**CHARACTERIZATION AND ANALYSIS OF SECONDARY METABOLITES
FROM FOLIAR FUNGAL ENDOPHYTES OF SPRUCE TREES**

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in partial fulfillment of the requirements for the degree of

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ABSTRACT

A collection of more than 2000 endophytes were isolated from natural white (*Picea glauca*) and red (*Picea rubens*) spruce trees located in New Brunswick, Nova Scotia and Maine, USA. A random selection of both white and red spruce endophytes were grown in one litre fermentations. Strains of interest due to their ability to grow and produce substantial amounts of secondary metabolites under laboratory conditions, were further screened by LC-MS. The extracts that showed interesting peaks in the chromatograms were tested for toxicity to spruce budworm (*Choristoneura fumiferana*) using diet incorporated bioassays and for antifungal properties to *Saccharomyces cerevisiae* using disc diffusion testing. The taxonomy of these strains that displayed toxicity were then examined using DNA sequencing to try and determine the identity of the endophytes present in the needles.

This study had two primary foci; the first was the characterization and structure elucidation of secondary metabolites from foliar fungal endophytes from both white and red spruce trees using MS and NMR. The work was highlighted by the discovery of two novel secondary metabolites, compounds **1** and **2a**; two new fungal metabolites, compounds **3** and **4**; and the known metabolites **5-10** from the three strains of white spruce endophytes studied in detail. Additionally, compounds **11** and **15** are reported as new structures, **12** and **13** as natural products for the first time, **14** is reported here for the first time as a fungal metabolite and **16-22** were identified as known fungal metabolites in new genera, from the six strains of red spruce endophytes examined. The second focus was on the analysis of the toxins and the presence of the endophyte in inoculated seedlings. Antibody based ELISA methods were developed for the detection of five

white spruce endophytes in needles, to provide a reliable method for determining inoculation success. Analytical methods were also developed using HPLC and LC-MS/MS in multiple reaction monitoring (MRM) mode to determine the presence and concentration of the characterized toxins in infected tree needles for three of the white spruce endophytes. The results indicated that needles of trees colonized with these endophytes contain the toxins. These toxins in turn are responsible for reducing the growth rate of spruce budworm larvae. The presence of the endophyte and its toxin is thought to increase the development time of larvae, put the populations out of reproductive synchrony and expose them to a greater risk of death from parasitoides, pathogens and predation.

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PUBLICATIONS

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Miller, J David; Cherid, Hafsa; Sumarah, Mark W; Adams, Greg W. **Horizontal transmission of the *Picea glauca* foliar endophyte *Phialocephala scopiformis*.** Fungal Ecology 2: 98-101. Sumarah, Mark W; Puniani, Eva; Blackwell, Barbara A, Miller, JD. **Characterization of Polyketide Metabolites from Foliar Endophytes of *Picea glauca*.** Journal of Natural Products (2008), 71(8), 1393–1398.

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Sumarah MW; Rand TG; Mason CD; Oulton M; MacDonald J; Anthes M. 1999. **Effects of *Stachybotrys chartarum* spores and toxin on alveolar surfactant phospholipid composition and concentration in mice.** In Johanning E (ed) Preceedings of the Third International Conference on Bioaerosols, Fungi and Mycotoxins. Eastern New York Occupational & Environmental Health Center, Albany, NY. pp. 444-452.

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CHAPTER I

GENERAL INTRODUCTION

Fungi

The kingdom Fungi includes three subdivisions; the Ascomycetes, Basidiomycetes and the Zygomycetes. Fungi form one of the largest groups of organisms of which some 100 000 are described species, with an estimated 1-1.5 million species in total (Hawksworth, 1991). This means that over 90% of all the fungi on earth have yet to be identified and classified. Many of these unidentified fungi are believed to be endophytes, meaning that they live within the plant. Fungi fulfil many roles including; in the production of alcoholic beverages and medicines such as penicillin, as food, biocontrol agents, recyclers of decaying matter, and as diseases of crops and animals. Fungi are heterotrophic organisms that are further classified as saprophytes, parasites or endophytes. The focus of this study will be on the last group, the endophytes.

In general, fungi are difficult to classify. Identifications are normally made by comparing morphological features, that vary with the culture technique used to prepare them (Söderström *et al.*, 1984). This had led to a great deal of work in the development of new methods for the identification of fungi. Methods currently being used include; ITS sequences, metabolite profiles and even lipid profiles (Boysen *et al.*, 2000; Dörge *et al.*, 2000). These advances have resulted in the separation of some species into new classes of fungi that can not be differentiated by even the best mycologists. Recently, the Assembling the Fungal Tree of Life (AFTOL) project in the USA, has provided a comprehensive overview of fungal taxonomy to the order level. This work is used as a baseline for identification of fungi using parts of the ribosomal repeat. However, many fungal groups including the endophytes remain poorly studied. Endophytes are especially difficult to identify using traditional methods. They lack sexual reproduction

structures and are non-sporulating in culture, resulting in their often poor characterization vs. other more common fungi. In recent years a push has been made to document in a database the ITS sequences of all isolated endophytes. This method is more definitive than using morphology alone but has its own pitfalls including the expense of the equipment required and the incorrect identification of strains deposited in databases.

Endophytes

The literal definition of endophyte is “within the plant” and it has been applied to many interactions involving algae, bacteria and fungi. The leaves of various plants including macroalgae, grasses, sedges and conifers are known to contain these symptomless infections. The definition used for the purposes of this project will be fungi that colonize plants without any visible symptoms of disease (Tan and Zou, 2001). Fungal endophytes consist of three major groups: mycorrhizal fungi, associated with plant roots, balansiaceous or the grass endophytes that occur above ground and the non-balansiaceous (Schulz and Boyle, 2005).

The grass endophytes are the most widely studied group and are the model for all other endophyte-host interaction. Grasses that are known to be infected by endophytes have been recognized to cause animal toxicoses since Biblical times (Clay, 1988). It is now well understood that toxic metabolites produced by grass endophytes greatly reduce populations of herbivorous insects attacking the plant. This has a large effect on plant fitness with a corresponding reduction of populations of herbivorous insects. The ability of the plant to defend itself against insect attack has a large effect on the survival of the

plant (Clay, 1988; Clay and Holah, 1999). The secondary metabolites produced by grasses include four classes of alkaloids: pyrrolizidines, ergot, indole diterpenes and pyrrolopyrazines (Figure 1.1). The ergot alkaloids and the indole diterpenes are responsible for the toxic effects observed in cattle and sheep; whereas, the pyrrolopyrazine and pyrrolizidine alkaloids are both insect deterrents (Bush *et al.*, 1997). Additionally, grass endophytes have been linked to increased tolerance to drought in the host plant. Grass endophytes are spread from the plant to the next generation via vertical transmission of the host seeds. They are found in both wild and cultivated grass and are very common in lawn and turf grass such as those used on golf courses. In tall fescue (*Festuca arundinacea*), it is known that there is a plant genetic component that determines the relationship with the fungus. Many endophyte-enhanced turf grass cultivars are registered in the USA and Canada and are used as a part of an integrated pest management strategy (Funk and White, 1997). Unlike man-made pesticides, these associations involve many toxins that act together to limit herbivory from a range of different defoliation insects. As noted above, endophyte-enhanced turf grasses are the dominant technology for lawns. Grass endophytes are not regulated in the US or Canada and their use has been promoted to reduce the amount of pesticides sprayed on golf courses and lawns.

Figure 1.1: Examples of common grass endophyte toxins; peramine, ergonovine and lolitrem B.

Carroll & Carroll (1978) first proposed that fungal endophytes recovered from coniferous needles might be mutualistic symbionts. They suggested a decrease in grazing by deforestation pests and a possible benefit for the host trees. An association between Douglas-fir and the needle endophyte *Rhizoctonia parkeri* has been demonstrated (Sherwood-Pike *et al.*, 1986; Todd, 1988). The fungal extract exhibited cytotoxicity to HeLa cells and resulted in reduced growth rates and mortalities when incorporated into synthetic diets of spruce budworm (Miller *et al.*, 1985). Royama (1984) published a comprehensive analysis of the population dynamics of the spruce budworm focusing on the period 1945 to 1983 in New Brunswick, Canada. One feature of this analysis was that he proposed a “fifth agent” referring to an unknown factor that was required to build models that best fit observed population changes. It was proposed that this unknown

factor may increase the response of the insect to known factors such as weather, predation and disease.

From 1984-1994 endophytes were collected from various conifers across NB. The needles of all mature conifers examined were colonized by several species of endophytes (Johnson and Whitney 1989; Wilson *et al.* 1994). Collections of endophytes from *Abies balsamea* (balsam fir), *Picea rubens* (red spruce), *Picea glauca* (white spruce) and *Picea mariana* (black spruce) were found to produce anti-insectan toxins (Calhoun *et al.*, 1992; Findlay *et al.*, 1995a, 1995b, 1997). One of the toxins, rugulosin (Figure 1.6), was obtained from cultures of *Homonema dermatioides*, derived from red spruce needles (Calhoun *et al.*, 1992). Subsequent studies determined that rugulosin was also produced by nonsporulating strains from white spruce. Rugulosin producers comprised about 20% of the toxigenic strains originating from NB conifer needles and two-thirds of strains originating from white spruce. It was shown that some strains of foliar endophytes of white spruce produce compounds that affect the survival of the spruce budworm *in vitro* (Calhoun *et al.*, 1992; Sumarah *et al.*, 2008a), under growth chamber conditions (Miller *et al.*, 2002) and under natural conditions (Miller *et al.*, 2008). These anti-insectan toxins reduce the growth rate of the insect and their resistance to parasites and natural bacterial pathogens.

The eastern spruce budworm (*Choristoneura fumiferana*) is one of the most economically-damaging insect pests in Canada (MacLean *et al.*, 2002; Royama *et al.*, 2005). The last time there was an epidemic in eastern North America, large-scale spraying of a chemical pesticide was undertaken at a level to prevent the trees from dying (Ireland, 1980). Approximately four million hectares were sprayed with a today's cost of

upwards of \$200 million. In the low period of the budworm cycle since the last epidemic, the chemical pesticides that had been sprayed have been subsequently deregistered for environmental and safety reasons (MacLean *et al.*, 2002).

Endophytes have also been shown to provide tolerance to white pine blister rust, an introduced pathogen that has nearly eliminated white pine in some areas of western Canada and the USA. White pine blister rust spreads by alternating between currant/gooseberry and white pine trees with infection via the needles. Blister rust then spreads into the branch and down the stem generally killing the infected tree within a few years. The impact of a warmer climate and the general push to fight forest fires has allowed pine beetles to survive over the winter months when they may normally have died off. These actions have resulted in more dead trees in a forest and an increased risk of insect epidemics.

Metabolism

Primary metabolism occurs in all organisms and is essential for growth and the existence of the organism. Betina (1995) described it as the sum of an interrelated series of enzyme-catalyzed chemical reactions that include processes such as glycolysis, Krebs cycle, amino and nucleic acid production. The intermediates and end products of these processes are called primary metabolites. These pathways result in the production of several primary metabolites and intermediates including acetyl-CoA, shikimic acid and mevalonic acid. These primary metabolites are then used in the production of secondary metabolites or compounds specific to the organism producing them. Acetyl CoA is

formed from the oxidative decarboxylation of pyruvic acid, via the glycolytic pathway and by the β -hydroxylation of fatty acids. Acetyl CoA is then used in the acetate pathway of secondary metabolism to produce phenols and fatty acid derivatives. Shikimic acid is produced by the combination of phosphoenolpyruvate (a glycolic pathway intermediate) and erythrose-4-phosphate (from the pentose phosphate cycle). It is then used in the shikimate pathway resulting in a variety of aromatic compounds including phenols, cinnamic acid derivatives, lignans and alkaloids. Mevalonic acid is formed from the condensation of three molecules of acetyl-CoA. The mevalonate pathway produces a large variety of terpenoids and steroids. Despite their diversity, secondary metabolites are typically produced from a few key intermediates of primary metabolism.

Secondary metabolism in fungi is a process that has received much debate. This process was best described by Demain (1989) when he stated that “It has always amazed me that the importance of secondary metabolites in ecological interactions between plant versus herbivore, insect versus insect and plant versus plant have been universally accepted, but their importance in microbial interactions has been almost universally denied”. Secondary metabolism is closely linked to primary metabolism and occurs in a wide variety of fungi and plants. Secondary metabolism is differentiated from primary metabolism in fungi by the fact that it usually occurs as a result of a stress on the organism such as a nutrient limitation (carbon, nitrogen, or even phosphorus). Additionally, secondary metabolism occurs if the fungus possesses the required genes (Miller, 1991).

The reason why the fungus produces these metabolites has been greatly debated. It was once believed that they were waste products of the cell, others proposed that they served no purpose and were only produced more or less “for fun”. It is now believed that they are probably not essential for growth, but they may play other roles such as cell regulators. The most widely accepted view today is that they are used for defense and their role has been labeled with the term “interference competition” (Janzen, 1977; Wicklow, 1981; Keller *et al.*, 2005). Fungi, plants and others organisms use these often-potent secondary metabolites as chemical warfare agents against other fungi, insects, bacteria and even animals.

Fungi occur in established niches where they live and continue to grow as long as nutrients are available. Problems occur when there is competition for the available nutrients and when these niches overlap. The competition for survival can take place in a variety of ways. The first is through interference competition which involves some form of behavioral or chemical interaction between the organisms before the resource is used (Wicklow, 1981). This form of competition among individuals influences the access of the competitor to the resource, thereby increasing its own probability of survival. The general mechanism of interference in fungi is antibiosis, and involves the use of toxic metabolites or antibiotics. Organisms also use exploration competition, this is where they deplete the resources without reducing the probability that others will compete for the remaining resources. Additionally, fungi colonize resources such as fruit, in an effort to make the resource unattractive to animals.

Fungal antagonism is a very common method of limiting competitor access to a resource. Early colonizers gain a foothold on the resources and then use the antibiotics and other

toxins they produce to slow and even prevent invasion by others. Further proof of the concept is provided from marine fungi where they have been shown to produce anti-fungal compounds with low water solubility. This idea is important because at first thought, they would simply leach out into the environment as they are produced (Miller *et al.*, 1985; Strongman *et al.*, 1997).

Fungal secondary metabolites are classified into four main groups: polyketides, terpenes, shikimate acid derivatives and peptides. Polyketides are similar to fatty acids as both proceed through the addition of C₂ units, with the exceptions that they contain alternating CO – CH₂ groups versus just CH₂ groups for fatty acids (Staunton and Weissman, 2001). They also have greater synthetic capacity, as evident by the large variety produced. Fungal polyketides are synthesized by type I polyketide synthases (PKSs), which are multi domain proteins that are similar to eukaryotic fatty-acid synthases. The active form of the first C₂ unit is the acetyl CoA with the subsequent C₂ units formed as malonyl CoA through carboxylation of acetyl CoA. Fungal PKSs are limited to one module, with which they can carry out repeated biosynthetic reactions. This differs from bacterial PKS type I enzymes that have separate modules for the addition of each methylmalonyl CoA (Staunton and Weissman, 2001; Hill, 2006; Keller *et al.*, 2005; Hoffmeister and Keller, 2006). The resulting straight chain can undergo various reactions including oxidation, reduction, methylation and rearrangement to form a variety of different compounds. The most common polyketides are derived from tetraketides, pentaketides, heptaketides and octaketides, leading to 8, 10, 12 and 14 carbon chain compounds respectively. After the initial cyclizations, polyketides undergo modifications, the most common of which are oxidations that lead to a wide variety of

secondary metabolites. These polyketides can occur as toxins, immunosuppressants, antibiotics or antiparasites. Examples of important fungal polyketides include zearalenone and aflatoxin (Figure 1.2) (Blackwell *et al.*, 1994; Steyn, 1998).

Figure 1.2: Aflatoxin, and zearalenone.

Terpenes are the second major class of metabolites produced by fungi. They are closely associated with the formation of sterols in primary metabolism with both using mevalonic acid as the key intermediate in their formation. Terpenes are characterized by addition of C₅ units and are classified accordingly as hemiterpenes (1 unit), monoterpenes (2 units), sesquiterpenes (3 units), diterpenes (4 units), sesterpenes (5 units) and triterpenes (6-C₅ units) (Keller *et al.*, 2005). Their biosynthesis involves the

intermediates isopentenyl pyrophosphate (IPP) and dimethylallylpyrophosphate (DMAPP) that are derived from mevalonic acid (MVA). C₅ units can be combined together, the most common being the condensation in a head to tail manner of a DMAPP molecule with a IPP to produce geranyl pyrophosphate (GPP), used for the production of the monoterpenes. This can be repeated through further reaction with IPP to form farnesyl pyrophosphate (FPP) which leads to the formation of the sesquiterpenoids. The process can be further repeated leading to GGPP and GFPP for the biosynthesis of diterpenoids and sesterterpenoids respectively. These processes result in the production of hundreds of structurally different secondary metabolites, the most economically important fungal metabolites in this class are the trichothecenes (Figure 1.3) (Steyn, 1998; Keller *et al.*, 2005).

Figure 1.3: Deoxynivalenol and T-2 toxin.

The shikimate pathway leads to the formation of the third major class of fungal metabolites. The biosynthesis of Shikimic acid begins with the condensation of erythrose-4-phosphate from the pentosephosphate cycle and phosphoenolpyruvic acid (PEP), from glycolysis. Shikimic acid is then used for the formation of important primary metabolites such as tryptophan, tyrosine and phenylalanine. These compounds are then used to form secondary metabolites such as roquefortine C (Figure 1.4), that is derived directly from tryptophan, histidine and dimethylallylpyrophosphate (Mantle *et al.*, 1983; Steyn, 1998; Keller *et al.*, 2005).

Figure 1.4: Roquefortine C.

The final class of fungal secondary metabolites are the peptides; these are the compounds derived from more than two amino acids. They can be formed from either a linear peptide, penicillin being a good example or cyclosporine which is a cyclic peptide (Figure 1.5).

Figure 1.5: Cyclosporin.

Fungi undergo a number of different phases throughout their life cycle. The first is the growth phase; this is defined by an increase in weight of the mycelium (dry). During this stage of laboratory fermentation, the organism is provided optimal conditions for growth with careful attention to pH, temperature, oxygen content and nutrients (Miller and Blackwell, 1986). The conditions for optimal growth vary among fungal species, but if provided, all fungi will undergo a similar growth stage. The fungal cells rapidly increase in number during this stage that corresponds to an increase in mycelium and also corresponds with the primary metabolism stage of the cells. If the optimal conditions could be provided indefinitely, the fungus would in theory continue in this stage forever.

The next stage occurs when one or more of the essential nutrients needed for growth are limited. Carbon, nitrogen and phosphorous are the most common limited resources (Wicklow, 1981). This stage is characterized by a decrease in the growth rate, as

determined by a slower increase or plateau in mycelium weight. This restriction on growth corresponds with an accumulation by the fungus of some primary metabolites. This is the point where secondary metabolism typically begins and the fungus utilizes these primary metabolites along with a series of enzymes to produce a wide variety of secondary metabolites.

The final stage involves the death of the cells, where the organism releases nitrogen and other nutrients back into the medium for consumption by other cells. The growth conditions obtained within a fermentor are unique because the cells are defined as being more or less “synchronous” meaning that all cells are at the same stage. This differs from still culture fermentations or in nature where fungal cells are at different stages at any given time.

The domestication and fermentation under laboratory conditions of fungal strains can lead to the production of different metabolites than are found from the organism in nature. In order to prevent this and to achieve optimal metabolite production in the laboratory, all factors that affect the growth and biosynthesis should be understood. It is difficult to achieve this optimal level of production, and if even one of the many conditions that affect growth and production of metabolites are not optimal, then the fermentation may not be successful.

The most important factor affecting the fermentation is the source of carbon used in the medium. Carbon provides the energy for the organism's essential processes, and it is also needed for synthesis of proteins, lipids and nucleic acids. Fungi that grow very rapidly may require a carbon source such as sucrose or maltose which is more difficult to process than glucose or fructose due to the complexity of the molecule. Other fungi

require a carbon source that can be metabolised more easily. Carbon is often the limiting factor in the fermentation, with limited secondary metabolism occurring until it has all been consumed by the organism. The use of malt extract with maltose as the carbon source was chosen for this study in an attempt to simplify and appeal to the potentially diverse species of endophytes isolated from spruce trees.

Nitrogen is the next important factor for secondary metabolite production; it is required for the biosynthesis of amino acids and metabolites derived from these primary metabolites. The carbon to nitrogen (C:N) ratio of the medium also influences secondary metabolite production (Miller and Blackwell, 1986). The source of nitrogen in the media can be ammonia, nitrates or nitrites, and even amino acids. Phosphorus is also a key nutrient consumed by fungi for production of DNA, RNA and ATP. Phosphorus can be from a number of sources, but the most common form is PO_4^{3-} . Oxygen is essential for all fermentations because filamentous fungi are aerobic. The oxygen tension in the growth medium affects the production of secondary metabolites. Trace metals also play important roles in the growth of many fungal species. The pH of the medium is important for fungal growth and secondary metabolism. It has been shown that a drop in pH can result in a complete stop of secondary metabolism (Blackwell *et al.*, 1985). Additionally, many reported compounds are sensitive to acid and require special attention during extraction to retain their original form.

Conifer endophytes have in recent years shown a pattern of protection for the host against deforestation. Studies have shown that endophytes isolated from conifer trees produce a number of secondary metabolites that are toxic to spruce budworm and other insects in bioassays (Miller *et al.*, 2002). It has also been shown that seedlings can be

inoculated with endophytes known to produce anti-insectan compounds such as rugulosin or vermiculin as a method of reducing the need for pesticides and protecting the tree against deforestation pests (Figure 1.6).

Rugulosin is an anthraquinone metabolite derived from acetate. It was first reported from *Penicillium rugulosum* (Bouhet *et al.*, 1976; Breen *et al.*, 1955) and has since been isolated from other species including many strains of conifer endophytes collected from eastern Canada. It is toxic to *C. fumiferana* larvae in an artificial diet (Calhoun *et al.*, 1992) and in needles in apparently similar concentrations (Miller *et al.*, 2002). Rugulosin has been reported as toxic to *Drosophila melanogaster* (Dobias *et al.*, 1980). It has shown low mammalian toxicity in mice and rats (Ueno *et al.*, 1971, 1980). Rugulosin was not cytotoxic to HepG2 cells (human hepatoma cells; Watts *et al.*, 2003). It has also been reported as an antibiotic to both Gram-positive and negative bacteria (Stark *et al.*, 1978) and is moderately antifungal (Breen *et al.*, 1955).

Vermiculin is a macrolide antibiotic also derived from the acetate pathway but composed principally of propionate units; it is characterized by its 16 membered macrocyclic lactone ring. Findlay (2003) reported that vermiculin was toxic to spruce budworm cells and it has also be reported to be cytotoxic to HeLa cells (Horakova *et al.*, 1976)

Figure 1.6: Rugulosin and vermiculin from conifer endophytes.

PROJECT AIM

The use of endophytes in grasses has been shown to be effective in preventing destruction of the plant by insects. The goal of this project was to use this grass / endophyte relationship as a model for study of the fungal endophytes of spruce trees. The first part involved the collection of a large sample of endophytes from Maine, Nova Scotia and New Brunswick. It was important for the collection to properly represent the biodiversity of endophytes and secondary metabolites present in the spruce trees from this area.

The next step was to isolate and characterize the secondary metabolites from strains that were screened based on DNA sequencing, toxicity to spruce budworm and metabolite production. These strains were grown in large scale fermentations from which secondary metabolites were isolated using a variety of techniques such as PTLC, column chromatography and HPLC. The characterization of the compounds requires analysis by LC-MS and NMR. The goal was to search for secondary metabolites other than the previously reported spruce budworm toxins vermiculin and rugulosin. The hope was to find strains of fungi that when used to inoculate spruce seedlings would provide protection to the tree against deforestation pests.

The last major goal of the project was to develop analytical methods for the detection of the endophytes and their toxins in inoculated spruce needles. The use of ELISA testing for the detection of the endophytes in needles was employed because of the ease of testing and the ability to further develop the technology for possible use in the

field. The analysis of the toxins in the needles required the development of methods using HPLC and LC-MS/MS.

CHAPTER II

ENDOPHYTE COLLECTION, GROWTH, SCREENING AND ANALYSIS

INTRODUCTION

Endophytes can be isolated from a wide variety of hosts, including algae, grasses and vascular plants. These endophytes can play important roles within the plant such as in defense or they can also cause harm to the host and in some cases, they appear to have only a very limited role. Recent evidence has shown that secondary metabolites produced by these endophytes may also play important roles in regulation of the host organism.

Grass endophytes are the best understood example of a beneficial symbiotic relationship with the host. These endophytes are found in both wild and cultivated grasses. Of note in grasses is the close relationship between the grass and the fungal endophytes as evident by the transmission of the endophytes in the seeds via vertical transmission. This interaction results in an almost 100% transmission of the endophyte to next generation. As previously noted some of these fungal endophytes produce metabolites that are used to help protect the host plant. They were originally discovered because of the toxicoses observed in animals grazing on these grasses. This was evident by reductions in weight gain, reduced milk production and other related problems. It is now well understood that toxic metabolites produced by grass endophytes also reduce destruction of the plant by herbivorous insects. This has a large effect on the health and prevalence of the plant (Clay, 1988; Clay and Holah, 1999). This has led to the widespread use of endophyte enhanced turf grass cultivars in Canada and the USA as a component of pest management.

It was first proposed by Carroll & Carroll (1978) that a similar relationship may occur in conifer trees. It was suggested that endophytes found within the needles may

decreased antagonism of herbivorous insects towards the host trees. Collections of conifer endophytes were made in Quebec and New Brunswick between 1984 and 1994. The endophytes present in needles were isolated, cultured and the secondary metabolites produced were isolated and characterized. From these collections of balsam fir, red spruce, white spruce and black spruce a number of anti-insectan toxins (Calhoun *et al.*, 1992; Clark *et al.*, 1989; Findlay *et al.*, 1995, 1997). The two major toxins identified from this work were rugulosin and vermiculin. The endophytes that produce them are currently being used to infect nursery seedlings as a means of helping to protect conifers in the forest. The search to find more strains that produce similar anti-insectan toxins is required to help increase the endophyte biodiversity in the reforested areas of Maine, NS and NB.

RESULTS and DISCUSSION

Toxicity Testing - Bioassays

The results of the diet incorporated bioassays testing for each extract of the red and white spruce endophyte against laboratory bred spruce budworms are displayed in Tables 2.1 and 2.2. The use of CH₂Cl₂ and plain diet as negative controls and rugulosin as a positive control were employed in this study. Each insect was weighed and its head capsule width was determined. Statistical analysis was performed using ANOVA, Fisher's LSD (SYSTAT v 10.2; San Jose, CA, USA) (Sumarah *et al.*, 2008). Extract toxicity against spruce budworm was shown in 16 of the red spruce and 10 of the white spruce strains tested. This was evident by reduced body weight, reductions in head

capsule size or both. We have postulated that significant differences in both of these important indicators of spruce budworm development are evidence that the presence of these endophytes and their toxins in the needles might put cohorts of spruce budworm out of reproductive synchrony and impact their ability to destroy trees.

Table 2.1: Bioassays of white spruce endophyte extracts with reductions in larval weight / head capsule size.

white spruce code	larval weight P=	head capsule width P=
05-037A	0.000	0.076
06-486D	0.010	0.000
06-485A	0.090	0.055
04-052B	0.028	0.058
06-011B	0.004	0.038
06-011D	0.025	0.038
06-012C	0.026	-
06-013A	0.029	-
06-014C	0.002	-
08-040B	0.013	-

Table 2.2: Bioassays of red spruce endophyte extracts with reductions in larval weight / head capsule size.

red spruce code	larval weight P=	head capsule width P=
06-264A	0.054	0.082
06-332A	0.057	0.026
06-268A	0.009	-
07-013D	0.008	-
08-011D	-	0.073
01-002A	0.082	-
04-002G	-	0.058
03-020B	0.070	0.078
04-012A	0.052	-
06-063D	0.011	0.039
02-002C	0.079	-
06-073C	0.008	-
06-094E	0.034	0.015
06-255A	0.015	0.016
06-097D	0.015	-
08-018A	-	0.019

Disc diffusion toxicity screening was also employed to test selected extracts for anti-fungal activity to *Saccharomyces cerevisiae*. The strains tested with anti-fungal activity were the three white spruce strains 05-037A, 06-486D and 06-485A. All three strains showed 1-1.5mm zones of inhibition that remained for well in excess of 10 days. The red spruce endophyte 06-097D also showed strong antifungal activity as indicated by 1.5-2mm zones of inhibition.

DNA Sequencing

The results of the DNA sequencing for the strains of interest are presented in appendix B and C. Cluster diagram B is a representation of the relationships for the ITS (internal transcribed spacer) sequence of each strain tested. Cluster diagram C presents the ITS sequences of many known fungi from all subdivisions to test the alignment of the endophyte sequences examined with known species. The ITS sequence and the closest identification matches for the strains listed in the Tables 2.1, 2.2 that displayed toxicity to spruce budworm are presented in appendix D. The results of the DNA sequencing and alignments for the 5 white spruce and 6 red spruce strains that the secondary metabolites were characterized from are listed below. All ITS sequences were edited to begin with the first occurrence of “CATTA”, the start of the primer chosen. The primer sits near the end of the 18S and therefore catches 10-20 bases usually at the end of the 18S. Sequences were also trimmed to this point for ease of use of the alignment algorithms.

DAOM 229536 (5WS22E1) - *Phialocephala scopiformis* (99% ITS similarity) (Kowalski and Kehr, 1995; Gruenig, personal communication) was reported as one of the most frequently isolated taxa from outer bark of *Picea abies* branches (Barklund and Kowalski, 1996). In addition, *P. scopiformis* was detected in spore traps laced in boreal forests (Kausrud *et al.*, 2005).

DAOM 229535 (5WS11I1) – Unidentified species of *Phialecephala*, closely related to *Phialocephala glacialis* (Menkis *et al.*, 2004; Gruenig, personal communication).

CBS 120381 (05-037A) – The ITS sequence (98% similarity) shows that it is in the *Xylariaceae* and close, but not identical to *Nemania serpens* (Vasiliauskas *et al.*, 2005, Sanchez-Ballesteros *et al.*, 2000).

CBS 120380 (06-485A) – Is a species of *Lophodermium* (98% ITS similarity) that is most closely related to unidentified fungal species isolated from black spruce in Quebec, Canada (Higgins *et al.*, 2007, Ganley and Newcombe, 2006).

CBS 120379 (06-486D) – Is a *Lophodermium* species (94% ITS similarity) and most similar to fungi in the *Rhytistimataceae*. The sequencing data indicate that it is most closely related to unidentified fungal species isolated from black spruce in Quebec, Canada (Higgins *et al.*, 2007, Ganley and Newcombe, 2006).

CBS 121942 (06-097D) - According to the DNA sequence (poor similarity) it is most closely related to *Pleopsidium chlorophanum* (Reeb *et al.*, 2007).

CBS 121944 (06-073C) – The ITS sequence shows that it is most similar to *Phaeoacremonium chlamydosporum* / *Phaeomoniella chlamydospora* (Groenewald *et al.*, 2001).

