Exploring how soil fungi can be used to restore native trees on reclaimed substrates containing petroleum hydrocarbons

by

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Abstract

As part of reclaiming landscapes mined for bitumen, ecosystems must be revegetated to restore self-sustaining ecosystems in the boreal forest of western Canada. Current reclamation practices can involve the construction of large landforms, sometimes from overburden containing up to 8% hydrocarbons, known as lean oil sand (LOS). This overburden is capped with a layer of salvaged soils, which acts as a base for revegetation. However, residual petroleum hydrocarbons can be present in these reclaimed landforms, and it is unclear what effect they may have on establishing native vegetation. In this same region, forests occur on natural surficial bitumen deposits and some vegetation persists on abandoned ore piles. Understanding how trees are able to survive in naturally occurring shallow-bituminous soils may be key to restoring forests on reclaimed landscapes, where residual petroleum hydrocarbons remain. The majority of the boreal forest plants interact with a group of root-associated fungi, namely mycorrhizal fungi. In addition to providing nutritional benefits, these fungi also have been found to increase a plant's tolerance to refined petroleum hydrocarbons. Exploring the interaction between trees and mycorrhizal fungi in naturally occurring bituminous soils provides a unique opportunity to investigate whether mycorrhizas promote plant tolerance to residual hydrocarbons in LOS. First, I tested whether LOS is detrimental to plants as a result of hydrocarbons and/or barriers including poor nutrient concentration, water availability, and air availability. I found even at low concentrations (0.88%); LOS reduces plant growth. I also found that none of the 'barrier modifications' alleviated the detrimental effects of LOS on plant biomass. Next, I surveyed soil fungi across sites varying in hydrocarbon concentrations and fractions. I then used small amounts of soil from the field sites as inoculum for two conifer species grown in pots. The effect of inoculum varied by origin and tree species suggesting that soil inoculum may be an effective method for establishing some tree species on reclaimed landscapes. This experiment was followed by further testing across several

experiments on the effect of LOS on fungal growth. I found that even at low concentrations (1.5%), LOS reduces fungal growth. Overall, my research provides evidence that even at low concentrations, if LOS is present within the rooting zone, plant and fungal growth may be impeded.

Preface

This thesis is an original work by James Franklin. No part of this thesis has been previously published

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Chapter 1: Thesis Introduction

1.1 Background

Canadian boreal forests cover 552 million hectares and make up approximately 55% of the landscape (Brandt, 2009). These forest ecosystems are often disturbed by non-anthropogenic agents including insect outbreaks, diseases, and forest fires (Audet et al., 2014). These forests are also rich in natural resources, including timber, minerals, natural gas, and oil, the extraction of which also cause large-scale disturbances (Pickell et al., 2015). One recent disturbance in western boreal forests is oil sands mining. The oil sands are found primarily in Northern Alberta within the boreal plain ecozone dominated by upland forests and wetlands (Audet et al., 2014). Oil sands mining disturbs entire ecosystems, with the process involving the removal of trees, understory vegetation, and surface soils, which are often stockpiled or used in reclamation projects, followed by the removal of overburden above the ore (Alberta Environment, 2010). Today mining companies are required by provincial law to reclaim the disturbed landscapes to equivalent land capability (Alberta Environment, 2010). This presents many challenging prospects including reconstructing landforms, developing suitable rooting mediums, replanting native vegetation, and restoring soil microbial communities and their activity (Macdonald et al., 2015). Given the magnitude, severity, and high cost of reclaiming these disturbed landscapes, it is necessary to develop effective and efficient techniques to meet provincial regulations, and use evidence-based research to develop new reclamation strategies.

1.2 History of Canadian oil sands

Oil sand is a mixture of quartz sand, clay, other trace minerals, and hydrocarbons (Larter and Head, 2014). Individual grains of sand are surrounded by a thin coat of water which is then surrounded by a layer of bitumen (Government of Alberta, 2009). Bitumen is a heavy crude oil that differs from other lighter oils by its high viscosity, density, and concentration of sulphur and heavy metals (Meyer and Attanasi et al., 2003). The most common theory for the origin of the oil sand in Canada is that during the late Cretaceous period, oil trapped in Mississippian to Jurassic sedimentary rock migrated northeast through permeable regions pushed by the Pacific plate crushing into the North American plate (Tozer *et al.,* 2014). In northeastern Alberta, the oil was trapped by the Clearwater shale deposit, forming a barrier to its movement (Czarnecki *et al.,* 2005). The lighter fraction of this oil was decomposed by microbes leaving behind viscous oil, which over time mixed with the quartz sand and clay (Zhou *et al.,* 2008).

The earliest European record of the Canadian oil sands was 1719 when samples were traded by Waupisoo of the Cree people to Henry Kelsey of the Hudson Bay Company (Stringham 2012). Further documentation of the oil sands followed including in 1790 when Sir Alexander Mackenzie travelled through the area, describing Indigenous people mixing bitumen with spruce gum to waterproof and patch their canoes (Stringham 2012). Over the next centuries, various surveys of the region noted bitumen seeping from outcrops but any attempt at drilling for the reservoirs, believed to be below the surface, was met without success (Chastko 2004). By the start of the 20th century, researchers began to attempt to extract bitumen from oil sand. In the 1920s, Dr. Karl Clark developed and patented a method of mixing oil sands with hot water and aerating the mixture to separate bitumen (Chastko 2004). The first commercial plant was built at Bitumount, Alberta by Robert Fitzsimmons, constructed North of Fort McMurray along the Athabasca River (Ferguson et al., 1985). Due to a lack of funding, the plant passed through various owners, with the Alberta government taking ownership in 1948 (Chastko 2004). The plant operated intermittently for the next decade until operations ceased permanently in 1958. In 1974, Bitumount was declared a provincial historic site, however, to ensure the area is preserved, public access was prohibited (Sweeney 2010). To date, no attempt has been made to reclaim the site. However, a variety of plant species, including *Pinus banksiana* grow on the abandoned ore pile.

1.3 Current mining practices

The Canadian oil sands are primarily located within northern Alberta and are made up of three main regions, Athabasca, Cold Lake, and Peace River. Combined these three regions cover an area of 142,000 km^2 (Stringham 2012). Acquisition of bitumen from the oil sands is separated into two major categories based on how far below the surface the oil sand is found. *In-situ* methods are used for deposits found > 75 meters below the surface and account for over 80% of bitumen recovery in Canada. This method is primarily used in the southern Athabasca, Peace River, and Cold Lake regions of Alberta where bitumen lies too deep beneath the surface to be accessed through conventional mining (Fung & Macyk, 2000). Currently, the most common *in-situ* recovery method is steam-assisted gravity drainage followed by cyclic steam stimulation (Johnson and Miyanishi, 2008, Oil Sands Magazine, 2020). These methods both utilize high-pressure steam heated to over 350 °C to melt and increase the flow of bitumen, which is then pumped to the surface. Alternatively, surface mining can be used to recover oil sands where they are < 75 meters below the surface (Alberta Environment, 2010). Surface mineable oil sands, found in the Athabasca oil sands region north of Fort McMurray, Alberta

account for 4,750 km^2 or approximately 3% of the entire area of the oil sands region. Today, approximately 20% of the oil reserves are thought to be accessible by open pit mining (Oil Sands Magazine, 2020).

Surfacing minable oil sand is accessed by first removing vegetation and surface soils. These surface soils are either stockpiled or used immediately in current reclamation projects (Alberta Environment, 2010). Next, substrates above the oil sand are removed. This geological material is often referred to as overburden and has an average depth of 50 meters (Oil Sands Magazine, 2020). This overburden is typically a mixture of sand, silt, clay, and shale, and often contains hydrocarbons in the form of bitumen. If hydrocarbons are present within the overburden, this material is collectively referred to as lean oil sands (LOS) (Visser, 2008, Rees et al., 2020). While LOS contains up to 8% hydrocarbons, it is not economical to extract with current technology. The exposed oil sands, which generally contain between 8% and up to 14% hydrocarbons are excavated with large shovels and hauled by trucks to machine crushers. The crushed material is mixed with hot water and the resulting slurry is pumped to an extraction plant. Gravity pulls the sand to the bottom of the extraction vessel while the lighter bitumen floats to the top (Oil Sands Magazine, 2020). This bitumen is treated with hydrocarbon solvents to further reduce viscosity and precipitate asphaltenes. The recovery of bitumen via this method is approximately 90%. The bitumen is upgraded, by applying heat and pressure, breaking down the large hydrocarbon chains into smaller fractions (Oil Sands Magazine, 2020). The newly generated synthetic crude oil can be further refined into petroleum products such as diesel and gasoline. The waste product, known as tailings, a mixture of sand, clay, water, and bitumen, is placed in large ponds and the water is reused.

1.4 Current reclamation practices

To date, over 4800 km^2 have been leased for surface mining, of which approximately 900 km^2 have been disturbed by oil sand mining operations (Government of Alberta, 2021). Mining companies are legally required by the Alberta provincial government to reclaim the disturbed lands to an equivalent land capability. Equivalent land capability is defined as "the ability of the land to support various land uses after conservation and reclamation is similar to the ability that existed before an activity being conducted on the land, but that the individual land uses will not necessarily be identical" (Alberta Environment, 2010). To date, 104 ha have been certified reclaimed with an additional 7,439 ha of terrestrial and 1,291 ha of wetlands and aquatic areas ready for certification (http://osip.alberta.ca/library/Dataset/Details/27).

Current reclamation practices involve the construction of land from overburden, which as previously mentioned, can contain up to 8% hydrocarbons (MacLennan et al., 2018). These landforms are capped with a layer of salvaged surface soils such as forest floor material or peat-mineral mix varying in-depth and populated with native vegetation (Alberta Environment 2010). However, the construction of landforms from overburden, including LOS, introduces the possibility of LOS being inadvertently mixed with the capping material, possibly leading to petroleum hydrocarbons being present in the rooting zone of establishing vegetation (Visser, 2008). Previous studies have shown LOS has many properties, including the presence of petroleum hydrocarbons, low concentration of nutrients, altered soil water regimes, and increased bulk density, which may have detrimental effects on plant establishment and growth (Visser 2008, 2011; Jung et al., 2014; Jamro et al., 2015; Pernistky et al., 2016; Neil and Si, 2019). However, to date, the exact mechanism(s) for such negative effects on plants is poorly understood, and this topic is explored in Thesis Chapter 2.

1.5 Petroleum hydrocarbons and plants

Overall petroleum hydrocarbons in soil present a major challenge in reclamation as they can, directly and indirectly, impact plant growth. Previous studies have found their presence can directly affect plant growth by inducing oxidative stress (Liu et al., 2009; Nardeli et al., 2016; Moradi et al., 2020), disrupting photosynthesis (Tomar et al., 2013; Cartmill et al., 2014; Han et al., 2016), and altering membrane permeability (Ali et al., 2020). Additionally, petroleum hydrocarbons have been found to disrupt metabolic and enzymatic activity in plant cells (Achuba 2006). Petroleum hydrocarbons may also indirectly affect plant growth through several mechanisms. For example, water movement and air flow of soils can be reduced because petroleum hydrocarbons increase the hydrophobicity of soils and fill soil (Neil and Si, 2019; Rees et al., 2020). These changes to soil properties along with creating a hydrophobic environment around plant roots limits nutrient availability (Devatha et al., 2019; Odukoya et al., 2019). Finally, the presence of hydrocarbons has also been shown to alter soil microbial communities, which play pivotal roles in nutrient cycling and plant growth (Feng et al., 2020; Galitskaya et al., 2021; Mafiana et al., 2021).

These direct and indirect effects of petroleum hydrocarbons on plants can reduce seed germination, decrease plant shoot and root biomass, reduce height, and decrease chlorophyll concentration in leaves (Haider et al., 2021). While petroleum hydrocarbons share many properties with crude and refined oils, the largest difference in LOS is that it is composed of a much larger fraction of high molecular weight hydrocarbons than crude and refined oils (Hein,

2017). Therefore, the effects of petroleum hydrocarbons products on plant growth observed in previous studies may differ from those imparted by LOS. To date, studies on the effects of petroleum hydrocarbons on plant growth have focused on crude oil and refined petroleum products (Haider et al., 2021). To my knowledge, only one report has investigated the effects of LOS on plant growth (Visser, 2008). As such, more information is needed to better understand how this material impacts the growth of plants and tree seedlings, including how the incorporation of these material in landforms may affect plant growth in the future.

1.6 Shallow bituminous soils

Shallow bituminous soils appear irregularly throughout northeastern Alberta down to 3 meters (Leskiw et al., 2006). The sporadic occurrence of the surface-level bitumen deposits throughout the area is hypothesized to be the result of an outburst flood from the glacial Lake Agassiz. The rapid release of the floodwaters likely carved large valleys and channels exposing deposits of bitumen (Fisher and Smith, 1994). To date, these surface-level bituminous soils have been found throughout the region supporting mature boreal forests dominated by tree species such as aspen (*Populus tremuloides*) and jack pine (*Pinus banksiana*) (Leskiw *et al*., 2005, 2006). Shallow-bituminous soils found in Northern Alberta provide a unique opportunity to investigate what mechanisms allow for the survival of native trees exposed to hydrocarbons. In particular, I focus on the role soil fungi may play in mediating establishment and survival of trees on shallow bituminous soils. Given that both shallow bituminous soils and the abandoned ore pile at Bitumount Alberta are unique landscapes and represent potential outcomes for unreclaimed or reclaimed landscapes, determining how the presence of petroleum hydrocarbons affects fungal communities in these locations may provide a greater insight into how mining practices in the region may affect soil-plant interactions through changes in microbial communities.

1.7 Soil fungi

Different fungal guilds play pivotal roles in boreal forest ecosystems as they influence organic matter decomposition, carbon and nutrient cycles, and plant performance. Saprotrophic fungi, for example, are key to these forest ecosystems' nutrient cycles as they obtain energy through the breakdown of organic matter and thus regulate the decomposition of recalcitrant carbon compounds (Lindahl et al., 2007). In addition to saprotrophic fungi, mycorrhizal fungi also play a major role in the biogeochemistry of the boreal forest (Finlay et al., 2006). Like many other forested ecosystems, the vast majority of plants in the boreal forest interact with

mycorrhizal fungi (Read *et al.*, 2004). Mycorrhizal fungi are typically known for facilitating soil-nutrient uptake, primarily of phosphorus and nitrogen, in exchange for sugars from photosynthesis (Smith and Read, 2008). Finally, pathogenic fungi influence plant communities through their negative effects on plant growth either by taking nutrients from living tissue (biotroph) or by damaging the tissue to extract the nutrients (Doehlemann et al., 2017).

The two most abundant types of mycorrhizal fungi are arbuscular mycorrhizal (AM) fungi and ectomycorrhizal (EM) fungi. Arbuscular mycorrhizal fungi and EM fungi differ in nutrient acquisition strategies, morphology, and the majority of plant species with which they interact (Smith and Read 2008). Arbuscular mycorrhizal fungi (phylum Glomeromycota), are the most common mycorrhizal fungi, which establish associations with approximately 72% of vascular plant species (Brundrett & Tedersoo, 2018). Within the boreal forest, AM fungi colonize roots of primarily understory vegetation as well a small number of tree and shrub species (Read *et al.*, 2004). These fungi are characterized by the presence of two unique fungal structures; arbuscules, and vesicles. Arbuscules, which form within the cortical cells of plant roots, are the main site of nutrient exchange (primarily phosphorus and nitrogen for carbon). The second distinct structure of AM are vesicles, which act as storage organs and can occur within and between cells (Smith and Read 2011).

Ectomycorrhizal fungi (Ascomycota and Basidiomycota) colonize the roots of only 2% of vascular plant species, though some plant species host both AM and EM fungi (e.g., *Populus tremuloides*) (Brundrett & Tedersoo, 2018). However, EM plants dominate in the boreal, temperate, and subtropical forests (McGuire *et al.*, 2013). These forests tend to have relatively slower rates of decomposition and therefore higher concentrations of soil organic matter. Ectomycorrhizal fungi, unlike AM fungi, produce enzymes capable of releasing nutrients, particularly nitrogen, from organic matter (Courty *et al.*, 2010). These fungi also have a distinct set of fungal structures, the mantle and Hartig net. The mantel sheaths the tip of lateral roots and forms a barrier between the rhizosphere and root tip. The Hartig net forms between the cortical and epidermal cells of the root, acting as the site of nutrient exchange (Smith and Read 2008).

Although the focus of my research is on mycorrhizal fungi, trees interact with a variety of other soil fungi and bacteria that may also play a role in plant-hydrocarbon tolerance. The mycorrhizal hyphal network and the surrounding rhizosphere are rich in nutrients and root/hyphal exudates which promotes microbial diversity (Jansa *et al.*, 2013). The liquid film that covers the fungal hyphal network has also been shown to aid bacteria in exploiting a larger volume of soil (Miquel Guennoc *et al.*, 2018). Consequently, the increased tolerance to

hydrocarbons attributed to mycorrhizal fungi may involve bacterial activity associated with breaking down the hydrocarbon molecules. Like fungi, bacteria also produce extracellular oxidative enzymes that can break down hydrocarbons (Seo *et al.*, 2009; Chauhan *et al.*, 2017). Community analyses of sites contaminated with hydrocarbons have found high abundances of bacteria known to degrade hydrocarbons (Voordouw *et al.*, 1996; Galitskaya et al., 2021). It is possible that shallow-bituminous soils are home to the same or functionally similar bacterial species as those found in sites contaminated with hydrocarbons and that the mycorrhizal hyphae found in these soils can provide a suitable environment for these bacteria. While bacteria produce enzymes capable of breaking down hydrocarbons, mycorrhizal fungi can create a physical barrier between roots and hydrocarbons, sequester hydrocarbons within the fungal tissue, produce oxidative enzymes, and increase nutrient uptake of plants. These features make mycorrhizal fungi ideal candidates for use as plan microbial partners to increase a plant's tolerance to hydrocarbons.

While mycorrhizal fungi are typically known for their ability to provide plants with nutrients, they have also been found to increase a plant's tolerance to stressors such as increased soil salinity (Muhsin & Zwiazek, 2002), heavy metals contamination (Hildebrandt *et al.*, 2007; Krznaric et al., 2009; Chot and Reddy; 2022), and the presence of petroleum hydrocarbons (Kuo *et al.*, 2014). Although not fully understood, there are a variety of hypotheses for how mycorrhizal fungi could increase a plant's tolerance to hydrocarbons in natural bituminous soils. For example, fungi may form a physical barrier between the plant's roots and the hydrocarbons, and/or harmful hydrocarbon molecules may be sequestered in fungal tissues (Gunderson *et al.*, 2007). Alternatively, non-specific fungal enzymes produced for the degradation of organic material may also degrade petroleum hydrocarbons (Robertson *et al*., 2007; Harms *et al.,* 2011). While AM fungi can take up organic forms of nutrients, they are unable to produce enzymes capable of breaking down organic matter (Whiteside *et al.*, 2012). Consequently, it is generally accepted that AM fungi have little to no ability to degrade organic material and thus no ability to degrade petroleum hydrocarbons (Michelsen *et al.*, 1996, 1998). Ectomycorrhizal fungi, however, are capable of taking up nutrients from organic matter, which are released by specific enzymes such as polyphenol oxidases. These enzymes have been shown to break down organic pollutants including petroleum hydrocarbons (Braun-Lüllemann *et al.*, 1999; Meharg & Cairney, 2000; Robertson *et al.*, 2007).

1.8 Mycorrhizal fungi and reclamation practices

Anthropogenic disturbances including mining, heavy metal contamination, farming, and clear-cut logging have been found to reduce the abundance and richness of mycorrhizal fungi (Maltz and Treseder, 2015; Policelli et al., 2020). Invasive plants and pesticides reduce available mycorrhizal hosts and excessive use of fertilizers, fungicides, and irrigation have also been shown to alter mycorrhizal abundance and diversity (Smith et al., 2000; Tian et al., 2009; Chen et al., 2014; Luneberg et al., 2019). As such, one cost-effective strategy for restoring disturbed landscapes may be to inoculate plants with mycorrhizal fungi. Previous research has shown mycorrhizal fungi can improve plant establishment and growth as well as the soil quality of these disturbed landscapes. The two main inoculation strategies that are often used consist of either inoculating seedlings with mycorrhizal spores or using native soils as inoculum before planting in the field. While both strategies have been successfully used to establish seedlings on disturbed landscapes (Greer et al., 2011; Onwuchekwa et al., 2014; Middleton et al., 2015; Kuziol et al., 2018; Vahter et al., 2020), pre-inoculating tree seedlings with soil inoculum rather than with specific strains of ectomycorrhizal fungi may be a more successful strategy as soils could be easily collected at nearby reference sites (Maltz and Treseder, 2015). Fungal specificity of plants that interact with ectomycorrhizal fungi is higher than that of AM plants (Molina et al., 1992). Therefore, the lack of host specific ectomycorrhizal species in the inoculum or the presence of those maladapted to the local environment could limit the success of these reclamation projects (Maltz and Treseder, 2015).

By managing soil fungal communities in the reclamation process, plant growth may be increased on sites containing residual hydrocarbons. Previous studies have shown mycorrhizal fungi can increase a plant's tolerance to petroleum hydrocarbons (Gunderson et al., 2007; Kuo et al., 2014; Nwoko, 2014; Xun et al., 2015). Despite mycorrhizal fungi being present in the capping material, previous studies have found the reclamation methods in the oil sand regions, including physically disturbing and stockpiling soils, reduce the abundance of fungal propagules (Danielson et al., 1983; Bois et al. 2005), thus reducing the soils' inoculum potential. While the inoculum potential of these reclaimed soils does increase with time, it appears to be a slow process (Bois et al., 2005). As such, inoculating seedlings with mycorrhizal fungi may be one method to help increase their establishment, survival, and growth on reclaimed landscapes. The few existing studies focusing on improving tree seedling performance in growth substrates common to oil sands reclamation (mineral soil and peat) report positive effects of ectomycorrhizal fungal inoculation (Greer et al., 2011; Onwuchekwa et al., 2014),

and these results varied with plant and fungal species combination (Onwuchekwa et al., 2014). However, to our knowledge, no studies have investigated the response of seedling growth to fungal communities from environments naturally containing hydrocarbons.

1.9 Thesis overview

This thesis consists of three research chapters. The main objective of my research was to investigate the effect of bituminous substrates on plant and fungal growth and determine whether the interactions between beneficial fungi and plants can be leveraged to increase seedling performance in soils containing residual hydrocarbons. In Chapter 2, I test whether lean oil sands are detrimental to plant growth through the presence of hydrocarbons and/or by creating barriers including low nutrient concentrations, water availability, or reduced aeration. In Chapter 3, I test the efficacy of soil inoculum collected from various sites ranging in hydrocarbon concentrations and fractions on the growth of two boreal tree species commonly used in reclamation, *Populus tremuloides* and *Pinus banksiana*. In Chapter 4, I first test how LOS influences growth responses of ectomycorrhizal fungi and *Pinus banksiana* seedlings separately and when mycorrhizas are formed between the two partners. Second, I test how fungal species, which vary in morphological traits and growth rates, differed in their direct response to LOS. Finally, in Chapter 5, I synthesize the results of the above-described experiments and provide management recommendations and directions for future research.

Chapter 2: 'You shall not pass.' Lean oil sand acts as a barrier to plant growth

2.1 Introduction

The oil sands region of northern Alberta, Canada contains the third largest proven oil reserves on the planet after Venezuela and Saudi Arabia (Government of Alberta, 2017). To date, oil sands mining has disturbed approximately 900 km^2 of boreal forest and, by law, this area must be reclaimed to self-sustaining ecosystems representative of the region (Government of Alberta, 2021). Oil sand deposits are accessed by removing vegetation, soils, and geologic material (overburden) and these materials are then used to reclaim mined landscapes. Overburden that contains up to 8% hydrocarbons by weight is known as lean oil sands (LOS), and, at this concentration, it is economically unviable to extract hydrocarbons using current technology (Visser, 2008). Lean oil sand is a mixture of quartz sand, clay, other trace minerals, and hydrocarbons (Leskiw *et al.,* 2006). Current land reclamation includes the construction of landforms using overburden including LOS, which is then capped with a layer of a peat–mineral mix and forest floor material of varying depth (Visser, 2008; Pernitsky et al., 2016). The use of LOS in constructing these landforms, may result in the presence of hydrocarbons in the vegetation's rooting zone (Visser, 2008). Previous studies indicate that overburden containing LOS may negatively influence plant growth on these reclaimed landscapes (Visser 2008, 2011; Jung et al., 2014; Jamro et al., 2015; Pernistky et al., 2016; Neil and Si, 2019). However, the mechanisms involved remain poorly understood.

The potential phytotoxicity of petroleum hydrocarbons within LOS likely acts as the most direct cause of plant growth inhibition. Petroleum hydrocarbons within LOS are of particular concern because they can reduce seed germination, negatively interfere with root development, metabolic activities such as photosynthesis, and shoot growth (Haider et al., 2021). However, LOS, like crude oil, is made up of a complex mixture of hydrocarbons, non-hydrocarbons, and heavy metals (Overton et al., 1994; Visser 2008). The most pronounced difference between LOS, crude oil, and refined petroleum products is that LOS is composed of a much greater fraction of high molecular weight hydrocarbons (Hein, 2017). As such, the effects of LOS on plant growth compared to those caused by soils contaminated with crude oil or petroleum products may differ. To date, the majority of research on the effects of petroleum hydrocarbons on plant growth have focused on crude oil and refined petroleum products (Kulakow et al., 2000; Tesar, 2002; Shirdam et al., 2008; Li et al., 2008; Njoku et al., 2009; Al-Moaika et al., 2012; Zamani et al., 2017; Ilyas et al., 2021). To my knowledge, only one study investigated the effects of LOS differing in the concentration of petroleum hydrocarbons

on plant growth, reporting a negative correlation between the two (Visser, 2008). Given that much of the reclamation in the oil sands region in the near-future will involve using LOS overburden, it is critical to understand how this material affects the establishing vegetation.

In addition to the petroleum hydrocarbons in LOS reducing plant growth, LOS may also inhibit plant growth owing to its lack of plant available nutrients and/or its negative effect on nutrient availability in soil. Lean oil sand typically contains little nitrogen and varies in plant available phosphorus (Visser 2008; Rees et al., 2020; Brown, 2020). While it is well established that a lack of available nutrients may reduce plant growth (Raich et al., 1994; Gleeson and Good, 2003), the presence of hydrocarbons may also inhibit nutrient availability. Ilyas et al. (2021) found that the presence of crude oil decreased soil available nitrogen, phosphorus, and potassium, possibly due to changes in soil pH, soil electrical conductivity (soil salinity), or plant-water-soil interactions. For example, if LOS reduces water availability to plants, this may reduce the uptake of many dissolved nutrients including nitrate, sulfate, calcium, and magnesium. While fertilization of reclaimed soils in the region may improve plant growth, studies conducted so far have only focused on the effects of fertilization of capping materials, such as peat-mineral mix and forest floor material (Rowland et al., 2009; Pinno et al., 2012; Errington and Pinno, 2015; Stack et al., 2021). Considering its direct and indirect effects on soil nutrients, adding nutrients to LOS may alleviate the effects of hydrocarbon toxicity on plant growth.

Reclamation practices may also contribute to reduced plant performance in presence of LOS. During reclamation, heavy equipment places and contours the overburden used to construct landforms, which can compact the substrate, increasing the potential for root mechanical impedance (Fung and Macyk, 2000). Compaction by heavy equipment can create distinct boundaries between soil layers placed during reclamation, which differ in density and texture (Jung et al., 2014). This can restrict root penetration (Jung et al., 2014; Duan et al., 2015; Jamro et al., 2015) and, as a result, limit plants from accessing resources (Tracy et al., 2012; Lipiec and Hatano, 2003; Jung et al., 2014; Duan et al., 2015; Jamro et al., 2015). To counteract this barrier, reclamation practices that contribute to increasing substrate permeability may help alleviate compaction of LOS and subsequent restriction on plant growth.

Finally, low water availability associated with LOS may also reduce plant growth. Previous work has found that the capping material above LOS has greater moisture content than surrounding soils (Flemming, 2012). Hydrophobicity of LOS or the creation of a physical seal by the compaction of LOS may reduce water availability (Leskiw et al., 2006; Rees et al., 2020). Previous studies suggest LOS with a bulk density of 1.5 g cm^{-3} and hydrocarbon

concentrations greater than 3.25 % decreases hydraulic conductivity and water holding capacity (Pernitsky et al., 2016). Furthermore, petroleum hydrocarbons can fill in substrate pores, which impedes water from saturating the material (Neil and Si, 2019). As a result, LOS may impede the downward movement of water through reclaimed landscapes (Rees et al., 2020). This decrease in downward flow of water may potentially alter plant water availability in LOS. Experimentally increasing the downward flow of water in LOS may be one approach to determine how decreased vertical water flow affects plant growth.

The objective of this experiment was to determine key factors underlying the effects of LOS on plant growth. I manipulated each factor in turn to isolate their effects on plant growth allowing me to investigate the indirect and direct effects of LOS on plant growth. The factors I manipulated included: the presence of petroleum hydrocarbons in LOS, soil nutrients, soil permeability, and water availability, to determine which factors may limit plant growth. Such knowledge is important to continue to develop best management practices to grow plants on reclaimed landforms containing this material.

2.2 Methods

2.2.1 Growth chamber experiment

To examine if LOS decreases plant growth, and to identify putative factors underlying its influence on plants, I conducted a growth chamber experiment using *Lactuca sativa* L. This species was used because it grows easily under controlled environment conditions, it has a short life cycle (~45–55 days), and is sensitive to the presence of hydrocarbons in soils (Ilyas et al., 2021). Plants were grown in a 3 × 2 × 2 × 2 factorial experiment with each factor chosen to represent possible mechanisms limiting plant growth in LOS. The total number of plants grown in this experiment was 192 (24 treatment combinations × 8 replicates). The first factor, 'Substrate' had three levels, (1) 0.88% ('Low LOS') and (2) 3.67% hydrocarbons in LOS ('High LOS'), and (3) free of hydrocarbons ('Field Soil'). Should hydrocarbons limit plant growth, LOS higher in hydrocarbons concentration is expected to decrease plant growth more than LOS with a lower concentration of hydrocarbons. The second factor, 'fertilization', had two levels, (1) fertilizer applied ('Yes') or (2) not ('No'). Should reduced nutrient availability of LOS be a main factor limiting plant growth, adding fertilizer should differentially increase plant growth depending on LOS treatment with greater plant growth responses in the relatively less toxic 'Low LOS'. The third factor, 'aeration' had two levels, (1) substrate aerated ('Yes') and not aerated ('No'). Should decreased substrate permeability limit plant growth through mechanical impedance as well as increase water flow through the substrate, then increasing permeability of substrates should increase plant growth in all substrate types. Finally, the fourth factor, 'water tile' had two levels, (1) present ('Yes') and absent ('No'). Should LOS decrease the downward movement of water and decrease the saturation of the material, adding water tiles to pots may encourage roots to grow into the LOS.

The two grades of LOS were provided by Syncrude Canada Ltd. containing 8,751 mg hydrocarbons kg^{-1} (0.88%; Low LOS) and 36,699 mg hydrocarbons kg^{-1} (3.67%; High LOS) (Appendix 2.1 Table S2.1). These grades were selected because they represent concentrations of hydrocarbons of LOS currently in use for reclamation. The LOS was collected on June 21st, 2018, from two locations within the Aurora North Mine Site, Fort McMurray, Alberta, Canada. Field soil was collected from three jack pine (*Pinus banksiana*) stands within 150 km from the mine site near Janvier, Alberta, Canada (55° 54' 24" N, 110° 44' 43" W). The forested sites are described in chapter 3. Field soil was selected as it represents a salvaged surface soil used to cap landforms in reclamation. To remove stones and roots, samples from all substrate types were passed through a 4 mm sieve and homogenized. The substrates were analysed for pH,

electrical conductivity, particle size, ammonia (NH_4-N), nitrate (NO_3-N), phosphate (PO_4-P), and extractable metals (Na, Mg, K, Ca, B, Li Mn, Fe, Zn, P, S) at the Natural Resources Analytical Laboratory, University of Alberta (Appendix 2.1 Table S2.2–S2.3).

Plants were grown in 100 cm^2 × 9 cm 'Greenhouse Pots' (Growers Solution, Cookville, Tennessee, USA) filled with 450 grams of either field soil, 'Low LOS', or 'High LOS'. These substrates were compressed to a bulk density of 1.5 g cm^{-3}, after which each pot was topped with 150 g of field soil, again compressed to a bulk density of 1.5 g cm^{-3}. This bulk density is within the range found in reclaimed soils (Alberta Innovates, 2013). I placed 1.5 cm of field soil at the surface of the substrates ('cap') to increase the probability of seedling germination and to emulate reclamation practices (Alberta Environment 2010).

The fertilizer treatment consisted of mixing 10 mg kg^{-1} of 16-16-16 controlled release fertilizer (Master Plant-Prod, Brampton, ON, Canada) with the substrate, leaving the cap unfertilized. However, because of the small amount of fertilizer each pot required, the fertilizer was powdered using a ball grinder and then mixed with the substrate. The concentration of fertilizer was selected as *L. sativa* uses approximately 1–2 mg of phosphorus per week as it grows (Buso and Bliss, 1988; Jackson and Smith 2002). The aeration treatment consisted of pressing a nail press (made using a 9.53 cm^2 × 1.91 cm thick plywood with nine 9.53 cm spiral framing nails spaced 2.54 cm apart in a square pattern) (Appendix 2.1 Fig. S2.1) into the substrates of each pot receiving the aerated substrate treatment. Substrate in these pots were then covered with the field soil cap. The tile drain treatment consisted of a PVC pipe (10.16 cm long and 1.91 cm in diameter), sealed at one end with aluminium foil using LePage Speed set epoxy. This tile, meant to allow water to drain below the soil cap, had four sets of three 0.32 cm holes 90° apart that were drilled into each pipe 1 cm below the soil cap layer (Appendix 2.1 Fig. S2.2). Plants with water tiles received the same amount of water as those in all other treatments, except that water was poured down the drain tiles.

After treatments were applied, substrates were saturated with water and ten seeds were sown in each pot. To prevent desiccation, during the first four days the pots were covered with plastic wrap. Plants were grown under 16/8 hours of light/dark cycles. Daytime and night-time temperatures were set at 22 °C and 18 °C, respectively. Light intensity within the growth chamber was approximately 300 µmol m^{-2} s^{-1} as measured by LI-250A light sensor (model LI-191/R, LI-COR Biosciences, Lincoln, Nebraska, USA). Each pot was randomly assigned a position on the growth chamber bench and moved to a new random position every week to reduce any potential effect of pot position within the growth chamber. Plants were thinned to one plant per pot after one week. Plants were watered by misting daily for the first week

followed by completely saturating the soil with 50 mL of water, except on days when fertilizer was applied. Two weeks after sowing, plants in the LOS treatments began to show signs of what appeared to be nutrient deficiency, based on yellowing leaves (McCauly et al., 2011). In response, all plants were fertilized with 50 mL of 5 mg kg^{-1} of 18-18-21 (N:P:K) on weeks 2 and 3. During the experiment two plants died and were not replaced; one grown in field soil with a water tile and the other in field soil with a water tile and fertilized soil.

Thirty-five days after seeds were sowed, plants were harvested. Plants were harvested earlier than 45 days, the length of a typical life cycle for this species, because the shoots in the LOS treatments began to exhibit severe chlorosis. At harvest, shoots were separated from roots and dried at 60 °C for 48 hours and weighed. Roots were separately harvested from the soil cap and the underlying substrate to determine whether they had grown into the LOS. All roots were washed with distilled (DI) H_2O and dried at 60 °C for 48 hours and weighed.

Previous research has shown that LOS is a hydrophobic material (Neil and Si, 2019). I hypothesized that because of the hydrophobicity of the LOS, the water holding capacity of the LOS would be less compared to sand. To examine if LOS decreases the water holding capacity of the substrate, I performed a separate experiment where water was poured through LOS and sand and the amount of water that was not retained by the substrate was measured. I also tested whether the increased movement of water through the substrate changed its water holding capacity by including water tile and aeration treatments. Finally, I tested whether the addition of a sand cap changed the water holding capacity of pots with water tiles (see Appendix 2.1 for complete methods).

2.2.2 Statistical analysis

All statistical analyses were conducted using R v.4.1.0 (R Development Core Team, 2021) statistical software with R Studio 1.3.1056. (R Studio Team, 2021) and visualized with *ggplot2* 3.3.5 package (Wickham, 2016). To evaluate whether shoot and root biomass responded to hydrocarbon concentration, fertilizer, water tile, or aeration, I used a four-way ANOVA. Shoot and root biomass were both log transformed to meet the ANOVA assumptions of normality and homogeneity of residual variance. Type III ANOVA was used to test for significant differences using the *Anova* function in the *car* 3.0-11 package (Fox et al., 2010). Means were compared using Tukey's honest significance difference (HDS) test ($P < 0.05$).

