Ectomycorrhizal fungal community response to disturbance and host phenology

by

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Abstract

The relationship between trees and ectomycorrhizal fungi is fundamental for tree growth and survival, particularly in the boreal forests of North America where low temperatures inhibit decomposition and consequently limit nutrient availability. The responses of ectomycorrhizal fungal communities to large-scale disturbances and host phenology are not well known, but are important for restoring and predicting carbon and nutrient cycling. To that end, I investigated the ectomycorrhizal fungal community present on roots of outplanted seedlings in sites that captured a gradient of above and belowground disturbances. Additionally, I monitored the extracellular enzyme secretions of ectomycorrhizal fungi during four phenological stages (host dormancy, leaf flush, full leaf expansion, leaf abscission) of mature *Populus tremuloides* stands to assess the potential decomposing activity of ectomycorrhizal fungi in relation to changes in tree physiology. Contrary to my prediction, there was no difference in ectomycorrhizal fungal community composition across sites that differed in extent of above and belowground disturbances; composition was instead primarily affected by the species of seedling used to assay the soils. Further, I found relatively constant levels of enzyme secretions by ectomycorrhizas across phenological stages irrespective of the amount of carbon stored in roots, suggesting the enzymes I measured may be secreted to acquire nitrogen or phosphorus locked within organic matter. Additionally, potential enzyme activity was better predicted by the foraging strategy of ectomycorrhizal fungi, highlighting the functional roles of species. These findings emphasize the importance of planting a diverse community of trees in reclaimed soils to yield a diverse community of belowground fungi. Moreover, differences in potential enzyme activity of exploration types throughout phenological stages point to unique functional roles among fungi, which may change seasonally. Consequently, this research stresses the importance of restoring functional diversity in reconstructed ecosystems.

Preface

A version of Chapter 3 of this thesis has been submitted for publication as S. F. Hupperts, Justine Karst, Karin Pritsch, and Simon M. Landhäusser, "Host phenology and potential saprotrophism of ectomycorrhizal fungi of aspen in the boreal forest," *Functional Ecology* (submitted 04 February 2016). I performed data collection, analysis, and preparing the manuscript. J. K. assisted with data collection and writing the manuscript. All authors contributed to experimental design and manuscript edits.

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Chapter 1: General Introduction

1.1 Ectomycorrhizal fungi

Trees require inorganic nutrients for growth and defense, yet depend on microbes to mineralize soil nutrients. Ectomycorrhizal fungi, in particular, are microbes that mediate resource uptake from soils through their symbiosis with most trees especially in the northern boreal forests, where cold temperatures reduce decomposition rates and consequently limit nutrient availability. Moreover, ectomycorrhizal fungi are especially efficient at mineralizing nitrogen and phosphorus from litter, a defining trait which drives nutrient cycling in boreal ecosystems and underlies the host-fungal symbiosis (Bidartondo *et al.* 2001; Read, Leake & Perez-Moreno 2004; Orwin *et al.* 2011). In exchange for inorganic nutrients, trees provide carbon from recent photosynthates to support the growth and defense of their ectomycorrhizal mycobionts (Marx 1972; Smith & Read 2008). When root symbionts die, their necromass contributes to soil organic matter. Consequently, the prevalence of ectomycorrhizal fungi in boreal soils contributes significantly to carbon sequestration in boreal forests (Clemmensen *et al.* 2013, 2015; Averill, Turner & Finzi 2014; Sterkenburg *et al.* 2015). Furthermore, the diversity of specific plant-fungal symbioses suggests a unique role ectomycorrhizal fungi have in driving plant diversity (van der Heijden *et al.* 1998; van der Heijden, Bardgett & van Straalen 2008; Rudawska, Leski & Stasińska 2011) and highlights the importance of maintaining and restoring ectomycorrhizal fungal communities in forest ecosystems.

1.2 Disturbance affects ectomycorrhizal fungal communities

Stand to landscape-scale disturbances affect the growth and development of ectomycorrhizal fungi (EMF) and may consequently impact the natural establishment of

seedlings and other vegetation (Jones, Durall & Cairney 2003; Barker *et al.* 2012). Bladed clear-cut stand harvesting, for example, has a much larger impact on above and belowground communities than single-tree selection harvesting, and may require more intensive site preparation to promote regeneration (Jurgensen *et al.* 1997; Hagerman *et al.* 1999; Johnson & Curtis 2001; Putz *et al.* 2008; Barker *et al.* 2012). Existing spore banks may be critical for EMF community establishment following disturbances and may impact seedling recruitment, depending on the colonization strategies of trees (i.e. seed germination vs. vegetative colonization). Hagerman *et al* (1999) examined the effect of cut size on the EMF community in soil cores taken from recently planted clear-cut subalpine *Picea engelmannii – Abies lasiocarpa* stands. Two growing seasons after planting, EMF diversity was significantly reduced with distance into the clear-cut, suggesting that propagule availability declines with increasing distance from existing vegetation (Hagerman *et al.* 1999). Another site preparation method, blading, removes the forest floor to enhance early seedling development by eliminating competition and increasing soil temperature (Lazaruk, Macdonald & Kernaghan 2008; Mackenzie 2011). Although beneficial for some tree species, blading may eliminate the EMF propagule bank and remove mycorrhizal networks, and as a consequence, decrease EMF community diversity (Hagerman *et al.* 1999; Barker *et al.* 2012). This leaves EMF spore dispersal and existing propagules in the mineral soil as the only means to reestablish ectomycorrhizas after blading. Assessing the EMF community on young (<1 year old) *Pseudotsuga mensiezii* seedlings in various disturbance treatments, Barker *et al* (2012) found that richness was unaffected by disturbance type, but EMF community composition was significantly altered in clear-cut and bladed stands when compared to undisturbed stands. Forest floor material is an important source of fungal propagules and its retention may ensure a fungal

community similar to that of an undisturbed forest. Lazuruk *et al* (2008) assessed the EMF community on *Picea glauca* seedlings planted in undisturbed sites, bladed sites where the forest floor had been removed, and sites where the organic layer had been mixed with the underlying mineral soil. After two growing seasons, seedlings planted in the 'mixed' treatment had an EMF community most similar to the undisturbed control, while the EMF community on seedlings planted in the bladed treatment was significantly different than the undisturbed control.

Severe disturbances such as glacier retreat, volcano eruptions, and surface mining create harsh site conditions including large diurnal temperature fluctuations, intense radiation and nutrient limitation. Moreover, the scarcity of organisms and organic material which were present prior to the disturbance means there are few biological legacies (Nara *et al* 2003; Dale, Swanson, & Crisafulli 2005; Ishida, Nara & Hogetsu 2007). Extensive research on glacier retreat has revealed that following colonization, EMF richness remains relatively stable over the course of site development following deglaciation, despite changes in EMF community composition (Jumpponen *et al.* 2012). Furthermore, the composition of EMF species in recently deglaciated sites was similar to that of nearby sites with greater time since deglaciation, highlighting the prominence of spore dispersal in recently disturbed areas with few biological legacies. These findings suggest that ruderal, pioneer EMF species are able to immediately colonize the bare and nutrient-poor soil, which then are joined or replaced by additional EMF species in the successional development of the area.

Surface mining presents another severe landscape-level disturbance which leaves few biological legacies (Johnson & Miyanishi 2008; Rowland *et al.* 2009; Mackenzie & Naeth 2010; Audet, Pinno & Thiffault 2015). Once mining has ceased, excavated sites are reclaimed and soil profiles reconstructed by placing soils directly or using stockpiled, salvaged material (Rowland

et al. 2009; Mackenzie & Naeth 2010). Reconstructed soils of reclaimed sites in boreal regions typically differ from local natural soils in their pH, nutrient composition, bulk density, and moisture content (Rowland *et al.* 2009; Mackenzie & Naeth 2010), factors which may influence EMF colonization and hyphal development (van der Heijden & Kuyper 2001; Dickie *et al.* 2013). For example, Rowland *et al* (2009) found lower rates of decomposition in reclamation treatments of coarse-textured subsoils when compared to soils capturing a range of natural variability, perhaps a result of lower microbial activity. Moreover, Brown and Naeth (2014) found lower microbial and mycorrhizal biomass in peat cover soil when compared to cover soils of salvaged forest floor material, highlighting how successional trajectories can be influenced by different reclamation strategies. While vegetation response has been widely examined in forest restoration, the composition of EMF communities has not been extensively investigated in the restoration of large-scale reclamation sites. Assessing the EMF community in reclamation cover soils of peat, forest floor material, and subsoil, Hankin, Karst & Landhäusser (2015) found early EMF community composition to be influenced by host species identity alone, with no effect of cover soil. These findings lay groundwork for further monitoring of EMF community development in large-scale reclamation sites.

Additionally, the use of relevant ecological benchmarks is necessary for comparisons of site development following reclamation (Sousa 1984; Rowland *et al.* 2009). For example, the highly severe nature of surface mining in the absence of reclamation pushes the ecosystem past its threshold of resilience. Reclamation and subsequent restoration of forests on these sites is an attempt to establish self-sustaining ecosystems integrated with the surrounding boreal forest[1].

[1] Reclamation is defined here as the stabilization, contouring, maintenance, conditioning, reconstruction, and revegetation of the surface of the land to a state that permanently returns the land to an equivalent land capability. (Alberta Energy Regulator). Restoration is defined here as the process of assisting the recovery of an ecosystem that has been degraded, damaged, or destroyed (Society for Ecological Restoration).

Selecting an appropriate benchmark for comparison of ecosystem development is necessary for effective restoration (White & Walker 1997; Harris *et al.* 2006). Moreover, previous research on ecosystem development following landscape disturbances such as harvesting is extensive (Hagerman *et al.* 1999; Jones *et al.* 2003; Rowland *et al.* 2009; Holden & Treseder 2013). If successional trajectories created by surface mining reclamation and restoration are similar to those created by harvesting disturbances, management of reclaimed sites could be informed by activities related to harvesting.

1.3 Role of ectomycorrhizal fungi in decomposition

Decomposition varies depending on soil temperature, moisture, and availability of organic matter (Davidson & Janssens 2006; Allison & Treseder 2011; Rineau *et al.* 2013), conditions which typically vary in different reclamation cover (Rowland *et al.* 2009; Dimitriu *et al.* 2010; Brown & Naeth 2014). Because ectomycorrhizas are the functional interface between forest productivity and decomposition, quantifying the role EMF play in carbon and nutrient cycling is critical to understanding underlying mechanisms in reclamation site development.

Ectomycorrhizal fungi secrete extracellular enzymes to degrade complex organic matter for nutrient acquisition. Chitinases and phosphatases are secreted to break down organic matter-protein complexes to acquire nitrogen and phosphorus, respectively (Hodge, Alexander & Gooday 1995; Pritsch & Garbaye 2011; Rineau *et al.* 2012). Although dependent on their host for carbon, recent work has suggested that EMF can also behave like saprotrophs during certain periods of the year, by releasing enzymes which decompose plant litter for use as a carbon source (Courty *et al.* 2006; Courty, Bréda & Garbaye 2007; Cullings & Courty 2009; Cullings *et al.* 2010; Bentzer *et al.* 2015). The emergence of enzyme activity profiling of root tips and

carbon isotope labeling has allowed researchers to assess the potential decomposing activity of ectomycorrhizal fungi (Pritsch *et al.* 2004, 2011; Pritsch & Garbaye 2011; Tedersoo *et al.* 2012). For example, Courty *et al* (2007) monitored extracellular enzyme secretions of *Quercus*-associated ectomycorrhizas before, during, and after leaf flush, and found an increase in several carbon-degrading enzymes just prior to and during leaf flush, perhaps indicating that EMF may be acting as saprotrophs during times of high carbon demand within the host tree. This work is further supported by work from Bréda *et al.* (2013) using isotopic evidence to conclude that EMF are able to mobilize carbon from plant litter. Furthermore, Cullings *et al* (2010) found similar results supporting saprotrophy of EMF during artificial defoliation of a *Pinus contorta* stand. However, the capacity for decomposition by EMF is debated (Treseder, Torn & Masiello 2006; Baldrian 2009; Cullings & Courty 2009; Moore *et al.* 2015). For example, Treseder *et al* (2006) found only 2% of labeled carbon from plant litter had been taken up by EMF, a negligible value equal to the detection limit. Additionally, the gene-based capacity for secretion of carbon-degrading enzymes is variable depending on the fungal species or guild (Morgenstern, Klopman & Hibbett 2008; Rineau *et al.* 2012; Bentzer *et al.* 2015; Kohler *et al.* 2015; Talbot *et al.* 2015).

Short growing seasons in boreal forests cause deciduous trees to undergo dramatic seasonal fluxes in carbon assimilation followed by extended winter dormancy, changes which may cause fluctuations of carbon allocation from host to fungi and consequently have cascading effects on soil carbon and nutrient cycling (Johansson 1993; Courty, Franc & Garbaye 2010). For example, trees store sugar and starch in roots and other organs to maintain metabolic processes during times when photosynthetically-derived carbon is limited. These sugars and starches, collectively termed nonstructural carbohydrates, fluctuate in response to seasonality and consequently induce changes in source-sink dynamics in trees (Chapin, Schulze & Mooney

1990; Hoch, Richter & Korner 2003; Körner 2003; Wiley & Helliker 2012), and may further affect carbon availability to ectomycorrhizal symbionts. Landhäusser & Lieffers (2003) found fine root sugar and starch concentrations of aspen (*Populus tremuloides*) to fluctuate from spring thaw to leaf abscission. These fluctuations, observed in many tree species (Gruber, Pirkebner & Oberhuber 2013; Da Silva *et al.* 2014; Dang *et al.* 2014), may determine how much sugar can be allocated to ectomycorrhizal fungi (Johansson 1993; Hoch *et al.* 2003). This allocation of sugar from host to symbiont may be indirectly quantified by monitoring invertase, a plant-derived enzyme secreted into the interfacial apoplast (Salzer & Hager 1993; Egger & Hampp 1993; Schaeffer *et al.* 1995; Parrent *et al.* 2009). By hydrolyzing sucrose, invertase makes glucose available to EMF; many species of EMF lack genes encoding invertase and are unable to absorb sucrose, consequently relying on the host for invertase synthesis and sucrose hydrolysis (Salzer & Hager 1993; Schaeffer *et al.* 1995). This mechanism may allow host trees to control photosynthate allocation to EMF by regulating their invertase activity. As seasonality may cause fluctuations in fine root NSC reserves, invertase levels may also fluctuate, determining how much glucose EMF are receiving from their host. Due to the critical role of EMF in nutrient foraging and soil carbon sequestration (Clemmensen *et al.* 2013, 2015; Treseder & Holden 2014; Averill *et al.* 2014), understanding their potential role in decomposition is essential for improving global carbon models and restoration processes (Landeweert *et al.* 2001; Moorhead & Sinsabaugh 2006; Chapin *et al.* 2009; Hobbie & Agerer 2010; Allison 2012; Treseder *et al.* 2012; Moore *et al.* 2015).

1.4 Objectives

To further our understanding of the role ectomycorrhizal fungi play in boreal forest ecosystems, the primary objective of this thesis is to assess the EMF community response to a variety of environmental changes. First, I investigate the recovery of EMF communities on outplanted seedlings following (1) the reconstruction of soils on reclaimed sites using peat, forest floor material, and subsoil, and (2) aboveground disturbances of different severity. Second, I assess the extracellular enzyme secretions of EMF during four host phenological stages, from dormancy to leaf abscission, to test the ability of EMF to decompose organic matter.

Chapter 2: Recovery of ectomycorrhizal fungal communities in intact and reclaimed soils of the boreal forest.

2.1 Introduction

The boreal forest of North America covers a vast area of the continent, providing economically important renewable resources such as timber and non-renewable resources such as minerals and oil. As a consequence, this area is subject to significant anthropogenic disturbances. Surface mining of resources requires the removal of vegetation, soil and geological material to access the underlying resource. When mining ceases, particularly in the boreal forest region of Alberta, the site is to be restored to a locally common, self-sustaining ecosystem (Alberta Environment 1999), a process which involves the reconstruction of soils and ecosystems including the restoration of plant-soil interactions.

A fundamental interaction that connects plants with soils in boreal forest is ectomycorrhizas. Ectomycorrhizal fungi (EMF) colonize tree roots, forming a symbiotic relationship with their hosts by providing mineral nutrients in exchange for photosynthetically-derived carbon. Moreover, EMF contribute significantly to soil carbon storage (Clemmensen et al. 2013) and have been described as a 'keystone' component of functioning ecosystems (Hawkins, Jones & Kranabetter 2015). The assembly of ectomycorrhizal fungal communities following a disturbance may depend on dispersal strategy, host presence and identity, and soil properties (Trowbridge & Jumpponen 2004; Smith & Read 2008; Peay, Kennedy & Bruns 2011; Peay *et al.* 2012). For example, fungal diversity on recently disturbed sites may be dependent on propagule availability (Nara *et al.* 2003; Cázares, Trappe & Jumpponen 2005; Ishida *et al.* 2008; Huang *et al.* 2014). Ectomycorrhizal fungal spores, dispersed via above or belowground fruit bodies, can persist in soil for many years and are consequently vital sources of propagation and

reproduction (Bruns *et al.* 2008; Ishida *et al.* 2008). Some EMF species are also specific to a certain host; species identity of trees comprising a stand may affect EMF community composition if the host tree selects for particular fungal symbionts (Molina & Trappe 1982; Cairney & Chambers 1999; Massicotte *et al.* 1999; Tedersoo *et al.* 2009). Host specificity is therefore important to consider when planting seedlings in a reclaimed site, as a diversity of tree species will likely yield a diverse community of ectomycorrhizal fungi. Reclamation and restoration practices following mining in particular, can also impact the establishment of EMF community on disturbed sites. Under current reclamation techniques, cover soils salvaged from surrounding areas are typically placed on reclaimed sites, and it is now widely accepted cover soils made of forest floor material (FFM) produce more suitable conditions for seedling establishment by retaining a richer propagule bank and providing higher microbial biomass (Mackenzie & Naeth 2010; Sorenson *et al.* 2011; Brown & Naeth 2014). Moreover, Brown & Naeth (2014) found higher mycorrhizal biomass in sites which received FFM than sites which did not receive FFM, suggesting that trees and other mycorrhizal vegetation may therefore have greater establishment success on sites with FFM used as cover soils. Following the application of cover soils, nursery-grown tree seedlings are planted to initiate forest development, a practice which could involve the inoculation of seedlings with ectomycorrhizal fungi, however inoculation may be unnecessary if the cover soils are able to retain viable fungal propagules from the time of salvage to the time of placement (Hankin, Karst & Landhäusser 2015).

The target forest type should also be considered when selecting soils for reclamation. For example, natural upland mesic sites in the boreal plains consist of Gray Luvisol soils supporting mixed *Populus tremuloides –Picea glauca* stands and a rich and diverse understory. Late-stage upland xeric sites consist of Brunisol soils supporting *Pinus banksiana* – dominated stands and a

sparse understory with fewer species (Beckingham & Archibald 1996). In contrast, lowland sites in the boreal plains are dominated by organic peatlands supporting *Picea mariana – Larix laricina* stands. These soils may be salvaged and placed as cover soils in reclaimed sites, and some evidence suggests soils from these forest types may harbor unique EMF communities (Courty *et al.* 2004; Awad 2012; Wubet *et al.* 2012; Goldmann *et al.* 2015). A mismatch between cover soils, their associated fungal community, and the forest type desired on a given reclamation site may not provide the most suitable conditions for that desired forest type.

Direct comparisons between surface mining reclamation sites and historically undisturbed sites may not be ecologically appropriate due to the unprecedented degree (e.g. lack of natural analogues) of disturbance generated by surface mining and the known disparities between recently disturbed and mature ecosystems (e.g. the target community) (Sousa 1984; Dickie *et al.* 2013). Evaluating early successional trajectories created by disturbances that are ecologically comparable will enhance our understanding of reclamation treatments on early development. For example, if plant-soil interactions function similarly after surface mining disturbance to those after harvesting disturbances, management of reclaimed sites could be informed by activities related to harvesting, as knowledge of community assembly of EMF following harvesting is relatively extensive (Hagerman *et al.* 1999; Jones, Durall & Cairney 2003; Rowland *et al.* 2009; Holden & Treseder 2013).

Due to the complex relationship between ectomycorrhizal fungi, host trees, and cover soil, evaluating their independent roles in boreal forest restoration remains difficult (Hankin, Karst & Landhäusser 2015). Consequently, the objectives of this research are 1) to understand how ectomycorrhizal fungal communities establish in reconstructed soils, and 2) how this compares to the development of fungal communities in disturbances where soils remain intact.

We assayed reconstructed soils in a reclaimed site in northern Alberta, Canada using three tree species native to the region which form ectomycorrhizas. The reconstruction of soils in this area is the result oil sands mining followed by reclamation. We focused on how ectomycorrhizal fungal communities establish in three cover soils often used in reclamation: peat, forest floor material and subsoil. Using the same tree species, we also assayed soils that had no history of mining or reclamation (i.e., benchmark sites) but experienced a range of aboveground disturbances including the removal of trees and organic soil horizons. To assay soils, we grew young tree seedlings of three native species (*Populus tremuloides*, *Picea glauca*, and *Pinus banksiana*) for two years across all sites and examined their roots for the occurrence of ectomycorrhizal fungi. We predicted host tree identity to affect EMF community composition and that cover soils harbored unique EMF communities. Furthermore, we predicted seedlings assaying soils of intact forests to be most diverse in their EMF community. We anticipated cover soils made of forest floor material to have an EMF community most similar to the benchmark with a removed canopy but intact forest floor, when compared to other cover soils. Additionally, we predicted the EMF community in subsoil to be most similar to the benchmark with a removed canopy and forest floor, when compared to other cover soils.