CBS 121943 (06-255A) and DAOM 239830 (06-268A) have virtually identical ITS sequences and morphology; both are species of *Mycosphaerella* (Crous *et al.*, 2007).

DAOM 239831 (06-063D) – The ITS sequence (94% similarity) to *Dwayaangam colodena* (Sokolski *et al.*, 2006).

DAOM 239833 (08-018A) - The ITS sequence (99% similarity) shows that it is most closely related to *Dwayaangam colodena* from streams in Quebec boreal and

mixed-wood forests, where it was shown to be a new aquatic hyphomycete species (Sokolski *et al.*, 2006).

The results of the initial DNA sequencing indicated a number of important points. First, it is clear that many of these fungi isolated are either very poorly classified or belong to the 90% group of undescribed fungal species proposed by many mycologists. It also highlights the difficulty in providing concrete identifications of the individual endophytes because of the wide diversity of species present in conifer trees. Finally it is evident that more work is required before we can confidently identify the species and groups studied. All of which adds to the difficulty of characterizing the secondary metabolites from these species, due to the limited availability of literature and reference standards. On the other hand, the probability of finding new compounds from strains of endophytes such as the ones isolated for this study is much higher than for the more commonly occurring and well described fungal species found on crops or in buildings. In other words, the productivity index for secondary metabolite discovery from endophytes is much higher than for other fungi. Finally rugulosin, the major metabolite isolated from a number of strains from the early work was not isolated or detected by LC-MS from any of the strains examined in the current study.

Figure 2.1: Flow chart outlining the screening process from 2250 endophytes down to the 9 used for metabolite isolation and characterization.

EXPERIMENTAL

Endophyte collection

The collection of branches from mature, superior natural trees from the forests of Maine, Nova Scotia and New Brunswick was conducted by a professional forester. Branches were selected from 648 *Picea rubens* (red spruce) trees and from 201 *Picea glauca* (white spruce) trees. They were immediately packed in ice and transported to the frozen storage facility located in Sussex, NB. The GPS coordinates of each tree was recorded so that they could be re-sampled in the future. The sample collected from each

tree was also given a code based on its location and the tree number from that area. Individual branches were then brought into the laboratory where 20 needles were removed and surfaced-disinfested by dipping in 70% ethanol for 1 min. The needles were then transferred to a 5 % sodium hypochlorite solution for 5 min. The needles were rinsed in sterile distilled water for 1 min and blotted dry on sterile tissue. The 20 needles were then placed in a sterile Petri dish and cut into two segments with the needle half that was attached to the branch (not the end with the point) plated on 2% malt-extract (ME) agar with equal spacing to allow growth of the endophytes. The plates were then incubated at 20° C and were examined each day to look for growth from within the needle. All fungi that grew from inside the cut end of the needle were then transferred to 2% malt-extract agar plates and allowed to grow for a further few weeks. At this point each plate was then examined and a determination was made as to whether each isolate was likely an endophyte or a contaminant such as common phylloplane fungi that are often present on needles. All that were determined to be endophytes by visual examination were then transferred to 2% malt-extract agar slants with screw caps for storage at 5° C and further analysis. The plating of all the white and red spruce branches collected resulted in the isolation of more than 250 white spruce endophytes and 2000 red spruce endophytes.

Endophyte Screening

Fermentation, Extraction and Analysis

A random selection of 125 white and 150 red spruce endophytes were chosen for further growth and analysis. The samples were selected to ensure that all morphology types were represented, and care was taken to make sure that endophytes collected from all 9 geographic locations in NS, NB and Maine were included. Each endophyte was first grown in still culture using a 250 mL Erlenmeyer flask containing 50 mL of 2% ME to ensure that it would grow in liquid culture and to help reduce potential contamination. For larger scale culture, 1 cm² of each endophyte was cut from the leading edge of the culture plate and mascerated in 30 mL of sterile water. The flasks were then inoculated with 2.5 mL (5%) of this solution using a sterile pipette. The flasks were blocked with a foam plug and allowed to grow for one week at 20 °C in darkness. After 7-10 days the flasks that showed the best growth and were free of contamination were then used to inoculate (5%) 1L Glaxo bottles containing 2% ME medium. They were grown for 3 months at 20 °C in darkness. The cultures were then harvested and filtered by vacuum using filter paper (Whatman #4) and a large Buchner funnel. The mycelium was frozen for later processing and the filtrate was extracted with 2 x EtOAc and dried by rotary evaporation, taken back up in a few mL of EtOAc and dried under N₂. The extracts were then weighed and dissolved in 1 mL of CH₃CN for analysis by HPLC. A 5 µL sample was tested by HPLC to determine if the endophyte produced any metabolites that may be of interest. Initial HPLC separation and preliminary screening of the extracts was performed on an Agilent 1100 system using a Phenomenex Synergi Max-RP column (250 mm x 4.6 mm) and a CH₃CN/H₂O gradient (100% water to 100 CH₃CN over 18 min with

a 2 min hold at 100% CH₃CN and return to the start conditions), this method was suitable for both subsequent LC-MS and LC-NMR analysis. The initial screening eliminated a number of the strains that did not produce high yields of cell mass under laboratory conditions or they had a very poor metabolite yield after 3 months. The screening process produced 24 white spruce endophytes and 49 red spruce endophytes that grew well in liquid culture and produced a substantial amount of extract that consisted of numerous HPLC peaks. These white and red spruce endophytes extracts were subjected to further analytical and toxicity screening to help determine which should be the focus of metabolite characterization.

Liquid Chromatography-UV-High-Resolution Mass Spectrometry

The 24 white and 49 red spruce endophyte extracts were then subjected to LC-MS analysis using 1-5 μ L of each extract per injection. The analysis was performed on an Agilent 1100 system equipped with a diode array detector (DAD) and a (50 mm x 2 mm), 3 μ m, Luna C18 II column (Phenomenex, Torrance, CA). The LC system was coupled to a Waters-Micromass (Manchester, UK) LCT orthogonal time-of-flight mass spectrometer, with a Z-spray electrospray ionization (ESI) source and a LockSpray probe. The LC system was operated using a H₂O/CH₃CN gradient systems starting from 5% CH₃CN and increasing to 100% CH₃CN in 16 min and maintaining for 5 min. Samples were analyzed in ESI⁺ mode at a resolution of >6500 using two scan functions with different cone potentials. The water was buffered with 10 mM ammonium formate and 20 mM formic acid and the CH₃CN with 20 mM formic acid. Samples were also analyzed in ESI⁻ mode at a resolution of >5000 using two scan functions. The CH₃CN was

unbuffered and water was buffered with 100 $\mu\text{L/L}$ formic acid. Leucine enkephalin was used as lock mass in both polarities to calibrate the instrument.

Analysis of LC-DAD-MS data

The UV, ESI+ and ESI- spectra from peaks not observed in blank media samples were matched against the Antibase 2008 (Wiley & Sons, Hoboken, NJ) database of known fungal and bacterial metabolites. The majority of metabolites present were not available as reference standards and required extensive deconvolution of the data by plotting all ions detected from background-subtracted MS spectra. Extracted ion chromatograms (m/z 0.01) were also reconstructed for the following ions; ESI+ data: $[M + H - H_2O]$, $[M + H]$, $[M + NH_4]$, $[M + Na]$, $[M + CH_3CN]$ and $[M + CH_3CN + Na]$, ESI- data $[M - H]$ and $[M + HCOO]$. The resulting high resolution mass data was used to determine the chemical formula of the major metabolites present in the extracts and were subsequently searched using SciFinder and the ACD database of fungal and bacterial metabolites.

Toxicity testing

Each extract was tested in diet-incorporated bioassays using disease-free *C. fumiferana* (purchased from Natural Resources Canada, Sault Ste. Marie, Ontario). The extract of each endophyte was dissolved in CH_2Cl_2 and incorporated into the spruce budworm synthetic diet at 400 $\mu\text{g/g}$ and allowed to evaporate overnight (Clark *et al.*,

1989). Ten second instar *C. fumiferana* larvae were added to each of 3 cups of diet prepared per strain. The test cups were held in a growth chamber at 25 °C and 55% relative humidity with 16 h light/day until the insects reached the fifth instar as determined by head capsule size (approximately 1 week). Each insect was then weighed and its head capsule width was determined. Statistical analysis was performed using ANOVA, Fisher's LSD on each with rugulosin as a positive control and CH₂Cl₂ as a negative control (SYSTAT v 10.2; San Jose, CA, USA).

Additionally, disc diffusion assays were employed for preliminary antifungal testing on a number of the strains. Dried endophyte extracts (50 mg) were dissolved in 1 mL of CH₂Cl₂ and 10 µL was pipetted onto each small filter under aseptic conditions and left over night to allow for solvent evaporation. The discs were then carefully placed onto 2% malt extract agar plates that were inoculated with a lawn of *Saccharomyces cerevisiae*. The plates were incubated at 25 °C and the resulting inhibition zones measured (Vincent and Vincent, 1944).

Sequencing / Fungal Identifications

Preliminary taxonomic identifications were made by extracting DNA from the fungal cultures growing on 2% MEA plates using the step by step procedure outlined in the UltraClean DNA Isolation Kit (MO BIO Laboratories 12224-250). The PCR and sequencing work was done by Laboratory Services, University of Guelph, Guelph, Ontario. The PCR primers used were ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') and ITS1F (5' CTT GGT CAT TTA GAG GAA GTA A 3') (White *et al.*, 1990). The

PCR fragments were subsequently sequenced and aligned using MAFFT (Katho *et al.*, 2002). They were compared to each other and the sequences of known fungi with phylogenetic analysis using parsimony (PAUP, Version 4. Sinauer Associates, Sunderland, Massachusetts). Individual sequences were also BLAST searched using the BLASTN algorithm against the NCBI nucleotide collection database to search for homologous sequences.

CHAPTER III

ISOLATION AND STRUCTURE ELUCIDATION OF SECONDARY METABOLITES FROM WHITE AND RED SPRUCE ENDOPHYTES

INTRODUCTION

The search for new bioactive compounds is of great importance for use by the pharmaceutical and agricultural industries. Toxicity screening against ecological outcomes (chemical ecology) as described in chapter II, is gaining popularity as the primary approach to drive forward the discovery of novel metabolites. It has been shown for endophytes that their productivity index for secondary metabolite production is much higher than for other more common fungi, especially with regard to novel compounds. The discovery of novel secondary metabolites from these species is of importance to mycology, forestry and chemistry.

There are a large variety of methods available for the analysis of fungal metabolites. The majority of today's methods involve the use of HPLC combined with mass spectrometry but few are effective at determining unknown compounds from fungal extracts. This has led to the development of new hyphenated methods that involves the coupling of several techniques including; LC, MS, NMR and SPE (solid phase extraction). These methods were originally developed for the pharmaceutical industry for the detection of trace or labile compounds, but they can be equally employed in the study of natural products. The need for rapid screening of fungal extracts is of the utmost importance to avoid spending countless hours isolating known or nontoxic compounds. In addition, the higher resolution and better experimental techniques available on current NMR spectrometers permit easier structure determination on much smaller quantities than was available in the past.

Structure elucidation of natural products involves a number of important steps that are employed in the current project. The first step is to grow fungi under the

conditions required to produce secondary metabolites, the key elements of which are described in chapter II. These then need to be carefully extracted and isolated in order to obtain relatively pure compounds. The first step is to acquire a high resolution mass spectrum (HRMS) of the compound. This provides the data required to determine the chemical formula and it also provides the number of double bond equivalents (rings and double bonds). NMR can then be used as the major structure elucidating tool. Each isolated compound is subjected to a complete NMR workup using a solvent carefully chosen so that the compound dissolves, is stable and allows for the observation of all spectral peaks. The use of solvents such as deuterated chloroform or benzene are not always optimal for compounds containing many aromatic peaks because of the location of the solvent peaks in the proton spectrum. In other situations, such as the analysis of sesquiterpenes that contain multiple CH₂ peaks and no aromatic peaks, the use of benzene is suitable. The primary NMR analysis involves the acquisition of ¹H and ¹³C spectra to help determine the number proton and carbon atoms present. Additionally, it provides some insight into type of protons or carbon atoms. The HRMS may be required to provide the total number of protons, this is important because exchangeable protons are often not observed by NMR. The use of integration will indicate which of the spectral peaks are CH's, CH₂ and CH₃'s. The NMR experiment most commonly used is the COSY experiment which provides the ¹H to ¹H connectivity through J_{H-H}. This information is useful for determining CH₂ groups and also adjacencies in the structure. The next set of NMR experiments involves the interaction of ¹H and ¹³C atoms in the compound. The use of HSQC/HMQC provides the direct ¹H and ¹³C correlations which allows for the connection of those proton and carbons. The HMBC experiment provides the long range interactions between ¹H and adjacent ¹³C atoms in the structure. The

HMBC is also useful for determining the presence of quaternary carbon atoms. In certain types of structures, there are insufficient protons to determine all of the quaternary carbons through HMBC connectivity. In these cases, there is a requirement for a high sensitivity ^{13}C direct observe experiment. The above listed experiments are usually adequate to elucidate the basic backbone of the structure but often do not address the relative stereochemistry of the molecule. In order to address this important structural feature of the compound the use of coupling constants and NOE experiments (eg NOESY) are required. The $^1\text{H} / ^1\text{H}$ coupling constants ($J_{\text{H-H}}$) measures the strength of the interaction between protons in the structure and can help with determining a *cis* vs. *trans* double bond, for example. The NOESY NMR data provide ^1H to ^1H interactions through space and combined with a model of the structure are used to determine the 3D orientation of the constituents of the structure. In some instances the use of other tools such as x-ray crystallography are required to determine absolute stereochemistry, but this requires substantial material and the ability to get it to crystallize.

RESULTS and DISCUSSION

Secondary Metabolite Isolation and Characterization

White Spruce Endophytes

In order to identify the toxic components, the crude EtOAc extract of each strain was first screened by LC-MS and LC-NMR and the major components were purified by column chromatography and/or preparative TLC (PTLC). In some cases, the extract was methylated with diazomethane in order to facilitate separation or to help solve structural issues. Structures were elucidated primarily by analysis of MS and NMR data, and confirmations were made by comparison with published data.

CBS 120380

Compound **1** was isolated as a white solid; HRMS m/z 208.0725 (calculated for $C_{11}H_{12}O_4$, 208.0736). EIMS m/z 208 $[M]^+$ (62), 207 (18), 194 (12), 193 (100), 190 (38), 165 (58), 162 (31), 161 (16), 135 (29), 43 (34). The 1H NMR resonances showed two OMe functions at δ 3.87 and δ 3.93, two *meta*-coupled aromatic protons at δ 6.38 ($J=1.8$ Hz, H-4) and δ 6.40 ($J=1.8$ Hz, H-6) and a secondary hydroxy function at δ 5.36 with an adjacent methyl at δ 1.56. The data are consistent with the structure for 5,7-dimethoxy-3-methylphthalide (Harris *et al.*, 1988; Watanabe *et al.*, 1989) (Figure 3.1). The ^{13}C chemical shift assignments are reported for the first time (Table 3.1). Compound **1** was previously reported as a synthetic product in the synthesis of key intermediates for mycophenolic acid (Watanabe *et al.*, 1989), in the selective nuclear lithiation of aromatic compounds (Uemura *et al.*, 1975) and the synthesis of pretetramides (Harris *et al.*, 1988).

It is of unknown toxicity and is reported here for the first time as a fungal metabolite.

The configuration at C-3 was not determined.

Figure 3.1: 5,7-dimethoxy-3-methylphthalide (**1**).

Table 3.1: ^1H (500 MHz, CDCl_3) and ^{13}C NMR (175 MHz, CDCl_3) for compound (**1**).

Position	δ_{H} (J, Hz)	δ_{C}
1		168.4
3	5.36 q (6.7)	76.2
3a		156.3
4	6.38 d (1.8)	98.7
5		166.7
6	6.40 d (1.8)	97.2
7		159.6
7a		106.6
5-OMe	3.93 s	56.0
7-OMe	3.87 s	55.9
3-Me	1.56 d (6.7)	20.5

Compound **2** was isolated as a white solid, HRMS m/z 138.0681 (calculated for $\text{C}_8\text{H}_{10}\text{O}_2$, 138.0681). EIMS m/z 138 $[\text{M}]^+$ (25), 124 (16), 107 (100), 91 (10), 77 (14), 69 (11), 55 (14), 43 (22), 41 (17). The ^1H NMR data (CDCl_3 , 400 MHz): δ 7.08 (d, $J=8.4$ Hz, 2H), 6.77 (d, $J=8.8$ Hz, 2H), 3.81 (t, $J=6.4$ Hz, 2H), 2.79 (t, $J=6.6$ Hz, 2H) agreed with the recently reported literature data for tyrosol (Takaya et al., 2007, Brenna *et al.*, 2003). Tyrosol is produced by a large variety of organisms including from *Xylaria*

longipes (Schneider *et al.*, 1996) and from the stomata of *Epichloe typhina* (Koshino *et al.*, 1988). Tyrosol is the only compound isolated from 06-485A not of polyketide origin, it is derived biosynthetically from tyrosine.

Figure 3.2: Tyrosol (**2**).

Compound **3** was isolated post treatment of the extract with diazomethane as a white solid. HRMS m/z 236.1059 (calculated for $C_{13}H_{16}O_4$, 236.1049). EIMS m/z 236 $[M]^+$ (83), 220 (34), 193 (42), 178 (57), 177 (33), 176 (34), 165 (48), 164 (65), 152 (100), 150 (36), 137 (34). The 1H NMR spectrum was in agreement with the published data for 3,4-dihydro-8-hydroxy-6-methoxy-(3*R*)-propylisocoumarin (Bohlmann *et al.*, 1984; Uchida *et al.*, 1998). The chirality at the C-3 position was determined to be *R* ($[\alpha]_D^{24} - 30$ (c 1, $CHCl_3$)) by comparison to the published data for both the *R* and *S* synthetic compounds (Uchida *et al.*, 1998). The ^{13}C NMR chemical shift assignments are reported for the first time (Table 3.2). LC-MS data on the pre-treated extract for **3** determined the mass to be 236, which is identical to the mass of the isolated compound. The LC-NMR analysis on the pre-treated extract also shows an identical 1H NMR spectrum to that of the isolated compound, including the presence of the OMe function at C-6 prior to treatment with diazomethane. This evidence proves that treatment of the extract with diazomethane had no effect on the isolated compound **3** which is identical to the natural

product produced by the endophyte. Compound **3** was previously isolated from the roots of *Solidago multiradiata* (Bohlmann *et al.*, 1984) with no reported configuration at C-3 and was later synthesized as both enantiomers (Uchida *et al.*, 1998). It is reported here for the first time as a fungal metabolite and is of unknown toxicity.

Figure 3.3: 3,4-dihydro-8-hydroxy-6-methoxy-(3*R*)-propylisocoumarin (**3**).

Table 3.2: ^1H (500 MHz, CDCl_3) and ^{13}C NMR (175 MHz) for compound **3**.

Position	δ_{H} (J, Hz)	δ_{C}
1		169.8
3	4.51 m	78.9
4	2.86 dd (16.2, 11.0) 2.81 dd (16.2, 4.4)	36.8
4a		141.0
5	6.34 d (2.3)	101.8
6		165.7
7	6.23 d (2.3)	99.4
8		164.5
8a		106.2
8-OH	11.22 s	
OMe	3.80 s	55.5
Me	0.99 t (7.5)	13.8
9	1.55 m 1.48 m	33.2
10	1.84 m 1.66 m	18.1

Compound **4b** was obtained as a colourless liquid following treatment with diazomethane, in an attempt to improve compound separation. The formula was determined to be $C_{12}H_{14}O_5$, HRMS m/z 238.0854 (calculated 238.0841) and the observed strong IR absorption at 1724 cm^{-1} confirmed the presence of ester groups. EIMS m/z 238 $[M]^+$ (37), 223 (92), 208 (18), 207 (100), 165 (5), 161 (9), 142 (7), 106 (6), 63 (5). ^1H NMR spectrum for **4b** contained two aromatic *meta*-coupled protons at δ 6.83 ($J=2.1\text{ Hz}$, H-3) and δ 6.62 ($J=2.1\text{ Hz}$, H-5), three methoxy singlets at δ 3.90, 3.88, 3.85 and one methyl singlet at δ 2.53. The ^{13}C NMR data for **4b** revealed a ketone (δ 198.2) and an ester carbon (δ 168.1) assigned to C-8 and C-7 respectively. A tetrasubstituted benzene ring system was evident from the six aromatic carbons, of which two were methines [δ 105.7 (C-3) and δ 101.7 (C-5)] and four were quaternary [δ 115.6 (C-1), 138.5 (C-2), 161.5 (C-4) and 158.4 (C-6)]. The high-field chemical shifts for C-4 and C-6 indicated that they were both oxygenated aromatic carbons. The presence of three methoxy groups (δ 56.3, 55.7, 52.3) and one methyl (δ 28.7) were also confirmed. The protonated carbons were assigned based on HSQC correlations, and the connectivity was confirmed by the analysis of key HMBC correlations (Table 3.3). Both of the aromatic protons H-3 and H-5 showed cross-peaks to C-1 and C-4 as well as to each other, suggesting that the methoxy ester substituent was located at C-1 while one methoxy group was at C-4. H-3 exhibited further correlations to C-8 (ketone), and the cross-peaks between the acetoxy methyl (8-Me) and C-2 established the connection of the acetoxy substituent to C-2. H-5 was further correlated to C-6, and the cross-peak between C-6 and the second aromatic methoxy (6-OMe) were consistent with the proposed structure for 2-acetyl-4,6-dimethoxybenzoic acid methyl ester (**2b**). Although **4a** was not isolated, its structure was deduced from the isolation and characterization of the methylated compound **4b**. LC-MS

data for **4a** (from the untreated extract) provided a mass of 224 corresponding to a difference of 14 mass units from **4b** (isolated compound). This mass difference of 14 shows that only one functionality, the free acid was methylated, corresponding to a loss of one proton and the addition of a methyl function. Further concrete evidence is provided by the LC-NMR data for **4a** which clearly shows two aromatic protons, one methyl and only two OMe functions vs. the three OMe's observed in the isolated compound **4b**. Thus the structure of the natural product **4a** was determined to be 2-acetyl-4,6-dimethoxybenzoic acid.

Figure 3.4: 2-acetyl-4,6-dimethoxybenzoic acid **4a**; methyl ester **4b**.

Table 3.3: ^1H (500 MHz, CDCl_3) and ^{13}C NMR (175 MHz) for compound **4b**.

Position	δ_{H} (J, Hz)	δ_{C}	HMBC
1		115.6	
2		138.6	
3	6.83 d (2.1)	105.7	C1, C4, C5, C8
4		161.5	
4-OMe	3.88 s	55.7	C4
5	6.62 d (2.1)	101.7	C1, C3, C4, C6
6		158.4	
6-OMe	3.85 s	56.3	C6
7		168.1	
7-OMe	3.90 s	52.6	C7
8		198.2	
8-Me	2.53 s	28.7	C8, C2

CBS 120379

The xanthone derived compound **5** was isolated as a white solid, whose composition $\text{C}_{16}\text{H}_{12}\text{O}_6$ was deduced by HRMS m/z 300.0662 (calculated, 300.0634), ^{13}C and ^1H NMR data analyses (Table 3.4). EIMS m/z 300 $[\text{M}]^+$ (21), 269 (21), 268 (100), 161 (9), 149 (18), 142 (7), 57 (11), 55 (8), 51 (7), 43 (11), 39 (10). Broad IR absorptions at 3363, 1738, and 1644 cm^{-1} indicated hydroxy and carbonyl groups respectively. The ^1H NMR spectrum of **5** displayed two downfield D_2O exchangeable singlets at δ 12.39 and at δ 7.75 attributed to the phenolic protons at C-8 and C-2 respectively. Four aromatic protons consisting of a one-proton singlet at δ 7.38 (H-4) and three vicinal protons as an AMX spin system at δ 7.57 (t, $J=8.0$ Hz, H-6), δ 6.90 (d, $J=8.1$ Hz, H-5) and at δ 6.79 (d, $J=8.0$ Hz, H-7) were also present. Additionally, a methoxy singlet at δ 3.99 and an aromatic methyl singlet at δ 2.43 were observed. These ^1H NMR chemical shifts were similar to that of mycoxanthone (however, disubstituted in ring C), previously isolated from *Mycosphaerella rosigena* (Gemma *et al.*, 1979). It is also comparable with

reported data typical of the xanthone skeleton (Barracclough *et al.*, 1970) and xanthone analogues reported from the endophytic fungus *Xylaria sp.* FRR 5657 (Davis and Pierens, 2006) the macrofungus *Xylaria sp.* (Healey *et al.*, 2004) and *Leptographium wagneri*. (Ayer *et al.*, 1989). The ^{13}C NMR spectrum of **5** revealed a xanthone carbon at δ 180.4, a polyaromatic system with 12 signals resonating between δ 106.8-161.4, a methyl at δ 17.0, as well as a carboxymethoxy moiety resonating at δ 53.1 (OCH_3) and δ 169.7 ($\text{C}=\text{O}$). The HSQC spectrum facilitated the assignment of the protonated carbons (Table 3.4). The strong HMBC correlations observed from 8-OH to δ 110.4 (C-7), δ 161.4 (C-8), and δ 109.3 (C-8a); from H-7 to δ 106.8 (C-5), δ 161.4 (C-8), δ 109.3 (C-8a); from H-6 to δ 161.4 (C-8) and δ 155.5 (C-10a); and from H-5 δ 110.4 (C-7), δ 109.3 (C-8a), and δ 155.5 (C-10a) established the structure of ring A. Diagnostic HMBC correlations observed between 2-OH and carbon resonances at δ 151.9 (C-2) and δ 137.2 (C-3); H-4 and carbon resonances at δ 151.9 (C-2), δ 116.5 (C-9a) and 150.7 (C-4a); and the methyl group and carbon resonances at δ 151.9 (C-2), δ 137.2 (C-3) and 122.5 (C-4) were consistent with the substitution pattern in ring C (Table 3.4). The positioning of the phenolic OH at C-2 was further supported by the base peak observed at m/z 268 [$\text{M}^+ - \text{MeOH}$], a dominant loss that is characteristic of salicylate esters, requiring the OH to be adjacent to the methylester substituent (Figure 3.6) as previously illustrated for a related compound where the phenolic methyl group was located in ring A (Moppett, 1971). The C-2 hydroxy group in this structure is also biosynthetically favoured (Henry and Townsend, 2005). On the basis of these data compound **5** was characterized as 2,8-dihydroxy-3-methyl-9-oxoxanthene-1-carboxylic acid methyl ester. This is the first report of this structure, although related structures have been previously reported as fungal metabolites (Moppett, 1971).

Figure 3.5: 2,8-dihydroxy-3-methyl-9-oxoxanthene-1-carboxylic acid methyl ester (**5**).

Table 3.4: ^1H (500 MHz, CDCl_3) and ^{13}C NMR (175 MHz) for compound **5**.

Position	δ_{H} (J, Hz)	δ_{C}	HMBC
1		130.8	
2		151.9	
2-OH	7.75 br s		C2, C3
3		137.2	
3-Me	2.43 s	17.0	C2, C3, C4
4	7.38 s	122.5	C2, C4a, C9a
4a		150.7	
5	6.90 d (8.1)	106.8	C7, C8a, C10a
6	7.57 t (8.0)	136.3	C8, C10a
7	6.79 d (8.0)	110.4	C5, C8, C8a
8		161.4	
8a		109.3	
8-OH	12.39 s		C7, C8, C8a
9		180.4	
9a		116.5	
10a		155.5	
11		169.7	
11-OMe	3.99 s	53.1	C11

Figure 3.6: Dominant loss of MeOH from compound **5**, characteristic of salicylate esters.

Compound **6** was isolated as a white solid with the formula $C_8H_{10}O_2$ determined by HRMS m/z 178.0638 (calculated, 178.0630). EIMS m/z 178 $[M]^+$ (59), 160 (20), 149 (15), 134 (37), 61 (19), 45 (16), 43 (100), 29 (16). 1H NMR ($CDCl_3$, 400 MHz): δ 11.04 (s, OH), 7.41 (dd, $J=8.2$ Hz, $J=7.6$ Hz, 1H), 6.89 (d, $J=8.4$ Hz, 1H), 6.69 (dd, $J=7.4$ Hz, $J=1.0$ Hz, 1H), 4.74 (sextet, $J=6.4$ Hz, 1H), 2.93 (d, $J=6.8$ Hz, 2H), 1.53 (d, $J=6.0$ Hz, 3H). The 1H NMR chemical shifts of **6** were in agreement with the literature values for (3*R*)-mellein (Kamisuki *et al.*, 2007; Uchida *et al.*, 2007; Floerke *et al.*, 2006; Efdi *et al.*, 2007; Islam *et al.*, 2006). Compound **6** was originally isolated from *Aspergillus melleus* (Nishikawa, 1933) and later was found to be produced by many fungi (Hill, 1986). It was also reported from endophytic fungi such as *Pezicula livida*, *Plectophomella sp.*, *Cryptosporiopsis malicoticis*, as well as *Geniculosporium sp.* (Floerke *et al.*, 2006; Krohn *et al.*, 1997). Mellein also occurs in plants (Rukachaisirikul *et al.*, 2005), in insects as part of their defense secretions (Gill, 1993) and as a trail pheromone component in the hindgut of *Lasius fuliginosus* (Kern *et al.*, 1997). It has been reported to be strongly fungicidal, herbicidal and algicidal (Schulz *et al.*, 1995).

Figure 3.7: Mellein (**6**).

Tyrosol **2** as previously described (CBS 120380) was also isolated as a polar metabolite from this endophyte.

CBS 120381

Compound **7** was isolated as a light orange liquid with the formula $C_9H_{14}O_2$, determined by HRMS m/z 154.1003 (calculated, 154.0994). EIMS m/z 154 $[M]^+$ (26), 139 (13), 125 (13), 112 (100), 55 (28), 41 (13). 1H NMR ($CDCl_3$, 400 MHz): δ 4.58 (s, 2H), 2.22 (t, $J=7.6$ Hz, 2H), 1.97 (s, 3H), 1.43 (quintet, $J=7.5$, 2H), 1.28 (sextet, $J=7.5$ Hz, 2H), 0.87 (t, $J=7.4$ Hz, 3H). The NMR data agreed with published data for 3-butyl-4-methyl-5*H*-furan-2-one (Edwards and Whalley, 1979; Shindo *et al.*, 2006; Campi *et al.*, 1992). Compound **7** was originally isolated from the fungus *Hypoxylon serpens*, which is closely related to our isolate, based on DNA sequencing (Edwards and Whalley, 1979). It has also appeared in some natural product syntheses (Shindo *et al.*, 2006; Campi *et al.*, 1992; Demnitz, 1989). Compound **7** is of unknown toxicity but was isolated as the major metabolite of CBS 120381.

Figure 3.8: 3-butyl-4-methyl-5*H*-furan-2-one (7).