2.3 Results

Roots were present below the soil cap in all treatments demonstrating they interacted with the substrates. Even though LOS decreased plant growth overall, the aeration, water tile, and fertilizer treatments did not increase plant biomass compared to plants grown in field soil. Instead, all specific barrier alleviation measures amplified the negative effects LOS on plant growth.

Compared to field soil, overall, Low and High LOS significantly reduced total plant biomass by 66% and 77%, respectively. However, across most barrier alleviation treatments the effect of LOS on plant growth did not differ by concentration. The only treatment that altered the sensitivity of total plant biomass to the two different concentrations of LOS was the presence/absence of the water tile. The negative effect of LOS on total biomass was higher when a water tile was present (Substrate × Water tile $P < 0.0001$; Appendix 2.1 Table S2.4). In the absence of a water tile, Low and High LOS similarly reduced total plant biomass by approximately 65% compared to plants grown in field soil (Fig. 2.1A). In the presence of water tile, Low and High LOS reduced total plant biomass by 35% and 56% respectively, compared to plants grown in the same substrate type without water tile (Fig. 2.1A). The water tile had no effect on plants grown in field soil.

The addition of fertilizer significantly affected total plant biomass across substrate treatments (Substrate × Fertilizer, $P = 0.003$, Appendix 2.1 Table S2.4). However, for each LOS substrate there was no difference between fertilized and unfertilized treatments (Fig. 2.1B). The addition of fertilizer increased shoot biomass by 58% in plants grown in field soil (Substrate × Fertilizer $P = 0.007$; Appendix 2.1 Table S2.5; Fig. 2.2). However, in the presence of LOS, total plant biomass was not affected by fertilizer application (Fig. 2.1). Substrate aeration did not significantly affect total plant biomass in any substrate type or in interaction with any other barrier modification (Appendix 2.1 Table S2.5, Fig. 2.1C).

The addition of water tile in combination with aeration significantly decreased total root biomass. However, this decrease was only observed in High LOS (Substrate × Water tile × Aerated $P = 0.023$; Appendix 2.1 Table S2.6; Fig. 2.3). Plant roots in aerated High LOS with a water tile had 57% less biomass than those grown in aerated High LOS without a water tile (Fig. 2.3). Additionally, water tile in combination with aeration significantly decreased total plant biomass (Substrate × Water tile × Aerated $P = 0.048$; Appendix 2.1 Table S2.4; Fig. 2.4).

I found LOS retained significantly less water than sand (Substrate, $P = >0.0001$, Appendix 2.1 Table S2.7, Fig.5). Overall, LOS retained 17% of the water poured through the

substrate, while sand retained 90%. In non-aerated LOS, the presence of a watertile significantly increased water retention compared to LOS treatments without a water tile (Substrate × Water tile × Aerated, P = 0.013, Appendix 2.1 Table S2.7, Fig. S2.3). The presence of a watertile in non-aerated LOS increased water retention of the material by 12%. Finally, the presence of a sand cap did not significantly increase the water holding capacity of LOS (Cap, P = 0.26, Appendix 2.1 Table S2.8).

2.4 Discussion

This study clearly showed that LOS reduces growth of *L. sativa*. In contrast to what I hypothesized, manipulating soil properties such as fertility or factors associated with water availability did not ameliorate the negative effects of LOS on plant growth. In the case of using water tile and aeration, the negative effects of LOS on plant growth were even amplified.

Despite LOS differing in hydrocarbon fractions compared to other petroleum products, the negative plant growth responses to LOS found in this study are consistent with those reported in a recent review on the effects of crude oil and refined petroleum products on plant growth across a variety of species (Haider et al., 2021). Furthermore, my results are similar to a recent study on *L. sativa*, showing that plant biomass was approximately 38% and 47% less when the soil was contaminated with 10% and 20% crude oil, respectively (Ilyas et al., 2021). Finally, the results of this study are consistent with the few reports that investigated the direct effect of LOS on plant growth across a variety of plant species. For example, Brown (2020) found that when petroleum hydrocarbon concentrations in LOS increased from 1.95% to 4.54% this decreased *Pinus banksiana* and *Populus tremuloides* seedling growth. However, the author did not compare between plant growth responses in LOS and field soil. Additionally, Visser (2008), reported that the growth of *Hordeum vulgare, Pinus banksiana, Populus tremuloides,* and *Picea glauca* seedlings significantly decreased with increasing petroleum hydrocarbon concentrations up to 5.3%. Taken as a whole, these results indicate that hydrocarbons in LOS act as a direct phytotoxin similarly to other petroleum products.

While LOS appears to directly reduce *L. sativa* growth, further manipulating the water dynamics of LOS using a water tile further reduced plant biomass. Various aspects of how LOS affects substrate water regimes may explain why the water tile affected plant growth. For example, water repellence and decreasing macroporosity are correlated with an increase in petroleum hydrocarbons (Neil and Si, 2019). It is possible that the water tile contributed to increasing hydrocarbon toxicity due to increased water movement into the LOS. This is supported by data showing the water tile increased the water retention of the LOS in my water

holding capacity test. However, decreased plant growth in the presence of the water tile despite the increased water retention suggests increased water movement through LOS creates an environment toxic to plant growth. Furthermore, aerating the substrate alone did not affect plant biomass but when aeration was coupled with water tile, plant root growth was further reduced, indicating that when combined, LOS surface area and water availability may be important predictors of plant growth responses to LOS. Future research will be needed to confirm the validity of these findings in boreal systems.

The addition of fertilizer did not reduce the growth-inhibiting effects of LOS even at low LOS concentrations. Plants only responded positively to the application of fertilizer when grown in field soil, although this effect was only observed for shoot biomass. This is likely the effect of plants allocating more resources to shoots rather than roots, which is consistent with studies showing that higher concentrations of fertilizer decrease root to shoot ratio (Lynch et al., 2012). My findings are also consistent with previous research which found the application of fertilizer did not improve plant growth in presence of petroleum hydrocarbons (Wyszkowski and Wyszkowska, 2005; Shirdam, 2008). One explanation for the lack of plant responses to fertilizer is that petroleum hydrocarbons present in the LOS can reduce nutrient bioavailability, especially nitrogen and phosphorus (Wyszkowski and Wyszkowska, 2005; Agbogidi and Eshegbeyi 2006; Wyszkowski and Ziółkowska 2008; Illyas et al., 2020). Multiple factors may play a role in reducing the availability of soil nutrients, including changes to physicochemical properties of the soil (Osuji et al., 2007; Wang et al., 2013) as well as petroleum hydrocarbons altering soil microbial community composition (Klimek et al., 2016). However, other studies on petroleum hydrocarbons have reported plant biomass increases with the application of fertilizer (Amadi et al., 1993; Tang et al., 2010; Wang et al., 2012). The inconsistency among previous studies is likely the result of using different fertilizers, different plant species, or differences in physicochemical properties of the soils tested.

While petroleum hydrocarbons change edaphic properties, changes in pH, EC, and texture were unlikely to explain the reduced plant growth in response to LOS observed in the current study. The field soil had a pH of 5.6 while both LOS substrates had neutral to slightly alkaline pH levels (Appendix 2.1 Table S2.2) As such, any reductions in soil nutrient availability would have been seen in the field soil. Soil EC likely did not affect soil nutrient availability as all substrate types had EC level below 1 dS m^{-1} (Appendix 2.1 Table S2.2). However, as LOS appears to affect the water holding capacity of the substrate, the change in water holding capacity could reduce plant nutrient uptake. Finally, field soil had a greater percentage of sand compared to both LOS types (Appendix 2.1 Table S2.2). As sandy soils

retain nutrients and water poorly, any reduction in nutrient availability would be observed in field soil.

In conclusion, LOS negatively impacted growth of *L. sativa* and this study elucidated some of the mechanisms involved. The results suggest that even if low concentrations of LOS are present within the rooting zone, plant growth may be reduced. Lean oil sand phytotoxicity appears to be associated with increasing concentrations of petroleum hydrocarbons and the addition of fertilizer may not alleviate such toxicity. Increasing water availability in LOS appears to further reduce plant growth and aerating LOS coupled with increased water movement further increased phytotoxicity. This suggests that any management practices to reduce the movement of water through the LOS in reclaimed sites should be cautiously evaluated. Overall, the results of this study contribute to develop best management strategies in oil sands reclamation campaigns.

Figures

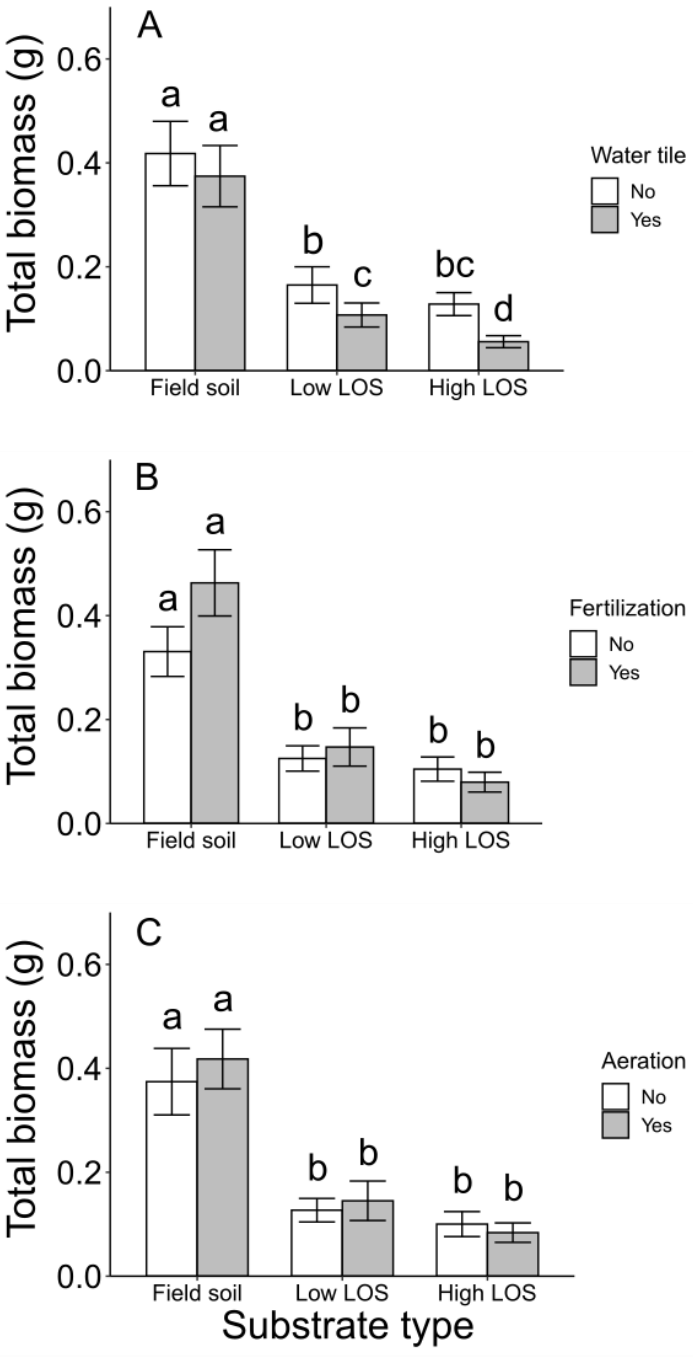

Figure 2.1 Mean total biomass (± 95% CI) of lettuce (*Lactuca sativa*) grown in field soil, lean oil sand containing 0.88% hydrocarbons (Low LOS), and lean oil sand containing 3.67% hydrocarbons (High LOS). Plants were grown with (grey) or without (white) a given treatment;

water tile in the pot (A), fertilizer (B), or aeration (C). Bars with different letters are significantly different (P <0.05) based on Tukey HSD test.

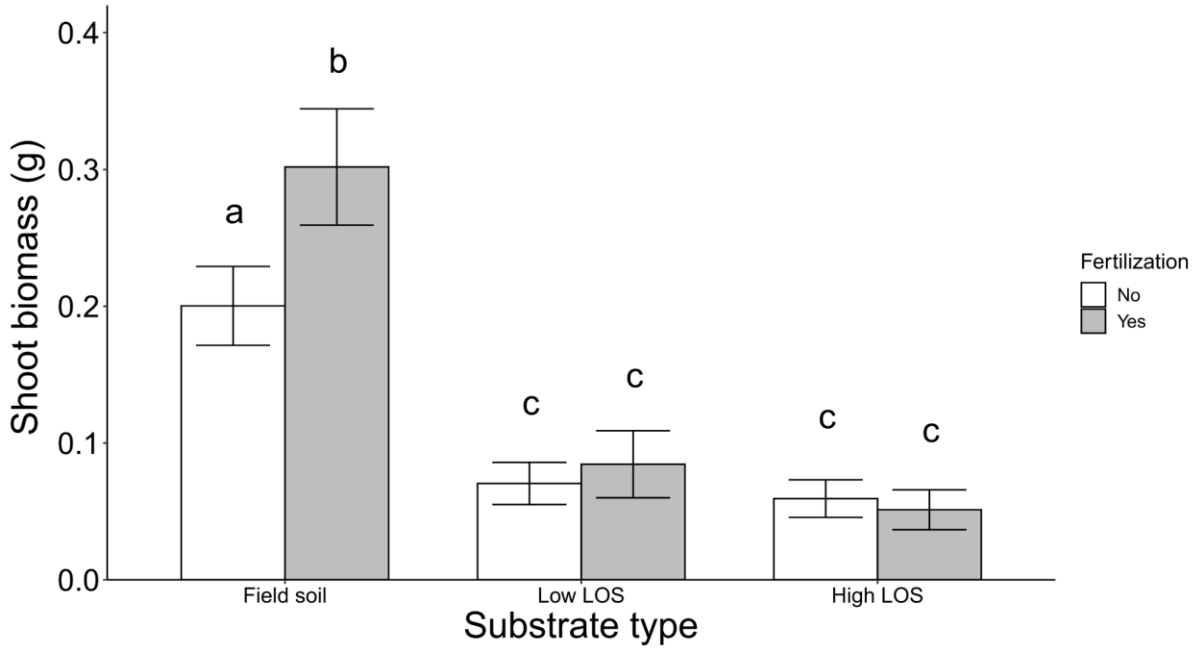

Figure 2.2 Mean shoot biomass (± 95% CI) of lettuce (*Lactuca sativa*) grown in field soil, lean oil sand containing 0.88% hydrocarbons (Low LOS), and lean oil sand containing 3.67% hydrocarbons (High LOS). Plants were grown with (grey) or without (white) fertilizer. Bars with different letters are significantly different (P <0.05) based on Tukey HSD test.

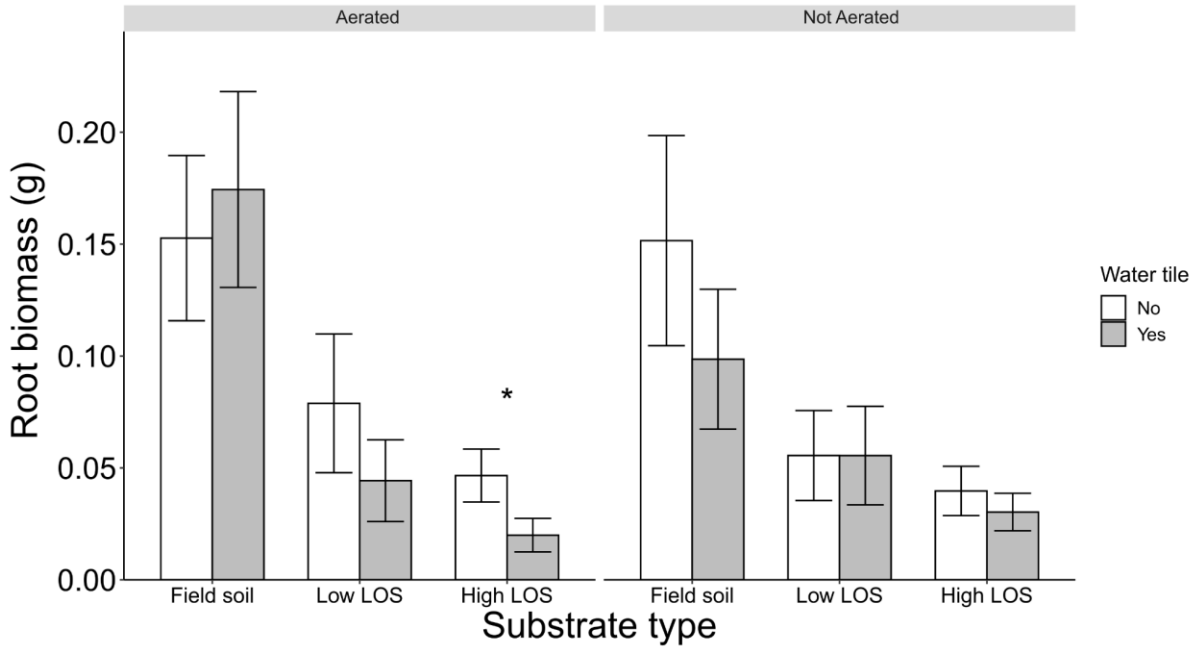

Figure 2.3 Mean root biomass (± 95% CI) of lettuce (*Lactuca sativa*) grown in field soil, lean oil sand containing 0.88% hydrocarbons (Low LOS), and lean oil sand containing 3.67% hydrocarbons (High LOS). Plants were grown with (grey) or without (white) water tiles and with or without aeration. Bars with asterisks are significantly different ($P < 0.05$) based on Tukey HSD test.

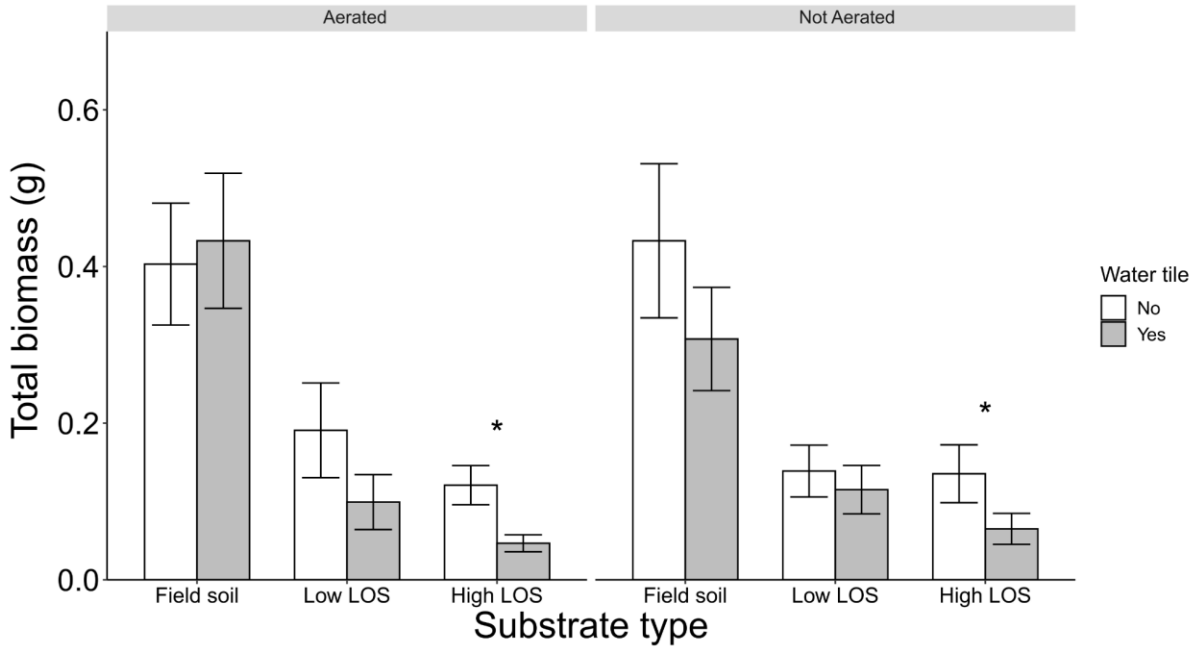

Figure 2.4 Mean total biomass (± 95% CI) of lettuce (*Lactuca sativa*) grown in field soil, lean oil sand containing 0.88% hydrocarbons (Low LOS), and lean oil sand containing 3.67% hydrocarbons (High LOS). Plants were grown with (grey) or without (white) water tiles and with or without aeration. Bars with asterisks are significantly different ($P < 0.05$) based on Tukey HSD test.

Chapter 3: Soil inoculation can improve tree seedling growth in reclamation substrates, but the effect varies by species and inoculum source

3.1 Introduction

Oil sands mining in northern Alberta, Canada, has disturbed approximately 900 km^2 of boreal forest (Government of Alberta, 2021). The government of Alberta requires mining companies to reclaim the disturbed lands to restore locally common, self-sustaining ecosystems (Government of Alberta, 2010). Current reclamation practices involve constructing landforms, sometimes from lean oil sands (LOS) overburden, which may contain up to 8% hydrocarbons (MacLennan et al., 2018). This overburden is capped with a layer of surface soils salvaged from the mined area and the depth of this cap varies across sites (Alberta Environment 2010). Bitumen, the form of hydrocarbons present in LOS, is heavily biodegraded crude oil, composed of hydrocarbon chains ranging in length from 10–50+ carbon atoms, with lighter volatile fractions than refined hydrocarbons meaning that benzene, toluene, ethylbenzene, and xylene are absent (Visser 2008). In addition to LOS, bitumen also occurs naturally in some soils of northeastern Alberta. Bitumen is a mixture of sand, clay, and water, and can be found mixed in soils at 0–3 m depth (Leskiw *et al*., 2005, 2006, Larter and Head, 2014). While previous studies have found that refined hydrocarbons in soil can reduce plant growth and soil microbial diversity and activity (Nicolotti & Egli, 1998; Franco-Ramírez *et al.*, 2007; Shirdam et al., 2008; Mitter *et al.*, 2017), naturally occurring soils containing bitumen support mature boreal forests dominated by tree species such as aspen (*Populus tremuloides* Michx.) and jack pine (*Pinus banksiana* Lamb.) (Leskiw *et al*., 2005, 2006). Research on these natural systems provides an opportunity to investigate how native trees grow in soil containing hydrocarbons with the goal of informing forest restoration in northern Alberta's mined landscapes, where residual hydrocarbons may remain.

Soil fungi, including mycorrhizal symbionts, saprotrophs, and plant pathogens may play key roles in influencing how trees can withstand hydrocarbons. For example, the majority of tree species in the Canadian boreal forest, including jack pine and aspen, establish associations with ectomycorrhizal fungi (Read *et al.*, 2004). While these fungi are typically known for their ability to provide plants with nutrients, they have also been found to increase plant tolerance to petroleum hydrocarbons (Gunderson et al., 2007; Kuo *et al.*, 2014; Nwoko, 2014; Xun et al., 2015). However, these past studies focused on plant responses in sites contaminated with crude oil or refined petroleum products, which compared to LOS and naturally occurring shallow-bituminous soil, contain greater concentrations of lighter

hydrocarbon fractions (Robertson et al., 2007; Visser 2008). Many of the understory plants and some tree species, including aspen, interact with arbuscular mycorrhizal fungi, which have been found to reduce oxidative stress caused by petroleum hydrocarbons (Lenoir et al., 2016). It is also possible that non-specific saprotrophic and ectomycorrhizal fungal enzymes involved in the degradation of organic material (e.g., polyphenol oxidases) degrade petroleum hydrocarbons (Braun-Lüllemann *et al.*, 1999; Meharg & Cairney, 2000; Robertson *et al.*, 2007; Harms *et al.,* 2011). Given such variability in nutrient acquisition strategies across trophic guilds in fungal communities associated with boreal forest trees, the degree of tolerance to hydrocarbons may result from the action of communities rather than single species effects. Therefore, it is important to understand the community composition of fungi in soils containing bitumen.

Although mycorrhizal fungi can occur in reclaimed soils, the inoculation potential of these soils appears to be low (Danielson and Visser1989; Bois et al. 2005; Hankin et al., 2015; Pec et al., 2019), albeit slowly increasing with the age of the site (Bois et al. 2005). Inoculating tree seedlings with small amounts of field soil may be one strategy to facilitate seedling growth on reclaimed sites with low inoculation potential. This method allows for the introduction of a diversity of fungal taxa, many of which may not be commercially available or culturable. This strategy has been successfully used to restore grasslands, in which most plant species form arbuscular mycorrhizas (Middleton et al., 2015; Neuenkamp et al. 2019; Vahter et al., 2020). However, fewer studies have investigated using soil inoculum to restore forested ecosystems (Policelli et al. 2020). A study by Karst et al. (2015) found the growth of lodgepole pine seedlings varied by soil inoculum source, while other studies reported no effect of soil inoculum on seedling growth (Beck et al., 2020; Wasyliw et al., 2022; Rodriguez-Ramos et al., 2022). Studies by Greer et al., (2011) and Onwuchekwa et al., (2014) found mycorrhizal fungi improved seedling growth on substrates similar to those used in oil sands reclamation (mineral soil and peat), although these studies used specific strains of fungi and the results varied by plant and fungal species combination (Onwuchekwa et al., 2014). The mixed results of these previous studies indicate more research is needed to provide information on the potential of soil inoculum in the restoration of forested ecosystems. Furthermore, no studies have investigated the effects of inoculating seedlings with soil inoculum from environments naturally containing hydrocarbons. Given the extreme environments post-mining generates, the use of soil inoculum presents a simple method that could be used in the restoration of these disturbed landscapes.

The goal of this study was to investigate whether beneficial relationships between soil fungi and trees can be leveraged to increase the performance of seedlings establishing on reclaimed sites containing residual hydrocarbons. I addressed this goal by evaluating the efficacy of soil inoculum collected from a range of sites varying in hydrocarbon concentrations and fractions on the performance of two tree species commonly used in reclamation. I first characterized the *in-situ* fungal community composition of the sites from which I sourced inoculum. Next, I inoculated seedlings and tracked their response to evaluate whether inoculation benefited seedlings and moreover, if the origin of inoculum differentially affected seedling outcomes.

3.2 Methods

3.2.1 In-situ characterization of fungi in sites sourced for soil inoculum

Soil inoculum was collected from sites in northeast Alberta, Canada ranging in hydrocarbon concentration and fractions, and dominated by jack pine (*Pinus banksiana*) (Appendix 3.1 Table S3.1, Fig. S3.1). The locations included: 1) sites with naturally occurring 'shallow-bituminous' soil (n = 3) identified using land surveys (Leskiw et al., 2005, 2006); 2) 'bituminous-free' soils (n = 3); and, 3) a site of recently exposed bitumen, Bitumount 'ore pile'. Fungal communities found in shallow-bituminous soils may be a promising source of inoculum for tree seedlings, however, these soils are more weathered than LOS. This means lighter fractions of hydrocarbons may have volatilized and are no longer present in shallow-bituminous soils. Recently exposed bitumen may contain fungal communities experiencing hydrocarbon concentrations and fractions more similar to those present in reclaimed LOS. Bitumount Alberta was the site of the first bitumen extraction from oil sands from 1925 to 1958. Since then, no oil sand processing has taken place at Bitumount, which was declared a historic site in 1974. To date, no attempt to reclaim the site has been made, and over the past decade a large ore pile of hardened bitumen has been colonized by trees, shrubs, grasses, and mosses (Appendix 3.1 Fig. S3.1). As the ore pile is a relatively homogeneous site at a unique location, site replication was not possible. Soils at the shallow-bituminous and bituminous-free sites were classified as Brunisols (Soil Classification Working Group, 1998). Soil texture of all sites was a loamy sand with the exception of one bituminous-free site, which was classified as sandy loam (Appendix 3.1 Table S3.1). The mean daily temperature in the region is 1 ˚C and average annual precipitation is 420 mm (Environment and Climate Change Canada, 2019).

To characterize fungal communities at each of the sites, I surveyed soils and tree roots. At each of the shallow-bituminous and bituminous-free sites, a 10 m^2 plot was delineated. Ten soil cores were collected from each plot using a step soil probe (1.9 cm diameter; 23 cm depth). Due to the hardness of the ore pile and sparseness of collectable soil, a 10 m^2 plot was not practical. Instead, ten 100 g soil samples were collected using a soil knife from 10 randomly selected locations across the entire ore pile. The step soil probe and soil knife were sterilized between samples with bleach. I also sampled the fine roots of three jack pine at each site by tracing three randomly selected roots from the bole of each tree (~50 cm) and collecting a cluster of fine roots (<2.0 mm in diameter). Root samples were pooled by tree. Soil and root samples were stored on ice after collection and then stored at -20 ℃ prior to processing. I assessed the understory plant composition of jack pine forests growing on shallow-bituminous

and bituminous-free sites. Specifically, within each 10 m^2 plot, four 1 m^2 plots were randomly distributed and all understory plants were identified to species and their percent cover was visually estimated. Due to the sparseness of vegetation growing on the ore pile, I visually identified plants across the entire ore pile and did not estimate their percent cover. Understory plant species were dominated by arbuscular mycorrhizal plants and a small amount of arbutoid and ericoid mycorrhizal plants (Appendix 3.1 Table S3.2). Tree age of the shallow-bituminous and bituminous-free sites were previously found to be similar, averaging 69 years and 81 years, respectively (La Flèche et al., 2021). Growth rates of trees at shallow-bituminous and bituminous-free were not significantly different (La Flèche et al., 2021). Age of trees at the ore pile could not be determined because pine trees were too small to core, and instead tree age was determined by counting whorls; these trees were on average 14 years old.

To measure the concentration of hydrocarbons in the rooting zone, two soil cores were taken within each of the plots 5 m apart in 10 cm increments to a depth of 40 cm using a 6.5 cm × 15 cm slide hammer soil corer (AMS, American Falls, Idaho, USA). Soil cores could not be taken at the ore pile due to the hardness of the bitumen. Instead, three ~250 g samples were taken using a soil knife. All soil samples were analyzed for hydrocarbon fractions 2–4 following reference methods set by the Canada-wide Standard for Petroleum in Soil (Canadian Council of Ministers of the Environment, 2001). Hydrocarbon fractions 2–4 represent F2 (>nC10 to nC16), F3 (>nC16 to nC34), and F4 (nC35 to nC50+) (Turle et al., 2007) (Appendix 3.1 Table S3.3). Soil cores were also analysed for pH, electrical conductivity, and particle size at the Natural Resources Analytical Laboratory, University of Alberta (Appendix 3.1 Table S3.1).

3.2.2 DNA extraction, sequencing, and bioinformatics

To prepare roots for DNA extraction, they were washed of adhering soil. Soil (n = 70) and root samples (n = 21) were freeze-dried (Labconco Corporation, Kansas City, USA) for 72 hours and then each sample ground twice using a Tissuelyser II (QIAGEN Inc.). Then, DNA from 250 mg was isolated using a DNeasy PowerSoil Kit (Qiagen, Hilden Germany) according to the manufacturer's instructions. Ten mg of roots were isolated using E.Z.N.A® Plant DNA Kits according to the manufacturer's instructions (Omega Bio-tek, Norcross, USA). The extracted DNA was prepared for Illumina Mi-Seq sequencing using a two-step PCR. Two sets of primers were used to amplify fungal rDNA. The internal transcribed spacer (ITS1) is commonly used to identify basidiomycetes and ascomycetes (Lekberg *et al.*, 2018), while the SSU region is used to identify AM fungi (Öpik *et al.*, 2013). In the first PCR step, I amplified

the 18S and ITS1 regions separately using primers WANDA - AML2 and ITS1F - ITS2, respectively (White et al., 1990; Gardes & Bruns; 1993; Lee et al., 2008; Dumbrell et al., 2011). Each primer used in 'PCR 1' had Illumina adapter overhangs attached: forward 5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG and reverse 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG (Illumina, San Diego, CA). The PCR reaction volumes consisted of 25 µL and contained 12.5 µL of Platinum SuperFi Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA), 1µL of DNA template, 1.25 µL of each 10 µM primer, and 9 µL of nuclease free H_2O. Reactions were conducted in a Mastercycler pro S Thermal Cycler (Eppendorf Canada, Mississauga, ON, Canada) under conditions specific to each target region (Appendix 3.1 Table S3.4). To ensure the target region had been amplified, I ran each sample on 2% agarose gels. PCR products were then purified using Mag-Bind Total Pure NGS magnetic beads (Omega Bio Tek, Norcross, GA, USA).

In the second PCR, individual Illumina index barcode primers, N7XX for forward and N5XX for reverse directions (Nextera XT Index, Illumina, San Diego) were attached to amplicons of each individual sample according to Illumina Mi-Seq system instructions (Amplicon et al., 2013). As before, the PCR products were purified using magnetic beads. Amplicons were quantified using a dsDNA HS Assay kit on a Qubit fluorometer (Invitrogen Carlsbad, USA) and pooled into equimolar concentrations. The pooled samples were analyzed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to verify amplicon sizes. Paired-end sequencing and demultiplexing was performed using a Mi-Seq reagent kit v3, 600-cycles (Illumina) at the University of Alberta Molecular Biology Service Unit.

Sequences were processed using the DADA2 ITS pipeline in R (Callahan *et al.*, 2016) using the standard filtering parameters. Primers, reverse complements, and Nextera adapters were removed using Cutadapt (Martin, 2011). Due to SSUs extended length, paired-end reads were not possible due to the low quality of the reverse reads and only forward reads were used, similar to Lekberg et al., (2018) and Rodriguez-Ramos et al., (2021). DADA2 inferred sequence variance using the error models, and denoised, merged, and removed chimeras (Appendix 3.1 Table S3.5). This filtering removed ambiguous sequences and restricted the number of expected errors to two. The DADA2 algorithm grouped sequences into amplicon sequence variants (ASVs). Finally, taxonomy was assigned using the UNITE database (Nilsson *et al.*, 2019) for ITS1 reads, while the Maarj*AM* database (Öpik et al., 2010) was used to assign taxonomy to the SSU sequences. To analyze and visualize the results, I used the phyloseq 1.28.0, vegan 2.5-6, and the ggplot 2 3.2.1 packages (McMurdie and Holmes 2013; Oksanen et al. 2019; Wickham 2016). Sequencing depth was assessed visually by generating rank

abundance curves. To normalize the number of reads due to uneven sequence depth, sequences were rarefied to 90% of the sample with the lowest read count (ITS1 soil 11,630; ITS1 roots 15,020; SSU soil 4,032; SSU roots 154,946).

To assign ASVs to function guilds, I used the FUNGuild database (Nguyen *et al.*, 2016), grouping ASVs according to life-style (i.e., ectomycorrhizal, saprotrophs, plant pathogens) using the defaults parameters (Nguyen *et al*., 2016). Only ASVs assigned with a confidence rating of probable or highly probable were given a guild assignment. All ASVs assigned to plant saprotrophs, soil saprotrophs, litter saprotrophs, undefined saprotrophs, and wood saprotrophs were pooled into the category, 'saprotrophs'. The ASVs assigned to guilds other than ectomycorrhizal, saprotrophs, plant pathogens or had dual or more assignments (such as symbiont/pathogen) were categorized as mixed/other, while those not assigned a confidence rating of probable or highly probable were categorized as unknowns.

3.2.3 Testing the effects of soil inoculum on seedling growth

To investigate the effect soil inoculum has on seedlings grown in various reclamation substrates, including soils containing hydrocarbons, and whether the origin of soil inoculum affects their growth, I performed a growth chamber experiment. I selected jack pine (*Pinus banksiana*) and trembling aspen (*Populus tremuloides)* as test species because they are common boreal forest trees that are frequently planted on reclaimed sites in this region. The experiment consisted of a completely randomized fully crossed factorial experiment with two main factors (Appendix 3.1 Fig. S3.2, S3.3). The first factor, 'substrate type' had five levels, each representing different substrates seedlings may encounter in reclaimed landforms: 1) LOS containing 0.88% hydrocarbons by weight ('Low LOS'), 2) LOS containing 3.67% hydrocarbons ('High LOS'), 3) shallow-bituminous field soil, 4) Bitumount ore pile ('Ore pile'), and 5) bituminous-free field soil. The second factor, 'soil inoculum origin' had three levels representing the sites from which inoculum was collected: 1) shallow-bituminous soils, 2) bituminous-free soils, and 3) ore pile. Each live soil was paired with a sterilized treatment as a control. As such, the experimental design consisted of two species × 30 treatment combinations × 10 replicates, for a total of 600 experimental units (pots) (Appendix 3.1 Fig. S3.2, S3.3).

