 (5.08)

Growth Inhibition Assays

Compounds **1-19** (Figure 3.10) were tested for antimicrobial activity towards *E. coli* (ATCC 67878), *B. subtilis* (ATCC 23857), *S. cerevisiae* and *M. violaceum*. The bacterial and *S. cerevisiae* data at 8 hours and *M. violaceum* data at 60 hours was used for statistical analysis by an ANOVA followed by Tukey's test for significant differences (Systat V13) compared to the negative control (DMSO). P-values below 0.05 were considered significantly different.

Figure 3.10: Secondary metabolites (**1-19**) characterized from foliar endophytes as part of this thesis.

Antibacterial activity towards the Gram-negative bacterium *E. coli* was observed in 8 of the isolated metabolites (Table 3.13). The isocoumarin (**11**) from the undescribed Rhytismatales species showed the greatest potency out of all of the metabolites tested, where it continued to inhibit *E. coli* growth at a concentration of 2.5µM. This activity was not conserved in the isocoumarin structures as compounds **1** and **2** were the only other isocoumarins showing activity, and were only active at 250µM. In addition **11**, the two new pyrone structures (**6**, **7**), one of the phthalides (**9**) and the tyrosol (**12**) metabolites isolated from the undescribed Rhytismatales endophyte all inhibited *E. coli* growth at 250µM. The *P. sporulosa* endophyte compound cryptosporiopsin (**16**) was the only metabolite that reduced *E. coli* growth, but showed activity at 25µM.

Table 3.13: Antibacterial activity of isolated metabolites towards *E. coli* ($p \leq 0.05$).

Compounds	Concentrations		
	250µM	25µM	2.5µM
1	+		
2	+		
6	+		
7	+		
9	+		
11	+	+	+
12	+		
16	+	+	

The metabolites isolated in this study showed limited bioactivity towards the Gram-negative bacterium *B. subtilis* (Table 3.14). Phomopsolide A (**13**) from was the most potent metabolite isolated, where it inhibited *B. subtilis* growth even at the lowest tested concentration (2.5µM). The new pyrone metabolite (**6**) from the undescribed Rhytismatales species and cryptosporiopsin (**16**) from the *P. sporulosa* endophyte both displayed antibacterial activity towards *B. subtilis* at 250µM. None of the isocoumarins (**1-5**) isolated from the *P. cf. gaeumannii* endophyte reduced the growth of *B. subtilis*. Additionally, the potent activity of compound **11** towards inhibiting *E. coli* was not observed when testing against *B. subtilis*.

Table 3.14: Antibacterial activity of isolated metabolites towards *B. subtilis* ($p \leq 0.05$).

Compounds	Concentrations		
	250µM	25µM	2.5µM
6	+		
13	+	+	+
16	+		

Antifungal activity towards the Ascomycete *S. cerevisiae* was observed in 8 of the isolated metabolites (Table 3.15). The metabolites originating from the undescribed Rhytismatales endophyte illustrated the highest potency. The new pyrone structure (**7**) inhibited *S. cerevisiae* growth even at the lowest tested concentration (2.5µM). The two isocoumarins (**10**, **11**) reduced *S. cerevisiae* growth at 250 and 25µM, respectively. One of the isolated phthalides (**9**) also showed activity at 250µM. Phomopsolides A (**13**) and B (**14**) from *D. maritima* showed antifungal activity

towards *S. cerevisiae* at 250µM. Lastly, one of the isocoumarins (**3**) from *P. cf. gaeumannii* and cryptosporiopsin (**16**) from *P. sporulosa* possessed antifungal activity towards *S. cerevisiae* at 250µM.

Table 3.15: Antifungal activity of isolated metabolites towards *S. cerevisiae* ($p \leq 0.05$).

Compounds	Concentrations		
	250µM	25µM	2.5µM
3	+		
7	+	+	+
9	+		
10	+		
11	+	+	
13	+		
14	+		
16	+		

Antifungal activity towards the Basidiomycete *M. violaceum* was observed in 7 of the isolated metabolites (Table 3.16). This activity was isolated to the undescribed Rhytismatales endophyte and the *D. maritima* endophyte. From the undescribed Rhytismatales species the two phthalides (**8**, **9**), two isocoumarins (**10**, **11**) and tyrosol (**12**) metabolites all showed antifungal activity towards *M. violaceum* at 250µM. Phomopsolide A (**13**) was the most potent metabolite tested, where it reduced the growth of *M. violaceum* at 25µM. Phomopsolide B (**14**) also reduced growth at 250µM.

Table 3.16: Antifungal activity of isolated metabolites towards *M. violaceum* ($p \leq 0.05$).

Compounds	Concentrations		
	250 μ M	25 μ M	2.5 μ M
8	+		
9	+		
10	+		
11	+		
12	+		
13	+	+	
14	+		

4. Discussion

Our current understanding of the endophytes present in conifer trees of the Acadian forest is still very limited. To date, all needles collected from conifer tree species of the Acadian forest have possessed endophytes. Initial screens of these endophytes revealed a modest proportion of toxigenic endophytes (~5%). Over the past 25 years, screening of old forestry stands in protected areas has shown that the proportion of toxigenic endophytes in the Acadian forest to be much larger than the original 5% estimate. Further, their ecological function has proven to be crucial to their conifer host. The *P. scopiformis* endophyte has been shown to produce, *in planta*, rugulosin above the effective concentration necessary to reduce *C. fumiferana* (Eastern spruce budworm) growth and herbivory (Miller et al., 2008; Frasz et al., 2014). By monitoring white spruce seedlings inoculated with *P. scopiformis* it was discovered that *P. scopiformis* can persist in the host tree for over 10 years, still producing rugulosin to deter herbivory by the cyclical spruce budworm pest (Miller et al., 2008; Miller, 2011, Frasz et al., 2014). Additionally, *P. scopiformis* was discovered to naturally infect other white spruce seedlings in close proximity of the original inoculated test tree. This horizontal transfer is why we are noticing high endophyte diversity throughout the undisturbed Acadian forest, yet low diversity in nursery conifers intended for reforestation projects (Sumarah et al., 2008a).