The known biosynthetically related metabolites **8-10** were also isolated during this investigation. (3*R*)-5-carbomethoxymellein **8** was identified by comparison of the ^1H NMR and MS data acquired with reported values (Anderson *et al.*, 1983). The ^{13}C NMR chemical shift assignments are reported for the first time (Table 3.5). Compound **8** was originally isolated as the major metabolite from *Hypoxylon mammatum*, a related species to our isolate according to the sequencing data (Anderson *et al.*, 1983). The (3*S*)-enantiomer has also been isolated from *Tubercularia sp.*, an endophytic fungus of *Taxus mairei* (Wang *et al.*, 2003a,b). (3*R*)-5-formylmellein **9** was first discovered in wood samples infested by fungi during storage (De Alvarenga *et al.*, 1978). Recently it was isolated from a culture of *Nodulisporium sp.* The ^1H NMR resonances observed here were consistent with literature values (Kamisuki *et al.*, 2007). (3*R*)-5-methylmellein **10** first appeared in the literature as the main phytotoxic metabolite of *Fusicoccum amygdale* (Ballio *et al.*, 1966). It has been reported from several endophytic fungi such as *Phomopsis sp.* from *Adenocarpus foliolosus* (Dai *et al.*, 2005), from mangrove (Zhu *et al.*, 2006) and from a marine-derived mangrove (Chen *et al.*, 2006). Additionally, it was reported to be produced by *Cephalosporium sp.* AL031 (Bi *et al.*, 2007), *Biscogniauxia*

mediterranea (Evidente *et al.*, 2005), and *Cytospora eucalypticola* (Kokubun *et al.*, 2003). It also occurs as one of the constituents from the heartwood of the Fijian species *E. fidjiana*. The ^1H NMR data for **10** matched the previously reported literature (Cambie *et al.*, 1991). According to Whalley (1985) *Nummulariella marginata* produces **9** and **10** as major secondary metabolites. Both compounds have also been detected from *Nummularia discrete* (Anderson *et al.*, 1983) and from the roots of *Rhus javanica* L. var. *roxburghiana* (Lee *et al.*, 2005). Tyrosol **2** described above (CBS 120380) was also isolated as a polar metabolite from this endophyte.

Compound **8** $[\alpha]_{\text{D}}^{24} - 151$ (*c* 1, CHCl_3). HRMS m/z 236.0676 (calculated for $\text{C}_{12}\text{H}_{12}\text{O}_5$, 236.0665). EIMS m/z 236 $[\text{M}]^+$ (33), 207 (16), 61 (15), 45 (15), 43 (100).

Table 3.5: ^1H (500 MHz, CDCl_3) and ^{13}C NMR (175 MHz) for compound **8**.

Position	δ_{H} (J, Hz)	δ_{C}
1		170.3
3	4.67 m	75.2
4	3.03 dd (18.0, 11.8)	32.6
4a		143.6
5		118.7
6	8.11 d (9.0)	138.3
7	6.92 d (9.0)	116.4
8		165.7
8a		109.1
8-OH	11.81 s	
OMe	3.86 s	51.7
CO ₂ Me		166.4
Me	1.54 d (6.4)	20.4

Figure 3.9: (3*R*)-5-Carbomethoxymellein (**8**).

Compound **9**: HRMS m/z 206.0566 (calculated for $C_{11}H_{10}O_4$, 206.0579). EIMS m/z 206 $[M]^+$ (100), 205 (16), 191 (20), 190 (16), 177 (23), 173 (26), 163 (48), 136 (45), 136 (45), 135 (18), 134 (30), 77 (17), 55 (15), 39 (17). 1H NMR ($CDCl_3$, 400 MHz): δ 11.94 (s, 1H, OH), 10.01 (s, CHO), 7.92 (d, $J=8.8$ Hz, 1H), 7.06 (d, $J=8.8$ Hz, 1H), 4.70 (m, 1H), 3.92 (dd, $J=17.6$ Hz, $J=3.2$ Hz, 1H), 3.02 (dd, $J=17.7$ Hz, $J=11.9$ Hz, 1H), 1.58 (d, $J=6.4$ Hz, 3H).

Figure 3.10: (3*R*)-5-Formylmellein (**9**).

Compound **10**: HRMS m/z 192.0793 (calculated for $C_{11}H_{12}O_3$, 192.0787). EIMS m/z 192 $[M]^+$ (100), 174 (17), 163 (25), 148 (19). 1H NMR ($CDCl_3$, 400 MHz): δ 11.02 (s, 1H, OH), 7.31 (d, $J=8.5$ Hz, 1H), 6.84 (d, $J=8.5$ Hz, 1H), 4.70 (m, 1H), 2.97 (dd, $J=16.7$ Hz, $J=3.4$ Hz, 1H), 2.74 (dd, $J=16.6$ Hz, $J=11.5$ Hz, 1H), 2.21 (s, 3H), 1.57 (d, $J=6.1$ Hz).

Figure 3.11: (3*R*)-5-Methylmellein (**10**).

The mellein-related metabolites **6**, **8-10** exhibited diagnostic features in their 1H NMR spectra which readily allow for their assignment and characterization. The appearance of a one-proton singlet at δ 11-12 is deshielded due to hydrogen bonding of this OH proton with the C=O group at C-1, and a one-proton multiplet at δ 4.5-4.7 is ascribed to H-3. Additionally, a methyl doublet was also observed at δ 1.5 ($J \sim 6$ Hz) for compounds **6**, **8-10**. The (3*R*)- absolute configuration is known to be common to this class of compounds (Findlay *et al.*, 1995; Uchida *et al.*, 2007) and was confirmed by measuring the optical rotation of **8**. The same configuration was assumed for **6**, **8-10** by

virtue of similar NMR data. In particular, H-4 has large couplings to H-3 ($J_{3,4}$ = 11-12 Hz), in which H-3 and H-4 are *trans*-diaxial.

Metabolites **1-10** represent the major compounds produced by the three white spruce endophytes studied, all of which showed similar toxicity to *C. fumiferana* and yeast cells. Of these compounds **4** and **5** are new metabolites albeit structurally related to known metabolites. Compound **1** is reported for the first time as a natural product, **3** has been previously reported as a plant metabolite and **2, 6-10** are known fungal metabolites. The only common metabolite produced by the three strains studied was tyrosol.

Metabolites **1, 3-10** all have a polyketide biosynthetic origin while tyrosol is derived from tyrosine. Compound **4a** appears to be derived from orsellinic acid and is a possible precursor to **1**, through reduction at C-8 and subsequent cyclization and elimination of water. Compounds **3, 6, 8-10** are all 3,4-dihydroxyisocoumarins derivatives of pentaketide origin.

Preliminary toxicity testing for tyrosol using the Hep3B cell line (ATCC# HB-8064) indicated that it was not a potent cytotoxin (data not shown). The toxicity of compounds **1, 3-5** and **7** is unknown. Mellein has been shown to be fungicidal, herbicidal and algicidal (Schulz *et al.*, 1995). Since **3, 8-10** are structurally similar to mellein with all possessing the isocoumarin ring system, these may also possess some toxicity.

Red Spruce Endophytes

The red spruce endophytes were treated using the same basic protocol as was followed for the white spruce endophytes. The crude EtOAc extract of each strain was first screened by LC-MS and LC-NMR and then purified by column chromatography

and/or preparatory TLC (PTLC). Structures were elucidated primarily by analyses of MS and NMR data and in the case of the known metabolites, confirmed by comparison with published data. CBS 121942 yielded the polyketide **11** and the biosynthetically related metabolites **12** and **13**, all are new fungal metabolites and the structure for compound **11** is reported for the first time; CBS 121944 yielded two sesquiterpenes; **14**, reported as fungal metabolite for the first time and **15**, a novel derivative of **14**; CBS 121943 and DAOM 239830 both yielded the known polyketides **16-20**; DAOM 239831 produced the known fungal metabolites **21** and **22**; and DAOM 239833 yielded four biosynthetically related metabolites **23-26** two of which **23** and **24** are isomers and are novel structures.

CBS 121942

Compound **11** was isolated as a white powder with the formula $C_{14}H_{18}O_5$, HRMS m/z 266.1153 (calculated, 266.1154), indicative of 6 double bond equivalents. EIMS m/z 266 $[M]^+$ (11), 180 (14), 115 (83), 85 (43), 68 (86), 41 (100). The 1H and ^{13}C NMR data for **11** are shown in Table 3.6. The structure of **11** was deduced from analysis of the NMR data which showed that this compound was biosynthetically related to seiricuprolide isolated by Ballio (1988) with a rearrangement of the constituents. The 1H NMR spectra showed 4 CH protons, indicative of two separate C=C double bonds: two double doublets at δ 5.95 (H-2) and 6.90 (H-3) in a *trans* orientation ($J_{2,3} = 15.9$ Hz) and two doublets of double doublets at δ 5.84 (H-5) and 5.25 (H-6) in a *cis* orientation ($J_{5,6} = 12.0$ Hz). A multiplet was observed at δ 5.15 (H-4) consistent with a secondary alcohol. Two adjacent CH-O- protons were evident at δ 3.65 (H-7) and 3.51 (H-8) with shifts and J values indicative of an epoxide ring. Compound **11** also showed the presence of 3

adjacent CH₂ groups (H-10, 11, and 12) as evident from the COSY. The last piece of evidence observed from the ¹H spectra was the presence of the Me doublet at δ (1.20) adjacent to a CH-O-C=O groups at δ 4.84. The HSQC data provided connectivity of these structural elements to their relevant carbons. Thus, two epoxide ring carbons occurred at δ 55.6 (C-7) and 58.9 (C-8). In the direct observe ¹³C, two C=O's were observed, one at δ 206.9 (C-9) indicative of a ketone and one at 165 ppm indicative of an ester linkage. The HMBC data provided the adjacencies of the structure at C-13 and C-4 and fixed the ketone at C-9. The relative stereochemistry of the substituents at C-4, C-13 and the C-7,8 epoxide ring could not be determined. The presence of the two C=C's, epoxide ring and two C=O's, indicates that the entire molecule contains only one ring to satisfy the six double bond equivalents. Compound **11** contains similar elements to seiricuprolide but was differentiated by the C=O at C-9 and the rearrangement of the epoxide ring and the C-5, C-6 double bond. The relative stereochemistry of the substituents at C-4, C-13 and the C-7,8 epoxide ring could not be determined. The need for a crystal structure to solve this was also evident for the related metabolite seiricuprolide (Ballio *et al.*, 1988; Bartolucci *et al.*, 1992). Compound **11** has a similar structural backbone to known fungal metabolites zearalenone and vermiculin, both of which are anti-fungal and therefore may account for some of the toxicity to spruce budworm observed from the extract (Utermark *et al.*, 2007; Kim *et al.*, 2002).

Table 3.6: ^1H NMR (CDCl_3 , 400 MHz) and ^{13}C NMR (CDCl_3 , 100 MHz) for compound

11.

Position	δ_{H} (J, Hz)	δ_{C}	HMBC
1		165.1	
2	5.95 dd (15.6, 1.2)	122.5	C1, C3, C4
3	6.90 dd (15.9, 6.3)	146.0	C1, C2, C4, C5
4	5.15 m	69.4	
5	5.84 ddd (12.0, 3.6, 1.2)	136.7	C3
6	5.25 ddd (12.0, 7.2, 2.4)	127.2	C4
7	3.65 dd (7.2, 1.8)	55.6	C5, C6, C8, C9
8	3.51 d (1.8)	58.9	C8, C9
9		206.9	
10	2.64 ddd (2.6, 7.2, 19.5)	38.5	C9, C11, C12
	2.57 ddd (2.6, 7.2, 19.5)		
11	1.82 m	18.7	C9, C10, C12, C13
	1.60 m		
12	1.70 m	32.5	C10, C12, C13, C14
13	4.84 (6.3)	72.5	C1, C14
14	1.20 d (6.0)	20.7	C12, C13

Figure 3.12: Compound 11.

Compounds 12 and 13 were also isolated as white powders from CBS 121942.

The formula for 12 was determined to be $\text{C}_{11}\text{H}_{12}\text{O}_3$ by HRMS m/z 192.0797 (calculated,

192.0787). EIMS m/z 192 $[M]^+$ (16), 101 (100), 92 (84), 91 (75), 73 (22), 65 (13), 29 (10). ^1H NMR (CDCl_3 , 400 MHz): δ 7.27 (m, 5H), 3.74 (s, 2H), 2.75 (t, $J=6.4$ Hz, 2H), 2.60 (t, $J=6.4$ Hz, 2H); ^{13}C NMR (CDCl_3 , 100 MHz): δ 207.1 (CO), 176.4 (CO_2H), 129.5, 128.8, 127.2, 50.0, 36.2, 27.5. The ^1H NMR data show 5 adjacent protons in a benzene ring, an isolated CH_2 and two adjacent CH_2 's in a chain. The ^{13}C data showed 10 of the required 11 carbons present in the compound with only one acidic carbonyl at 176.4 ppm, which was also not observed in the HMBC due to hydrogen bonding with the acid function. The ketone function at C-4 was only observed after treatment of the compound with diazomethane to convert the acid function into a CO_2Me thus removing the hydrogen bonding and allowing for the observation of the carbon at δ 207.1. The NMR data for compound **12** were in agreement with the known metabolite 4-oxo-5-phenylpentanoic acid (Ketterer *et al.*, 2006). They described compound **12** as an intermediate in the synthesis of enantiomerically pure tricyclic benzomorphan analogues; it is reported here for the first time as a fungal metabolite and is of unknown toxicity.

Figure 3.13: 4-oxo-5-phenylpentanoic acid (**12**).

The HRMS m/z 176.0849 (calculated, 176.0838) for **13** determined the formula to be $\text{C}_{11}\text{H}_{12}\text{O}_2$ indicating a loss of an oxygen from **12** and 6 double bond equivalents.

EIMS m/z 176 $[M]^+$ (40), 91 (33), 85 (100), 29 (22). ^1H NMR (CDCl_3 , 400 MHz): δ 7.28 (m, 5H), 4.74 (quintet, $J=6.7$ Hz, 1H), 3.08 (dd, $J=14.0$ Hz, $J=6.0$ Hz, 1H), 2.93 (dd, $J=14.0$ Hz, $J=6.3$ Hz, 1H), 2.42 (m, 2H), 2.24 (m, 1H), 1.96 (m, 1H); ^{13}C NMR (CDCl_3 , 100 MHz): δ 176.9, 135.9, 129.5, 128.7, 127.0, 80.8, 41.4, 28.7, 27.1. The ^1H NMR data for compound **13** show 5 adjacent protons in a benzene ring as was present in **12**, a CH, an isolated CH_2 and two adjacent CH_2 's. The ^{13}C data shows all of the 11 carbons present. The NMR data for compound **13** also showed good agreement with the next step in the above synthesis of compound **12** (Ketterer *et al.*, 2006); it was determined to be 5-benzyl-dihydro-furan-2-one (**13**). Therefore compound **13** is also a new natural product of unknown toxicity. The relationship between **12** & **13** suggests that **12** would be a precursor of **13** through cyclization between C-4 and the terminal OH with a loss of oxygen.

Figure 3.14: 5-benzyl-dihydro-furan-2-one (**13**).

CBS 121944

The structurally related metabolites **14** and **15** were both isolated as white powders from CBS 121944. The formula of each was determined by HRMS m/z

234.1612 (calculated for $C_{15}H_{22}O_2$, 234.1614) and m/z 250.1548 (calculated for $C_{15}H_{22}O_3$, 250.1569), respectively. The HRMS data showed that compound **14** had the same formula as **15** with the addition of an oxygen. The EIMS m/z 234 $[M]^+$ (100), 201 (19), 166 (74), 162 (16), 161 (48), 149 (21), 123 (17), 122 (53), 94 (24), 91 (33), 83 (23), 79 (20), 77 (18), 59 (23), 55 (21), 43 (55), 39 (19) for compound **14** and m/z 250 $[M]^+$ (46), 189 (26), 182 (26), 161 (26), 151 (100), 134 (29), 91 (32), 83 (17), 77 (17), 41 (23) for compound **15**. The 1H NMR data for both compounds **14** and **15** are very similar except that the methyl at δ 0.82 in **14** and is replaced by a hydroxy methylene at δ 3.40 and 3.62 in compound **15** indicative of an oxidation (Table 3.7). The 1H NMR data for both were characterized by a C=CH at δ ~5.9, a gem-dimethyl at δ 1.55 and 2.27 and an additional methyl singlet at δ 1.1-1.2. The compounds also contained two adjacent CH_2 groups and an additional isolated CH_2 as evident from the COSY. The assignment of each proton to carbon was accomplished from the HMQC and the adjacencies were determined from the HMBC data. The chemical shifts of all 15 carbons were also obtained from the 2D NMR data including the C=O at δ ~191. The data for compound **14** was in agreement with the basic structure of isopetasol (Sugama *et al.*, 1983). Compound **15** was determined to be similar with an oxidation of the methyl at C-15 to a CH_2OH . However, examination of the NOE data for **14** revealed that the relative stereochemistry at C-3 was different from that reported for isopetasol. The same stereochemistry at C-3 for compound **15** was also observed from the NOE data. The NOE data for compound **15** provided further support for the stereochemistry at C-3, as would be expected if they are related by a simple oxidation. The structure of **14** was subsequently determined to be that of the previously described plant metabolite 3-epiisopetasol (Barrero *et al.*, 1991; Bohlmann *et al.*, 1982) and from synthetic studies

(Yamakawa *et al.*, 1979, 1974). Compound **15** was determined to be the novel structure, 15-OH-3-epiisopetasol. The observed stereochemistry of the hydroxy at C-3 was also present for related sesquiterpenes isolated from a different fungal species (Sorensen *et al.*, 2007). The toxicity of compounds **14** and **15** is unknown.

Table 3.7: NMR data for compounds **14** and **15**, C₆D₆ (600 MHz).

Position	14		15	
	δ_{H} (J, Hz)	δ_{C}	δ_{H} (J, Hz)	δ_{C}
1	α 1.69 dd (3, 14.5) β 2.52 dt (2, 4.6, 14.5)	26.9	α 1.70 dt (4, 13.2) β 2.59 dt (2, 5.3, 14.5)	26.8
2	α 1.43 dd (2.6, 13.8) β 1.19 dt (3, 13.8)	34.1	α 1.50 dt (2, 13.8) β 1.17 dt (4, 13.8)	21.9
3	3.26 br s	71.0	3.88 br s	67.7
4	1.05 dt (2.6, 7.8)	45.4	0.95 m	52.0
5		43.2		41.4
6	α 1.89 dd (13.2) β 2.71 d (13.2)	42.4	α 1.90 dd (13.2) β 2.77 d (13.8)	42.5
7		129.0		128.6
8		191.1		190.7
9	5.95 br s	127.0	5.91 br s	126.9
10		167.0		166.5
11		142.0		142.5
12	2.27 d (2)	22.8	2.26 d (2.6)	22.8
13	1.55 br s	21.9	1.54 br s	22.0
14	1.13 s	19.3	1.21 s	20.8
15	0.82 d (7.3)	12.2	3.62 dd (4, 10.5) 3.40 dd (4, 10.5)	61.1

Figure 3.15 - 16: 3-epiisopetasol (**14**) and 15-OH-3-epiisopetasol (**15**).

CBS 121943 and DAOM 239830

CBS 121943 and DAOM 239830 as previously noted are closely related if not identical species based on DNA sequencing, morphology and their metabolite profiles. Compounds **16**, **17**, **18** and **20** were isolated and characterized from DAOM 23983 and the NMR data for each were in agreement with NMR data for compounds isolated by Findlay (1995). The NMR and mass spec data for each are presented below. LC-MS and LC-NMR were used to confirm the presence of these compounds in the crude extract of CBS 121943. In addition, compound **19** was not isolated from either strain but was identified in both by LC-MS and LC-NMR (Islam *et al.*, 2007). All five of these known biosynthetically related metabolites were previously isolated from a fungal endophyte of black spruce (Findlay *et al.*, 1995). The strain isolated from the black spruce tree is

probably similar if not the same as our isolates, but the earlier strain was not deposited in a culture collection so an absolute comparison was not possible. Previous toxicity testing showed that compounds **16** and **17** were toxic to spruce budworm larvae and that compound **16** was also toxic to spruce budworm cells (Findlay *et al.*, 1995).

Compound **16**: (3*R*, 4*S*, 4*aR*), 4,8-Dihydroxy-3-methyl-3,4,4*a*,5-tetrahydro-1*H*-2-benzopyran-1-one: HRMS m/z 196.0746 (calculated for $C_{10}H_{12}O_4$, 196.0736). EIMS m/z 196 $[M]^+$ (3.1), 121 (16), 61 (21), 45 (17), 43 (100). 1H NMR ($CDCl_3$, 400 MHz): δ 12.85 (s, 1H, 8-OH), 6.51 (ddd, $J=9.5$ Hz, $J=8.1$ Hz, $J=2.4$ Hz, 1H, H6), 6.09 (dd, $J=9.9$ Hz, $J=3.2$ Hz, 1H, H7), 4.16 (dq, $J=9.4$ Hz, $J=3.2$ Hz, 1H, H3), 3.47 (t, $J=9.6$ Hz, 1H, H4), 2.77 (m, 1H, H4*a*), 2.66 (m, 1H, H5), 2.0 (m, 1H, H5), 1.46 (d, $J=6.2$ Hz, 3H, Me). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 170.8, 169.1, 140.1, 124.5, 90.5, 78.0, 74.5, 37.6, 27.7, 17.8.

Figure 3.17: (3*R*, 4*S*, 4*aR*), 4,8-dihydroxy-3-methyl-3,4,4*a*,5-tetrahydro-1*H*-2-benzopyran-1-one (**16**).

Compound **17**: (3*R*, 4*aS*, 8*S*, 8*aR*)-8-Hydroxy-3-methyl-3,4,4*a*,5,6,7,8,8*a*-octahydro-1*H*-2-benzopyran-1-one: HRMS m/z 184.1081 (calculated for $C_{10}H_{16}O_3$, 184.1099). EIMS m/z 184 $[M]^+$ (0.8), 156 (75), 135 (19), 114 (23), 113 (100), 97 (15),

95 (21), 81 (19), 80 (31), 79 (16), 70 (15), 55 (16), 43 (23), 41 (24). ^1H NMR (CDCl_3 , 400 MHz): δ 4.43 (m, 1H, H3), 3.63 (m, 1H, H8), 2.99 (t, $J=5.3$ Hz, H8a), 2.27 (m, 1H, H4), 2.17 (m, 1H, H4a), 1.91 (m, 1H, H7), 1.75 (m, 1H, H7), 1.75 (m, 1H, H6), 1.64 (m, 1H, H5), 1.29 (m, 1H, H6), 1.26 (m, 1H, H4), 1.36 (d, $J=6.2$ Hz, 3H, Me), 0.97 (m, 1H, H5). ^{13}C NMR (CDCl_3 , 100 MHz): δ 175.2, 73.4, 70.2, 44.2, 36.6, 33.0, 31.9, 30.9, 23.6, 20.7.

Figure 3.18: (3*R*, 4*aS*, 8*S*, 8*aR*)-8-Hydroxy-3-methyl-3,4,4*a*,5,6,7,8,8*a*-octahydro-1*H*-2-benzopyran-1-one (**17**).

Compound **18**: (3*R*, 4*S*)- 3,4,4*a*,5,6,7-Hexahydro-4,8-dihydroxy-3-methyl-1*H*-2-benzopyran-1-one 1*H*-2-Benzopyran-1-one: HRMS m/z 198.0895 (calculated for $\text{C}_{10}\text{H}_{14}\text{O}_4$, 198.0892). EIMS m/z 198 $[\text{M}]^+$ (16), 141 (34), 123 (100), 92 (16), 58 (15). ^1H NMR (CDCl_3 , 400 MHz): δ 13.29 (s, 1H, 8-OH), 4.19 (dq, $J=9.5$ Hz, $J=6.3$ Hz, 1H, H3), 3.21 (ddd, $J=9.7$ Hz, $J=4.9$ Hz, 1H, H4), 2.39 (m, 1H, H4*a*), 2.39 (m, 2H, H5), 2.20 (m, 1H, H7), 1.97 (m, 1H, H6), 1.97 (m, 1H, H7), 1.60 (m, 1H, H6), 1.46 (d, $J=6.3$, 3H, Me). ^{13}C NMR (CDCl_3 , 100 MHz): δ 176.2, 170.7, 94.4, 78.9, 73.6, 40.0, 28.9, 26.0, 20.5, 18.4.

Figure 3.19: (3*R*, 4*S*)- 3,4,4a,5,6,7-Hexahydro-4,8-dihydroxy-3-methyl-1*H*-2-benzopyran-1-one 1*H*-2-Benzopyran-1-one (**18**).

The known metabolite ramulosin **19** was identified by comparison of the LCMS and LC-NMR data to that published in the literature for the synthesis of the compound (Islam *et al.*, 2007). The data are presented below along with the structure. HRMS m/z 182.0951 (calculated for $C_{10}H_{14}O_3$, 182.0942). 1H NMR (CD_3CN , 600 MHz): δ 1.26 (d, 3H), δ 4.45 (m, 1H) δ 2.39 (m, 2H), 2.46 (m, 1H), δ 1.83 (m, 2H), 1.56 (m, 1H), 1.07 (dd, 1H), 1.19 (dd, 1H).

Figure 3.20: Ramulosin **19**.

Compound **20**: 4-Hydroxy-3-methyl-2-oxabicyclo[3.3.1]non-6-one: HRMS m/z 170.0932 (calculated for $C_9H_{14}O_3$, 170.0943). EIMS m/z 170 $[M]^+$ (10), 126 (17), 85 (20), 69 (34), 61 (15), 61 (15), 56 (35), 45 (18), 43 (100), 41 (28). 1H NMR ($CDCl_3$, 400 MHz): δ 4.35 (m, 1H, H1), 3.46 (dd, $J=9.6$ Hz, $J=4.4$ Hz, 1H, H4), 3.34 (dq, $J=9.7$ Hz, $J=5.8$ Hz, 1H, H3), 2.94 (dq, $J=17.6$ Hz, $J=2.0$ Hz, H7), 2.76 (dq, $J=17.7$ Hz, $J=2.2$ Hz, 1H, H8), 2.51 (m 1H, H5), 2.48 (dd, $J=17.8$ Hz, $J=4.6$ Hz, 1H, H8), 2.35 (dd, $J=17.6$ Hz, $J=5.8$ Hz, 1H, H7), 2.10 (m, 1H, H9), 1.97 (ddd, $J=13.6$ Hz, $J=3.5$ Hz, 1H, H9), 1.24 (d, $J=5.7$ Hz, 3H, Me). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 209.3, 74.2, 69.4, 68.7, 47.0, 40.3, 34.2, 31.6, 18.8.

Figure 3.21: 4-Hydroxy-3-methyl-2-oxabicyclo[3.3.1]non-6-one (**20**).

DAOM 239831

Compounds **21** and **22** were isolated as white solids from the endophyte DAOM 239831. HRMS m/z 152.0466 (Calculated, 152.0473) determined the formula of **21** to be $C_8H_8O_3$. EIMS m/z 152 $[M]^+$ (29), 142 (13), 107 (100), 57 (13), 43 (10). 1H NMR ($CDCl_3$, 400 MHz): δ 7.15 (d, $J=8.6$ Hz, 2H), 6.79 (d, $J=8.6$ Hz, 2H), 3.60 (s, 2H). The 1H NMR for **21** showed 4 aromatic protons in addition to an isolated CH_2 . Compound **21**

was determined to be the known fungal metabolite *p*-hydroxyphenylacetic acid, from examination of the spectral data and comparison to an authentic standard (Sigma). Compound **21** was previously isolated from a variety of organisms including; a *Cladosporium* sp. (Ding, 2008) that appears to be in the *Davidiellaceae* family, *Epichloe typhina* (Koshino, 1988) and *Burkholderia* sp. (Mao, 2006). It is derived biosynthetically from tyrosine and is reported to be antifungal (Koshino, 1988; Mao, 2006).

Figure 3.22: *p*-hydroxyphenylacetic acid **21**.

The ^1H NMR data for compound **22** are presented below and showed a $\text{CH}_2\text{-OH}$ at δ 4.71, two adjacent $\text{HC}=\text{C}$ protons at δ 7.21 and 6.50 ppm and an aldehyde proton at δ 9.59 ppm. HRMS m/z 126.0311 (calculated for $\text{C}_6\text{H}_6\text{O}_3$, 126.0317). EIMS m/z 126 $[\text{M}]^+$ (29), 142 (13), 107 (100), 57 (13), 43 (10). ^1H NMR (CDCl_3 , 400 MHz): δ 9.59 (s, 1H, CHO), 7.21 (d, $J=3.6$ Hz, 1H), 6.50 (d, $J=3.6$ Hz, 1H), 4.71 (s, 2H); ^{13}C NMR (CDCl_3 , 100 MHz): δ 177.6 (CHO), 160.3 (C2), 152.5 (C5), 123.1 (C3), 110.0 (C4), 57.7 (C6). The ^{13}C data showed the presence of the aldehyde $\text{C}=\text{O}$ at δ 177.6 ppm. The structure was determined from the HMBC data which showed good agreement with the literature for the previously described fungal metabolite 5-hydroxymethyl-2-furaldehyde (Miyazawa *et al.*, 2003; Etse, 1988). It has been isolated from plants (Miyazawa *et al.*,

2003; Etse *et al.*, 1988), and from *Verruculina enalia* (Lin *et al.*, 2002). It is reported to be insecticidal against *Drosophila melanogaster* (Miyazawa *et al.*, 2003), has shown limited toxicity to rats and dogs (Ulbricht *et al.*, 1984) and has been studied for its antitumor potential (Michail *et al.*, 2007).

Figure 3.23: 5-Hydroxymethyl-2-furaldehyde **22**.

DAOM 239833

Compounds **23-26** were isolated using LC-MS-SPE in microgram amounts and were characterized by NMR following elution (CD₃CN) of the compounds from the SPE cartridges. The isolated metabolites were first analyzed by LC-MS in both ESI+ and ESI- mode to determine the molecular formula of each (Figures 3.24, 3.25). Compounds **23** and **24** have identical mass spectra, in ESI+ mode both are characterized by the 431 [M+Na], 391 [M+H-H₂O], 373 [M+H-2(H₂O)], 426 [M+NH₄] and 839 [2M+Na], the molecular ion of 408 (409 M+H) is not observed (Figure 3.24). In ESI- mode a large 407 [M-H] peak was observed confirming the mass of 408.1415, which gives a molecular formula of C₂₀H₂₄O₉ (calculated, 408.1420) for both indicating 9 double bond equivalents.

Figure 3.24: ESI+ spectra for compound **23** and **24**.

Figure 3.25: ESI- spectra for compound **23** and **24**.