Soil from shallow-bituminous and bituminous-free field locations was collected from each of the three sites surveyed for *in-situ* fungi (see above). At each site, a one-meter square of forest floor was randomly selected and soil was collected to a depth of 25 cm (~30 kg per plot). Soils from each site type were then homogenized to remove variation among sites in soil

nutrients, pH, and soil electrical conductivity (EC). Next, I collected soil from the ore pile. As previously mentioned, due to the hardness of the ore pile, a soil pit could not be dug, therefore ~90 kg of bitumen was collected from five, one-meter square plots randomly selected across the ore pile. To obtain soil inoculum, I collected five soil samples from random locations within each site, each to a depth of 25 cm (except at the ore pile, 10 cm) to capture within-stand variation. This soil was transported on ice to the laboratory, refrigerated and used within 10 days of being collected. Soil from each location was pooled to be representative of the fungal community composition in each soil type. Finally, two grades of LOS were provided by Syncrude Canada Ltd. containing 8,751 mg kg^{-1} ('Low LOS') and 36,699 mg kg^{-1} ('High LOS') (Appendix 3.1 Table S3.6). These grades were selected because they represent concentrations of hydrocarbons of overburden used in reclamation landforms. The LOS was collected on June 21st, 2018, from two locations within the Aurora North Mine Site, Alberta, Canada. All substrates used in the experiment (Bituminous-free, Shallow bituminous, Ore pile, Low LOS, and High LOS) were analysed for pH, EC, particle size, ammonium (NH_4), nitrate (NO_3), phosphate (PO_4), and extractable metals (Na, Mg, K, Ca, B, Li Mn, Fe, Zn, P, S) at the Natural Resources Analytical Laboratory, University of Alberta (Appendix 3.1 Table S3.7, S3.8). To remove stones and roots, soils were passed through a 4 mm sieve. Bituminous, bituminous-free soils, and ore pile substrates were autoclaved, (121°C for 90 minutes) twice with a 24-hour period between each cycle. Autoclaving can increase concentrations of polycyclic aromatic hydrocarbons but not petroleum hydrocarbons (Zamani et al., 2015). The High LOS and substrate from the ore pile were tested for polycyclic aromatic hydrocarbons and found to contain levels below detection limits (Appendix 3.1 Table S3.9). Due to safety concerns, the Low LOS and High LOS were not autoclaved. To ensure microbes present in the LOS were distributed throughout all treatments, I created a microbial wash by mixing one kilogram of Low LOS and one kilogram of High LOS with two litres of sterile DI H_2O and adding 1 mL of this solution to each pot.

Seedlings were grown in 656 mL Deepots (D40 Stuewe & Sons, Tangent, OR, USA) filled with 620 mL of one of the substrate types, which was compressed to a bulk density of 1.3 g mL^{-1}. This bulk density is within the range found in reclaimed soils (Drozdowski et al., 2010). To increase germination success and prevent cross-contamination, soils were capped with 222 mL of sterilized forest floor (LFH layer), collected from bituminous-free sites. The soil cap also emulates placing salvaged soils over mined overburden, a standard practice in reclamation projects in Northern Alberta (Alberta Environment 2010). Pots were inoculated (5% by volume of the pot) with either shallow-bituminous soil, bituminous-free soil, or ore pile

substrate. Non-inoculated controls received the same volume of steam-sterilized field soil of each soil inoculum type. Pots received inoculum two days before the seeds were planted. The soil cap and soil inoculum were also compressed to a bulk density of 1.3 g mL^{-1}. To prevent the loss of soil from pots, each pot was lined with 1-mm-mesh fiberglass window screen, sterilized in 10% bleach for ten minutes.

To determine if 5% soil inoculum contained an adequate amount of inoculum (i.e., fungal propagules) capable of influencing plant growth, I grew an additional set of jack pine and aspen seedlings in unsterilized (100% soil inoculum) shallow-bituminous, bituminous-free soils, and ore pile substrates. This consisted of growing two species × three soil inoculation origins × 10 replicates for a total of 60 experimental units (pots). These plants were grown at the same time and with the same growing conditions as the other seedlings grown during this experiment. To examine whether total seedling biomass in soil with 5% soil inoculum differed from that of seedlings grown in 100% soil inoculum, I used one-way ANOVAs. I found the biomass of jack pine and aspen seedlings that received 5% soil inoculum did not significantly differ from those which received 100% soil inoculum (Appendix 3.1 Table S3.10-S3.15). This result indicated that 5% soil inoculum was an adequate amount of soil inoculum to mimic soil microbiome function in its capacity to influence their growth.

Pine seeds were sourced from seed lot SYN 26-96-10-4-2008 PJ, zone CM 2.1, Smokey Lake, Alberta, matching the location from where the soils were collected. Pine seeds were surface sterilized with 5% bleach for 15 minutes and rinsed in sterile deionized H_2O. Seeds were soaked in sterile deionized H_2O for 24 hrs and cold stratified at 4°C for 14 days. I was unable to source aspen seeds from the relevant seed zone (CM2.1), so instead we collected seeds from trees near the University of Alberta, Alberta, Canada (seed zone CP 1.1), following established protocols (Smreciu *et al*., 2013). Aspen seeds were surface sterilized in 1% commercial bleach for 15 minutes and rinsed in sterile deionized H_2O. One percent bleach was used as it was enough to sterilize the seeds, since 5% was found to kill aspen seeds. Five seeds of either pine or 20 seeds of aspen were planted per pot. Pine and aspen were grown in separate growth chambers. Seedlings were grown in 16 hours of light and 8 hours of dark. Daytime and night time temperatures were set at 20 °C and 18 °C, respectively for pine. Daytime and night time temperatures were set at 22 °C and 18 °C, for aspen. Light intensity in both chambers was approximately 320 μmol m^{-2} s^{-1} as measured by LI-250A light sensor (model LI-191/R, LI-COR Biosciences, Lincoln, Nebraska, USA). Following sowing, pots were randomly assigned a location on a single growth chamber bench. To reduce the effect of pot position within the growth chamber, pots were moved to a new random location every two weeks. Seedlings were

watered by misting for the first two weeks. To prevent seedling desiccation for the first two weeks of growth the pots were covered with plastic wrap. After two weeks, aspen seedlings were watered every second day and pine seedlings every third day, except on days when fertilizer was applied. After 3 weeks of growth, seedlings were thinned to one plant per pot.

On week 4, pine seedlings in the LOS treatments began to exhibit symptoms of nitrogen deficiency consisting of yellowing needles (Landis 1989; McCauly et al., 2011). To ensure plants had adequate nitrogen, all pine seedlings were fertilized with 50 mL of a 30-10-10 fertilizer at a concentration of 25 ppm per pot every three weeks, resolving the signs of nitrogen deficiency. By week 4, aspen seedlings in LOS treatments began to show signs of phosphorus deficiency based on red and purple leaves (McCauly et al., 2011). Aspen seedlings were fertilized with 50 mL of 25 ppm of P (10-52-10) every two weeks, resolving the signs of phosphorus deficiency. Finally, after three and half months, the pine seedlings began to exhibit signs of magnesium deficiency based on needle tip chlorosis (Landis, 1989). Pine seedlings were fertilized with 24.3 ppm of $MgSO_4*7H_2O$, resolving the signs of magnesium deficiency. During the experiment, two pine and five aspen seedlings died and were not replaced (Appendix 3.1 Table S3.16).

I harvested pine and aspen five and half, and four months after germination, respectively. Shoots of each plant were dried at 65 °C for 72 hours and weighed. Roots were washed and frozen at -20 °C before being measured for fungal colonization and then dried at 65 °C for 72 hours. Ectomycorrhizal fungal root colonization was estimated by cutting fine roots into 1 cm sections and assessing 100 root tips per seedling for fungal colonization under 20 × magnification. Root tips were considered colonized when a mantle was present and root hairs were absent. A sub-selection of colonized and uncolonized root tips from each sample were checked under 200 × magnification for the presence of a mantle. Root colonization by ectomycorrhizal fungi of all pine seedlings was measured. No colonization was observed in pine seedlings which received sterile soil inoculum. Owing to the limited response to soil inoculum of aspen seedlings (see Results), only three seedlings from each treatment were checked for colonization. As aspen can be dual-mycorrhizal (Karst et al., 2021), I estimated arbuscular mycorrhizal colonization of three randomly selected aspen seedlings from each treatment combination, using a modified method by Neville el al., (2002). I randomly selected 15-1 cm fragments of root per seedling and cleared them in 10% w/v KOH 90 °C for three hours. The roots were placed in fresh 10% KOH overnight. To remove the dark pigment of the aspen roots, the roots were bleached (30% H_2O_2 and 0.05% NH_4OH by volume) for 15 minutes. Roots were stained with an ink-vinegar solution and then mounted on glass microscope slides

(Vierheilig et al., 1998). Due to the lack of colonization, each 1 cm section of root was checked for colonization. No colonization of aspen roots by ectomycorrhizal fungi or arbuscular mycorrhizal fungi was observed, including aspen grown in 100% soil inoculum.

3.2.4 Statistical analysis

All statistical analyses were conducted using R v.4.1.0 (R Development Core Team, 2021) statistical software with R Studio 1.3.1056. (R Studio Team, 2021) and visualized with *ggplot2* 3.3.5 package (Wickham, 2016).

In-situ characterization of fungi in sites sourced for soil inoculum

To test for differences in composition of the entire fungal communities among sites, I performed permutational multivariate analysis of variance (perMANOVA) based on Bray-Curtis dissimilarities with 999 permutations using the *adonis* function in the vegan 2.5.6 package (Oksanen et al., 2019). Because the study design was unbalanced, perMANOVA was only run if no significant difference in dispersion was detected between sites as differences in dispersion can affect the outcome of perMANOVA tests (Anderson and Walsh, 2013). To test beta dispersion among sites, I used the betadisper function in the vegan package, with a bias.adjust = TRUE to account for variations in sample size. To account for the multiple plots per site, plot was nested within site and treated as a fixed effect. To determine if plots within sites were spatially autocorrelated, I performed a Mantel test using the *mantel* function in the *vegan* package. perMANOVAs were followed by pairwise comparisons with a false-discovery rate p-correction using *pairwise.perm.manova* function in the *RVaideMemoire* 0.9-77 package (Hervé, 2020). I note that spatial autocorrelation was detected within shallow-bituminous (r = 0.26, P = 0.001) and bituminous-free sites (r = 0.33, P = 0.001). However, given the uniqueness of shallow-bituminous sites this could not be avoided. To identify fungal ASVs that could potentially be used as markers for soils containing petroleum hydrocarbons, I used Indicator Species Analysis. The analysis was performed using the *multipatt* function in the *indicspecies* package (De Cáceres & Legendre, 2009). To correct for uneven numbers of groups, I used the *r.g* function within the same package. Fungal communities and guilds were visualized using Bray-Curtis dissimilarities to generate non-metric multidimensional spacing (NMDS) ordinations using the *nmd* function in the *vegan* package.

Testing the effect of soil inoculum on seedling growth

To determine the response of the pine and aspen seedlings to soil inoculum, I calculated the log response ratios of the biomass of seedlings, which received live soil inoculum to those which received sterilized soil inoculum (Hoeksema et al., 2010). Log response ratio 95% confidence intervals were calculated according to Hedges et al. (1999). If 95% confidence intervals are above or below zero, this indicates a significant effect of soil inoculum on seedling biomass. If confidence intervals cross zero, there is no significant effect. To determine if the origin of soil inoculum influenced seedling growth across the substrate types, I used two-way ANOVAs. Because of the low number of replicates per treatment, live and sterile soil inoculum were compared separately per species. Sterile jack pine biomass was log transformed to meet the assumption of normality. Means were compared using Tukey's honest significance difference (HSD) test ($P < 0.05$). I present the untransformed means but used the transformed means to determine if differences were present between the treatments.

3.3 Results

3.3.1 In-situ characterization of fungi in sites sourced for soil inoculum

I obtained a total of 2,974,718 (ITS1) and 1,326,519 (SSU) sequences, averaging 42,496 (± 7969 SD) and 14,485 (± 3255 SD) per soil sample, respectively. After processing the sequences through the DADA2 pipeline, 2,289,052 (77%) (ITS1) and 1,013,977 (76%) (SSU) sequences remained (Appendix 3.1 Table S3.5). These sequences were assigned to 4104 ASVs (ITS1) and 2352 ASVs (SSU). In total, 1932 of the 4104 ITS1 ASVs (47%), representing 71% of all reads, were assigned to the functional guilds, ectomycorrhizal, saprotrophs, plant pathogens, or mixed/other. Fungi forming ectomycorrhizas represented 22% of all assigned ASVs. Saprotrophic ASVs represented 36% of the assigned ASVs, plant pathogens represented 5%, while mixed/other represented 37%. The remaining 2172 ASVs, representing 29% of total reads, were categorized as unknown. Fungal composition, based on ITS1 sequences, significantly differed among all three sites at the whole fungal community level as well as at guild level (Fig. 3.1, Appendix 3.1 Table S3.17-S3.22). Specifically, Archaeorhizomycetaceae was found in much greater abundance in shallow-bituminous and bituminous-free soils than in the ore pile (Table 3.1). Tricholomataceae and Cortinariaceae were found in much greater amounts in bituminous-free soils than in shallow-bituminous soils or the ore pile, while the abundance of fungi in the Trimorphomycetaceae and Piskurozymaceae families was highest in substrates collected from the ore pile (Table 3.1). However, some families, including Russulaceae and Atheliaceae were found in high abundance at all sites (Table 3.1). A total of 1264 out of 2352 SSU ASVs (54%), representing 58% of all reads, were assigned to the functional guild, arbuscular mycorrhizal fungi. Amplicon sequence variants from SSU were not assigned to any other guild. Of the reads that were assigned, the most common genera based on SSU read abundance were *Glomus* (67%) and *Paraglomus* (12%). The remaining reads were assigned to unknown genera (21%), and *Ambispora*, *Archaeospora*, and *Claroideoglomus* together represented <1% of reads. The abundance of the assigned genera and of the unknowns was similar across all three sites (Table 3.3).

From pine roots collected in the field, I obtained a total of 874,325 (ITS1) and 639,415 (SSU) sequences averaging 41,625 (±9866 SD) and 30,448 (±3,467 SD) sequences per sample, respectively. After processing, 607,345 sequences (70%) (ITS1) and 489, 595 (77%) remained (Appendix 3.1 Table S3.5). In total, 645 of the 1116 ASVs (58%) were assigned to a functional guild, representing 82% of all reads. Ectomycorrhizal fungi represented 25% of assigned ASVs, saprotrophs represented 26%, plant pathogens only accounted for 1%, and mixed/other

represented 48%. A total of 577 out of 897 SSU ASVs (64%), representing (90%) of all reads, were assigned to functional guilds. Of the reads assigned, the most common genera based on SSU read abundance was *Glomus* (90%) with the second most abundant genera *Paraglomus* representing only 0.3% of assigned reads. Unlike the composition of soil fungal communities, which differed across sites at all fungal guild levels, pine root fungal communities did not differ significantly across sites. Based on ITS1 sequences, fungal composition differed among the three sites at the whole fungal community level as well as mixed/other guild levels (Fig. 3.1, Appendix 3.1 Table S3.23, S3.24). However, root-ectomycorrhizal fungal communities from bituminous-free soils differed from substrates collected from the ore pile (Fig. 3.1, Appendix 3.1 Table S3.25). Saprotrophic fungi associated with pine roots collected from the ore pile differed from those collected from shallow-bituminous and bituminous-free sites (Fig. 3.1, Appendix 3.1 Table S3.26). Arbuscular mycorrhizal fungal communities from shallow-bituminous soils differed from those present in bituminous-free and ore pile substrates (Fig. 3.1, Appendix 3.1 Table S3.27). Finally, unknown fungal ITS1 communities from the ore pile differed from those associated with roots collected in shallow-bituminous soils (Fig. 3.1, Appendix 3.1 Table S2.28). Thelephoraceae and Pilodermataceae families were in the greatest abundance on roots from shallow-bituminous soils, while Bankeraceae and Tylosporaceae were found in the largest amounts on roots from bituminous-free soils (Table 3.2). The family Hyaloscyphaceae was found in high amounts on pine roots from the ore pile (Table 3.2). The most common families by ITS1 read abundance were Vibrisseaceae, Russulaceae, and Thelephoraceae (Table 3.2).

In total, 352 ITS1 ASVs from soils were found to be indicator species; 234 from the ore pile, 55 from shallow-bituminous soils, and 57 from bituminous-free soils. Fungi not assigned to a guild represented the majority of the indicator species across all sites (Fig. 3.2). Saprotrophic fungi were the next most common type of indicator species in shallow-bituminous soils and ore pile substrates, while ectomycorrhizal, saprotrophic, and mixed/other each made up 18% of the ASVs found in bituminous-free soils. Shallow-bituminous and bituminous-free soils shared six indicator species, four of which were saprotrophic fungi, with the last two being categorized as mixed/other and unknown. The two ASVs assigned as indicator species and the greatest in read abundance from shallow-bituminous and bituminous-free soils were both assigned as *Archaeorhizomyces* (Appendix 3.1 Table S3.29). The most abundant ASV from ore pile substrates was assigned to *Saitozyma podzolica*.

Compared to soils, considerably less ITS1 ASVs associated with pine roots were found to be indicator species. A total of 24 ASVs were indicator species, with 13 from roots collected

from the ore pile, 10 from shallow-bituminous soils, and finally one from bituminous-free soils (Appendix 3.1 Table S3.30). Similar to soil indicator species, fungi not assigned to a guild made up the majority of indicator species. The two ASVs that were the greatest in read abundance from shallow-bituminous roots and ore pile soils were both assigned to *Phialocephala fortinii*. The single ASV found to be an indicator of fungi associated with roots collected from bituminous-free soils was categorized as an unknown Ascomycota.

3.3.2 The effect of soil inoculum on seedling growth

The effect of soil inoculum on pine seedling biomass depended on substrate type (Appendix 3.1 Table S3.31, S3.32). Overall, live soil inoculum varied in promoting pine seedling growth (Appendix 3.1 Table S3.31, Fig. 3.3A). Pine seedling biomass responded positively to at least one inoculation treatment relative to sterilized controls in all substrate types except when pine was grown in LOS (0.88%) (Fig. 3.3C). Pine seedlings grown in bituminous-free soil, inoculated with shallow-bituminous live soil were 49% larger than those that received sterilized inoculum of the same origin (Fig. 3.3C). Pine seedlings grown in shallow-bituminous, ore pile, and High LOS and inoculated with ore pile soil were 128%, 62%, and 64% larger than those that received sterile inoculum from the same origin. Finally, pine seedlings grown in High LOS and inoculated with bituminous-free soil were 49% larger than those that received sterile inoculum from the same source (Fig. 3.3C). Pine seedlings inoculated with sterilized soil inoculum varied in response depending on the origin of the sterile inoculum (Appendix 3.1 Table S3.32, Fig. 3.3B). This result appears to be primarily driven by a decrease in pine seedling biomass when inoculated with sterile ore pile substrate. Biomass of pine seedlings grown in ore pile substrate and inoculated with sterilized ore pile inoculum were significantly smaller than seedlings that received live inoculum. However, the biomass of seedlings that received live inoculum did not differ from those grown in 100% ore pile live inoculum. This result indicates the soil biota from the ore pile may be increasing pine seedlings' tolerance to the ore pile's negative effects on seedling growth.

Live soil inoculum did not increase the growth of aspen seedlings in any of the tested soil types (Appendix 3.1 Table S3.33; Fig. 3.4C) and growth responses varied irrespective of substrate type, (Appendix 3.1 Table S3.33), (Fig. 3.5). The biomass of aspen seedlings that received live inoculum from the ore pile was 18% smaller than that of seedlings inoculated with bituminous-free soil. Seedlings inoculated with live shallow-bituminous soil did not differ from those inoculated with bituminous-free soil (Fig. 3.5). Aspen inoculated with sterile soil did not differ in biomass irrespective of inoculum origin (Appendix 3.1 Table S3.34). The type

of substrate aspen seedlings were grown in significantly affected their growth (Appendix 3.1 Table S3.33, S3.34). When compared to aspen grown in bituminous-free field soil, biomass of aspen was lower in the presence of LOS. Biomass of aspen decreased by 39% and 23% in Low LOS and High LOS, respectively (Fig. 3.4A). Similarly, when compared to aspen grown in bituminous-free field soil, biomass of aspen in sterile soil decreased by 13%, 40%, and 23% in ore pile substrates, Low LOS and High LOS, respectively (Fig. 3.4B).

Overall, the origin of soil inoculum significantly affected pine seedling colonization by ectomycorrhizal fungi regardless of substrate type (Appendix 3.1 Table S3.35). However, the difference in pine root colonization among inoculum treatments was minor. Pine seedling root tips were 8% colonized when inoculated with soil from bituminous-free soil, 8% when inoculated with soil from bituminous soil, and 4% when inoculated with substrates from the ore pile.

3.4 Discussion

I evaluated the efficacy of soil inoculum collected from a range of field sites varying in hydrocarbon concentrations and fractions on the performance of two tree species grown in a range of substrates, including LOS, which is present in some reclamation landforms in the Alberta oil sands region of Canada. I evaluated soil inoculum from natural systems as an opportunity to investigate how representative native trees growing in soil containing hydrocarbons may inform forest restoration in northern Alberta's mined landscapes, where residual hydrocarbons may remain. The field survey demonstrated that soil and tree roots across the different sites differed in fungal community composition indicating that site specific fungi may be important if local soils are used as a source of inoculum. Indeed, soil inoculum increased the growth of *P. banksiana,* and the benefits varied by inoculum origin. However, the growth of *P. tremuloides* was insensitive to soil inoculum regardless of origin.

To evaluate how soil inoculum may vary across this region, I first characterized the *in-situ* fungal community composition of the sites from which I sourced inoculum. At the family level, based on sequence read number, Russulaceae and Thelephoraceae, were the most abundant ectomycorrhizal forming families found on the roots of pine trees across all field sites (Table 3.2). While these families were shared across all field sites, at the species level, very little overlap occurred among ectomycorrhizal fungi (Appendix 3.1 Table S3.36, S3.37). Despite similar concentrations of petroleum hydrocarbons (Appendix 3.1 Table S3.3) present in soils at the ore pile and shallow-bituminous sites, I did not find specific fungal species that were commonly high in abundance in soils from the ore pile or shallow-bituminous sites and in pine tree roots growing in these sites. However, shallow-bituminous and bituminous-free soils and roots from these soils shared a number of highly abundant ASVs including *Russula vinososordida* and *Archaeorhizomyces finlayi* (Appendix 3.1 Table S3.36, S3.37). This result is likely due to these sites having trees of similar age (~ 69 to 81 years old) as pine trees at the ore pile were much younger (~ 15 years old). A number of fungal species were found to be unique to ore pile roots and soils including *Tomentella subclavigera,* which was found to be the most abundant ectomycorrhizal fungus found on pine roots from the ore pile (Appendix 3.1 Table S3.36). Despite having a global distribution (Lilleskov and Bruns 2005), fungi in this genus appear to be associated with post-mining reclaimed soils in northern Alberta (Krpata et al. 2008; Stefani et al., 2018; Trofymow et al., 2020). I also found *Thelephora terrestris* in high abundance in ore pile substrates and root tips. This is an ectomycorrhizal fungus often

associated with colonizing young seedlings and shown to increase jack pine growth on reclaimed soils (Danielson and Visser, 1989a; Danielson and Visser, 1989b).

Jack pine seedling biomass responded to the soil inoculum, however, it varied by substrate type in which seedlings were grown and by origin of the soil inoculum. The results of this study along with those of Karst at al., 2015 not only demonstrate that soil inoculum can improve pine seedling growth but also that the location from where the soil inoculum is obtained is important as it can determine plant growth responses. However, while I observed increased pine growth in response to soil inoculum, I could not identify particular taxa present in the soil's fungal communities that could explicitly predict an increase in plant biomass. Given that shallow-bituminous and bituminous-free soils shared a number of the most abundant ASVs (Appendix 3.1 Table S3.36, S3.37)) as well as a number of indicator species, perhaps these soil inoculations could impact pine seedling growth similarly, however, this was not the case as seedlings inoculated with these soils varied considerably in growth response (Fig. 3.3C). These observed differences in growth response of pine seedlings to soil inoculum may be the result varying bacterial communities among shallow-bituminous and bituminous-free inoculums. For example, a recent study by Padda et al., 2021 found plant growth promoting bacteria increased pine seedling growth in a disturbed environment. On the other hand, inoculum collected from the ore pile was the most beneficial to pine seedling growth (Fig. 3.3C). This observed increase in growth, coupled with the substrates and roots of the ore pile containing a drastically unique fungal community indicate the soils of this ore pile deserve further study. As previously mentioned, *T. subclavigera* and *T. terrestris* were found in high abundance in ore pile soils and roots. Given that *T. terrestris* has been shown to increase pine seedling growth in reclaimed soils in the region (Danielson and Visser, 1989a; Danielson and Visser, 1989b) and *T. subclavigera* can be present in soils disturbed by mining (Krpata et al. 2008; Stefani et al., 2018; Trofymow et al., 2020), these fungal species may be viable candidates for inoculum application and present an opportunity for further research. This future work should focus on isolating fungal cultures from the ore pile at Bitumount and evaluating whether inoculation by either fungal species increases pine seedling tolerance to petroleum hydrocarbons.

While I found the use of soil inoculum improved pine seedling growth in various substrate types, this was not the case with aspen. Aspen seedlings did not respond positively to any soil inoculum indicating they were insensitive to the origin of the inoculum and inoculum *per se*. Further, roots of aspen seedlings were not colonized by ectomycorrhizal or arbuscular mycorrhizal fungi. This lack of growth response to soil inoculum may be the result of collecting

soil from pine-dominated stands and thus, any fungi specific to aspen may be absent. While various fungal genera previously reported on the roots of mature aspen, including, *Glomus, Cennococum, Lactarius, Piloderma, Hygrophorus* (Karst et al., 2021) were detected in the field survey, they may have not survived in the inoculum. Second, the collection of soil inoculum may have acted as a filter, where fungi specific to aspen did not survive and only generalist fungi or those specific to pine species were left in the soil inoculum. For example, Quoreshi and Khasa (2008) found *Suillus tomentosus* and *Rhizopogon vinicolor,* ectomycorrhizal fungi specific to Pinaceae, did not colonize the roots of aspen seedlings. As previous studies have found positive growth results of aspen inoculated with ectomycorrhizal fungi after six and 10 weeks (Yi et al 2008; Quoreshi and Khasa 2008), extending the duration of the experiment was unlikely to yield different results. To my knowledge this is the first report testing soil inoculum on the growth of aspen seedlings. Taken together, future research evaluating the efficacy of soil inoculum on aspen seedling performance should acquire soil inoculum from aspen dominated stands to determine if the lack of growth response to soil inoculum observed in this experiment was the result of a lack of aspen specific mycorrhizal fungi.

For both aspen and pine, biomass was lower in the presence of LOS. These results are consistent with a recent review on the effects of crude oil and refined petroleum products on plant growth across a variety of plant species (Haider et al. 2021). However, the negative effects of petroleum products on tree seedlings specifically, appears to vary with species (Nicolotti and Egli, 1998; Bes et al., 2019; Buzmakov et al., 2021). The results of the current study follow a similar trend of the few experiments that have investigated the direct effect of LOS on tree seedlings. For example, Visser (2008) and Brown (2020) both reported LOS with increasing concentrations of hydrocarbons reduced *P. banksiana* and *P. tremuloides* seedling growth. While in my growth chamber experiment, *P. banksiana* and *P. tremuloides* seedlings grew smaller in the presence of LOS, I did not observe bigger growth reductions as hydrocarbon concentration increased. I found that seedlings of both species grew the least in Low LOS rather than High LOS. The discrepancy between my results and those reported in previous studies may be explained by differences in how different hydrocarbons concentrations in the material were achieved. Visser (2008) and Brown (2020) diluted LOS with sand in order to create a gradient of hydrocarbons, while the LOS used in this experiment was taken from two locations at the Aurora North Mine Site in order to keep the LOS material as operationally relevant as possible. This resulted in the two grades of LOS used in my experiment differing in soil texture (Appendix 3.1 Table S3.7), with the High LOS having substantially less sand (64%) than Low LOS (83.4%). Given this difference in texture, I hypothesize that differential water-

hydrocarbon interactions were responsible for the greater plant growth reductions observed in Low LOS. This is supported by the findings associated with the experiment using water-tiles reported in chapter 2. The greater the surface area of interaction between the substrate and water the greater the release of hydrocarbons.

While petroleum hydrocarbons and a sandier soil texture likely contributed to decrease plant growth in LOS, other properties, including pH and EC, were unlikely to account for the decrease in seedling growth. The field soils as well as substrates from the ore pile had pH levels ranging from 4.98 to 5.34 while both LOS substrates had pH levels were neutral to slightly alkaline (Appendix 3.1 Table S3.7). This means that if pH were to play a role in reducing plant growth it would be expected to occur in the field soil. Finally, soil EC likely did not affect plant growth as the EC of all substrate types was below 1 dS m^{-1} (Appendix 3.1 Table S3.7).

In conclusion, my results show soil inoculum may be an effective method of establishing some tree species on reclaimed areas containing residual hydrocarbons. The characterization of the *in-situ* fungal communities demonstrated these sites differed in fungal community composition both of soil and plant roots, suggesting that soils used for inoculum likely differed in the fungi present. The results of this study also demonstrate that soil inoculum likely cannot be collected from various locations and simply applied with the expectation of increasing seedling growth. Thus, if reclamation practices are to incorporate the use of soil inoculum in the establishment of seedlings on reclaimed soils, more research will be needed to determine if soil inoculum collected from stands of the same species increases the effectiveness of soil inoculation.

Figures

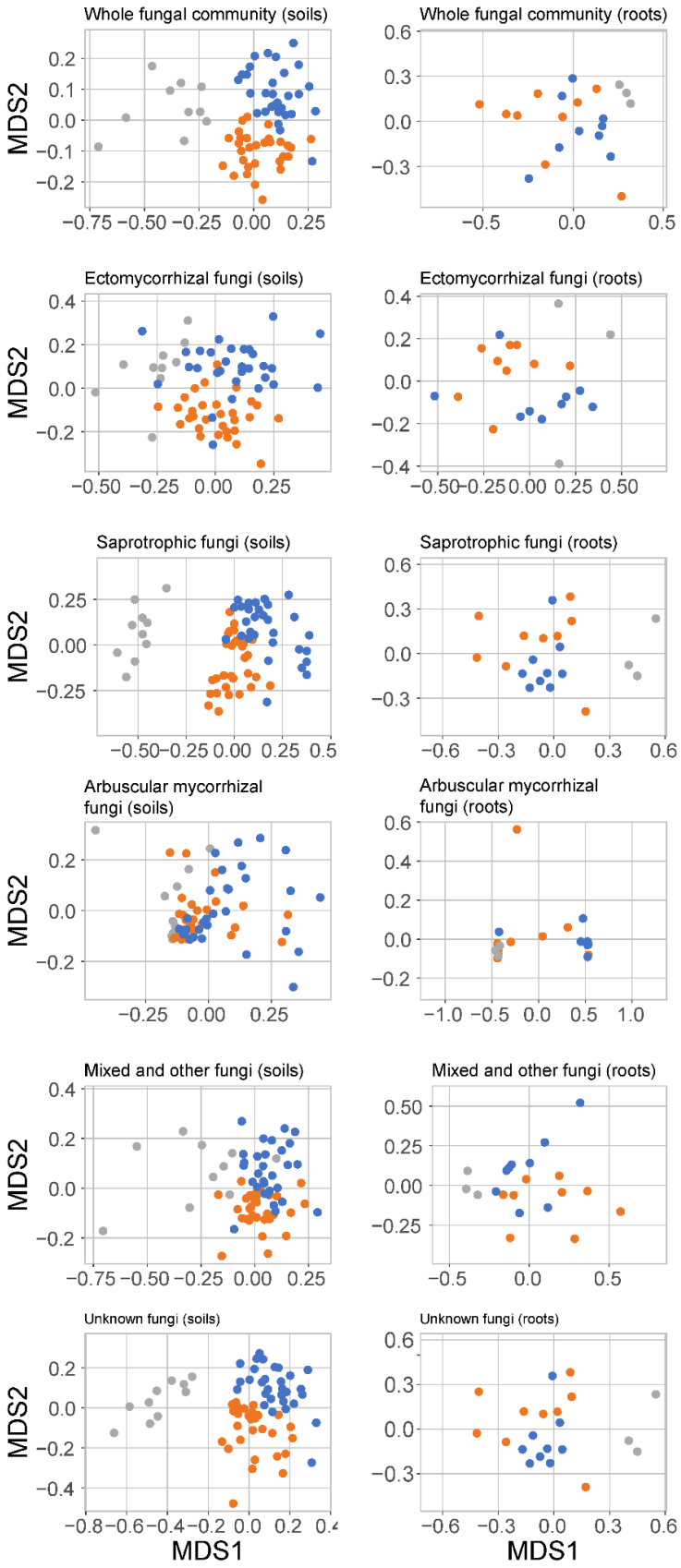
Whole fungal community (soils)
Whole fungal community (roots)
Ectomycorrhizal fungi (soils)
Ectomycorrhizal fungi (roots)
Saprotrophic fungi (soils)
Saprotrophic fungi (roots)
Arbuscular mycorrhizal fungi (soils)
Arbuscular mycorrhizal fungi (roots)
Mixed and other fungi (soils)
Mixed and other fungi (roots)
Unknown fungi (soils)
Unknown fungi (roots)
MDS1
MDS2

Figure 3.1 A non-metric multidimensional scaling (NMDS) ordination of amplicon sequence variants (ASVs) from soil and *Pinus banksiana* root samples representing the whole fungal community, ectomycorrhizal fungal, saprotrophic fungal guilds, arbuscular mycorrhizal fungal guild, mixed or other guilds, and fungi not assigned to a guild from shallow-bituminous soils (blue) bituminous-free (orange) and from the ore pile (grey). Non-metric multidimensional scaling ordinations were calculated using 500 permutations and the lowest stress permutations were selected.

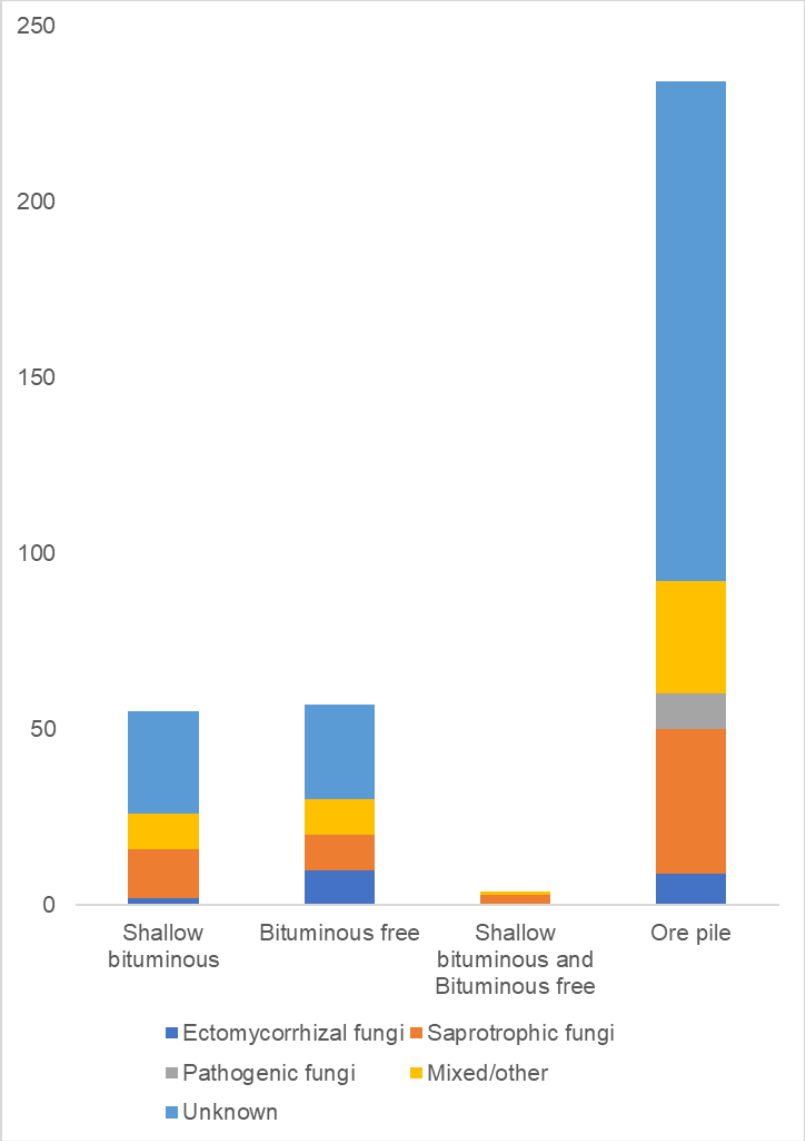

Figure 3.2 The distribution of indicator amplicon sequence variants (ASVs) by functional guild for soil samples collected from bituminous-free, shallow-bituminous-free soils, and the ore pile in northeast Alberta, Canada. The FUNGuild database was used to assign functional guilds to the ASVs (Nguyen et al. 2016).