2.2 Materials and Methods

2.2.1 Site description

The overall study area is located within the Boreal Mixedwood ecological area (Beckingham and Archibald 1996), where the receding Laurentide ice sheet exposed loamy till and coarse-textured glaciofluvial and lacustrine deposits which developed into a mosaic of forested uplands and wetlands (Johnson & Miyanishi 2008). Uplands are characterized by nutrient-rich Gray Luvisolic soils underlying *Populus tremuloides* Michx. - *Picea glauca* (Moench) Voss stands with understories of *Cornus sericea* L. and *Rosa acicularis* Lindl., whereas nutrient-poor Brunisol soils underlie *Pinus banksiana* Lamb.-dominated stands (Beckingham & Archibald 1996). Soils are characterized by a thin eluvial A horizon, a distinct Bm (Brunisols) or Bt horizon (Luvisols), and a C horizon. Abundant, nutrient-poor peatlands typically consist of *Larix laricina* (Due Roi)- and *Picea mariana* (Mill.)-dominated fens interspersed with *P. mariana*-dominated bogs. Productivity in the boreal mixedwood is influenced by long, harsh winters and short, warm summers (Beckingham & Archibald 1996). Specifically, growing season (June-August) temperatures at the study area in 2013, the year sampling was conducted, ranged from 6.2°C to 33.7°C, with total growing season rainfall of 218.7 mm (data collected by O'Kane Consultants).

To evaluate a variety of reclamation practices, the 36 ha Aurora Soil Capping Study (hereafter 'Reclamation Site') was constructed in 2011 at the Syncrude Canada Ltd.-Aurora mine lease, approximately 75 km north of Fort McMurray, Alberta, Canada (57°19'20"N, 111°30'24"W). During the winter prior to planting (2011/12), surface forest floor material (FFM) and 'subsoil' (i.e., material below the FFM) was salvaged from upland *P. banksiana*-dominated stands, with peat material salvaged from *P. mariana*-dominated lowlands, and all

cover soils were directly placed on the Reclamation Site without stockpiling to preserve soil structure and vegetative and microbial propagules. Specifically, FFM was salvaged to a depth of approximately 15 cm, subsoil (including both B and C horizons) was salvaged to a depth of 100 cm, and peat was harvested to a depth of approximately 200 cm. Soil physical properties and the origin of cover soils are described in detail by Hankin, Karst & Landhäusser (2015). Briefly, peat material had a mean pH of 7.4 (min: 5.0 max: 7.8) and comprised, on average, 34.1% organic matter (min: 0.9 max: 61.7). Forest floor material had a mean pH of 5.6 (min: 4.9 max: 7.1) and mean organic matter content of 2.6% (min: 1.5 max: 5.6). Subsoil material had a mean pH of 7.2 (min: 6.2 max: 7.9) and average organic matter content of 1.0% (min: 0.5, max: 1.6) (North Wind Land Resources Inc. 2013). Underlying the cover soils was lean oil sand overburden.

Each 1 ha cover soil treatment, replicated three times, contained three 25 x 25 m single-species tree plots (Appendix I) planted at a density of 10,000 stems per hectare in May 2012 with 1-year old nursery-grown container stock of *P. tremuloides* (6 cm plug diameter, 15 cm depth), *P. glauca* (6 cm plug diameter, 15 cm depth), or *P. banksiana* (4 cm plug diameter, 12 cm depth) Seedlings from mixed open-pollinated seed collected from several populations near Fort McMurray were grown at Smoky Lake Forest Nursery (Smoky Lake, Alberta). Based on a subsample of 20 seedlings, seedlings had an initial mean seedling height prior to outplanting of 30 cm (±1.9 S.E.), 18 cm (±0.6), and 29 cm (±1.2) for *P. tremuloides, P. banksiana,* and *P. glauca,* respectively.

We compared ectomycorrhizal fungal communities assayed by seedlings planted at the Reclamation Site to those assayed by seedlings planted into a 'Benchmark Site' located approximately 5 km (57° 21' 49.1" N, 111° 25' 45.6" W) from the Reclamation Site (Appendix II). The Benchmark Site was within a mature (~63 years old) *P. banksiana* forest overlying

Eutric Brunisol soils, with an *Arctostaphylos uva-ursi – Vaccinium* understory, and consisted of three treatments ranging in aboveground disturbance intensity over otherwise intact soils: 1) Undisturbed: canopy and forest floor intact; 2) Trees removed: forest floor left intact following a clear-cut harvest approximately 17 years prior; and 3) Trees removed + forest floor removed: following the same clear-cut harvest, sites were bladed to remove the forest floor. Treatments were replicated three times and separated by at least 20 m. Each treatment contained three 2.5 × 2.5 m plots separated by at least 2 m. The Undisturbed treatment had a mean pH of 5.5 (min: 4.9 max: 6.0), the 'trees removed' treatment had a mean pH of 5.7 (min: 4.6 max: 6.4), and the 'trees + forest floor removed' treatment had a mean pH of 5.9 (min: 5.5 max 6.3). In May 2012 at the same time as planting of the Reclamation Site, each plot was planted with 6-8 seedlings each of *P. tremuloides, P. banksiana,* and *P. glauca*, resulting in 18-24 total seedlings per plot, using the same seedling stock planted at the Reclamation Site and planted approximately 70 cm apart in rows.

2.2.2 Ectomycorrhiza sample collection

Roots of seedlings at the Reclamation Site were harvested in August 2013, two growing seasons after planting. Five trees per 25 × 25 m single-species tree plot were randomly selected, a lateral root identified, and approximately 300 fine roots were collected from roots of approximately 20 cm in length. Fine roots were placed in a sealed bag containing moist paper towels, kept on ice for approximately 48 hours and shipped cold to the University of Alberta where they were stored at -20 °C until further examination. Three seedlings per species were harvested from each plot in the Benchmark Site during the same week (total number of seedlings = 379). Roots from Benchmark Site seedlings were separated from shoots and placed in sealed

bags with moist paper towels, stored on ice for 48 hours and shipped cold to the University of Alberta where they were stored at -20 °C until further examination.

Harvested roots were thawed, gently washed with tap water over a 1.2 mm sieve to remove soil and debris, and cut into 1-2 cm fragments. Root fragments were mixed thoroughly in a container filled with deionized water using forceps for approximately 30 seconds. Sterilized forceps were used to randomly select root tips, which were placed on a petri dish with deionized water and examined under a dissecting microscope at 100× magnification. Root tips were identified as mycorrhizal by appearance of hyphae, mantle structure, color, and texture (Goodman *et al.* 1996). At least five root tips per seedling were collected for immediate DNA extraction, and subsequently stored at -20 °C until downstream polymerase chain reactions (PCR) amplification.

2.2.3 Molecular identification

Two root tips per tree, per plot for each Reclamation Site treatment (peat, FFM, subsoil) and Benchmark Site treatment (undisturbed, trees removed, trees + forest floor removed) replicate were selected for molecular identification (total number of seedlings = 108). DNA of colonized root tips selected for molecular analysis was extracted using Sigma Extraction Buffer and Neutralization Solution B according to the manufacturer's protocol (Sigma, Gillingham, Dorset, UK). Extractions were amplified via PCR using the fungal-specific combination ITS1-F (5'-cttggtcatttagaggaagtaa-3') and ITS4 (5'- tcctccgcttattgatatgc -3') forward and reverse primers, respectively (Innis *et al.* 1990; Gardes & Bruns, T 1993). Following extraction, 1.0 µL DNA extract was added to a solution of 5.4 µL MilliQ H_2O, 8.0 µL RedTaq (Sigma-Aldrich, St. Louis), 0.8 µL of 10 µM/L ITS1-F and 0.8 µL of 10 µM/L ITS4. PCR was conducted with an

initial denaturation at 95 °C for 5 minutes followed by 40 cycles of (95°C for 1.5 minutes, 57°C for 1 minute, 72°C for 1.5 minute) and a final extension at 72°C for 10 minutes.

The ITS1-F/ITS4 fungal primer pair has the potential to amplify the internal transcribed spacer (ITS) region of all ascomycetous, basidiomycetous and zygomycetous fungi, therefore it was necessary to use primers with greater specificity when PCR products were consistently yielding amplifications of multiple species. For these cases, extractions were amplified using a nested PCR with the fungal specific primer combinations NSA3/NLC2 (5'-aaactctgtcgtgctggggata-3'/5'- gagctgcattcccaaacaactc-3') and NSI1/NLB4 (5'-gattgaatggcttagtgagg-3'/5'- ggattctcaccctctatgac-3') (Martin & Rygiewicz 2005). Template was first amplified using the outer primers (NSA3/NLC2), followed by amplification with the inner primers (NSI1/NLB4). The outer primer pair PCR solution contained 1.0 µL DNA extract, 5.4 µL MilliQ H_2O, 8.0 µL RedTaq (Sigma-Aldrich, St. Louis), 0.8 µL of 10 µM/L NSA3 and 0.8 µL of 10 µM/L NLC2. The inner primer pair PCR solution consisted of 1 µL product from the outer primer pair PCR product, 5.4 µL MilliQ H_2O, 8.0 µL RedTaq (Sigma-Aldrich, St. Louis), 0.8 µL of 10 µM/L NSI1 and 0.8 µL of 10 µM/L NLB4. The outer primer pair PCR solution was run with an initial denaturation at 95°C for 5 minutes followed by 30 cycles of (95°C for 1.5 minutes, 67°C for 1 minutes, 72°C for 1.5 minutes) and a final extension at 72°C for 10 minutes. Following the outer primer amplification, the inner primer amplification was run at an initial denaturation of 95°C for 5 minutes followed by 27 cycles of (95°C for 1.5 minutes, 55°C for 1 minutes, 72°C for 1.5 minutes) and a final extension at 72°C for 10 minutes.

Confirmation of successful PCR reaction product was visualized using gel electrophoresis. Gels of 1.7% agar were run at 100 volts for 60 minutes. If gels failed to produce clear fragment bands, voltage was decreased and run time increased. A QIAquick Gel Extraction

Kit (Qiagen, Valencia, California) was used to extract bands from a subsample of PCR products which produced multiple gel bands. Once confirmed by gel electrophoresis, PCR product was purified by adding 5 µL ExoSAP-IT (USB, Cleveland, Ohio, USA) to 2 µL PCR product. Solutions were incubated at 37 °C for 30 minutes, then denatured at 80 °C for 20 minutes. Following purification, a bi-directional sequencing reaction was performed with BIGDYE v3.1 (Applied Biosystems, Foster City, California, USA) using the ITS1-F/ITS4 or NSI1/NLB4 primer pair, matching the corresponding primers used for PCR, and subsequently precipitated with EDTA and ethanol. Sequences were read by an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, California, USA).

Raw sequences were edited with Geneious software (BioMatters, Auckland, New Zealand). Specifically, ends were trimmed with an error probability limit of 3% and complementary sequences were then assembled using DeNovo assembly to create a consensus sequence. Following assembly, phred scores below 20 were changed to 'N.' Single direction reads were subjected to the same criteria. The resulting sequences (consensus and single) were clustered into operational taxonomic units using the CAP3 plugin with the following settings: ≥ 97% identity; overlap percentage identity cutoff = 97; maximum overhang percentage length = 60; match score factor = 5; clipping range = 6. Operational taxonomic units (OTUs) were run through the GenBank database (National Center for Biotechnology Information, Bethesda, Maryland) using BLASTn to identify the best match. Identity was assigned to an OTU if percent identity was ≥ 97 and query coverage was ≥ 80%.

2.2.4 Statistical Analyses

Within the Reclamation Site, the effect of cover soil, host identity, and their interaction on estimated EMF richness was tested using a two-way ANOVA. Due to uneven samples sizes, raw OTU abundance values were rarefied using EsimateS software (Colwell 2013), based on Colwell et al. (2012) to produce estimated values of richness for each treatment. The effect of cover soil, host identity, and their interaction on EMF community composition was examined with a permutational multivariate analysis (perMANOVA) of variance using the Adonis function from the vegan package, and visualized with a nonmetric multidimensional scaling (NMDS) ordination using the metaMDS function from the vegan package in R (R version 3.2.2, R Core Development Team, 2015). Significant main effects ($p < 0.05$) were followed by Tukey tests to examine differences among treatments, while interactions were examined with pairwise comparisons using the Holm adjustment. Similar analyses were used to assess EMF richness and composition at the Benchmark Site; however the effect of cover soil was replaced with aboveground disturbance type in the analysis. Indicator species were found using the multipatt function from the indicspecies package in R by testing the Reclamation Site and Benchmark Site separately.

2.3 Results

Across 331 seedlings, DNA was amplified with a success rate of 78%. Of those samples amplified from 273 seedlings, 27% contained double-bands, i.e., multiple fungi were amplified from a single root tip. A total of 825 root tips, including 12 producing double bands, were sequenced; quality filtering yielded 345 sequences which were clustered into 33 operational taxonomic units, 27 of which met quality criteria for taxon identity (OTUs, Table 2-1). Of the 331 initially sampled seedlings, 149 seedlings yielded successful amplifications producing quality sequences of root-associated fungi.

The most abundant OTUs matched an uncultured *Hebeloma* clone, a *Thelephora terrestris* isolate, and an uncultured *Amphinema* clone, comprising approximately 14, 10, and 10 percent of sequences, respectively (Figs 2-1, 2-2). The *Hebeloma* and *Amphinema* OTUs occurred in all cover soils at the Reclamation Site and in all intact soils assayed at the Benchmark Site. *Thelephora terrestris* was in all Reclamation Site and Benchmark Site treatments except peat. Successfully amplified bands from samples containing multiple bands were most commonly matched to a *Hebeloma velutipes* clone or, to a lesser extent, an uncultured *Amphinema* clone. Twenty OTUs were found in the Reclamation Site and 20 in the Benchmark Site (Table 2-1).

Within the Reclamation Site, there was no effect of cover soil or host species on estimated OTU richness (Table 2-2); however ectomycorrhizal community composition was affected by host ($p < 0.01$, Table 2-3), cover soil ($p < 0.05$, Table 2-3) and marginally affected by the interaction of host × cover soil ($p = 0.057$, Table 2-3). Specifically, each host species assayed a different EMF community in subsoil; however there was no effect of host species in peat or FFM. Furthermore, the EMF community assayed by *P. tremuloides* grown in FFM differed than

that assayed in subsoil, and EMF community composition assayed by pine grown in FFM was different from pine grown in peat. The effect of cover soil was explained by the difference of EMF communities assayed in FFM from peat ($p = 0.07$) and subsoil ($p = 0.07$). The effect of host species identity was explained by the difference of EMF communities assayed by *P. tremuloides* from *P. banksiana* ($p = 0.01$). The EMF community assayed by *P. tremuloides* was marginally different from that assayed by *P. glauca* ($p = 0.08$), but the EMF communities assayed on *P. banksiana* and *P. glauca* were not different.

Within the Benchmark Site, there was no effect of disturbance or host species on estimated OTU richness (Table 2-4). The EMF community composition within the Benchmark was affected by host ($p < 0.001$, Table 2-5), but there was no effect of disturbance or host × disturbance interaction. *Populus tremuloides, P. banksiana,* and *P. glauca* each assayed different EMF communities.

Nonmetric multidimensional scaling ordinations illustrate the effect of host species on EMF community composition in the Reclamation Site and Benchmark Site (Fig. 2-3), along with the effect of Reclamation Site cover soil on EMF community composition (Figure 2-3a). In the Reclamation Site, host identity affected the abundance of *Tomentella* 1 and Pezizales 2, while the abundances of *Thelephora terrestris* and Pyronemataceae 1 were driven by the effect of cover soil (Fig. 2-3a). In the Benchmark Site, the direction and magnitude of arrows depicted in Fig. 2-3b indicate the effect of host identity on the abundance of *Hebeloma* 1, *Amphinema* 1, *Rhizopogon pseudoroseolus*, *Suillus variegatus*, and *Suillus brevipes*. Using a species indicator analysis we did not detect a correlation between any ectomycorrhizal OTUs and the Benchmark Site disturbance treatments, highlighting that EMF community composition was unaffected by disturbance type in the Benchmark Site.

2.4 Discussion

We assayed ectomycorrhizal fungal communities in reconstructed soils of reclaimed areas mined for oil sands and in intact soils capturing a gradient of aboveground disturbance. This is a continuation of a research project initialized and described in Hankin, Karst & Landhäusser (2015). Building on those findings we found: (1) after two growing seasons there were more ectomycorrhizal OTUs; however the ectomycorrhizal fungal community composition continued to be primarily influenced by the tree species used to assay the soils, and (2) outplanted seedlings interact with similar ectomycorrhizal fungal communities regardless of above or belowground disturbance severity.

2.4.1 Ectomycorrhizal fungal community: Reclamation Site

Across all Reclamation Site cover soil treatments, we found a total of twenty EMF operational taxonomic units (OTUs), compared with five found one year prior, although there was no effect of host species, cover soil type, nor an interaction between the two on estimated EMF species richness, consistent with the previous year (Hankin *et al.* 2015). The increased richness in the second year compared to the first is consistent with prior work on EMF succession (Kipfer *et al.* 2011; Dickie *et al.* 2013). For example, research on glacier forefronts on sites with coarse mineral substrates and limited organic content also showed increases in EMF richness with time since glaciation, suggesting the influx of propagules from wind dispersion and animals (Jumpponen, Trappe & Cázares 2002; Blaalid *et al.* 2012). Ectomycorrhizal fungi present in recently disturbed areas have been described as ‘early’ stage fungi, that are joined or replaced by ‘mid’ and ‘late’ stage fungi over time (Peay et al 2011, Dickie et al 2013.).

Consequently, as species richness increases with the arrival of mid-stage fungi, EMF community composition likely changes (Dickie et al 2013).

In the first year after planting, host was the single influence on EMF community composition three months after seedlings had been planted (Hankin, Karst & Landhäusser 2015). Two years later ectomycorrhizal fungal community composition continued to be affected by host identity; however, there appears to be a transformation occuring where, depending on the host species, community types were affected by cover soil (i.e., a cover soil × host interaction). This is also supported by the nonmetric multidimensional scaling ordination, which shows considerable overlap in the EMF communities assayed by seedlings in different cover soils. Our findings are consistent with research on long-term succession of both EM and arbuscular mycorrhizal fungi, which have shown host species as a stronger predictor of mycorrhizal community composition than soil properties during early ecosystem development (Cázares *et al.* 2005; Hankin *et al.* 2015; Martinez-Garcia *et al.* 2015).

Though the host × cover soil interaction was only marginally significant with an alpha of 0.05, we found several interesting trends suggesting that differences in Reclamation Site cover soil may only affect the EMF community assayed by certain host species. For example, subsoil was the only cover soil with distinct EMF communities assayed by each host species. Additionally, *Populus tremuloides* grown in FFM assayed an EMF community distinct from *P. tremuloides* grown in subsoil, indicating that the addition of FFM may have provided EMF not present in the subsoil. Moreover, *Pinus banksiana* grown in FFM assayed a different EMF community from those grown in peat, again indicating that FFM may have provided distinct viable propagules of fungal species not present in the other cover soils. These trends may

become more pronounced with time as seedling root systems develop and interact with other propagules in the soil.

The dispersal and colonization strategies of different EMF species likely played a role in structuring the fungal community at the Reclamation Site. Mycelial networks are limited in reconstructed soils owing to the high degree of disturbance, a condition which favors colonization through spore dispersal (Ishida *et al.* 2008; Peay *et al.* 2011; Peay & Bruns 2014). Species with high spore dispersal such as *Thelephora terrestris*, *Wilcoxina mikolae,* and *Cenococcum geophilum* may therefore have higher abundances in reconstructed soils, a trend confirmed by Hankin, Karst & Landhäusser (2015). Higher spore reactivity, or the rate at which spores germinate, provides an additional advantage when colonizing disturbed sites (Peay *et al.* 2011). For example, *Rhizopogon* species are found to have more reactive spores than *Suillus* species, and therefore are able to outcompete *Suillus* species in recently disturbed sites (Peay *et al.* 2011). This supports the findings of Hankin, Karst & Landhäusser (2015), where *Rhizopogon* species were far more abundant than *Suillus* species after one growing season at the Reclamation Site. *Suillus* species disperse via spores from aboveground sporocarps, a carbon expense that ectomycorrhizal seedlings may not have been able to support with only one growing season (Dickie *et al.* 2013). Moreover, *Rhizopogon* species have belowground sporocarps which may have persisted in the soil during the reclamation process when materials used for cover soils were moved from donor to recipient sites. After two growing seasons, we found a higher abundance of *Suillus* compared to *Rhizopogon* species, suggesting that once established, *Suillus* may be able to outcompete *Rhizopogon* species. Additionally, we found *T. terrestris* to be an indicator for FFM, however this particular species is ubiquitous; it is commonly found in nurseries, disturbed, and intact forests across many stages of succession (Visser 1995; Cairney &

Chambers 1999). Its status as an indicator species for FFM is perhaps a reflection of high spore retention in the coarse debris and microsites provided by the addition of FFM.

We found seven EMF taxa occurring only in the Reclamation Site, five of which belong to the family Pezizales or Helotiales and were primarily assayed by seedlings planted in subsoil and FFM. This is consistent with previous work, which has shown these families to be common in disturbed sites characterized by soils with high pH and low organic matter (Hansen & Pfister 2006). Subsoil and FFM cover soils both have very low organic matter content when compared to peat. Moreover, these taxa can form other types of root mutualisms, such as 'dark septate endophytes', which previous work has found to be dominant over ectomycorrhizal relationships in soils with limited organic matter, such as those exposed by recently retreating glaciers (Jumpponen *et al.* 2002; Cázares *et al.* 2005).