This thesis is part of a much larger project that is investigating the endophytes producing biologically active metabolites that are hiding throughout the Acadian forest. There are many important aspects to this work. Investigating these cryptic fungal species is providing information about their biodiversity and their ecological importance to the host tree as well as in forest

dynamics. While describing these new fungal endophytes, novel metabolites with various antibiotic, antifungal and antiinsectan properties are being characterized. By inoculating nursery seedlings with these toxigenic endophytes we are increasing the fitness of replanted stands towards common pests and pathogens and we are restoring some endophyte diversity. Another important aspect of this research is in finding various, phylogenetically distinct endophytes, the risk imposed by common pests and pathogens developing tolerance to this increased diversity of endophyte toxins is reduced.

While sampling the Acadian forest, conifer needles are obtained from multiple trees in various regions in order to get a proper representation of the endophyte diversity. This generates a large collection of endophytes that are studied by our taxonomist collaborators for morphological features and ITS sequences in order to get a phylogenetic understanding of the fungal endophyte community. Doing so allows the selection of undescribed endophytes for metabolite screening. In this thesis, 61 endophytes were selected for screening, in which the crude extracts produced by one litre fermentations were tested for antifungal activity towards *S. cerevisiae* and *M. violaceum* (see appendix II) and analyzed by LC-UV-MS for complexity of the metabolite profiles. Based on LC-UV-MS chromatograms, bioassay result and phylogenetic analysis two *P. rubens* (red spruce) and two *P. mariana* (black spruce) endophytes were selected for secondary metabolite characterization studies.

A currently undescribed *Phaeocryptopus* cf. *gaeumannii* (DAOM 250863) was isolated as an endophyte of *P. mariana* (black spruce). Unlike many of the fungal endophytes studied here for natural products, the crude extract obtained from its one litre screening fermentation was adequate

for metabolite isolations, particularly since the LC-UV-MS chromatogram only displayed six major metabolites. Endophytes of black spruce had not received extensive metabolite attention in the Miller group compared to white spruce and red spruce endophytes, prompting their investigation (Sumarah et al., 2008b; 2010). The crude extract of DAOM 250863 inhibited the growth of *M. violaceum* and afforded five structurally similar isocoumarins (**1-5**; Figure 3.1) including a new metabolite (**1**). The ramulosin related isocoumarins **2-5** were previously characterized from an undescribed endophyte of black spruce called *Canoplea elegantula* (Cooke) along with several structural analogs (Findlay et al., 1995b). This black spruce endophyte is most likely closely related or the same species as DAOM 250863 studied here; however, it was not deposited in a culture collection, thus preventing direct comparison. Based on sequencing of a portion of the ITS region, DAOM 250863 is 100% identical to the red spruce endophytes CBS 121943 and DAOM 239830 (Sumarah & Miller, 2009). Compound (**4**) and four other biosynthetically related metabolites characterized by Findlay et al. (1995b) were produced by these red spruce endophytes. The crude culture filtrate extracts of CBS 121943 and DAOM 239830 were toxic to spruce budworm in feeding assays (Sumarah & Miller, 2009). The culture filtrate of the unidentified black spruce endophyte and several isocoumarins, including compound **4**, showed toxicity towards spruce budworm larvae and cells (Findlay et al., 1995b). In this study, compounds **1** and **2** showed modest antibacterial activity towards *E. coli* at 250µM (Table 3.13) and compound **3** showed modest antifungal activity to *S. cerevisiae* at 250µM (Table 3.15). Despite this, *in vitro* spruce budworm assays is warranted to evaluate the toxicity of the new isocoumarin (**1**) as well as the extract itself. As mentioned previously, the low activity of individual compounds may not be truly representative as it is believed endophytes produce an array of toxic metabolites that have synergistic toxicity (Miller, 1986).

The culture filtrate extract of an undescribed black spruce endophyte (NB236-1A) from the Order Rhytismatales yielded several different chemical classes (Figures 3.3, 3.5-3.7) including pyrones (6-7), phthalides (8-9), isocoumarins (10-11) and the amino acid derived tyrosol metabolite (12). The pyrones (6-7) characterized by HRMS, NMR and other spectroscopic methods are reported here as new structures. Their structures share the same 3-acyl-4-hydroxy-5,6-dihydro-2-pyrone core structure as alternaric acid originally isolated from *Alternaria solani* (Brian et al., 1949; Tabuchi et al., 1994) and podoblastins A-C obtained from the medicinal plant *Podophyllum* (Miyakado et al., 1982; Tanabe et al., 1982). The methylated pyrone (7) showed potent antifungal activity as it inhibited the growth of *S. cerevisiae* even at the lowest concentration tested, 2.5 μ M (Table 3.15). Alternaric acid is not appreciably antibiotic; however, is antifungal in the low μ g mL⁻¹ range (Brian et al., 1949). Podoblastins were identified as the antifungal constituents of a *Podophyllum* extract that inhibited the growth of *Pyricularia oryzae*, the causative agent of rice blast, in the low μ g mL⁻¹ range (Miyakado et al., 1982).