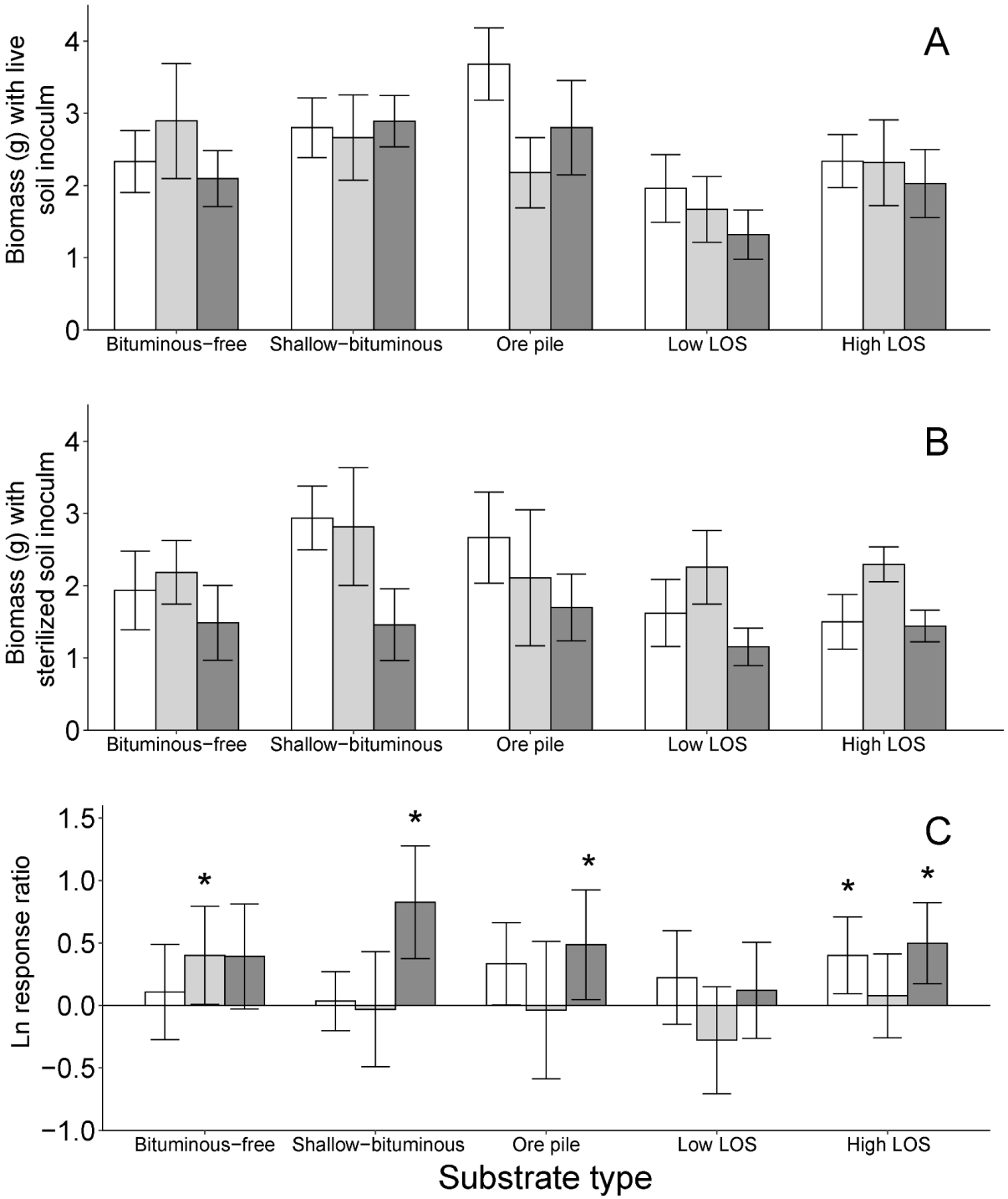

Figure 3.3 Mean biomass (± 95% CI) of jack pine (*Pinus banksiana*) grown with live soil inoculum (A) or sterile soil inoculum (B) in bituminous-free soil, shallow-bituminous soil, ore pile substrate, lean oil sand containing 0.88% hydrocarbons (Low LOS), and lean oil sand containing 3.67% hydrocarbons (High LOS). Soil inoculum collected from bituminous-free pine stands (white), shallow-bituminous pine stands (light grey), or ore pile (dark grey). Bars with different letters are significantly different ($P < 0.05$) based on Tukey HSD test. Response ratio of jack pine biomass to soil inoculation (C). If 95% confidence intervals are above or

below zero (y axis) this indicates a significant effect of soil inoculum on seedling biomass, indicated by *. If confidence intervals cross the zero line there is no significant effect.

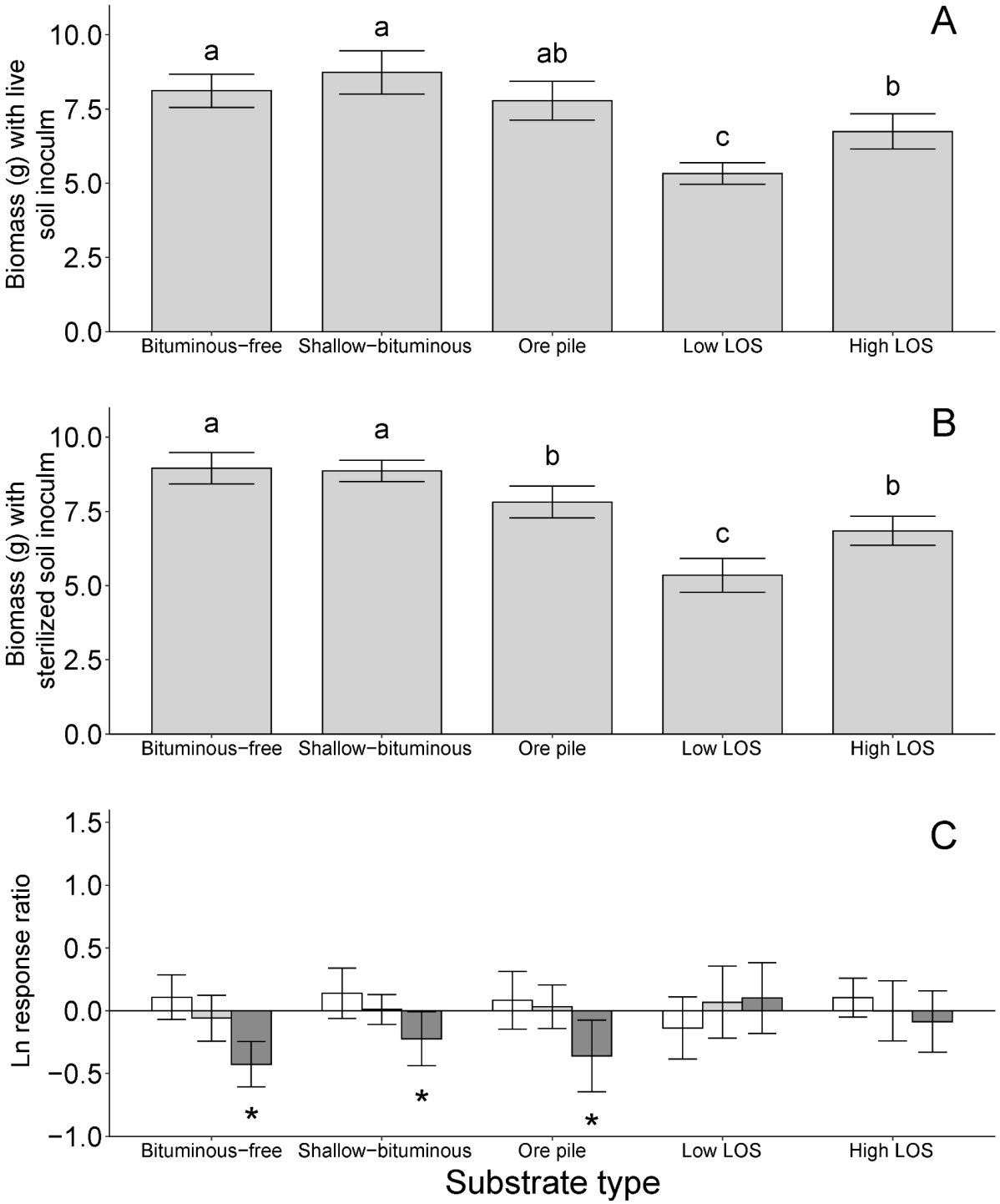

Figure 3.4 Mean biomass (± 95% CI) of aspen (*Populus tremuloides*) grown with live soil inoculum (A) or sterile soil inoculum (B) in bituminous-free soil, shallow-bituminous soil, ore pile substrate, lean oil sand containing 0.88% hydrocarbons (Low LOS), and lean oil sand containing 3.67% hydrocarbons (High LOS). Soil inoculum collected from bituminous-free pine stands (white), shallow-bituminous pine stands (light grey), or ore pile (dark grey). Bars with different letters are significantly different ($P < 0.05$) based on Tukey HSD test. Response ratio of jack pine biomass to soil inoculation (C). If 95% confidence intervals are above or

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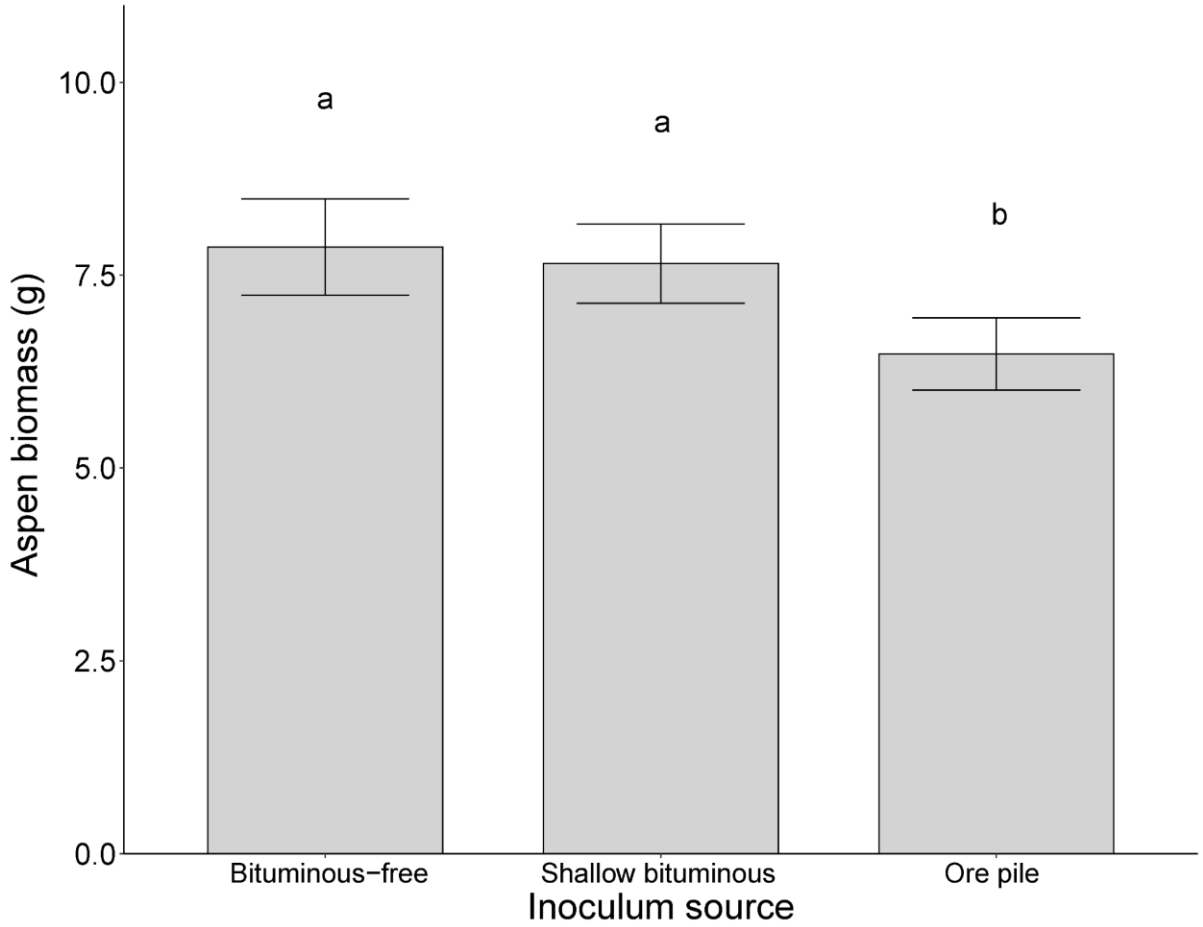

Figure 3.5 Mean biomass (± 95% CI) of aspen (*Populus tremuloides*) inoculated with live soil inoculum collected from bituminous-free pine stands, shallow-bituminous jack pine stands (*Pinus banksiana*), or Bitumount Alberta, Canada, ore pile. Bars with different letters are significantly different ($P < 0.05$) based on Tukey HSD test.

Tables

Table 3.1 Relative abundance of ITS1 amplicon sequence variance (ASV) for the top ten most abundant families from shallow-bituminous, bituminous-free soils, and the ore pile from northeast Alberta, Canada. The percentage is relative to the total number of ITS1 reads per substrate type. Sequences that were not assigned a family are denoted by NA.

		Shallow-bituminous	Bituminous-free	Ore pile
Ascomycota		48.59	39.70	47.72
	Archaeorhizomycetaceae	26.84	18.70	< 0.01
	Vibrisseaceae	2.63	4.93	3.41
	Hyaloscyphaceae	2.22	0.81	4.04
	Helotiaceae	2.12	2.10	1.05
	Saccharomycetales_fam_Incertae_sedis	1.72	< 0.01	0.00
	Helotiales_fam_Incertae_sedis	1.13	1.26	1.76
	Herpotrichiellaceae	1.09	1.47	3.75
	Gloniaceae	1.07	0.54	0.00
	Lipomycetaceae	0.58	0.33	0.00
	Ophiocordycipitaceae	0.11	0.85	0.04
	Pleosporaceae	0.00	0.50	0.14
	Leotiaceae	0.18	0.13	8.07
	Phacidiaceae	0.07	0.05	4.52
	Sordariaceae	0.00	0.00	3.92
	Trichomeriaceae	0.05	< 0.01	2.01
	Nectriaceae	< 0.01	< 0.01	0.99
	NA	7.20	6.08	8.07
Basidiomycota		44.85	51.08	42.00
	Russulaceae	19.21	16.62	9.26
	Atheliaceae	8.36	5.67	2.55
	Thelephoraceae	2.08	1.89	5.03
	Hygrophoraceae	1.82	0.95	< 0.01
	Trichosporonaceae	1.17	0.35	0.03
	Tricholomataceae	0.94	5.37	0.44
	Serendipitaceae	0.91	1.33	2.35
	Tritirachiaceae	0.91	1.63	0.00
	Trimorphomycetaceae	0.80	0.63	6.15
	Cortinariaceae	0.23	4.34	0.15
	Geminibasidiaceae	0.03	2.89	< 0.01
	Piskurozymaceae	0.15	1.38	6.13
	Cantharellales_fam_Incertae_sedis	0.37	1.21	1.01

Inocybaceae	0.06	1.00	0.79
Ceratobasidiaceae	0.11	0.25	0.68
NA	5.84	2.55	3.02

Table 3.2 Relative abundance of ITS1 amplicon sequence variance (ASV) for the top ten most abundant families present in jackpine (*Pinus banksiana*) roots collected from shallow-bituminous, bituminous-free soils, and the ore pile. The percentage is relative to the total number of ITS1 reads per substrate type. Sequences that were not assigned a family are denoted by NA.

		Shallow-bituminous	Bituminous-free	Ore pile
Ascomycota		55.93	42.54	60
	Vibrisseaceae	27.23	12.44	37.02
	Gloniaceae	9.14	1.36	< 0.01
	Hyaloscyphaceae	4.44	2.84	8.44
	Archaeorhizomycetaceae	4.32	16.99	1.04
	Dothideomycetes_fam_Incertae_sedis	2	1.3	0.19
	Pyronemataceae	1.83	1.13	NA
	Herpotrichiellaceae	0.87	0.95	0.58
	Helotiaceae	0.69	0.55	1.91
	Helotiales_fam_Incertae_sedis	0.46	0.42	0.3
	Sarcosomataceae	0.19	1.86	0.03
	Dermateaceae	0.05	0.01	0.09
	Ascocorticiaceae	NA	NA	0.07
	NA	3.54	2.23	10.13
Basidiomycota		42.71	55.84	38.33
	Russulaceae	14.25	23.77	12.62
	Thelephoraceae	9.1	4.4	14.62
	Pilodermataceae	8.77	3.87	NA
	Cortinariaceae	4.65	2.85	NA
	Tricholomataceae	1.79	5.38	5.21
	Serendipitaceae	0.65	1.81	0.26
	Tylosporaceae	0.57	3.84	NA
	Suillaceae	0.31	0.03	0.06
	Syzygosporaceae	0.25	0.01	0.01
	Bankeraceae	NA	4.91	< 0.01
	Clavulinaceae	0.01	1.8	0.01

Trimorphomycetaceae	0.14	0.04	1.32
Dacrymycetaceae	NA	NA	0.16
Piskurozymaceae	< 0.01	< 0.01	0.02
Suillaceae	0.03	0.01	0.02
NA	1.29	0.96	3.99

Table 3.3 Relative abundance of SSU amplicon sequence variance (ASV) assigned to arbuscular mycorrhizal fungal genera present in shallow-bituminous, bituminous-free soils, and ore pile substates from northeast Alberta, Canada. The percentage is relative to the total number of SSU reads per substrate type. Sequences that were not assigned a genus are denoted by NA.

	Bituminous soils	Bituminous-free soils	Ore pile
Ambispora	0.19	0	0
Archaeospora	0.61	1.01	0.79
Claroideoglomus	0	0	0
Glomus	66.92	66.19	68.24
Paraglomus	12.27	9.56	16.79
NA	20.01	23.23	14.18

Chapter 4: Lean oil sand influences the growth of ectomycorrhizal fungi

4.1 Introduction

Surface mining for oil sands in Canada involves the removal of vegetation, soils, and overburden to access the ore below. Overburden containing up to 8% hydrocarbons, which is referred to as lean oil sands (LOS), can therefore become exposed as part of the reclaimed landscape. At present, it is not economically viable to extract hydrocarbons from LOS.

Upon mine closure, according to provincial law, mined oil sands sites must be returned to a state of land capability equivalent to that found prior to disturbance, which typically includes boreal forest uplands and wetlands (Government of Alberta 2021). Current reclamation practices involve the placement of overburden into large landforms, which are then capped with substrates including peat or mineral soils. As such, LOS may interact with the capping material, possibly leading to petroleum hydrocarbons being present in the rooting zone of establishing vegetation (Visser, 2008). Properties of LOS such as the presence of petroleum hydrocarbons and low soil nutrient concentrations (see Chapter 2, 3), may inhibit plant growth (Visser, 2008; Rees et al., 2020). Growth inhibition may be the result of direct toxic effects on plants or indirect effects on plant-soil interactions. Furthermore, LOS may also have a negative affect on mutualistic fungi many plants found in the boreal forests interact with. However, to what extent LOS affects the growth of fungi is poorly understood.

Soils in the boreal forest are dominated by fungi, which affect plant growth (Read *et al.*, 2004). For example, the majority of tree species in the boreal forest interact with ectomycorrhizal fungi (Read *et al.*, 2004). These symbiotic fungi form associations with plant roots and provide their hosts with soil-derived nutrients in exchange of sugars from photosynthesis (Smith and Read, 2008). Ectomycorrhizal fungi can improve plant growth in reclaimed soils (Greer et al., 2011; Onwuchekwa et al., 2014). However, whether the inhibitory effect of LOS on plant growth could be even stronger if these fungi were absent is unknown. Furthermore, soil management practices that can disrupt ectomycorrhizas may contribute to the inhibitory effects of LOS on plant growth. While ectomycorrhizal fungi are generally widespread and, as such, present in the soil used as capping material, stockpiling and physical disturbances of these soils during reclamation can reduce ectomycorrhizal fungal abundance (Danielson et al., 1983; Bois et al. 2005), and, consequently, soil inoculum potential (i.e., the percentage of ectomycorrhizal root colonization in a bioassay plant) (Brundrett and Abbott, 1994; Danielson and Visser, 1989; Bois et al. 2005; Hankin et al., 2015). While there is evidence that the inoculum potential of disturbed soils increases with the age of the site, this

appears to be a slow process (Bois et al. 2005; Trofymow et al., 2020). As such, data are needed to determine whether artificial inoculation may be required in the restoration of these sites.

Inoculation of seedlings with locally adapted ectomycorrhizal fungi is expected to yield positive growth responses greater than those inoculated with fungi sourced elsewhere (Nadeau et alk., 2018; Repas et al., 2017; Vahter et al., 2020). As such, inoculating seedlings and shrubs with ectomycorrhizal fungi obtained from LOS and, consequently, potentially tolerant to LOS, may increase seedling survival and growth in reclaimed soils containing hydrocarbons. There are various mechanisms by which ectomycorrhizal fungi could alleviate the negative effects of hydrocarbons on plant growth. First, since LOS often contain low concentrations of nitrogen (N) and vary in phosphorus (P) concentrations (Visser 2008; Rees et al., 2020; Brown, 2020), the capacity of ectomycorrhizal fungi to access organic forms of N and P may be key. Second, hydrocarbon molecules may be sequestered in the mantle and other fungal tissues, which both contribute to increasing the root's surface area and act as a protective barrier (Gunderson *et al.*, 2007). Third, non-specific fungal enzymes such as polyphenol oxidases produced for the degradation of organic materials may contribute to degrade petroleum hydrocarbons (Braun-Lüllemann *et al.*, 1999; Meharg & Cairney, 2000; Robertson *et al*., 2007; Harms *et al.,* 2011).

Native trees and shrubs are planted to revegetate reclaimed landforms. While host specificity in the ectomycorrhizal symbiosis may limit the number of taxa available, identifying the most suitable fungal species and strains for use as inoculants in the reclamation of LOS contaminated sites is an area requiring further investigation. Prior to greenhouse or small-scale field trials, *in vitro* experiments can be used as a first step for screening and selecting suitable ectomycorrhizal fungal taxa (Bois and Coughlan, 2009). While the fungal response *in vitro* may differ from that in symbiosis in soil, *in vitro* experiments are a valid starting point for identifying candidate species. Previous studies investigating the role of ectomycorrhizal fungi on plant tolerance to metals (Formina et al., 2005), salt (Bois et al., 2006), and refined petroleum (Nicolotti and Egli, 1998; Mohsenzade et al., 2009) found broad interspecific variation in response to these stressors. However, little work has been conducted to identify fungal species that are tolerant to LOS.

Selection of appropriate ectomycorrhizal fungal taxa to use as inoculant is important, as species vary in their degree of hydrocarbon degradation (Braun-Lüllemann *et al.*, 1999; Joner et al., 2006, Gunderson et al., 2007). Additionally, other fungal characteristics may play a role in how these fungi tolerate the presence of hydrocarbons. Melanin, for example, is a complex macromolecule made up of phenolic or indolic monomers which gives fungal cell walls their color (Butler and Day, 1998). Melanin can increase fungal tolerance to

environmental stressors including, salts (although see Gaber et al., 2020), heavy metals, increased UV radiation, and water stress (Gessler et al., 2014). As melanin is localized in fungal cell walls, it may serve as a barrier against petroleum hydrocarbons. However, to my knowledge, the potential association between fungal melanin concentration and an increased tolerance to hydrocarbons has not been investigated. If fungi with greater melanin concentration are indeed more tolerant to the presence of LOS, measuring fungal melanin concentration may be a simple screening method for selecting suitable fungal inoculants.

I conducted an *in vitro* experiment to determine first how different ectomycorrhizal fungal species respond to the presence of LOS with or without a host seedling. I hypothesized that: 1) hydrocarbons found in LOS reduce plant and fungal growth, and 2) seedlings and ectomycorrhizal fungi in symbiosis are better able to tolerate hydrocarbons than when grown alone. Next, I investigated how various fungal species found in boreal forest soils of northern Alberta respond to the presence of LOS. I hypothesized that: 3) there is interspecific variation in fungal response to the presence of LOS; and 4) fungi with greater concentrations of melanin are relatively more tolerant to the presence of LOS. Taken together, these experiments will inform how land reclamation with substrates containing hydrocarbons affects plant-microbe interactions, and whether fungal inoculants hold promise for use in reclamation campaigns.

4.2 Methods

4.2.1 Sterilization of lean oil sand

The LOS used in both microcosm experiments (see below) was collected at the Aurora North Mine Site, Alberta, Canada on June 21st, 2018. In the laboratory, this LOS was ground with a mortar and pestle and passed through a 1 mm sieve. The LOS was analyzed for hydrocarbon fractions 2–4 following reference methods set by the Canada-wide Standard for Petroleum in Soil (Canadian Council of Ministers of the Environment, 2014). Hydrocarbon fractions 2–4 represent F2 (>nC10 to nC16), F3 (>nC16 to nC34), and F4 (nC35 to nC50+) (Turle et al., 2007) (Appendix Table S4.1). Analysis of hydrocarbon concentrations was performed by the Bureau Veritas Environmental Services Laboratory in Edmonton, Alberta. Owing to safety concerns (i.e., release of organic volatile compounds and fire hazards), the LOS could not be sterilized by autoclaving; instead, gamma irradiation was used. Eight kilograms of LOS were divided into 1 kg portions 1 cm thick and vacuumed sealed in plastic bags before receiving four passes of electron beam radiation at 82.1 kGy (Iotron Industries Canada Ltd., Port Coquitlam, British Columbia).

4.2.2 Experiment #1: How does symbiosis modulate ectomycorrhizal fungal responses to lean oil sand?

I first tested the effects of LOS on the growth of two species of ectomycorrhizal fungi alone and in symbiosis with jack pine (*Pinus banksiana* Lamb.) seedlings. Construction of microcosms was adapted from Jones *et al*., 2013. Jack pine was selected because it is a common tree species in the region and is often planted in areas reclaimed after oil sand mining (Farnden et al., 2013). The two species of ectomycorrhizal fungi, *Cenococcum geophillum* Fr. and *Suillus tomentosus* Kauffman. were selected based on my previous research (Thesis Chapter 3) and that of others (Danielson, 1984 and Visser 1995) demonstrating that these fungi are common in soils of the region. Specifically, I found that *C. geophillum* represented the most abundant number of ectomycorrhizal DNA sequences present in the roots of mature jack pine trees growing in shallow-bituminous soils (Thesis Chapter 3). *Suillus tomentosus* was selected because of its host specificity on *Pinus* sp. (Molina et al., 1992). Additionally, *Cenococcum geophillum* and *S. tomentosus,* have been found to interact with jack pine in reclaimed soils (Calvo-Polanco *et al*., 2009, Onwuchekwa *et al*., 2014). *Cenococcum geophillum* (UAMH 5512) was provided by the University of Alberta Microfungus Collection and Herbarium (Edmonton, Alberta). *Suillus tomentosus* was provided by Dr. Roland Treu (Athabasca University, Athabasca, Alberta). The treatment combinations for this experiment (Inoculum

type) included jack pine seedlings either inoculated or not with *C. geophillum* or *S. tomentosus*. In addition, each of these fungal species was grown without a seedling. These treatment combinations were grown on four different microcosm plates containing different amounts of hydrocarbons expected to affect seedling growth (Visser 2008, Thesis Chapter 2, Thesis Chapter 3). These treatments included agar only ('Agar'), agar mixed with LOS for a total concentration of 0.5% hydrocarbons ('LOS 0.5%'), and agar mixed with LOS for a total concentration of 1.5% hydrocarbons ('LOS 1.5%'). An additional treatment with agar and glass ('Glass') was included to control for any potential physical effects LOS on the symbionts. In summary, the experimental design included five pine/fungal species combinations × 4 levels of hydrocarbons × 15 replicates, for a total of 300 experimental units (i.e., microcosms).

Microcosms contained the following medium: Melin-Norkrans Medium, modified without agar (bioWorld #30627028) and 1.7% bacto agar (Fisher Scientific #DF0140-01-0) per litre. The pH of the medium was then adjusted to 5.8 with 10% KOH. To inhibit bacterial growth after autoclaving the solution, I added 10 mL per litre of 0.1 μm filtered penicillin-streptomycin after allowing it to cool to approximately 60 °C (MilliporeSigma #P4333). Forty-four point one millilitres of agar were poured into 100 mm × 15 mm Petri plates. Plates with 0.5% hydrocarbons had 1.96 g of LOS added, while plates with 1.5% hydrocarbons received 5.89 g of LOS (See supplemental material for calculation). The crushed glass ('Glass' treatment) was as an inert substance similar in size to the LOS and was prepared by crushing 2.5 cm × 7.5 cm glass slides (Fisherbrand # 12-549-3) with a mortar and pestle and passing the fragments through a 1 mm sieve. The crushed glass was steam sterilized at 121 °C (30 minutes, repeated twice) and 5.89 g were mixed into the poured agar. Plates which received a seedling had a 5 mm hole melted on one side (Appendix Figure S4.1).

Pinus banksiana seeds were sourced from seed lot SYN 26-96-10-4-2008 PJ, zone CM 2.1, Smokey Lake, Alberta. *Pinus banksiana* seeds were surface sterilized with 30% hydrogen peroxide for 15 minutes and rinsed in sterile deionized H_2O. Seeds were soaked in sterile deionized H_2O for 24 hours and cold stratified at 4 °C for 14 days. Seeds were then spread on Melin-Norkrans Medium (MMN) agar plates (bioWorld #30627028) and allowed to germinate for five days. Seedlings with approximately 2 cm long radicles were selected haphazardly and transplanted into a randomly selected microcosm with the radicle sitting against the agar and the cotyledon on the exterior of the edge of the plate (Figure S1). The hole around the hypocotyl of each seedling was sealed with steam sterilized lanolin (MilliporeSigma #L7387). Plates were inoculated with fungi by subculturing a 5 mm plug from the edge of actively growing cultures. Each plug was placed in the centre of each plate. To ensure the seedlings' roots and fungal

cultures were grown in darkness, all plates were sealed with parafilm and individually wrapped with aluminium foil. The completed microcosms were placed in a growth cabinet set at 16/8 hours of light/darkness, 20/18 °C daytime/nighttime temperatures and 65% relative humidity. To minimize any positioning effects within the growth cabinet, microcosms were randomly rearranged weekly.

To assess the growth rate of pine roots and fungal mycelium, plates were scanned every 10 days with an Epson Perfection V600 Photo scanner (Epson Canada Limited, Markham, Canada) set to 800 dpi resolution. Root length and fungal surface area were measured using ImageJ 1.53k 6 software (Schneider et al. 2012). Fifty days following the placement of germinated seedlings in the microcosms, seedlings were harvested. Shoots of each plant were dried at 65 °C for 72 hours and weighed. Roots were separated from the agar and fungal tissue and frozen at -20 °C before being measured for fungal colonization. I estimated ectomycorrhizal fungal root colonization of all root tips for each inoculated seedling under 20 × magnification. Root tips were considered colonized when a mantle was present and root hairs were absent.

Throughout the duration of the experiment, I observed minor puddling/condensation of water at the edge of plates with LOS. I also found pine seedlings' root growth was highest in plates with 1.5% hydrocarbons. To help answer why pine seedling root growth was highest in the presence of 1.5% hydrocarbons, I grew additional seedlings on agar plates with free standing water (Methods provided in supplemental material). I found the presence of free-standing water at the bottom of the microcosm did not increase root growth. While I detected a significant interaction between time and the presence of water (Water × Time, $P = 0.016$, Table S4.2), pine roots with or without water treatments did not differ on day 10, 20, or 30 (Fig. S2).

4.2.3 Experiment #2: Is there interspecific variation in ectomycorrhizal fungal response to the presence of lean oil sand?

To test how fungal species found in the boreal forest of northern Alberta respond to the presence of LOS, I performed a second *in vitro* experiment. Nine species of fungi were used in this experiment encompassing a range of growth rates and morphologies. These species included: *Cenococcum geophillum, Coltricia confluens* Keizer*, Laccaria bicolor* Maire (Orton)*, Nectria mauritiicola* (Henn.) Seifert & Samuels*, Phellinus tremulae* (Bondartsev) Bondartsev & PN Borisov*, Rhizopogon pseudoroseolus* Sm.*, Suillus brevipes* (Peck) Kuntze*, Suillus tomentosus, Wilcoxina mikolae* (Chin S. Yang & H.E. Wilcox) Chin S. Yang & Korf. Of these,

seven are ectomycorrhizal and two are plant pathogens. The two pathogenic fungal species were included to examine if fungi with a nutrient acquisition strategy other than ectomycorrhizal differ in their response to LOS. Fungal cultures were obtained from different sources: *Suillus tomentosus* was provided by Dr. Roland Treu (Athabasca University, Athabasca, Alberta). *Laccaria bicolor* was provided by Dr. Melanie Jones (University of British Columbia Okanagan Campus, Kelowna, British Columbia). *Cenococcum geophillum* (UAMH 5512), *Nectria mauritiicola* (UAMH 5838), *Suillus brevipes* (UAMH 5287), *Rhizopogon pseudoroseolus* (UAMH 5188), *Phellinus tremulae* (UAMH 6266), *Coltricia confluens* (UAMH 8961), and *Wilcoxina mikolae* (UAMH 6703), were provided by the University of Alberta Microfungus Collection and Herbarium (Edmonton, Alberta). Subcultures of *C. geophillum* and *S. tomentosus* used in experiments #1 and #2 were subcultured from the same original agar plate. To obtain enough mycelium for the experiment, fungal cultures were grown by subculturing 5 mm diameter plugs of actively growing culture onto MMN agar plates (100 mm × 15 mm). These subcultures were grown in permanent darkness for 3 weeks at 22 °C.

To confirm fungal species identity, I used Sanger sequencing. DNA was extracted from one milligram of tissue collected from each culture and placed separately in REDExtract-N-Amp buffer (MilliporeSigma, St. Louis, USA) and heat-extracted following the manufacturer's instructions. The internal transcribed spacer (ITS) region was amplified using the primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). All reaction volumes in this first amplification were 25 µL and contained 12.5 µL EconoTaq PLUS 2× Master Mix (Lucigen Corporation, Middleton, Wisconsin, USA), 2.0 µL of DNA template, 2.5 µL of each µM primer, and 5.5 µL of nuclease free H_2O. Reactions were conducted in a Mastercycler pro S Thermal Cycler (Eppendorf Canada, Mississauga, ON, Canada). Reaction conditions were 95 °C for 5 minutes; followed by 35 cycles of 95 °C for 60 s, 53 °C for 60 s, 72 °C for 90 s; and a final extension of 72 °C for 10 minutes. To verify if the target region had been amplified, I ran each sample on a 2% agarose gel. PCR products were then purified using Mag-Bind Total Pure NGS magnetic beads (Omega Bio Tek, Norcrsoss, GA, USA). Sanger sequencing was performed at the University of Alberta Molecular Biology Service Unit. Sequences were edited and aligned with Geneious Prime 2021.0.3 (Kearse et al., 2012). The resulting sequences were compared to National Center for Biotechnology Information (NCBI) GenBank databases using the MEGA BLAST program with standard settings (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences that produced significant alignments with >97% identity or the top ten sequences with the greatest bit score with >97% identity are shown in Table S4.3

Fungi were grown on two agar plate types, containing either agar with glass ('Glass') or agar mixed with LOS with a total concentration of 1.5% hydrocarbons. The total number of agar plates constructed for this experiment was 320 (8 fungal species × 2 plate types × 20 replicates). Melin-Norkrans Medium agar plates were prepared with sterile crushed glass and LOS as described above. As before, plates were inoculated with fungi by subculturing a 5 mm plug from the edge of actively growing cultures. Each plate was sealed with parafilm and individually wrapped with aluminium foil. Plates were placed in a growth cabinet in permanent darkness for 40 days at 22 °C. To assess the growth rate of fungi, every 10 days I scanned each plate with an Epson Perfection V600 Photo scanner (Epson Canada Limited, Markham, Canada) set to 800 dpi resolution. By day 30, *C. confluens, L. bicolor, N. mauritiicola, P. tremulae, and W. mikolae* colonies had neared or reached the edge of the plates, at which time they were harvested. Slower growing fungi, *C. geophillum, R. pseudoroseolus, S. brevipes*, and *S. tomentosus* were grown for an additional 10 days. Measurements of the fungal surface area were completed using ImageJ 1.53k 6 software (Schneider et al. 2012).