2.4.2 Ectomycorrhizal fungal community: Benchmark Site

Contrary to our prediction, we found no effect of host species, extent of aboveground disturbance, or their interaction on EMF richness in soils assayed by seedlings of the same species and age used at the Reclamation Site. This finding is consistent with previous research which found *Pseudotsuga mensiezii* seedlings in clear-cut, forest floor removed, and undisturbed stands of interior *P. mensiezii* in British Columbia, Canada to have similar EMF richness (Barker *et al.* 2012). Moreover, Hagerman *et al*. (1999) found no difference in EMF diversity with increasing distance into clear-cuts from a forest edge one growing season after tree removal in mature *Picea engelmannii – Abies laciocarpa* stands in British Columbia, Canada.

We were surprised to see no effect of aboveground disturbance on EMF community composition due to the wealth of information documenting a shift in composition following

aboveground disturbances (Visser, Maynard & Danielson 1998; Hagerman *et al.* 1999; Dickie, Koide & Steiner 2002; Rosling *et al.* 2003; Cázares *et al.* 2005; Barker *et al.* 2012). The forest floor harbors many EMF species, and its removal can drastically alter the EMF community (Dickie, Koide & Steiner 2002; Rosling *et al.* 2003). In our study, however, there was no effect of forest floor removal on the assayed EMF community. The EMF communities we found in intact soils of 'trees removed' and 'trees + forest floor removed' treatments is, however, consistent with work which has shown a relatively small number of ubiquitous, pioneer species to dominate recently disturbed sites (Ishida *et al.* 2008; Blaalid *et al.* 2012), though we also found these species in the undisturbed site. For example, *Amphinema byssoides* and *Thelephora terrestris* are noted as ubiquitous fungal species found commonly in nurseries and disturbed sites (Cairney & Chambers 1999; Smith *et al.* 2011), but we found these particular species across all Reclamation Site and Benchmark Site treatments, including undisturbed. This pattern may be a reflection of dispersal and foraging strategy of EMF, as Peay *et al* (2011) proposed that EMF community composition in primary succession is determined by spore abundance and fitness. Fungi with high "reactivity," or ability to establish quickly, will dominate a young site. As the site matures, fungal community composition shifts from species with a strategy of spore establishment to species which expand through mycelial growth. When the distance between roots declines, reproduction via mycelium may become more advantageous (Peay *et al.* 2011). Consequently, pioneer-type fungi may be joined by late-stage fungi as the forest matures, litter input changes soil nutrient composition, and root systems become more complex (Peay *et al.* 2011; Dickie *et al.* 2013).

Previous work has shown large differences in EMF community composition between recently disturbed sites and sites with an intact canopy (Hagerman *et al.* 1999; Jones *et al.* 2003;

Cázares, Trappe & Jumpponen 2005; Lazaruk *et al.* 2008; Barker *et al.* 2012; Dickie *et al.* 2013; LeDuc *et al.* 2013). When a site develops an established canopy, the subsequent input of organic matter from plant and fungal necromass increases and may consequently alter EMF community composition (Ekblad *et al.* 2013; LeDuc *et al.* 2013; Sterkenburg *et al.* 2015). Research on glacier forefronts has found that pioneer-type fungal species are able to establish on nutrient-poor soils of the most recently exposed sites along with non-mycorrhizal plant species (Cázares *et al.* 2005). These early colonizers improve the soil conditions by contributing nutrients via exudates and necromass, enabling tree seedling germination and establishment. Furthermore, EMF propagule input from surrounding sources increases the viable spore bank over time, additionally improving seedling establishment (Cázares *et al.* 2005; Ishida *et al.* 2008; Peay & Bruns 2014). We therefore predicted the undisturbed Benchmark Site treatment to harbor a distinct EMF community when compared to the other Benchmark Site treatments (trees removed, trees + forest floor removed) and Reclamation Site cover soil treatments (peat, forest floor material, subsoil), but found no such effect.

Our results, which show no effect of aboveground disturbance on the EMF community of outplanted seedlings, are perhaps due to the harvesting and processing of entire root systems from tree seedlings grown in the Benchmark Site treatments, while lateral roots were collected from seedlings in the Reclamation Site cover soil treatments. This may have allowed a large number of EMF from within the root plug, and consequently legacies of the nursery, to influence the observed community composition of seedlings grown in the Benchmark Site treatments. For example, Hankin, Karst & Landhäusser (2015) found several EMF taxa present on seedling roots both before planting and three months after planting. Alternatively, it may be possible that 1-year old seedlings of the same species planted in Brunisol soil may simply select for similar EMF

communities, regardless of other late-stage propagules which may be present, such as those in undisturbed forests. For example, Walker & Jones (2013) found no difference in EMF community composition on young *P. engelmannii* × *Picea glauca* seedlings outplanted into mineral soil with or without coarse woody debris, further suggesting that young outplanted seedlings recover similar EMF communities regardless of microsite conditions. On the other hand, the lack of differences in EMF community composition in our study may simply be due to inadequate sampling depth.

2.4.3 Conclusion

This study was conducted two growing seasons after seedlings were planted, and our results suggest that all cover soil treatments remain in the early stages of community development, with ruderal taxa dominating the EMF community. Based on our findings, all cover soils and disturbance types have similar EMF communities when assayed with planted seedlings, with interesting trends demonstrating a marginal interaction of host identity and cover soil. Contrary to our predictions, the extent of above and belowground disturbance had no effect on the EMF community colonizing planted seedlings, an unexpected result which may have important implications for future reclamation sites. Moreover, seedlings planted in mature, intact forests yield similar EMF communities after two growing seasons as seedlings planted in clear-cut sites. Ectomycorrhizal fungal community composition in the Benchmark Site and Reclamation Site was primarily influenced by host identity, but within the Reclamation Site only the subsoil - a sandy, coarse-textured soil salvaged from a *Pinus banksiana* stand - yielded distinct differences in EMF communities on each host species. This might suggest that seedlings of the same species outplanted in Brunisol soils are unaffected by the presence of mid- or late-

stage fungi; they will harbor the same EMF community despite aboveground disturbances, a pattern which should be further investigated.

Tables

Table 2-1. Operational taxonomic units with BLAST and UNITE species hypothesis (SH) matches, assembled from quality filtered sequences from amplified fungal rDNA. Reconstructed (Rec: Reclamation Site) and intact soils, differing in aboveground disturbance (Ben: Benchmark Site), of northern Alberta, Canada were assayed for ectomycorrhizal fungi with seedlings of *Picea glauca*, *Pinus banksiana*, and *Populus tremuloides* grown in 2012-2013.

Best match	BLAST ID	Query length	Max score	Query cover	Percent identity	UNITE SH	UNITE Accession	Rec	Ben
***Tuber pacificum* isolate**	JQ712002	636	1166	100	99	*Tuber pacificum*	SH202491.07FU		X
Uncultured *Cadophora* clone	GU289410	897	1548	98	98	Uncultured *Cadophora*	SH214265.07FU	X	
Uncultured Helotiales clone	FJ553656	886	1509	98	98	Uncultured Helotiales	SH215265.07FU	X	
Cadophora luteo-olivacea	HM116747	654	1103	99	99	*Cadophora luteo olivacea*	SH186775.07FU	X	
Uncultured Pyronemataceae clone 1	EU726302	812	1168	85	97	Uncultured Pyronemataceae	SH222143.07FU	X	
Uncultured *Wilcoxina* clone	HM146894	620	1101	99	98	Uncultured *Wilcoxina*	SH211927.07FU	X	X
***Thelephora terrestris* isolate**	JQ711777	881	1581	98	99	*Thelephora terrestris*	SH184510.07FU	X	X
***Tomentella ellisii* clone**	DQ06897	692	1051	89	97	*Tomentella ellisii*	SH189381.07FU	X	X
***Rhizopogon pseudoroseolus* voucher**	GQ267483	896	1391	85	99	*Rhizopogon pseudoroseolus*	SH221091.07FU	X	X
***Suillus brevipes* voucher**	FJ845440	887	1618	100	99	*Suillus brevipes*	SH176743.07FU	X	X
***Hebeloma velutipes* ectomycorrhiza**	AF430254	728	1290	98	99	*Hebeloma velutipes*	SH215995.07FU		X
Uncultured *Amphinema* clone	HM044498	859	1304	82	99	Uncultured *Amphinema*	SH197944.07FU	X	X
***Cortinarius erythrinus* voucher**	AY669690	578	972	97	97	*Cortinarius erythrinus*	SH188477.07FU		X
***Russula declorans* voucher**	FJ845432	908	1644	100	99	*Russula declorans*	SH219855.07FU		X
***Russula laccata* isolate**	HQ604844	711	1290	100	99	*Russula laccata*	SH218421.07FU	X	
Tomentella sp.	U83482	693	1236	98	99	*Tomentella* sp LT56	SH177816.07FU		X
Uncultured *Hebeloma* clone	JX135070	920	1511	94	97	Uncultured *Hebeloma*	SH215994.07FU	X	X
***Tomentellopsis sp*. voucher**	HM190009	683	1107	90	99	*Tomentellopsis* sp BB 2010	SH184845.07FU	X	X
Suillus variegatus	JQ711926	721	1282	99	99	*Suillus variegatus*	SH176741.07FU		X
***Piloderma olivaceum* isolate**	JQ711806	629	1127	100	99	*Piloderma olivaceum*	SH203891.07FU		X
Uncultured Pezizales clone	HM146844	655	1024	85	99	Uncultured Pezizales	SH010197.07FU	X	
Uncultured Pezizales clone	JN704819	757	1133	81	99	Uncultured Pezizales	SH212010.07FU	X	X
***Wilcoxina mikolae* voucer**	GQ267499	631	1138	99	99	*Wilcoxina mikolae*	SH194156.07FU	X	X
Uncultured *Wilcoxina* isolate	EU668262	636	1140	98	99	Uncultured *Wilcoxina*	SH194158.07FU	X	
***Cenococcum geophilum* isolate**	JQ711896	557	1022	99	99	*Cenococcum geophilum*	SH199612.07FU	X	X
***Phialocephala fortinii* isolate**	JQ711853	822	1482	99	99	*Phialocephala fortinii*	SH204986.07FU	X	X
Uncultured ectomycorrhiza (Helotiaceae) isolate	EF218791	748	1066	82	97	Uncultured ectomycorrhizal fungus	SH181125.07FU	X	X

Table 2-2. ANOVA of the effect of cover soil and host species on estimated operational taxonomic unit richness of assayed ectomycorrhizal fungi. Reconstructed soils of a reclaimed site in northern Alberta, Canada were assayed for fungi with seedlings of *Picea glauca*, *Pinus banksiana*, and *Populus tremuloides* grown in 2012-2013.

Source	**df**	**Sum. Sq**	**Mean Sq**	***F*-value**	***P*-value**
Cover soil	2	0.180	0.090	1.771	0.249
Residuals	6	0.305	0.051		
Host species	2	2.890	1.445	2.598	0.168
Residuals	5	2.781	0.556		

df: degrees of freedom, Sum Sq: sum of squares, Mean Sq: Mean Square

Table 2-3. Permutational multivariate analysis of variance of host tree species, cover soil, and the host tree species × cover soil interaction on ectomycorrhizal community composition (n = 3). Reconstructed soils of a reclaimed site in northern Alberta, Canada were assayed by seedlings of *Picea glauca*, *Pinus banksiana*, and *Populus tremuloides* grown in 2012-2013.

	df	**Sum Sq.**	**Mean Sq.**	**F. Model**	R^2	***P* value**
Host	2	1.426	0.713	2.060	0.132	0.003
Cover soil	2	1.185	0.592	1.711	0.110	0.027
Host x Cover soil	4	1.937	0.484	1.399	0.180	0.057
Residuals	18	6.353	0.353		0.591	

df: degrees of freedom, Sum Sq: Sum of Squares, Mean Sq: Mean Square

Table 2-4. Analysis of variance of the effect of disturbance and host species on estimated operational taxonomic unit richness of assayed ectomycorrhizal fungi. Intact soils differing in extent of aboveground disturbance in northern Alberta, Canada were assayed by seedlings of *Picea glauca*, *Pinus banksiana*, and *Populus tremuloides* grown in 2012-2013.

Source	**df**	**Sum. Sq**	**Mean Sq**	***F*-value**	***P*-value**
Disturbance	2	0.014	0.007	0.372	0.704
Residuals	6	0.113	0.019		
Host species	2	1.165	0.583	1.478	0.301
Residuals	6	2.365	0.394		

df: degrees of freedom, Sum Sq: Sum of Squares, Mean Sq: Mean Square

Table 2-5. Permutational multivariate analysis of variance using distance matrices (Adonis) of host tree species, disturbance, and the host tree species × disturbance interaction on ectomycorrhizal community composition (n = 3). Intact soils differing in extent of aboveground disturbance in northern Alberta, Canada were assayed by seedlings of *Picea glauca*, *Pinus banksiana*, and *Populus tremuloides* grown in 2012-2013.

	df	**Sum Sq.**	**Mean Sq.**	**F. Model**	R^2	***P* value**
Host	2	2.576	1.288	3.801	0.241	0.001
Disturbance	2	0.561	0.280	0.827	0.052	0.697
Host x Disturbance	4	1.456	0.364	1.074	0.136	0.358
Residuals	18	6.100	0.339		0.570	

df: degrees of freedom, Sum Sq: Sum of Squares, Mean Sq: Mean Square

Table 2-6. Indicator species analysis using operational taxonomic units of ectomycorrhizal fungi ($\alpha = 0.05$). Ectomycorrhizal roots were collected in 2013 from seedlings of *Picea glauca*, *Pinus banksiana*, and *Populus tremuloides* grown in three cover soils (peat, forest floor material (FFM), subsoil) used to reconstruct soils in a reclaimed site and intact soils differing in extent of aboveground disturbance (undisturbed, trees removed, trees + forest floor removed) in northern Alberta, Canada. This analysis reveals *Thelephora terrestris* as an indicator of forest floor material, and Pyronemataceae 1 as an indicator of subsoil material.

Cover Soil	**OTU**	**Stat**	***P* value**
FFM	*Thelephora terrestris*	0.756	0.014
Subsoil	Pyronemataceae 1	0.720	0.022

Figures

Figure 2-1. Relative abundance of operational taxonomic units of ectomycorrhizal fungi colonizing roots of tree seedlings used to assay three reconstructed soils using peat, forest floor material, or subsoil as cover soils, and intact soils differing in aboveground disturbance (undisturbed, trees removed, trees removed + forest floor removed) (n = 3) in northern Alberta, Canada. Only taxa which occurred in more than one plot are included. Seedlings of *Picea glauca*, *Pinus banksiana*, and *Populus tremuloides* grown from 2012-2013 were used to assay soils. Abbreviations: TFFR: trees and forest floor removed; TR: trees removed; Und: undisturbed.

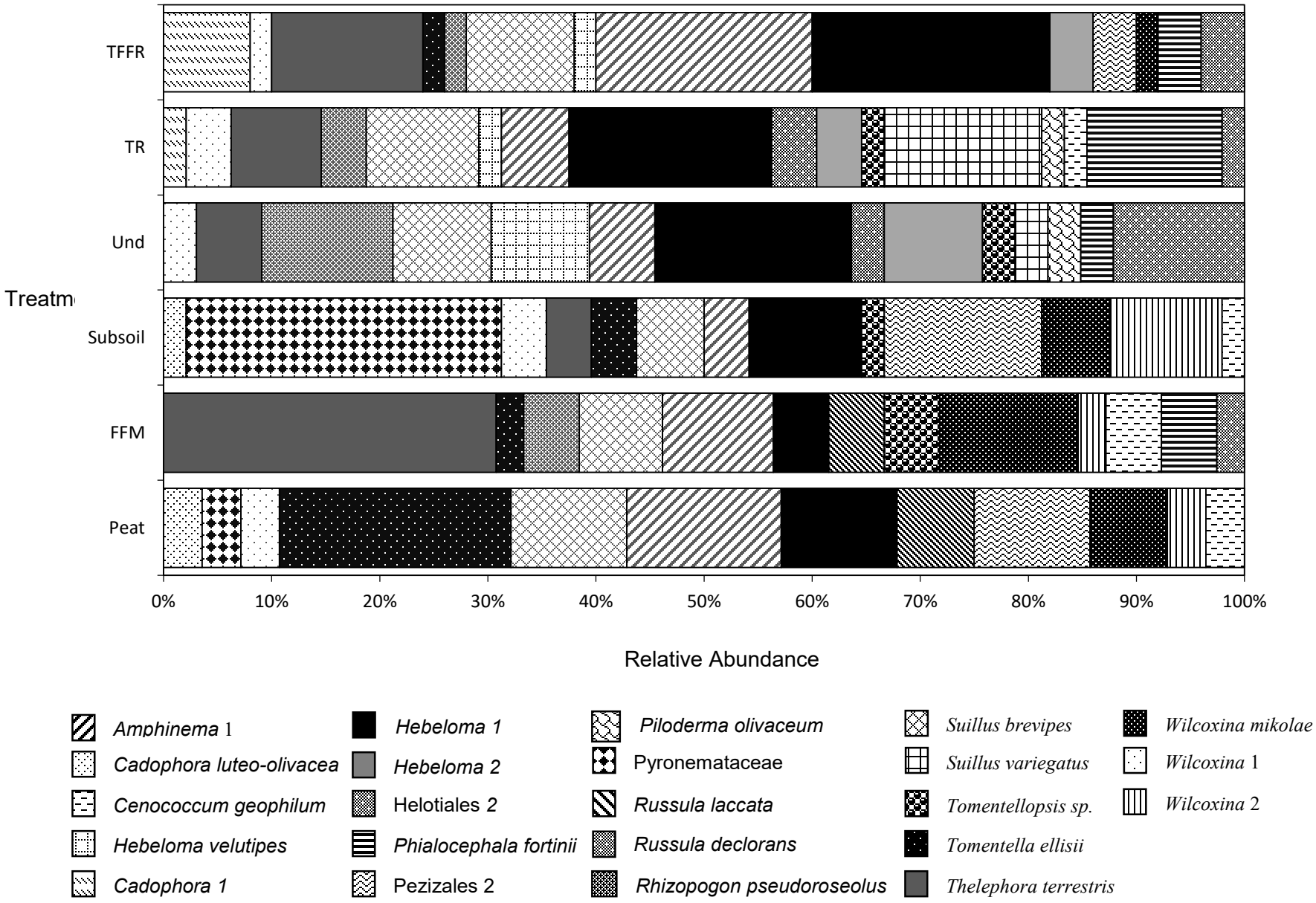
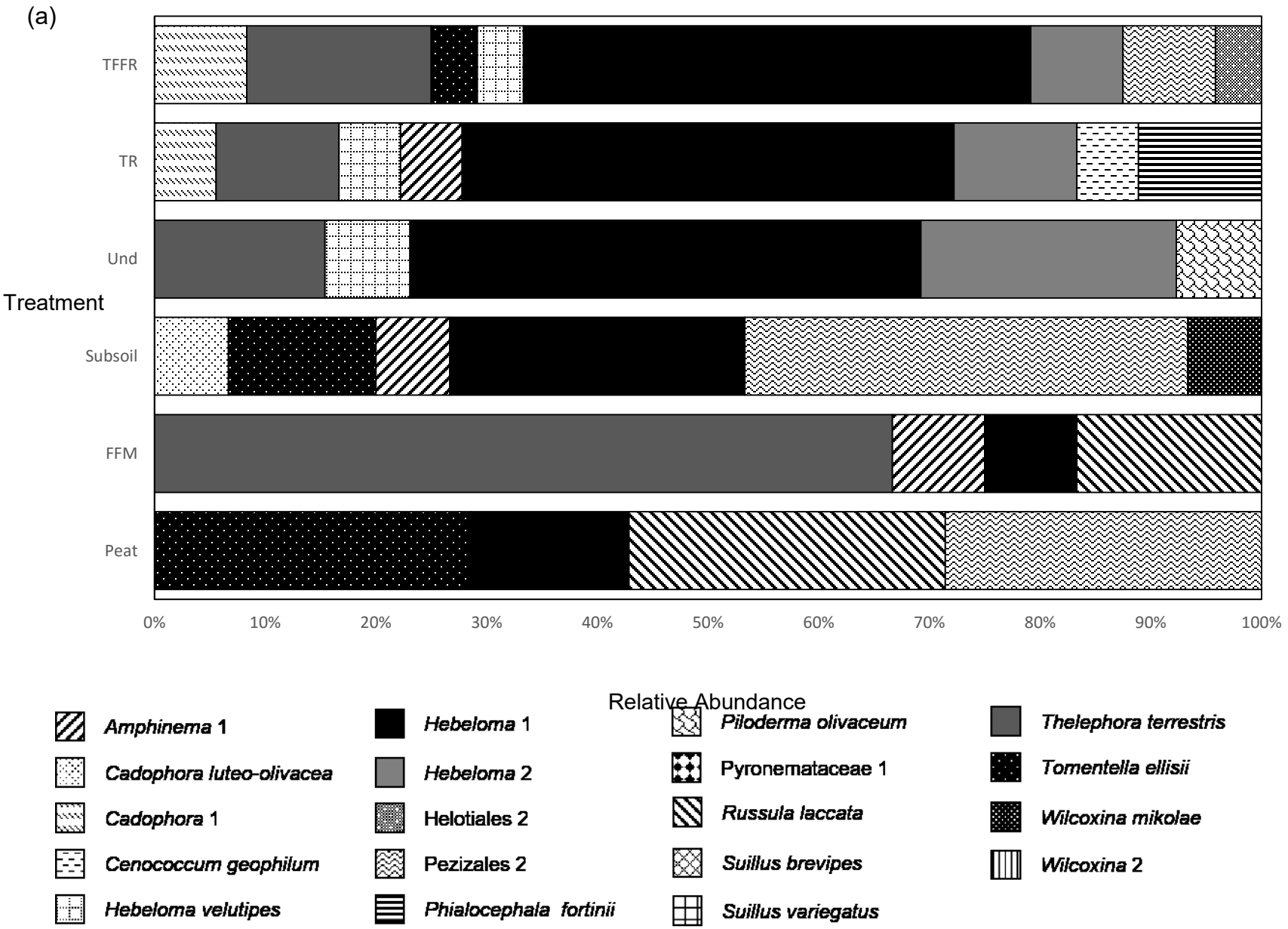
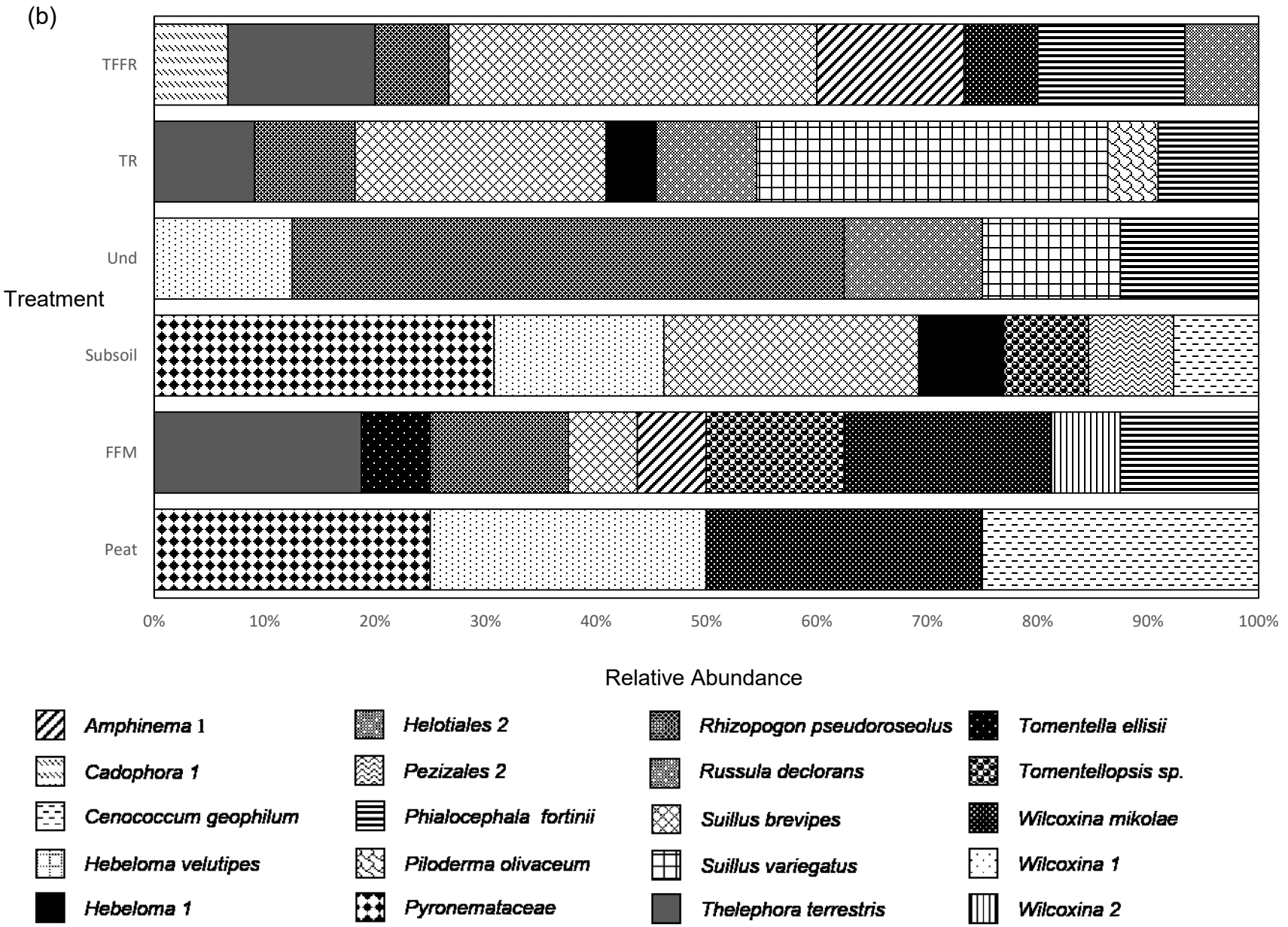