The phthalide, 5-hydroxy-7-methoxy-4,6-dimethylphthalide (8), was previously characterized from *Microsphaeropsis* obtained from the foliage of *Garcinia hombroniana* (seashore mangosteen; Sommart et al., 2012) and a wood associated *Lachnum papyraceum* strain (Shan et al., 1997; Stadler et al., 1993). It was observed in this study to have modest (250 μ M) antifungal activity towards the rust fungus *M. violaceum*. Compound 9, 5-hydroxy-4-(hydroxymethyl)-7-methoxy-6-methylphthalide, was prepared as a reduced derivative of cyclopaldic acid in a structure activity relationship study examining mosquito bite deterrent compounds (Cimmino et al., 2013). This phthalide (9) was active in the *Aedes aegypti* (dengue fever insect vector) bite deterrent assay.

In this study, **9** showed modest (250µM) activity towards *E. coli* and both test fungal species (Tables 3.13, 3.15, 3.16).

8-hydroxy-6-methoxy-3-methyisocoumarin (**10**) was previously isolated as a fungal metabolite with a related isocoumarin, indole-3-acetic acid, tyrosol (**12**) and several other para-hydroxyphenolic metabolites (Stossel, 1969) and from the bacterium *Streptomyces mobaraensis* (Eaton & Hutchinson, 1971). Compound **10** inhibited the growth of a *Fusarium* species (minimum inhibitory concentration (MIC) of 200 µg mL⁻¹ or *ca.* 970µM) and was prepared during the synthesis of a related chlorinated isocoumarin produced by a *Periconia* species (Giles & Turner, 1969). In this study, compound **10** showed antifungal activity at 250µM towards *S. cerevisiae* (Table 3.15) and *M. violaceum* (Table 3.16). (*R*)-8-hydroxy-6-methoxy-3-propylisochromanone (**11**) was identified as a fungal natural product from an unidentified white spruce endophyte also originating from the Order Rhytismatales (CBS 120380; Sumarah et al., 2008b). Although structurally similar to **10**, this isocoumarin (**11**) showed potent (2.5µM) antibacterial activity to *E. coli* as well as antifungal activity to *S. cerevisiae* at 250µM and *M. violaceum* at 25µM.

The remaining secondary metabolite from the undescribed Rhytismatales species (NB236-1A) is tyrosol (**12**), which showed modest (250µM) antimicrobial activity to *E. coli* and *M. violaceum*. Tyrosol (**12**) was also identified from three white spruce endophytes (CBS 120379-120381) and has been reported from numerous fungal genera including *Diaporthe*, *Xylaria* and *Epichloë* (Claydon et al., 1985; Koshino et al., 1988; Schneider et al., 1996; Sumarah et al., 2008b). The white spruce endophytes CBS 120379 and 120380 are closely related to an unidentified black spruce endophyte isolated in Quebec based on ITS sequence comparisons (Ganley & Newcombe,

2006). A phylogenetic analysis of Rhytismatales endophytes obtained from the Acadian forest determined that the undescribed Rhytismatales species (NB236-1A) studied here and CBS 120380 are two distinct species; however, closely related (Joey Tanney, personal communication). Their secondary metabolite profiles were also quite similar. An isocoumarin (**11**) and tyrosol (**12**) were isolated from both extracts and a phthalide, 5,7-dimethoxy-3-methylphthalide, structurally similar to compounds (**8-9**) were also characterized (Sumarah et al., 2008b). No dihydropyrones related to compounds (**6-7**) were identified from CBS 120380. Incorporation of the culture filtrate extracts of CBS 120380 significantly reduced the weight and head capsule size of spruce budworm larvae (Sumarah et al., 2008b). The similarity of their metabolite profiles suggests the undescribed Rhytismatales endophyte studied here (NB236-1A) synthesizes metabolites toxic to spruce budworm, warranting further investigation.

During this survey of endophyte of the Acadian forest, a fungus belonging to the *Diaporthe eres* species complex was frequently recovered from healthy surface-sterilized black and red spruce needles. In a complementary investigation, this abundant foliar endophyte was recently described as new species, *Diaporthe maritima*, using morphological characteristics and multiple gene phylogenetic analysis (Tanney et al., 2016b). The crude extract of *D. maritima* DAOM 628553 was shown to inhibit the growth of the biotrophic fungal pathogen *M. violaceum*. The culture filtrate extract of this strain afforded phomopsolides A (**13**), B (**14**) and C (**15**) (Figure 3.8). Phomopsolides A (**13**) and B (**14**) were originally isolated from *Diaporthe eres* (as *Phomopsis oblonga*), a fungal species commonly associated with the outer bark of healthy *Ulmus* (elm) trees (Grove, 1985). These strains have been shown to invade the phloem tissue of elm trees infected with the fungus *Ophiostoma ulmi*, the causative agent of Dutch elm disease (Webber, 1981).