To determine if fungi with greater concentration of melanin within their tissue are more tolerant of LOS or how the presence of LOS affects fungal melanin concentration, I measured the concentration of melanin within each fungal species using a colorimetric assay (Fernandez & Koide, 2014). The method is based on the strong binding affinity Azure A has for melanin. The change in absorbance of Azure A when in contact with melanin allows for the quantification of melanin in fungal tissue. Azure A (MilliporeSigma #861049) was dissolved in 0.1 M HCl, and vacuum filtered through Whatman 1 filter to remove any undissolved particles. The Azure A solution was diluted to an absorbance of 0.650 at 610 nm. To generate a standard curve, pure melanin was first isolated from *C. geophilum* tissue. *Cenococcum geophillum* has a very high concentration of melanin (20–30% by weight). To isolate melanin, fungal tissue was dried at 65 °C for 48 hours and then placed in 6 M HCl at 80 °C for 4 days. Melanin is acid insoluble, allowing for the selective isolation of melanin from the fungal tissue. The melanin was collected by vacuum filtering and then washed several times with DI H_2O. The melanin was then lyophilized for 72 hours and stored at -20 °C. To generate a standard curve, 0.1 to 5 mg of melanin was placed in 3 mL of Azure A for 90 minutes. The solution was then filtered through 0.45 mm syringe tip filters (MilliporeSigma #WHA67841304). The absorbance of the filtrate was measured at 610 nm and the change in absorbance was used to generate a standard curve. Fungal tissue samples from each species were dried at 65 °C and placed in the Azure A solution for 90 minutes. The solution was again passed through 0.45 mm

syringe tip filters and the absorbance measured at 610 nm. To calculate the melanin concentration, I used the change in absorbance and compared it to the standard curve.

4.2.4 Statistical analysis

All statistical analyses were conducted using R v.4.1.0 (R Development Core Team, 2021) statistical software with R Studio 1.3.1056. (R Studio Team, 2021) and visualized with *ggplot2* 3.3.5 package (Wickham, 2016).

Experiment #1:

The response of seedlings and fungi to LOS was measured by shoot biomass and root length for the seedlings, and surface area of mycelium for the fungi. To evaluate how root length responded to the presence of LOS over time, I ran a three-way mixed ANOVA using the *ezANOVA* function in the *ez* package 0.28-1 (Lawrence 2016). Time was the within-factor while plate-type and inoculum type were the between-factors. Root length was natural log transformed to meet normality and homogeneity assumptions. To evaluate how the fungal surface of *C. geophillum* and *S. tomentosus* responded to the presence of LOS and a pine seedling, I ran a three-way mixed ANOVA. Time was the within-factor while plate-type and seedling presence were the between-factors. For the mixed ANOVAs the assumption of sphericity was tested using the Mauchly's test. If violations of sphericity occurred, degrees of freedom were adjusted using the Greenhouse-Geisser or Huynh-Feldt correction, depending on the epsilon value. Type III ANOVA was used to test for significant differences using the *Anova* function in the *car* 3.0-11 package (Fox and Weisberg., 2019). To complete post-hoc comparisons, I computed the estimated marginal means using the *emmeans* function in the *emmeans* package 4.4-0 (Lenth, 2021). To compare specific time points and treatments, I used P-value protected contrast analyses. To evaluate whether shoot biomass responded to inoculation with *C. geophillum* or *S. tomentosus* when grown in LOS, I ran a two-way ANOVA with plate type and inoculum type as factors. Shoot biomass was log transformed to meet the ANOVA assumptions of normality and homogeneity of residual variance. Means were compared using Tukey's honest significance difference (HDS) test ($P < 0.05$).

Experiment #2:

To evaluate how various fungal species responded to the presence of LOS over time, I ran a three-way mixed ANOVA with fungal surface area as the dependent variable. Time was the within-factor while plate-type and fungal species were the between-factors. This analysis of

surface area was conducted on measurements taken from day 10 to 30. To determine whether fungi grown up to day 40 responded to the presence of LOS, I ran a two-way ANOVA with plate-type and fungal species as factors. Surface area was box-cox transformed to meet the ANOVA assumptions of normality and homogeneity of residual variance. Means were compared using Tukey's honest significance difference (HDS) test ($P < 0.05$).

To determine if the melanin concentration of fungal tissue responded to the presence of LOS and whether fungal species varied in melanin concentration, I used a two-way ANOVA with plate-type and fungal species as factors. Type III ANOVA was used to test for significant differences. Melanin concentration was log transformed to meet the ANOVA assumptions of normality and homogeneity of residual variance. Means were compared using Tukey's honest significance difference (HDS) test ($P < 0.05$).

Linear regression was used to determine whether there was a relationship between the concentration of melanin in fungal tissue and fungal growth area in the presence of LOS. To standardize fungal growth, I divided the growth area of each fungal species grown on plates containing LOS by those grown without LOS. Finally, melanin concentration was log transformed to improve data visualization.

4.3 RESULTS

4.3.1 Experiment #1: How does symbiosis modulate ectomycorrhizal fungal responses to lean oil sand?

The presence of LOS positively affected pine seedling shoot growth (Plate type, P = 0.0001, Table S4.4). Measured at the end of the experiment, shoot biomass of pine seedlings grown in microcosms containing LOS (1.5%) hydrocarbons was 64% larger than shoot biomass in any other treatment (Fig. 4.1A). Irrespective of hydrocarbon treatment, pine seedling shoot biomass varied in response to inoculum type (Inoc type, P = <0.0001, Table S4.4). Seedlings inoculated with *S. tomentosus* were 35% smaller than those in non-inoculated controls, while seedlings inoculated with *C. geophillum* did not differ from those in the non-inoculated controls (Fig. 4.1B). I did not detect a significant plate type × inoculum type interaction (Plate type × Inoculation type P = 0.846, Table S4.4).

Throughout the duration of the experiment, pine seedling root lengths were significantly affected by the presence of LOS and inoculum type (Inoc type × Plate type × Time, P = <0.0001, Table S4.5). While root length was the highest between days 20 and 50 in plates containing LOS (1.5%), uninoculated seedlings and those inoculated with *C. geophillum* (Fig. 4.2A, 4.2B) had significantly longer root lengths than seedlings inoculated with *S. tomentosus* (Fig. 4.2C). By day 50, roots of uninoculated controls and those inoculated with *C. geophillum* grown on plates containing LOS (1.5%) were, respectively, 324% and 387% longer than those in the glass controls. However, the roots of seedlings grown on LOS (1.5%) and inoculated with *S. tomentosus* were 67% longer than those inoculated with *S. tomentosus* grown on glass controls.

Overall, while there was a significant interaction between plate type and pine seedling on the surface area of *C. geophillum* throughout this experiment (Pine × Plate type × Time, P <0.0001, Table S4.6), the presence of a host did not alleviate the negative effect of hydrocarbons on *C. geophillum* surface area. Pine seedling presence only increased *C. geophillum* surface area on agar plates (Fig. 4.3A). This increase was observed on days 30–50 and by day 50 fungal surface area was 26% larger when a pine seedling was present than in the controls without a host. The 1.5% LOS (1.5%) significantly reduced fungal surface area by day 20 and this continued for the duration of the experiment (Fig. 4.3A). By day 50, the growth of *C. geophillum* in LOS (1.5%) decreased by 55% when compared to growth on glass plates. *C. geophillum* surface area was also reduced on 0.5% LOS plates. However, this reduction was

only observed on days 20 through 40 (Fig. 4.3A). By day 50, *C. geophillum* grown on LOS (0.5%) plates did not significantly differ from those grown on glass plates.

Although I detected a significant interaction between plate type and pine seedling on the surface area of *S. tomentosus* throughout this experiment (Pine × Plate type × Time, P = 0.001, Table S4.7), I found the presence of LOS did not reduce the surface area of *S. tomentosus* and presence of a pine seedling did not increase the fungus' tolerance to the presence of hydrocarbons. *S. tomentosus* mycelium growth on the LOS (1.5%) plates was not significantly different than that on glass plates throughout the duration of the experiment (Fig. 4.3B, Table S4.7). The interaction appeared to be primarily driven by fungi growing 31% larger on glass plates when pine seedlings were present (Fig. 4.3B).

4.3.2 Is there interspecific variation in ectomycorrhizal fungalresponse to the presence of lean oil sand?

Overall, throughout the duration of the experiment fungal area varied significantly by species in response to LOS (Fungal species × Plate type × Time, P <0.0001, Fig. 4.4, Table S4.8, S4.9). By the end of the experiment, the surface area of *C. geophillum* was reduced by 47%*, C. confluens* (-58%), *L. bicolor* (-19%), *N. mauritiicola* (-10%), *P. tremulae* (-49%), *R. pseudoroseolus* (-51%) in response to LOS. In contrast, *S. brevipes* and *W. mikolae* were not significantly affected by the presence of LOS and by day 40 *S. tomentosus* surface area was 46% larger in the presence of LOS.

I found no significant relationship between fungal surface area and fungi with greater melanin concentration. Melanin concentration did not affect the growth of different fungi in response to LOS ($F_{1,7}$ =0.927, slope = -0.396, P = 0.368, Fig. S3). However, how LOS affected fungal tissue melanin concentration varied by species (Plate type × Fungal species P = <0.0001, Fig. 4.5; Table S4.10). The melanin concentration of *C. geophillum, P. tremulae, R. pseudoroseolus, S. tomentosus,* and *W. mikolae* was significantly reduced by the presence of LOS (Fig. 4.5), while the melanin concentration of *C. confluens, L. bicolor, N. mauritiicola*, and *S. brevipes* was not significantly affected by the presence of LOS (Fig. 4.5).

4.4 Discussion

This study clearly indicated that LOS can reduce growth in various ectomycorrhizal fungal species. However, in contrast to what I hypothesized, the growth pine seedlings were significantly greater in the presence of LOS. Furthermore, in contrast to what I hypothesized, the presence of a pine seedling did not increase fungal tolerance to LOS. I also found no relationship between fungal tissue melanin concentration and growth response to LOS. This latter result indicates fungi with greater concentrations of melanin in their tissue are not more tolerant to LOS. Finally, opposite to what I hypothesized, I found that LOS reduced fungal tissue melanin in some fungal species but not others.

4.4.1 Ectomycorrhizal fungi and pine seedlings response to lean oil sand

In contrast to what I hypothesized; overall pine seedling growth was not reduced by the presence of LOS. Pine shoot mass and root length were largest in the presence of LOS (1.5%). This contrasts with previous research on jack pine seedlings' response to LOS, which found LOS at similar concentrations reduced jack pine seedling growth (Thesis Chapter 3, Visser 2008). While I have no explanation for this increase in seedling growth, the increased root growth may indicate the seedlings were searching for nutrients (Cahill and McNickle, 2011).

I also found the interaction between fungi and seedlings did not lead to an increase in seedling growth or fungal tolerance to the presence of LOS. Non-inoculated seedlings and those inoculated with *C. geophillum* did not significantly differ in growth of shoots or roots indicating seedlings did not benefit from the presence of *C. geophillum*. However, seedling growth was reduced when grown in LOS (1.5%) and inoculated with *S. tomentosus,* indicating when LOS is present *S. tomentosus* acts as a parasite rather than a mutualist (Figure 1B, 2C). While ectomycorrhizal fungi are typically known for stimulating seedling growth this may be the result of publication biases from underreporting of non-significant or negative growth responses (Karst et al, 2008).

4.4.2 Interspecific variation in fungal response to the presence of lean oil sand

The results of both *in vitro* experiments clearly demonstrated that fungal species varied in response to LOS. However, this appears to be dependent on the concentration of petroleum hydrocarbons the fungi are exposed to, as the growth of *C. geophilum* during experiment #1 was only significantly reduced in plates containing LOS (1.5%) when compared to fungi grown on plates with no LOS. Although LOS differs in hydrocarbon fractions compared to other petroleum products, the variation in fungal growth response to the presence LOS found in this

study is consistent with previous studies which used microcosms to screen fungi for tolerance to petroleum products (Nicolotti and Egli, 1998; Mohsenzade et al., 2009). However, only one species, *L. bicolor,* used in my study overlapped with those previously cited. Nicolotti and Egli, 1998 reported *L. bicolor* responded positively to the presence of crude oil, while I found the presence of LOS negatively affected the growth of *L. bicolor*. This might be due to the high degree of intraspecific variation in fungal tolerance to stressors (Colpaert et al., 2000). Colpaert et al., 2000 also found fungi isolated from contaminated sites were more tolerant to the presence of the pollutants than those from uncontaminated sites. The variation between the results of my study and Nicolotti and Egli, 1998 suggests that screening specific isolates for tolerance to LOS is important. Future work will need to determine whether fungal genotypes isolated from natural bituminous soils or recently disturbed soils (Chapter 3) are more tolerant to the presence of LOS.

The complex molecular composition of petroleum hydrocarbons found in LOS makes isolating the exact component responsible for the direct effect of reduced mycelium growth difficult. However, previous studies suggest that growth reductions in the presence of petroleum hydrocarbons may be the result of the disruption of microbial membrane integrity (Miller and Herman, 1997). The yeast *Saccharomyces cerevisiae* has previously been found to alter the composition of cell membranes, increasing hydrophilicity and thus repelling hydrophobic compounds (Park et al., 1988). While this was not measured during my study, it may explain why certain species were more tolerant to the presence of LOS. It is also possible that fungi that were not affected or grew better in the presence of LOS may have utilized the LOS as a source of carbon, as previously suggested in petroleum studies that observed fungi growing better in the presence of hydrocarbons (Nicolotti and Egli, 1998; Mohsenzade et al., 2009; Patil et al., 2012; Rad et al., 2014).

4.4.3Fungal melanin and response to lean oil sand

In contrast to what I hypothesized, the melanin concentration of fungi did not increase in response to LOS. Instead, while melanin production in response to LOS was species specific, melanin production significantly decreased in five out of the nine species tested. This is a similar result reported by (Gaber et al., 2020) who found, at 100 mM of NaCl, melanin concentration of fungal hyphae increased but decreased at 500 mM. However, consistently with my data, (Gaber et al., 2020) reported species specific responses. It is possible, if a lower concentration of LOS had been used, I may have observed an increase in melanin concentration

across these fungi. This is especially probable, given during experiment number #1, *C. geophillum* surface area was not reduced by the presence of LOS (0.5%).

Contrary to my hypothesis, I did not detect a relationship between melanin concentration and the growth response of fungi to LOS. The evidence to whether melanin confers tolerance to environmental stressors appears to be mixed. For example, in contrast to my results, previous studies have found melanin increases fungal tolerance to stressors including UV radiation (Singaravelan et al., 2008), osmotic stress (Kogej et al., 2006), and heavy metals (Gadd and de Rome, 1988). However, other studies have reported no increased tolerance to salt (Gaber et al., 2020) or heavy metals (Berthelot et., 2020). Additionally, one recent review by Cordero et al., 2017 reported numerous studies which found highly melanized fungi across various extreme environments and stressors. However, many of the studies reported in this review focus on long-term stressors to which the fungi may have adapted to, including extreme temperatures, increased osmotic stress, and increased radiation, and increased acidity. Petroleum hydrocarbons are likely to be a novel stressor to the fungi used here. However, little information is available on how petroleum hydrocarbons affect the growth of fungi. Further examining how and why petroleum hydrocarbons affect fungal growth may help determine why increased melanin within fungal tissue does not confer an increased tolerance to petroleum hydrocarbons,

In conclusion, the results of this study show fungal species vary in response to LOS. The data suggest, however, that the concentration of LOS likely plays a pivotal role in whether fungi are negatively affected by the presence of LOS. This study further shows that *in vitro* microcosms are a viable method to test fungal responses to the presence of soils containing LOS. Future research is needed to determine whether fungal taxa which exhibit tolerance to LOS in microcosms also exhibit this tolerance in soils containing LOS. Greenhouse or field studies will also be needed to examine whether fungi more tolerant to LOS are able to increase a host's tolerance to the presence of LOS.

Figures

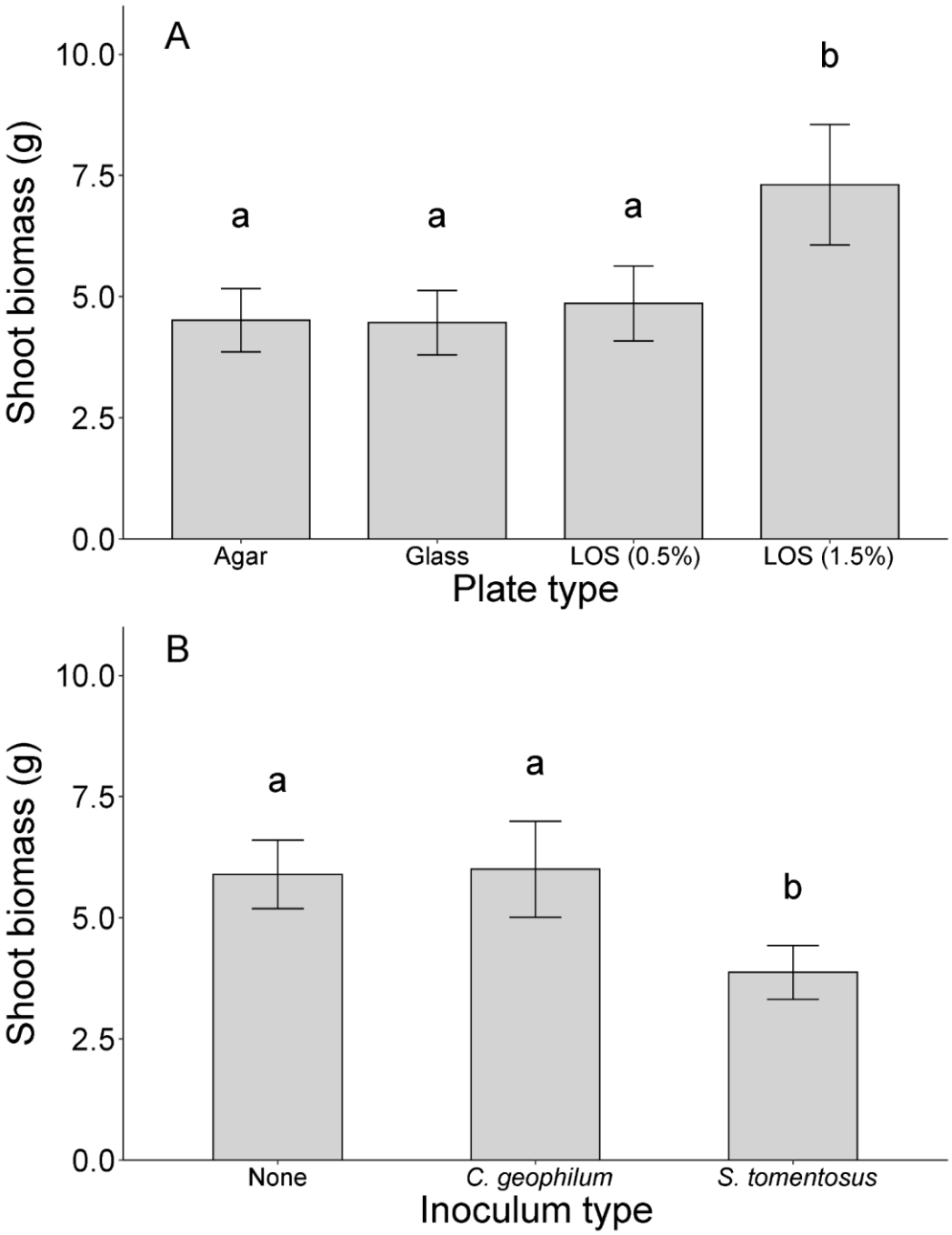

Figure 4.1 Mean shoot biomass (± 95% CI) of jack pine (*Pinus banksiana*). Plate type (A) includes agar only ('Agar' control), agar with glass ('Glass' control), agar mixed with lean oil sand with a total concentration of 0.5% hydrocarbons ('LOS 0.5%'), and agar mixed with lean oil sand with a total concentration of 1.5% hydrocarbons ('LOS 1.5%'). Inoculum type (B) includes none, *Cenococcum geophillum,* or *Suillus tomentosus*. Bars with different letters are significantly different (P <0.05) based on the Tukey HSD test.

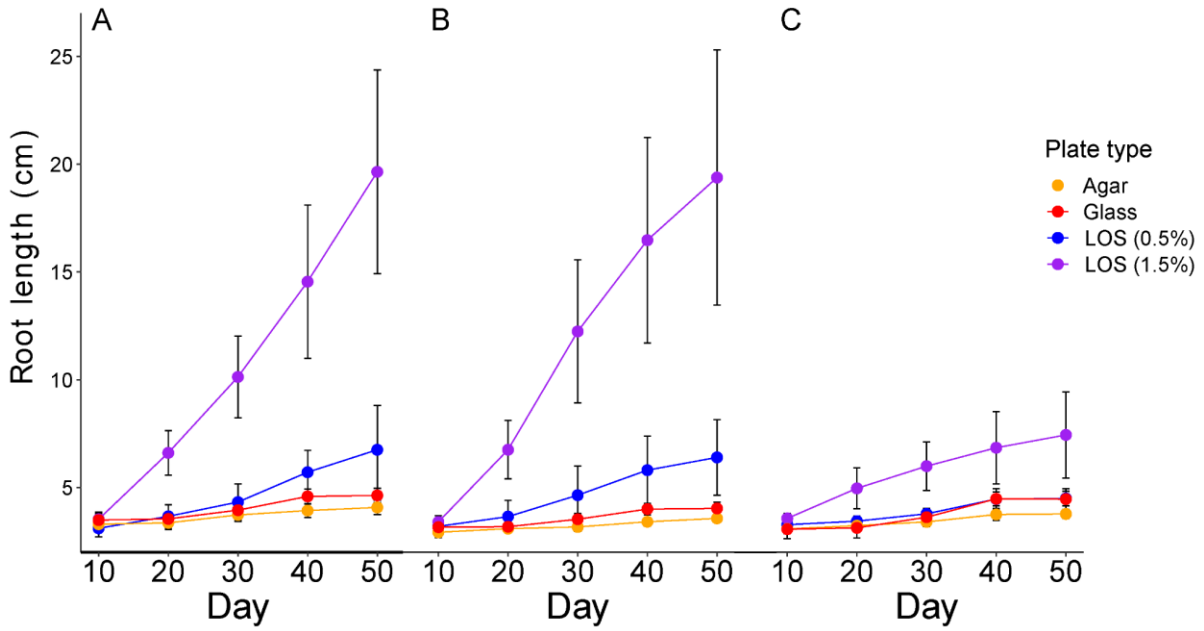

Figure 4.2 Mean root length (± 95% CI) of jack pine (*Pinus banksiana*) seedlings. Inoculum types include A) none, B) *Cenococcum geophillum,* or C) *Suillus tomentosus*. Plate type (A) includes agar only ('Agar' control), agar with glass ('Glass' control), agar mixed with lean oil sand with a total concentration of 0.5% hydrocarbons ('LOS 0.5%'), and agar mixed with lean oil sand with a total concentration of 1.5% hydrocarbons ('LOS 1.5%'). (n = 15).

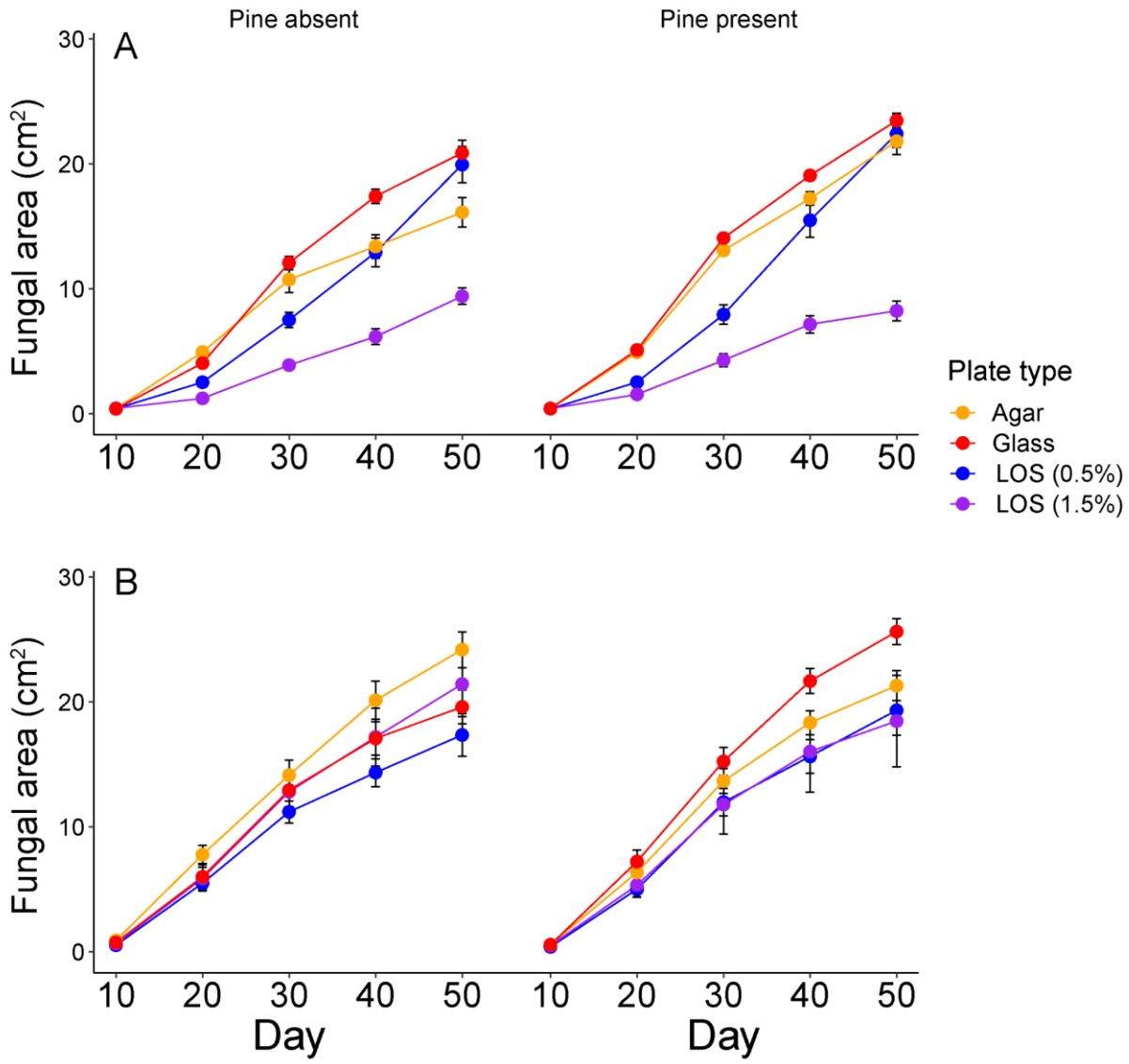

Figure 4.3 Mean surface area of *Cenococcum geophillum* (A) and *Suillus tomentosus* (B) (± 95% CI) with jack pine (*Pinus banksiana*) seedlings absent or present. Plate type (A) includes agar only ('Agar' control), agar with glass ('Glass' control), agar mixed with lean oil sand with a total concentration of 0.5% hydrocarbons ('LOS 0.5%'), and agar mixed with lean oil sand with a total concentration of 1.5% hydrocarbons ('LOS 1.5%'). (n = 15).

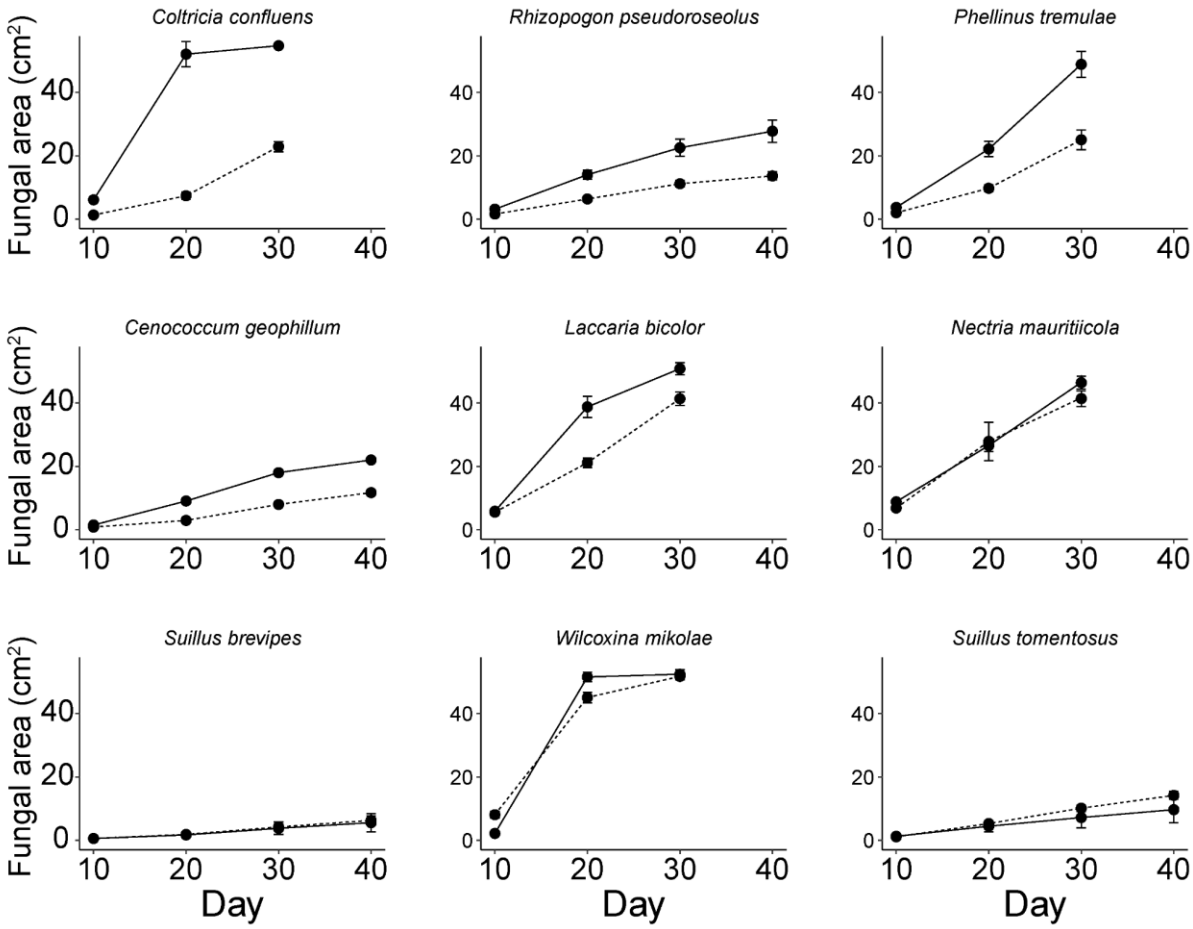

Figure 4.4 Mean growth area (± 95% CI) of fungal species. Fungi grown on agar with glass ('Glass' control) (solid line) or agar mixed with lean oil sand (dashed line) with a total concentration of 1.5% hydrocarbons. Measurements taken every 10 days for 30 days for all fungal species and an additional 10 days for slower growing species, including *Cenococcum geophillum, Coltricia confluens, Laccaria bicolor, Nectria mauritiicola, Phellinus tremulae, Rhizopogon pseudoroseolus, Suillus brevipes, Suillus tomentosus, Wilcoxina mikolae* (n = 20). Species are ordered by effect size, beginning with those fungal species most negatively affected by lean oil sand.

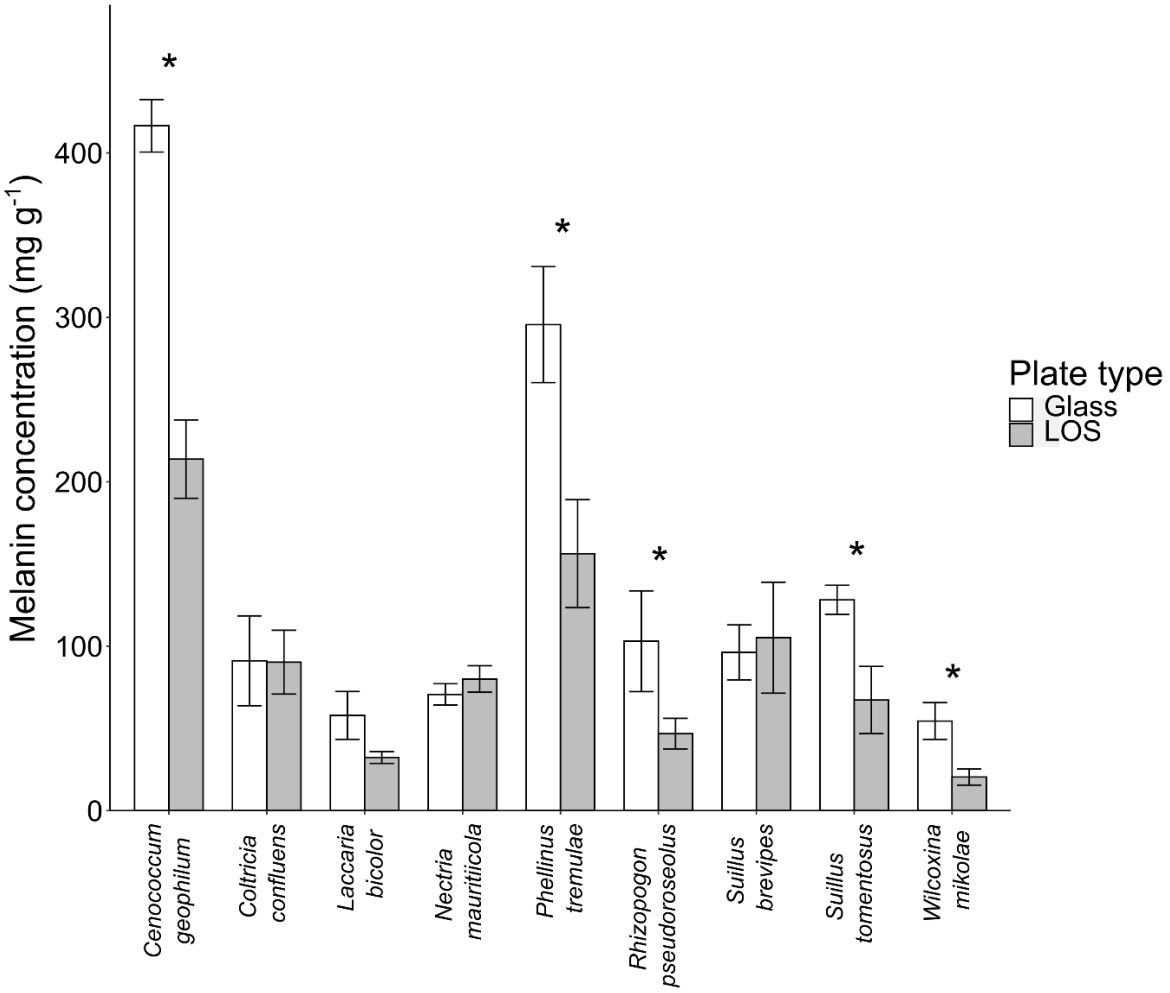

Figure 4.5 Mean melanin concentrations of fungal species (± 95% CI) used in experiment 2. Plate type includes agar with glass ('Glass' control) and agar mixed with lean oil sand with a total concentration of 1.5% hydrocarbons ('LOS'). (n = 4). Bars with an asterix are significantly different (P <0.05) based on Tukey HSD test.

Chapter 5: General discussion and conclusion

5.1 Summary

Landscapes mined for bitumen must be reclaimed and revegetated to restore self-sustaining ecosystems in the boreal forest of western Canada. Residual hydrocarbons can be present in reclaimed landforms, and it is unclear what effect they may have on establishing native vegetation. In this same region, forests occur on shallow bitumen deposits and some vegetation persists on abandoned ore piles. The main goal of my thesis was to investigate the effect of bituminous substrates including shallow-bituminous field soil, Bitumount Alberta ore pile soil, and lean oil sand (LOS) on plant and fungal growth and determine whether the interactions between beneficial fungi and plants can be leveraged to increase seedling performance in soils containing residual hydrocarbons.

In my first research chapter, I tested whether 'lean oil sand' is detrimental to plants as a result of hydrocarbons and/or barriers such as poor nutrient concentrations, water availability, and air availability. I used a fully randomized factorial experiment with hydrocarbon level (i.e., control, 0.88%, and 3.67% hydrocarbons) and 'barrier modifications' (i.e., nutrient availability, water availability, aeration, and respective controls) as main factors. I found that plant biomass was significantly lower in soil containing 0.88% or 3.67% hydrocarbons compared with plants grown in soil containing no hydrocarbons. None of the 'barrier modifications' alleviated the detrimental effects of lean oil sand on plant biomass. Fertilization had no effect on plant biomass when grown in lean oil sand while increasing subsurface watering and aeration decreased plant growth in lean oil sand. Consequently, the results of this study suggest that low concentrations of petroleum hydrocarbons in lean oil sand can impede plant growth, and specific measures to alleviate this may not be effective in reclamation campaigns.

In my second chapter, I examined the efficacy of soil inoculum collected from a range of sites varying in hydrocarbon concentrations and fractions on the performance of two tree species commonly used in reclamation. I first characterized soil fungal community composition of the sites from which I sourced soil inoculum using Illumina sequencing. Next, I inoculated seedlings of *Populus tremuloides* and *Pinus banksiana* and tracked their response to evaluate whether soil inoculation benefited seedlings and moreover if the origin of inoculum differentially affected seedling growth. Seedlings were grown in substrates used in reclamation either inoculated or not with soils collected from my survey. I found that field sites differed in composition of soil and root-associated fungal communities indicating that soils used as inoculum likely differed in the fungi present. In my growth chamber experiment, I found that

the presence of hydrocarbons reduced the growth of *P. tremuloides* and *P. banksiana*. Soil inoculation increased the growth of *P. banksiana* but the benefits varied by inoculum origin. For example, when *P. banksiana* was grown in lean oil sand, growth was highest when inoculated with soils from forests free of bitumen and the abandoned ore pile, while inoculum from forests on natural bitumen deposits had no effect. The growth of *P. tremuloides* was insensitive to soil inoculation regardless of origin. My results show soil inoculum may be an effective method of establishing some tree species on reclaimed areas.