TFFR
TR
Und
Treatm
Subsoil
FFM
Peat
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Relative Abundance
Amphinema 1
Cadophora luteo-olivacea
Cenococcum geophilum
Hebeloma velutipes
Cadophora 1
Hebeloma 1
Hebeloma 2
Helotiales 2
Phialocephala fortinii
Pezizales 2
Piloderma olivaceum
Pyronemataceae
Russula laccata
Russula declorans
Rhizopogon pseudoroseolus
Suillus brevipes
Suillus variegatus
Tomentellopsis sp.
Tomentella ellisii
Thelephora terrestris
Wilcoxina mikolae
Wilcoxina 1
Wilcoxina 2

Figure 2-2. Relative abundance of operational taxonomic units of ectomycorrhizal fungi colonizing roots of (a) *Populus tremuloidces,* (b) *Pinus banksiana*, and (c) *Picea glauca* seedlings used to assay three reconstructed soils using peat, forest floor material, or subsoil as cover soils, and intact soils differing in aboveground disturbance (undisturbed, trees removed, trees removed + forest floor removed) (n = 3) in northern Alberta, Canada. Only taxa which occurred in more than one plot are included. Seedlings of *Picea glauca*, *Pinus banksiana*, and *Populus tremuloides* grown from 2012-2013 were used to assay soils. Abbreviations: TFFR: trees and forest floor removed; TR: trees removed; Und: undisturbed.


(a)
TFFR
TR
Und
Subsoil
FFM
Peat
Treatment
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Relative Abundance
Amphinema 1
Cadophora luteo-olivacea
Cadophora 1
Cenococcum geophilum
Hebeloma velutipes
Hebeloma 1
Hebeloma 2
Helotiales 2
Pezizales 2
Phialocephala fortinii
Piloderma olivaceum
Pyronemataceae 1
Russula laccata
Suillus brevipes
Suillus variegatus
Thelephora terrestris
Tomentella ellisii
Wilcoxina mikolae
Wilcoxina 2


(b)
TFFR
TR
Und
Treatment
Subsoil
FFM
Peat
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Relative Abundance
Amphinema 1
Cadophora 1
Cenococcum geophilum
Hebeloma velutipes
Hebeloma 1
Helotiales 2
Pezizales 2
Phialocephala fortinii
Piloderma olivaceum
Pyronemataceae
Rhizopogon pseudoroseolus
Russula declorans
Suillus brevipes
Suillus variegatus
Thelephora terrestris
Tomentella ellisii
Tomentellopsis sp.
Wilcoxina mikolae
Wilcoxina 1
Wilcoxina 2

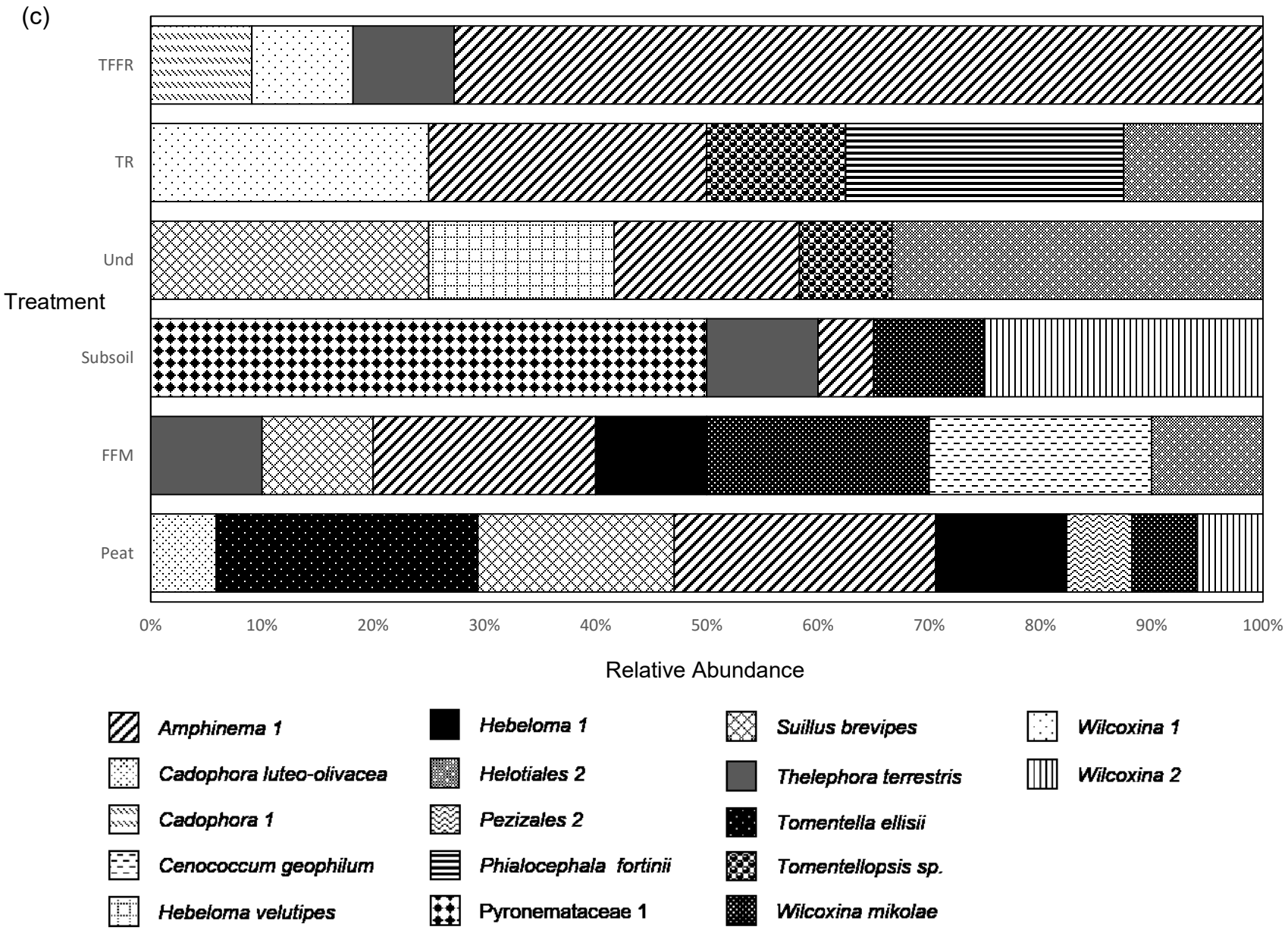
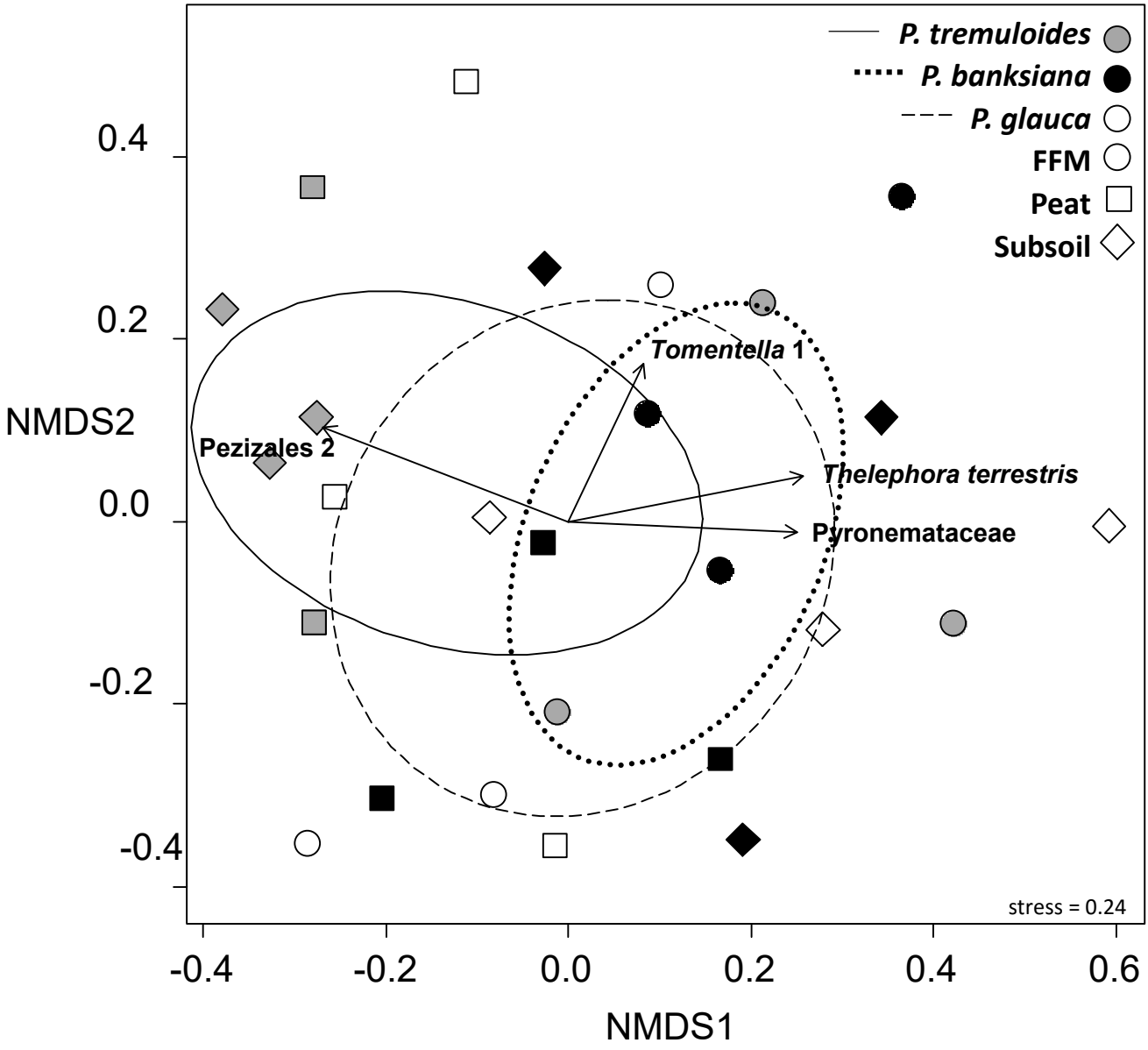
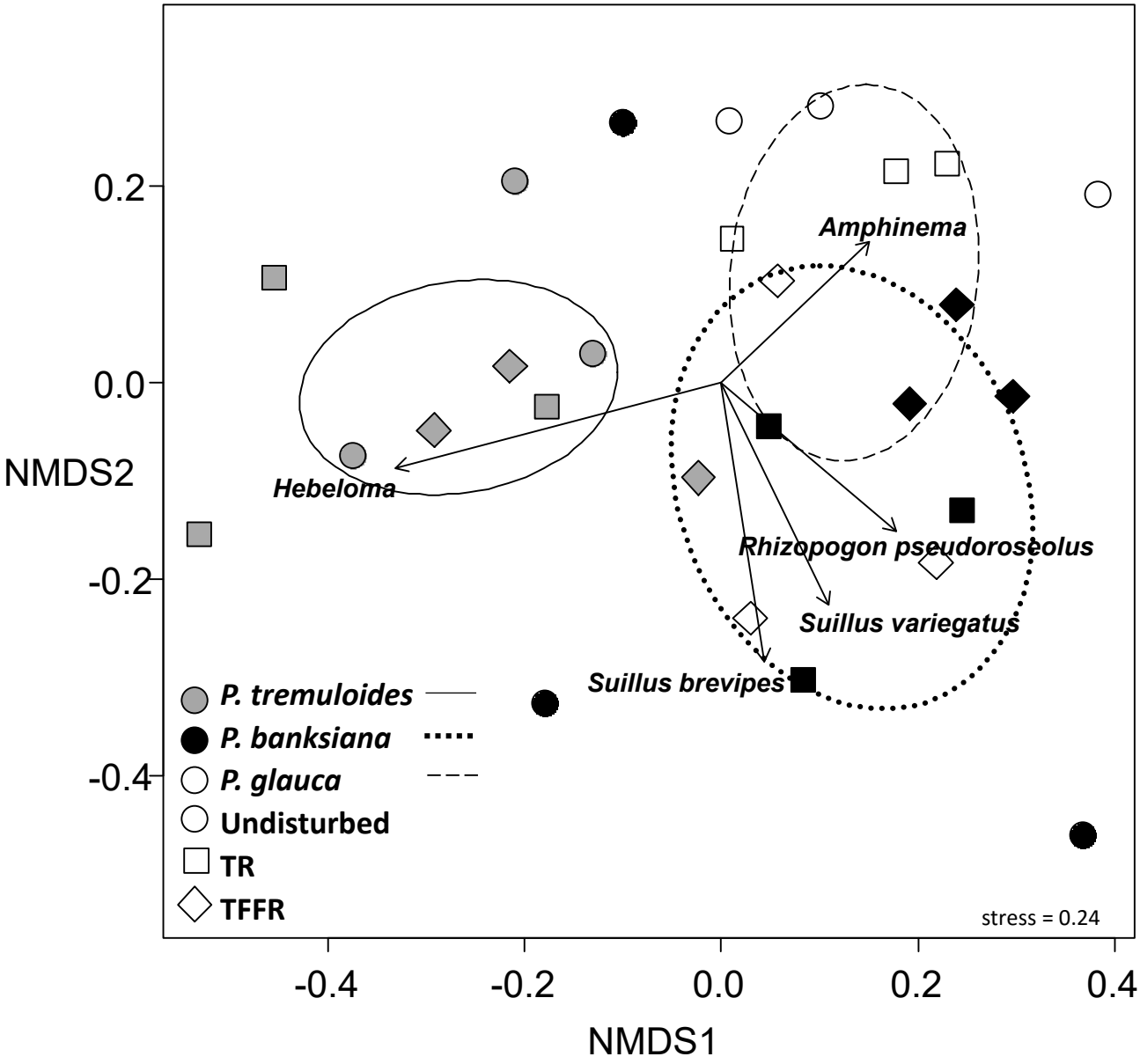

(c)
TFFR
TR
Und
Subsoil
FFM
Peat
Treatment
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Relative Abundance
Amphinema 1
Cadophora luteo-olivacea
Cadophora 1
Cenococcum geophilum
Hebeloma velutipes
Hebeloma 1
Helotiales 2
Pezizales 2
Phialocephala fortinii
Pyronemataceae 1
Suillus brevipes
Thelephora terrestris
Tomentella ellisii
Tomentellopsis sp.
Wilcoxina mikolae
Wilcoxina 1
Wilcoxina 2

Figure 2-3. Nonmetric multidimensional scaling ordination of operational taxonomic unit (OTU) abundance of ectomycorrhizal fungi assayed by *Populus tremuloides, Pinus banksiana,* and *Picea glauca* grown from 2012-2013 in (a) cover soils used in reclamation, and (b) intact soils differing in extent of aboveground disturbance all located in northern Alberta, Canada. Abundance was measured as the relative frequency of each OTU per plot for three host tree species. Each point represents the EMF community at a single plot. Points closer together are more similar in community composition. The direction and length of vectors indicate the influence of each OTU on the ectomycorrhizal community composition. Only OTUs which were marginal indicators of host, cover soil or aboveground disturbances are shown ($p < 0.10$). Ellipses illustrate grouping of seedlings assayed by different host species using the standard deviation of point scores. Abbreviations: forest floor material (FFM), undisturbed (Und), trees removed (TR), and trees removed + forest floor removed (TFFR).

(a)


P. tremuloides
P. banksiana
P. glauca
FFM
Peat
Subsoil
Tomentella 1
Pezizales 2
Thelephora terrestris
Pyronemataceae
stress = 0.24
NMDS1
NMDS2
-0.4
-0.2
0.0
0.2
0.4
0.6

(b)


0.2
0.0
-0.2
-0.4
NMDS2
-0.4
-0.2
0.0
0.2
0.4
NMDS1
Amphinema
Hebeloma
Rhizopogon pseudoroseolus
Suillus variegatus
Suillus brevipes
P. tremuloides
P. banksiana
P. glauca
Undisturbed
TR
TFFR
stress = 0.24

Chapter 3: Potential decomposition by ectomycorrhizal fungi across phenological stages of mature *Populus tremuloides*

3.1 Introduction

Ectomycorrhizal fungi form a symbiotic relationship with trees, functionally extending the roots of their host tree and providing mineral nutrients in exchange for photosynthetically-derived carbon. This symbiosis is particularly important in boreal forests, where slow decomposition rates drive nutrient limitation (Read *et al.* 2004; Toljander *et al.* 2006; McGuire *et al.* 2013). Moreover, trees in the boreal forest undergo dramatic fluxes in carbon assimilation due to short growing seasons followed by long periods of winter dormancy. The subsequent fluctuations of carbon allocation from host to fungi may have cascading effects on soil carbon and nutrient cycling (Johansson 1993; Lloyd & Taylor 1994; Buée *et al.* 2007).