Phomopsolides A (**13**) and B (**14**) were shown to elicit anti-feedant and anti-boring activity against adult female bark beetles (*Scolytus scolytus*), an insect vector of *O. ulmi* (Claydon et al., 1985). It was hypothesized that these *Diaporthe* species naturally controlled Dutch elm disease as they deterred boring and in turn, the subsequent mating of bark beetles, insect vectors for this fungal pathogen (Webber, 1981; Webber & Gibbs, 1984).

Subsequent to the original discovery of phomopsolides from species within the *Diaporthe eres* species complex, this class of secondary metabolites has been reported from related and phylogenetically distant fungi associated with various host species. Phomopsolide C (**15**), the *trans* isomer of **13**, was originally isolated from a *Penicillium* species (NRRL 21208) obtained from the inner bark of *Taxus brevifolia* (Pacific yew). This *Penicillium* strain was shown to produce Phomopsolides A-E as well as a furanone structure, all of which had strong inhibitory activity towards *Staphylococcus aureus* with potencies comparable to the known antibiotics tetracycline and streptomycin (Stierle et al., 1997). Phomopsolide A (**13**) isolated from the *D. maritima* endophyte in this study had strong antimicrobial activity towards *B. subtilis*, where it remained effective at the lowest concentration of 2.5µM (Table 3.14). Phomopsolide A (**13**) was also observed to have antifungal activity towards *S. cerevisiae* at 250µM and *M. violaceum* at 25µM (Tables 3.15 and 3.16, respectively). Phomopsolide B (**2**) was recently characterized as the dominant metabolite from an undescribed *Diaporthe* species (GenBank DQ272498) isolated from surface sterilized twig tissue of *Camptotheca acuminata* in China (Yuan et al., 2009). Nine *Diaporthe* strains associated with grapevines growing in Switzerland synthesized several different mixtures of secondary metabolites. Characterized natural products included phomopsolide B, four furanones, the xanthonenes sydownin A and sydowninol, and cytosporone (Goddard et al., 2014). A

large proportion of North American and European *Diaporthe* endophytes of *Silax* (willow) trees produced phomopsolide B whereas phomodiol biosynthesis varied (Horn et al., 1996).

Diaporthe species associated with woody plants produce a diverse array of biologically active natural products. Several of these chemicals are directly implicated in host tree-pest chemical ecologies. From these chemical investigations, phomopsolide B appears as the most commonly reported metabolite from *Diaporthe* species (Claydon et al., 1985; Grove, 1985; Horn et al. 1996). The secondary metabolites of European isolates within the DESC associated with *Fraxinus* (Ash), *Platanus*, and *Ulmus* (Elm) trees were studied because of the known antiinsectan activity of their natural products such as the phomopsolides (Claydon et al. 1985; Grove, 1985). Chemical differences were observed between morphologically distinct elm-derived isolates. *Diaporthe* isolates from each of the host trees produced phomopsolide B (**14**) and oblongolide (Begley & Grove, 1985; Claydon et al., 1985; Grove, 1985). The elm-derived *Diaporthe* species also synthesized various pyrones, orsellinic acids, aromatics, and isocoumarins including (*R*)-5-methylmellein, tyrosol, and mellein. Interestingly, several of these secondary metabolites were also reported from endophytes of white spruce in the Acadian forest of North America (Sumarah et al., 2008b). Culture filtrate extracts of these white spruce endophytes, when incorporated into the diets of spruce budworm larvae, significantly reduced both the weight and head capsule size (Sumarah et al., 2008b). Phomopsolides A (**13**) and B (**14**), (*R*)-5-methylmellein, tyrosol, and mellein all displayed *in vitro* antiinsectan activities, where **13** was the most active towards the elm 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. These data further

demonstrate the production of antagonistic mixtures of secondary metabolites by tree-associated and endophytic fungi toward host insect pests. Further investigations into the antiinsectan activity of the *D. maritima* (DAOM 628553) extract as well as the isolated phomopsolides (**13-15**) towards spruce budworm larvae is warranted.

From the fermentation broth of *Pezicula sporulosa* DAOM 250862, three chlorinated metabolites (**16-18**) were characterized and (*R*)-mellein (**19**) was detected using an in-house standard (Figure 4). (+)-Cryptosporiopsin (**16**) and related *chlorinated cyclopentenone* analogs were originally isolated from a coprophilous *Sporormia* sp. (McGahren et al. 1969), a *Periconia* sp. (Giles & Turner 1969) and *Cryptosporiopsis* sp. (Strunz et al. 1969) at approximately the same time. Several biosynthetically related chlorinated dihydroisocoumarins not isolated here or previously from *Cryptosporiopsis* were characterized from the other cryptosporiopsin producing fungi. The structure of (+)-cryptosporiopsin (**16**) was confirmed by x-ray crystallography (McGahren et al. 1969) and has the *S* configuration at positions 1 and 5. It is not surprising that (+)-cryptosporiopsin (**16**) was isolated from a *P. sporulosa* endophyte since one of the original reports of this metabolite was from its anamorphic state, *Cryptosporiopsis*, isolated from decaying *Betula alleghaniensis* (yellow birch; Stillwell et al. 1969; Strunz et al. 1969). Interestingly, the *P. sporulosa* endophyte studied here was collected from as an endophyte of red spruce in a yellow birch and red maple stand. In a study of *Pezicula* endophytes isolated from asymptomatic deciduous and coniferous tree branches in Northern Germany, (+)-cryptosporiopsin (**16**) was characterized from six of the eighty five strains studied (Schulz et al. 1995). This chlorinated natural product possesses antifungal activity against several other wood rot fungi and is antibiotic (Stillwell et al. 1969). The authors suggested this biologically active metabolite could be applied to prevent the deterioration

of forest products or control diseases of agriculture and forests. This agrees with the findings in this study, where cryptosporiopsin (**16**) was antibacterial towards *E. coli* and *B. subtilis* at 25 μ M and 250 μ M, respectively, as well as inhibited the growth of *S. cerevisiae* at 250 μ M.