The experiment presented in the third chapter tested the effect of lean oil sand on the growth of *P. banksiana* seedlings either inoculated or not with two ectomycorrhizal fungi; *Cenococcum geophilum* and *Suillus tomentosus*. I found that the growth of *C. geophilum* was decreased in presence of lean oil sand, while the growth of *S. tomentosus* was not affected. Seedling shoot and root growth were significantly greater in the presence of lean oil sand than lean oil sand-free treatments. The presence of *Cenococcum geophilum* did not change the growth response of *P. banksiana* to LOS. However, seedlings inoculated with *S. tomentosus* and grown in LOS were significantly smaller indicating that when LOS is present this species of fungi may act as a parasite. To further determine how ectomycorrhizal fungal species varied in their direct response to lean oil sand, I then tested the effect of lean oil sand on nine species varying in morphological traits and growth rates. I found that the growth of the majority of fungi was lower in the presence of lean oil sand. However, I did not detect a relationship between fungal tissue melanin concentration and the growth response to LOS, indicating no relationship between tissue concentrations of melanin and tolerance to petroleum hydrocarbons. The results of this study suggest, however, that the concentration of LOS likely plays a pivotal role in its fungal toxicity. Furthermore, my research shows that *in vitro* microcosms are a viable method to test fungal responses to the presence of soils containing LOS.

5.2 Synthesis

Taken as a whole, my dissertation provides multiple lines of evidence that LOS limits plant growth. These findings are consistent with a recent review of the effects of petroleum hydrocarbons on plant growth (Haider et al., 2021) as well as previous research specifically focusing on the effects of LOS on plant growth (Visser 2008; Brown 2020). While I found LOS reduced plant biomass, ore pile soils did not reduce seedling growth to the same extent despite containing greater concentrations of petroleum hydrocarbons than my 0.88% LOS treatment. I also found that shallow-bituminous soils did not significantly reduce plant growth when compared to bituminous free soils. These results are further supported by a recent study by La Flèche et al., 2021 who found that the growth rate of mature trees at shallow-bituminous and bituminous-free sites were not significantly different. This may be the result of petroleum hydrocarbons present in both the ore pile and shallow-bituminous soils being older and therefore more weathered. As such, the lighter more phytotoxic petroleum hydrocarbons may have volatilized from these soils while still being present in freshly mined LOS. However, in chapter 4 when pine seedlings were grown in microcosms containing LOS, seedling growth was significantly greater. I can only speculate about why increase in growth occurred. Perhaps the seedlings may have been searching for nutrients (Cahill and McNickle, 2011). Lean oil sand is a complex mixture of various high molecular weight hydrocarbons (Hein, 2017), and the discrepancy in results along my thesis chapters illustrates the difficulty of determining which properties of LOS affect plant growth.

The presence of petroleum hydrocarbons is likely not the only factor affecting plant growth in soils containing petroleum hydrocarbons. As per the results of my second chapter, LOS has a significantly lower water holding capacity than pure sand free of hydrocarbons. This result along with previous research, (Pernistky et al., 2016; Neil and Si, 2019) suggests petroleum hydrocarbons found in LOS likely have a major limiting effect on the water holding capacity of any landform containing LOS. Furthermore, while petroleum hydrocarbons in LOS may reduce plant growth, previous research has found that LOS with a bulk density of greater than 1.6 g cm^{-3} will restrict the downward movement of water and nutrients, thus increasing the amount of water and nutrients stored in the overlaying capping material (Pernistky et al., 2016, Rees et al., 2020). This would suggest that plants grown in capping material placed over LOS with a high bulk density may not be limited by water and soil nutrients. Therefore, the depth and selection of cover soils to provide an appropriate water regime for native vegetation

will greatly depend on the concentration of petroleum hydrocarbons in the LOS if it is to be used in future reclamation projects.

The water regime in shallow bituminous soils may also be affected by the presence of petroleum hydrocarbons. However, this is less likely when compared to reclaimed landscapes containing LOS. There likely are two reasons for this, 1) typically, shallow bituminous soils have lower concentrations of petroleum hydrocarbons than LOS. 2) The bitumen found at the shallow bituminous sites can vary in depth 0–3 meters and texture, from small particles to tar balls (Leskiw et al., 2006). Therefore, the amount of shallow-bituminous soils restricts the downward movement of water and nutrients likely vary within and among these sites. The water regime of the ore pile at Bitumount Alberta is likely affected by the presence of hydrocarbons. The ore pile at Bitumount is a hard asphalt-like material and water pools on the surface. With little to no soil capping the ore pile, water quickly evaporates or flows from the site. Future research will be needed to determine whether the phytotoxicity of LOS or the effect LOS has on the water regime of Bitumount Alberta, plays a greater role in affecting the growth of plants growing on the ore pile.

While my dissertation shows plant growth can be limited by the presence of LOS, I also found the effect of LOS on plant growth may be mitigated by soil inoculation, but the benefits varied by soil inoculum origin. Given shallow-bituminous, and bituminous-free soils shared a number of the top 20 most abundant ASVs, I expected these soil inoculations from these locations would affect pine seedling growth similarly, but this was not the case. This indicates factors other than fungi, may be affecting plant growth at sites containing petroleum hydrocarbons. For example, previous research on root endophytic bacteria at Bitumount, Alberta found that these communities contained hydrocarbon-degrading genes (Blain et al., 2017). It is possible the positive effect of Bitumount soil inoculation on pine seedlings, is not only driven by the distinct fungal community of these substrates but also by the presence of these hydrocarbon-degrading bacteria. As such, future research on shallow-bituminous soils should focus on also characterizing the bacterial communities to investigate whether bacteria capable of degrading hydrocarbons are also present.

Although soil inoculation has been found to be a convenient and effective method of restoring degraded grassland ecosystems (Middleton et al., 2015; Wubs et al., 2016; Koziol et al., 2018; Neuenkamp et al. 2019; Vahter et al., 2020), the effectiveness of soil inoculum on ectomycorrhizal plant communities appears to be much more variable (Karst et al., 2015; Beck et al., 2020; Policelli et al. 2020; Wasyliw et al., 2022; Chapter 3). This may be the result of the collection process of soil inoculum acting as a filter, where only fungal propagules resistant

to disruptions survive. For example, Wasyliw et al., 2022 reported soil inoculums had far lower fungal richness than field soils. Additionally, the species present in the soil inoculum likely play a pivotal role in the effectiveness of increasing a plant's tolerance to LOS as fungal species vary in response to LOS (Chapter 4). Overall, the variation of pine seedlings' response to soil inoculum indicates seedling inoculation is not a “silver bullet” for restoring plant communities on soils containing petroleum hydrocarbons. However, my study does demonstrate that characterization of fungal communities through the use of next-generation sequencing is the first step into understanding how petroleum hydrocarbons present at these locations may shape fungal communities. In the future, these surveys in conjunction with culturing and microcosm experiments to screen fungi for petroleum hydrocarbon tolerance may be an effective method for measuring the potential success of soil inoculums without the need for large-scale greenhouse experiments.

5.3 Limitations of research

The main limitations of this work are the limited number of sites and species that were studied as well as the relatively short growing time. Many of the plant species used in reclamation are long-lived perennials. Future research should focus on longer observations over multiple growing seasons to determine if the effects of soil inoculation change with time and how other species of trees, plants, and fungi respond to LOS. Furthermore, while shallow bituminous and Bitumount ore pile soils provide a unique opportunity to investigate how the presence of hydrocarbons affects microbial communities and tree seedling growth, they are not perfect analogs for reclaimed landscapes. These sites, especially the shallow bituminous soil sites, are much older, and as such, lighter fractions of petroleum hydrocarbons may have volatilized and are no longer present. Factors such as differences in site age and the differences in hydrocarbon fractions make comparisons of these sites to reclaimed soils more difficult. Finally, the use of growth chambers to complete these studies may also introduce some limitations as the results of these studies may not directly translate to field studies. However, the use of growth chambers enabled me to investigate the effects of LOS on plant growth and soil inoculum applications under controlled conditions. Future field trials over multiple growing seasons may help to further determine whether soil inoculum would improve seedling growth in soils containing petroleum hydrocarbons.

5.4 Management implications

My research can provide information to help inform industry and government about the impacts of LOS on the growth of native plant and fungal species found in the oil sands region of Northern Alberta, Canada. Overall, my research provides evidence that even at low concentrations, if LOS is present within the rooting zone, plant and fungal growth can be impeded. As such, I suggest that if LOS is to be used as a reclamation material, care should be taken during the placement, grading, and capping of these large landforms to ensure no LOS is incorporated within the rooting zone of young seedlings. My results corroborate current practices where LOS is placed under a subsoil and capped with suitable material, thus limiting its interaction with roots (North Wind Land Resources Inc., 2013; Rees et al., 2020). However, current reclamation practices may result in soils containing up to 1% petroleum hydrocarbons (Visser, 2008) which in both chapters 2 and 3 is shown to be a high enough concentration to reduce plant growth. My research further demonstrates that if the interaction between petroleum hydrocarbons and seedlings is possible in these reclaimed soils, soil inoculum may be an effective method of increasing a seedling's tolerance to these hydrocarbons. However, where this soil inoculum is collected from and which species it is applied determine how it affects plant growth. Given the results of my study, I can only recommend the use of soil inoculum on *P. banksiana* seedlings.

Next, my work suggests that if LOS is present in the rooting zone the addition of fertilizer will not alleviate the negative effect LOS has on plant growth. Therefore, if fertilizer is to be used to improve plant growth on these reclaimed landscapes, I recommend ensuring the capping material is placed at an adequate depth so that plant root systems are unable to interact with the material. I also recommend testing the capping material for petroleum hydrocarbons to ensure no LOS was incorporated in the capping material. Furthermore, the results of chapter 2 suggest the hydrophobicity of LOS reduces plants' ability to grow in this material. If LOS is to be used in reclaimed landscapes as a base layer, I suggest ensuring the overlying soil profile, including the subsoils and capping material, has re-established and appropriate soil moisture regimes to support native plant species and disturbances causing increased movement of water through the LOS base layer is avoided.

Finally, my work clearly shows the presence of LOS can limit the growth of ectomycorrhizal fungi, however, this varies by species. If ectomycorrhizal fungi are to be used in future reclamation practices, my research provides a method for screening fungal species for tolerance to the presence of LOS. The results of my final data chapter suggest *Suillus brevipes*

and *Wilcoxina mikolae* are tolerant to the presence of LOS and further studies should be conducted to determine if inoculation of tree seedlings with either species increases the seedling's tolerance to LOS.

5.5 Recommendations for future research

Several future studies could expand the findings of this dissertation. While this research found soil inoculum can increase *P. banksiana* seedling growth in the presence of petroleum hydrocarbons, the benefits varied by inoculum origin. Furthermore, *P. tremuloides* seedlings were insensitive to soil inoculum regardless of where it originated from. However, this may be the result of collecting soil inoculum from pine-dominated stands. Future research is needed to investigate whether soil inoculum collected from *P. tremuloides* dominated stands improves seedling growth. Additionally, *Tomentella subclavigera* and *Thelephora terrestris* were two species of ectomycorrhizal fungi found in high abundance in the soils and roots of the ore pile. Future research should focus on isolating these species from Bitumount and determine if they alone can improve plants' tolerance to the presence of LOS. Isolated cultures of these species could be compared to cultures isolated from various regions to determine if fungi isolated from Bitumount have adapted to the presence of petroleum hydrocarbons. Additionally, molecular tools such as quantitative PCR could also be incorporated into future studies of these soils to either quantitatively determine the abundance of specific fungal species or detect fungi with genes capable of producing enzymes capable of degrading hydrocarbons. Furthermore, the results of my research suggest if LOS is used as a base layer and present in the rooting zone of plants, plant growth may be reduced. As LOS can affect the water regime of the growth medium, future research should focus on determining if drought-tolerant native plant species are more tolerant to the presence of LOS. Finally, while I found various fungal species were tolerant to the presence of LOS, future research will be needed to determine whether the inoculation of those particular species increases a plant host's tolerance to LOS.

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Appendices

Appendix 2.1

Table S2.1 Mean hydrocarbon concentrations for Low LOS and High LOS used in the growth chamber experiment. The lean oil sand (LOS) was collected from two locations within the Aurora North Mine Site, Fort McMurray, Alberta, Canada. Hydrocarbon fractions 2–4 represent F2 (>nC10 to nC16), F3 (>nC16 to nC34) and, F4 (nC35 to nC50+) (Turle et al., 2007). No hydrocarbons were detected in field soil.

Substrate type	F2 mg kg^{-1}	F3 mg kg^{-1}	F4 mg kg^{-1}	F4G+ mg kg^{-1}	F Total mg kg^{-1}
Low LOS	84	2033	1267	5367	8751
High LOS	2166	10,600	4600	19,333	36,699

Table S2.2 Physical and chemical properties of substrates used in the growth chamber experiment. Substrates included Low LOS, High LOW, and field soil. The lean oil sand (LOS) was collected from two locations within the Aurora North Mine Site, Fort McMurray, Alberta, Canada. Field soil was collected from three jack pine (*Pinus banksiana*) stands near Janvier, Alberta, Canada (55° 54' 24" N, 110° 44' 43" W).

Substrate type	pH	Electrical Conductivity dS m^{-1}	Sand %	Silt %	Clay %	PO_4-P (mg kg^{-1})	NH_4-N (mg kg^{-1})	NO_3-N (mg kg^{-1})	NO_2-N (mg $kg^{-1)}$
Field soil	5.6	0.033	87.85	8.7	3.45	26.56	1.8	0.93	Below limit of detection
Low LOS	7.76	0.322	83.4	10.51	6.08	0.54	0.74	Below limit of detection	Below limit of detection
High LOS	7	0.165	64.28	32.45	3.27	0.76	1.03	Below limit of detection	Below limit of detection

Table S2.3 Concentration of extractable metals of substrates used in the growth chamber experiment. Substrates included Low LOS, High LOS, and field soil. The lean oil sand (LOS) was collected from two locations within the Aurora North Mine Site, Fort McMurray, Alberta, Canada. Field soil was collected from three jack pine (*Pinus banksiana*) stands near Janvier, Alberta, Canada (55° 54' 24" N, 110° 44' 43" W).

Substrate type	Na	Mg	K	Ca	B	Li	Mn	Fe	Zn	P	S
	µg g-1	µg g-1	µg g-1	µg g-1	µg g-1	µg g-1	µg g-1	µg g-1	µg g-1	µg g-1	µg g-1
Field soil	32.18	308.82	188.75	284.54	1.44	1.63	48.9	3533.66	14.79	270.59	24.71
Low LOS	69.26	3574.21	567.7	25101.09	4.27	4.73	207.44	14474.1	14.75	186.8	3748.7
High LOS	40.53	561.83	863.01	804.54	10.06	7.14	245.63	5478.67	23.3	89	2489.58

Table S2.4 Four-way ANOVA table showing the response of lettuce (*Lactuca sativa*) total biomass (ln transformed) to substrate type (Substrate), Fertilization (Fertilizer), water availability (Water tile), and increased permeability (Aerated). Degrees of freedom [df], Sums of squares [SS], *F*-statistic, and *P*-value are shown).

Term	df	SS	*F*	*P*
Substrate	2	82.45	153.296	**<0.0001**
Fertilizer	1	0.17	0.631	0.428
Water tile	1	10.05	37.368	**<0.0001**
Aerated	1	0	0.011	0.915
Substrate × Fertilizer	2	3.28	6.096	**0.003**
Substrate × Water tile	2	3.87	7.201	**0.001**
Fertilizer × Water tile	1	0.18	0.66	0.418
Substrate × Aerated	2	0.89	1.664	0.193
Fertilizer × Aerated	1	0.06	0.22	0.64
Water tile × Aerated	1	0.26	0.985	0.323
Substrate × Fertilizer × Water tile	2	0.61	1.134	0.324
Substrate × Fertilizer × Aerated	2	0.77	1.433	0.242
Substrate × Water tile × Aerated	2	1.66	3.082	**0.048**
Fertilizer × Water tile × Aerated	1	0.13	0.485	0.487
Substrate × Fertilizer × Water tile × Aerated	2	0.96	1.782	0.172
Error	166	44.64		

Table S2.5 Four-way ANOVA table showing the response of lettuce (*Lactuca sativa*) shoot biomass (ln transformed) to substrate type (Substrate), Fertilization (Fertilizer), water availability (Water tile), and increased permeability (Aerated). Degrees of freedom [df], Sums of squares [SS], *F*-statistic, and *P*-value are shown).

Term	df	SS	*F*	*P*
Substrate	2	97.25	147.796	**<0.0001**
Fertilizer	1	0.79	2.388	0.1241947
Water tile	1	13.19	40.085	**<0.0001**
Aerated	1	0.02	0.070	0.792
Substrate × Fertilizer	2	3.4	5.168	**0.007**
Substrate × Water tile	2	5.56	8.453	**<0.0001**
Fertilizer × Water tile	1	0.13	0.393	0.532
Substrate × Aerated	2	0.51	0.779	0.460
Fertilizer × Aerated	1	0.02	0.053	0.818
Water tile × Aerated	1	0.03	0.106	0.745
Substrate × Fertilizer × Water tile	2	1.51	2.291	0.104
Substrate × Fertilizer × Aerated	2	0.4	0.611	0.544
Substrate × Water tile × Aerated	2	0.92	1.399	0.250
Fertilizer × Water tile × Aerated	1	0.12	0.353	0.553
Substrate × Fertilizer × Water tile × Aerated	2	1.32	2.001	0.138
Error	166	54.61		

Table S2.6 Four-way ANOVA table showing the response of lettuce (*Lactuca sativa*) root biomass (ln transformed) to substrate type (Substrate), Fertilization (Fertilizer), water availability (Water tile), and increased permeability (Aerated). Degrees of freedom [df], Sums of squares [SS], *F*-statistic, and *P*-value are shown).

Term	df	SS	*F*	*P*
Substrate	2	70.59	88.582	**<0.0001**
Fertilizer	1	0	0.012	0.915
Water tile	1	5.66	14.209	**<0.0001**
Aerated	1	0.18	0.460	0.499
Substrate × Fertilizer	2	2.34	2.936	0.056
Substrate × Water tile	2	1.47	1.840	0.162
Fertilizer × Water tile	1	0.15	0.388	0.534
Substrate × Aerated	2	1.95	2.453	0.089
Fertilizer × Aerated	1	0.36	0.916	0.34
Water tile × Aerated	1	0.76	1.919	0.168
Substrate × Fertilizer × Water tile	2	0.29	0.369	0.692
Substrate × Fertilizer × Aerated	2	1.14	1.433	0.242
Substrate × Water tile × Aerated	2	3.08	3.871	**0.023**
Fertilizer × Water tile × Aerated	1	0.5	1.255	0.264
Substrate × Fertilizer × Water tile × Aerated	2	0.67	0.840	0.434
Error	166	66.14		

Table S2.7 Three-way ANOVA table showing the response of water holding capacity to substrate type (Substrate), water availability (Water tile), and increased permeability (Aerated). Degrees of freedom [df], Sums of squares [SS], *F*-statistic, and *P*-value are shown).

Term	df	SS	*F*	*P*
Substrate	1	31753	5952.566	**<0.0001**
Water tile	1	13	2.428	0.139
Aerated	1	0	0.008	0.931
Substrate × Water tile	1	80	14.978	**0.001**
Substrate × Aerated	1	11	1.992	0.177
Water tile × Aerated	1	45	8.46	**0.01**
Substrate × Water tile × Aerated	1	41	7.736	**0.013**
Error	16	85		

Table S2.8 One-way ANOVA table showing the response of water holding capacity of lean oil sand to soil cap (Cap). Degrees of freedom [df], Sums of squares [SS], *F*-statistic, and *P*-value are shown).

Term	df	SS	*F*	*P*
Cap	1	28.34	1.721	0.26
Error	4	65.845		

Figure S2.1 Lettuce (*Lactuca sativa*) grown in greenhouse pots (Growers Solution, Cookville, Tennessee, USA) filled with lean oil sand and capped with field soil. Top image: aerated substrate covered with soil cap. Bottom image: greenhouse pots filled with aerated field soil before soil cap was added.

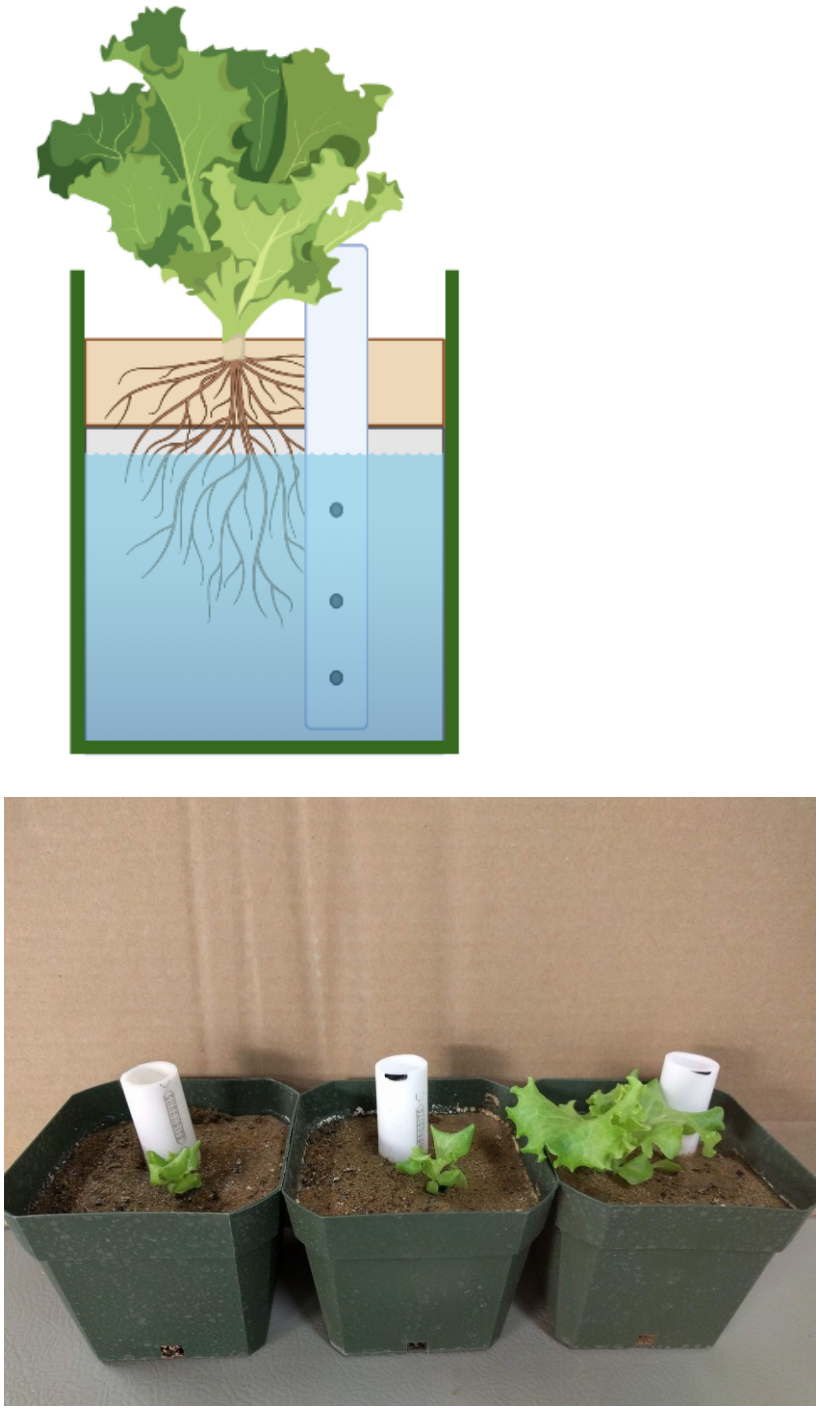

Figure S2.2 Lettuce (*Lactuca sativa*) grown in greenhouse pots (Growers Solution, Cookville, Tennessee, USA) filled with lean oil sand (LOS) and capped with field soil. Top image shows a water tile allowing the water to drain below the soil cap. Bottom image shows greenhouse pots with water tiles filled with High LOS, Low LOS, or field soil (left to right).

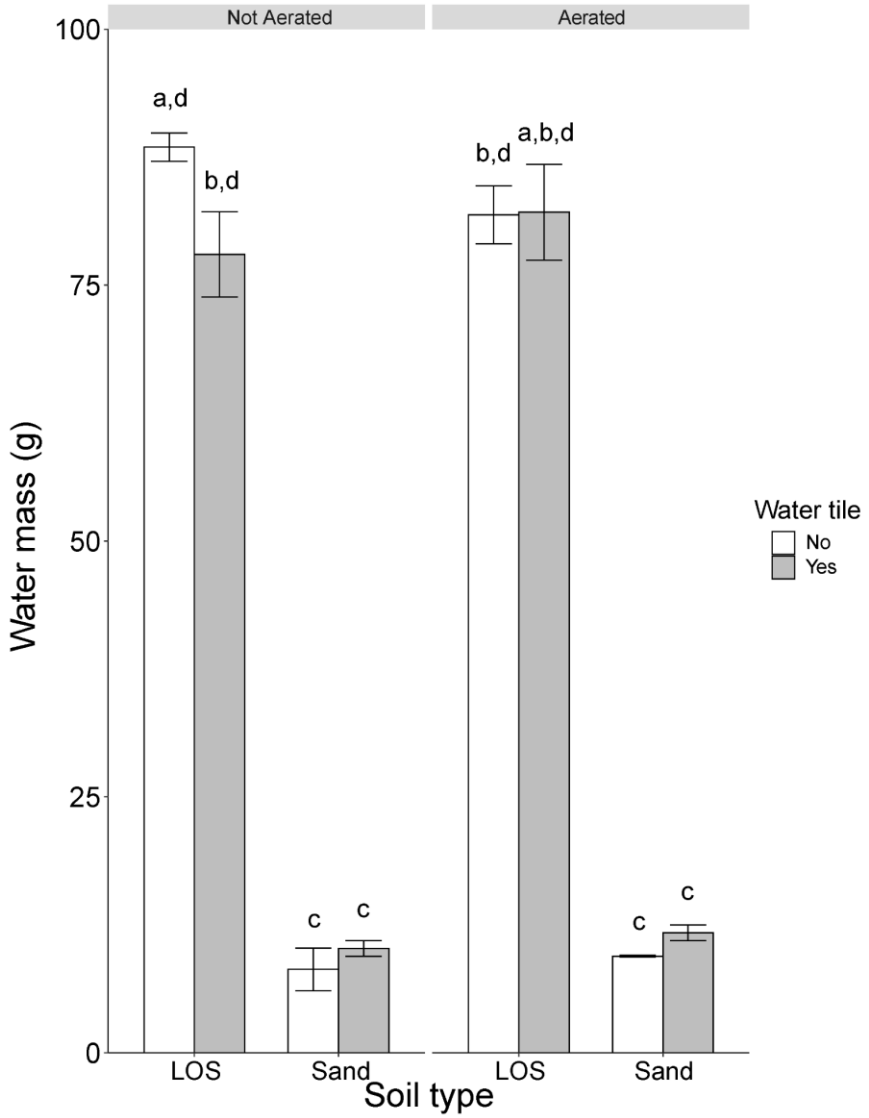

Figure S2.3 Mean mass of water (± 95% CI) not retained by either sand or lean oil sand (LOS). Soils were treated with (grey) or without (white) watertiles and with or without aeration. Bars with different letters are significantly different ($P < 0.05$) based on Tukey HSD test.

Chapter 2: Supplemental methods

Water holding capacity of lean oil sand

Previous research showed that lean oil sand (LOS) is hydrophobic (Neil and Si, 2019). I hypothesized that because of the hydrophobicity of the LOS, the water holding capacity of the LOS would be less compared to sand. To examine if LOS decreases the water holding capacity of the substrate, I performed an experiment where water was poured through LOS and sand, and the amount of water that was not retained by the substrate was measured. I also tested whether the increased movement of water through the each of the substates changed their water holding capacity by including water tile and aeration treatments. This experiment had a 2 × 2 × 2 factorial design with each factor being one used in the main experiment that may have affected water holding capacity. The total number of pots filled with substrate in this experiment was 24 (8 treatment combinations × 3 replicates). The first factor 'substrate type' had two levels, (1) sand, and (2) 12.73% LOS. The second factor, 'water tile', had two levels, (1) present ('Yes'), and absent ('No'). Finally, the third factor 'aeration' had two levels, (1) substrate aerated ('Yes'), and not aerated ('No').

The same pots 'Greenhouse Pots' used in the main experiment (see Chapter 2) were filled with 450 grams of either 12.73% LOS or silica sand (Garden Sand, Kott Holdings Ltd., Edmonton, Alberta, Canada). Sand and 12.73% LOS was used in this experiment rather than field soil and 3.67% LOS, as an adequate amount of this material was not retained to complete this experiment. These substrates were compressed to a bulk density of 1.5 g cm^{-3}. The water tile and aeration treatments were the same as described in the main experiment (see Chapter 2). I poured 100 millilitres into each pot and collected all the water that drained through the substrates for ten minutes, after which the water collected was weighed. The mass of the water that passed through the substrate was the amount of water not held by the substrate.

To determine whether the water holding capacity of the substrate responded to either the presence of hydrocarbons, water tile, or aeration, I used a three-way ANOVA. Means were compared using a Tukey's honest significance difference (HDS) test ($P < 0.05$).

Water holding capacity of lean oil sand with a sand cap.

As the water tile affected how much water passed through the LOS, I also tested whether adding a sand cap to LOS increased the water holding capacity of the material. Again, pots were filled with 450 grams of 12.73% LOS with a water tile. These pots either received a 150 gram sand cap or an additional 150 grams of LOS. The 150 grams of LOS was added to

ensure the volume of substrate in each pot did not affect the test. Three replicates of each treatment were used. To determine whether the water holding capacity of the substrate responded to the presence of a sand cap, I used a one-way ANOVA. Means were compared using a Tukey's honest significance difference (HDS) test ($P < 0.05$).

Appendix 3.1

Table S3.1 Characteristics of soils including depth of soil sample, pH, electrical conductivity, texture, and location. Shallow bituminous sites located near the Hammerstone Quarry north of Fort MacKay Alberta, Canada. Bituminous-free sites were located near Janvier, Alberta, Canada. The ore pile was located at Bitumount Alberta, Canada.

Site	Depth	pH	Electrical Conductivity	Sand	Silt	Clay	Latitude	Longitude
	cm		mS cm^{-1}	%	%	%		
Shallow bituminous soil: Site #1	10	4.3	0.012	86.63	9.25	4.13	57.165884	-111.540825
	20	4.1	0.01	83.55	10.51	5.95		
	30	4	0.004	75.64	16.07	8.29		
	40	4.1	0.004	79.16	14.12	6.72		
Shallow bituminous soil: Site #2	10	5.4	0.009	81.78	13.25	4.97	57.172111	-111.540733
	20	4.6	0.009	77.5	14.77	7.74		
	30	4.5	0.009	79.34	13.41	7.26		
	40	4.6	0.003	85	10.66	4.35		
Shallow bituminous soil: Site #3	10	5	0.004	90.26	7.41	2.33	57.157077	-111.541146
	20	4.7	0.001	78.26	13.92	7.83		
	30	4.6	0.002	80.02	11.94	8.05		
	40	4.7	0.001	71.22	19.03	9.75		
Bituminous free soil: Site #1	10	5.5	0.009	68.52	23.36	8.12	55.814664	-110.742461
	20	5.5	0.003	58.3	25.99	15.71		
	30	5.3	0.004	60.01	25.02	14.97		
	40	5.1	0.008	60.83	23.36	15.81		
Bituminous free soil: Site #2	10	5.1	0.001	88.11	4.87	7.02	55.831949	-110.850396
	20	5.5	0.001	89.78	5.81	4.41		
	30	5.2	0.007	89.15	6.35	4.5		
	40	5.1	0.002	86.77	9.01	4.22		
Bituminous	10	5.4	0.01	83.85	11.38	4.77	55.866734	-110.819993

free soil: Site #3	20	5.4	0.005	83.35	8.44	8.21		
	30	5.7	0.003	88.94	5.31	5.75		
	40	5.6	0.004	91.14	3.73	5.14		
Ore pile	NA	5.3	0.051	93.04	5.39	1.56	57.384506	-111.646535

Table S1: 2 Understory plant species recorded study sites located in *Pinus banksiana* stands in north eastern Alberta, Canada. Shallow bituminous sites located near the Hammerstone Quarry north of Fort MacKay Alberta, Canada. Bituminous-free sites were located near Janvier, Alberta, Canada. The ore pile was located at Bitumount Alberta, Canada. Asterix indicates mycorrhizal status of the plant species is listed as undetermined in the Fungal root database (Soudzilovskaia et al., 2021).

Shallow bituminous soils	Bituminous free soils	Ore pile
Amelanchier alnifolia (AM)	*Amelanchier alnifolia* (AM)	*Alnus viridis* (EM, AM*)
Arctostaphylos uva-ursi (ArM)	*Arctostaphylos uva-ursi* (ArM)	*Arctostaphylos uva-ursi* (ArM)
Ceratodon purpureus	*Cladina mitis*	*Betula glandulosa* (EM, AM*)
Cladina mitis	*Cornus canadensis* (AM)	*Carex spp.*
Cladonia spp.	*Dicranum polysetum*	*Castilleja spp.*
Cladonia stellaris	*Diphasiastrum digitatum* (AM)	*Ceratodon purpureus*
Cornus canadensis (AM)	*Fragaria virginiana* (AM)	*Cladina mitis*
Cypripedium acaule	*Hylocomium splendens*	*Cladonia coccifera*
Dicranum polysetum	*Linnaea borealis* (AM)	*Cornus stolonifera*
Dicranum spp.	*Maianthemum canadense* (AM)	*Epilobium angustifolium* (AM)
Galium triflorum (AM)	*Peltigera aphthosa*	*Fragaria virginiana* (AM)
Hylocomium splendens	*Pleurozium schreberi*	*Koeleria macrantha* (AM)
Linnaea borealis (AM)	*Populus tremuloides* (EM, AM)	*Larix laricina* (EM, AM*)
Maianthemum canadense (AM)	*Ptilium crista-castrensis*	*Melilotus officinalis* (AM)
Peltigera aphthosa	*Usnea lapponica*	*Phleum pratense* (AM)
Pleurozium schreberi	*Vaccinium myrtilloides* (ErM)	*Picea mariana* (EM)

Ptilium crista-castrensis	*Vaccinium oxycoccos* (ErM)	*Populus tremuloides* (EM, AM)
Rhododendron groenlandicum (ErM)	*Viola adunca*	*Racomitrium spp.*
Rosa acicularis (AM)		*Rhododendron groenlandicum* (ErM)
Vaccinium myrtilloides (ErM)		*Salix spp.*
Vaccinium oxycoccos (ErM)		*Shepherdia canadensis*
		Taraxicum officinale
		Trifolium hybridum (AM)
		Trifolium repens (AM)
		Typha latifolia
		Vaccinium oxycoccos (ErM)

At each site I recorded the percent cover of plant species found in four 1 m^2. Each square meter was randomly assigned to a random position within each plot, and percent cover was assessed by one recorder. Moss, grass and lichen were also observed but could not be identified. Mycorrhizal status of plant species, arbuscular mycorrhizal fungi (AM), arbutoid mycorrhizal fungi (ArM) ectomycorrhizal fungi (EM), ericoid mycorrhizal fungi (ErM) listed after name. Soudzilovskaia N A, Vaessen S, Barcelo M, He J, Rahimlou S, Abarenkov K, Brundrett M C, Gomes S, Merckx V, Martinez-Suz L, Tedersoo L. Taxon occurrence data for the FungalRoot database. PlutoF. Occurrence dataset https://doi.org/10.15468/a7ujmj accessed via GBIF.org on 2021-08-12.

Table S3.3 Hydrocarbon concentrations of soils from study sites located in *Pinus banksiana* stands in northeastern Alberta, Canada. No hydrocarbons were detected at bituminous-free sites. Hydrocarbon fractions 2–4 represent F2 (>nC10 to nC16), F3 (>nC16 to nC34) and F4 (nC35 to nC50+) (Turle et al., 2007).