Though ectomycorrhizal fungi depend on living hosts for carbon, their capacity for saprotrophism has recently been debated (Baldrian 2009; Cullings & Courty 2009; Lindahl & Tunlid 2014). Ectomycorrhizal fungi (EMF) release a suite of extracellular enzymes to break down complex organic matter for nutrient acquisition in exchange for glucose derived from their plant hosts. Chitinases and phosphatases, for example, are released to degrade organic matter-protein complexes to acquire nitrogen and phosphorus, respectively (Pritsch & Garbaye 2011; Rineau et al. 2012). However, recent discoveries indicate that EMF also release enzymes which can decompose plant litter. Several findings have demonstrated that EMF secrete carbon-degrading enzymes to mobilize glucose (Courty *et al.* 2007; Cullings, Ishkhanova & Henson 2008; Rineau *et al.* 2012), a trait historically only attributed to free-living saprotrophic fungi (Koide *et al.* 2008; Baldrian 2009; Talbot *et al.* 2013). Moreover, laboratory studies have shown the potential of EMF to depolymerize carbon compounds for glucose acquisition, albeit at low rates compared to their saprotrophic counterparts (Burke, Smemo & Hewins 2014).

The purpose of direct decomposition of organic carbon compounds by EMF is debated, but two general models have been proposed. The first model, hereafter termed the 'nutrient acquisition model' (Talbot, Allison & Treseder 2008; Lindahl & Tunlid 2014; Moore *et al.* 2015), describes decomposition by EMF as a byproduct of releasing nutrients locked in organic matter. Enzymes are secreted to break down carbon complexes for accessing nitrogen or phosphorus within the complexes, and glucose mobilization may simply be a secondary process, not the goal of decomposition. With this model, the level of enzyme secretion is not inversely dependent on glucose availability from the host tree, rather enzymes are secreted at a relatively consistent rate. The second model, hereafter termed the 'saprotrophy model' (Talbot et al. 2008; Moore et al. 2015), proposes that the ability of EMF to decompose carbon compounds is inversely related to carbon allocation from the host tree. When allocation is high, enzymes for decomposition decrease and carbon mobilization from soil decreases. Likewise, when allocation is low, enzymes for decomposition increase, and carbon mobilization from soil increases. Due to the important role of EMF in soil carbon sequestration (Clemmensen et al. 2013; Averill, Turner & Finzi 2014), saprotrophism would represent a large carbon loss often misattributed in global carbon models (Moorhead & Sinsabaugh 2006; Allison 2012; Treseder et al. 2012).

Under the saprotrophy model, decomposition by EMF may be driven by the phenology of the host trees and the associated changes in carbon inputs to their symbionts. Deciduous trees in boreal forests spend most of the year dormant followed by a relatively short period for leaf flush, expansion and abscission - phenological stages which may directly influence the host-symbiont relationship as well as leaf litter input. Many trees species reserve sugar and starch for metabolism when photosynthetically-derived carbon is limited. These sugars and starches, collectively termed nonstructural carbohydrates (NSCs), fluctuate in response to seasonality and

subsequently induce changes in source-sink dynamics in trees (Chapin, Schulze & Mooney 1990). For example, when Landhäusser and Lieffers (2003) monitored the fine root sugar and starch content of aspen (*Populus tremuloides*) from spring thaw to autumn frost, they found starch reserves initially low but increasing as the growing season progressed. Following leaf abscission however, starch levels dramatically declined towards the winter dormancy. Sugar concentration was inversely related, with high levels observed during thaw and bud flush, decreasing as the growing season progressed, but increasing again during leaf abscission and ground frost. This pattern, observed in many tree species (Gruber, Pirkebner & Oberhuber 2013; Da Silva et al. 2014; Dang et al. 2014), may determine how much sugar is allocated to ectomycorrhizal fungi (Johansson 1993; Hoch, Richter & Korner 2003).

The mechanism of sugar transfer from host to EMF is poorly understood, but may be indirectly measured by monitoring invertase activity (Salzer & Hager 1993; Parrent et al. 2009). This plant-derived enzyme is secreted into the interfacial apoplast where it hydrolyzes exuded sucrose into glucose and fructose, both of which can be absorbed by the EMF, however glucose is preferred (Smith & Read 2008). Most EMF lack genes encoding invertase and are unable to absorb sucrose, therefore they rely on the host for invertase synthesis and sucrose hydrolysis (Salzer & Hager 1993; Schaeffer et al. 1995). Hosts may consequently control the amount of photosynthate allocated to associated symbionts by regulating invertase activity. As seasonality may cause fluctuations in fine root NSC reserves, invertase levels may also fluctuate, determining how much glucose EMF are receiving from their host.

When glucose is available, by processes represented by either the saprotrophy model or the nutrient acquisition model, EMF are able to forage for nutrients. Foraging strategy is often dependent on physical and functional characteristics of emanating hyphae, and emphasis has

recently been placed on EMF exploration type, rather than lineage, to better indicate functional characteristics (Hobbie & Agerer 2010; Peay *et al.* 2011; Tedersoo *et al.* 2013; Fernandez & Kennedy 2015). Agerer (2001) was the first to classify EMF into exploration types based on the presence and length of emanating hyphae: contact types are characterized by a smooth mantle with few to no emanating hyphae, while medium and long-distance types have increasing rhizomorph lengths. Exploration type may predict foraging patterns of EMF species in addition to extracellular enzyme secretions (Tedersoo et al. 2012; Lindahl & Tunlid 2014). For example, contact types have been found to secrete greater levels of lignin-degrading enzymes, while long-distance exploration types have been found secrete greater levels of cellulose-degrading enzymes (Tedersoo et al. 2012; Burke et al. 2014). EMF exploration types may therefore determine the set of functions performed by an EMF community (Rudawska, Leski & Stasińska 2011; Tedersoo et al. 2012; Clemmensen et al. 2015) and may have distinct responses to changes in host phenology.

The relationship among the phenology-dependent storage of NSCs, root invertase activity and EMF-secreted carbon-degrading enzymes is not well understood, but we anticipate these processes to be intimately linked due to the interdependence between trees and ectomycorrhizal fungi. The objective of this study is to quantify the potential carbon-degrading ability of *Populus tremuloides* ectomycorrhizas from host dormancy through leaf abscission. We tested whether phenological shifts in fine root carbon reserves of mature *P. tremuloides* affect the activity of EM-derived carbon-degrading enzymes, predicting that decomposing abilities of EMF would be dependent on host phenology and follow the saprotrophy model. Specifically, we hypothesized that root NSC reserves and invertase activity would be lowest during leaf flush and leaf abscission, when host photosynthesis is low but EMF are active. We predicted that in response,

EMF-secreted carbon-degrading enzymes would be highest during these phenological stages, and lowest during host dormancy and full leaf expansion.

3.2 Materials and Methods

3.2.1 Site description

To test the relationship between host phenology and EMF-derived carbon-degrading enzyme secretions, eight mature stands (~64 years old) of aspen (*Populus tremuloides*) were chosen near Conklin, northeastern Alberta, Canada (55°38'N, 111°07'W) within the Boreal Mixedwood Forest. Sites were chosen to be approximately 1 hectare in size and separated by at least 500 m, up to several kilometers. Aspen basal area averaged 98%, ranging from a minimum of 90% to 100% of total stand basal area (Table 3-1). Sites have a *Viburnum edule* (Michx.) Raf.-*Rosa acicularis* Lindl. understory and Orthic Gray Luvisol soils. Mean precipitation for the area is 419 mm with a mean high air temperature of 16.8 °C in July and mean low temperature of -18.8 °C in January (1981-2010, Fig. 3-1). During each collection, roots were harvested for nonstructural carbohydrate (NSC) concentration, potential invertase activity, and EMF-derived enzyme secretion analysis (see below).

3.2.2 Field sampling

Collection times of ectomycorrhizas and fine roots were determined by the phenological stage of the host tree. The four stages for collection were during: (1) tree dormancy and soil partially frozen (April 14/15, 2014); (2) leaf flush (May 27/28, 2014); (3) late growing season (August 7/8, 2014); and (4) leaf abscission (Sept 27/28, 2014). Phenological stages were determined by visual observations.

For the EMF-derived enzyme secretion analysis, fine roots were harvested from three mature trees by digging four holes in the four cardinal directions, approximately 15 cm deep and within 0.5 m of the host stem. For each of three trees, fine roots, traced to the host tree, were

collected and pooled. Roots were stored with the surrounding soil in a plastic bag and placed on ice. For invertase activity analysis, additional fine roots were collected from one mature tree also used for EMF-derived enzyme root collection, stored in separate plastic bags without surrounding soil and immediately placed on dry ice upon harvesting. For NSC analysis, fine root (≤ 2 mm diameter) samples were collected from the same mature aspen used for invertase activity analysis for each site (n = 8). Roots of approximately 20 cm long were harvested within 0.5 m of the stem and immediately placed on ice. All samples were transported within 48 hours to the University of Alberta. Roots for EMF-derived enzyme analyses were stored at 4-6 °C, while roots for invertase enzyme and NSC analyses were stored at -20 °C, and all roots collected for enzyme analyses processed within 28 days.

Soil temperature and volumetric soil water content were also recorded at each site. During the dormancy stage collection, soils were frozen and soil temperature was recorded using a UE DT130 digital thermometer with Omega Probe (Universal Enterprises, Inc., Beaverton, Oregon) at a depth of 10 cm. After soil had thawed, three Hobo temperature pendants (Onset Computer Corporation, Bourne, Massachusetts) were installed at each site at a depth of 10 cm, approximately 25 m apart recording temperature at two hour intervals. Soil temperature ranged from a minimum of 0 °C during host dormancy to a maximum of 14.50 °C during full leaf expansion (Fig. 3-1).

Volumetric soil water content was measured around a target tree for each site, at a depth of 6 cm using a Theta Probe Soil Moisture Sensor (Delta-T Devices Ltd., Cambridge UK), at each cardinal direction approximately 0.5 m from the stem. Volumetric soil water content ranged from a minimum of 4.6% during full leaf expansion to a maximum of 53.4% during leaf flush. The mean soil water content was 12.7% (± 1.10) during host dormancy, 39.9% (± 2.25) during

leaf flush, 12.2 % (± 1.24) during full leaf expansion, and 29.1% (± 0.82) during leaf abscission. Collections of roots during leaf flush and leaf abscission took place during periods of high precipitation in the area, which likely increased soil moisture (Fig. 3-1).

3.2.3 Fine root nonstructural carbohydrates

Fine roots (≤ 2 mm) were thawed and gently washed over a 1.2 mm sieve to remove all soil and debris and subsequently oven-dried for one hour at 75 °C followed by 60 °C for one week. After drying, roots were weighed to calculate mass, then ground through a 40 μm mesh screen in a Wiley mill. Starch and sugar concentrations were then measured following the protocol of Chow & Landhausser (2004). Briefly, sugars and starches were extracted with 80% hot ethanol. Sugar concentration was subsequently measured colorimetrically using phenol-sulfuric acid with a phenol concentration of 2%; absorbance was read with an optimized wavelength between 465 and 505 nm. For starch concentrations, an enzyme digestion mixture of 1000 U α-amylase and 5 U amyloglucosidase was used on plant tissue samples. A peroxidase-glucose oxidase/*o*-diansidine reagent was used to measure glucose hydrolyzate obtained, followed by the addition of sulfuric acid. Sample absorbance was read with a wavelength of 525 nm.

3.2.4 Root invertase activity

Harvested roots were gently washed with tap water over a 1.2 mm sieve to remove soil and debris, and cut into 1-2 cm fragments. Root fragments were mixed thoroughly in a container filled with deionized water using forceps. Root tips were placed in a petri dish with deionized water and examined under a dissecting microscope at 100× magnification. Ectomycorrhizal root

tips were morphotyped by appearance of hyphae, mantle structure, color, and texture (Goodman et al. 1996).

Assays of invertase activity on the mycorrhizal roots were performed using materials and methods provided by Sigma-Aldrich (Invertase Assay Kit, Catalog Number MAK118, Sigma-Aldrich, St. Louis). Briefly, up to 75 ectomycorrhizal root tips per site were placed in separate wells of a 96-well plate. Four remaining wells were reserved for the calibration curve and eight wells for negative controls. To account for possible EMF-derived invertase activity, additional emanating hyphae were separated from ectomycorrhizal root tips and placed in up to twelve wells for a positive control. Each well received 5 µL of a 1× sucrose solution and dark incubated at 30 °C for 20 minutes. Following incubation, a solution of buffer, enzyme mix and dye reagent was added to each well. The plate was shaken horizontally to mix and dark incubated again for 20 minutes at 22 °C. Following incubation, the solution was immediately vacuum-transferred to an empty black measurement plate and placed in a Synergy HT microplate reader (BioTek Instruments, Winooski, Vermont), where fluorescence was read at 535 (±40) nm excitation and 587 (±40) nm emission. Following enzyme assays, root tips were scanned and projected area was measured with WinRHIZO Pro 2009b software (Regent Instruments Inc., Quebec, Canada). Potential root invertase activity was calculated using the following equation:

$$\text{Invertase Activity} = \frac{\text{sample} - \text{negative}}{a \cdot \text{pa} \cdot t}$$

"Sample" is the measured fluorescence value of the sample, whereas "negative" is the value of the negative control; pa is the projection area of the root tips (mm^2); t is the incubation time (min); and a equals the slope of the calibration curve regression line, per mole.

3.2.5 Potential ectomycorrhizal enzyme secretion

Harvested roots were first morphotyped using the same procedure described above. A maximum of seven roots tips were collected for up to four EMF morphotypes per tree, for three trees per site, corresponding to a maximum of 84 root tips assayed per site. Activity of four enzymes was measured: β-glucuronidase (EC 3.2.1.31), β-glucosidase (EC 3.2.1.3), *N*-acetylglucosaminidase (EC 3.2.1.52), and laccase (EC 1.10.3.2). β-glucuronidase, a hemicellulase, hydrolyzes the bond between glucuronic acid and an organic complex, releasing glucuronic acid for further degradation to glucose. β-glucosidase is a cellulase which degrades plant cell wall material by hydrolyzing the bond between two glucose molecules. A chitinase, *N*-acetylglucosaminidase releases nitrogen by hydrolyzing the glycosidic bonds in chitin. Laccase contributes to plant cell wall decomposition by oxidizing the linked phenols in lignin.

Ectomycorrhizal enzyme secretion assays were conducted using the procedures described by Pritsch et al (2011). Briefly, solutions of 4-methylumbelliferone (MU) and diammonium 2,2'-azinobis-3-ethylbenzothiazoline (ABTS) were made for the assay substrates, corresponding to the fluorometric assay used for β-glucuronidase, β-glucosidase, and *N*-acetylglucosaminidase, and colorimetric assay for laccase, respectively. Root tips were placed in 84 wells of a 96-well filter plate (AcroPrep™ Advance Plate with 30-40 µm PP/PE non-woven media; Pall Life Sciences, Port Washington, New York). Six remaining wells were used as negative controls, and six used for calibration. Root tips were rinsed with buffer, which was then vacuum-removed and disposed. Following buffer disposal, 150 µL of incubation solution was added to each well for the fluorometric assay, and 120 µL of incubation solution added for the colorimetric assay, excluding the negative control wells. The plate was shaken horizontally and incubated at room

temperature for 15 minutes (β-glucosidase and *N*-acetylglucosaminidase), 30 minutes (β-glucuronidase), or 60 minutes (laccase).

Following incubation, the incubation solution was vacuum-transferred to either a clear (colorimetric) or black (fluorometric) measurement plate and immediately measured in a Synergy HT microplate reader (BioTek Instruments, Winooski, Vermont). Fluorometric assays were read at 360 nm (±40) nm excitation and 460 (±40) nm emission; colorimetric assays at 420 nm. The root tips were subsequently transferred from the measurement plate to a clear plate with 50 µL dH_2O and scanned using WinRHIZO Pro 2009b software (Regent Instruments Inc., Quebec, Canada) to determine projected surface area. Enzyme activity of the fluorometric assays was calculated using the same equation used to calculate invertase activity, however, the ABTS test uses the following equation to calculate *a*:

$$a = \frac{\mathcal{E}_{425} \cdot \text{pl}}{\text{vol}}$$

Where $\mathcal{E}_{425}$ is the molar coefficient of extinction for ABTS ($\mathcal{E}_{425} = 3.6\text{x}10^4$ cm^2 mol^{-1}), pl is equal to the path length (cm) of the well, and vol is the incubation volume (liters). Enzyme activity is expressed as (moles per square millimeter per minute) of released MU or ABTS.

3.2.6 Identification of ectomycorrhizal fungi

Ectomycorrhizal morphotypes were classified as either "contact" or "distance" exploration types based on the presence and length of emanating hyphae (Table 3-2). These two broad categories were chosen to eliminate inconsistencies in mycelial length observations. Once assays were completed, two specimens of each morphotype per tree were collected for DNA extraction and identification, for a total of up to twenty-four root tips per site. Fungal DNA of

colonized root tips selected for molecular analysis was extracted using Sigma Extraction Buffer and Neutralization Solution B according to the manufacturer's protocol (Sigma, Gillingham, Dorset, UK). Extractions were amplified via PCR using the fungal-specific combination ITS1-F (5'-cttggtcatttagaggaagtaa-3') and ITS4 (5'- tcctccgcttattgatatgc -3') forward and reverse primers, respectively (Innis et al. 1990; Gardes & Bruns, T 1993). Following extraction, 1.0 µL DNA extract was added to a solution of 5.4 µL MilliQ H_2O, 8.0 µL RedTaq (Sigma-Aldrich, St. Louis), 0.8 µL ITS1-F and 0.8 µL ITS4. PCR was conducted with an initial denaturation at 95 °C for 5 minutes followed by 40 cycles of (95 °C for 1.5 minutes, 57 °C for 1 minute, 72 °C for 1.5 minute) and a final extension at 72 °C for 10 minutes.

Confirmation of successful PCR reaction product was visualized using gel electrophoresis. Gels of 1.7% agar were run at 100 volts for 60 minutes. If gels failed to produce clear fragment bands, voltage was decreased and run time increased. Once confirmed by gel electrophoresis, PCR product was purified by adding 5 µL ExoSAP-IT (USB, Cleveland, Ohio, USA) to 2 µL PCR product. Solutions were incubated at 37 °C for 30 minutes, then denatured at 80 °C for 20 minutes. Following purification, a bi-directional sequencing reaction was performed with BIGDYE v3.1 (Applied Biosystems, Foster City, California, USA) using the ITS1-F/ITS4 primer pair and subsequently precipitated with EDTA and ethanol. Sequences were read by an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, California, USA).

Raw sequences were edited with Geneious software (BioMatters, Auckland, New Zealand). Specifically, ends were trimmed with an error probability limit of 3% and complementary sequences were then assembled using DeNovo assembly to create a consensus sequence. Following assembly, phred scores below 20 were changed to 'N.' Single direction reads were subjected to the same criteria. The resulting sequences (consensus and single) were

clustered into operational taxonomic units using the CAP3 plugin with the following settings: ≥ 97% identity; overlap percentage identity cutoff = 97; maximum overhang percentage length = 60; match score factor = 5; clipping range = 6. Operational taxonomic units (OTUs) were run through the GenBank database (National Center for Biotechnology Information, Bethesda, Maryland) using BLASTn to identify the best match. Identity was assigned to an OTU if percent identity was ≥ 97 and query coverage was ≥ 80%.

3.2.7 Statistical analyses

Nonstructural carbohydrate (NSC) concentration, root invertase activity, and potential enzyme activity was averaged for each site (n = 8), and the effect of phenological stage was tested with one-way ANOVAs. Values were transformed as necessary to meet assumptions of normality prior to analysis. Significant effects ($p < 0.05$) were followed by Tukey tests to examine differences among phenological stages ($\alpha = 0.05$). Pearson correlation coefficients were calculated between potential enzyme activities, NSC concentrations, soil temperature, and volumetric soil water content. The effects of ectomycorrhizal exploration type, host phenology and their interaction on potential enzyme activity were tested using a two-way ANOVA when assumptions were met, and a permutational ANOVA when assumptions were not met. The effect of phenological stage on the ratio of contact to distance exploration types, and on the relative abundance of contact and distance exploration types, was tested with a one-way ANOVA. Significant effects were followed by Tukey tests to examine differences among phenological stages ($\alpha = 0.05$). All statistical tests were performed with R software (R Development Core Team 2011).

3.3 Results

3.3.1 Host phenology and root nonstructural carbohydrate status

Phenology affected the total nonstructural carbohydrate (NSC; soluble sugars and starch) concentration of fine roots (Table 3-3, Fig. 3-2). Specifically, mean total NSC concentration of fine roots was 6.0% during host dormancy, increased to 10.1% during leaf flush, further increasing to13.9% during full leaf expansion, which was maintained during leaf abscission. Each of the components of NSCs followed a similar trend. Fine root sugar concentrations were affected by phenology ($p < 0.001$, Table 3-3, Fig. 3-2), with sugar concentration increasing from 5.7% during host dormancy to 9.0% during leaf flush and remaining constant across the remaining phenological stages. Over the same phenological stages fine root starch concentrations increased from 0.3% during host dormancy to 1.4% during leaf flush, to approximately 5% during full leaf expansion and leaf abscission stages ($p < 0.001$, Table 3-3, Fig. 3-2). Sugar concentration of fine roots was positively correlated with soil temperature ($r^2_{(8)} = 0.55$, $p < 0.001$, Table 3-4) and soil water content ($r^2_{(8)} = 0.44$, $p<0.05$, Table 3-4). Starch was positively correlated with soil temperature ($r^2_{(8)} = 0.82$, $p < 0.001$, Table 3-4).