The 5-hydroxy cryptosporiopsin analog (**17**) was originally isolated from the fermentation broth of a wood derived *Cryptosporiopsis* along with cryptosporiopsin (**16**). Chemical derivatization and comparison of the spectroscopic data of compounds **16** and **17** determined that both metabolites were 5*S*; however, **17** was 1*R* as opposed to **16**, which was 1*S* (Strunz et al. 1974). Compound **17** did not display any considerable *in vitro* antifungal activity when assayed by Strunz et al. (1974), nor in this present investigation. This suggests the C-5 chlorine is necessary for appreciable antifungal activity.

An in house standard of (*R*)-mellein (**19**) isolated from an undescribed Rhytismatales (Order) foliar endophyte of white spruce (Sumarah et al., 2008b) was utilized to confirm its production by *P. sporulosa* (DAOM 250862). At the appropriate retention index, an $[M+H]^+$ ion at m/z 179.0702 was observed, identifying the simple isocoumarin within the culture filtrate extract. In a survey of the secondary metabolites produced by endophytic *Pezicula* species, (*R*)-mellein (**19**) was detected from all eighty five extracts (Schulz et al., 1995). While (*R*)-mellein (**19**) could be characteristic of the genus, the antifungal metabolites cryptosporiopsin (**16**), (-)-mycorrhizin A and 4-epi-ethiosolide appear to be more species specific (Schulz et al., 1995). Metabolite profiles were also influenced by culture conditions. Several other structurally related non-chlorinated isocoumarins have been characterized from endophytic *Pezicula* species (reported as *Cryptosporiopsis*; Krohn et al. 1997). This metabolite was originally reported from *Aspergillus melleus* (Nishikawa, 1933)

and subsequently from several fungal genera, plants and insects (Nago & Matsumoto, 1993; Sumarah et al., 2008b). (*R*)-mellein (**19**) possesses antifungal, herbicidal, algicidal and antiinsectan activities (Claydon et al., 1985; Schulz et al., 1995). As noted previously, this metabolite has been characterized from a white spruce endophyte extract that displayed antiinsectan activity towards the spruce budworm.

The *P. sporulosa* endophyte of red spruce examined here and the several *Pezicula* species investigated by Schulz et al. (1995) for natural products were all isolated from needles of healthy tree branches. The synthesis of antifungal metabolites such as cryptosporiopsin (**16**) that are active towards plant pathogens hints at a host-endophyte mutualistic relationship existing (Schulz et al., 1995; Stillwell et al., 1969). Since these *Pezicula* endophytes were reported to be recovered from a high percentage of healthy foliage, it is suspected that at the very least they have low virulence towards their host. A *Xylaria* species producing the potentially antifungal metabolite was recovered as an endophyte of white pine needles and *Vaccinium angustifolium* (low bush blueberry) stems (Richardson et al., 2014). Interestingly, the same species was also recovered growing on decaying *Acer saccharum* (sugar maple) from the same environment. A similar observation was made for the *Cryptosporiopsis* sp. producing cryptosporiopsin (**16**) that was isolated from decaying yellow birch (Strunz et al., 1969; Stillwell et al., 1969). The cryptic life cycles of fungal endophytes are currently poorly understood despite their important ecological roles. It is conceivable that specific foliar endophytes complete a portion of their lifecycle as mutualistic symbionts which is supported by their energy expenditure synthesizing metabolites toxic to host pests *in planta* (Miller et al. 2008). During this latency period, they maintain an equilibrium with their host. The production of herbicidal metabolites (**19**) or low pathogenicity towards their host

may represent a dispersal technique whereby the foliage provides a vector enabling endophytes to colonize new microclimates not accessible by typical spore dispersion. This notion was proposed and termed the foraging ascomycete hypothesis by Prof. George C. Carroll (Thomas et al., 2016). This notion is additionally supported by the discovery of foliar endophytes infecting trees close in proximity by horizontal transmission (Miller et al. 2009). Decaying yellow birch may represent a secondary host for *Pezicula* species as sugar maple does for the griseofulvin producing *Xylaria*. Continuing to investigate the *in planta* production of metabolites of taxonomically distant endophytes would contribute to a better understanding of plant-endophytes relationships, their biodiversity, cryptic lifecycles and ecological roles.

5. Conclusion

The cyclical pest Eastern spruce budworm defoliates millions of hectares of coniferous forest during each outbreak. One of the only available options to mitigate this damage is to spray the naturally occurring bacterium *Bacillus thuringiensis* var. *Kurstaki* (Btk). This bacterium produces a toxic crystal that is activated by the alkaline intestines of insect pests. The problem with spraying Btk is that it is expensive, and the total forestry region affected during spruce budworm outbreaks is vast. Our research group aims to improve the fitness of reforested stands by inoculating them with toxigenic endophytes.