Site	Dept cm	F2 mg kg^{-1}	F3 mg kg^{-1}	F4 mg kg^{-1}	F4G+ mg kg^{-1}	F Total mg kg^{-1}
Shallow bituminous soil: Site #1	10	0	745	950	N/A	1705
	20	7	1450	1615	N/A	3091.5
	30	18	3000	3000	N/A	6048
	40	27	3600	3450	N/A	7116.5
Shallow bituminous soil: Site #2	10	8	457.5	586.5	N/A	1061.5
	20	26	1295	2000	N/A	3341
	30	37	2700	3500	N/A	6267
	40	76	3550	4150	N/A	7816
Shallow bituminous soil: Site #3	10	0	0	0	N/A	10
	20	10	430	650	N/A	1109.5
	30	15	1085	1480	N/A	2610
	40	0	850	970	N/A	1860
Ore pile	NA	67	3367	2400	8167	14001

Table S3.4 Description of 'PCR1' thermocycler parameters.

Target region	Primers	PCR conditions
SSU	WANDA (Dumbrell et al. 2011) AML2 (Lee et al. 2008)	2 min 95℃ → 35 × (1 min 95℃ → 1 min 54℃ → 1 min 72℃) → 10 min 72℃
ITS1	ITS1F (Gardes and Bruns. 1993) ITS2 (White et al. 1990)	2 min 94℃ → 35 × (30 sec 94℃ → 30 sec 58℃ → 1 min 68℃) → 7 min 68℃

Table S3.5 Summary statistics of fungal rDNA for ITS1 and SSU sequences before and after DADA2 pipeline (Callahan et al. 2016). (n=70 soil samples, n=21 jack pine (*Pinus banksiana*) root samples).

Sequences before processing

Sample type	Region	Total	Average	Standard Deviation	Min	Max
Soil	ITS1	2974718	42496	7969	18029	58263
Roots	ITS1	874325	41635	9866	22114	60468
Soil	SSU	1326519	18950	3870	5237	25852
Roots	SSU	639415	30448	3467	25297	37841

After DADA 2

Sample type	Region	Total	Average	Standard Deviation	Min	Max
Soil	ITS1	2289052	32701	6664	12923	47361
Roots	ITS1	607345	28921	8224	16689	47331
Soil	SSU	1013977	14485	3255	4480	20938
Roots	SSU	489595	23314	3325	17718	28803

Table S3.6 Mean hydrocarbon concentrations for shallow bituminous soil, ore pile, Low LOS, and High LOS of substrate types used in the growth chamber experiment. Hydrocarbon fractions 2–4 represent F2 (>nC10 to nC16), F3 (>nC16 to nC34) and, F4 (nC35 to nC50+) (Turle et al., 2007). No hydrocarbons were detected in bituminous-free soils. LOS: lean oil sand

Substrate type	F2 mg kg^{-1}	F3 mg kg^{-1}	F4 mg kg^{-1}	F4G+ mg kg^{-1}	F Total mg kg^{-1}
Shallow bituminous soil	3.5	1098	1283	N/A	2385
Ore pile	67	3367	2400	8167	14001
Low LOS	84	2033	1267	5367	8751
High LOS	2166	10600	4600	19333	36699

Table S3.7 Physical and chemical properties of substrates used in the growth chamber experiment. Shallow bituminous sites located near the Hammerstone Quarry north of Fort MacKay Alberta, Canada. Bituminous free sites were located near Janvier Alberta, Canada. The abandoned ore pile was located at Bitumount Alberta, Canada. The two grades of lean oil sand were provided by Syncrude Canada Ltd. containing 8,751 mg kg^{-1} ('Low LOS') and 36,699 mg kg^{-1} ('High LOS')

Substrate type	pH	Electrical Conductivity	Sand	Silt	Clay	PO_4-P	NH_4-N	NO_3-N	NO_2-N
		uS cm^{-1}	%	%	%	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})
Shallow bituminous soil	4.98	49.4	93.19	5.17	1.64	4.28	1.48	Below limit of detection	Below limit of detection
Bituminous free soil	5.6	33	87.85	8.7	3.45	34.62	1.5	Below limit of detection	Below limit of detection
Ore pile	5.34	51.9	93.04	5.39	1.56	0.68	1.46	Below limit of detection	Below limit of detection
Low LOS	7.76	322	83.4	10.51	6.08	0.54	0.74	Below limit of detection	Below limit of detection
High LOS	7.00	165	64.28	32.45	3.27	0.76	1.03	Below limit of detection	Below limit of detection

Table S3.8 Concentration of extractable metals of substrates used in the growth chamber experiment. Shallow bituminous sites located near the Hammerstone Quarry north of Fort MacKay Alberta, Canada. Bituminous-free sites were located near Janvier, Alberta, Canada. The ore pile was located at Bitumount Alberta, Canada. The two grades of lean oil sand were provided by Syncrude Canada Ltd. containing 8,751 mg kg^{-1} ('Low LOS') and 36,699 mg kg^{-1} ('High LOS'). LOS: lean oil sand

Substrate type	Na	Mg	K	Ca	B	Li	Mn	Fe	Zn	P	S
	µg g^{-1}	µg g^{-1}	µg g^{-1}	µg g^{-1}	µg g^{-1}	µg g^{-1}	µg g^{-1}	µg g^{-1}	µg g^{-1}	µg g^{-1}	µg g^{-1}
Shallow bituminous soil	10.59	72.82	76.61	501.31	1.14	0.34	24.66	1878.98	2.04	32.81	229.97
Bituminous free soil	27.41	246.90	171.21	676.43	1.44	1.63	287.04	2089.15	15.22	140.86	64.43
Ore pile	18.42	176.24	180.49	555.74	1.08	0.60	40.14	5036.50	6.51	151.25	2392.71
Low LOS	69.26	3574.21	567.70	25101.09	4.27	4.73	207.44	14474.10	14.75	186.80	3748.70
High LOS	40.53	561.83	863.01	804.54	10.06	7.14	245.63	5478.67	23.30	89.00	2489.58

Table S3.9 Polycyclic aromatic hydrocarbons concentrations of High LOS and soil from the ore pile at Bitumount Alberta, Canada. Note, RDL = reportable detection limit. LOS: lean oil sand

Polycyclic Aromatics	High LOS (mg kg^{-1})	Ore pile (mg kg^{-1})	RDL (mg kg^{-1})
Acenaphthene	<0.50	<0.050	0.50
Benzo[a]pyrene equivalency	<0.71	<0.071	0.71
Acenaphthylene	<0.50	<0.050	0.50
Acridine	<1.0	<0.10	1.0
Anthracene	<0.40	<0.040	0.40
Benzo(a)anthracene	<0.50	<0.050	0.50
Benzo(b&j)fluoranthene	<0.50	0.067	0.50
Benzo(k)fluoranthene	<0.50	<0.050	0.50
Benzo(g,h,i)perylene	<0.50	<0.050	0.50
Benzo(c)phenanthrene	<0.50	<0.050	0.50
Benzo(a)pyrene	<0.50	<0.050	0.50
Benzo[e]pyrene	<0.50	<0.50	0.50
Chrysene	<0.50	<0.050	0.50
Dibenz(a,h)anthracene	<0.50	<0.050	0.50
Fluoranthene	<0.50	<0.050	0.50
Fluorene	<0.50	<0.050	0.50
Indeno(1,2,3-cd)pyrene	<0.50	<0.050	0.50
1-Methylnaphthalene	<0.50	<0.050	0.50
2-Methylnaphthalene	<0.50	<0.050	0.50
Naphthalene	<0.50	<0.050	0.50
Phenanthrene	<0.50	<0.050	0.50
Perylene	<0.50	<0.050	0.50
Pyrene	<0.50	<0.050	0.50
Quinoline	<1.0	<1.0	1.0

Table S3.10 One way ANOVA table showing the response of jack pine (*Pinus banksiana*) biomass to the amount of shallow-bituminous soil inoculum (Inoculum) used for inoculation. Sums of squares [SS], degrees of freedom [df], *F*-statistic, and *P*-value are shown).

Term	df	SS	*F*	*P*
Inoculum	1	0.725	0.917	0.351
Error	18	14.233		

Table S3.11 One way ANOVA table showing the response of jack pine (*Pinus banksiana*) biomass to the amount of bituminous-free soil inoculum (Inoculum) used for inoculation. Sums of squares [SS], degrees of freedom [df], *F*-statistic, and *P*-value are shown).

Term	df	SS	*F*	*P*
Inoculum	1	1.596	1.913	0.185
Error	17	14.184		

Table S3.12 One way ANOVA table showing the response of jack pine (*Pinus banksiana*) biomass to the amount of ore pile inoculum (Inoculum) used for inoculation. Sums of squares [SS], degrees of freedom [df], *F*-statistic, and *P*-value are shown).

Term	df	SS	*F*	*P*
Inoculum	1	<0.0001	<0.0001	0.997
Error	18	0.158		

Table S3.13 One way ANOVA table showing the response of trembling aspen (*Populus tremuloides*) biomass to the amount of shallow-bituminous soil inoculum (Inoculum) used for inoculation. Sums of squares [SS], degrees of freedom [df], *F*-statistic, and *P*-value are shown).

Term	df	SS	*F*	*P*
Inoculum	1	5.511	3.266	0.090
Error	16	26.997		

Table S3.14 One way ANOVA table showing the response of trembling aspen (*Populus tremuloides*) biomass to the amount of bituminous-free soil inoculum (Inoculum) used for inoculation. Sums of squares [SS], degrees of freedom [df], *F*-statistic, and *P*-value are shown).

Term	df	SS	*F*	*P*
Inoculum	1	7.095	4.185	0.056
Error	18	30.517		

Table S3.14 One way ANOVA table showing the response of trembling aspen (*Populus tremuloides*) biomass to the amount of ore pile inoculum (Inoculum) used for inoculation. Sums of squares [SS], degrees of freedom [df], *F*-statistic, and *P*-value are shown).

Term	df	SS	*F*	*P*
Inoculum	1	0.024	0.011	0.919
Error	18	40.664		

Table S3.16 *Pinus banksiana* (Pine) and *Populus tremuloides* (Aspen) seedlings that died during the experiment. Substrate type indicates the substrate each seedling was grown in while inoculum origin indicates which location the soil inoculum was collected from and whether the inoculum was live or sterile. LOS: lean oil sand

Species	Substrate type	Inoculum origin
Pine	Bituminous-free	Ore pile (live)
Pine	Ore pile	Shallow bituminous (sterile)
Aspen	Shallow bituminous	Ore pile (live)
Aspen	Ore pile	Shallow bituminous (live)
Aspen	High LOS	Bituminous-free (live)
Aspen	Low LOS	Ore pile (sterile)
Aspen	Low LOS	Ore pile (sterile)

Table S3.17 The results of a permutational analysis of variance (PERMANOVA) on the whole fungal community of shallow-bituminous, bituminous-free soils, and ore pile. A pairwise PERMANOVA with a false discovery-rate p-adjustment was used for determining differences between sites.

Term	df	SS	MS	*F*	R^2	*P*
Site	2	3.131	1.566	4.142	0.105	0.001
Site:Plot	2	2.157	1.078	2.852	0.072	0.001
Error	65	24.572	0.378		0.823	

	Pairwise comparison of locations (P- values)	
	Shallow-bituminous soils	Bituminous-free soils
Bituminous-free soils	0.001	
Ore pile	0.001	0.001

Table S3.18 The results of a permutational analysis of variance (PERMANOVA) on the ectomycorrhizal fungi of shallow-bituminous, bituminous-free soils, and ore pile. A pairwise PERMANOVA with a false discovery-rate p-adjustment was used for determining differences between sites.

Term	df	SS	MS	F	R^2	P
Site	2	2.637	1.318	3.093	0.081	0.001
Site:Plot	2	2.086	1.043	2.446	0.064	0.001
Error	65	27.711	0.426		0.854	

	Pairwise comparison of locations (P- values)	
	Shallow-bituminous soils	Bituminous-free soils
Bituminous-free soils	0.001	
Ore pile	0.001	0.001

Table S3.19 The results of a permutational analysis of variance (PERMANOVA) on the saprotrophic fungi of shallow-bituminous, bituminous-free soils, and ore pile. A pairwise PERMANOVA with a false discovery-rate p-adjustment was used for determining differences between sites.

Term	df	SS	MS	F	R^2	P
Site	2	3.244	1.622	4.284	0.108	0.001
Site:Plot	2	2.281	1.14	3.012	0.076	0.001
Error	65	24.607	0.379		0.817	

	Pairwise comparison of locations (P- values)	
	Shallow-bituminous soils	Bituminous-free soils
Bituminous-free soils	0.001	
Ore pile	0.001	0.001

Table S3.20 The results of a permutational analysis of variance (PERMANOVA) on the arbuscular mycorrhizal fungi of shallow-bituminous, bituminous-free soils, and ore pile. A pairwise PERMANOVA with a false discovery-rate p-adjustment was used for determining differences between sites

Term	df	SS	MS	F	R^2	P
Site	2	1.675	0.837	3.622	0.097	0.001
Site:Plot	2	0.541	0.27	1.17	0.031	0.257
Error	65	15.024	0.231		0.871	

	Pairwise comparison of locations (P- values)	
	Shallow-bituminous soils	Bituminous-free soils
Bituminous-free soils	0.003	
Ore pile	0.003	0.003

Table S3.21 The results of a permutational analysis of variance (PERMANOVA) on the other/mixed fungal guilds from shallow-bituminous, bituminous-free soils, and ore pile. A pairwise PERMANOVA with a false discovery-rate p-adjustment was used for determining differences between sites.

Term	df	SS	MS	F	R^2	P
Site	2	2.474	1.237	3.561	0.094	0.001
Site:Plot	2	1.216	0.608	1.751	0.046	0.001
Error	65	22.573	0.347		0.86	

	Pairwise comparison of locations (P- values)	
	Shallow-bituminous soils	Bituminous-free soils
Bituminous-free soils	0.001	
Ore pile	0.001	0.001

Table S3.22 The results of a permutational analysis of variance (PERMANOVA) on the unknown fungal guilds from shallow-bituminous, bituminous-free soils, and ore pile. A pairwise PERMANOVA with a false discovery-rate p-adjustment was used for determining differences between sites.

Term	df	SS	MS	F	R^2	P
Site	2	3.454	1.727	4.709	0.118	0.001
Site:Plot	2	1.934	0.967	2.637	0.066	0.001
Error	65	23.841	0.367		0.816	

	Pairwise comparison of locations (P- values)	
	Shallow-bituminous soils	Bituminous-free soils
Bituminous-free soils	0.001	
Ore pile	0.001	0.001

Table S3.23 The results of a permutational analysis of variance (PERMANOVA) on the whole fungal community of jack pine (*Pinus banksiana*) root samples collected from shallow-bituminous, bituminous-free soils, and ore pile. A pairwise PERMANOVA with a false discovery-rate p-adjustment was used for determining differences between sites.

Term	df	SS	MS	F	R^2	P
Site	2	1.3	0.65	2.17	0.17	0
Site:Plot	4	2.33	0.58	1.94	0.3	0
Error	14	4.2	0.3		0.54	

	Pairwise comparison of locations (P- values)	
	Shallow-bituminous roots	Bituminous-free roots
Bituminous-free roots	0.039	
Ore pile roots	0.024	0.021

Table S3.24 The results of a permutational analysis of variance (PERMANOVA) on the other/mixed fungal guilds of jack pine (*Pinus banksiana*) root samples collected from shallow-bituminous, bituminous-free soils, and ore pile. A pairwise PERMANOVA with a false discovery-rate p-adjustment was used for determining differences between sites.

Term	df	SS	MS	F	R^2	P
Site	2	1.098	0.549	2.476	0.186	0.001
Site:Plot	4	1.714	0.429	1.933	0.29	0.002
Error	14	3.104	0.222		0.525	

	Pairwise comparison of locations (P- values)	
	Shallow-bituminous roots	Bituminous-free roots
Bituminous-free roots	0.031	
Ore pile roots	0.031	0.031

Table S3.25 The results of a permutational analysis of variance (PERMANOVA) on the ectomycorrhizal fungi of jack pine (*Pinus banksiana*) root samples collected from shallow bituminous, bituminous-free soils, and ore pile. A pairwise PERMANOVA with a false discovery-rate p-adjustment was used for determining differences between sites.

Term	df	SS	MS	F	R^2	P
Site	2	1.383	0.691	1.908	0.15	0.002
Site:Plot	4	2.76	0.69	1.904	0.299	0.001
Error	14	5.073	0.362		0.551	

	Pairwise comparison of locations (P- values)	
	Shallow-bituminous roots	Bituminous-free roots
Bituminous-free roots	0.051	
Ore pile roots	0.081	0.048

Table S3.26 The results of a permutational analysis of variance (PERMANOVA) on the saprotrophic fungi of jack pine (*Pinus banksiana*) root samples collected from shallow-bituminous, bituminous-free soils, and ore pile. A pairwise PERMANOVA with a false discovery-rate p-adjustment was used for determining differences between sites.

Term	df	SS	MS	F	R^2	P
Site	2	1.374	0.687	1.625	0.155	0.007
Site:Plot	4	1.563	0.391	0.924	0.176	0.703
Error	14	5.919	0.423		0.668	

	Pairwise comparison of locations (P- values)	
	Shallow-bituminous roots	Bituminous-free roots
Bituminous-free roots	0.104	
Ore pile roots	0.039	0.046

Table S3.27 The results of a permutational analysis of variance (PERMANOVA) on the arbuscular mycorrhizal fungi of jack pine (*Pinus banksiana*) root samples collected from shallow-bituminous, bituminous-free soils, and ore pile. A pairwise PERMANOVA with a false discovery-rate p-adjustment was used for determining differences between sites.

Term	df	SS	MS	F	R^2	P
Site	2	1.697	0.848	4.7	0.35	0.009
Site:Plot	2	0.261	0.131	0.724	0.054	0.53
Error	16	2.888	0.181		0.596	

	Pairwise comparison of locations (P- values)	
	Shallow-bituminous roots	Bituminous-free roots
Bituminous-free roots	0.038	
Ore pile roots	0.038	0.274

Table S3.28 The results of a permutational analysis of variance (PERMANOVA) on the unknown fungal guilds of jack pine (*Pinus banksiana*) root samples collected from shallow-bituminous, bituminous-free soils, and ore pile. A pairwise PERMANOVA with a false discovery-rate p-adjustment was used for determining differences between sites.

Term	df	SS	MS	F	R^2	P
Site	2	1.234	0.617	1.394	0.13	0.007
Site:Plot	4	2.03	0.507	1.146	0.215	0.07
Error	14	6.196	0.443		0.655	

	Pairwise comparison of locations (P- values)	
	Shallow-bituminous roots	Bituminous-free roots
Bituminous-free roots	0.064	
Ore pile roots	0.018	0.064

Table S3.29 The top 10 most abundundant indicator amplicon sequence variants (ASVs) sourced from shallow bituminous, bituminous-free soils, and the ore pile Alberta, Canada. The ASVs assigned as indicators are associated with soils from a specific location. Proportional read abundance is calculated as a percentage of the total number of reads per soil type. The indicative value (IndVal) of each ASV was calculated using (De Cáceres & Legendre, 2009) and is a measure of the association between an ASV and soil type. Functional guilds were assigned to each ASV using the FUNGuild database (Nguyen et al., 2016). An NA in the guild column indicates the confidence of the guild assignment was below highly probable or probable. An NA within the taxonomy column indicates the ASV is unknown at that taxonomic level.

Soil type	ASV ID	Abundance	Indicator stat	P value	Guild	Taxonomy
Shallow bituminous	ASV0004	3.794	0.305	0.038	Soil Saprotroph	Ascomycota; Archaeorhizomycetes; Archaeorhizomycetales; Archaeorhizomycetaceae; Archaeorhizomyces; NA
Shallow bituminous	ASV0009	3.094	0.329	0.013	NA	Basidiomycota; Agaricomycetes; Cantharellales; NA; NA; NA
Shallow bituminous	ASV0013	2.766	0.462	0.001	Soil Saprotroph	Ascomycota; Archaeorhizomycetes; Archaeorhizomycetales; Archaeorhizomycetaceae; Archaeorhizomyces; NA
Shallow bituminous	ASV0012	2.070	0.371	0.009	NA	Ascomycota; Leotiomycetes; Helotiales; NA; NA; NA
Shallow bituminous	ASV0030	1.967	0.232	0.037	Ectomycorrhizal	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Lactarius; rufus

Shallow bituminous	ASV0031	1.680	0.289	0.027	Undefined Saprotroph	Ascomycota; Saccharomycetes; Saccharomycetales; Saccharomycetales_fam_Incertae_sedis; Myxozyma; NA
Shallow bituminous	ASV0029	1.517	0.240	0.022	Soil Saprotroph	Ascomycota; Archaeorhizomycetes; Archaeorhizomycetales; Archaeorhizomycetaceae; Archaeorhizomyces; NA
Shallow bituminous	ASV0055	1.144	0.291	0.039	Ectomycorrhizal	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Lactarius; mammosus
Shallow bituminous	ASV0051	0.964	0.360	0.009	NA	NA; NA; NA; NA; NA; NA
Shallow bituminous	ASV0046	0.804	0.404	0.005	Endophyte-Litter Saprotroph-Soil Saprotroph-Undefined Saprotroph	Mortierellomycota; Mortierellomycetes; Mortierellales; Mortierellaceae; Mortierella; globulifera
Bituminous free	ASV0003	6.458	0.315	0.014	Soil Saprotroph	Ascomycota; Archaeorhizomycetes; Archaeorhizomycetales; Archaeorhizomycetaceae; Archaeorhizomyces; NA
Bituminous free	ASV0006	4.032	0.310	0.026	Ectomycorrhizal	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Russula; farinipes
Bituminous free	ASV0021	2.167	0.269	0.046	Ectomycorrhizal	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Russula; roseipes

Bituminous free	ASV0024	2.020	0.395	0.004	Ectomycorrhizal	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Russula; chloroides
Bituminous free	ASV0034	1.657	0.229	0.045	Ectomycorrhizal	Basidiomycota; Agaricomycetes; Atheliales; Atheliaceae; Piloderma; bicolor
Bituminous free	ASV0023	1.592	0.324	0.033	Endophyte	Ascomycota; Leotiomycetes; Helotiales; Vibrisseaceae; Phialocephala; fortinii
Bituminous free	ASV0057	1.057	0.243	0.034	Ectomycorrhizal	Basidiomycota; Agaricomycetes; Agaricales; Cortinariaceae; Cortinarius; NA
Bituminous free	ASV0056	0.998	0.302	0.024	Undefined Saprotroph	Basidiomycota; Geminibasidiomycetes; Geminibasidiales; Geminibasidiaceae; Geminibasidium; NA
Bituminous free	ASV0069	0.929	0.319	0.029	Ectomycorrhizal-Fungal Parasite	Basidiomycota; Agaricomycetes; Agaricales; Tricholomataceae; Tricholoma; portentosum
Bituminous free	ASV0067	0.917	0.487	0.000	Undefined Saprotroph	Basidiomycota; Geminibasidiomycetes; Geminibasidiales; Geminibasidiaceae; Geminibasidium; NA
Ore pile	ASV0036	6.117	0.524	0.000	Fungal Parasite-Undefined Saprotroph	Basidiomycota; Tremellomycetes; Tremellales; Trimorphomycetaceae; Saitozyma; podzolica

Ore pile	ASV0041	5.862	0.400	0.000	Bryophyte Parasite-Ectomycorrhizal-Ericoid Mycorrhizal-Undefined Saprotroph-Wood Saprotroph	Ascomycota; Leotiomycetes; Helotiales; Leotiaceae; Pezoloma; ericae
Ore pile	ASV0014	5.575	0.365	0.007	NA	Basidiomycota; Tremellomycetes; Filobasidiales; Piskurozymaceae; Solicoccozyma; terricola
Ore pile	ASV0038	4.516	0.264	0.002	Plant Pathogen	Ascomycota; Leotiomycetes; Phacidiales; Phacidiaceae; Phacidium; pseudophacidioides
Ore pile	ASV0068	3.805	0.263	0.020	Dung Saprotroph-Plant Saprotroph	Ascomycota; Sordariomycetes; Sordariales; Sordariaceae; NA; NA
Ore pile	ASV0066	2.879	0.658	0.000	NA	Ascomycota; Dothideomycetes; Pleosporales; NA; NA; NA
Ore pile	ASV0058	2.797	0.421	0.001	Ectomycorrhizal	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Russula; velenovskyi
Ore pile	ASV0110	1.927	0.301	0.001	NA	Ascomycota; NA;NA; NA; NA; NA
Ore pile	ASV0129	1.342	0.427	0.000	NA	NA; NA; NA; NA; NA; NA
Ore pile	ASV00154	1.069	0.291	0.000	Undefined Saprotroph	Ascomycota; Eurotiomycetes; Chaetothyriales; Herpotrichiellaceae; Cladophialophora; NA

Shallow bituminous and Bituminous free	ASV0001	7.238	0.346	0.021	Soil Saprotroph	Ascomycota; Archaeorhizomycetes; Archaeorhizomycetales; Archaeorhizomycetaceae; Archaeorhizomyces; NA
Shallow bituminous and Bituminous free	ASV0022	1.122	0.323	0.031	Endophyte	Ascomycota; Leotiomycetes; Helotiales; Vibrisseaceae; Phialocephala; fortinii
Shallow bituminous and Bituminous free	ASV0076	0.412	0.312	0.030	Undefined Saprotroph	Basidiomycota; Tritirachiomycetes; Tritirachiales; Tritirachiaceae; Paratritirachium; NA
Shallow bituminous and Bituminous free	ASV0082	0.387	0.357	0.013	Undefined Saprotroph	Ascomycota; Saccharomycetes; Saccharomycetales; Lipomycetaceae; NA; NA
Shallow bituminous and Bituminous free	ASV0093	0.326	0.393	0.010	Undefined Saprotroph	Basidiomycota; Tritirachiomycetes; Tritirachiales; Tritirachiaceae; Paratritirachium; NA

Shallow bituminous and Bituminous free	ASV0132	0.202	0.388	0.009	NA	Ascomycota; Lecanoromycetes; NA; NA; NA; NA

Table S3.30 The complete list of amplicon sequence variants (ASVs) from jack pine (*Pinus banksiana*) roots sourced from shallow bituminous, bituminous-free soils, and the ore pile Alberta, Canada. The ASVs assigned as indicators are associated with soils from a specific location. Proportional read abundance is calculated as a percentage of the total number of reads per soil type. The indicative value (IndVal) of each ASV was calculated using (De Cáceres & Legendre, 2009) and is a measure of the association between an ASV and soil type. Functional guilds were assigned to each ASV using the FUNGuild database (Nguyen et al., 2016). An NA in the guild column indicates the confidence of the guild assignment was below highly probable or probable. An NA within the taxonomy column indicates the ASV is unknown at that taxonomic level.

Soil type	ASV ID	Abundance	Indicator stat	P value	Guild	Taxonomy
Shallow bituminous	ASV0003	10.088	0.624	0.023	Endophyte	Ascomycota; Leotiomycetes; Helotiales; Vibrisseaceae; Phialocephala; fortinii
Shallow bituminous	ASV0050	0.700	0.546	0.032	Undefined Saprotroph	Ascomycota; Leotiomycetes; Helotiales; Hyaloscyphaceae; Hyaloscypha; NA
Shallow bituminous	ASV0073	0.479	0.553	0.045	Undefined Saprotroph	Ascomycota; Leotiomycetes; Helotiales; Hyaloscyphaceae; Hyaloscypha; variabilis

Shallow bituminous	ASV0106	0.376	0.457	0.033	NA	Ascomycota; Leotiomycetes; Helotiales; NA; NA; NA
Shallow bituminous	ASV0122	0.235	0.639	0.017	Fungal Parasite-Lichen Parasite	Basidiomycota; Tremellomycetes; Tremellales; Syzygosporaceae; Syzygospora; effibulata
Shallow bituminous	ASV0130	0.219	0.613	0.009	NA	Ascomycota; Dothideomycetes; Capnodiales; NA; NA; NA
Shallow bituminous	ASV0189	0.096	0.620	0.017	NA	Rozellomycota; NA; NA; NA; NA; NA
Shallow bituminous	ASV0208	0.088	0.485	0.043	NA	Ascomycota; NA; NA; NA; NA; NA
Shallow bituminous	ASV0215	0.082	0.607	0.034	NA	Ascomycota; Leotiomycetes; Helotiales; NA; NA; NA

Shallow bituminous	ASV0271	0.063	0.575	0.022	Endophyte-Litter Saprotroph-Soil Saprotroph-Undefined Saprotroph	Mortierellomycota; Mortierellomycetes; Mortierellales; Mortierellaceae; Mortierella; pulchella
Bituminous free	ASV0175	0.126	0.506	0.018	NA	Ascomycota; NA; NA; NA; NA; NA
Ore pile	ASV0002	26.767	0.693	0.007	Endophyte	Ascomycota; Leotiomycetes; Helotiales; Vibrisseaceae; Phialocephala; fortinii
Ore pile	ASV0032	4.035	0.740	0.017	Undefined Saprotroph	Ascomycota; Leotiomycetes; Helotiales; Hyaloscyphaceae; Hyaloscypha; bicolor
Ore pile	ASV0086	1.427	0.712	0.014	NA	Ascomycota; Leotiomycetes; Helotiales; NA; NA; NA

Ore pile	ASV0084	1.314	0.561	0.002	NA	Basidiomycota; Tremellomycetes; Tremellales; Trimorphomycetaceae; Saitozyma; podzolica
Ore pile	ASV0132	0.630	0.555	0.017	Ectomycorrhizal	Basidiomycota; Agaricomycetes; Thelephorales; Thelephoraceae; Tomentella; ellisii
Ore pile	ASV0191	0.399	0.502	0.042	Endophyte-Litter Saprotroph-Soil Saprotroph-Undefined Saprotroph	Mortierellomycota; Mortierellomycetes; Mortierellales; Mortierellaceae; Mortierella; basiparvispora
Ore pile	ASV0198	0.297	0.572	0.017	Undefined Saprotroph	Ascomycota; Leotiomycetes; Helotiales; Helotiales_fam_Incertae_sedis; Xenopolyscytalum; pinea
Ore pile	ASV0334	0.171	0.530	0.017	NA	Basidiomycota; Agaricomycetes; GS29; NA; NA; NA

Ore pile	ASV0390	0.093	0.755	0.014	NA	Rozellomycota; NA; NA; NA; NA; NA
Ore pile	ASV0410	0.073	0.633	0.017	NA	Ascomycota; NA; NA; NA; NA; NA
Ore pile	ASV0480	0.053	0.603	0.014	Wood Saprotroph	Ascomycota; Leotiomycetes; Helotiales; Ascocorticiaceae; Ascocorticium; NA
Ore pile	ASV0740	0.022	0.756	0.017	Arbuscular Mycorrhizal	Glomeromycota; Glomeromycetes; Diversisporales; Diversisporales_fam_Incertae_sedis; Entrophospora; infrequens
Ore pile	ASV0780	0.009	0.629	0.028	NA	Ascomycota; Dothideomycetes; Pleosporales; NA; NA; NA

Table S3.31 Two-way ANOVA table showing the response of jack pine (*Pinus banksiana*) biomass to substrate type (Substrate) and live soil inoculation (Inoculation). Sums of squares [SS], degrees of freedom [df], *F*-statistic, and *P*-value are shown).

Term	df	SS	*F*	*P*
Substrate	4	29.377	10.772	<0.0001
Inoculation	2	4.927	3.613	0.0296
Substrate×Inoculation	8	14.535	2.665	0.00951
Error	135	92.041		

Table S3.32 Two-way ANOVA table showing the response of jack pine (*Pinus banksiana*) biomass (ln transformed) to substrate type (Substrate) and sterile soil inoculation (Inoculation). Sums of squares [SS], degrees of freedom [df], *F*-statistic, and *P*-value are shown).

Term	df	SS	*F*	*P*
Substrate	4	2.114	2.59	0.04
Inoculation	2	5.956	14.595	<0.0001
Substrate×Inoculation	8	3.519	2.156	0.035
Error	134	27.343		

Table S3.33 Two-way ANOVA table showing the response of aspen (*Populus tremuloides*) biomass to substrate type (Substrate) and live soil inoculation (Inoculation). Sums of squares [SS], degrees of freedom [df], *F*-statistic, and *P*-value are shown).

Term	df	SS	*F*	*P*
Substrate	4	208.4	22.857	<0.0001
Inoculation	2	53.4	11.78	<0.0001
Substrate×Inoculation	8	24	1.314	0.242
Error	132	300.836		

Table S3.34 Two-way ANOVA table showing the response of aspen (*Populus tremuloides*) biomass to substrate type (Substrate) and sterile soil inoculation (Inoculation). Sums of squares [SS], degrees of freedom [df], *F*-statistic, and *P*-value are shown).

Term	df	SS	*F*	*P*
Substrate	4	265.3	34.227	<0.0001
Inoculation	2	2.5	0.639	0.53
Substrate×Inoculation	8	17.6	1.134	0.345
Error	133	257.7		

Table S3.35 Two-way ANOVA table showing the response of jack pine (*Pinus banksiana*) root tip colonization (ln transformed) to substrate type (Substrate)) and soil inoculation (Inoculation). Sums of squares [SS], degrees of freedom [df], *F*-statistic, and *P*-value are shown).

Term	df	SS	*F*	*P*
Substrate	4	1.06	0.519	0.726
Inoculation	2	8.036	7.930	0.001
Substrate × Inoculation	8	6.17	1.505	0.161
Error	134	68.636		

Table S3.36 Top 20 most abundant amplicon sequence variants (ASVs) of *Pinus banksiana* roots associated with each site . The first number is the ASV ranking and the number within the parenthesis is the proportional read abundance. A NA within the soil type indicates no sequences were found within samples from that soil type. A NA within Taxonomy indicates the ASV is unknown at that taxonomic level. ASVs were assigned to taxonomic guilds using the FUNGuild database (Nguyen et al., 2016).