3.3.2 Root invertase activity

Host phenology affected root invertase activity (Table 3-5, Fig. 3-3). Specifically, the mean (± 1 standard error) potential root invertase activity was 125.1 pmol mm^{-2} min^{-1} (± 28.6) during host dormancy, which increased to 190.0 pmol mm^{-2} min^{-1} (± 46.7) during leaf flush, and after that did not change significantly during stages of full leaf expansion and leaf abscission with 151.3 pmol mm^{-2} min^{-1} (± 23.3), and 210.5 pmol mm^{-2} min^{-1} (± 21.8), respectively. Root invertase activity was positively correlated with fine root sugar concentrations ($r^2_{(8)} = 0.49$, p

<0.01, Table 3-4), soil temperature ($r^2_{(8)} = 0.38$, $p < 0.05$, Table 3-4) and soil water content ($r^2_{(8)} = 0.42$, $p < 0.01$, Table 3-4). Potential invertase activity from isolated fungal hyphae was 54.7 pmol mm^{-2} min^{-1} (± 20.2) during host dormancy, 1617.8 pmol mm^{-2} min^{-1} (± 486.3) during leaf flush, 2305.6 pmol mm^{-2} min^{-1} (± 840.5) during full leaf expansion, and 333.0 pmol mm^{-2} min^{-1} (± 27.9) during leaf abscission. Removing hyphae from colonized root tips may have released intracellular invertases due to the highly disruptive nature of this procedure. These values were consequently disregarded.

3.3.3 Potential ectomycorrhizal enzyme secretion

Of the measured enzymes secreted by ectomycorrhizas, host phenology affected the activity of β-glucuronidase only (Table 3-5). The most pronounced difference in the activity of β-glucuronidase was between dormancy and leaf flush (Fig. 3-3). Potential secretions of β-glucosidase, laccase and *N*-acetylglucosaminidase did not differ by host phenology (Table 3-5, Fig. 3-3). Potential secretions of β-glucosidase, *N*-acetylglucosaminidase, and laccase were correlated (Table 3-4), while fine root sugar concentration and secretion of β-glucosidase were negatively correlated (Table 3-4). Despite a trend showing an inverse relationship between β-glucuronidase and invertase (Fig. 3-3), there was no significant correlation (Table 3-4).

3.3.4 Taxa and exploration types of ectomycorrhizal fungi

Across the four phenological stages, DNA of 62 enzyme-assayed root tips was amplified with a success rate of 60%. The DNA of the resulting 37 root tips was sequenced and quality filtering yielded 26 sequences clustered into four operational taxonomic units (OTUs) and 14 remaining singletons (Table 3-6). In total, 18 ectomycorrhizal fungal taxa were recorded; the

most common taxa were *Russula* occurring at all phenological stages, and *Cenococcum geophilum* which was observed during three of the four phenological stages (Fig. 3-4). When ectomycorrhizal root tips were categorized by exploration type, we found that exploration type affected potential activity of all enzymes but β-glucosidase (Table 3-7, Fig. 3-5). Overall, distance exploration types had higher potential activity of β-glucuronidase, particularly during the phenological stages other than leaf flush (Table 3-7, Fig. 3-5). Potential *N*-acetylglucosaminidase activity was also higher in distance exploration types (Table 3-7, Fig. 3-5). Secretions of laccase were marginally higher in contact exploration types (Table 3-7, Fig. 3-5), but there was no effect of phenology on secretions of β-glucosidase, *N*-acetylglucosaminidase, or laccase from either exploration type. Contact exploration types included OTUs belonging to the *Russula* genus (Morphotypes E4, E12), an uncultured *Thelephora* clone (Morphotype E10), and a *Phialocephala fortinii* strain (E12, Table 3-2). Distance exploration types included OTUs matching sequences of a *Cenococcum geophilum* isolate (Morphotypes E1, E2), a *Russula* sp. isolate (Morphotype E2), a *Boletus subglabripes* isolate (Morphotype E2), a *Cortinarius cedriolens* isolate (Morphotype E2), an uncultured *Cortinarius* clone (Morphotype E2), a *Piloderma lanatum* clone (Morphotype E2), a *Leccinum populinum* isolate (E11), and a *Cortinarius subexitiosus* clone (Morphotype E11, Table 3-2).

The ratio of contact to distance exploration types did not change across phenological stages ($F_{(3,28)} = 0.94$, $p = 0.44$); however phenological stage affected the relative abundance of distance exploration type ectomycorrhizal fungi ($p < 0.01$, Table 3-8, Fig. 3-6). Leaf flush coincided with a lowered relative abundance of distance exploration types, but was unchanged across the other phenological stages. The relative abundance of contact exploration types remained similar across each phenological stage.

3.4 Discussion

We predicted host phenology to effect the secretion of carbon-degrading enzymes by ectomycorrhizal fungi due to changes in root nonstructural carbohydrates and correlated fluctuations in root invertase activity. Though levels of root nonstructural carbohydrates changed seasonally, we found that aside from β-glucuronidase the potential activity of other carbon-degrading enzymes were unaffected by host phenological stage. Ectomycorrhizal exploration type was a stronger predictor of potential enzyme secretion than phenology with distance exploration types having higher activity of two of the four enzymes. Moreover, the relative abundance of distance exploration types declined during leaf flush.

3.4.1 Potential ectomycorrhizal enzyme secretion

Contrary to our prediction, we found no relationship between host phenology and overall secretions of β-glucosidase, *N*-acetylglucosaminidase, or laccase. Interestingly however, phenology affected secretions of β-glucuronidase, which was highest during host dormancy and lowest during leaf flush. β-glucuronidase hydrolyzes the β-O- bond between glucuronic acid and the linked organic compound, a process which releases glucuronic acid for further breakdown into glucose. The high activity during dormancy may indicate a greater need by ectomycorrhizal fungi to (1) release glucuronic acid for further glucose mobilization as part of the saprotrophy model, or (2) access nutrients locked within the organic compounds linked to β-glucuronidase as part of the nutrient acquisition model. In relation to phenology, Courty *et al*. (2007) recorded a spike in potential β-glucuronidase activity of ectomycorrhizas shortly after leaf flush in a temperate oak forest, and suggested its role in leaf development, a finding that could support the

nutrient acquisition model. Our study did not sample frequently enough to capture potential enzyme activity immediately following leaf flush, but β-glucuronidase was marginally higher during full leaf expansion than leaf flush, perhaps a reflection of its role in leaf maintenance throughout the growing season and supporting the nutrient acquisition model. Alternatively, microbial respiration is known to continue through the winter, even in frozen soil (Mikan, Schimel & Doyle 2002); however because most water is frozen, extracellular secretions diffuse at a much slower rate, and enzymatic reactions proceed at a much slower rate, than in unfrozen soil (Davidson & Janssens 2006). This low rate of diffusion and reaction may cause EMF to compensate by secreting enzymes at higher rates, suggesting a new model during periods of freezing.

The trends in potential β-glucuronidase, β-glucosidase, *N*-acetylglucosaminidase, and laccase activity we observed differ from what we predicted. They also differ from patterns in previous work, which was conducted in a temperate oak forest and reporting that seasonality had a large effect on potential activity of β-glucuronidase, β-glucosidase, *N*-acetylglucosaminidase, and laccase, which we did not find (Courty *et al*. 2007). To our knowledge however, the work presented here is the first to monitor seasonal changes of enzyme secretions of ectomycorrhizas in the boreal forest. Defoliation has also been found to induce elevated levels of β-glucosidase and laccase (Cullings *et al*. 2008), suggesting that when the supply of photosynthates decreases, EMF are able to increase mobilization of soil carbon. We anticipated a similar outcome in response to leaf abscission, but found no such effect.

In contrast to most enzymes we monitored, the negative correlation between potential activity of β-glucosidase and fine root sugar concentration is consistent with the saprotrophy model that predicts EMF increase the degradation of cellulose from plant litter when host carbon

allocation to symbionts is low. Additionally, potential activity of β-glucosidase was positively correlated with *N*-acetylglucosaminidase, laccase, and marginally correlated with β-glucuronidase, suggesting these enzymes work in tandem to break apart complex carbon compounds, supporting the nutrient acquisition model. This finding is consistent with Courty *et al.* (2010), who found a correlation between β-glucosidase, β-glucuronidase, and *N*-acetylglucosaminidase. Courty *et al*. (2007), however, found no correlation within this set of enzymes when monitoring seasonal activity in oak ectomycorrhizas.

To our knowledge, this is the first study to examine the seasonal relationship between root invertase and EMF enzyme secretions. Contrary to our hypothesis, our results suggest no relation between root invertase activity and EMF-secreted carbon-degrading enzymes, supporting the nutrient acquisition model; however an inverse trend between invertase and β-glucuronidase warrants further investigation. Leaf litter input during fall provides a rich supply of easily accessible carbon (soluble sugars) and organic matter for decomposition (Landhäusser & Lieffers 2003). Our initial hypothesis, based on the saprotrophy model, was that EMF-derived enzyme activity would be significantly higher during this period due to loss of photosynthetic leaf area, and subsequent reduced carbon allocation to root symbionts. Under the nutrient acquisition model, enzyme activity is predicted to be lower during this stage, as the EMF may be receiving less carbon from their host to forage for nutrients. However, the lack of change in total fine root NSC and invertase levels from full leaf expansion to leaf abscission may account for the unchanged rates of EMF-derived enzyme secretions; carbon supply to mycorrhizas may have been similar at these phenological stages, suggesting a relationship may still exist.

The potential activities of β-glucuronidase and β-glucosidase were negatively correlated with volumetric soil water content, a finding which differs from previous work comparing EMF

enzyme activity and soil moisture or drought (Courty *et al.* 2007; Herzog *et al.* 2013) which found no correlation or lower enzyme activity during drought, respectively. Our findings may indicate that as volumetric soil water content decreases, the rates of enzyme diffusion in soil decrease, which may necessitate higher rates of enzyme secretion. On the other hand, proteolytic activity of enzymes in soil may be inhibited by low temperatures, consequently resulting in an accumulation of enzymes (Wallenstein, Mcmahon & Schimel 2009). Wallenstein et al (2009) observed high levels of *N*-acetylglucosaminidase and β-glucosidase in bulk Arctic soil during late winter with decreasing levels during the growing season, suggesting that microbial enzyme production continues to be active despite frozen soil.

3.4.2 Host phenology and nonstructural carbohydrates

We predicted potential enzyme activity of ectomycorrhizas to be explained by changes in nonstructural carbohydrates, which we found to be affected by host phenology. Host fine root non-structural carbohydrate concentrations were lowest during dormancy and increased during leaf flush and full leaf expansion while remaining high during leaf abscission, demonstrating increased storage as photosynthetic organs become active. Although leaf abscission dramatically decreases photosynthesis, most NSC accumulation occurred earlier on in the growing season allowing trees to prepare for dormancy (Chapin et al. 1990). During dormancy, NSC concentrations were almost entirely comprised of sugars; starch concentrations made up only ~5% of total NSCs. This is consistent with previous findings of mature *P. tremuloides* during dormancy (Landhäusser and Lieffers 2003), who also found starch concentrations to increase during leaf flush and full leaf expansion, and remain stable until leaf abscission We recorded an increase in sugar concentrations during leaf flush, which then remained stable during full leaf

expansion and leaf abscission. This pattern differs from the work of Landhäusser and Lieffers (2003), who found fine root sugar concentrations to remain constant before and during leaf flush, then decrease by the conclusion of shoot elongation. On the other hand, the low sugar concentrations we found during host dormancy may be a result of leakage. New fine roots grown during the previous fall likely had high sugar concentrations, and thawing soil may have resulted allowed sugar to leak from the fine roots (Landhäusser & Lieffers 2003).

Though fine root non-structural carbohydrates were not correlated to most of carbon-degrading enzymes, changes in root growth and its effects on NSC storage and demand may have been influential. In general, root growth and root NSC storage is lower in the spring than later in the season (Gruber et al. 2013; Dang et al. 2014). During dormancy, root NSC storage is low and frozen soil likely results in low carbon export to EMF and subsequently higher secretions of β-glucuronidase. During leaf flush, root sugar concentration is higher, but limited root growth may result in more carbon allocation to EMF and subsequently lower secretions of β-glucuronidase. Root growth increases when leaves are fully expanded and during leaf abscission, creating a higher demand for NSCs, and may subsequently lower carbon allocation to EMF. This may explain why β-glucuronidase was slightly higher during these two stages when compared to leaf flush. On the other hand, we pooled enzyme activity from the roots of three trees per stand while NSCs were only measure on one tree per stand, a procedure which may have impacted our results.

3.4.3 Root invertase activity

We predicted root invertase activity to be positively correlated with fine root sugar concentrations. Our results support this hypothesis, and suggest that higher fine root sugar

concentration may translate into higher glucose levels in the plant-fungus interface. In other words, via invertase activity the host tree may allocate more carbon to EMF when fine root sugar concentrations are high. We also predicted host phenology to effect root invertase activity, and found this to be true. Although we predicted invertase activity to change over time, we anticipated levels to be lowest during leaf abscission and highest during full leaf expansion. Our results may reflect the host's investment in EMF even during carbon-expensive periods such as leaf flush. This could benefit the host if EMF nutrient foraging remains high during this phenological stage (Smith & Read 2008). On the other hand, bacteria can secrete extracellular invertase (Parrent et al. 2009) and may have contributed to the values we observed if they were not effectively washed off during sample preparation. Additionally, our method of measuring invertase activity from severed fine roots may have captured a large portion of intracellular invertases, which are important for plant growth and development (Egger & Hampp 1993). If that is the case, high invertase levels during leaf flush, full leaf expansion, and leaf abscission may not necessarily reflect higher carbon delivery to fungi. However, if carbon export to EMF is diffusive, our method would be an accurate proxy for detecting the level of glucose becoming available in the interfacial apoplast for EMF uptake.

3.4.4 Ectomycorrhizal exploration types

The relative abundance of ectomycorrhizal exploration types varied during the growing season, perhaps as a direct result of changes in host carbon inputs. The abundance of distance exploration types was significantly lower during leaf flush, while there was no change in the abundance of contact exploration types. Rhizomorphs and emanating mycelium require a greater carbon investment by the host than contact exploration types (Agerer, 2001). Moreover, leaf

flush is a carbon-expensive period when trees are not at full photosynthetic capacity and fine root NSC levels are lower than later in the growing season. This may cause the host to conserve NSCs for leaf development and in turn allocate less carbon to distance exploration types, a finding consistent with previous defoliation studies (Saikkonen *et al.* 1999; Saravesi *et al.* 2008).

We found exploration type to be a better predictor of enzyme secretion than host phenological stage. Potential activity of β-glucuronidase and *N*-acetylglucosaminidase was higher in distance types, with a more pronounced effect of phenological stage on β-glucuronidase secretions. In a tropical rainforest, Tedersoo et al (2012) found potential activity of β-glucuronidase in long-distance exploration types higher than in contact and short-distance exploration types, and in general found exploration type a better indicator of potential enzyme activity than EMF phylogeny. On the other hand, the potential activity of β-glucuronidase in mycorrhizal roots was similar to nonmycorrhizal roots (Tedersoo et al. 2012). We also found potential *N*-acetylglucosaminidase higher in distance exploration types, suggesting greater chitin-degrading abilities than in contact exploration types. Hobbie & Agerer (2010) found that high biomass mycorrhizas, i.e., long-distance exploration types, had greater sporocarp ^{15}N enrichment than low biomass mycorrhizas such as contact and short-distance exploration types, perhaps demonstrating enhanced nitrogen foraging characteristics of distance exploration types. Although not significantly different, potential activity of *N*-acetylglucosaminidase in distance exploration types was elevated during leaf flush and full leaf expansion, suggesting higher host nutrient demand and consequently more nitrogen foraging during these stages, according to the nutrient acquisition model. Alternatively, the saprotophy model predicts there may be a greater carbon demand by the EMF during these stages. Contact exploration types had marginally higher potential laccase activity than distance exploration types, a finding consistent with previous work

(Tedersoo et al. 2012). For example several *Russula* species, a contact exploration type present on our roots, have recorded greater phenol oxidase (laccase) secretions in similar studies (Burke et al. 2014). The saprotrophy model would predict that contact exploration types are mobilizing more carbon during periods of low availability from the host, but the nutrient-acquisition model would predict that contact exploration types simply have a greater ability to access nutrients locked inside carbon complexes. Because there was no effect of host phenology, fine root NSC concentration, or root invertase activity on potential laccase activity, what we observed is aligned with predictions of the nutrient acquisition model. From a plant-centric view, (Koide *et al.* 2008) proposed that hosts may select for an EMF community with a particular set of functions, and this selection could have evolved into host specificity if the plant allocates more carbon to a particular species. The higher levels of potential laccase activity in contact exploration types gives them an important role in decomposing phenol-protein complexes, while distance exploration types have an important role in decomposing hemicelluloses and chitin. A diverse set of exploration types may therefore supply the host with various specific benefits in nutrient acquisition.

In conclusion, only β-glucuronidase was significantly affected by host phenology, but not in the pattern we predicted based on the saprotrophy model. Observed trends warrant further investigation into the biotrophy-saprotrophy continuum, however our evidence suggests that the secretion of carbon-degrading enzymes by EMF is primarily driven by nutrient acquisition. The enzymes we measured may be utilized in tandem by EMF to break apart organic material and therefore acquiring nitrogen or phosphorus locked within carbon compounds. In addition, exploration type determined the secretion levels of β-glucuronidase, *N*-acetylglucosaminidase

and, to a smaller extent, laccase. Distance exploration types had higher potential activity of β-glucuronidase and *N*-acetylglucosaminidase, while contact exploration types had marginally higher potential laccase activity. Differences in potential enzyme activity among contact and distance exploration types throughout phenological stages point to unique functional roles which may change seasonally. These functional roles necessitate further investigation to better predict temporal patterns of carbon and nutrient cycling in boreal forests.

Tables

Table 3-1. Total basal area index, *Populus tremuloides* basal area, and percent *P. tremuloides* for eight mature *P. tremuloides* stands in northern Alberta, Canada of approximately one hectare each.

Site	Total basal area[Ψ]	*P. tremuloides* basal area[Ψ]	*P. tremuloides* (%)
1	216.81 (± 2.33)	194.75 (± 6.19)	89.82 (± 3.82)
2	202.54 (± 9.95)	202.54 (± 9.95)	100
3	222.03 (± 3.36)	220.52 (± 3.89)	99.32 (± 0.25)
4	168.96 (± 10.42)	159.71 (± 11.64)	94.53 (± 1.08)
5	217.64 (± 9.38)	212.96 (± 11.57)	97.85 (± 1.11)
6	200.73 (± 6.45)	200.73 (± 6.45)	100
7	192.49 (± 5.43)	191.70 (± 5.45)	99.59 (± 0.02)
8	244.72 (± 8.60)	244.68 (± 8.61)	99.99 (± 0.01)

[Ψ] $meters^{2-}$ $hectare^{1-}$

Table 3-2. Descriptions of observed morphotypes comprising contact and distance exploration types of aspen ectomycorrhizal root tips, based on Agerer (2001). Roots tips were morphotyped using a dissecting microscope (100x magnification). Roots were collected from eight mature *Populus tremuloides* stands in northern Alberta, Canada.

Exploration Type	Morphotype	Description
Contact	E4	Tan; smooth
	E5	Brown; rough; grainy; no emanating hyphae
	E12	Very swollen; light tan; smooth; no emanating hyphae
	E13	Darker brown; beaded shape; very small emanating hyphae
	E20	Brown; grainy; cylindrical; few small hyphae
Distance	E1	Cenococcum-like; black; grainy texture; wiry black hyphae
	E2	Small; white; fuzzy; emanating white hyphae
	E11	White; fuzzy; thick rope-like rhizomorphs.
	E21	Very branched, grey, grainy, black emanating hyphae
	E25	Yellow; fuzzy; yellow rope-like rhizomorphs

Table 3-3. Analysis of variance for the effect of host phenological stage on total nonstructural carbohydrate concentration, sugar concentration, and starch concentration. Fine roots were collected from mature *Populus tremuloides* stands (n = 8) in northern Alberta, Canada. Host phenological stages includes dormancy, leaf flush, full leaf expansion, and leaf abscission.

Source	**Df**	**Sum. Sq**	***F*-value**	***P*-value**
Total NSC[1]	3	344.800	47.240	<0.0001
Residuals	21	51.100	2.430	
Sugar[2]	3	66.520	13.91	<0.0001
Residuals	21	33.480	1.594	
Starch[3]	3	13.630	4.543	<0.0001
Residuals	21	0.771	0.037	

[1]Nonstructural carbohydrate concentration (percent dry mass)
[2]Sugar concentration (percent dry weight)
[3]Starch concentration (percent dry weight)
df: degrees of freedom, Sum Sq: sum of squares

Table 3-4. Pearson correlation coefficients among potential enzyme activities of ectomycorrhizal root tips, fine root sugar concentrations, and soil conditions across four phenological stages (dormancy, leaf flush, full leaf expansion, and leaf abscission) of *Populus tremuloides*. Roots were collected from mature *P. tremuloides* stands in northern Alberta, Canada. Bold values denote a significant correlation ($p < 0.05$, n = 8 sites). Bold, italic values denote a marginally significant correlation ($p < 0.10$). Abbreviations: Gls: *β*-glucosidase; Glr: *β*-glucuronidase; Nag: *N*-acetylglucosaminidase; Lac: laccase; Inv: root invertase; Temp: soil temperature; SWC: soil water content.