The basis of this research was demonstrated with the white spruce endophyte *P. scopiformis*. After inoculating this toxigenic endophyte into white spruce seedlings it was monitored for over 10 years. Despite natural needle shedding, this endophyte remained present in the inoculated white spruce. Additionally, it continued to produce its major toxin rugulosin at concentrations that were

effective to reduce spruce budworm growth. This was demonstrated by white spruce inoculated with *P. scopiformis* having more tolerance towards spruce budworm herbivory compared to uninoculated white spruce. The ongoing effort of our group is to expand the number of toxigenic endophytes that can be inoculated into conifer trees as this will allow the endophyte diversity in replanted forests to be restored. Additionally, the phylogenetic diversity of these endophytes translates to the production of unique arrays of secondary metabolites. By inoculating conifers with genetically distinct toxigenic endophytes, we may also be limiting the risk of spruce budworm developing a tolerance.

This thesis focused on four conifer needle endophytes, two isolated from red spruce and two from black spruce. From the *Phaeocryptopus* cf. *gaeumannii* endophyte of black spruce 5 isocoumarin structures were characterized, where compound **1** was a new structure. Although the antibiotic and antifungal activity of these isocoumarins was modest, previous reports have shown some of these isocoumarins are toxic towards spruce budworm larvae and cells (Findlay et al., 1995b). From an undescribed Rhytismatales endophyte also from black spruce, two new pyrones (**6**, **7**) structures were elucidated together with two phthalides (**8**, **9**), two isocoumarins (**10**, **11**) and the amino acid derived metabolite tyrosol (**12**). These metabolites represent an array of structural variability. Additionally, a number of these metabolites and related structures have documented antiinsectan activity towards spruce budworm larvae and mosquitos (Cimmino et al., 2013; Sumarah et al., 2008b). From the red spruce endophyte *D. maritima*, phomopsolides A-C (**13-15**) were characterized. In addition to the potent antimicrobial activity observed for **13** and **14** in this study, these phomopsolides disrupt the insect vector of Dutch elm disease (Claydon et al., 1985). Lastly, from a *P. sporulosa* endophyte of red spruce, cryptosporiopsinol (**16**), 5-hydroxyl cryptosporiopsin

(**17**), cryptosporiopsinol (**18**) and mellein (**19**) were identified. Cryptosporiopsin and mellein are metabolites known for their broad spectrum biological activities (Schulz et al., 1995). Taken together, the experimentally observed antimicrobial activities and the literature reports on the antiinsectan activities of these metabolites warrants further testing towards spruce budworm *in vitro* and potentially *in planta* thereafter by inoculating these toxigenic endophytes into spruce trees.

6. References

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Appendix I – ^1H and ^{13}C NMR spectra of isolated endophyte metabolites

^1H NMR spectrum of compound 1 (400MHz, CD_3OD)

^{13}C NMR spectrum of compound 1 (100 MHz, CD_3OD)

¹H NMR spectrum of compound **2** (400MHz, CD₃OD)

¹³C NMR spectrum of compound **2** (100 MHz, CD₃OD)

¹H NMR spectrum of compound **3** (400MHz, CD₃OD)

BG-250-4H-5-cleaned oct 2015.1.fid
Bruker AVANCE 400 proton

¹³C NMR spectrum of compound **3** (100 MHz, CD₃OD)

BG-250-4H-5-cleaned oct 2015.3.fid
Bruker AVANCE 400 carbon

¹H NMR spectrum of compound **4** (400 MHz, CD₃OD)

BG-250-4H-3-cleaned oct 2015.1.fid
Bruker AVANCE 400 proton

¹³C NMR spectrum of compound **4** (100 MHz, CD₃OD)

BG-250-4H-3-cleaned oct 2015.3.fid
Bruker AVANCE 400 carbon

¹H NMR spectrum of compound **5** (400 MHz, CD₃OD)

BG-250-4H-4-clean and full oct 2015.1.fid
Bruker AVANCE 400 proton

¹³C NMR spectrum of compound **5** (100 MHz, CD₃OD)

BG-250-4H-4-clean and full oct 2015.3.fid
Bruker AVANCE 400 carbon

^1H NMR spectrum of compound **6** (400 MHz, CD_3OD)

^{13}C NMR spectrum of compound **6** (100 MHz, CD_3OD)

^1H NMR spectrum of compound **7** (400 MHz, CD_3OD)

BG-NB236-1A-Fr9-TLC-2-4 methanol-full.1.fid
Bruker AVANCE 400 proton

^{13}C NMR spectrum of compound **7** (100 MHz, CD_3OD)

BG-NB236-1A-Fr9-TLC-2-4 methanol-full.3.fid
1D C13 Experiment

¹H NMR spectrum of compound **8** (400 MHz, CD₃OD)

BG-236-1A-F4-2-full.1.fid
Bruker AVANCE 400 proton

¹³C NMR spectrum of compound **8** (100 MHz, CD₃OD)

BG-236-1A-F4-2-full.3.fid
1D C13 Experiment

^1H NMR spectrum of compound **9** (400 MHz, CD_3OD)

^{13}C NMR spectrum of compound **9** (100 MHz, CD_3OD)

^1H NMR spectrum of compound **10** (400 MHz, CD_3OD)

BG-236-1A-F1-1-full.1.fid
Bruker AVANCE 400 proton

^{13}C NMR spectrum of compound **10** (100 MHz, CD_3OD)

BG-236-1A-F1-1-full.3.fid
1D C13 Experiment

¹H NMR spectrum of compound **11** (400 MHz, CD₃OD)