From the ^1H spectra of both (Appendix F) 21 of the 24 expected protons are observed, including; three methine protons above 5.5 ppm, two methyl (triplets) indicating $\text{CH}_2\text{-CH}_3$'s at δ 3.2 and 3.23 ppm, two CH's and numerous CH_2 's. The five CH_2 functions were identified from the COSY spectrum (Figure 3.26) in addition to the relationships between all other adjacent protons. The HMQC spectrum (Figure 3.27) was used to make the 12 ^1H - ^{13}C direct bond correlations observed in the compound, which

are displayed in Table 3.8. The HMBC spectra (Figure 3.28) helped to determine chemical shifts of 5 out of the 8 of the quaternary carbons, leaving 3 carbonyls (C-16, C-17 and C-18) that were not observed by any 2D NMR method. Direct ^{13}C NMR (Appendix F) was then required in order to observe the 3 additional C=O functions and to assign all 5 carbonyls of which 3 must be free acids in order to satisfy the molecular formula and the NMR data. The number of C=O functions in addition to the double bonds observed leaves only one ring function to satisfy the 9 double bond equivalents. These data were used to determine the structure of the molecule as shown in Figure 3.29. From a full set of specific NOEs the orientation of H-6 was determined to be *trans* to the C-16 acid function on C-5. An NOE was observed between H-6 and H-4 as well as H-3 suggests that C-15, C-16 was stabilized by hydrogen bonding. From the structure of **23**, there are only 2 possible stereochemical centres, namely C-4 and C-7. The spectra of **23** and **24** are virtually identical, except for the chemical shift of H-7 and H-4. However, the C-7 shift remains identical, as do the ^{13}C and ^1H shifts of positions 8 and 19, indicating the difference is likely the orientation of the substituents at C-4. Further analysis of **25** and comparison of all metabolites (**23-26**) and their apparent biosynthetic relationship helps confirm this hypothesis. The toxicity of compounds **23-26** is unknown.

Figure 3.26: COSY spectra for compound 23.

Figure 3.27: HMQC spectra for compound 23.

Figure 3.28: HMBC spectra for compound 23.

Table 3.8: NMR data for compounds **23**, **24** and **25** (^1H – 600 MHz CD_3CN , ^{13}C 175

	23		24		25	
	δ_{H} (J, Hz)	δ_{C}	δ_{H} (J, Hz)	δ_{C}	δ_{H} (J, Hz)	δ_{C}
1		174.2		174.2		173.4
2	2.24 m	31.7	2.22 m	31.8	2.70 m	20.6
3	2.06 m 1.79 m	26.5	2.03 m 1.76 m	26.0	2.70 m 1.79 m	31.3
4	3.20 dd (7.5)	49.3	3.26 dd (7.5)	48.9		144.9
5		132.2		132.4		145.4
6	5.67 d (10.0)	146.6	5.70 d (10.0)	145.8	2.41 m, 2.58 m	29.3
7	3.23 m	41.2	3.20 m	41.2	2.15 m	39.1
8	2.62 dd (4.0, 14.0) 2.48 dd (10.0, 13.0)	29.2	2.60 dd (5.0, 13.0) 2.49 dd (10.0, 13.0)	29.3	2.43 m 2.54 m	28.7
9		138.8		138.8		139.1
10		138.3		138.3		139.4
11	6.30 dt (2.0, 16.0)	117.7	6.31 dt (2.0, 16.0)	117.6	6.36 dt (1.7, 16.2)	117.6
12	7.09 dt (7.0, 16.0)	148.8	7.12 dt (6.5, 16.0)	149.1	7.18 dt (6.4, 16.2)	149.8
13	2.28 m	27.7	2.29 m	27.7	2.31 m	27.9
14	1.07 t (7.5)	12.8	1.08 t (7.5)	12.7	1.09 t (7.5)	12.9
15		174.1		173.9		165.7
16		168.1		168.2		167.5
17		167.3		167.2		27.1
18		165.8		165.8		167.2
19	1.59 m 1.38 m	29.0	1.59 m 1.39 m	29.0	1.34 m 1.38 m	166.8
20	0.87 t (7.5)	12.0	0.86 t (7.5)	12.0	0.93 t (7.5)	11.1

MHz).

Figure 3.29: Compounds **23** and **24**.

Compounds **25** and **26** were also analyzed in both ESI+ and ESI- mode, compound **25** was characterized in ESI+ mode by the 413 [M+Na], 391 [M+H], 408 [M+NH₄] and 803 [2M+Na]. In ESI- mode a large 345 [M-H-CH₃CHO] peak is observed as well as a 779 [2M-H] to confirm the mass of 390.1308 which gives a molecular formula of C₂₀H₂₂O₈ (calculated, 390.1314). Compound **26** was characterized in ESI+ mode by the 557 [M+H], 574 [M+NH₄] and 579 [M+Na]. In ESI- mode a 555 [M-H] peak is observed as well as a 511 [M-H-CH₃CHO] to confirm the mass of 556.1945 which gives a molecular formula of C₂₉H₃₂O₁₁ (calculated, 556.1944). The full set of ¹H, ¹³C and 2D NMR data was acquired as before for compounds **25** (Table 3.8) and **26**. ¹H NMR (CD₃CN, 600 MHz): δ 7.18 (1H), 6.35 (1H), 2.70 (2H), 2.60 (2H), 2.53 (4H), 2.39 (4H), 2.31 (2H), 1.96 (2H), 1.34 (4H), 1.08 (3H), 0.93 (3H), 0.92 (3H). ¹³C NMR (CD₃CN, 175 MHz): δ 172.3, 166.4, 166.1, 166.0, 165.7, 164.6, 159.9, 148.9, 148.8, 144.6, 144.6, 144.2, 143.8, 138.4, 138.0, 116.5, 37.9, 37.6, 30.3, 28.6, 28.5, 28.2, 27.7, 26.9, 26.1, 26.0, 19.6, 11.8, 10.0. This information along with the molecular

formula and a search of the metabolite database allowed for the determination of the compounds to be the known fungal metabolites cordyanhydrides A and B respectively (Isaka *et al.*, 2000; Yamanishi *et al.*, 2000) (Figures 3.30, 3.31).

Figure 3.30: Compound **25**.

Figure 3.31: Compound **26**.

Metabolites **11-26** represent the major compounds produced by the six red spruce endophytes studied, of which **11-13** showed toxicity to *C. fumiferana* and to yeast cells (06-097D). The six strains were also selected to provide a representation of geographic, taxonomic and secondary metabolite diversity. Of these compounds **11, 15, 23** and **24** are new metabolites albeit structurally related to known metabolites. Compounds **12** and **13** are reported for the first time as natural products, **14** as a new fungal metabolite and the remaining compounds have been reported as fungal metabolites. Unlike the observation of tyrosol in all selected white spruce species (Sumarah et al., 2008), no common metabolite was observed in the selected red spruce strains. However, the clear similarities between tyrosol and **21** and subsequently **12** and **13** suggest similar biosynthetic pathways are used by these species.

Metabolites **11, 16-20** all have a polyketide biosynthetic origin. Compounds **14** and **15** are sesquiterpenes, derived biosynthetically from the condensation of three C₅ units from mevalonic acid. Compounds **23-26** are nonadrides bearing two and three maleic anhydride moieties derived from either two or three 9 carbon units. Compound **21** is derived from shikimic acid.

The toxicity of compounds **11-15**, and **23-26** is unknown. Compounds **21** and **22** have shown some antifungal and insecticidal properties. The previously described metabolites **16-20** (Findlay *et al.*, 1995) have been tested against spruce budworm larvae and cells, with toxicity observed to both for compound **16** and larvae for **17**. These metabolites are all isocoumarin derivatives and may possess similar toxicity to the isocoumarin derivatives of mellein isolated and described above from white spruce endophytes.

EXPERIMENTAL

Isolation of Secondary Metabolites

White spruce metabolites

Three white spruce strains from the original ten that exhibited toxicity towards spruce budworm and yeast cells were selected for further study based on growth in culture and secondary metabolite production. These three strains were further processed to isolate and determine the structures of the major metabolites. The fungal strains tested were CBS 120379 and CBS 120380, isolated from *Picea glauca* (white spruce) needles near Sussex, NB, Canada and CBS 120381 which was isolated from the needles of a *P. glauca* tree in St. George, New Brunswick, Canada. The 3 strains were then fermented from starter cultures (as previously described) in groups of 5 Glaxo bottles each containing 1L of 2% ME medium for 3 months at 20 °C and extracted as previously described. The resulting extracts were first screened by LC-MS using electrospray ionization in both positive and negative ion mode to acquire MS data on the compounds. Each extract was subsequently analysed by LC-NMR using a Varian 9010 HPLC system with UV detector attached to a Varian 600 MHz INOVA spectrometer using a 5 mm broadband cryoprobe with a 60µL (the active volume of the NMR probe) LC-NMR insert. Varian LCStar software controlled the coordination of the LC pump, NMR analyses were performed in stop-flow mode where ¹H NMR data were acquired for each major HPLC peak to crudely evaluate the individual secondary metabolite structure. Each extract was then subjected to column chromatography, TLC, or both to isolate the compounds and NMR and MS were used to deduce the structures of the individual metabolites. NMR data were acquired on either a Bruker Avance 400 MHz spectrometer

with a 5 mm autotuning broadband probe (Z gradient), a Bruker ARX 500 MHz spectrometer with a 3 mm inverse probe or a Bruker AVANCE 700 MHz with a 5mm cryogenic (TCI) probehead (both ^1H and X channels). HRMS data on the isolated compounds were obtained on a Kratos Concept instrument (University of Ottawa, Mass Spectrometry Centre). Optical rotation was measured on a Perkin-Elmer 141 Polarimeter.

CBS 120380

The crude EtOAc extract (40 mg) from the culture filtrate was first subjected to PTLC (preparative thin layer chromatography) (1000 μm) eluting with 10% MeOH/ CHCl_3 (100 mL). The main UV active band (254nm) was scrapped off the plate and extracted with 100% EtOAc (50 mL) to furnished compound **1** as a white solid (0.7 mg). Purification of a second portion of the crude filtrate extract (70 mg) was performed by column chromatography on Si gel eluting first with hexanes/ CHCl_3 mixtures (0-100%, 100 mL aliquots), followed by MeOH/ CHCl_3 solvent gradient (0-15%, 100 mL aliquots). The major fraction was again subjected to PTLC using multiple elutions [MeOH/ CHCl_3 1%, 3% and 5%, 100 mL aliquots] to afford compound **2** (1 mg). In an attempt to isolate the polar compounds, another portion of the crude filtrate extract (110 mg) was methylated with diazomethane prior to purification. The extract was stirred at 0°C in CH_2Cl_2 (20 mL) with five drops of MEOH to aid solubility. Excess yellow ether diazomethane solution was added and stirring continued for 1h at 0°C before it was warmed to room temperature [diazomethane solution was prepared by adding

nitrosomethylurea (200 mg) to a solution of 20% KOH (2 mL) and Et₂O (6 mL) at 0°C]. The reaction mixture was washed with H₂O (2 x 5 mL) and the solvent evaporated *in vacuo* before it was purified by Si gel chromatography, eluting sequentially with mixtures of EtOAc-hexanes (0-100%, 100 mL portions) and MeOH/CHCl₃ solvent gradients (5 and 10%, 100 mL portions). The two most promising fractions by TLC were subjected to further purification. Fraction 1 was applied to a small Si gel column eluting with EtOAc-hexanes solvent gradient (0-30%, 10 mL each) to afford compound **3** (1 mg). Multiple elution of fraction 2 by PTLC (15%, 30%, 35% EtOAc-hexanes, 100 mL each) followed by Si gel column chromatography [100% hexanes, 10%, 20%, 30% EtOAc-hexanes (20 mL each)] provided compound **4** (1 mg).

CBS 120379

The crude EtOAc (74 mg) from the culture filtrate was subjected to Si gel chromatography eluting consecutively with hexanes-CHCl₃ (0-100%, 100 mL each), and MeOH-CHCl₃ solvent gradients (1-10%, 100 mL each). The major fraction was further purified twice by PTLC: first using 30% EtOAc-hexanes (100 mL) as eluants followed by eluting successively with 50% CHCl₃-hexanes and 100% CHCl₃ and 1% MeOH-CHCl₃ (100 mL each), to afford compound **5** as a white solid (1 mg). Another crude filtrate extract (20 mg) was fractionated by PTLC, eluting first with 5% MeOH-CHCl₃ followed by 10% MeOH-CHCl₃ solvent systems (100 mL each). The main fraction was further purified by PTLC eluting with 15% EtOAc-hexanes and then 30% EtOAc-hexanes (100 mL each), to afford compound **6** (0.5 mg). Fractionation of the crude

filtrate extract (50 mg) was also performed by column chromatography over Si gel eluting successively with EtOAc-hexanes (0-20%, 50 mL aliquots) and MeOH-CHCl₃ solvent gradients (0-15%, 50 mL aliquots). The main fraction was separated by PTLC using 10% MeOH-CHCl₃ (100 mL) as the eluant, providing compound **2** (1 mg).

CBS 120381

The crude filtrate extract (590 mg) was purified by column chromatography on Si gel eluting successively with EtOAc/hexanes solvent gradient (0-100% EtOAc, 100 mL portions) to afford compound **7** as a light orange liquid (165.8 mg), and a mixture of compounds **8-10** (25 mg). Separation of **8-10** was achieved by PTLC eluting first with 10% EtOAc/hexanes followed by 15% EtOAc/hexanes (100 mL each). The UV active bands were extracted with 100% EtOAc to provide compound **8** (3.5 mg), compound **9** (2.8 mg), and compound **10** (3 mg). Further purification of the polar fractions was carried out by PTLC using 10% MeOH/CHCl₃ (100 mL) as eluant, to yield compound **2** (2 mg). Purification of the crude cell extract (260 mg) was performed on Si gel column using hexanes/CHCl₃ and MeOH/CHCl₃ solvent gradients as eluants (0-100% and 1-10% respectively, 100 mL each) to afford additional compound **7** (5 mg).

Red spruce metabolites

Six red spruce strains from the original 16 that showed toxicity were selected for further study based on growth in culture, secondary metabolite production and toxicity to spruce budworm. These six strains were then further processed to isolate and determine the structures of the major metabolites. The fungal strains CBS 121942, CBS 121944, CBS 121943, DAOM 239830 and DAOM 239831 were isolated from the needles of different *Picea rubens* (red spruce) located in the area surrounding Sussex, New Brunswick, Canada. DAOM 239833 was isolated from a red spruce tree located in Western Nova Scotia. All isolated compounds were subject to HRMS analysis and to complete NMR structural analysis.

CBS 121942

Fractionation of the crude filtrate extract (160 mg) was performed by column chromatography on Si gel eluting first with EtOAc-hexanes solvent gradient (0-70%, 100 mL each) then MeOH-CHCl₃ solvent gradient (0-20%, 50 mL each) to provide two major fractions. The first major fraction (10 mg) was re-purified by PTLC eluting with 5% MeOH-CHCl₃ to afford compound **11** (1 mg). Fraction 2 (40 mg) was re-applied to a Si gel column eluting sequentially with CHCl₃-hexanes solvent gradient (0-100%, 100 mL each) followed by MeOH-CHCl₃ solvent gradient (0-20%), yielded compound **12** (7 mg) and compound **13** (1 mg). A 3 mg portion of compound **12** was methylated with diazomethane to confirm the presence of the acid group. It was stirred at 0°C in CH₂Cl₂ (20 mL), excess ether diazomethane solution was added and stirring continued for 1h at

0°C before it was warmed to room temperature [diazomethane solution was prepared by adding nitrosomethylurea (100 mg) to a solution of 20% KOH (2 mL) and diethyl ether (5 mL) at 0°C]. The reaction mixture was washed with water (2 x 5 mL) and the solvent evaporated *in vacuo*. The crude product was purified by PTLC using 35% EtOAc-hexanes (100 mL) as eluants, to furnish compound **12b**, the methylated version, used only for the purposes of solving the structure of **12**.

CBS 121944

The crude filtrate extract (20 mg) was fractionated by PTLC using 10% MeOH-CHCl₃ (100 mL) as eluants, resulting in one fraction of interest. This major fraction was further purified by PTLC by eluting twice with 5% MeOH to afford two major bands that were scraped and re-dissolved in EtOAc to produce compounds **14** (1 mg) and **15** (1 mg). Additionally small amounts of both compound **14** and **15** were isolated by LC-MS-SPE using the conditions to be described for DAOM 239833. These higher purity samples allowed for confirmation of the mass data and also for complete characterization of the compounds by NMR in C₆D₆.

DAOM 239830

The crude filtrate extract (30 mg) was dissolved in CH₂Cl₂ (0.5 mL) with three drops of MeOH and purified using PTLC, eluted successively with 25% EtOAc-hexanes, 1% MeOH-CHCl₃ and 2% MeOH-CHCl₃. The major band (10 mg) was re-purified by Si

gel small column chromatography using CHCl_3 -hexanes solvent gradient (0-100%, 10 mL each) followed by 1% MeOH- CHCl_3 (10 mL) to afford **16** (1.5 mg) as a white solid. The remaining bands were combined (5 mg) and re-purified by Si gel column chromatography eluting with CHCl_3 -hexanes solvent gradient (0-100%, 10 mL each) followed by 1% MeOH- CHCl_3 (10 mL) and 2% MeOH- CHCl_3 (10 mL) to afford **17** (1 mg) and **18** (1 mg), both as white solids. Compound **19** was not isolated from the extract of either 06-255A or 06-268A but its presence in both was confirmed by analysis of the LC-MS and LC-NMR data compared to the literature. The non-polar fractions (5 mg) were re-purified by Si gel chromatography eluting with CHCl_3 -hexanes solvent gradient (0-100%, 10 mL each) to afford **20** (0.5 mg) as a white solid.

DAOM 239831

The crude filtrate extract (50 mg) was dissolved in a minimum amount of CH_2Cl_2 with a few drops of MeOH before it was purified by PTLC eluting first with 2% MeOH- CHCl_3 (100 mL) then twice with 5% MeOH- CHCl_3 (100 mL). The first main fraction was determined to be **21** (2 mg). The other more polar fraction (5 mg) was further purified by PTLC eluting first with 2% MeOH- CHCl_3 (100 mL) and then three times with 5% MeOH- CHCl_3 to afford **22**.

DAOM 239833

The crude filtrate extract (80 mg) was analyzed using LC-MS-SPE/NMR in triple trap mode with emphasis on the major peaks in the chromatogram. The LC-MS-SPE system consisted of an Agilent 1100 series HPLC, with an Accurate ICP-04-20 flow-splitter by LC Packings, Bruker Esquire 4000 ion trap mass spectrometer, and a Bruker/Spark Prospekt II LC-SPE-NMR interface module with N₂-blanketing of the SPE compartment, all managed by Bruker HyStar/EsquireControl software. Separation was achieved on a Phenomenex Synergi Max-RP 4 μ m, 250 x 4.6 mm HPLC column with a gradient of CH₃CN/ H₂O with 5 mM formic acid (0.5 mL/min; 0:100 to 100:0 over 20 min). A postcolumn makeup flow of H₂O (1.5 mL/min) was added to reduce the solvent strength for trapping on Spark Hysphere Resin GP 10 x 2 mm SPE cartridges. The samples were filtered through 0.45 μ m GHP Acrodisc (13 mm) syringe filters prior to injections. The non-polar compounds **23**, **24**, **25** and **26** were all trapped, isolated and collected using this method. The isolated peaks were then transferred directly from the SPE cartridges to 3 mm NMR tubes using CD₃CN where complete NMR workups were obtained on each at 600 MHz.

CHAPTER IV

DETECTION OF ENDOPHYTES AND ANALYSIS OF TOXINS IN NEEDLES

INTRODUCTION

The development of methods for the analysis of the endophytes and their toxins in inoculated conifer needles was required. The ability to quantify the toxin concentrations in individual needle samples allows for many future experiments on the spread and persistence of the toxins over years. Additionally, toxicity experiments can be conducted using deforestation pests to produce dose response curves for toxin concentration vs. effects such as reduced body weight and head capsule size.

Methods for the detection of fungal endophytes and toxins in grasses include the use of direct microscopy using transmission electron microscopy (TEM) or light microscopy both of which require the preparation of the tissue samples. Assays using antibody-based methods for the detection of grass endophytes have also been developed for several endophyte species. These methods include ELISA tests to both the chemicals produced by the endophytes and also to the endophyte proteins. (Gwinn *et al.*, 1991; Johnson and Whitney, 1992; Reddick and Collins, 1998). The last method for their detection involves the use of PCR, which has also been shown to be effective at detecting the endophytes.

The detection of endophytes in conifer trees has mainly been through plating surface sterilized needles and examining them for the growth of the endophyte. This method is time consuming, requires a fungal taxonomist and is very susceptible to false negatives due to contamination from competing fungi on and in the needles. The plating method also provides no information as to the presence and concentration of the toxins in the needles. The use of ELISA for the detection of the endophyte proteins is examined as a way of rapidly and more accurately determining success for inoculations. Additionally,

the use of analytical techniques such as HPLC and LC-MS/MS are also examined with a variety of different extraction techniques to determine the concentration of the toxins in the needles.

RESULTS and DISCUSSION

ELISA testing and development for 5WS22E1

The limit of quantification for the target endophyte 5WS22E1 was determined to be 30 –60 ng cells per well; the limit of detection was 30 ng. Over a concentration range of one log, the antibody demonstrated a linear response to 60 ng of target endophyte (Figure 4.1) (results of triplicate experiments presented). Relative cross-reactivity to 15, 30, 60, 120 and 240 ng cells of the phyloplane species was moderate (8%). Over the same range, there was slightly greater cross-reactivity to the cells of the two white spruce endophytes tested (~15%), one of which produced rugulosin. Even at 240 ng cells, the response was below the one absorbance unit threshold used. The response of the polyclonal to the above range of the nontarget fungal cells was not linear across a range of antibody concentrations. The response to 60, 100 and 500 ng of white spruce cells again across a range of antibody concentrations was moderate (~6%) and nonlinear. A comparison of the response to 60 ng of nontarget cells to the target endophyte is provided (Figure 4.1); average relative standard deviation of replicates included in these data was 6.3%.

Figure 4.1: Response of serial dilutions of polyclonal antibody to target endophyte 5WS22E1 (best fit curve with LOWESS procedure) together with comparisons at 60 ng/well of cells of some white spruce endophytes (WS331L1, WS1111), some fungi common on the outside of the seedlings (*A. alternata*; *A. fumigatus*, *C. cladosporioides*, *Phoma* species, as well as control powdered freeze dried white spruce needles (see text).

Quantification of the target endophyte was not affected by the presence of larger amounts of powdered freeze dried needles. By ANOVA with Fishers LSD test, the response for 500 ng needle material plus 30 ng target endophyte was significantly greater than that for the 15 ng combination ($P = 0.008$). The value for 30 and 60 ng were not significantly different ($P = 0.312$) indicating that the limit of quantification was between these values in the presence of needle material. All remaining p values between endophyte needle cell additions were >0.003 . Absorbance values for 30, 60, 120 and 240 ng target endophyte plus needle material and the fungus alone were highly correlated $r =$

0.951 ($P > 0.000$) indicating that the presence of the needle did not affect the linearity of the assay (Figure 4.2) (results of triplicate experiments presented).

Figure 4.2: Response of polyclonal antibody to 30, 60, 120 and 240 ng 5WS22E1 cells and to the same amounts added to 500 ng powdered white spruce cells (mean plus standard error).

Of the 1235 trees tested, only 40 were clearly positive for 5WS22E1 by plating analysis (i.e. the fungus grew from the cut end of $>15/20$ of the needles per test plate). The majority of the ca. 25 000 surface-disinfested needle segments exhibited the growth of non-endophyte fungi comprising those previously observed (Miller *et al.*, 2002). As before the colonies of these taxa typically arose from the sides of the needles rather than the cut ends. When the same samples were analyzed by the antibody method, 330 or 27% were positive. All samples where the fungus was seen in culture were positive by the antibody assay.

Needle and chemical analyses for rugulosin

Of the 113 samples tested for rugulosin by HPLC from the 330 antibody positive needles, 101 (90%) were positive at the limit of quantification. The range of concentrations was 0.15 –24.8 $\mu\text{g/g}$ needle. The distribution of values (assuming half the detection limit for the non-detects) is shown in Figure 4.3). The geometric mean needle rugulosin concentration was 1.02 $\mu\text{g/g}$. Mean frozen weight of 100 representative needles was 2.6 $\mu\text{g/needle}$. Freeze dried weight was 1.08 $\mu\text{g/needle}$.

Figure 4.3: The distribution of rugulosin concentrations in needles from 113 seedlings, assuming half the detection limit for the nondetects (with normal smoother).

We previously demonstrated that the needles of seedlings leaving a production facility were not colonized by needle endophytes. Small seedlings (~7 weeks post germination), wound-inoculated and grown under laboratory conditions, could be colonized successfully with the rugulosin-producing endophyte 5WS22E1, albeit with

apparently a low success rate. Colonized needles contained toxic concentrations of rugulosin and this resulted in reduced growth of 3rd instar *Choristoneura fumiferana* (spruce budworm) larvae. The primary screening method for detecting successful colonization depended on the culture of surface disinfested needles, followed by lengthy incubation and microscopic examination. We speculated that the percentage of successfully colonized seedlings was possibly higher for the following reasons. The culture method has many limitations (Miller *et al.*, 2002). It was known that the endophyte grew and colonized the seedling slowly. The three-month incubation might not have been sufficient to permit the seedling to be colonized to a detectable level. In addition it was not clear in what direction the fungus would spread from the point of colonization. These latter factors made it impossible to know whether the needles harvested for the test could reasonably be expected to be colonized at all. The polyclonal assay developed for the target endophyte 5WS22E1 was of comparable sensitivity to similar assays for grass endophytes (Gwinn *et al.*, 1991; Johnson and Whitney, 1992; Reddick and Collins, 1998). This was achieved despite the greater difficulties of the conifer needle matrix compared to grass leaves. Cuttings of the latter can be placed directly in microplates whereas the tough, hydrophobic conifer needles must be ground to expose fungal cells to the antibody. Cross-reactivity to the potentially competing fungi was acceptable (Figure 4.1). Epiphytic biomass is typically low in young needles (Carroll, 1979) and is comprised mostly of fungi (Swisher and Carroll, 1980). Based on an extrapolation of our data on their data (Swisher and Carroll, 1980 their TABLE II), the fungal epiphytic biomass on these 19 month old needles would have $< 3 \mu\text{g/g}$. This means the presence of such fungi in our needle samples had no effect on the antibody response in these assays. In addition, relatively large amounts of powdered white spruce

needles compared to fungal cells did not affect the reliable detection of 30–60 ng cells of target endophyte (Figure 4.2). Non-endophyte fungi grew from nearly all the surface-disinfested needles of these nursery-grown seedlings. This was in contrast to the situation from the younger, growth chamber-grown seedlings where this was observed previously in half the needles (Miller *et al.*, 2002). The presence of these faster growing fungi made it much more difficult to recognize the presence of the target endophyte. When the same needle samples were tested by the antibody method, the rate of colonization was eight times higher or 27%. The present data cannot completely resolve the reason for this difference, but it is clear that a major factor was the insensitivity of culture method. It also is likely that endophyte spread and biomass were greater than we could detect and this will be the subject of further study. Most (90%) of the needles shown to be endophyte positive by the antibody method contained rugulosin concentrations above the detection limit. This provides additional evidence of the reliability of the antibody method. The mean (1 µg/g), range and distribution of rugulosin concentrations in these needles (Figure 4.3) were similar to that found in growth chamber-grown seedlings (Miller *et al.*, 2002). The analytical method in the present study included examination of the full scan UV spectrum of the rugulosin peak. This provides additional confirmation of the presence of this compound compared to the previous HPLC UV and TLC analyses (Miller *et al.*, 2002). The analyses were done with replicate 500 ng subsamples obtained from a 20 needle sample. The conservative assumption used in the scoring of the 330 positive seedlings was that they contained the limit of quantification. Using this assumption, each needle would contain 60 µg endophyte biomass/g needle or ~6%. For comparison Swisher and Carroll (1980) report that 1-4 year old Douglas-fir needles have ~10 µg epiphyte biomass/g needle. This

comprised mainly fungi but including algae and bacteria in older needles. This measurement enabled another comparison to be made; namely the amount of rugulosin per weight of fungal cells. Several studies have been made of the production of mycotoxins in living plants with culture and ergosterol to assess fungal biomass. A representative example is a study of deoxynivalenol in experimentally inoculated preharvest corn with corresponding measurements of ergosterol and viable fungi among other data (Miller *et al.*, 1983). With the ergosterol-fungal biomass conversion discussed in Gessner and Newell (2002), it is possible to estimate that *in planta* deoxynivalenol concentration corresponded to ~3% of the fungal biomass. With the mean rugulosin concentration, the ratio was ~2%. In summary the polyclonal assay developed for the target endophyte 5WS22E1 reliably detected the fungus in 500 ng subsamples of colonized needles. Nineteen months post-inoculation, rates of colonization detected were much higher than that documented from earlier chamber studies. Analysis showed most colonized needles (90%) contained detectable concentrations of the 5WS22E1 anti-insectan compound rugulosin. The range and distribution of needle rugulosin concentrations was similar to that found in earlier tests done in growth chambers.

Other Endophyte ELISA Development

Results for the development of detection methods for the four other white spruce endophytes; 5WS1111; 05-037A, 06-485A and 06-486D are displayed in Figures 4.4-4.11. Using the methods and techniques describe for 5WS22E1, ELISA methods were successfully developed for the detection of the four other white spruce endophytes.

The ELISA method for the detection of the 5WS11I1 showed that the optimal primary and secondary antibody dilutions were identical to that of 5WS22E1 (4000 and 5000 respectively). The use of 60 ng of cells was also used as the control. The cross reactivity to other endophytes and other common epiphyte fungi is minimal as shown in Figure 4.4. The impact of needles on the ELISA test is displayed in Figure 4.5. The results of the validating testing for this method showed that 125 out of 633 (24%) of the seedlings inoculated with 5WS11I1 were positive by ELISA at 6 months post inoculation. Other tests using 5WS11I1 in cut and spray experiments showed similar results, indicating that it is as effective as the ELISA test for 5WS22E1.

Figure 4.4: Response of serial dilutions of polyclonal antibody to target endophyte 5WS11I1, together with comparisons at 60 ng/well of cells of endophytes and other fungi as well as control powdered freeze dried white spruce needles.

Figure 4.5: Response of polyclonal antibody to 30, 60, 120 and 240 ng 5WS1111 cells and to the same amounts added to 500 ng powdered white spruce cells.

The development of an ELISA method for the detection of the 05-037A determined the optimal primary antibody dilution to be 4 000 and the secondary antibody dilution to be 10 000. The use of 15 ng of cells was also used as the control which is much more sensitive than for the first two endophytes, this is probably the result of injecting the goat with extracted protein from the endophyte. The cross reactivity to other endophytes and other common epiphyte fungi is minimal as shown in Figure 4.6. The impact of needles on the ELISA test is displayed in Figure 4.7. The results of the validating testing for this method in two separate trials showed that 71/340 (21%) and 193/538 (17%) of the seedlings inoculated with 05-037A were positive by ELISA at 6 months post inoculation.

Figure 4.6: Response of serial dilutions of polyclonal antibody to target endophyte 05-037A, together with comparisons at 60 ng/well of cells of endophytes and other fungi.

Figure 4.7: Response of polyclonal antibody to 30, 60, 120 and 240 ng 05-037A cells and to the same amounts added to 500 ng powdered white spruce cells.

The development of an ELISA method for the detection of 06-485A determined the optimal primary antibody dilution to be 2000 and the secondary antibody dilution to be 2 000. The use of 60 ng of cells was also used as the control which is the same as for the first two endophytes. The cross reactivity to other endophytes and other common epiphyte fungi is minimal as shown in Figure 4.8. The impact of needles on the ELISA test is displayed in Figure 4.9. The results of the validating testing for this method showed that 20.5% of the seedlings inoculated with 06-485A were positive by ELISA at 6 months post inoculation.