	Gls	Glr	Nag	Lac	Inv	Sugar	Starch	Temp
Gls								
Glr	***0.34***							
Nag	**0.68**	0.19						
Lac	**0.54**	0.17	**0.45**					
Inv	-0.14	-0.21	-0.08	0.11				
Sugar	**-0.47**	-0.18	-0.22	-0.10	**0.49**			
Starch	-0.17	-0.10	0.16	0.18	0.27	**0.47**		
Temp	-0.19	-0.20	0.17	0.00	**0.38**	**0.55**	**0.82**	
SWC	**-0.31**	**-0.42**	-0.29	-0.22	**0.42**	**0.44**	-0.15	-0.08

Table 3-5. Analysis of variance for the effect of host phenological stage on potential enzyme activity of ectomycorrhizal root tips collected from mature *Populus tremuloides* stands (n=8) in northern Alberta, Canada. Host phenological stage includes dormancy, leaf flush, full leaf expansion, and leaf abscission.

Source	**Df**	**Sum. Sq**	**Mean Sq.**	***F*-value**	***P*-value**
β-glucuronidase	3	1.199	0.400	3.269	0.042
Residuals	21	2.566	0.122		
β-glucosidase	3	23.56	7.852	1.308	0.298
Residuals	21	126.04	6.002		
N-acetylglucosaminidase	3	96.0	31.990	1.298	0.301
Residuals	21	517.6	24.650		
Laccase	3	718766	260589	2.586	0.080
Residuals	21	2115805	100753		
Invertase	3	17.538	5.846	12.82	<0.0001
Residuals	21	9.573	0.456		

df: degrees of freedom, Sum Sq: sum of squares, Mean Sq: Mean Square

Table 3-6. Operational taxonomic units of ectomycorrhizal fungi with BLAST and UNITE species hypothesis (SH) matches, and presence during host phenological stages (dormancy: D, leaf flush: LF, full leaf expansion: LE, and leaf abscission: LA). Operational taxonomic units were assembled with quality filtered sequences from amplified fungal rDNA. Roots were collected from mature *Populus tremuloides* stands (n = 8) in northern Alberta.

Best match	BLAST ID	Query length	Max score	Query cover	UNITE SH	UNITE Accession	Distance to closest SH	D	LF	LE	LA
Russula xerampelina voucher	FJ845433	747	1339	100	*Russula xerampelina*	SH176552.07FU	1.5	X	X	X	
Cenococcum geophilum clone	JN129390	1025	1749	98	*Cencococcum geophilum*	SH199612.07FU	1.5	X	X		X
Uncultured *Thelephora* clone	HQ204725	691	1064	95	Uncultured *Thelephora*	SH189427.07FU	1.5				X
Russula bicolor voucher	GU966633	452	804	99	*Russula bicolor*	SH218422.07FU	1.5		X	X	
Uncultured ectomycorrhizal (*Tomentella*) isolate	EF218835	674	1181	100	Uncultured *Tomentella*	SH184521.07FU	1.5	X			
Thelephoraceae sp.	U83467	687	1253	99	Thelephoraceae	SH177833.07FU	1.5			X	
Leccinum populinum	KM248965	814	1430	97	*Leccinum populinum*	SH181314.07FU	1.5		X		
Cortinarius subexitiosus voucher	KP165574	613	1122	100	Uncultured *Cortinarius*	SH188571.07FU	1.5			X	
Uncultured *Russula* clone	JQ393112	645	1147	99	Uncultured *Russulaceae*	SH187182.07FU	1.5				X
Uncultured ectomycorrhiza (*Russula*) isolate	EF218804	735	1260	97	Uncultured *Russula*	SH219258.07FU	1.5				X
Uncultured *Tomentella*/*Thelephora* clone	JQ346862	670	1195	100	Uncultured *Tomentella*/*Thelephora*	SH189369.07FU	1.5		X		
Phialocephala fortinii strain	KM460828	501	880	98	*Phialocephala fortinii*	SH204986.07FU	1.5	X			
Russula sp.	GU981742	657	1094	95	*Russula* sp.	SH180254.07FU	1.5		X		
Boletus subglabripes isolate	KM248936	838	1447	94	*Boletus* sp.	SH209318.07FU	1.5				X
Cortinarius cedriolens isolate	HQ604729	582	1035	96	*Cortinarius cedriolens*	SH188478.07FU	1.5			X	
Uncultured *Cortinarius* clone	FJ554223	490	857	99	Uncultured *Cortinarius*	SH188478.07FU	1.5			X	
Piloderma lanatum isolate	JQ711873	370	660	100	*Piloderma lanatum*	SH212907.07FU	1.5		X		
Russula brevipes voucher	FJ845429	721	1328	100	*Russula brevipes*	SH220517.07FU	1.5			X	

Table 3-7. Effects of ectomycorrhizal exploration type, host phenology and their interaction on potential enzyme activity of ectomycorrhizal root tips collected from mature *Populus tremuloides* stands in northern Alberta, Canada (n = 8). Host phenological stages include dormancy, leaf abscission, full leaf expansion, and leaf abscission. Exploration type includes contact and distance ectomycorrhizal fungi.

Source	**df**	**Sum Sq**	***F*-value**	***P*-value**
β-glucuronidase [1]				
exploration type	1	0.679	5.084	0.028
host phenology	3	2.256	5.633	0.002
exploration type x host phenology	3	0.078	0.196	0.899
residuals	56	7.476		
β-glucosidase [1]				
exploration type	1	0.200	0.009	0.926
host phenology	3	85.000	1.468	0.233
exploration type x host phenology	3	8.500	0.147	0.931
residuals	56	1080.500		
N-acetylglucosaminidase [1]				
exploration type	1	1.636	5.399	0.024
host phenology	3	0.923	1.015	0.393
exploration type x host phenology	3	0.253	0.278	0.841
residuals	56	16.973		
	df	**Chi-square**		***P*-value**
Laccase [2]				
exploration type	1	3.739		0.053
host phenology	3	3.034		0.387

[1]Calculated with two-way analysis of variance.
[2]Calculated with Kruskal-Wallis test.
df: degrees of freedom, Sum Sq: sum of squares

Table 3-8. Effects of host phenological stage on the relative abundance of contact and distance ectomycorrhizal exploration types. Ectomycorrhizal root tips were collected from mature *Populus tremuloides* stands in northern Alberta, Canada (n = 8). Host phenological stages include dormancy, leaf abscission, full leaf expansion, and leaf abscission.

	df	**Sum. Sq**	**Mean Sq**	***F*-value**	***P*-value**
Contact	3	0.014	0.005	0.269	0.847
Residuals	28	0.483	0.017		
Distance	3	0.272	0.091	6.011	0.003
Residuals	28	0.422	0.015		

df: degrees of freedom, Sum Sq: sum of squares, Mean Sq: Mean square

Figures

Figure 3-1. (a) Mean daily air temperature (°C) for the Conklin, Alberta, Canada area and mean daily soil temperature (°C) for the eight mature *Populus tremuloides* stands. Air temperature was retrieved from the nearest weather station in Fort McMurray, Alberta, Canada. (b) Total precipitation (mm) for the Conklin, Alberta, Canada area and volumetric water content (%) for the eight mature *P. tremuloides* stands. Total precipitation was retrieved from the nearest weather station in Fort McMurray, Alberta, Canada. Arrows indicate sampling dates.

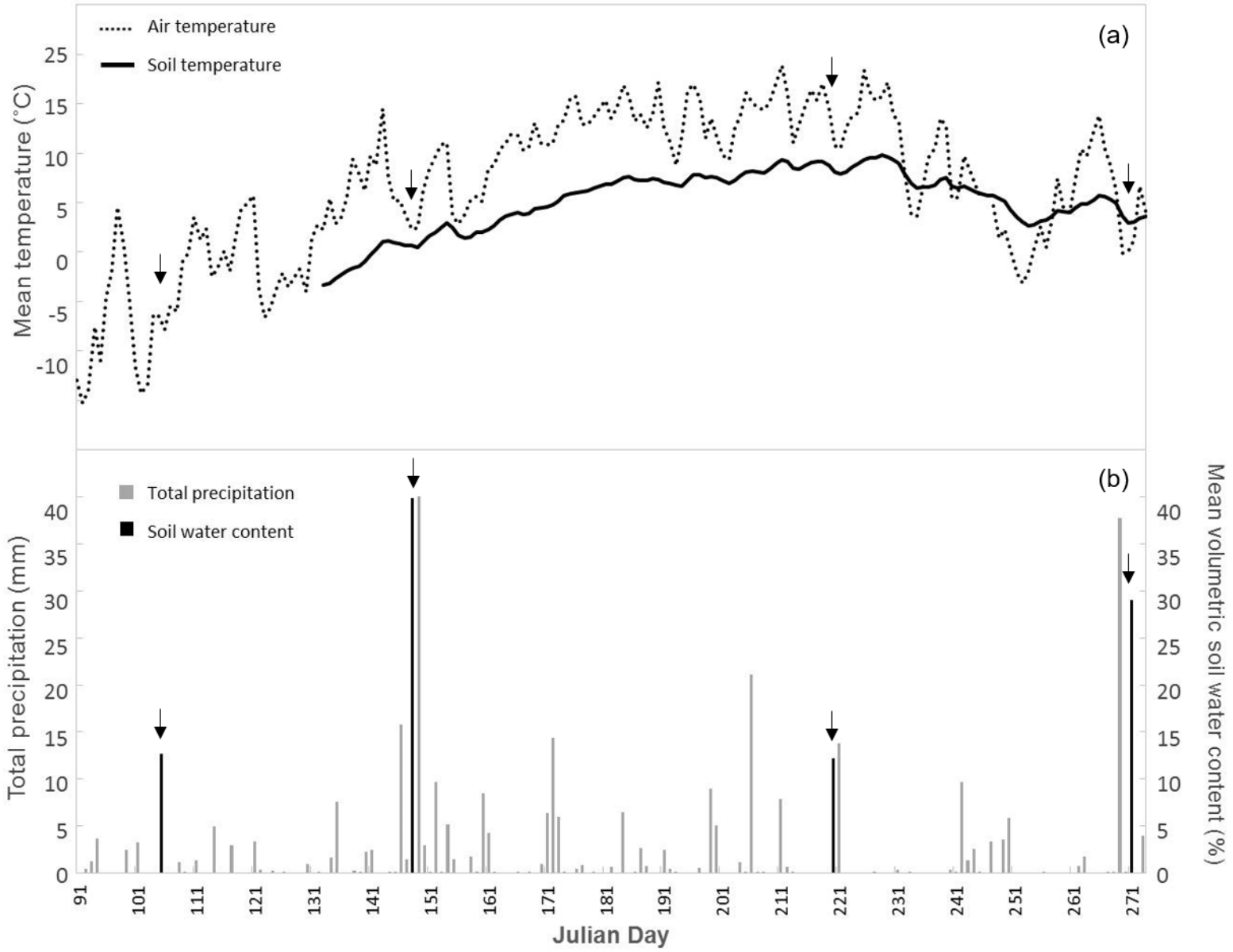

Air temperature
Soil temperature
(a)
Mean temperature (°C)
25
15
10
5
0
-5
-10
Total precipitation
Soil water content
(b)
Total precipitation (mm)
Mean volumetric soil water content (%)
40
35
30
25
20
15
10
5
0
91
101
111
121
131
141
151
161
171
181
191
201
211
221
231
241
251
261
271
Julian Day

Figure 3-2. Mean nonstructural carbohydrate concentration of fine roots collected from mature *Populus tremuloides* stands (n = 8) during four phenological stages (D: dormancy, LF: leaf flush, LE: full leaf expansion, LA: leaf abscission). Across phenological stages upper and lowercase letters denote significant differences among means (± 1 standard error) of starch and sugar concentrations, respectively (α = 0.05).

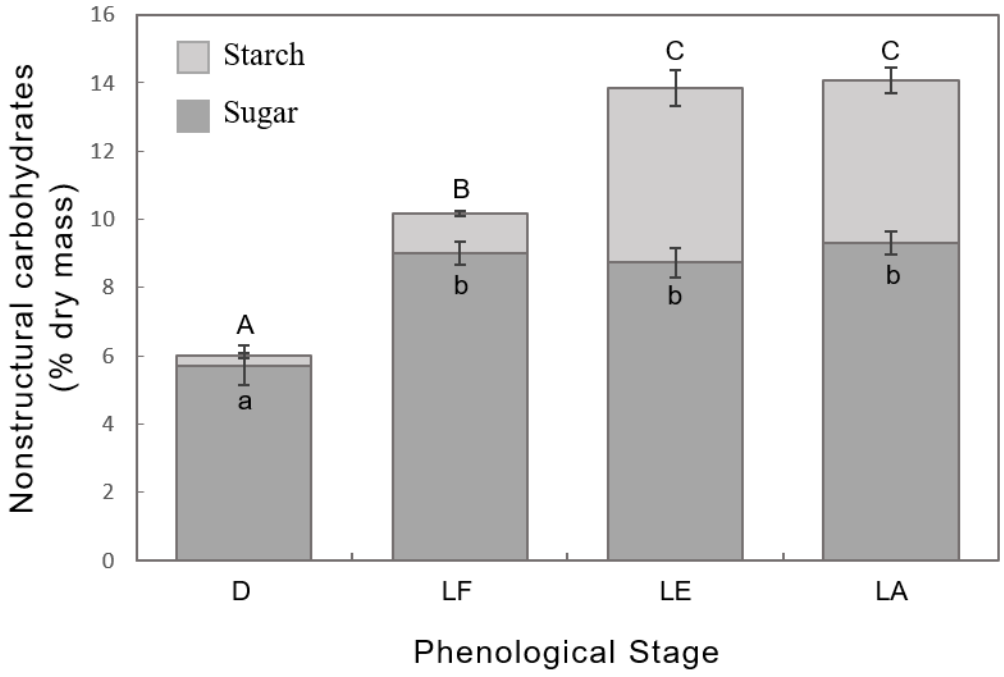


Figure 3-3. Mean (± 1 standard error) potential activity of (a) β-glucuronidase (Glr), (b) β-glucosidase (Gls), (c) *N*-acetylglucosaminidase (Nag), (d) laccase (Lac), and (e) root invertase (Inv) of ectomycorrhizal root tips across four phenological stages of mature *Populus tremuoides* (D: dormancy, LF: leaf flush, LE: full leaf expansion, LA: leaf abscission). Ectomycorrhizal roots were collected from *P. tremuloides* stands (n = 8) in northern Alberta, Canada. Letters denote significant differences in activity among phenological stages ($\alpha = 0.05$). All enzyme values are reported in in (pmol mm^{-2} min^{-1}).

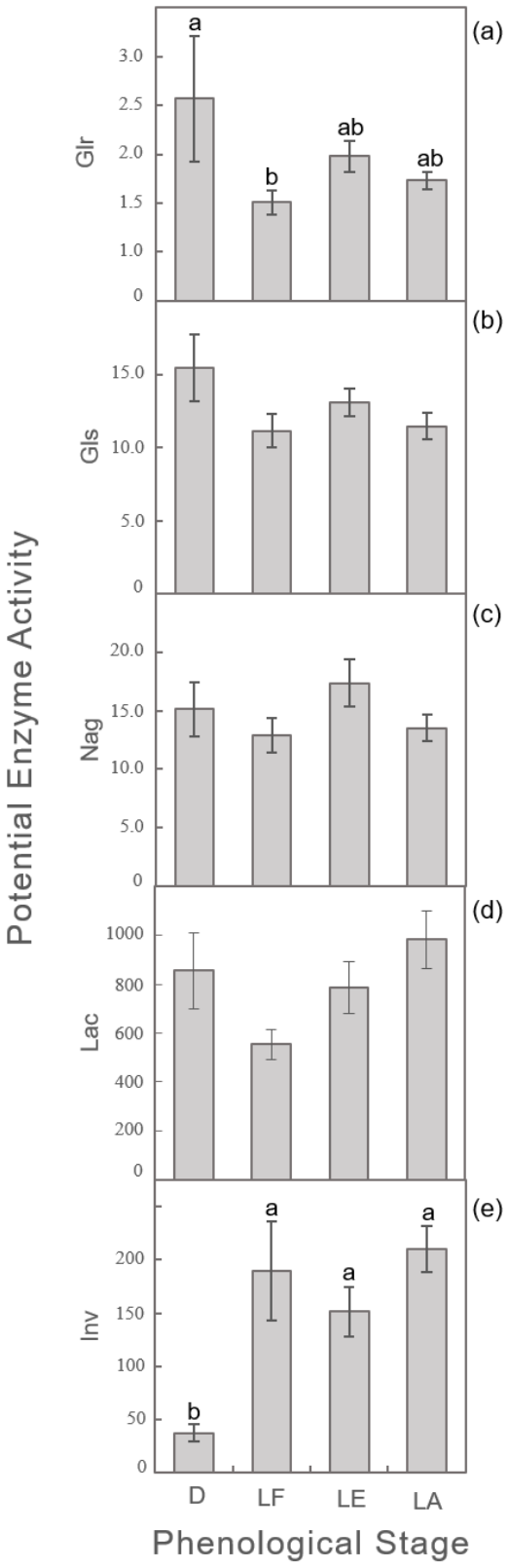

Potential Enzyme Activity
(a)
Glr
3.0
2.5
2.0
1.5
1.0
0
a
b
ab
ab
(b)
Gls
15.0
10.0
5.0
0
(c)
Nag
20.0
15.0
10.0
5.0
0
(d)
Lac
1000
800
600
400
200
0
(e)
Inv
200
150
100
50
0
b
a
a
a
D
LF
LE
LA
Phenological Stage

Figure 3-4. Relative abundance of ectomycorrhizal fungal taxa. Roots were collected from mature *Populus tremuloides* stands (n=8) in northern Alberta, Canada during host dormancy (D), leaf flush (LF), full leaf expansion (LE), and leaf abscission (LA). Owing to differences in sequencing success, fungi forming ectomycorrhizas were not able to be identified at four and two stands during host dormancy and leaf abscission, respectively.

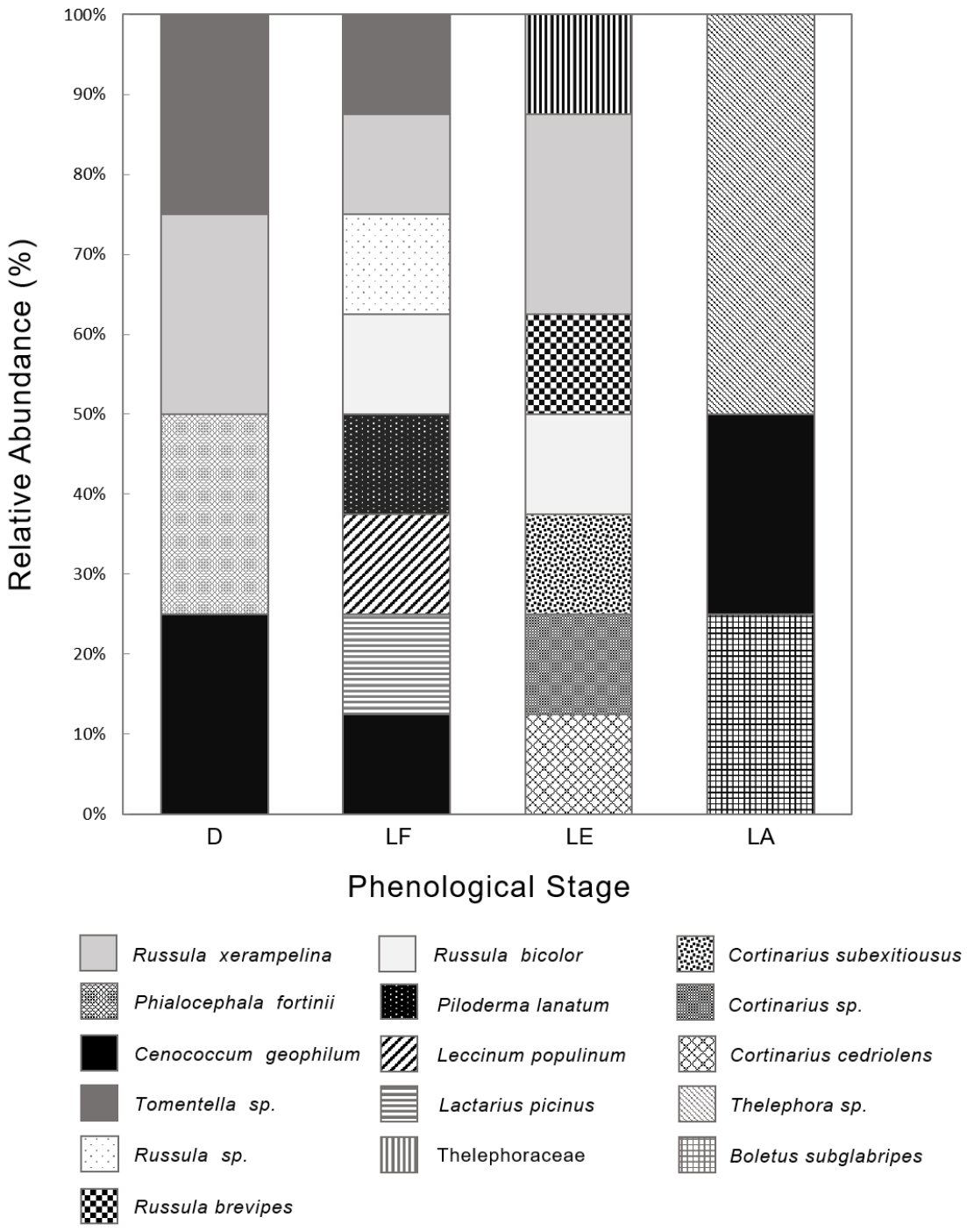

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative Abundance (%)
D
LF
LE
LA
Phenological Stage
Russula xerampelina
Russula bicolor
Cortinarius subexitiousus
Phialocephala fortinii
Piloderma lanatum
Cortinarius sp.
Cenococcum geophilum
Leccinum populinum
Cortinarius cedriolens
Tomentella sp.
Lactarius picinus
Thelephora sp.
Russula sp.
Thelephoraceae
Boletus subglabripes
Russula brevipes

Figure 3-5. Mean (± 1 standard error) potential activity of (a) β-glucuronidase (Glr), (b) β-glucosidase (Gls), (c) *N*-acetylglucosaminidase (Nag), and (d) laccase (Lac) of contact and distance exploration-type ectomycorrhizal root tips. Roots were collected from mature *Populus tremuloides* stands (n = 8) in northern Alberta, Canada during host dormancy (D), leaf flush (LF), full leaf expansion (LE), and leaf abscission (LA). All enzyme values are reported in (pmol mm^{-2} min^{-1}). Upper and lowercase letters denote significant differences in activity among exploration types and phenological periods, respectively ($\alpha = 0.05$).