BG-236-1A-F1-2-full.1.fid
Bruker AVANCE 400 proton

¹³C NMR spectrum of compound **11** (100 MHz, CD₃OD)

BG-236-1A-F1-2-full.3.fid
1D C13 Experiment

^1H NMR spectrum of compound **12** (400 MHz, CD_3OD)

BG-236-1A-F4-1-full.1.fid
Bruker AVANCE 400 proton

^{13}C NMR spectrum of compound **12** (100 MHz, CD_3OD)

BG-236-1A-F4-1-full.3.fid
1D C13 Experiment

^1H NMR spectrum of compound **13** (400 MHz, CD_3OD)

BG-365-711-Fr3-4.1.fid
Bruker AVANCE 400 proton

^{13}C NMR spectrum of compound **13** (100 MHz, CD_3OD)

BG-365-711-Fr3-4.2.fid
Bruker AVANCE 400 carbon

¹H NMR spectrum of compound **14** (400 MHz, CD₃OD)

BG-365-711-Fr5-6-3 (B).1.fid
Bruker AVANCE 400 proton

¹³C NMR spectrum of compound **14** (100 MHz, CD₃OD)

BG-365-711-Fr5-6-3 (B).3.fid
Bruker AVANCE 400 carbon

¹H NMR spectrum of compound **15** (400 MHz, CD₃OD)

BG-NB-365-711-AC3-full workup-repeat.1.fid
Bruker AVANCE 400 proton

¹³C NMR spectrum of compound **15** (100 MHz, CD₃OD)

BG-NB-365-711-AC3-full workup-repeat.3.fid
Bruker AVANCE 400 carbon

^1H NMR spectrum of compound **16** (400 MHz, CD_3OD)

BG-382-3D-F2-6-full.1.fid
Bruker AVANCE 400 proton

^{13}C NMR spectrum of compound **16** (100 MHz, CD_3OD)

BG-382-3D-F2-6-full.3.fid
Bruker AVANCE 400 carbon

^1H NMR spectrum of compound **17** (400 MHz, CD_3OD)

BG-382-3D-F4-2-full.1.fid
Bruker AVANCE 400 proton

^{13}C NMR spectrum of compound **17** (100 MHz, CD_3OD)

BG-382-3D-F4-2-full.3.fid
1D C13 Experiment

^1H NMR spectrum of compound **18** (400 MHz, CD_3OD)

BG-382-3D-F5-8.1.fid
Bruker AVANCE 400 proton

^{13}C NMR spectrum of compound **18** (100 MHz, CD_3OD)

BG-382-3D-F5-8.3.fid
Bruker AVANCE 400 carbon

Appendix II – Strain, host, collection site and preliminary antifungal screening results

Strain Code	Species	Host/Source	Collection Site	Bioassay Results*		Extract Mass (mg)
				<i>S. cerevisiae</i>	<i>M. violaceum</i>	
NB626-12A	<i>Thysanophora</i> sp.	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	1	53.4
NB625-9H	<i>Rhizosphaera kalkhoffi</i>	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	0	17.8
NB236-7B	cf. <i>Rosasphaeria</i>	<i>Picea mariana</i> endophyte	Grand Lake Meadow, NB	0	2	49.6
W64103	<i>Thysanophora</i> sp. 1a	Isolated from cave wall	Anticosti Island, QC	0	0	57.1
NB660	<i>Mollisia</i> sp.	on very old decorticated decayed hardwood log	Aylmer, QC	0	0	14.3
RS10-5F	<i>Phialocephala</i> sp. nov.	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	2	44.5
NB626-11B	<i>Teratosphaeria Bryochiton</i> sp.	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	0	10.3
DAOM63909	<i>Thysanophora</i> sp. 5			2	2	44.7
CBS612.84	<i>Meloderma desmazierei</i>	<i>Pinus ayacahuite</i> needle	Mexico	0	2	80.5

NB437-6B	<i>Dermateaceae</i> sp.	<i>Picea rubens</i> endophyte	Little Lepreau, NB	0	0	17.4
NB643	<i>Mollisia</i> sp.	Rotten hardwood log	Prescott-Russell, ON	2	2	28.8
NB625-5J	<i>Leotiomyces</i> sp.	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	0	11.2
RS10-14G	<i>Thysanophora</i> sp. 3	<i>Picea rubens</i> endophyte	Fundy National Park, NB	2	2	26.9
RS10-12A	<i>Thysanophora</i> sp.	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	2	54.2
06-236C	<i>Phaeomoniella</i> sp. nov. 1	<i>Picea rubens</i> endophyte	St. Martins, NB	0	0	19.3
NB625-7H	<i>Phialocephala</i> sp.	<i>Picea rubens</i> endophyte	Fundy National Park, NB	1	0	19.0
NB639	<i>Mollisia</i> sp.	on rotten hardwood branch	Orleans, ON	0	0	12.1
NB625-6C	<i>Phialocephala</i> sp.	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	2	18.6
NB365-71F	<i>Rhytismataceae</i> sp.	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	1	19.5
NB488-6B	<i>Lachnellula</i> cf. <i>subtillissima</i>	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	1	56.4
NB487-2H	<i>Trybliopsis</i> sp. nov.	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	2	318.0