Figure 4.8: Response of serial dilutions of polyclonal antibody to target endophyte 06-485A, together with comparisons at 60 ng/well of cells of endophytes and other fungi.

Figure 4.9: Response of polyclonal antibody to 30, 60, 120 and 240 ng 06-485A cells and to the same amounts added to 500 ng powdered white spruce cells.

The development of an ELISA method for the detection of 06-486D determined the optimal primary antibody dilution to be 2 000 and the secondary antibody dilution to be 2 000. The use of 30 ng of cells was also used as the control which is half as much as is used for the first two endophytes. The cross reactivity to other endophytes and other common epiphyte fungi is minimal as shown in Figure 4.10. The impact of needles on the ELISA test is displayed in Figure 4.11. The results of the validating testing for this method showed that 21% of the seedlings inoculated with 06-486D were positive by ELISA at 6 months post inoculation.

Figure 4.10: Response of serial dilutions of polyclonal antibody to target endophyte 06-486D, together with comparisons at 60 ng/well of cells of endophytes and other fungi.

Figure 4.11: Response of polyclonal antibody to 30, 60, 120 and 240 ng 06-486D cells and to the same amounts added to 500 ng powdered white spruce cells.

Other Endophyte Toxin Analysis

5WS11I1 – Because vermiculin has an unremarkable UV spectrum, quantification was more difficult than for rugulosin. During this work, improvements to the analytical method had to be made as naturally contaminated samples became available for the first time. The original work was done on control needles spiked with the toxin.

The HPLC analysis for vermiculin of the ELISA positive samples showed that 30% contained vermiculin with the note that the limit of detection of the current method is not as sensitive as for rugulosin. This indicates that other positive samples may have been missed. Analysis of control samples showed no vermiculin peak in the chromatograms so the risk of having false positives is low.

05-037A – The results of the LC-MS/MS analysis of the ELISA positive samples of 05-037A in a limited study confirmed the presence of 3-butyl-4-methyl-5*H*-furan-2-one in 7 samples. The sample number was small and the samples were taken from young seedlings, where it has been more difficult to detect toxins than in older trees. The fact that 3-butyl-4-methyl-5*H*-furan-2-one was detected shows the sensitivity of the method and is a clear indicator that LC-MS/MS (MRM) is the method of choice for all future analysis of toxins in a needle matrix.

Additional Toxicity Testing

Other Insects – Diet Incorporated Bioassays

Spruce budworm larvae fed $>25 \mu\text{M}$ rugulosin weighed significantly less than the respective controls by analysis of variance (ANOVA; $25 \mu\text{M}$: $P = 0.027$, $50 \mu\text{M}$: $P = 0.001$ and $100 \mu\text{M}$: $P < 0.001$). Larvae fed $100 \mu\text{M}$ dietary rugulosin also had a significantly reduced head capsule ($P = 0.008$). Significance values given are for the second trial, which were similar to the duplicate test.

Hemlock looper larvae fed $>50 \mu\text{M}$ weighed significantly less than the respective controls. Significant differences were not observed for the first trial. During the second test, the weights of hemlock looper fed rugulosin were significantly lower than controls (ANOVA, $50 \mu\text{M}$: $P = 0.030$, $100 \mu\text{M}$: $P = 0.081$ and $150 \mu\text{M}$: $P = 0.001$). For the third trial the results were similar ($50 \mu\text{M}$: $P = 0.034$, $100 \mu\text{M}$: $P = 0.046$ and $150 \mu\text{M}$: $P = 0.001$). Larvae fed $150 \mu\text{M}$ dietary rugulosin also had a significantly reduced head capsule ($P = 0.028$).

Spruce bud moth larvae did not perform as consistently, presumably because these were collected in the wild. Many (60 %) succumbed to fungal infections (*Aspergillus fumigatus*) and other unknown causes over the course of rearing. Larval weights between controls and the treatment groups (10, 50, 100 and $150 \mu\text{M}$ rugulosin) were not significantly different by ANOVA [least significant difference (LSD), Tukey]. However, using the Wilcoxon matched-pairs signed-ranks test, the larvae fed 100 or $150 \mu\text{M}$ rugulosin in the diet were lighter than those fed the $10 \mu\text{M}$ rugulosin and control diets ($P < 0.10$). Because of the high percentage of *A. fumigatus*-contaminated larvae in the experiment, an unanticipated result was the antifungal activity observed above $50 \mu\text{M}$ few vials had fungal growth in the tests at 100 and $150 \mu\text{M}$ rugulosin. Breen *et al.* (1955) reported that rugulosin was moderately antifungal.

EXPERIMENTAL

The need to develop methods to detect the endophytes and their toxins in inoculated seedlings was required to help move the project from the laboratory to the nursery and ultimately to the forest. The strain *Phialocephala scopiformis* DAOM 229536 (5WS22E1) was isolated before the start of this project. It has been previously described as a rugulosin producer (Findlay *et al.*, 2003) and it is the main strain used for validation testing in this project.

Seedlings selected for the inoculation testing with DAOM 229536 were all control-pollinated full-sib families of white spruce. They were chosen to provide diversity among the set of genotypes. The parents were selected from the J.D. Irving Ltd. genetic improvement program and originated within a range of 45N to 47.5N latitude and 65W to 70W longitude in New Brunswick and Maine. The DAOM 229536 cultures used for inoculation were grown on 9 cm plates containing 2% malt-extract agar (Difco) at 20° C for 8 weeks. After the incubation period, 5 mL sterile water was added to the agar surface, which was then rubbed gently with a sterile bent glass rod. The resulting suspension was taken up with a sterile pipette, macerated and diluted with sterile water to deliver an average of three fungal hyphal fragments per drop (6 µL) from a sterile 1 mL syringe with a 0.45 mm needle as determined by counting with a hemocytometer. The wound inoculation of 1235 seedlings was performed in a laminar flow hood by injecting 6 µL into the unlignified tissue of the stem typically 10 mm away from the terminal shoot (Miller *et al.* 2002). The trees were then allowed to grow in trays in a greenhouse for 6 months, at which point they were planted into pots and left in a shaded area with irrigation until sampling.

Determination of infection

Plating method

At the time of sampling, average tree height was 12.9 cm. Needles from each tree were carefully removed noting the inoculation point, they were then placed in sterile plastic bags and immediately frozen for transport to the laboratory. Once in the laboratory approximately 20 needles were removed from each sample. The needles were surfaced-disinfested by dipping in 70% ethanol for 1 min, rinsing in sterile distilled water for 1 min and blotted dry on sterile tissue. The needles were then placed in a sterile Petri dish and cut into two segments with the needle half that was attached to the stem then being plated on a 2% malt-extract agar plate. They were incubated at 20° C for 6 weeks and were inspected regularly by microscopy for DAOM 229536 growth (Miller *et al.*, 2002).

Enzyme-Linked ImmunoSorbent Assay Development

The need for a more quantitative and a higher throughput method led to the development of an ELISA method for the detection of DAOM 229536 infection in seedlings. Cells of DAOM 229536 were grown on irradiated, young uninoculated control white spruce needles. The needles were irradiated with 25kGy (MDS Nordion, Montreal, PQ) and 200 mg was placed in a sterile glass Petri dish containing a filter paper followed by the addition of 1 mL sterile water. After 24 h, the culture was inoculated with a small piece of culture taken from the leading edge of a 2% malt-extract agar plate. They were also grown on glucose/sucrose mineral salts medium (1 g/L KH_2PO_4 , 1 g/L KNO_3 , 0.5

g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g/L KCl, 0.2 g/L glucose, 0.2 g/L sucrose) and on 2% malt-extract. An aliquot (50 mL) of each medium was dispensed into 250 mL Erlenmeyer flasks, respectively and autoclaved. An agar culture was macerated in 100 mL sterile water under aseptic conditions. An aliquot (2.5 mL) was used to inoculate the flasks. All cultures were incubated at 20° C for 3 months. At the end of the incubation period, the cells growing on the needles were carefully scraped off with a scalpel and freeze dried. Cells from the liquid cultures were filtered and washed several times with sterile distilled water and freeze dried.

Polyclonal antibody production was performed in goats at Cedarlane Laboratories Limited, Hornby, Ontario. This laboratory meets the requirements of the Canadian Council on Animal Care. Freeze-dried cells from each medium were ground up and each diluted in sterile PBS to a concentration of 20 mg/mL for the antigen solution. A total of 0.5 mL of this solution was emulsified with 0.5 mL of complete Freund's adjuvant (Brenntag Biosector, Denmark) for the primary immunization. A total of 0.5 mL of incomplete adjuvant was used for the subsequent boosts. A pre-immune sample was obtained from the jugular vein of each goat with a needle and vacutainer before the primary immunization. Each goat then was injected with a 21-gauge needle intramuscularly in the hind quarter at four sites with 0.25 mL of the emulsified antigen solution per injection site. After 28 d the goat received its first boost as described above, its second boost at day 53 and a test bleed was taken at day 66.

The antibodies produced from the three goats were tested to determine their avidity and cross reactivity with powdered, freeze-dried young uninoculated spruce needle cells, as well as cells of the most common needle phylloplane fungi isolated from

these needles (*Alternaria alternata*, *Phoma herbarum*, *Cladosporium cladosporioides* and *Aspergillus fumigatus*; Miller *et al.*, 1985; 2002), and other white spruce conifer endophytes: DAOM 229535 and 5WS331L1. In addition a number of balsam fir endophytes were tested (data not reported here because they would not occur in white spruce needles). In the case of the needle endophytes, cells were produced on irradiated needles as above. The phylloplane species tested were grown in shake culture with a maltose, yeast extract, peptone medium, filtered, washed and freeze dried as above. This method was shown to be suitable for antigen production by such fungi in unrelated studies.

Cells were ground to a fine powder using a small mortar and carefully weighed. Suspensions of known concentration were made in TBS (0.8 g/L NaCl, 0.2 g/L KCl, 1.89 g/L Tris-HCl, and 1.57 g/L Tris base, pH 7.5) in vials and vortexed. Avidity and cross reactivity experiments were conducted on sera from the goats treated with the various immunogens in a similar fashion, first optimizing cell additions/serum dilutions, and then conducting cross-reactivity experiments. As needed, aliquots of cells were diluted in 0.1 M carbonate buffer pH 9.6 (Sigma) coating buffer to defined concentrations and pipetted into 96 well Nunc brand microplates. The plates were covered with an acetate-sealing sheet and placed on a rotary shaker for 4 h at room temperature. The plate was removed, turned upside down and shaken to remove all of the coating solution in the sink. A total of 200 μ L of Blotto (10 g of nonfat dry milk/L of TBS) was added to each well, covered and placed in a refrigerator at 5 C overnight. The plate was removed and washed with a Molecular Devices Skan Washer 400 to remove the Blotto solution. The washing solution used was TTBS (0.5 mL of Tween-20/L of TBS) with a washing program of

three cycles of soaking, washing and rinsing. Various dilutions of goat serum in Blotto were made from which 100 μ L was added to the microplate wells. The plate was covered and placed on a rotary shaker 1 h at room temperature; it was removed and washed with TTBS as described above. A total of 100 μ L of antigoat IgG-horseradish peroxidase conjugate (Sigma) diluted 5 000 times in Blotto was added to each microplate well. The plate was covered and incubated at room temperature 1 h. The plate was washed for the final time and substrate was added. A total of 100 μ L of TMB (Tetramethly benzidine, Sigma) was added to each well. The plate was covered and incubated at room temperature 30 min. The reaction was stopped with 50 μ L of 0.5 M sulfuric acid. The plate was read immediately at 450 nm with subtraction of 630 nm on a Molecular Devices Spectra Max 340PC reader.

The polyclonal antibody produced with DAOM 229536 cells grown on the defined medium had low avidity and was not studied further. The antibody from the 2% MEA medium had acceptable avidity but unacceptable cross-reactivity. The polyclonal antibody to the cells cultured on irradiated needles was used in all further studies. A 4 000-fold dilution from the latter serum with a 5 000 dilution of the secondary antibody was determined to be optimal for tests with DAOM 229536 cells, allowing a preliminary estimate of the sensitivity of the assay to be made. Tests with this and the other conifer endophytes were done with cell weights of 15–240 ng over a fivefold range in antibody concentration. Using a serum dilution of 4000, response to 15, 30, 60, 120 and 240 ng cells of the phyloplane species and 60, 100 and 500 ng freeze dried white spruce needles was determined. Powdered freeze-dried white spruce needles (500 ng/well) were spiked with additions of DAOM 229536 cells over the above range.

Enzyme-Linked ImmunoSorbent Assay Testing

Twenty freeze-dried needles from each frozen needle sample were removed, ground to a fine powder in a vial with a Spex- Certiprep grinder-mixer (model 5100) and 10 mg weighted out. One mL of TBS (0.8 g/L NaCl, 0.2 g/L KCl, 1.89 g/L Tris-HCl, and 1.57 g/L Tris base) was added to each vial and placed on the vortex until completely mixed (ca. 1 min). The samples were assigned codes unrelated to the tree codes and randomized to ensure that samples from individual trees were analyzed across many plates. All were diluted in 0.1 M carbonate buffer pH 9.6 (Sigma) coating buffer to concentrations of 100 and 500 ng of needles per 100 μ L well and pipetted in duplicate onto 96-well microplate. The remaining steps in the analysis were as described above. In each trial 60 ng of DAOM 229536 cells were used as positive control to assess the performance of the assay; relative standard deviation of the net value of 25 representative experiments was 8.7%. Unless an acceptable positive control result was obtained, the results from individual plates were redone. Samples with high absorbance values in both 100 ng and 500 ng tests were rejected as indicating dilution problems. Results were scored as positive when absorbance of the 500 ng sample was greater than the lowest absorbance value above 1 (1.000) plus the mean absorbance value of 30 ng of the target endophyte on that plate.

Chemical analyses

A method for the detection of rugulosin, the major secondary metabolite produced by DAOM 229536 was available (Miller *et al.*, 2002). A new modified HPLC method

was developed for improved sensitivity. Rugulosin (purity > 95%) used for standards was a gift from Dr John A. Findlay, University of New Brunswick. The presence of rugulosin was analyzed in a subsample of 113 randomly selected trees of the 330 trees determined to be endophyte positive by antibody. A 100 mg sample of freeze-dried needles was ground to a fine powder as described above and extracted with 10 mL of ice-cold petroleum ether by stirring for 45 min under low light. The flask was kept on ice during the extracting and was covered with aluminium foil to prevent degradation by light. The suspension was filtered by suction and discarded. The needles were returned to the flask and extracted with 10 mL of room temperature chloroform for 45 min as above for petroleum ether. The new suspension was filtered by suction and retained while the needles were discarded. The chloroform extract was washed with 10 mL of 5% NaHCO₃ in a separator funnel. This first chloroform layer was discarded, the pH was acidified to pH 3 with 1 N HCl, and a new 10 mL of chloroform was added to the funnel and extracted. The chloroform was removed and dried in an amber vial under a gentle stream of nitrogen. The dried extracts were redissolved in 50 mL of acetonitrile, 10 µL was removed and injected into an 1100 series Agilent Technologies HPLC-DAD, with a Synergi Max RP 80A, 250 mm x 4.6 column (Phenomenex) and a gradient method adapted from Frisvad and Thrane (1987). The gradient started at 90% water with 0.05% TFA and 10% acetonitrile and changed to 10% water with 0.05% TFA and 90% acetonitrile over the 20 min run. Samples were analyzed at 389 nm, the maximum UV/VIS absorption for rugulosin and peak identity was confirmed by full spectrum data from the diode array detector. The limit of quantification was 150 ng/g freeze dried powdered material; recoveries from spiked needles averaged 75%. Statistical analyses were done with SYSTAT v. 10.2 (Point Richmond, California).

Other endophyte ELISA development

Similar methods for detection of the endophyte were developed for the other white spruce strains; 5WS11I1, 05-037A, 06-485A and 06-486D.

5WS11I1 was grown as irradiated needles as described above for DAOM 229536 and used to inoculate a goat. The resulting serum was tested in a similar manner to that above in order to develop a second method for endophyte detection and to provide additional evidence to support the results obtained for DAOM 229536. The cross reactivity was also tested as above along with the impact of needles on the test. The sensitivity and the specificity of the test were also determined.

None of the three other white spruce endophytes grew effectively on irradiated needles so all three were grown on 2% ME for cell production to inoculate goats with. A mixture of ground cells and extracted protein from the filtrate were injected in the goats for both 05-037A and 06-486D. The goat injected with 06-485A used only cells grown on 2% ME that were ground up and diluted in sterile PBS to a concentration of 20 mg/mL for the antigen solution. The optimal dilutions used for the primary and secondary antibodies for the 3 endophytes were determined as described above. The cross reactivity, sensitivity and specificity of each test were also determined as previously described for DAOM 229536.

Other Endophyte Chemical Analysis

A method for the analysis of vermiculin from 5WS11I1 was also developed using CH₃CN as the extraction solvent. The samples were then dried under a gentle stream of nitrogen, re-suspended in 50 µL of CH₃CN and mixed for injection in the HPLC using the above described gradient conditions for rugulosin. The vermiculin peak eluted off at 10.5 min with the DAD detector was set to detect at 222 nm (max for vermiculin).

A method for the analysis of 3-butyl-4-methyl-5*H*-furan-2-one in 05-037A infected needles was developed. Needles were removed and freeze-dried from seedlings inoculated with the white spruce endophyte 05-037A (CBS 120381). Each sample (100 mg) was then ground using a 5100 mixer/mill to a fine power. The needle samples were then extracted with 10 mL of acetonitrile and dried under nitrogen. The extract was then loaded onto a strata x reverse phase SPE column and washed first with water and then successive 10% increase in acetonitrile. All were discarded up to 40% organic, the fraction where the metabolite of interest elutes off the column. The 40% fraction (1 mL) was then analyzed by HPLC with a 50 mm reverse phase column (Phenomenex) using a 7 min run starting with 20% water and changing to 100% CH₃CN over 5.5 min. The peak of interest eluted at 3.4 min using this program, full scan UV spectra were used to confirm the metabolite as compared to a reference standard. Further confirmation of the presence of 3-butyl-4-methyl-5*H*-furan-2-one in inoculated needles comes from the use of LC-MS/MS in MRM (multiple reaction monitoring) mode. The samples were analyzed using a Micromass triple quad mass spectrometer with APCI+ as the ionization mode. The APCI probe temperature was set to 350 °C, the source block temperature was set to 100 °C and the cone voltage used was 30, all other MS parameters used were

standard settings. The compound was detected using the MS loss of 100, i.e the jump from the molecular ion of M^+ (155) to 55 to confirm its presence. The dissociation of the protonated molecule M^+ (155) to form the m/z 55 fragment ($C_3H_3O^+$) is likely formed from as a cascade fragmentation product in the mass spectrometer. Methods for the analysis of chemicals produced by 06-485A and 06-486D have yet to be developed.

Effects of dietary rugulosin on various insect larvae

Choristoneura fumiferana larvae (spruce budworm) were obtained from Insect Production Services, Forestry Canada (Sault Ste. Marie, ON) and stored at 5 °C. For each test, a sufficient number of larvae were hatched and put in creamer cups containing 15 mL artificial diet. The diet was prepared in-house (McMorran, 1965). The cups were placed in a growth chamber at 22 °C, 55 % RH with 16 hour light/day until the larvae reached second/third instar (McGugan, 1954). Previous tests suggested the effective concentration of rugulosin for *C. fumiferana* growth limitation was *ca* 10 μ M (Calhoun *et al.*, 1992). A suitable amount of diet was prepared, and four aliquots were dispensed into a flask. Dilutions of pure rugulosin were made in 95% ethanol to provide the required concentrations (540 μ l ethanol solution plus the vehicle control) and added to the flasks. The diet was mixed with a stir bar and two drops of hot liquid diet were each added with a 10 ml sterile pipette (0.1 g D.W.) to a 4 ml tapered plastic sample cup (Fisher Scientific). Vials containing diet and toxin were freeze-dried to eliminate the ethanol and rehydrated with 60 μ l sterile water. One larva was placed in each vial, which was returned to the growth chamber to feed for four days. After four days, all larvae were frozen and weighed on a Mettler 163 analytical balance ($\pm$ 0.02 mg). Previous studies

demonstrated that the frozen wet weight was correlated with dry weight ($P < 0.001$). Head capsule widths were determined using a stereo microscope at $\times 40$ with ocular and stage micrometers. *C. fumiferana* larvae were tested in two treatments, the first with rugulosin concentrations of 5, 10 and 50 μM (75 insects per concentration tested) and the second with concentrations of 25, 50 and 100 μM (75 insects per concentration, tested in duplicate).

Lambdina fiscellaria eggs (hemlock looper) were purchased from Forestry Canada in three lots. The eggs were incubated at room temperature for 10 days until larvae emerged. Larvae from each batch emerged within two days of each other and were immediately put on diet in cups as above. After they had grown to second instar (*ca* one week), larvae were placed in test vials following the same procedure as for *C. fumiferana* with a concentration range of 5, 10 and 50 μM rugulosin for the first test and 10, 50, 100 and 150 μM for the second test (~ 67 larvae per concentration, tested in duplicate). After one week on the test diets, the larvae were frozen, weighed, and head capsule width determined.

Zeiraphera canadensis larvae (spruce budmoth) were collected from the wild near Sussex, NB, by mid June (received 13 June 2002 on spruce buds). They were immediately put in test vials and tested at rugulosin concentrations of 10, 50, 100, and 150 μM (75 per concentration, collected in the wild, therefore tested only once). After seven days, surviving larvae were frozen and measured.

CHAPTER V

GENERAL DISCUSSION

Endophyte Collection, Growth, Screening and Analysis

The knowledge that grasses contain endophytes and that these endophytes play a variety of roles within the plant is well understood. A similar mutualistic relationship has also been demonstrated in conifers (Sieber, 2007). The need for these fungi and the toxins they produce to help protect the tree from deforestation pests is extremely important. It is also understood that seedlings are infected by spores from cast needles via horizontal transmission which differs from grasses which keep their endophytes in the seeds from one generation to the next (vertical transmission). This is important for the reforestation process which involves the planting of seedlings from a greenhouse. Studies have shown that seedlings from a greenhouse contain reduced levels or no endophytes (Miller *et al.*, 2002). This means that as a forest company harvests a stand of natural trees and replaces them with seedlings from a seed production facility, they have inadvertently diminished the trees natural defense. As previously discussed, spruce budworm outbreaks occur in cycles that have historically occurred every 20 years. It is safe to assume that the next outbreak will cause even greater damage than the previous ones. The outbreak will also be impacted by the deregulation of chemical pesticides that during the previous outbreaks were used to help save the trees and also by the cost of such a spraying (MacLean *et al.* 2002). The proof that the pesticide use was effective is evident by the complete destruction of spruce and fir trees in Cape Breton Highlands National Park where spraying was prohibited vs. in NB where trees were sprayed and remained “green”. Public and private resources have been invested to infect seedlings in the greenhouse with natural strains of endophytes that have been shown to produce compounds that are toxic to spruce budworm and other deforestation pests.

Aside from small collections made in 1984 (noted in Miller *et al.* 1985) and 1987-88 (Johnson and Whitney 1989; 1992), a large survey had been done for conifer endophytes in the region. In 1992, branches were collected from superior trees from Crown lands that covered the area from near the southern border of New Brunswick with Maine to outside Quebec City in a north south transect representing 3 500 isolates (Miller *et al.* 2002; Wilson *et al.* 1994). Studies from these collections isolated and characterized compounds that showed toxicity to spruce budworm (Findlay *et al.*, 2003). A need for more strains to help increase the biodiversity of replanted forests was demonstrated. This led to the decision to produce a new collection of endophytes from the forests of Maine, NS and NB, with the added benefit of being able to record the GPS coordinates of each tree sampled. In contrast to the 1992 collection, this was done on J.D. Irving land and in an east – west direction. The undertaking of a collection of this size (greater than 2 000 endophytes) from nine distinct areas from this region was needed to address a number of problems. The collection needed to be big enough to ensure that we received a proper representation of the families of endophytes from this area and also subsequently ensured representation of the toxins produced by endophytes in this region. The collection is also of value for future studies on the taxonomy of these endophytes because so much is known about these strains. The compounds identified are of interest for studies on the chemical ecology of protecting trees from insects and fungal diseases. The possibility to discover new compounds and to explore their biosynthetic pathways is the basis for many synthetic experiments and as leads for drug discovery.

The strains collected were placed into groups based on morphology for the purposes of classifying and identifying them for further screening. The use of 2% malt

extract was chosen because for more than 100 years it has been used to culture fungi. It has a C:N:P ratio that is comparable to nature and complex plant polysaccharides (versus simple sugars). Growing all cultures on 2% malt extract also allowed for comparison of similar strains with respect to their secondary metabolite production. From a biological perspective it is plausible that the endophytes grow very slowly due to the fact that no evolutionary benefit could be obtained from growing quickly and rapid growth would lead to needle damage (Johnson and Whitney, 1989).

The DNA sequencing work for the selected endophytes showed large diversity among the strains. The DNA data on the strains used for metabolite characterization suggest that many of them are poorly classified or are new species. Based on the DNA sequence information (provided after the publication of Miller *et al.* 2008), Dr. Christoph Gruenig (Swiss Federal Institute of Technology) was able to determine that DAOM 229536 (5WS22E1) was *Phialocephala scopiformis*. This was done by comparison to data in an unpublished database of this genus and related genera. This species has been reported as endophytic in Norway and from some other conifers in cool areas (Grunig *et al.* 2002). Another strain DAOM 229536 (5WS11I1) based on DNA sequence information was also thought to be a *Phialocephala*, but probably is a new species. The identification of these two strains demonstrates the difficulty in determining the taxonomy of each individual strain in the collection.

The selection of the strains for metabolite isolation and characterization was partially based on toxicity data. The use of bioassays is common for the isolation of natural products, as a technique for narrowing down the source of the activity in the extract. The individual toxicity of the compounds isolated was not tested because of the

large amount of pure compound required for these tests. It is also common with fungi that the toxic impact observed from a strain is not the result of a single toxin, but for multiple toxins. These effects have been demonstrated by Dowd where it was shown that the presence of a mycotoxin (fusaric acid) synergized the toxicity of allelochemicals resulting in increased mortality to corn earworm (Dowd, 1989) and similarly in caterpillars (Dowd, 1988). In the case of cattle toxicosis due to the consumption of silage contaminated with *Penicillium* species, a variety of metabolites are produced including; immunosuppressants, tremorgens and other toxins all of which may play a role in affecting the cattle (Nielsen *et al.*, 2006). Disc diffusion tests employed in this study are also commonly employed when searching for compounds with a specific activity towards other fungi. The use of antifungal compounds in medicine is of great importance and interest to the pharmaceutical industry.

Over the past decade the use of LC-MS in the screening and identification of metabolites from fungal extracts has become the technique of choice (Nielsen and Smedsgaard, 2003). LC-MS provides the ability to obtain high resolution mass spectrum data on the compounds from an extract in a 20 minute gradient HPLC run. For fungi where standards are available metabolite confirmations can be made from the mass spectrum data alone. In the case of fungi such as the endophytes from this study where little or no published information is available, screening by LC-MS is still useful to help determine which extracts may contain compounds for further study and to determine the chemical formula of each. The LC-MS screening of the extracts also helped to eliminate strains that produced little or no compounds as evident from the HPLC and total ion chromatograms.

Isolation and Structure Elucidation of Secondary Metabolites from White and Red Spruce Endophytes

The screening of a selection of red and white spruce endophytes from different geographic regions through out the Maritimes and Maine revealed a number of important findings. The first observation was that none of the strains examined produced either rugulosin or vermiculin in detectable quantities (Miller *et al.*, 2002). There are several explanations as to why when collections were made in the 1980's and 1990's that these compounds were isolated from multiple strains and are not observed today. The first is that they are simply no longer present in the trees from this area. The probability of this reason being correct is low as evident by the re-discovery of compounds **16 – 20** in strains from the current collection. With the exception of ramulosin (**19**) these compounds have either not been reported or occur rarely in the literature other than from the black spruce endophyte reported by Findlay *et al.* (1995). The last explanation and the most probable as to why rugulosin and vermiculin were not detected in the extracts from the current collection has to do with the sampling area. The original large collection (3 500 isolates) was made in a north south direction and from public and Crown land. The current collection was made in an east west direction and from J.D. Irving managed lands. These differences in climate, tree age and provenance affect the endophytes isolated and could explain the consequent lack of detection of these compounds from the current collection.

The metabolites isolated from the white and red spruce strains can be classified according to their biosynthetic origins. Most were polyketides that includes metabolites **1, 3-11** and **16-20**. Polyketides are among the most common secondary metabolites

produced by fungi, representing many diverse structures. The study of endophytes presented here showed that even with the large amount of literature available on fungal polyketides, compounds **4**, **5** and **11** are reported as new structures. The results show that one in five of the polyketides isolated during this study were reported for the first time. This provides further evidence for the theory that the productivity index for new compounds from endophytes is much higher than for other fungi (Miller, 1991). Compound **1** is reported for the first time as a natural product and compound **3** is reported for the first time as a fungal metabolite. The majority of the other previously described polyketides that have only been presented in a few literature reports.

The second class of metabolites isolated during this study are the sesquiterpenes; compound **14** was previously reported as a plant metabolite and **15** was reported for the first time and the toxicity of both are unknown. The third group was compounds derived from amino acids, these include compounds **2**, **12**, **13** and **21** that show clear similarities between their biosynthetic pathways. The last group isolated was the nonadrides bearing two and three maleic anhydride moieties derived from either two or three 9-carbon units. Compounds **23** and **24** are reported for the first time, but their structures suggest that they are likely biosynthetic precursors to the known metabolites **25** and **26**.

The isocoumarin backbone was observed in the majority of the polyketide metabolites isolated from both white and red spruce endophytes during this study. The similarity of the structures is not only relevant with respect to their biosynthetic origin but also to the observed toxicity. Many of the compounds isolated in this study are novel or are very poorly characterized in the literature and as a result very little is known about their toxicity. Mellein (**6**) is one of the compounds isolated that has been reported from

numerous sources and is well described, including toxicity, in the literature. Schulz *et al.* (1995) showed that it had fungicidal, herbicidal and algicidal activity, and it would be safe to assume that the other related structures may exhibit similar toxicity. This point was further emphasized from the isocoumarins isolated from the red spruce, where metabolites were characterized that had previously shown toxicity to spruce budworm cells comparable to vomitoxin (Findlay *et al.*, 1995). The relevance of the individual toxins in the needles and also the importance of families of these toxins to help protect the tree from deforestation pests were evident. The insect toxicity in the extracts containing the two sesquiterpenes (**14** and **15**), and in the extract with the four maleic anhydride compounds (**23-26**) remains to be explained. The insect toxicity of tyrosol and the related amino acid derived metabolites isolated during this study do not explain the effects on spruce budworm from the diet incorporated bioassays. Similar effects on spruce budworm development and growth were observed from many endophyte metabolites. This is important and addresses the point that a forestry company would not want to only use one or even two strains to protect trees against deforestation pests. It also appears from the survey of the endophytes collected that a similar situation occurs in nature where there are many different strains that produced a wide variety of metabolites.