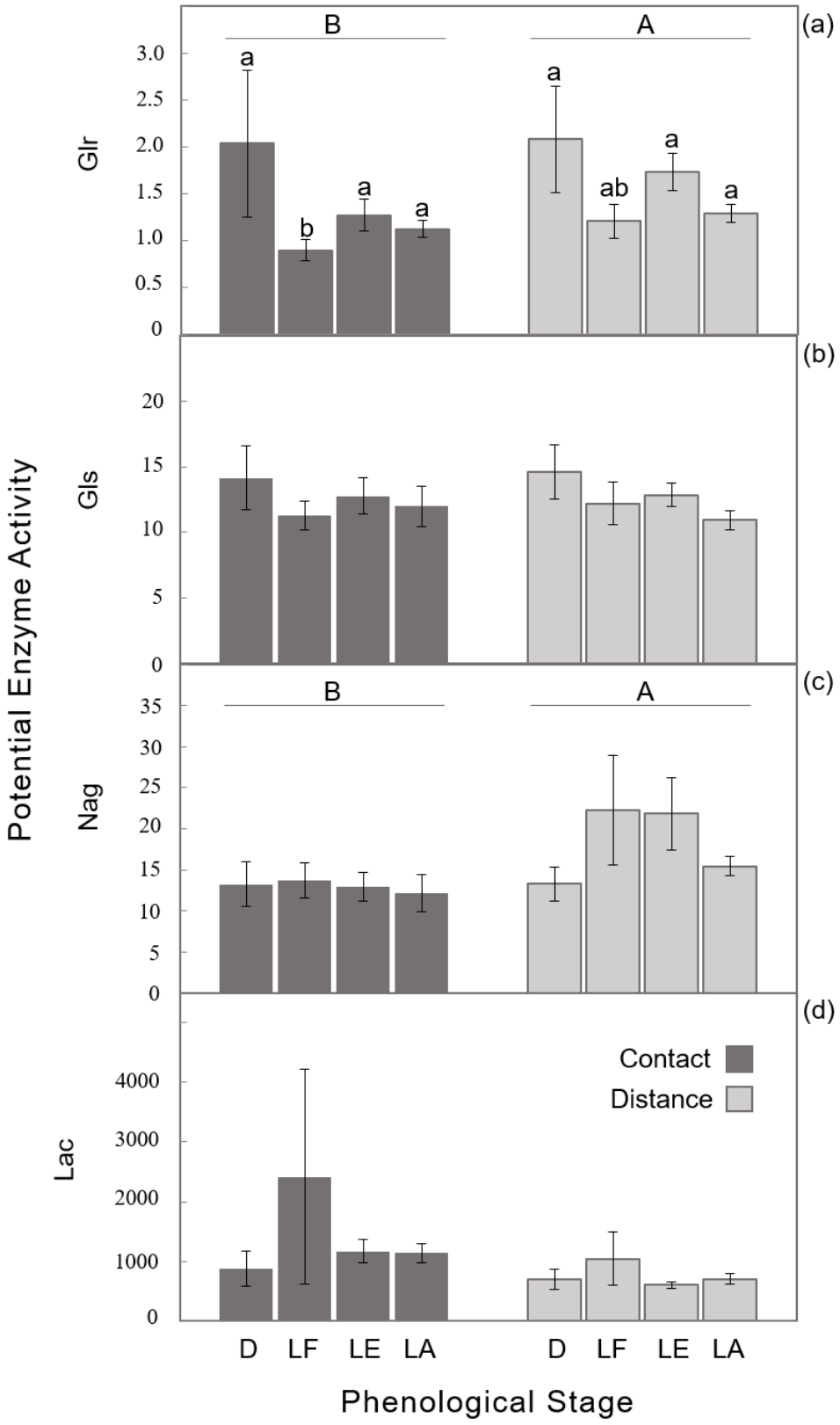
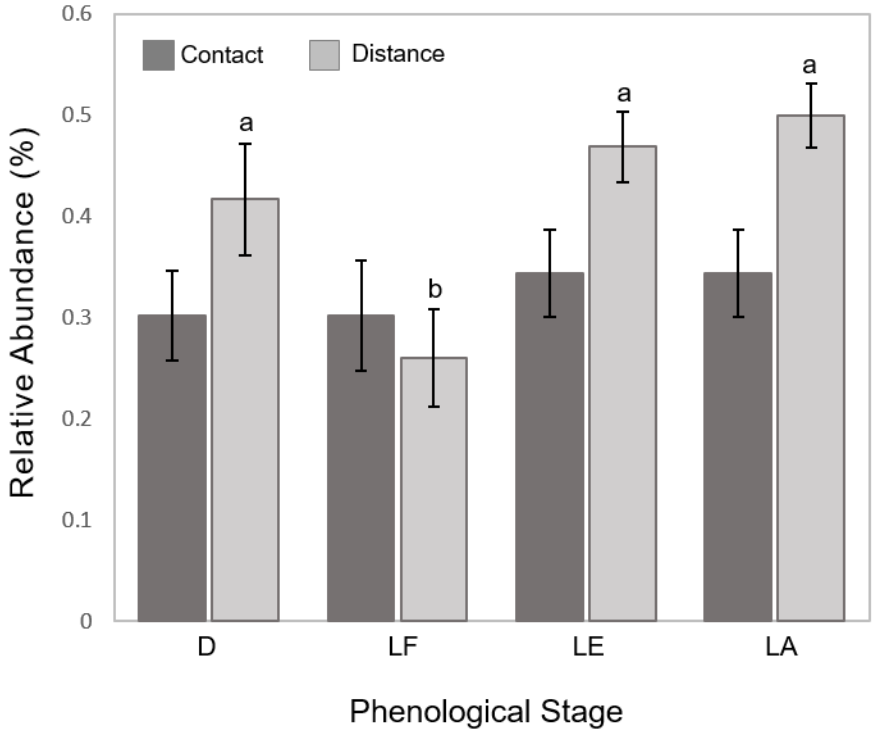

Potential Enzyme Activity
Glr
Gls
Nag
Lac
(a)
(b)
(c)
(d)
B
A
a
b
ab
Contact
Distance
D
LF
LE
LA
Phenological Stage

Figure 3-6. Relative abundance of contact and distance ectomycorrhizal fungal exploration types across four phenological stages of mature *Populus tremuoides*. Phenological stages include dormancy (D), leaf flush (LF), full leaf expansion (LE) and leaf abscission (LA). Ectomycorrhizal roots were collected from mature *Populus tremuloides* stands (n = 8) in northern Alberta, Canada. Letters denote significant (α = 0.05) differences in relative abundance among phenological stages.



Chapter 4: General Discussion and Conclusion

4.1 Research Summary

Given that ectomycorrhizal fungi influence tree growth and survival, and nutrient cycling in the boreal biome (Read *et al.* 2004; van der Heijden *et al.* 2008; Clemmensen *et al.* 2013), understanding current shifts in ectomycorrhizal fungal communities in response to environmental change (anthropogenic and natural) is fundamental to predicting the future structure and function of boreal forests. The goals of my research were to (1) examine the recovery of ectomycorrhizal fungi (EMF) following a gradient of above and belowground anthropogenic disturbances, and (2) monitor the response of EMF communities to phenology-driven changes in host carbon allocation. Toward addressing the first goal, EM fungal community composition was characterized in three cover soils commonly used in upland boreal forest restoration and compared to a range of ecologically relevant benchmarks to identify potential successional trajectories created by common reclamation practices. To address the second goal, to determine the potential role EMF play in organic matter decomposition, we measured the secretion of organic matter-degrading enzymes of ectomycorrhizal roots during four phenological stages of mature *Populus tremuloides*.

The integral relationships between trees, soil, and ectomycorrhizal fungi generate challenges when evaluating their independent roles in disturbances (Onwuchekwa *et al.* 2014; Hankin, Karst & Landhäusser 2015). I decoupled the effects of tree species from those of soils on EMF communities by assaying fungi in three different cover soils using three native species of outplanted seedlings. To evaluate a range of above and belowground disturbances on EMF community recovery, I then compared my findings from the reclaimed site to those from sites where soils remain intact but a gradient of aboveground disturbance existed. I predicted that

EMF community composition would be affected by cover soil and disturbance type, with the most diverse community found in undisturbed forests. Moreover, I anticipated that salvaged forest floor material used in reclamation would retain an EMF community most similar to those in soils of harvested stands.

Contrary to my predictions, however, all cover soils (peat, forest floor material, subsoil) and aboveground disturbance benchmarks (undisturbed, trees removed, trees + forest floor removed) have a similar EMF community when assayed with outplanted seedlings. However, we found a host × cover soil interaction, indicating the effect of cover soil on EM fungal community composition depends on host species. Our results imply that young, outplanted seedlings may select for similar EMF communities regardless of the extent of above or belowground disturbance. Moreover, the fungi colonizing roots of young, outplanted seedlings are insensitive to the extent of above or belowground disturbance. Consistent with the previous survey by Hankin, Karst & Landhäusser (2015), was the effect of host species identity on EMF community composition. Planting reclaimed sites with a diverse set of tree species may therefore lead to a more diverse EMF community. On the other hand, two growing seasons may not be an adequate timeframe to observe deviations in successional trajectories created by different reclamation practices. Although our sampling intensity of mature *P. tremuloides* in Chapter 3 was less than that of outplanted seedlings in Chapter 2, comparing the EMF community present on outplanted *P. tremuloides* seedlings with the EMF community present on mature *P. tremuloides* reveals notable differences. Specifically, all outplanted seedlings retained high abundances of *Hebeloma* species and, to a lesser extent, *Russula laccata* and EMF in the Pezizales order. These taxa were not found on mature *P. tremuloides*, which were instead colonized by high abundances of *Russula xerampelina*, *Russula brevipes*, *Russula bicolor*, and several *Cortinarius* species. This

might further support the pattern of EMF community assembly we observed on outplanted seedlings.

My second objective for this thesis was to test the potential decomposing activity of the EMF community in response to phenology-driven fluctuations in host carbon allocation. During the stages of host dormancy, leaf flush, full leaf expansion, and leaf abscission, I measured potential organic matter-degrading enzyme activity of ectomycorrhizal root tips, in addition to fine root nonstructural carbohydrate concentrations and the potential level of carbon made available to EMF in the plant-fungus interface. I predicted that EMF would secrete higher levels of organic matter degrading enzymes during leaf flush and leaf abscission in response to anticipated decreases in host carbon acquisition, following a 'saprotrophy model'. Potential enzyme activity did not follow the predicted trend, with phenology affecting only one enzyme, β-glucuronidase. Moreover, the potential activity of β-glucuronidase was not in the pattern we predicted based on the saprotrophy model. These results suggest that these enzymes are secreted in tandem by EMF to break apart organic material and release nitrogen or phosphorus locked within carbon compounds, and subsequent carbon acquisition is a secondary outcome. Moreover, EMF exploration types may have different abilities to decompose organic matter. We found distance types to have higher potential activity of β-glucuronidase and *N*-acetylglucosaminidase, while contact exploration types had marginally higher potential laccase activity. These differences highlight the unique roles exploration types have in organic matter decomposition throughout phenological stages of host trees.

Using exploration type to infer decomposing activity may be useful in predicting carbon and nutrient pathways in the Reclamation Site. Previous work has suggested that young, undeveloped soils will support a higher abundance of long-distance exploration types than short-

distance or contact types, due to enhanced foraging ability in nutrient-poor sites and low root density (Peay *et al.* 2011; Suz *et al.* 2014; Clemmensen *et al.* 2015; Dickie *et al.* 2015). If this is also true in boreal forests colonized by *P. tremuloides*, an EMF community in a recently disturbed site dominated by long-distance exploration types may indicate higher degradation of cellulose- and chitin-containing organic material, while the EMF community in a mature *P. tremuloides* stand is dominated by lignin-degrading contact exploration types. In an old-growth Northern hardwood stand, Burke *et al.* (2014) found high levels of phenol oxidase activity by *Russula* species. Perhaps this indicates the shifting role of the EMF community, from cellulose and chitin degraders to lignin degraders, as the site develops into a mature forest.

On the other hand, the EMF community of *P. tremuloides* seedlings in the Reclamation Site had a higher abundance of EMF taxa typically classified as contact or short-distance exploration types (Agerer 2001), suggesting that in highly disturbed sites, the EMF community has a larger role in lignin degradation than cellulose or chitin degradation. These findings may further support the need for identifying appropriate ecological benchmarks in restoration practices due to the unexpected composition of EMF exploration types observed in the Reclamation Site.

4.2 Experimental Limitations and Future Directions

Assessing EMF diversity in bulk soil with high-throughput sequencing techniques has been shown to yield a high number of species when compared to sampling root tips (Taylor *et al.* 2014) suggesting that our method of root-tip level sampling may not have captured the entire community colonizing seedling roots. High-throughput sequencing of bulk soil may further be useful when assessing the propagule bank retained by soil reconstruction. Additionally, Sanger

sequencing often yielded more than one fungal species on a single root tip, an outcome that may be avoided if sequencing is performed using high-throughput sequencing. To best capture the EMF community at a given site, bulk soil sampling may provide the most robust sampling method. Additionally, the presence of ectomycorrhizal fungal propagules present in the reconstructed soils prior to seedling outplanting was not assessed. A pre-treatment record of initial EMF community composition would reveal any differences in that obtained by outplanted seedlings and the initial soil propagule bank. Aerial dispersal of ectomycorrhizal fungal spores has also been found to vary through space and time (Peay & Bruns 2014), and therefore may warrant investigation when monitoring community assembly in a disturbed site.

When monitoring the potential decomposing abilities of EMF throughout phenological stages we used potential enzyme activity to infer how carbon is transferred in the plant-fungus-soil continuum, an indirect but useful sampling procedure supported by previous work (Courty *et al.* 2007; Pritsch *et al.* 2011; Tedersoo *et al.* 2012). Carbon labelling may be a more direct and accurate method for monitoring the flow of carbon through ecosystems (Treseder *et al.* 2006; Bréda *et al.* 2013), although its feasibility may be limited in large field sites requiring multiple sample collections. Further, it may be difficult to assess the net amount of carbon moving through a system, and identifying carbon in the rhizosphere as litter-derived or tree-derived may prove challenging (Singh *et al.* 2004; Jones, Nguyen & Finlay 2009). Additionally, soil microbial enzyme activity is quite variable, and attributing our measured values to EMF alone may not be an accurate representation of the microbial community. Moreover, the ability of EMF to secrete the particular set of enzymes measured in my study has been debated (Baldrian 2009; Cullings & Courty 2009), and the difficulty in directly isolating and measuring EMF tissue without disrupting enzymatic pathways remains a challenge. Genome sequencing has aided in

our understanding of whether EMF possess the genes which encode for these particular enzymes (Parrent *et al.* 2009; Kohler *et al.* 2015), however the number of species with sequenced genomes is limited. Further investigations into the functional traits of ectomycorrhizal fungal species and their role in the boreal forest are critical for the restoration of the plant-soil continuum.

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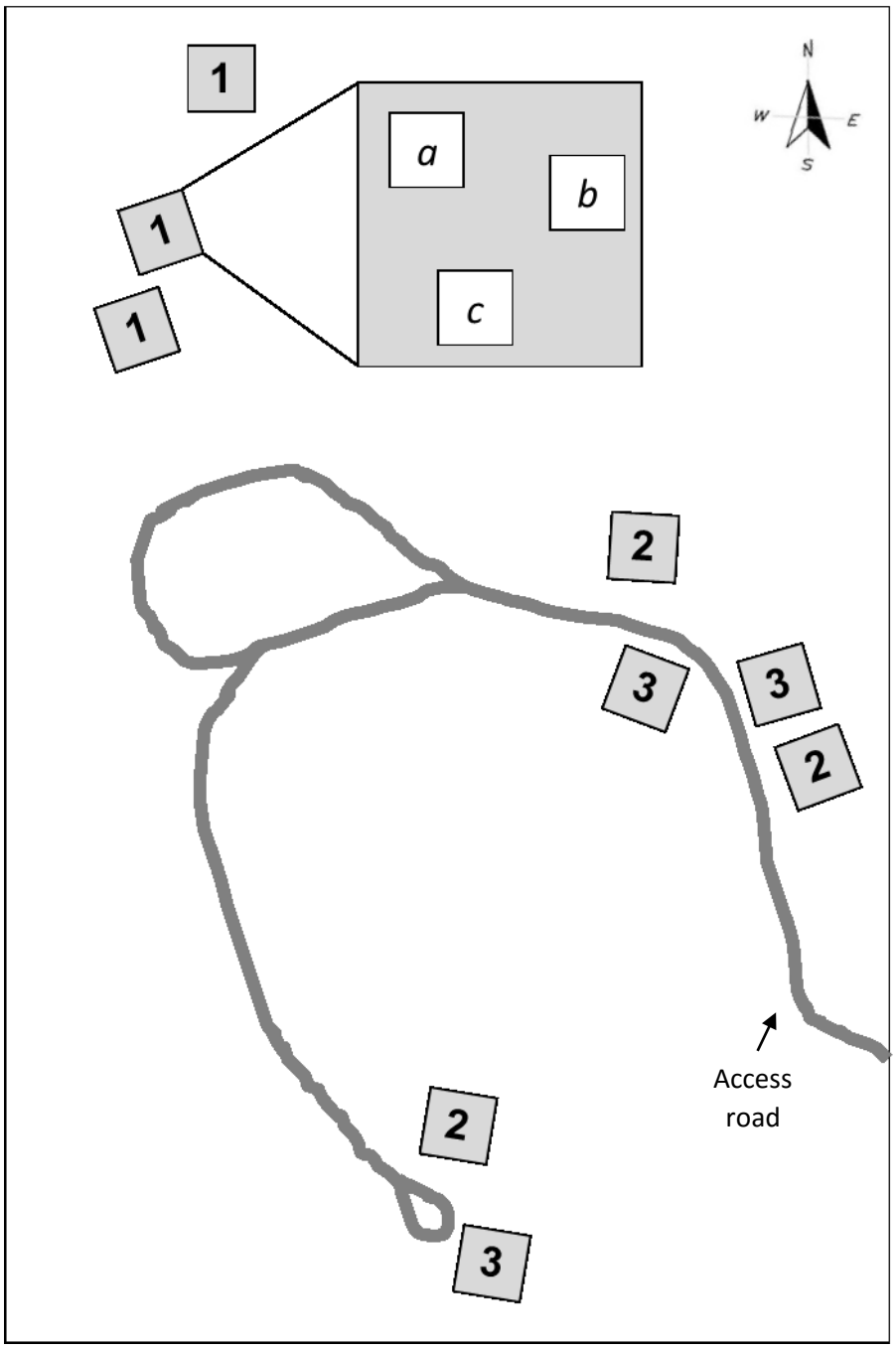
Appendices

Appendix I. Layout of the Reclamation Site, showing soil capping treatments of peat, forest floor material (FFM), and subsoil (n=3). Each replicate contains a 25 x 25 m single-species tree plot of *Populus tremuloides, Pinus banksiana,* and *Picea glauca* planted at 10,000 stems per hectare used to assay the ectomycorrhizal fungal community. Tree plots are labeled as "A" (*P.tremuloides*), "P" (*P. banksiana*), or "S" (*P. glauca*) (Hankin, Karst & Landhäusser 2015).

Peat
P
S
A
Subsoil
P
S
A
FFM
S
P
A
Subsoil
P
S
A
FFM
P
A
S
Peat
P
A
S
FFM
A
S
P
Peat
S
P
A
Subsoil
S
A
P
N
E
S
W
0
10
25
50
100
250
500
scale in meters

Appendix II. Layout of plots at the 'Benchmark Site' used to compare ectomycorrhizal fungal communities with those assayed at the Aurora Soil Capping Study. The Benchmark Site was located approximately 5 km northeast of the Aurora Soil Capping Study on the Syncrude Canada Ltd. – Aurora mine site. Treatments of undisturbed (1), trees removed (2), and trees + forest floor removed (3) are replicated three times, with each replicate containing three 2.5 x 2.5 m plots (*a* – *c*) separated by at least 2 m. In May 2012, each plot was planted with 6-8 seedlings of *Populus tremuloides, Pinus banksiana,* and *Picea glauca* from the same seedling stock used for the Reclamation Site.


1
1
1
a
b
c
N
W
E
S
2
3
3
2
2
3
Access
road