NB627-9CC	<i>Rhizosphaera</i> cf. <i>pini</i>	<i>Picea rubens</i> endophyte	Fundy National Park, NB	2	2	129.0
NB371A	<i>Ombrophila</i> <i>hemiamyloides</i>	Isolated from apothecia on hardwood branch in stream	Fundy National Park, NB	1	0	12.4
NB285-4J	<i>Rhytismatales</i> sp.	<i>Picea rubens</i> endophyte	Doaktown, NB	1	2	16.2
NB625-9A	<i>Phialocephala</i> sp.	<i>Picea rubens</i> endophyte	Fundy National Park, NB	1	0	33.2
NB642	<i>Coccomyces strobi</i>	Dead corticated attached branches on living <i>Pinus</i> <i>strobus</i>	Prescott-Russell, ON	2	2	105.1
RS10-5G	<i>Thysanophora</i> sp.	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	2	43.1
NB437-10G	<i>Peziza varia</i>	<i>Picea rubens</i> endophyte	Little Lepreau, NB	0	0	7.6
NB505-9I	Unknown <i>Rhytismataceae</i> gen./sp.	<i>Picea rubens</i> endophyte	Fundy National Park, NB	1	1	109.9
NB625-10K	<i>Allantophomopsis</i> cf. <i>lycopodina</i>	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	0	5.0
NB437-8C	cf. <i>Gremmeniella</i>	<i>Picea rubens</i> endophyte	Little Lepreau, NB	2	0	55.7

NB464-1G	<i>Tryblidiopsis</i> sp. nov.	<i>Picea rubens</i> endophyte	Little Lepreau, NB	2	1	228.1
NB464-12E	<i>Leotiomyces</i> sp.	<i>Picea rubens</i> endophyte	Little Lepreau, NB	2	0	213.5
RS9-5D	<i>Phacidiales</i> sp. 5	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	2	76.0
NB467	<i>Phaeomollisia</i> sp. nov.	Fallen <i>Acer saccharum</i> branch	Little Lepreau, NB	0	2	38.7
RS10-10B	<i>Phacidiales</i> sp. 3	<i>Picea rubens</i> endophyte	Fundy National Park, NB	2	0	14.5
NB423	<i>Phialocephala</i> <i>dimorphospora</i>	Isolated from conidiophores on hardwood	Orleans, ON	1	2	115.1
NB623	<i>Xylaria</i>	Fallen <i>Acer saccharum</i> log	Fundy National Park, NB	1	0	25.9
RS10-8E	<i>Pseudoplectania</i> sp.	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	1	8.8
RS10-3B	<i>Lachnellula</i> cf. <i>calyciformis</i>	<i>Picea rubens</i> endophyte	Fundy National Park, NB	1	2	120.3
CBS114583	<i>Lophodermium</i> <i>piceae</i> (European strain)	<i>Picea abies</i>	Norway	2	0	49.0

NB589	<i>Thysanophora</i> sp.	Isolated from conidiophores on <i>Picea rubens</i> needle	Fundy National Park, NB	0	0	136.7
RS9-6A	<i>Phacidiales</i> sp. 1	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	0	43.6
RS10-5H	<i>Peyronellaea</i> cf. <i>pinodella</i>	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	0	9.7
RS10-2E	Gen. nov. cf. <i>Phoma</i>	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	1	23.2
NB437-10B	cf. <i>Gremmeniella</i>	<i>Picea rubens</i> endophyte	Little Lepreau, NB	0	0	17.4
RS10-3J	<i>Phacidiales</i> sp. 1	<i>Picea rubens</i> endophyte	Fundy National Park, NB	1	0	8.9
RS10-11C	Unidentified species	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	1	33.4
NB437-2A	<i>Pseudoplectania</i> cf. <i>nigrella</i>	<i>Picea rubens</i> endophyte	Little Lepreau, NB	0	0	9.5
RS9-5Q	<i>Phacidiales</i> sp. 4	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	2	63.4
RS17-1L	<i>Phacidiales</i> sp. 1	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	1	33.0

NB487-3K	<i>Mycosphaerella</i> cf. <i>berberidis</i>	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	1	20.1
RS9-7P	<i>Sclerotiniaceae</i> sp.	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	2	76.7
NB437-6A	<i>Tryblidiopsis</i> sp. nov.	<i>Picea rubens</i> endophyte	Little Lepreau, NB	0	2	173.1
06-313B	<i>Phaeomoniella</i> sp. nov. 2	<i>Picea rubens</i> endophyte	Parkindale, NB	0	0	25.4
RS9-10K	<i>Phacidiales</i> sp. 2	<i>Picea rubens</i> endophyte	Fundy National Park, NB	0	0	9.4
RS17-9W	<i>Thysanophora</i> sp.	<i>Picea rubens</i> endophyte	Fundy National Park, NB	1	0	38.0
DAOM 628553	<i>Diaporthe maritima</i>	<i>Picea rubens</i> endophyte	Alma, NB	0	2	89.5
NB236-1A	<i>Rhytismatales</i> sp.	<i>Picea mariana</i> endophyte	Gagetown, NB	2	2	143.4
DAOM 250862	<i>Pezicula sporulosa</i>	Needle isolations from 6cm DBH red spruce in yellow birch and red maple stand	Alma, NB	0	2	93.1
DAOM 250863	<i>Phaeocryptopus</i> cf. <i>gaeumannii</i>	<i>Picea mariana</i> endophyte	Doaktown, NB	0	2	582.3

*-0 indicates no zone of inhibition, 1 indicates a zone between 0.1-2cm, 2 indicates a zone >2cm.