None of the compounds isolated from any of the strains contained nitrogen, chlorine or sulfur as are found in many other common fungal metabolites. The compounds isolated do have different biosynthetic origins such as polyketide, sesquiterpenes and shikimic acid, indicating that they are not limited to one particular pathway. Also of note is that the size of the majority of the compounds isolated is small i.e MW < 300, with the exception of the compounds isolated from 08-018A. Elucidation

of the secondary metabolites from these endophytes contributes to studies on the polyphasic taxonomy for this group.

The relatively new technique combining LC-MS-SPE/NMR was used to isolate and characterize compounds **14**, **15** and **23-26**. The technique allows for the isolation of small quantities of pure metabolites and helps to avoid the problematic possibility that compounds are altered during the process of isolation, a hazard in traditional natural product research. The use of the same LC conditions as for other analysis combined with real time mass spectrum data on the individual peaks in the chromatogram enables the collection of compounds of interest on SPE cartridges. This is very important for compounds that are sensitive to light and acid for example, which is common for fungal metabolites. The technique allows for the direct elution from the SPE cartridges into 3 mm NMR tubes for analysis. It was only made possible by advances in NMR technology i.e. cryo-probes that require much less material than in the past. The downside of the technique is in the determination of the relative stereochemistry of compounds that are not rigid or do not contain sufficient protons to characterize it by NOE experiments. This problem is addressed in a recent paper where it was suggested that CD (circular dichroism) analysis may be able solve this problem (Sprogøe, 2008).

Detection of Endophytes and Analysis of Toxins in Needles

The development of the ELISA and the HPLC based methods for the detection of the endophytes and their toxins in the needles was important for the current and all future aspects of this project. The primary method used for detecting successful colonization by

5WS22E1 involved the plating of surface disinfested needles, followed by lengthy incubation and microscopic examination (Miller *et al.*, 2002). It was speculated that the percentage of successfully colonized seedlings was possibly higher than observed for the following reasons. The culture method has many limitations, including contamination from other faster growing epiphytes (Miller *et al.*, 2002). Additionally, it was known that the endophyte grew and colonized the seedling slowly and that plating the needles with three months of incubation might not have been sufficient to permit the seedling to be colonized to a detectable level. It was also not clear how the fungus would spread from the point of colonization. These factors made it impossible to know whether the needles tested could reasonably be expected to be colonized at all. Thus it became clear that better methods were required for the detection of the endophyte and the toxins in needles. These methods were required to not only determine a yes/no infection question, but also to address the issue of the spread of the endophyte within the tree and to adjacent trees; as well the persistence over time in the tree. Additionally, there was a requirement for toxin quantification for *in planta* dose response toxicity testing.

For the purposes of this study a method was developed and validated for the detection of 5WS22E1 in needles that were inoculated by injection. The analyses were done using replicate 500 ng sub-samples obtained from a 20 needle sample. Based on the results, each needle would contain ~60 µg endophyte biomass per g needle or ~6%. For comparison, Swisher and Carroll (1980) reported that 1-4 year old Douglas fir needles have ~10 µg epiphyte biomass per gram of needle. This was mainly comprised of fungi but including algae and bacteria in older needles. The concentration of rugulosin in the needle samples was also quantified to help validate the ELISA data for 5WS22E1

infection in the needles (Sumarah *et al.*, 2005). Rugulosin needle concentrations were approximately $1 \mu\text{g g}^{-1}$, this concentration is on the order of the $10 \mu\text{M}$. Compared to the data on the effective concentrations *in vitro*, this concentration is in approximately the right order of magnitude for the predicted effect on insect growth, consistent with the results obtained in growth chambers (Miller *et al.*, 2002; 2008).

Endophyte Spread within the Seedling

The spread of the endophyte within the seedling was evaluated by removing all of the branches from 20 trees that were infected and left for 15 months in a nursery. These trees were all inoculated but half of them (10) tested negative by ELISA at three months post inoculation. The branches were individually mapped and analyzed by both ELISA and for rugulosin as described in chapter IV. Almost all of branches from both the 10 positive and 10 negative trees at three months were found at 15 months to be positive by ELISA and positive for the presence of rugulosin. The choice of waiting three months post-inoculation before assaying for rugulosin and the endophyte was based on practical considerations but it was understood that because fungal endophytes grow and colonized the seedling very slowly it was prone to false negatives (Miller *et al.*, 2002; Sumarah *et al.*, 2005). As noted, previous studies had shown the problem of false-negatives with plating because of the slow rate of growth of the fungus (Miller *et al.*, 2002; Sumarah *et al.*, 2005). In addition, there was evidence for the first time that there was variation in the performance of the ELISA due to differences in the needles from spruce trees (Sumarah *et al.*, 2008). The rate of false negatives by ELISA was greater in the trees that tested

negative at three months. In summary, the endophyte and its toxin were shown to be well distributed between branches 15 months post inoculation.

Endophyte Spread and Persistence at the Field Site

The persistence of the endophyte and rugulosin in infected trees was tested using trees planted in a test field site. Needle samples were collected from trees approximately 3.5 and 4.5 years post-inoculation. Needles from one, two and three year age classes were tested by ELISA and for rugulosin as described. All of the needle samples from the branches were positive by ELISA and 96 % contained detectable rugulosin at one year in the field and 3.5 years post inoculation. Mean rugulosin concentration corrected for recovery was $1.0 \mu\text{g g}^{-1}$; rugulosin concentrations from each age class of needles were virtually identical. From the trees collected as above 4.5 years after inoculation, 40 % of the samples were positive by ELISA but 100% contained rugulosin. Mean rugulosin concentration corrected for recovery was $0.7 \mu\text{g g}^{-1}$; rugulosin concentrations from each age class of needles were again virtually identical. The data showed that virtually all of the branches from each age class were positive for the endophyte, rugulosin or both (Sumarah *et al.*, 2008). This includes needles that were not present on the tree at the time of inoculation. It is important to note that spruce trees normally add their needles over a 3 year cycle, meaning that new needles which arose from the branches within the last year are also infected. This demonstrates that although the spread and growth of the endophyte in the tree is slow, it is persistent.

The second aspect was the replication of natural events in a field site, where the spread of the endophyte to small spruce seedlings planted around the endophyte positive trees and to all natural regeneration was tested. This included testing for the presence of 5WS22E1 by ELISA of all non-target species such as: other spruce; white pine; and willow trees (Miller *et al.*, 2009). The results of testing using the ELISA method developed for 5WS22E1 showed that 40 % of the small spruce seedlings planted immediately under the trees (i.e. in the zone where cast needles would fall) were colonized. This is a further confirmation of the horizontal transmission of the endophyte in conifers which is in contrast to the grass endophytes, where the transmission is vertical (vegetative or through the seed; Clay and Schardl, 2002). *P. scopiformis* was not detected in the non-target plants. Of the 53 natural regenerated spruce seedlings collected, 5 (9%) were positive for *P. scopiformis* or something very closely related. Cross-reactivity to very similar strains is possible but is not material between other endophytes (Sumarah *et al.*, 2005).

Effect of 5WS22E1 Inoculated Seedlings *in planta*

The availability of endophyte-positive trees in the field site allows for a determination of the impact on spruce budworm *in planta*. Two aspects were examined: first the effect of rugulosin in needles was studied by comparing the growth and development of spruce budworm that were placed on infected and uninfected trees. The second was to use the concentration of rugulosin in needles from an infected tree to serve as its own control (Miller *et al.*, 2008). This strategy is typically used in toxicology to eliminate possible confounding variables arising from intra-individual variance. The

intention here was to look for evidence of a dose-response to rugulosin. The trees used in the present studies represented a diverse genetic population used for reforestation in eastern Canada and Maine.

The results of the first experiment showed that there was a statistically significant difference in budworm weights between the two groups. As in the growth chamber, the presence of the fungus and its toxin reduced spruce budworm growth. The second experiment showed that the presence of 5WS22E1 and rugulosin in trees as determined using the describe methods, resulted in reduced spruce budworm growth in a dose dependent manner. In addition, these experiments show that the effect is primarily explained by rugulosin (Miller *et al.*, 2008). These two studies provide strong evidence as to the usefulness of infecting spruce trees with endophytes that produce compounds that are toxic to spruce budworm, for protecting the tree.

In a monograph on spruce budworm population dynamics in New Brunswick, Royama (1984) discussed the cyclical nature of budworm epidemics. He postulated an unknown factor to modulate upwards the observed population changes in relation to his model. The central characteristic of the unknown factor was that it increased the response of the insect populations to known factors such as predation and disease. Since the primary effect of rugulosin *in vitro* and in the plant is to slow growth rate, the animals would be exposed to these environmental and biotic factors for longer durations. We have also postulated that this effect might put cohorts of the insects out of reproductive synchrony.

In summary, the results from the ELISA and rugulosin data were supported by plating the same samples. These methods were further validated from the dose response

evidence and the experiments on spread and persistence of the endophyte and toxin. Once inoculated, the test endophyte spreads throughout the developing tree and persists under field conditions for at least four years. The rugulosin needle concentration data was determined to be approximately $1 \mu\text{g g}^{-1}$ (Sumarah *et al.* 2005, 2008, Miller *et al.* 2002, 2008). Compared to the data on the effective concentrations *in vitro*, this concentration is in approximately the right order of magnitude for the predicted effect on insect growth, consistent with the results obtained in growth chambers (Miller *et al.* 2002). In synthetic diet (Calhoun *et al.* 1992; Sumarah *et al.* 2008), the presence of approximately 10-25 μM rugulosin resulted in the reduction in *C. fumerana* growth rate (ranging to 50 μM for some other herbivorous insect species). Using the weight of evidence rule in toxicology, the effects seen are largely explained by the toxin, and are consistent with other endophyte-interactions that have been studied.

CONCLUSIONS

The isolation and characterization of secondary metabolites from white and red spruce endophytes was highlighted by the discovery of six novel compounds. Additionally, three compounds are reported for the first time as natural products and two for the first time as a fungal metabolites. All of these compounds either alone or as mixtures in the natural extract of these endophytes were toxic to spruce budworm.

ELISA methods effective at detecting five of the white spruce endophytes in needles have been developed. For three of these endophytes, analytical methods for the detection of the major toxins produced by each endophyte in the needles have been developed and validated.

FUTURE WORK

The next step for the research would be to continue studying the red spruce endophytes in the collection to identify additional strains that produce compounds that are toxic to spruce budworm, other than those reported here. This use of multiple strains that produce a wide variety of metabolites are needed to help increase and maintain the biodiversity of endophytes in the forests of Maine, NS and NB. In addition, the toxicity of the individual isolated metabolites should be examined to explain the toxicity observed from the extract and to look for a possible mechanism.

Further work on the identification of the endophytes isolated in this study is required. The DNA sequencing work presented was for the most part inconclusive for identification purposes but demonstrated that identifications are very difficult to make and that many of the strains isolated are poorly characterized species that required further study.

The red spruce strains characterized in this study as showing toxicity to spruce budworm need to be incorporated into J.D. Irving's tree improvement program. This requires the development of similar methods for the analysis of the endophytes and the identified toxins in needles.

The final experiment that should be conducted involves using the analytical methods developed during this study to go back (using the GPS data) and look for the isolated metabolites in the host tree from which they were collected. The ability to detect the toxins in the original tree is the final proof that the idea of infecting seedlings with these endophytes is a viable method of providing resistance to deforestation pests.

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APPENDIX

Section A: Total list of trees sampled including species and location

TREE #	SPECIES	LATITUDE	LONGITUDE	COMMENTS	MAP #
01-001	WS	47.28.00.91	67.24.25.66		3720
01-002	RS	47.26.54.45	67.27.01.42		3721
01-003	RS	47.23.05.48	67.35.28.57		3622
01-004	WS	47.13.38.16	37.37.03.35		3525
01-005	RS	47.13.54.19	37.34.33.36		3625
01-006	RS	47.13.58.26	67.34.13.28		3625
01-007	RS	47.13.50.25	67.33.32.33		3625
01-008	RS	47.13.16.82	67.33.09.57		3625
01-009	RS	47.14.36.37	67.33.23.04		3625
01-010	RS	47.11.56.00	67.28.08.91		3726
01-011	RS	47.12.03.02	67.28.13.53		3725
01-012	RS	47.14.02.64	67.33.51.58		3725
01-013	RS	47.15.05.50	67.37.58.97		3524
01-014	RS	47.14.12.03	67.41.41.55		3525
01-015	RS	47.13.56.38	67.42.49.98		3425
01-016	RS	47.13.54.74	67.43.00.32		3425
01-017	RS	47.13.46.74	67.43.33.49		3425
01-018	RS	47.13.30.06	67.44.10.09		3425
01-019	RS	47.13.06.67	67.44.56.47		3425
01-020	RS	47.13.18.23	67.46.00.33		3425
01-021	RS	47.13.37.88	67.46.26.71		3425
01-022	RS	47.14.52.05	67.48.05.39		3325
01-023	WS	47.15.34.73	67.47.46.59		3424
01-024	RS	47.16.08.35	67.46.50.81		3424
01-025	RS	47.17.01.58	67.45.12.59		3424
01-026	RS	47.20.04.26	67.44.11.65		3423
01-027	RS	47.20.45.57	67.45.45.60		3423
01-028	RS	47.21.56.33	67.48.21.27		3322
02-001	RS	46.04.56.03	67.00.49.09		4148
02-002	RS	46.08.13.65	67.06.44.64		4047
02-003	RS	46.11.01.00	67.05.24.77		4146
02-004	RS	46.11.51.74	67.04.37.57		4146
02-005	RS	46.13.14.67	67.02.52.85		4145
02-006	RS	46.15.16.73	67.01.46.69		4144
02-007	RS	46.19.14.42	67.01.04.70		4143
02-008	RS	46.20.25.71	66.57.44.73		4243
02-009	RS	46.21.17.06	66.53.33.66		4342
02-010	RS	46.22.02.36	66.39.50.35		4542
02-011	RS	46.23.31.47	66.38.11.02		4542
02-012	RS	46.26.23.86	66.33.29.51		4641
02-013	RS			KI Road #1	
02-014	RS			E Br. Kensington River	

02-015	RS			New Rd D.D SV For. Rd	
02-016	RS			D20 Road #1	
02-017	RS			Red G Nth Rdg D.D.Sth	
02-018	RS			D20 Road #1	
02-019	RS			Red G Nth Rdg D.D.Sth	
02-020	RS			New Rd D.D SV For. Rd	
02-021	RS			D20 Road #1	
02-022	RS			New Rd D.D SV For. Rd	
02-023	RS			E Br.Keswick River	
02-024	RS			E Br.Keswick River	
02-025	RS			D20 Road # 2	
03-001	RS	46.27.36.44	66.27.00.22		4740
03-002	RS	46.27.28.79	66.15.51.93		4940
03-003	RS	46.28.55.56	66.11.00.76		5040
03-004	RS	46.30.34.15	66.07.50.58		5039
03-005	RS	46.29.29.01	66.06.45.30		5040
03-006	RS	46.29.46.02	66.04.23.86		5140
03-007	RS	46.21.48.01	65.59.27.26		5242
03-008	RS	46.33.38.21	66.02.59.39		5138
03-009	RS	46.33.24.87	66.03.06.22		5138
03-010	RS	46.33.04.46	66.03.54.37		5138
03-011	RS	46.33.37.23	66.05.01.30		5138
03-012	RS	46.32.39.56	66.13.14.84		4939
03-013	RS	46.32.55.91	66.14.26.17		4939
03-014	RS	46.33.34.77	66.16.40.59		4938
03-015	RS	46.34.46.26	66.18.48.53		4838
03-016	RS	46.37.57.77	66.19.26.82		4837
03-017	RS	46.36.57.68	66.19.33.75		4837
03-018	RS	46.36.21.33	66.17.14.93		4937
03-019	RS	46.36.30.07	66.14.40.89		4937
03-020	RS	46.36.00.83	66.12.24.99		4937
03-021	RS	46.36.11.89	66.10.40.10		5037
03-022	RS	46.27.14.7	66.24.01.4		
03-023	RS	46.26.54.4	66.22.50.7		
03-024	RS	46.26.40.4	66.22.28.3		
03-025	RS	46.26.35.6	66.22.26.3		
03-026	RS	46.25.59.9	66.22.04.5		
03-027	RS	46.25.52.6	66.21.51.9		
03-028	RS	46.25.48.9	66.21.42.4		
03-029	RS	46.25.45.3	66.21.13.2		
03-030	RS	46.25.34.6	66.20.49.3		
03-031	RS	46.25.02.1	66.19.52.2		
03-032	RS	46.24.54.7	66.19.49.7		
03-033	RS	46.24.46.9	66.19.47.8		
03-034	RS	46.24.34.9	66.19.48.4		
03-035	RS	46.23.38.4	66.19.35.1		
03-036	RS	46.22.13.5	66.19.33.1		
03-037	RS			#1 Porter Cove	
03-038	RS			#2 Porter Cove	
03-039	RS			#3 Porter Cove	
03-040	RS			#4 Porter Cove	

03-041	RS	#5 Porter Cove
03-042	RS	#6 Porter Cove
03-043	RS	#7 Porter Cove
03-044	RS	#8 Porter Cove
03-045	RS	# 9 Porter Cove
03-046	RS	#11 Porter Cove
03-047	RS	#12 Porter Cove
03-048	RS	#13 Porter Cove
03-049	RS	#14 Porter Cove
03-050	RS	#15 Porter Cove
03-051	RS	Airstrip
03-052	RS	CrookBrook Rd. "A"
03-053	RS	Pink Panther Rd.
03-054	RS	CrookBrook 1st Br. "B"
03-055	RS	CrookBrook 1st Br. "B"
03-056	RS	CrookBrook Rd. "A"
03-057	RS	Airstrip
03-058	RS	Pink Panther Rd.
03-059	RS	HazeltonCross by pwline
03-060	RS	Banford Rd.
03-061	RS	HazeltonCross by pwline
03-062	RS	HazeltonCross by pwline
03-063	RS	Airstrip
03-064	RS	CrookBrook Rd. "C"
03-065	RS	Pink Panther Rd.
03-066	RS	Banford Rd.
03-067	RS	Banford Rd.
03-068	RS	CrookBrook 1st Br."B"
03-069	RS	CrookBrook 1st Br."C"
03-070	RS	CrookBrook Rd. "C"
03-071	RS	CrookBrook Rd. "A"
03-072	RS	Crk.Br.Rd. 6-7km mkr.
03-073	RS	Crk.Br.Rd. 6-7km mkr.
03-074	RS	Banford Rd.
03-075	RS	Banford Rd.
03-076	RS	Banford Rd.
03-077	RS	Banford Rd.
03-078	RS	Banford Rd.
03-079	RS	Banford Rd.
03-080	RS	Banford Rd.
03-081	RS	Banford Rd.
03-082	RS	Banford Rd.
03-083	RS	Banford Rd.
03-084	RS	Banford Rd.
03-085	RS	Banford Rd.
03-086	RS	Banford Rd.
03-087	RS	Banford Rd.
03-088	RS	Banford Rd.
03-089	RS	Banford Rd.
03-090	RS	Banford Rd.
03-091	RS	Banford Rd.

03-092	RS			Banford Rd.	
03-093	RS			Crook Br.Rd. 6-7km mkr	
03-094	RS			Crook Br.Rd. 6-7km mkr	
03-095	RS			Crook Br.Rd. 6-7km mkr	
03-096	RS			Crook Br.Rd. 6-7km mkr	
03-097	RS			CrookBr.Rd.10-12km mkr	
03-098	RS			CrookBr.Rd.10-12km mkr	
03-099	RS			CrookBr.Rd.10-12km mkr	
03-100	RS			CrookBr.Rd.10-12km mkr	
04-001	RS	46.08.13.34	66.30.46.46		4847
04-002	RS	46.11.12.21	66.03.02.84		5146
04-003	RS	46.11.29.94	66.04.45.96		4146
04-004	RS	46.12.33.52	66.06.41.49		5046
04-005	RS	46.12.35.78	66.09.43.44		5045
04-006	RS	46.13.18.42	66.12.41.06		4945
04-007	RS	46.13.32.75	66.15.25.10		4945
04-008	RS	46.12.53.43	66.17.55.97		4945
04-009	RS	46.12.23.24	66.19.40.16		4845
04-010	RS	46.13.34.63	66.23.08.41		4845
04-011	RS	46.15.14.46	66.26.43.86		4744
04-012	RS	46.17.30.74	66.26.38.77		4744
04-013	RS	46.18.53.34	66.24.09.32		4743
04-014	RS	46.18.15.44	66.23.40.39		4843
04-015	RS	46.13.07.25	66.25.02.57		4745
04-016	RS	46.10.55.54	66.21.31.71.		4846
04-017	RS	46.09.47.34	66.22.47.52		4846
04-018	RS	46.08.19.15	66.23.48.41		4847
04-019	RS	46.07.37.87	66.26.10.94		4747
04-020	RS			#1 Bronson/Harley Rd	
04-021	RS			#2 Bronson/Harley Rd	
04-022	RS			#3 Bronson/Harley Rd	
04-023	RS			#4 Bronson/Harley Rd	
04-024	RS			#5 Bronson/Harley Rd	
04-025	RS			#6 Bronson/Harley Rd	
04-026	RS			#7 Bronson/Harley Rd	
04-027	RS			#8 Bronson/Harley Rd	
04-028	RS			#9 Bronson/Harley Rd	
04-029	RS			#10 Bronson/Harley Rd	
04-030	RS			#11 Bronson/Harley Rd	
04-031	RS			#12 Bronson/Harley Rd	
04-032	WS			#13 Bronson/Harley Rd	
04-033	RS			#14 Bronson/Harley Rd	
04-034	RS			#15 Bronson/Harley Rd	
04-035	RS			#16 Bronson/Harley Rd	
04-036	RS			#17 Bronson/Harley Rd	
04-037	RS			#18 Bronson/Harley Rd	
04-038	RS			#19 Bronson/Harley Rd	
04-039	RS			#20 Bronson/Harley Rd	
04-040	RS			#21 Bronson/Harley Rd	
04-041	RS			#22 Bronson/Harley Rd	
04-042	RS			#23 Bronson/Harley Rd	

04-043	RS			#24 Bronson/Harley Rd	
04-044	RS			#25 Bronson/Harley Rd	
04-045	RS			#26 Bronson/Harley Rd	
04-046	RS			#27 Bronson/Harley Rd	
04-047	RS			#28 Bronson/Harley Rd	
04-048	RS			#29 Bronson/Harley Rd	
04-049	RS			#30 Bronson/Harley Rd	
04-050	BS			#23 Bronson/Harley Rd	
04-051	WS			#1 Indian Point/Ripples	
04-052	WS			#2 Indian Point/Ripples	
04-053	RS			#3 Indian Point/Ripples	
04-054	WS			#4 Indian Point/Ripples	
04-055	RS			#5 Indian Point/Ripples	
04-056	RS			#6 Indian Point/Ripples	
04-057	RS			#7 Indian Point/Ripples	
04-058	RS			#8 Indian Point/Ripples	
04-059	RS			#9 Indian Point/Ripples	
04-060	WS			#10 Indian Point/Ripples	
05-001	RS	45.12.12.56	66.19.24.86		4865
05-002	RS	45.11.14.3	66.28.07.4		4766
05-003	RS	45.08.49.13	66.44.14.42		4467
05-004	RS	45.15.46.06	66.46.16.04		4464
05-005	RS	45.18.38.26	66.44.04.39		4463
05-006	RS	45.21.40.55	66.45.47.82		4462
05-007	RS	45.24.54.17	66.47.44.53		4461
05-008	RS	45.27.45.89	66.46.48.85		4460
05-009	RS	45.34.19.48	66.41.21.13		4558
05-010	RS	45.37.43.89	66.36.04.01		4557
05-011	RS	45.26.07.78	66.40.26.11		4561
05-012	RS	45.29.00.40	66.27.24.22		4760
05-013	RS	45.26.11.28	66.26.15.76		4761
05-014	RS	45.15.01.67	66.11.18.69		5064
05-015	RS	45.43.03.62	66.36.19.46		4555
05-016	WS	46.02.54.11	66.32.12.08		4649
05-017	WS			#1 Nerepis	
05-018	WS			#2 Nerepis	
05-019	WS			#3 Nerepis	
05-020	WS			#4 Nerepis	
05-021	WS			#5 Nerepis	
05-022	WS			#6 Nerepis	
05-023	RS			#7 Nerepis	
05-024	WS			#8 Nerepis	
05-025	WS			#9 Nerepis	
05-026	WS			#10 Nerepis	
05-027	WS			#11 Nerepis	
05-028	WS			#12 Nerepis	
05-029	RS			#13 Nerepis	
05-030	RS			#14 Nerepis	
05-031	RS			#15 Nerepis	
05-032	RS			#16 Nerepis	
05-033	RS			#17 Nerepis	

05-034	RS			#18 Nerepis	
05-035	RS			#19 Nerepis	
05-036	RS			#20 Nerepis	
05-037	WS			#21 Nerepis	
05-038	WS			#22 Nerepis	
06-001	WS	45.56.48.62	64.49.08.01		6351
06-002	RS	45.53.43.71	64.48.10.13		6352
06-003	RS	45.52.16.7	64.35.59.1		6552
06-004	RS	45.39.27.7	64.52.21.2		6356
06-005	RS	45.36.57.0	65.01.27.6		6157
06-006	RS	45.43.41.8	65.13.26.9		5955
06-007	RS	45.34.50.1	65.18.04.48		5858
06-008	RS	45.37.31.8	65.27.58.1		5757
06-009	RS	45.32.01.89	65.31.02.4		5659
06-010	RS	45.43.31.0	65.56.18.27		5255
06-011	WS	45.46.58.55	65.57.55.19		5254
06-012	WS	45.47.45.5	66.03.20.27		5154
06-013	WS	45.52.40.2	65.55.30.6		5252
06-014	WS	45.56.42.3	65.47.56.07		5451
06-015	WS	46.01.39.1	65.32.29.46		5649
06-016	WS	46.02.31.8	65.25.57.28		5749
06-017	RS	46.01.50.46	65.23.00.87		5849
06-018	RS	45.52.06.31	65.03.57.98		6152
06-019	RS	45.43.56.54	65.21.46.48		5855
06-020	RS	45.49.00.93	65.20.52.69		6245
06-021	RS	46.13.22.93	64.58.30.34		6243
06-022	RS	46.19.23.38	64.54.26.52		6140
06-023	RS	46.28.46.16	65.03.13.95		4865
06-024	RS	45.51.41.05	65.42.54.41		5452
06-025	WS	45.48.35.77	64.58.17.78		6253
06-026	RS	45.47.45.12	64.59.01.73		6254
06-027	RS	45.45.43.44	65.12.28.97		5954
06-028	RS	45.44.51.97	65.16.03.93		5955
06-029	RS	45.50.13.27	65.03.20.16		6153
06-030	RS	45.44.37.83	65.00.10.67		6155
06-031	RS	45.42.52.77	64.57.39.02		6255
06-032	RS	45.50.27.63	64.53.39.62		6353
06-033	RS	45.52.53.57	64.54.40.58		6252
06-034	RS	45.53.22.41	64.52.34.66		6352
06-035	RS	45.58.03.75	64.54.37.04		6250
06-036	RS	45.23.15.20	65.27.51.44		5762
06-037	RS	45.24.27.21	65.28.28.23		5761
06-038	RS	45.18.01.40	65.42.23.34		5463
06-039		45.16.43.71	65.43.15.37		5464
06-040	RS	45.19.25.44	65.46.51.67		5463
06-041	RS	45.23.06.96	65.45.38.96		5462
06-042	RS			Row 1 Tree 7 (1876) STN	
06-043	RS			Row 4 Tree 7 (1260) STN	
06-044	RS			Row 4 Tree 5 (1624) STN	
06-045	RS			Row 4 Tree 4 (1530) STN	
06-046	RS			Row 4 Tree 3 (1534) STN	

06-047	RS	Row 4 Tree 2 (1648) STN
06-048	RS	Row 4 Tree 1 (1769) STN
06-049	RS	Row 3 Tree 1 (1217) STN
06-050	RS	Row 3 Tree 2 (1761) STN
06-051	RS	Row 3 Tree 3 (1371) STN
06-052	RS	Row 29 Tree 28 (1811)STN
06-053	RS	Row 3 Tree 7 (1776) STN
06-054	RS	Row 3 Tree 8 (1551) STN
06-055	RS	Row 3 Tree 9 (1012) STN
06-056	RS	Row 3 Tree 10 (1238) STN
06-057	RS	Row 2 Tree 10 (1243) STN
06-058	RS	Row 2 Tree 9 (1787) STN
06-059	RS	Row 2 Tree 7 (1116) STN
06-060	RS	Row 2 Tree 6 (1960) STN
06-061	RS	Row 2 Tree 5 (3012) STN
06-062	RS	Row 2 Tree 4 (1666) STN
06-063	RS	Row 2 Tree 3 (1542) STN
06-064	RS	Row 2 Tree 2 (1550) STN
06-065	RS	Row 5 Tree 1 (1647) STN
06-066	RS	Row 5 Tree 2 (1876) STN
06-067	RS	Row 5 Tree 3 (1625) STN
06-068	RS	Row 15 Tree 1 (1884)STN
06-069	RS	Row 15 Tree 2 (1494) STN
06-070	RS	Row 15 Tree 3 (1674) STN
06-071	RS	Row 15 Tree 4 (1140) STN
06-072	RS	Row 15 Tree 5 (1759) STN
06-073	RS	Row 15 Tree 6 (1551) STN
06-074	RS	Row 15 Tree 7 (1871) STN
06-075	RS	Row 15 Tree 8 (1639) STN
06-076	RS	Row 14 Tree 9 (1426) STN
06-077	RS	Row 14 Tree 10 (1877)STN
06-078	RS	Row 14 Tree 11 (1239)STN
06-079	RS	Row 14 Tree 12 (1983)STN
06-080	RS	Row 14 Tree 15 (1195)STN
06-081	RS	Row 21 Tree 18 (1963)STN
06-082	RS	Row 38 Tree 4 (1420) STN
06-083	RS	Row 31 Tree 3 (1214)STN
06-084	RS	Row 31 Tree 4 (1535)STN
06-085	RS	Row 31 Tree 5 (1805)STN
06-086	RS	Row 31 Tree 6 (1266)STN
06-087	RS	Row 31 Tree 8 (1624)STN
06-088	RS	Row 31 Tree 9 (1555)STN
06-089	RS	Row 38 Tree 21(1608)STN
06-090	RS	Row 31 Tree 12 (1004)STN
06-091	RS	Row 31 Tree 15 (1961)STN
06-092	RS	Row 31 Tree 16 (1782)STN
06-093	RS	Row 31 Tree 17 (1400)STN
06-094	RS	Row 31 Tree 22 (1196)STN
06-095	RS	Row 31 Tree 24 (1494)STN
06-096	RS	Row 31 Tree 25 (1791)STN
06-097	RS	Row 31 Tree 27 (1988)STN

06-098	RS	Row 32 Tree 1 (1632)STN
06-099	RS	Row 32 Tree 2 (1760)STN
06-100	RS	Row 32 Tree 5 (1540)STN
06-101	RS	Row 32 Tree 6 (1764)STN
06-102	RS	Row 32 Tree 8 (1260)STN
06-103	RS	Row 32 Tree 9 (1339)STN
06-104	RS	Row 32 Tree 12 (1369)STN
06-105	RS	Row 32 Tree 13 (1143)STN
06-106	RS	Row 32 Tree 14 (1632)STN
06-107	RS	Row 32 Tree 15 (1101)STN
06-108	RS	Row 32 Tree 16 (1630)STN
06-109	RS	Row 32 Tree 19 (1024)STN
06-110	RS	Row 35 Tree 35(1238)STN
06-111	WS	Row 1 Tree 2 (06-001) STN
06-112	WS	Row 1 Tree 5 (06-002) STN
06-113	WS	Row 1 Tree 9 (06-003) STN
06-114	WS	Row 1 Tree 10(06-004)STN
06-115	WS	Row 1 Tree 15(06-005)STN
06-116	WS	Row 1 Tree 16(06-006)STN
06-117	RS	#1 Goshen
06-118	RS	#2 Goshen
06-119	RS	#3 Goshen
06-120	RS	#4 Goshen
06-121	RS	#5 Goshen
06-122	RS	#6 Goshen
06-123	RS	#7 Goshen
06-124	RS	#8 Goshen
06-125	WS	#9 Goshen
06-126	RS	#10 Goshen
06-127	RS	#11 Marshall Hill
06-128	RS	#12 Marshall Hill
06-129	RS	#13 Marshall Hill
06-130	RS	#14 Marshall Hill
06-131	RS	#15 Marshall Hill
06-132	RS	#16 Marshall Hill
06-133	RS	#17 Marshall Hill
06-134	RS	#18 Marshall Hill
06-135	RS	#19 Marshall Hill
06-136	RS	#20 Marshall Hill
06-137	RS	#21 Marshall Hill
06-138	RS	#22 Marshall ahill
06-139	RS	#23 Jeffries Corner
06-140	RS	#24 Jeffries Corner
06-141	RS	#25 Jeffries Corner
06-142	RS	#26 Jeffries Corner
06-143	RS	#27 Jeffries Corner
06-144	RS	#28 Jeffries Corner
06-145	RS	#29 Jeffries Corner
06-146	RS	#30 Jeffries Corner
06-147	RS	#31 Jeffries Corner
06-148	RS	#32 Jeffries Corner

06-149	RS	#33 Jeffries Corner
06-150	RS	#34 Jeffries Corner
06-151	RS	#35 Jeffries Corner
06-152	RS	#36 Jeffries Corner
06-153	RS	#37 Jeffries Corner
06-154	RS	#38 Jeffries Corner
06-155	RS	#39 Jeffries Corner
06-156	RS	#40 Jeffries Corner
06-157	RS	#41 Jeffries Corner
06-158	RS	#42 Jeffries Corner
06-159	RS	#43 Jeffries Corner
06-160	RS	#44 Jeffries Corner
06-161	RS	#45 Jeffries Corner
06-162	RS	#46 Jeffries Corner
06-163	RS	#47 Jeffries Corner
06-164	RS	#48 Jeffries Corner
06-165	RS	#49 Jeffries Corner
06-166	RS	#50 Jeffries Corner
06-167	RS	#51 Jeffries Corner
06-168	RS	#52 Jeffries Corner
06-169	WS	Row 1 Tree 20(06-007)STN
06-170	WS	Row 1 Tree 24(06-008)STN
06-171	WS	Row 1 Tree 27(06-009)STN
06-172	WS	Row 1 Tree 29(06-010)STN
06-173	WS	Row 1 Tree 34(06-012)STN
06-174	WS	Row 1 Tree 37(06-013)STN
06-175	WS	Row 2 Tree 1(06-014)STN
06-176	WS	Row 2 Tree 4(06-015)STN
06-177	WS	Row 2 Tree 7(06-016)STN
06-178	WS	Row 2 Tree 10(06-017)STN
06-179	WS	Row 2 Tree 13(06-018)STN
06-180	WS	Row 2 Tree 18(06-019)STN
06-181	WS	Row 2 Tree 20(06-020)STN
06-182	WS	Row 2 Tree 24(06-021)STN
06-183	WS	Row 2 Tree 27(06-022)STN
06-184	RS	#53 Martin Head
06-185	RS	#54 Martin Head
06-186	RS	#55 Martin Head
06-187	RS	#56 Martin Head
06-188	RS	#57 Martin Head
06-189	RS	#58 Martin Head
06-190	RS	#59 Martin Head
06-191	RS	#60 Martin Head
06-192	RS	#61 Martin Head
06-193	RS	#62 Martin Head
06-194	RS	#63 Martin Head
06-195	RS	#64 Martin Head
06-196	RS	#65 Martin Head
06-197	RS	#66 Martin Head
06-198	RS	#67 Martin Head
06-199	RS	#68 Martin Head

06-200	RS	#69 Martin Head
06-201	RS	#70 Martin Head
06-202	RS	#71 Martin Head
06-203	RS	#72 Martin Head
06-204	RS	#73 Martin Head
06-205	RS	#74 Martin Head
06-206	RS	#75 Martin Head
06-207	RS	#76 Martin Head
06-208	RS	#77 Martin Head
06-209		#78 No Tree
06-210		#79 No Tree
06-211	RS	#80 St Martins
06-212	RS	#81 St. Martins
06-213	RS	#82 St. Martins
06-214	RS	#83 St. Martins
06-215	RS	#84 St. Martins
06-216	RS	#85 St. Martins
06-217	RS	#86 St. Martins
06-218	RS	#87 St. Martins
06-219	RS	#88 St. Martins
06-220	RS	#89 St. Martins
06-221	RS	#90 St. Martins
06-222	RS	#91 St. Martins
06-223	RS	#92 St. Martins
06-224	RS	#93 St. Martins
06-225	RS	#94 St. Martins
06-226	RS	#95 St. Martins
06-227	RS	#96 St. Martins
06-228	RS	#97 St. Martins
06-229	RS	#98 St. Martins
06-230	RS	#99 St. Martins
06-231	RS	#100 St, Martins
06-232	RS	#101 St. Martins
06-233	RS	#102 St. Martins
06-234	RS	#103 St. Martins
06-235	RS	#104 St. Martins
06-236	RS	#105 St. Martins
06-237	RS	#1 Hammondvale
06-238	WS	#2 Hammondvale
06-239	WS	#3 Hammondvale
06-240	WS	#4 Hammondvale
06-241	RS	#5 Hammondvale
06-242	WS	#6 Hammondvale
06-243	RS	#7 Hammondvale
06-244	RS	#8 Hammondvale
06-245	RS	#9 Hammondvale
06-246	RS	#10 Hammondvale
06-247	RS	#11 Hammondvale
06-248	RS	#12 Hammondvale
06-249	RS	#13 Hammondvale
06-250	RS	#14 Hammondvale

06-251	RS	#15 Hammondvale
06-252	RS	#16 Hammondvale
06-253	RS	#17 Hammondvale
06-254	RS	#18 Hammondvale
06-255	RS	#19 Marshall Hill/Drury's Cove
06-256	RS	#20 Marshall Hill/Drury's Cove
06-257	RS	#21 Marshall Hill/Drury's Cove
06-258	RS	#22 Marshall Hill/Drury's Cove
06-259	WS	#23 Marshall Hill/Drury's Cove
06-260	WS	#24 Marshall Hill/Drury's Cove
06-261	RS	#25 Marshall Hill/Drury's Cove
06-262	RS	#26 Marshall Hill/Drury's Cove
06-263	RS	#27 Marshall Hill/Drury's Cove
06-264	RS	#28 Marshall Hill/Drury's Cove
06-265	RS	#29 Marshall Hill/Drury's Cove
06-266	RS	#30 Goose Creek
06-267	RS	#31 Goose Creek
06-268	RS	#32 Goose Creek
06-269	RS	#33 Goose Creek
06-270	RS	#34 Goose Creek
06-271	RS	#35 Goose Creek
06-272	RS	#36 Goose Creek
06-273	RS	#37 Goose Creek
06-274	RS	#38 Goose Creek
06-275	RS	#39 Goose Creek
06-276	RS	#40 Goose Creek
06-277	RS	#41 Goose Creek
06-278	RS	#42 Goose Creek
06-279	RS	#43 Goose Creek
06-280	RS	#44 Goose Creek
06-281	RS	#45 Goose Creek
06-282	WS	#101 Saltsprings
06-283	WS	#102 Saltsprings
06-284	WS	#103 Saltsprings
06-285	WS	#104 Saltsprings
06-286	WS	#105 Saltsprings
06-287	RS	#106 Saltsprings
06-288	RS	#107 Saltsprings
06-289	WS	#108 Saltsprings
06-290	RS	#109 Saltsprings
06-291	RS	#110 Saltsprings
06-292	RS	#111 Saltsprings
06-293	WS	#112 Saltsprings
06-294	RS	#113 Parkindale
06-295	RS	#114 Parkindale
06-296	RS	#115 Parkindale
06-297	RS	#116 Parkindale
06-298	RS	#117 Parkindale
06-299	RS	#118 Parkindale
06-300	RS	#119 Parkindale
06-301	RS	#120 Parkindale

06-302	RS	#121 Parkindale
06-303	WS	#122 Parkindale
06-304	WS	#123 Parkindale
06-305	RS	#124 Parkindale
06-306	RS	#125 Parkindale
06-307	RS	#126 Parkindale
06-308	RS	#127 Parkindale
06-309	RS	#128 Parkindale
06-310	RS	#129 Parkindale
06-311	RS	#130 Parkindale
06-312	RS	#131 Parkindale
06-313	RS	#132 Parkindale
06-314	RS	#133 Parkindale
06-315	RS	#134 Parkindale
06-316	WS	#135 Parkindale
06-317	RS	#136 Parkindale
06-318	RS	#137 Parkindale
06-319	WS	#138 Parkindale
06-320	RS	#139 Parkindale
06-321	RS	#140 Parkindale
06-322	WS	#141 Parkindale
06-323	RS	#1 Waterloo Corner
06-324	RS	#2 Waterloo Corner
06-325	RS	#3 Waterloo Corner
06-326	RS	#4 Waterloo Corner
06-327	RS	#5 Waterloo Corner
06-328	RS	#6 Waterloo Corner
06-329	RS	#7 Waterloo Corner
06-330	RS	#8 Waterloo Corner
06-331	RS	#9 Waterloo Corner
06-332	RS	#10 Waterloo Corner
06-333	RS	#11 Waterloo Corner
06-334	RS	#12 Waterloo Corner
06-335	RS	#13 Waterloo Corner
06-336	RS	#14 Waterloo Corner
06-337	WS	#15 Waterloo Corner
06-338	WS	#16 Waterloo Corner
06-339	RS	#17 Waterloo Corner
06-340	RS	#18 Waterloo Corner
06-341	RS	#19 Waterloo Corner
06-342	RS	#20 Waterloo Corner
06-343	WS	Row 2 Tree 29(06-023)STN
06-344	WS	Row 2 Tree 33(06-025)STN
06-345	WS	Row 2 Tree 35(06-026)STN
06-346	WS	Row 2 Tree 39(06-027)STN
06-347	WS	Row 3 Tree 1(06-028)STN
06-348	WS	Row 3 Tree 5(06-029)STN
06-349	WS	Row 3 Tree 10(06-031)STN
06-350	WS	Row 3 Tree15(06-1000)STN
06-351	WS	Row 3 Tree18(06-1001)STN
06-352	WS	Row 3 Tree24(06-1003)STN

06-353	WS	Row 3 Tree27(06-1004)STN	
06-354	WS	Row 3 Tree30(06-1005)STN	
06-355	WS	Row 3 Tree32(06-1006)STN	
06-356	WS	Row 3 Tree36(06-1007)STN	
06-357	WS	Row 3 Tree37(06-1008)STN	
06-358	WS	Row 4 Tree 3(06-1009)STN	
06-359	WS	Row 4 Tree 5(06-1010)STN	
06-360	WS	Row 4 Tree 8(06-1011)STN	
06-361	WS	Row 4 Tree10(06-1012)STN	
06-362	WS	Row 4 Tree13(06-1013)STN	
06-363	WS	Row 4 Tree18(06-1014)STN	
06-364	WS	Row 4 Tree21(06-1015)STN	
06-365	WS	#1 Springhill/Dubee	
06-366	WS	#2 Springhill/Dubee	
06-367	WS	#3 Springhill/Dubee	
06-368	WS	#4 Springhill/Dubee	
06-369	RS	#5 Springhill/Dubee	
06-370	RS	#6 Springhill/Dubee	
06-371	RS	#7 Springhill/Dubee	
06-372	WS	#8 Springhill/Dubee	
06-373	WS	#9 Springhill/Dubee	
06-374	RS	#10 Springhill/Dubee	
06-375	RS	#11 Springhill/Dubee	
06-376	WS	#12 Springhill/Dubee	
06-377	RS	#13 Springhill/Dubee	
06-378	RS	#14 Springhill/Dubee	
06-379	RS	#15 Springhill/Dubee	
06-380	RS	#16 Springhill/Dubee	
06-381	RS	#17 Springhill/Dubee	
03-382	RS	#18 Springhill/Dubee	
06-383	WS	#19 Springhill/Dubee	
06-384	WS	#20 Springhill/Dubee	
06-385	WS	Row 4 Tree24(06-1016)STN	
06-386	WS	Row 4 Tree26(06-1017)STN	
06-387	WS	Row 4 Tree30(06-1018)STN	
06-388	WS	Row 4 Tree31(06-1019)STN	
06-389	WS	Row 4 Tree35(06-1020)STN	
06-390	WS	Row 4 Tree37(06-1021)STN	
06-391	WS	Row 5 Tree 1(06-1022)STN	
06-392	WS	Row 5 Tree 6(06-1023)STN	
06-393	WS	Row 5 Tree 9(07-02-0)STN	
06-394	WS	Row 5 Tree17(07-02-1)STN	
06-395	WS	Row 5 Tree26(07-02-2)STN	
06-396	WS	Row 5 Tree35(NB1P18)STN	
06-397	WS	Row 6 Tree 1(SP-SW-81-5)	STN
06-398	WS	Row 6 Tree 20 (10-01) STN	
06-399	WS	Row 6 Tree 27 (10-07) STN	
06-400	WS	#1 Plumsweep	
06-401	WS	#2 Plumsweep	
06-402	WS	#3 Plumsweep	
06-403	RS	#4 Plumsweep	

06-404	WS	#5 Plumsweep
06-405	WS	#6 Plumsweep
06-406	WS	#7 Plumsweep
06-407	WS	#8 Plumsweep
06-408	WS	#9 Plumsweep
06-409	WS	#10 Plumsweep
06-410	WS	#11 Picadilly
06-411	WS	#12 Picadilly
06-412	WS	#13 Picadilly
06-413	WS	#14 Picadilly
06-414	WS	#15 Picadilly
06-415	WS	#16 Picadilly
06-416	WS	#17 Picadilly
06-417	WS	#18 Picadilly
06-418	WS	#19 Picadilly
06-419	WS	#20 Picadilly
06-420	WS	#21 Picadilly
06-421	WS	#1 Snider Mountain
06-422	WS	#2 Snider Mountain
06-423	WS	#3 Snider Mountain
06-424	WS	#4 Snider Mountain
06-425	WS	#5 Snider Mountain
06-426	WS	#6 Snider Mountain
06-427	WS	#7 Snider Mountain
06-428	WS	#8 Snider Mountain
06-429	WS	#9 Snider Mountain
06-430	WS	#10 Snider Mountain
06-431	WS	#11 Snider Mountain
06-432	WS	#12 Snider Mountain
06-433	WS	#13 Snider Mountain
06-434	RS	#14 Snider Mountain
06-435	WS	#15 Snider Mountain
06-436	WS	#16 Snider Mountain
06-437	WS	#17 Snider Mountain
06-438	RS	#18 Snider Mountain
06-439	WS	#19 Snider Mountain
06-440	RS	#20 Snider Mountain
06-441	WS	#21 Snider Mountain
06-442	WS	#22 Snider Mountain
06-443	WS	#23 Snider Mountain
06-444	WS	#1 Henderson Settlement
06-445	RS	#2 Henderson Settlement
06-446	RS	#3 Henderson Settlement
06-447	RS	#4 Henderson Settlement
06-448	RS	#5 Henderson Settlement
06-449	RS	#6 Henderson Settlement
06-450	RS	#7 Henderson Settlement
06-451	RS	#8 Henderson Settlement
06-452	RS	#9 Henderson Settlement
06-453	RS	#10 Henderson Settlement
06-454	WS	#11 Henderson Settlement

06-455	WS			#12 Henderson Settlement	
06-456	WS			#1 Springhill/Salaem	
06-457	RS			#2 Springhill/Salaem	
06-458	WS			#3 Springhill/Salaem	
06-459	RS			#4 Springhill/Salaem	
06-460	RS			#5 Springhill/Salaem	
06-461	WS			#6 Springhill/Salaem	
06-462	WS			#7 Springhill/Salaem	
06-463	WS			#8 Springhill/Salaem	
06-464	WS			#9 Springhill/Salaem	
06-465	WS			#10 Springhill/Salaem	
06-466	WS			#11 Springhill/Salaem	
06-467	WS			#12 Springhill/Salaem	
06-468	WS			#13 Springhill/Salaem	
06-469	WS			#14 Springhill/Salaem	
06-470	WS			#15 Springhill/Salaem	
06-471	WS			#16 Springhill/Salaem	
06-472	WS			#17 Springhill/Salaem	
06-473	WS			#18 Springhill/Salaem	
06-474	WS			#19 Springhill/Salaem	
06-475	WS			#20 Springhill/Salaem	
06-476	WS			#21 Springhill/Salaem	
06-477	WS			#22 Springhill/Salaem	
06-478	WS			#23 Springhill/Salaem	
06-479					no tree
06-480	WS			#25 Springhill/Salaem	
06-481	RS			#26 Springhill/Salaem	
06-482	RS			#27 Springhill/Salaem	
06-483	WS			#28 Springhill/Salaem	
06-484	WS			#29 Springhill/Salaem	
06-485	WS			#30 Springhill/Salaem	
06-486	WS			Fill Rep 2 R1 P1 T1 Test 161	
06-487	WS			8380 Rep 2 R3 P1 T1 Test 161	
06-488	WS			8620 Rep 2 R2 P1 T1 Test 161	
06-489	WS			Row 50 Tree 7 (PSO) 06-004	
06-490	WS			Row 50 Tree 6 (PSO) 07-02-11	
06-491	WS			Row 49 Tree 4 (PSO) SP-SW-81	
06-492	WS			Row 45 Tree 2 (PSO) 06-015	
06-493	WS			Row 49 Tree 12 (PSO) 07-02-25	
06-494	WS			Row 49 Tree 5 (PSO) 06-008	
06-495	WS			Row 49 Tree 3 (PSO) 10-06	
06-496	WS			Row 49 Tree 2 (PSO) 06-001	
06-497	WS			Row 46 Tree 2 (PSO) 06-012	
06-498	WS			Row 47 Tree 2 (PSO) 10-01	
06-499	WS			Row 46 Tree 3 (PSO) 0-017	
06-500	WS			Row 50 Tree 17 (PSO) IP-SW-81	
07-001	RS	45.53.48.56	64.04.39.47	Tyndall Road	
07-002	RS	45.53.57.28	64.05.18.07	Tyndall Road	
07-003	RS	45.54.13.94	64.06.11.98	Tyndall Road	
07-004	RS	45.55.07.36	64.06.00.06	Tyndall Road	
07-005	RS	45.54.31.24	64.06.28.46	Tyndall Road	

07-006	RS	45.52.48.73	64.07.31.81	Tyndall Road	
07-007	RS	45.41.40.90	64.10.41.49	Athol	
07-008	RS	45.34.21.22	64.14.58.03	South Hampton	
07-009	RS	45.39.35.43	64.11.25.55	Athol	
07-010	RS	45.41.46.76	64.07.14.34	Athol	
07-011	RS	45.40.30.28	64.01.49.49	Springhill	
07-012	RS	45.37.54.69	63.49.03.48	Millvale	
07-013	RS	45.39.41.87	63.48.38.68	Millvale	
07-014	RS	45.22.29.57	63.11.44.33	Bible Hill	
08-001	RS	44.30.22.36	65.35.49.68		5679
08-002	RS	44.30.01.25	65.36.29.89		5579
08-003	RS	44.29.34.94	65.36.27.61		5580
08-004	RS	44.29.08.61	65.36.02.54		5580
08-005	RS	44.28.43.50	65.35.37.84		5680
08-006	RS	44.28.11.68	65.35.05.83		5680
08-007	RS	44.27.39.29	65.34.42.27		5680
08-008	RS	44.27.26.76	65.34.24.04		5680
08-009	RS	44.27.13.20	65.33.40.55		5680
08-010	RS	44.26.45.57	65.33.10.29		5680
08-011	RS	44.26.59.42	65.35.20.25		5680
08-012	RS	44.26.36.62	65.35.47.85		5681
08-013	RS	44.26.07.15	65.35.56.11		5681
08-014	RS	44.25.38.66	65.36.02.88		5681
08-015	RS	44.25.21.19	65.36.07.20		5581
08-016	RS	45.25.04.48	65.36.33.91		5581
08-017	RS	44.25.01.10	65.37.09.90		5581
08-018	RS	44.24.56.87	65.37.55.74		5581
08-019	RS	44.24.52.45	65.38.35.20		5581
08-020	RS	44.24.48.57	65.39.24.22		5581
08-021	RS	44.24.45.68	65.39.54.16		5581
08-022	RS	44.24.22.01	65.41.07.95		5581
08-023	RS	44.24.10.97	65.41.32.35		5482
08-024	RS	44.24.00.68	65.41.55.56		5482
08-025	RS	44.23.49.65	65.42.19.87		5482
08-026	RS	44.23.43.06	65.42.34.48		5482
08-027	RS	44.23.29.48	65.43.04.15		5482
08-028	RS	44.23.04.81	65.43.58.25		5482
08-029	RS	44.22.48.98	65.44.32.65		5482
08-030	RS	44.22.32.24	65.44.49.22		5482
08-031	RS	44.22.27.35	65.45.10.75		5482
08-032	RS	44.22.05.94	65.46.41.02		5482
08-033	RS	44.22.00.21	65.47.05.11		5482
08-034	RS	44.21.39.49	65.48.24.29		5382
08-035	RS	44.21.49.47	65.49.32.45		5382
08-036	RS	44.22.00.10	65.50.19.81		5382
08-037	RS	44.21.39.07	65.50.48.55		5382
08-038	RS	44.19.43.19	65.52.01.28		5383
08-039	RS	44.19.31.88	65.51.17.69		5383
08-040	RS	44.19.23.48	65.50.43.42		5383
08-041	WS	44.19.15.86	65.49.54.94		5383
08-042	RS	44.18.50.01	65.49.26.57		5383

08-043	RS	44.18.13.67	65.48.59.32	5383
08-044	RS	44.17.55.40	65.48.45.18	5383
08-045	RS	44.17.32.91	65.48.27.85	5384
08-046	RS	44.17.04.66	65.48.06.06	5384
08-047	RS	44.16.30.51	65.48.14.85	5384
08-048	RS	44.16.05.25	65.48.24.49	5384
08-049	RS	44.15.07.01	65.48.47.09	5384
08-050	RS	44.13.59.22	65.49.12.92	5384
08-051	RS	44.13.26.15	65.49.23.53	5385
08-052	RS	44.12.21.46	65.49.20.60	5385
08-053	WS	44.10.35.74	65.49.29.75	5385
09-001	RS		T 7 R 5	
09-002	RS		T 7 R 5	
09-003	RS		T 7 R 5	
09-004	RS		T 7 R 5	
09-005	RS		T 7 R 5	
09-006	RS		T 7 R 5	
09-007	RS		T 7 R 5	
09-008	RS		T 7 R 5	
09-009	RS		T 7 R 5	
09-010	RS		T 7 R 5	
09-011	RS		T 7 R 5	
09-012	RS		T 7 R 5	
09-013	RS		T 7 R 5	
09-014	RS		T 7 R 5	
09-015	RS		T 7 R 5	
09-016	RS		T 7 R 5	
09-017	RS		T 7 R 5	
09-018	RS		T 7 R 5	
09-019	RS		T 7 R 5	
09-020	RS		T 7 R 5	
09-021	RS		T 7 R 5	
09-022	RS		T 7 R 5	
09-023	RS		T 7 R 5	
09-024	RS		T 7 R 5	
09-025	RS		Umcolcus Lake Rd.	
09-026	RS		Umcolcus Lake Rd.	
09-027	RS		Umcolcus Lake Rd.	
09-028	RS		Umcolcus Lake Rd.	
09-029	RS		Umcolcus Lake Rd.	
09-030	RS		Umcolcus Lake Rd.	
09-031	RS		Umcolcus Lake Rd.	
09-032	RS		Umcolcus Lake Rd.	
09-033	RS		Umcolcus Lake Rd.	
09-034	RS		Umcolcus Lake Rd.	

Section B – Cluster diagram of red and white spruce endophytes

Section C – Cluster diagram of red and white spruce endophytes (endophytes from our collection are identified by “XXXXXX” at the end of the code) with other species of fungi

P cyclopium gi3925712
 P expansum gi14349129
 P tricolor gi3925748
 P venetum gi3925745
 P discolor gi3925728
 P solitum gi3925743
 P echinulatum gi2668713
 P camemberti gi3925726
 P commune gi3925707
 P crustosum gi2668712
 P fennelliae CCFC214803
 gi21666910 P commune
 P coprophilum gi2668709
 P coprobium ATCC58615
 P expansum gi2668719
 P gladioli gi2668720
 1506063DITSIF 09XXXXXX
 P glandicola NRRL2036
 P chrysogenum gi14349130
 2506188AITSIF 10XXXXXX
 P griseofulvum gi2668708
 P dipodomys gi3925735
 P nalgioense gi3925733
 P aethiopicum gi3925705
 P viridicatum gi2668630
 Eupen crustaceum gi2668706
 Eupen egyptiacum gi2668707
 P clavigerum CCFC214797
 P vulpinum gi437635
 P clavigerum gi437637
 P concentricum CBS 47775
 P sclerotigenum gi2668710
 P digitatum gi2668711
 Eupen baarnense gi2668721
 P turbatum gi3169897
 Eupen sp gi8248470
 P atramentosum gi2668723
 P madriti gi2668722
 Hemicarpenteles paradoxus gi2668
 P kojigenum gi2668729
 P steckii CBS 26055
 P swiecickii gi2668730
 P lanosum CCFC214775
 P raistrickii gi2668731
 P soppii gi2668728
 P scabrosum CCFC214786
 P brevicompactum gi14349131
 P alberechii gi7158995
 P vulpinum CCFC229825
 Asp crystallinus gi2668726
 Asp malodoratus gi2668725
 Eupen tularensis gi2668727
 P atrovenetum gi2668732
 P coralligerum
 P canescens gi2668733
 P janczewskii KAS1588
 P chermesinum gi2668653
 melanospora pascuensis gi 510275
 Eupen brefeldianum gi2668675
 P janthinellum gi2668674
 P ochrochloron 34412 R31
 P raperi gi2668673
 P velutinum 96M8 350
 Eupen levitum gi2668676
 Eupen ehrlichii gi2668672
 P daleae gi2668682
 P piscarium afl178514
 Eupen javanicum gi639446
 Eupen reticulisporum gi266867
 P rolfsii gi2668679
 P simplicissimum gi6010602
 P pulvillorum gi6010614
 P paraherquei gi6010598
 gi 6010598 Pen paraherquei
 P skirjabinii gi6010605
 P gastrivorus gi7158991
 P brasilianum gi6010610
 Penicillium sp gi6010611
 P ochrochloron gi6010603
 P pulvillorum gi6010604
 P ciegliei gi6010600

Section D – Insect toxicity data for red and white spruce endophytes.

Notes: * indicate significance difference by ANOVA. Endophytes are grouped according to testing on individual days with controls.

	Weight	SD	HC width	SD
control	0.043559	0.025544	1.405	0.170895
01-010A	0.046944	0.035027	1.411111	0.254116
01-012D	0.05325	0.030918	1.461538	0.144435
01-017A	0.038038	0.025003	1.409524	0.211907
02-002C	0.029358 *	0.022995	1.354545	0.211488
02-008A	0.038755	0.031542	1.36	0.243656
03-001D	0.036471	0.024359	1.338095	0.243877
03-007A	0.030025	0.016537	1.28	0.302844
03-020B	0.0345 *	0.025429	1.246667	* 0.247463
03-028B	0.035564	0.019182	1.356	0.208327
03-032A	0.041274	0.030673	1.369565	0.265321
03-047A	0.039491	0.02608	1.447826	0.23716
04-002C	0.030947	0.025135	1.3	0.342783
04-002G	0.032795	0.016185	1.258824	* 0.280755
04-008A	0.0381	0.021671	1.366667	0.266369
04-010A	0.0452	0.032088	1.426316	0.294094
04-012A	0.02537 *	0.018041	1.29375	0.229401
04-048D	0.041787	0.028061	1.326087	0.176371

	Weight	SD	HC width	SD
control	0.041143	0.026264	1.37561	0.2071
04-052B	0.036295 *	0.021587	1.233333	* 0.231969

	Weight	SD	HC width	SD
control	0.025368	0.024489	0.975	0.327187
06-011B	0.012848 *	0.012124	0.918519	* 0.243432
06-011D	0.014765 *	0.013568	0.91	* 0.25526
06-012C	0.014835 *	0.016154	0.94	0.283586
06-013A	0.018286 *	0.01317	1.028571	0.272292
06-014C	0.011472 *	0.010753	0.943478	0.318842

	Weight	SD	HC width	SD
control	0.025825	0.019206	1.010811	0.312502
06-023A	0.022792	0.013467	0.957143	0.31713
06-023B	0.021467	0.016243	0.947826	0.334215
06-052C	0.021359	0.015549	0.954545	0.272077
06-054A	0.022707	0.018294	0.973333	0.328344
06-063A	0.020713	0.024369	0.956	0.32414
06-063D	0.015206 *	0.012453	0.911765	* 0.266651
06-073C	0.015393 *	0.015381	0.926316	0.280559
06-083B	0.030968	0.038715	1.045455	0.330551
06-094D	0.032379	0.032154	1.047368	0.309782

	Weight		SD	HC width		SD
control	0.032353		0.03575	1.137931		0.351352
06-094E	0.015012	*	0.014053	0.956	*	0.264701
06-097D	0.018409	*	0.010297	1.143478		0.314536
06-130D	0.023726		0.01596	1.135		0.268083
06-184A	0.025631		0.019594	1.11875		0.322942
06-188A	0.02315		0.017609	1.161111		0.314622
06-219A	0.020421		0.021912	0.975		0.325859
06-254B	0.0327		0.077971	1.020833		0.270232
06-255A	0.018356	*	0.032974	0.864706	*	0.323924
06-255B	0.021265		0.022097	0.988235		0.365517
06-264A	0.017913	*	0.022543	0.916667	*	0.164225

	Weight		SD	HC width		SD
control	0.03233		0.023884	1.484091		0.277818
06-265B	0.033711		0.030927	1.333333		0.360555
06-268A	0.01098	*	0.010573	1.266667		0.320416
06-271A	0.037458		0.021393	1.494737		0.22967
06-317A	0.033892		0.024199	1.45		0.27187
06-321A	0.040232		0.027894	1.477273		0.259912
06-332A	0.018485	*	0.01259	1.265	*	0.346828
07-013D	0.02561	*	0.008886	1.584211		0.197943
08-011D	0.028364		0.012328	1.31875	*	0.314576
08-018A	0.042689		0.023431	1.313333	*	0.311372
01-002A	0.023269	*	0.01237	1.526087		0.202748

	Weight		SD	HC width		SD
control	0.032353		0.03575	1.203774		0.288872
06-485A	0.014138	*	0.015518	1.036364	*	0.369521
06-486D	0.017979	*	0.013499	1.0625	*	0.258444

	Weight		SD	HC width		SD
control	0.03233		0.024062	1.484091		0.277818
06-396D	0.041037		0.029493	1.473684		0.282532
06-424B	0.042071		0.02747	1.414286		0.388007
06-430A	0.035053		0.025833	1.482353		0.316692
06-431A	0.039578		0.027189	1.438889		0.250033
06-436B	0.039817		0.031112	1.416667		0.35887
08-040B	0.02405	*	0.0189	1.366667		0.286972
08-040D	0.03898		0.020648	1.429412		0.261641
05-037A	0.029757	*	0.023921	1.205882	*	0.411507

Section E - ITS sequences of strains tested for toxicity and metabolites

5WS22E1 - *Phialocephala scopiformis*

CATTAATAAGGATGCTGGAAGGGGGTGAGCCACGCCTCCCTAGACGGTATACCCACCCGTGTCTATCTACTCTTGT
TGCTTTGGCAGGCCGTGGCTCCACTGTGGGCTCTGCCTGCATGTGCCTGCCAGAGGATTAAACTCTGAATTTTAGTGAT
GTCTGAGAACTATCTAATAGTTAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG
CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTGTGGTATTCCGCAGG
GCATGCCTGTTTCGAGCGTCATTATAACCACTCAAGCCTGGCTTGGTGTGGGGTTTGGCGTTCCGCAGCCCTAAAATC
AGTGGCGGTGGCGTCTACGCGTAGTAAATCTCCTCGCGATTGAGTTCCCCGGTGGCCTGCCAGAACCCCAA
TTTTTACAGGTTG

5WS11I1 – Unknown species of *Phialocephala* (closely related to *Phialocephala glacialis*)

CATTACAAGGTTCTTAGTGACATCTCGAAAGAGATCCCTCCCAAGTATCCCACCCGTGTCTATATACTCTTGTGCTTTG
GCGGGGCGTGGCCTCCACTGCGGGCTCTGCTCGTACGTACCCGCCAGAGGACAACTCTGAATGTTAGTGATGTCTGA
GTACTATATAATAGTTAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAA
GTAAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCGTGGTATTCCGCGGGGCATGC
CTGTTTCGAGCGTCATTATAACCACTCAAGCCTAGCTTGGTGTGGGGCACGCGTTTCGCGGCCCTCAAATCCAGTGGN
GGCGCCGGTGGGCTCTAAGCGTAGTAACTTCTCGCTTCAGGGTCCCTCGGTGGCTCGCCAGAACCTCCAACCTTT
TAAGGTTGACCTCGGAT

05-037A – *Xylariaceae* and close, but not identical to *Nemania serpens* (98% ITS sequence similarity)

CATTACAGAGTTATCAAACTCCCAAACCCATGTGAACATACCTCGCGTTGCTCGGAGGTGGCGTCTCCCCGTAAGAAC
CTACCCTGTAGGACCTTACCCGGTAGACGACCCTGCCGACGGCCCCGAACTCTGTTTTATAGCATTAACTTCTGAAA
ATATAACTAAATAAGTTAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT
AAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCACTAGTATTCTGGTGGGCAT
GCCTGTTTCGAGCGTCATTTCAACCCCTAAGCCCTGTTGCTTAGCGTTAGGAGCTTACCGGAACTCTCTGGTAGCTCCCC
AAAGTCAGTGGCGGAGGTTTCGCACTCCAGACGTAGTAGCTTTTACACGTCGCCTGTAGCGCGGGCCGGTCCCCTGCCGT
AAAACACCCCAATTTTATAGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACCTAAGCATATCAATAAGCGGAG
GAA

06-485A – *Lophodermium* (98% ITS similarity)

CATTAAAGAATATCAGGCTTCACAGCCCTATTCTCACCCATATGTATTGAACTTCGTTGCTTTGGCAGGCCCCCTGGCTT
CGGCTGGACAGCGCCTGCCAGAGGATCTAAACCCCTGAATCTCTGCTGTCTGAGTACTACACAATAGTTAACTTTCA
ACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTG
AATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGGGGGCATGCCTGTTTCGAGCGTCATTACAACCCCTC
ACGCTCTGCGTGGTGTGGGCTGCCCTAAAGGGCTTGCCTCAAAATCAGTGGCGGCCGCTGTTTCGACCCCTAAGCGTA
GTAA
TTATTCTGTCGCTTCTGGGCTAGAGCAAGCCTGCTAGGAACCCCACTTCAAAGGTTGACCTCGGATCAGGTAGGGATA
CCCCTGAACCTAAGCATATCATNANGCGGANGAAA

06-486D – *Lophodermium* (94% ITS similarity) and most similar to fungi in the *Rhytistimataceae*

CATTAAAGAATACAAAGCCCTCCGGGCACTATTCTCACCCATATGTTTACCAAACCTTTGTTGCTTTGGCGGAAACGCAG
AGGACCGAAACCCCTGAATCTCTGCCGTCTGAGTCACTATATAATAGTTAACTTTCAACAACGGATCTCTTGGTTCT
GGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
CACATTGCGCCCTTGGCATTCCGGAGGCATGCCTGTTTCGAGCGTCATTACAACCCCTCAAGCTCTGCTTGGTATTGGCT
GGGCTCTTCCAAAGGGCTGCCCTCAAAATAGTGGCGGCACCGTCTGACCTTAAGCGCAGTAATGCTCGCTCGCTTCTTA
GGTTCCGGGGCGGCGCTTGCTAACAACCCCACTTATCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGACTTAT
CTATCATTA

06-097D – Most closely related to *Pleopsidium chlorophanum*

CATTA AAAAGTTGGCCGGGGTTTCGCCCCGCACGCCTCCATCCTATGAGTACTTACCCAGTTGCTTTGGCGAGCCGGGG
TGTTGCGGCCCGGGGCTCTGGCTGCCAGCGCCGTCAGAGGTCAATCAAACCTTGCTTAACGGTGAAGTCTGATAA
CTATTATGAATAGCTAAAACCTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAG
TAATGTGAATTGCAGAAATCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCGGGGGGCATGCGT
GTTGAGCGTCAATACAACCTCAAGCTCCGCTTGGTATTGGTGGCTCGCCCTACCCGGGGCGGCCCGAAAATCAGCGG
CACAGCTGCCCGACCTAAGCGTAGTAACATTCTCGCAAGAGGTGCTCGGGTGGTCTGCGGCCGACAACTATCAC
ATCAATTGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACCTAAGCATATCATAAAGCGGGAGAGGA

06-073C – Related to *Phaeoacremonium chlamydosporum* / *Phaeomoniella chlamydospora*

CATTACTGAGTTAGGGTTCTTCTNAGCCCAACCTCCAACCTTTGTTAATTTACCTTGTTGCTTTGGCAGGCCCGCCTTT
CGGGGCGCGGGGATGTTCAAGTCATCTCTGGCCAGTGCTTGCCAGTAGCCACTTCAAATCTTTTTAACTATGTCTGCT
AAACAATTAATCAAAATTTAATTAACCTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAA
ATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCGGA
AGGGCATGCCTGTTGAGCGTCAATATCAACCCTCAAGCCCGGCTTGTATTGGGTCCTGTATCGTTAAAAGATAGGCC
CGAAAGATAATGGCGGCGTCAAGATAGACCCAGGTGCAGCGAGCTTGCTAAGCATACACTGAGGTGGTCTGCTTGGC
CTGGCCCAACCCCAATTTTCTAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACCTAAGCATATCATTAAAGC
GGAGGAAA

06-255A – Species of *Mycosphaerella*

CATTACCGAGTGAGGGCTTCGGTCCGACCTCCAACCTTGATGTGAACCAAACCTTGTGCTTCGGGGGCGACCCTGCCGC
CTCGGCGGCGCGGCGCCCCGAAGGCCATCAAACACTGCATCATTGCGTCGGAGTCGAAGTAAATCAAATAAACTTT
CAACAACGGATCTCTTGGTTCCAGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
TGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGGGGGCATGCCTGTTGAGCGTCAATTCACCACT
CAAGCCTGGCTTGGTATTGGGCGTCGCGGCTCCGCGCGCCTCAAAGTCTTCCGCTGAGCTGTCCGTCTCTACGCGTTG
TGGCAACTATTGCGGTGGGAGGTGCGGCGGCGCGGCCGTTAAACCTTTACAGGTTGACCTCGGATCAGGTAGGGAT
ACCCGCTGAACCTAAGCATATCAATAAGCGGAGGAA

06-268A – Species of *Mycosphaerella*

CATTACCGAGTGAGGGCTTCGGTCCGACCTCCAACCTTGATGTGAACCAAACCTTGTGCTTCGGGGGCGACCCTGCCGC
TCGGCGGCGCGGCGCCCCGAAGGCCATCAAACACTGCATCATTGCGTCGGAGTCGAAGTAAATCAAATAAACTTT
AACAACGGATCTCTTGGTTCCAGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGT
GAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGGGGGCATGCCTGTTGAGCGTCAATTCACCACT
AAGCCTGGCTTGGTATTGGGCGTCGCGGCTCCGCGCGCCTCAAAGTCTTCCGCTGAGCTGTCCGTCTCTACGCGTTG
GGCAACTATTGCGGTGGGAGGTGCGGCGGCGCGGCCGTTAAACCTTTACAGGTTGACCTCGGATCAGGTAGGGATA
CCCGCTGAACCTAAGCATATCATAAAGCGGAGGAA

06-063D – Similar to *Dwayaangam colodena*

CATTAGAATGAGTTCCGGACCATGTCGTAGGTGAAAGCCTACACGTGCCAGTAAGCGTCCGTAAACCTCCACCCTTGTA
TATTATTCTTTGTTGCTTTGGTAGGCCGCGAGCTTTGGGCGAGCGCTGGCTCCGGCTAGAGAGTGCTGCCAGAGGAC
CCAACCTCTGTAATCTAGTGATGTCTGAGTACTATTAAATAGTTAAACCTTTCAACAACGGATCTCTTGGTTCTGGCATC
ATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTG
CGCCCTCTGGTATTCCGGAGGGCATGCCTGTTGAGCGTCAATTTCAACCAATCACGCGAGTGGTGTGGGGCTTGCTAC
ACAGCATCCCTTAAAAACAGTGGCGGTGCTATAGGGCTCTCAGCGTAGTAATTATCCGCTCTTGAACCTAGACACAC
CTGTCATAACCCCAATTTTCTAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACCTAAGCTATC

06-264A – Similar to *Dwayaangam colodena*

CATTAGAATGAGAGCCGGACCATGTCGTGGGCGAAAGCCCAAACGTGCCAGTAAGCGCCCGTAAACCTCCACCCTTG
ATATTATTCTTTGTTGCTTTGGTGGGCGCAAGCCTTCGGGCGAGCACCGGCTTCGGCTGGAGAGTGCTGCCAGAGGA
CCCAACTCTGTAATTTAGTGATGTCTGAGTACTATTAAATAGTTAAACCTTTCAACAACGGATCTCTTGGTTCTGGCATC
GATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATT
GCGCCCTCTGGTATTCCGGANGGCATGCCTGTTGAGCGTCAATATAACCAATCACGCANGTGGTTTGGGGCTTGCTA
TCTAGCATCCCTTAAAAACAGTGGCGGTGCTATAGGGCTCTCAGCGTAGTAATTATCCGCTTTGAACCTAGACACACC
TGTCAGAACCCCAATTTTAAAGGTTGACCTCGGATCAGGTAGGATACCCGCTGAACCTAAGCATATCAATAAGCGGAGG
AAA

06-332A – Related to *Phaeoacremonium chlamydosporum* / *Phaeomoniella chlamydospora*

CATTACTGAGTTAGGGTTCTTCNGAGCCCAACCNCAACCCTTTGTTAATTTACCTTGTTGCTTNGGCAGGCCCGCCTT
TCGGGGCCGCCGGGGATGTTTCAGTCATCTCTGGCCAGTGCTTGCCAGTAGCCACTTCAAATTTCTTTTAACTATGTCGTC
TAAACAATTAATAAAAAATTTAATTAACACITTCACAAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAA
ATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCGA
AGGGCATGCCTGTTTCGAGCGTCATTATCAACCCTCAAGCCCGGCTTGTTATTGGGTCCTGTATCGTTAAAGATAGGCC
CGAAAGATAATGGCGGCGTCNAGATAGACCCAGGTGCAGCGAGCTTGCTAAGCATACACTGAGGTGGTCTGCTTGGC
CTGGCCCAACCCCAATTTTCTAAGGTTGACCTCGGATCAGGTAGGAATACCCNCTGANCTTAAGCATATCAATAAGGG
GAGGAA

07-013D – Similar to *Dwayaangam colodena*

CATTAGAATGAGAGCCGGACCATGTCTGTTGGGTGAAAGCCCAACCGTGCCAGTAAGCGCCCGTAAACCTCCACCCTTGT
ATATTATCTTTGTTGCTTTGGTGGGCGCAAGCCTTCGGCGAGCACCGGCTTCGGCTGGAGAGTGCCTGCCAGAGGAC
CCAACCTCTGTAATTTAGTGATGTCTGAGTACTATTAATAGTTAAACCTTTCAACAACGGATCTCTTGGTTCTGGCATCG
ATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTG
CGCCCTCTGGTATTCCGGAGGCATGCCTGTTTCGAGCGTCATTATAACCAATCACGCGAGTGGTTTTGGGGCTTGCTATCT
AGCATCCCTTAAAAACAGTGGCGGTGCTATAGGGCTCTCAGCGTAGTAATTATCCGCTTTGAAACCTAGACACACCT
GTCAGAACCCCAATTTTAAAGGTTGACCTCGGATCAGGAGGATACCCGCTGAACCTTAAGCATATCATAAGGCGGAGG
AAA

08-018A – Similar to *Dwayaangam colodena*

CATTAGAATGAGAGCCGGACCATGTCTGTTGGGCGAAAGCCCAACCGTGCCAGTAAGCGCCCGTAAACCTCCACCCTTG
TATATTATCTTTGTTGCTTTGGTGGGCGCAAGCCTTCGGGCGAGCACCGGCTTCGGCTGGAGAGTGCCTGCCAGAGG
ACCCAACCTCTGTAATTTAGTGATGTCTGAGTACTATTAATAGTTAAACCTTTCAACAACGGATCTCTTGGTTCTGGCAT
CGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACAT
TGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTTCGAGCGTCATTATAACCAATCACGCAAGTGGTTTTGGGGCTTGCTT
ATCTAGCATCCCTTAAAAACAGTGGCGGTGCTATAGGGCTCTCAGCGTAGTAATTATCCGCTTTTGAACCTAGACAC
ACCTGTCAGAACCCCAATTTTAAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACCTTAAGCATNTAAAAAN
NCNGGAAGGAAA

08-011D – Related to *Phaeoacremonium chlamydosporum* / *Phaeomoniella chlamydospora*

CATTACTGAGTTAGGGTTCTTCCCNCCCAACCTGGAAAAAATTTGTTAATTTACCTTGTTGCTTTGCCAGGCCCGCCT
TTCGGGGCCGCCGGGGATGTTTCAGTCATCTCTGGCCAGTGCTTGCCAGTAGCCACTTCAAATTTCTTTTAACTATGNCNT
CTAAACAATTAATAAAAAATTTAATTAACCTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGA
AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCG
AAGGGCATGCCTGTTTCGAGCGTCATTATCAACCCTCAAGCCCGGCTTGTTATTGGGTCCTGTATCGTTAAAGATAGGC
CCGAAAGATAATGGCGGCGTCAAGATAGACCCAGGTGCAGCGAGCTTGCTAAGCATACACTGAGGTGGTCTGCTTGG
CCTGGCCCAACCCCAATTTTCTAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACCTTAAGCATATCAATAAGC
GGAGGAAA

01-002A – *Nectria mauritiicola*

CATTACCAGAGTGCCCTAGGCTCTCAACCCATTGTGAACATACCTATCGTTCCTCGGCGGGCTCAGCGCGCGGTGCC
TCCGGGCTCCGGGCGTCCGCCGGGACAACCAAACCTCTGATTTTATTGTGAATCTCTGAGGGGCGAAAGCCGAAAAC
AAAATGAATCAAACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAAT
GTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCGGCACTCCGGCGGGCATGCCGTGTC
CGAGCGTCATTTCAACCCCTCAGGACCCCTTTCCGGGGGGGACCTGGTGTGGGGATCAGCGGCCCTCCGGGCCCCCTGTCC
CCCAAATTGAGTGGCGGTCGCGCCCGAGCCTCCCTGCGTAGTAGCACACCTCGCACCGGAGAGCGGCTCGGCCACGC
CGTGAAACCCCAATTTTAAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACCTTAAGCATATCATTAAAGCGG
AGGAA

04-002G – Related to *Phaeoacremonium chlamydosporum* / *Phaeomoniella chlamydospora*

CATTACTGAGTTAGGGTTCTTCTGAGCCCAACCTCCAACCCCTTTGTTAATTTACCTTGTTGCTTTGGCAGGCCCGCCTTT
CGGGGCCGCGGGGATGTTTCAGTCATCTCTGGCCAGTGCTTGCCAGTAGCCACTTCAAATTTCTTTTAACTATGTCGTCT
AAACAATTAATCAAAATTTAATTAATACTTTCAACAACGGATCTCTGGTTCTGGCATCGATGAAGAACGCAGCGAA
ATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCGA
AGGGCATGCCTGTTTCGAGCGTCATTATCAACCCCTCAAGCCCGGCTTGTTATTGGGTCCTGTATCGTTAAAAGATAGGCC
CGAAAGATAATGGCGGCGTCAAGATAGACCCAGGTGCAGCGAGCTTGCTAAGCATACTGAGGTGGTTCGTCTTGGC
CTGGCCCAACCCCAATTTTCTAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACCTAAGCATATCATAAAGCG
GAGGAA

03-020B – Related to *Phaeoacremonium chlamydosporum* / *Phaeomoniella chlamydospora*

CATTACTGAGTTAGGGTTCTTCCNAGCCCAACCTCAAACCCCTTTGTTAATTTACCTTGTTGCTTTGGCAGGCCCGCCTTT
CGGGGCCGCGGGGATGTTTCAGTCATCTCTGGCCAGTGCTTGCCAGTAGCCACTTCAAATTTCTTTTAACTATGNCGTCT
AAACAATTAATCAAAATTTAATTAATACTTTCAACAACGGATCTCTGGTTCTGGCATCGATGAAGAACGCAGCGAA
ATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCGA
AGGGCATGCCTGTTTCGAGCGTCATTATCAACCCCTCAAGCCCGGCTTGTTATTGGGTCCTGTATCGTTAAAAGATAGGCC
CGAAAGATAATGGCGGCGTCAAGATAGACCCAGGTGCAGCGAGCTTGCTAAGCATACTGAGGTGGTTCGTCTTGGC
CTGGCCCAACCCCAATTTTCTAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACCTAAGCATATCAATNAAGC
GGAGGAAA

04-012A – Related to *Phaeoacremonium chlamydosporum* / *Phaeomoniella chlamydospora*

CATTACTGAGTTAGGGTTCTTCTNAGCCCAACCTCCAACCCCTTTGTTAATTTACCTTGTTGCTTTGGCAGGCCCGCCTTT
CGGGGCCGCGGGGATGTTTCAGTCATCTCTGGCCAGTGCTTGCCAGTAGCCACTTCAAATTTCTTTTAACTATGNCGTCT
AAACAATTAATCAAAATTTAATTAATACTTTCAACAACGGATCTCTGGTTCTGGCATCGATGAAGAACGCAGCGAA
ATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCGA
AGGGCATGCCTGTTTCGAGCGTCATTATCAACCCCTCAAGCCCGGCTTGTTATTGGGTCCTGTATCGTTAAAAGATAGGCC
CCGAAAGATAATGGCGGCGTCAAGATAGACCCAGGTGCAGCGAGCTTGCTAAGCATACTGAGGTGGTTCGTCTTGG
CCTGGCCCAACCCCAATTTTCTAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACCTAAGCATATCAATNAAG
CGGAGGAA

02-002C – Related to *Phaeoacremonium chlamydosporum* / *Phaeomoniella chlamydospora*

CATTACTGAGTTAGGGTTCTTCTGAGCCCAACCTCCAACCCCTTTGTTAATTTACCTTGTTGCTTTGGCAGGCCCGCCTTT
CGGGGCCGCGGGGATGTTTCAGTCATCTCTGGCCAGTGCTTGCCAGTAGCCACTTCAAATTTCTTTTAACTATGTCGTCT
AAACAATTAATCAAAATTTAATTAATACTTTCAACAACGGATCTCTGGTTCTGGCATCGATGAAGAACGCAGCGAA
ATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCGA
AGGGCATGCCTGTTTCGAGCGTCATTATCAACCCCTCAAGCCCGGCTTGTTATTGGGTCCTGTATCGTTAAAAGATAGGCC
CCGAAAGATAATGGCGGCGTCAAGATAGACCCAGGTGCAGCGAGCTTGCTAAGCATACTGAGGTGGTTCGTCTTGG
CCTGGCCCAACCCCAATTTTCTAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACCTAAGCATATCATTAAAGC
GGAGGAA

06-094E – *Nectria mauritiicola*

CATTACCAGAGTGCCCTAGGCTCTCCAACCCATTGTGAACATACCTATCGTTCCCTCGGCGGGCTCAGCGCGCGGTGCC
TCCGGGCTCCGGGCGTCCGCCGGGACAACCAAACCTCTGATTTTATTGTGAATCTCTGAGGGGCGAAAGCCCGAAAAC
AAAATGAATCAAACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAAT
GTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCGGCACTCCGGCGGGCATGCCTGTC
CGAGCGTCATTTCACCCCTCAGGACCCCTTTCGGGGGGGACCTGGTGCTGGGGATCAGCGGCCCTCCGGGCCCCGTGCC
CCCAAATTGAGTGGCGGTGCGCGCGCAGCCTCCCTGCGTAGTAGCACCTCGCACCGGAGAGCGGCTCGGCCACGC
CGTGAAACCCCAATTTTCTAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACCTAAGCATATCAATAAGCGCA
AGGAA

04-052B – Unidentified (new) fungal species

CATTACAGAGAAGTTGCCCTTCCCCGTAGATCTCAAAANCTTTGTTTACATTACCCTTGTTGCTTTGGCAGGCCCGCTCT
CTCGGGACCGCCGGCTTCGGCTGGCCCGCGCCTGCCAGAGGATCTTAAACTCTTGTTTAAATGTCGTCTGAGTACTNTA
TAATAAGTTAAACCTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGT
GAATTGCAGAATTTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGGGGGCATGCCCTGTTTCG
AGCGTCATTACAACCTCAAGCTCTGCTTGGTATTGGGCGTCACCGCTTCGGTGCGCTTAAATCAGTGGCGGTGCCA
TCCGGCTTCAAGCGTAGTAATTCTCTCGCTCTGGGGATCCGGGTGTGTGCTTGCCAGCAACCCCCAATTATCAAAGGT
TGACCTCGGATCAGGTAGGGATACCCGCTGAACCTTAAGCATATCAATAAGCGGAGGAA

06-011B – Related to *Phaeoacremonium chlamydosporum* / *Phaeomoniella chlamydospora*

CATTACTGAGTTAGGGTTCTTCCNAGCCCAACCTCCAACCCCTTGTTTAAATTTACCTTGTTGCTTTGGCAGGCCCGCTT
TCGGGGCCGCGGGGATGTTTCAGTCATCTCTGGCCAGTGCTTGCCAGTAGCCACTTCAAATCTTTTTAACTATGTCGTC
TAAACAATTAAATCAAAATTTAATTAATACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAA
ATGCGATAAGTAATGTGAATTGCAGAAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCGA
AGGGCATGCCCTGTTTCGAGCGTCATTATCAACCCCTCAAGCCCGGCTTGTTATTGGGTCTGTATCGTTAAAAAGATAGGCC
CGAAAGATAATGGCGGCGTCAAGATAGACCCAGGTGCAGCGAGCTTGCTAAGCATACACTGAGGTGGTCTGCTTGGC
CTGGCCCAACCCCAATTTTCTAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACCTTAAGCATATCATTAAGCG
GAGGAA

06-011D – Related to *Phaeoacremonium chlamydosporum* / *Phaeomoniella chlamydospora*

CATTACTGAGTTAGGGTTCTTCTCAGCCCAACCTCCAACCCCTTGTTTAAATTTACCTTGTTGCTTTGGCAGGCCCGCTT
CGGGGCCGCGGGGATGTTTCAGTCATCTCTGGCCAGTGCTTGCCAGTAGCCACTTCAAATCTTTTTAACTATGTCGTC
AAACAATTAAATCAAAATTTAATTAATACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAA
ATGCGATAAGTAATGTGAATTGCAGAAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCGA
AGGGCATGCCCTGTTTCGAGCGTCATTATCAACCCCTCAAGCCCGGCTTGTTATTGGGTCTGTATCGTTAAAAAGATAGGCC
CCGAAAGATAATGGCGGCGTCAAGATAGACCCAGGTGCAGCGAGCTTGCTAAGCATACACTGAGGTGGTCTGCTTGG
CCTGGCCCAACCCCAATTTTCTAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACCTTAAGCATATCAATTAAG
CGGAGGAA

06-012C – Similar to *Dwayaangam colodena*

CATTAGAATGAGAGCCGGAACCATNCTCGTGGGTGCAAGCNCNAAACGTGCCAGNTAAGCGCCCGTAAACCTCCACCC
TTGTATATTATTCTTTGTTGCTTTGGTGGGCGCAAGCCTTCGGGTGAGCACC GGCTTCGGCTGGAGAGTGCCTGCCAGA
GGACCCAACTCTGTAAATTTAGTGATGTCTGAGTACTATTAAATAGTTAAACCTTCAACAACGGATCTCTTGGTTCTGGC
ATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAAATTCAGTGAATCATCGAATCTTTGAACGCAC
ATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTTCGAGCGTCATTATAACCAATCAGCAAGTGTTTGGGGCTTG
CTATCTAGCATCCCTTAAAAACAGTGGCGGTGCTATAGGGCTCTCAGCGTAGTAATTATCCGCTTTTGAACCTAGAC
ACACCTGTCAAAACCCCAANTCTTTTAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACCTTAAGCATATCAAT
AAANCGGAGGAA

06-013A – Similar to *Dwayaangam colodena*

CATTAGAATGAGAGCCGGACCATGTCGTGGGCGAAAAACGTGCCAGTAAGCGCCCGTAAACCTCCACCCCTTGATAT
TATTCTTTGTTGCTTTGGTGGGCGCAAGCCTTCGGCGAGCACC GGCTTCGGCTGGAGAGTGCCTGCCAGAGGACCCAA
CTCTGTAAATTTAGTGATGTCTGAGTACTATTAAATAGTTAAACCTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGA
AGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC
CTCTGGTATTCCGGAGGCATGCCTGTTTCGAGCGTCATTATAACCAATCAGCAAGTGTTTGGGGCTTGCTATCTAGC
ATCCCTTAAAAACAGTGGCGGTGCTATAGGGCTCTCAGCGTAGTAATCATTCCGCTTTTGAACCTAGACACACCTGTC
AAAACCCCAATCTTTTAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACCTTAAGCATATCATAAAGCGGAG
GAGA

06-014C – Unidentified (new) fungal species

CATTAATGGTCNATTGTGGAGCCNTCAGAAGGTATGCTACGGCGTCATTTCGCAGTGGGACCAATAGAATCCACGTATCA
GGGTGGACCCCTGGAACCCCTACCCGGGTTCTCACGAGTTCGGGGCGTGAGTCTCTGGCCCTATAGACGGGCCCTAAG
AGCGGCCGGGGTTTACAGCCCCAATACCGACATAGTCCTTGGCCCGCCAAGCGAGCCGATGCGTCCACGCTGGCGCT
CCCTTTCCAAACCCCAACCCTTGAATACCTCACCTCTGTTGCCTCGGCGGGTACGCTCGCCGGTGGACGCAAACCAAAC
TCTGTCTTAACGGTGTAGTCTGAGCAAACAAAGTAATAGTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCG
ATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTG
CGCCCTTTGGCATCCCGAAGGGCATGCCTGTTGAGCGTCATTTCACCTCTCAAGCGCAGCTTGGTGTGGAGGACGTC
CCTTCCTATAGAGGGGCCCTCCTAAAATCATCGGCGATTCTAT

08-040B – Similar to *Dwayaangam colodena*

CATTAGAATGAGAGCCGGACCATGTCGTGGGTGAAAGCCCAAACGTGCCAGTAAGCGCCCGTAAACCTCCACCCTTGT
ATATTATTCTTTGTTGCTTTGGTGGGCCGCAAGCCTTCGGGCGAGCACCGGCTTCGGCTGGAGAGTGCCTGCCAGAGGA
CCCAACTCTGTAATTTAGTGATGTCTGAGTACTATTAAATAGTTAAACCTTTCAACAACGGATCTCTTGGTTCTGGCATC
GATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATT
GCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTGAGCGTCATTATAACCAATCACGCGAGTGGTTTTGGGGCTTGCTA
TCTAGCATCCCTTAAACAGTGGCGGTGCTATAGGGCTCTCAGCGTAGTAATTATTCCCGCTTTTGAAACCTAGACACA
CCTGTCAGAACCCCAATTTTAAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACCTTAAGCTTCATTTAAAGCN
GGAAGAAAA

Section F – HPLC chromatogram spectra for white and red spruce endophytes **06-485A – HPLC chromatogram** **06-486D – HPLC chromatogram**

06-097D – HPLC chromatogram **06-073C – HPLC chromatogram** **06-268A & 06-255A – HPLC chromatogram**

06-063D – HPLC chromatogram **08-018A – HPLC chromatogram**

Section G – Spectral data for compounds 1-26

Compound 1

Compound 2

Compound 3

Compound 4

Compound 5

Compound 6

Compound 7

Compound 8

Compound 9

Compound 10

Compound 11

Compound 12

Compound 13

Compound 14

Compound 15

Compound 16

Compound 17

Compound 18

Compound 19

Compound 20

Compound 21

Compound 22

Compound 23

Compound 24

Compound 25

Compound 26

Section H – Structures of compounds 1-10 from white spruce endophytes

1

2

3

4a R = H
4b R = Me

5

7

6 R = H
8 R = CO₂Me
9 R = COH
10 R = Me

Section I – Structures of compounds 11-26 from red spruce endophytes