ASV	Shallow-bituminous soil	Bituminos-free soil	Ore pile	Taxonomy	Guild	Confidence
ASV0002	1 (11.36)	2 (7.25)	1 (26.77)	Ascomycota; Leotiomycetes; Helotiales; Vibrisseaceae; Phialocephala; fortinii	Endophyte	Highly Probable
ASV0003	2 (10.09)	13 (1.48)	49 (1.00)	Ascomycota; Leotiomycetes; Helotiales; Vibrisseaceae; Phialocephala; fortinii	Endophyte	Highly Probable
ASV0006	3 (7.48)	54 (0.22)	166 (< 0.001)	Ascomycota; Dothideomycetes; Mytilinidales; Gloniaceae; Cenococcum; geophilum	Ectomycorrhizal	Highly Probable
ASV0008	4 (5.28)	NA	NA	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Lactarius; rufus	Ectomycorrhizal	Highly Probable
ASV0001	5 (4.23)	1 (21.51)	NA	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Russula; vinososordida	Ectomycorrhizal	Highly Probable
ASV0007	6 (3.02)	7 (2.80)	NA	Ascomycota; Leotiomycetes; Helotiales; Vibrisseaceae; Phialocephala; fortinii	Endophyte	Highly Probable
ASV0009	7 (2.87)	NA	NA	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Russula; vinososordida	Ectomycorrhizal	Highly Probable

ASV0011	8 (2.83)	85 (0.12)	NA	Basidiomycota; Agaricomycetes; Atheliales; Pilodermataceae; Piloderma; sphaerosporum	Ectomycorrhizal	Highly Probable
ASV0017	9 (2.68)	NA	NA	Basidiomycota; Agaricomycetes; Agaricales; Cortinariaceae; Cortinarius; junghuhnii	Ectomycorrhizal	Highly Probable
ASV0004	10 (2.60)	4 (5.36)	NA	Ascomycota; Archaeorhizomycetes; Archaeorhizomycetales; Archaeorhizomycetaceae; Archaeorhizomyces; finlayi	NA	NA
ASV0022	11 (2.60)	205 (0.02)	NA	Basidiomycota; Agaricomycetes; Thelephorales; Thelephoraceae; NA; NA	Ectomycorrhizal-Undefined Saprotroph	Probable
ASV0018	12 (2.34)	46 (0.32)	NA	Basidiomycota; Agaricomycetes; Atheliales; Pilodermataceae; Piloderma; bicolor	Ectomycorrhizal	Highly Probable
ASV0027	13 (1.97)	NA	NA	Basidiomycota; Agaricomycetes; Atheliales; Pilodermataceae; Piloderma; olivaceum	Ectomycorrhizal	Highly Probable
ASV0020	14 (1.51)	44 (0.32)	9 (2.49)	Ascomycota; Pezizomycetes; Pezizales; Pyronemataceae; Wilcoxina; rehmii	Ectomycorrhizal	Highly Probable
ASV0029	15 (1.45)	NA	NA	Ascomycota; Leotiomycetes; Helotiales; Vibrisseaceae; Phialocephala; fortinii	Endophyte	Highly Probable
ASV0025	16 (1.03)	35 (0.43)	NA	Ascomycota; Dothideomycetes; Dothideomycetes_ord_Incertae_sedis; Dothideomycetes_fam_Incertae_sedis; Septonema; fasciculare	Wood Saprotroph	Highly Probable
ASV0042	17 (0.91)	107 (0.07)	NA	Basidiomycota; Agaricomycetes; Thelephorales; Thelephoraceae; NA; NA	Ectomycorrhizal-Undefined Saprotroph	Probable

ASV0046	18 (0.89)	NA	NA	Ascomycota; Dothideomycetes; Mytilinidales; Gloniaceae; NA; NA	NA	NA
ASV0045	19 (0.85)	140 (0.05)	NA	Basidiomycota; Agaricomycetes; Auriculariales; NA; NA; NA	NA	NA
ASV0064	20 (0.82)	217 (0.02)	NA	Basidiomycota; Agaricomycetes; Thelephorales; Thelephoraceae; NA; NA	Ectomycorrhizal-Undefined Saprotroph	Probable
ASV0001	5 (4.23)	1 (21.51)	NA	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Russula; vinososordida	Ectomycorrhizal	Highly Probable
ASV0002	1 (11.36)	2 (7.25)	1 (26.77)	Ascomycota; Leotiomycetes; Helotiales; Vibrisseaceae; Phialocephala; fortinii	Endophyte	Highly Probable
ASV0005	41(0.40)	3 (6.39)	NA	Ascomycota; Archaeorhizomycetes; Archaeorhizomycetales; Archaeorhizomycetaceae; Archaeorhizomyces; NA	NA	NA
ASV0004	10 (2.60)	4 (5.36)	NA	Ascomycota; Archaeorhizomycetes; Archaeorhizomycetales; Archaeorhizomycetaceae; Archaeorhizomyces; finlayi	NA	NA
ASV0013	45 (0.35)	5 (4.80)	127 (0.01)	Ascomycota; Archaeorhizomycetes; Archaeorhizomycetales; Archaeorhizomycetaceae; Archaeorhizomyces; NA	NA	NA
ASV0010	34 (0.56)	6 (3.61)	NA	Basidiomycota; Agaricomycetes; Atheliales; Tylosporaceae; Amphinema; byssoides	Ectomycorrhizal	Highly Probable
ASV0007	6 (3.02)	7 (2.80)	NA	Ascomycota; Leotiomycetes; Helotiales; Vibrisseaceae; Phialocephala; fortinii	Endophyte	Highly Probable

ASV0026	NA	8 (2.55)	167 (<0.01)	Basidiomycota; Agaricomycetes; Thelephorales; Bankeraceae; Hydnellum; glaucopus	Ectomycorrhizal	Highly Probable
ASV0014	NA	9 (2.13)	NA	Basidiomycota; Agaricomycetes; Thelephorales; Bankeraceae; Phellodon; tomentosus	Ectomycorrhizal	Highly Probable
ASV0019	NA	10 (1.97)	NA	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Russula; versicolor	Ectomycorrhizal	Highly Probable
ASV0039	389 (0.01)	11 (1.8)	155 (0.01)	Basidiomycota; Agaricomycetes; Cantharellales; Clavulinaceae; NA; NA	NA	NA
ASV0023	200 (0.02)	12 (1.75)	NA	Basidiomycota; Agaricomycetes; Agaricales; Tricholomataceae; Tricholoma; equestre	Ectomycorrhizal-Fungal Parasite	Highly Probable
ASV0003	2 (10.09)	13 (1.48)	49 (0.10)	Ascomycota; Leotiomycetes; Helotiales; Vibrisseaceae; Phialocephala; fortinii	Endophyte	Highly Probable
ASV0030	81 (0.15)	14 (1.27)	102 (0.03)	Ascomycota; Pezizomycetes; Pezizales; Sarcosomataceae; Plectania; melastoma	Undefined Saprotroph	Probable
ASV0028	NA	15 (1.26)	NA	Basidiomycota; Agaricomycetes; Agaricales; Cortinariaceae; Cortinarius; millaresensis	Ectomycorrhizal	Highly Probable
ASV0031	NA	16 (1.24)	NA	Basidiomycota; Agaricomycetes; Agaricales; Tricholomataceae; Mycena; flavoalba	Leaf Saprotroph-Plant Pathogen-Undefined Saprotroph-Wood Saprotroph	Probable
ASV0037	NA	17 (1.23)	NA	Basidiomycota; Agaricomycetes; Atheliales; Pilodermataceae; Piloderma; olivaceum	Ectomycorrhizal	Highly Probable

ASV0035	NA	18 (1.13)	NA	Basidiomycota; Agaricomycetes; Agaricales; Cortinariaceae; Cortinarius; alpinus	Ectomycorrhizal	Highly Probable
ASV0041	141 (0.06)	19 (0.91)	NA	Ascomycota; Dothideomycetes; Mytilinidales; Gloniaceae; NA; NA	NA	NA
ASV0036	70 (0.19)	20 (0.87)	NA	Ascomycota; Leotiomycetes; Helotiales; Hyaloscyphaceae; NA; NA	Plant Saprotroph-Wood Saprotroph	Probable
ASV0002	1 (11.36)	2 (7.25)	1 (26.77)	Ascomycota; Leotiomycetes; Helotiales; Vibrisseaceae; Phialocephala; fortinii	Endophyte	Highly Probable
ASV0012	NA	NA	2 (9.47)	Ascomycota; Leotiomycetes; Helotiales; Vibrisseaceae; Phialocephala; fortinii	Endophyte	Highly Probable
ASV0015	NA	NA	3 (7.88)	Basidiomycota; Agaricomycetes; Thelephorales; Thelephoraceae; Tomentella; subclavigera	Ectomycorrhizal	Highly Probable
ASV0016	35 (0.56)	NA	4 (7.10)	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Russula; emetica	Ectomycorrhizal	Highly Probable
ASV0021	NA	NA	5 (5.78)	Ascomycota; NA; NA; NA; NA; NA	NA	NA
ASV0040	NA	NA	6 (5.47)	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Russula; emetica	Ectomycorrhizal	Highly Probable
ASV0032	NA	72 (0.14)	7 (4.03)	Ascomycota; Leotiomycetes; Helotiales; Hyaloscyphaceae; Hyaloscypha; bicolor	Undefined Saprotroph	Probable
ASV0038	NA	NA	8 (3.41)	Basidiomycota; Agaricomycetes; Thelephorales; Thelephoraceae; Thelephora; terrestris	Ectomycorrhizal-Undefined Saprotroph	Probable

ASV0044	198 (0.03)	211 (0.02)	9 (2.49)	Ascomycota; Leotiomycetes; Helotiales; Hyaloscyphaceae; Hyaloscypha; variabilis	Undefined Saprotroph	Probable
ASV0090	NA	NA	10 (1.91)	Basidiomycota; Agaricomycetes; Agaricales; Tricholomataceae; Mycena; zephirus	Leaf Saprotroph-Plant Pathogen-Undefined Saprotroph-Wood Saprotroph	Probable
ASV0092	NA	NA	11 (1.83)	Basidiomycota; Agaricomycetes; Trechisporales; NA; NA; NA	NA	NA
ASV0096	NA	NA	12 (1.70)	Basidiomycota; Agaricomycetes; Thelephorales; Thelephoraceae; NA; NA	Ectomycorrhizal-Undefined Saprotroph	Probable
ASV0061	274 (0.01)	408 (<0.01)	13 (1.68)	Basidiomycota; Agaricomycetes; Agaricales; Tricholomataceae; Mycena; NA	Leaf Saprotroph-Plant Pathogen- Undefined Saprotroph-Wood Saprotroph	Probable
ASV0099	361 (0.01)	272 (0.01)	14 (1.53)	Ascomycota; Leotiomycetes; Helotiales; NA; NA; NA	NA	NA
ASV0086	NA	NA	15 (1.43)	Ascomycota; Leotiomycetes; Helotiales; NA; NA; NA	NA	NA
ASV0067	144 (0.05)	143 (0.05)	16 (1.36)	Ascomycota; Leotiomycetes; Helotiales; Helotiaceae; Meliniomyces; NA	Ectomycorrhizal-Endophyte-Ericoid Mycorrhizal-Litter Saprotroph-Orchid Mycorrhizal	Probable
ASV0084	NA	190 (0.02)	17 (1.31)	Basidiomycota; Tremellomycetes; Tremellales; Trimorphomycetaceae; Saitozyma; podzolica	NA	NA

ASV0087	NA	NA	18 (1.17)	Basidiomycota; Agaricomycetes; GS29; NA; NA; NA	NA	NA
ASV0093	NA	NA	19 (1.01)	Ascomycota; Archaeorhizomycetes; Archaeorhizomycetales; Archaeorhizomycetaceae; Archaeorhizomyces; NA	NA	NA
ASV0105	NA	NA	20 (0.83)	Ascomycota; Leotiomycetes; Helotiales; Hyaloscyphaceae; Hyaloscypha; bicolor	Undefined Saprotroph	Probable

Table S3.37 Top 20 most abundant amplicon sequence variants (ASVs) in each site. The first number is the ASV ranking and the number within the parenthesis is the proportional read abundance. A NA within the soil type indicates no sequences were found within samples from that soil type. A NA within Taxonomy indicates the ASV is unknown at that taxonomic level. ASVs were assigned to taxonomic guilds using the FUNGuild database (Nguyen et al., 2016).

ASV	Shallow - bituminous soil	Bituminous-free soil	Ore pile	Taxonomy	Guild	Confidence
ASV0001	1 (6.27)	1 (8.20)	NA	Ascomycota; Archaeorhizomycetes; Archaeorhizomycetales; Archaeorhizomycetaceae; Archaeorhizomyces; NA	Soil Saprotroph	Probable
ASV0002	2 (4.72)	3 (4.51)	525 (<0.01)	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Russula; vinososordida	Ectomycorrhizal	Highly Probable
ASV0004	3 (3.79)	36 (0.54)	NA	Ascomycota; Archaeorhizomycetes; Archaeorhizomycetales; Archaeorhizomycetaceae; Archaeorhizomyces; NA	Soil Saprotroph	Probable
ASV0005	4 (3.76)	NA	NA	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Russula; NA	Ectomycorrhizal	Highly Probable
ASV0009	5 (3.09)	NA	NA	Basidiomycota; Agaricomycetes; Cantharellales; NA; NA; NA	NA	NA

ASV0013	6 (2.77)	NA	NA	Ascomycota; Archaeorhizomycetes; Archaeorhizomycetales; Archaeorhizomycetaceae; Archaeorhizomyces; NA	Soil Saprotroph	Probable
ASV0010	7 (2.23)	NA	NA	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Russula; pubescens	Ectomycorrhizal	Highly Probable
ASV0012	8 (2.07)	37 (0.52)	39 (0.56)	Ascomycota; Leotiomycetes; Helotiales; NA; NA; NA	NA	NA
ASV0030	9 (1.97)	NA	NA	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Lactarius; rufus	Ectomycorrhizal	Highly Probable
ASV0018	10 (1.92)	44 (0.47)	NA	Basidiomycota; Agaricomycetes; Atheliales; Atheliaceae; Piloderma; olivaceum	Ectomycorrhizal	Highly Probable
ASV0015	11 (1.82)	53 (0.37)	NA	Ascomycota; Archaeorhizomycetes; Archaeorhizomycetales; Archaeorhizomycetaceae; Archaeorhizomyces; finlayi	Soil Saprotroph	Probable
ASV0025	12 (1.81)	163 (0.08)	NA	Basidiomycota; Agaricomycetes; Agaricales; Hygrophoraceae; Hygrophorus; hypothejus	Ectomycorrhizal	Highly Probable

ASV0026	13 (1.75)	159 (0.08)	NA	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Lactarius; albocarneus	Ectomycorrhizal	Highly Probable
ASV0031	14 (1.68)	711 (0.01)	NA	Ascomycota; Saccharomycetes; Saccharomycetales; Saccharomycetales_fam_Incertae_sedis; Myxozyma; NA	Undefined Saprotroph	Possible
ASV0029	15 (1.52)	NA	NA	Ascomycota; Archaeorhizomycetes; Archaeorhizomycetales; Archaeorhizomycetaceae; Archaeorhizomyces; NA	Soil Saprotroph	Probable
ASV0007	16 (1.23)	545 (0.01)	41 (0.53)	Basidiomycota; Agaricomycetes; Atheliales; Atheliaceae; Amphinema; byssoides	Ectomycorrhizal	Highly Probable
ASV0019	17 (1.16)	57 (0.33)	1028 (<0.01)	Basidiomycota; Tremellomycetes; Trichosporonales; Trichosporonaceae; Apiotrichum; porosum	Soil Saprotroph	Possible
ASV0055	18 (1.14)	NA	NA	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Lactarius; mammosus	Ectomycorrhizal	Highly Probable
ASV0045	19 (1.13)	123 (0.12)	NA	Basidiomycota; Agaricomycetes; Atheliales; Atheliaceae; Piloderma; bicolor	Ectomycorrhizal	Highly Probable

ASV0011	20 (1.07)	14 (1.34)	20 (1.04)	Ascomycota; Leotiomycetes; Helotiales; Vibrisseaceae; Phialocephala; fortinii	Endophyte	Highly Probable
ASV0001	1 (6.27)	1 (8.20)	NA	Ascomycota; Archaeorhizomycetes; Archaeorhizomycetales; Archaeorhizomycetaceae; Archaeorhizomyces; NA	Soil Saprotroph	Probable
ASV0003	NA	2 (6.46)	NA	Ascomycota; Archaeorhizomycetes; Archaeorhizomycetales; Archaeorhizomycetaceae; Archaeorhizomyces; NA	Soil Saprotroph	Probable
ASV0002	2 (4.72)	3 (4.51)	525 (0.01)	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Russula; vinososordida	Ectomycorrhizal	Highly Probable
ASV0006	54 (0.42)	4 (4.03)	NA	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Russula; farinipes	Ectomycorrhizal	Highly Probable
ASV0017	115 (0.12)	5 (2.32)	NA	NA; NA; NA; NA; NA; NA	NA	NA
ASV0021	NA	6 (2.17)	NA	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Russula; roseipes	Ectomycorrhizal	Highly Probable

ASV0024	NA	7 (2.02)	NA	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Russula; chloroides	Ectomycorrhizal	Highly Probable
ASV0028	NA	8 (1.82)	NA	Ascomycota; Leotiomycetes; Helotiales; NA; NA; NA	NA	NA
ASV0034	NA	9 (1.66)	NA	Basidiomycota; Agaricomycetes; Atheliales; Atheliaceae; Piloderma; bicolor	Ectomycorrhizal	Highly Probable
ASV0043	257 (0.03)	10 (1.62)	NA	Ascomycota; Archaeorhizomycetes; Archaeorhizomycetales; Archaeorhizomycetaceae; Archaeorhizomyces; NA	Soil Saprotroph	Probable
ASV0023	57 (0.41)	11 (1.59)	37 (0.61)	Ascomycota; Leotiomycetes; Helotiales; Vibrisseaceae; Phialocephala; fortinii	Endophyte	Highly Probable
ASV0037	433 (0.01)	12 (1.51)	NA	Ascomycota; Leotiomycetes; Helotiales; Helotiaceae; Hymenoscyphus; NA	Bryophyte Parasite-Ectomycorrhizal-Ericoid Mycorrhizal-Undefined Saprotroph	Possible
ASV0022	26 (0.90)	13 (1.34)	210 (0.05)	Ascomycota; Leotiomycetes; Helotiales; Vibrisseaceae; Phialocephala; fortinii	Endophyte	Highly Probable

ASV0011	20 (1.07)	14 1.34)	20 (1.11)	Ascomycota; Leotiomycetes; Helotiales; Vibrisseaceae; Phialocephala; fortinii	Endophyte	Highly Probable
ASV0042	NA	15 (1.30)	1027 (<0.01)	Basidiomycota; Agaricomycetes; Agaricales; Tricholomataceae; Mycena; aetites	Leaf Saprotroph-Plant Pathogen-Undefined Saprotroph-Wood Saprotroph	Probable
ASV0040	NA	16 (1.25)	728 (<0.01)	Basidiomycota; Agaricomycetes; Agaricales; Cortinariaceae; Cortinarius; athabascus	Ectomycorrhizal	Highly Probable
ASV0014	102 (0.14)	17 (1.16)	3 (5.58)	Basidiomycota; Tremellomycetes; Filobasidiales; Piskurozymaceae; Solicoccozyma; terricola	NA	NA
ASV0057	NA	18 (1.06)	NA	Basidiomycota; Agaricomycetes; Agaricales; Cortinariaceae; Cortinarius; NA	Ectomycorrhizal	Highly Probable
ASV0060	NA	19 (1.04)	NA	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Russula; farinipes	Ectomycorrhizal	Highly Probable
ASV0032	218 (0.04)	20 (1.04)	10 (1.64)	Mortierellomycota; Mortierellomycetes; Mortierellales; Mortierellaceae; Mortierella; basiparvispora	Endophyte-Litter Saprotroph-Soil Saprotroph-Undefined Saprotroph	Possible

ASV0036	90 (0.17)	186 (0.07)	1 (6.12)	Basidiomycota; Tremellomycetes; Tremellales; Trimorphomycetaceae; Saitozyma; podzolica	Fungal Parasite-Undefined Saprotroph	Possible
ASV0041	NA	NA	2 (5.86)	Ascomycota; Leotiomycetes; Helotiales; Leotiaceae; Pezoloma; ericae	Bryophyte Parasite-Ectomycorrhizal-Ericoid Mycorrhizal-Undefined Saprotroph-Wood Saprotroph	Possible
ASV0014	102 (0.14)	17 (1.16)	3 (5.58)	Basidiomycota; Tremellomycetes; Filobasidiales; Piskurozymaceae; Solicoccozyma; terricola	NA	NA
ASV0038	1765 (<0.01)	NA	4 (4.52)	Ascomycota; Leotiomycetes; Phacidiales; Phacidiaceae; Phacidium; pseudophacidioides	Plant Pathogen	Probable
ASV0068	NA	NA	5 (3.80)	Ascomycota; Sordariomycetes; Sordariales; Sordariaceae; NA; NA	Dung Saprotroph-Plant Saprotroph	Probable
ASV0033	39 (0.65)	96 (0.16)	6 (3.40)	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Russula; versicolor	Ectomycorrhizal	Highly Probable
ASV0066	370 (0.02)	NA	7 (2.88)	Ascomycota; Dothideomycetes; Pleosporales; NA; NA; NA	NA	NA

ASV0058	70 (0.30)	1525 (<0.01)	8 (2.80)	Basidiomycota; Agaricomycetes; Russulales; Russulaceae; Russula; velenovskyi	Ectomycorrhizal	Highly Probable
ASV0110	NA	1223 (<0.01)	9 (1.93)	Ascomycota; NA; NA; NA; NA; NA	NA	NA
ASV0032	218 (0.04)	20 (1.04)	10 (1.64)	Mortierellomycota; Mortierellomycetes; Mortierellales; Mortierellaceae; Mortierella; basiparvispora	Endophyte-Litter Saprotroph-Soil Saprotroph-Undefined Saprotroph	Possible
ASV0134	532 (0.01)	NA	11 (1.62)	Basidiomycota; Agaricomycetes; Thelephorales; Thelephoraceae; Thelephora; terrestris	Ectomycorrhizal	Highly Probable
ASV0152	NA	NA	12 (1.54)	Basidiomycota; Agaricomycetes; Atheliales; Atheliaceae; Tylospora; asterophora	Ectomycorrhizal	Highly Probable
ASV0139	247 (0.03)	1402 (<0.01)	13 (1.53)	Ascomycota; Leotiomycetes; Helotiales; Helotiales_fam_Incertae_sedis; Eleutheromyces; subulatus	Undefined Saprotroph	Possible
ASV0153	NA	NA	14 (1.49)	Ascomycota; Leotiomycetes; Helotiales; Leotiaceae; Pezoloma; ericae	Bryophyte Parasite-Ectomycorrhizal-Ericoid Mycorrhizal-Undefined Saprotroph-Wood Saprotroph	Possible

ASV0129	NA	NA	15 (1.34)	NA; NA; NA; NA; NA; NA	NA	NA
ASV0179	NA	NA	16 (1.26)	Ascomycota; Leotiomycetes; Helotiales; Hyaloscyphaceae; Hyaloscypha; variabilis	Undefined Saprotroph	Probable
ASV0274	NA	NA	17 (1.19)	Basidiomycota; Agaricomycetes; Sebacinales; Serendipitaceae; Serendipita; NA	Orchid Mycorrhizal	Highly Probable
ASV0283	NA	NA	18 (1.16)	Ascomycota; Leotiomycetes; Helotiales; Vibrisseaceae; Phialocephala; fortinii	Endophyte	Highly Probable
ASV0181	NA	NA	19 (1.12)	Basidiomycota; Agaricomycetes; Thelephorales; Thelephoraceae; Thelephora; terrestris	Ectomycorrhizal	Highly Probable
ASV0011	20 (1.07)	14 (1.34)	20 (1.11)	Ascomycota; Leotiomycetes; Helotiales; Vibrisseaceae; Phialocephala; fortinii	Endophyte	Highly Probable

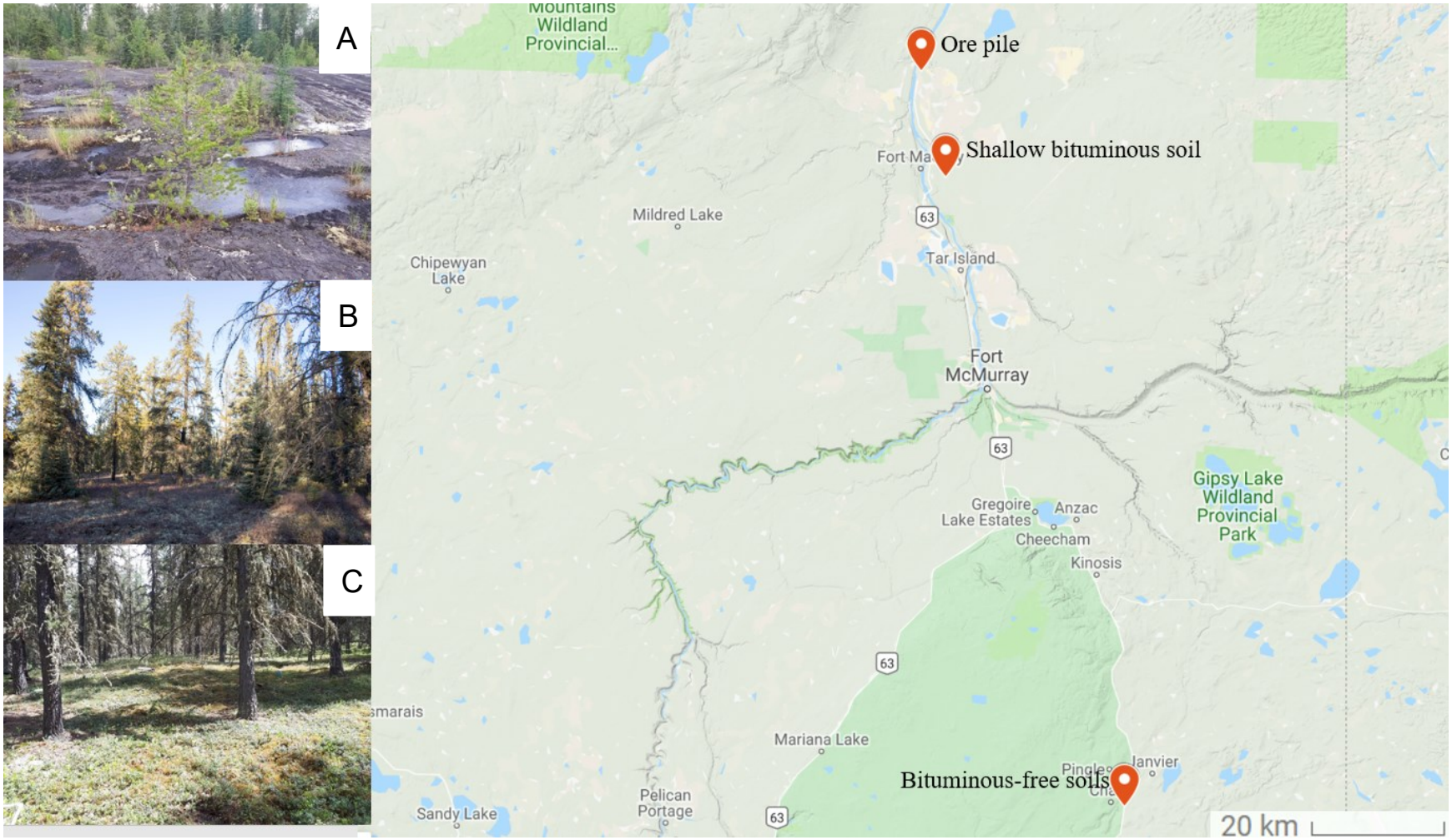

Figure S1: 1 Photo of *Pinus banksiana* and surrounding vegetation on the ore pile at Bitumount Alberta, Canada (A); a shallow-bituminous site (B); and a bituminous-free site (C), where soil and root and soil samples were collected

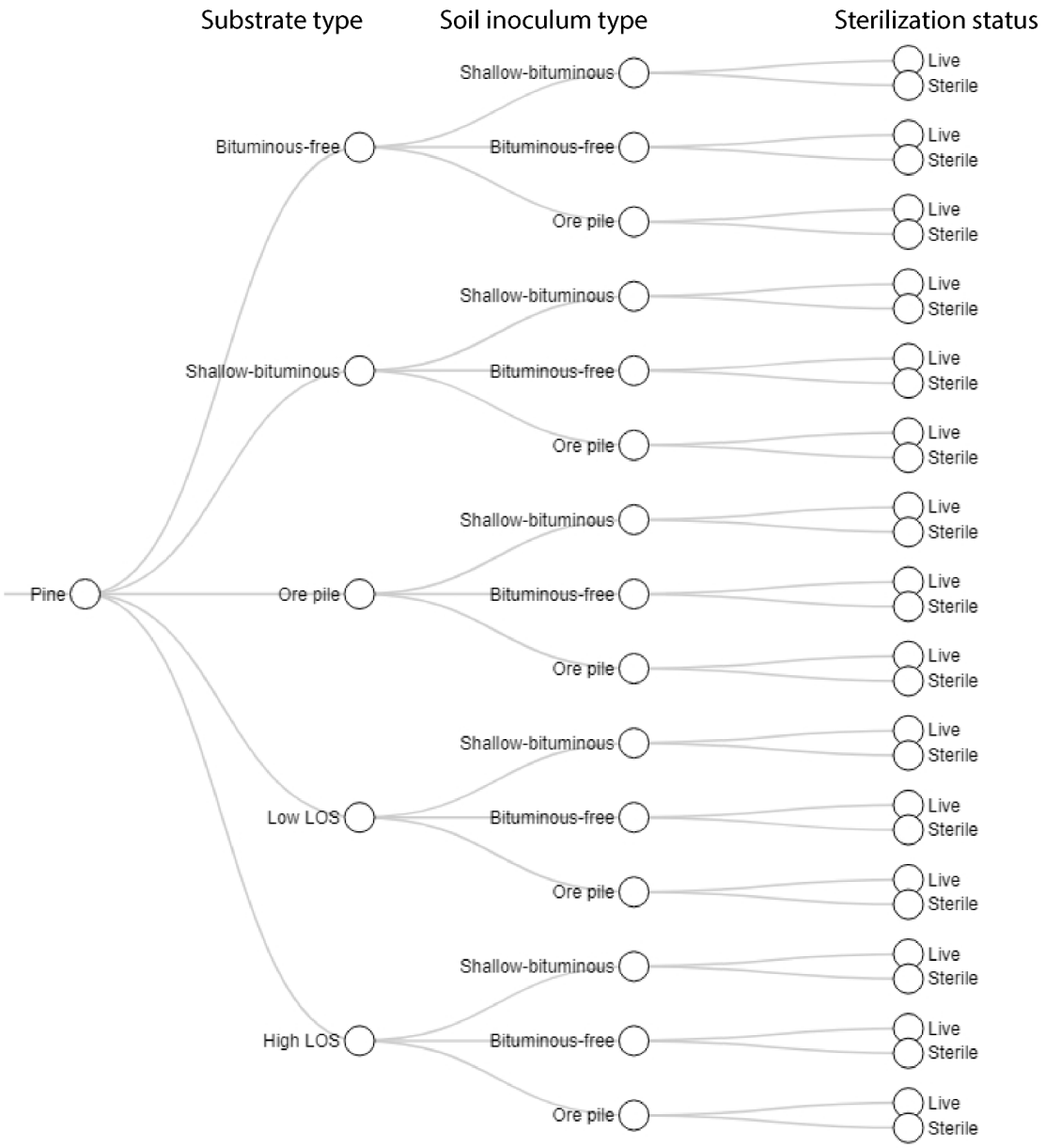

Figure S3.3 Experimental design of growth chamber experiment. *Pinus banksiana* and *Populus tremuloides* were grown in five substrate types: soils from Shallow-bituminous sites located near the Hammerstone Quarry north of Fort MacKay Alberta, Canada; soils from Bituminous-free sites located near Janvier, Alberta, Canada; substates from the ore pile located at Bitumount Alberta, Canada; two grades of lean oil sand provided by Syncrude Canada Ltd. containing 8,751 mg kg^{-1} ('Low LOS') and 36,699 mg kg^{-1} ('High LOS'). Tree seedlings were inoculated with either live or sterile soil inoculum collected from Shallow bituminous sites, bituminous free sites, or the ore pile. Each treatment combination had a n =10.

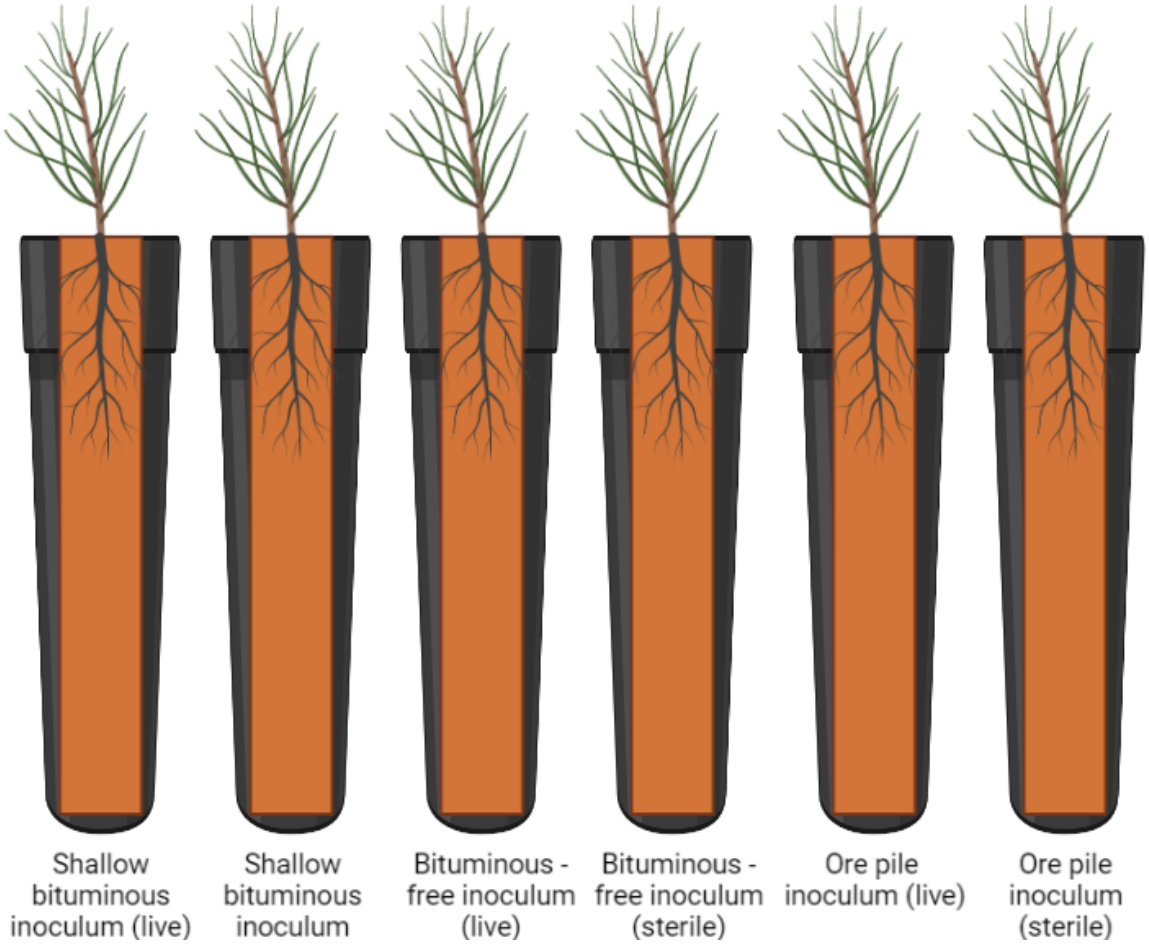

Figure S3.3 Experimental design of growth chamber experiment. Jack pine (*Pinus banksiana*) seedlings inoculated with soil inoculum (live or sterile) collected from shallow bituminous, bituminous-free, or the abandoned ore pile locations. Seedlings were grown in five different substrate types they may encounter in reclamation (bituminous-free shown here).

Appendix 4.1

Table S4.1 Hydrocarbon concentrations of lean oil sand material collected from Aurora North Mine Site, Alberta, Canada. Hydrocarbon fractions 2–4 represent F2 (>nC10 to nC16), F3 (>nC16 to nC34) and, F4 (nC35 to nC50+) (n = 3) (Turle et al., 2007).

F2 mg kg^{-1}	F3 mg kg^{-1}	F4 mg kg^{-1}	F4G+ mg kg^{-1}	F Total mg kg^{-1}
5667	36667	16000	69000	127333

Table S4.2 Two-way mixed ANOVA table showing the response of jack pine (*Pinus banksiana*) root length to the presence of water in the microcosm (Water type) and Time. Degrees of freedom of the numerator [DFn], Degrees of freedom of the numerator [DFd], *F*-statistic, and *P*-value are shown, *P-value* of the test of sphericity [W p value], *P*-value of the term after the Greenhouse-Geisser correction has been applied p [GG].

Term	DFn	DFd	F statistic	p value	W p value	p [GG]
Water	1	28	4.288	**0.048**	-	-
Time	2	56	61.441	**<0.0001**	**0.0006**	**<0.0001**
Water × Time	2	56	5.309	**0.008**	**0.0006**	**0.016**

Table S4.3 BLAST sequence similarity of the ITS PCR products obtained from cultured fungi. The top ten sequences represented by their accession numbers from National Centre for Biotechnology Information (NCBI) with the greatest bit score with over >97% identity are shown.

Cultured fungi	Bit score rank	Phylum	Query length	BLAST description	E-value	Percent query coverage	Percent Identity	Accession
Cenococcum geophilum	1	Ascomycota	571	Uncultured fungus clone 10 18S ribosomal RNA gene	0	94.07%	100.00%	KJ938039
	2	Ascomycota	571	Uncultured fungus clone otu28_gjp_network2016 small subunit ribosomal RNA gene	0	94.07%	99.80%	KX498057
	3	Ascomycota	571	Cenococcum geophilum isolate FFP888 18S ribosomal RNA gene	0	94.07%	99.80%	JQ711896
	4	Ascomycota	571	Cenococcum geophilum isolate FFP820 18S ribosomal RNA gene	0	94.07%	99.80%	JQ711879

5	Ascomycota	571	Cenococcum geophilum isolate FFP528 18S ribosomal RNA gene	0	94.07%	99.80%	JQ711854
6	Ascomycota	571	Cenococcum geophilum isolate FFP481 18S ribosomal RNA gene	0	94.07%	99.80%	JQ711848
7	Ascomycota	571	Uncultured Cenococcum clone FON_b07 internal transcribed spacer 1	0	94.07%	99.80%	HM488458
8	Ascomycota	571	Uncultured Cenococcum clone UBCOFE383A 18S ribosomal RNA gene	0	94.07%	99.80%	GU452521

	9	Ascomycota	571	Cenococcum geophilum isolate FFP758 18S ribosomal RNA gene	0	94.07%	99.70%	JQ711872
	10	Ascomycota	571	Cenococcum geophilum isolate FFP584 18S ribosomal RNA gene	0	94.07%	99.60%	JQ711861
Coltricia confluens	1	Basidiomycota	795	Coltricia confluens isolate RA717-1 internal transcribed spacer 1	0	100.00%	98.60%	MK217436
	2	Basidiomycota	795	Uncultured fungus clone R98 18S ribosomal RNA gene	0	100.00%	98.60%	JQ975954
	3	Basidiomycota	795	Coltricia confluens ITS1, 5.8S rRNA gene	0	100.00%	98.50%	AM412241

4	Basidiomycota	781	Coltricia confluens voucher GO-2009-444 18S ribosomal RNA gene	0	100.00%	97.50%	KC152085
5	Basidiomycota	784	Coltricia confluens voucher MushroomObserver.org/236441 internal transcribed spacer 1	0	98.62%	98.30%	MG966154
6	Basidiomycota	797	Uncultured Coltricia clone UBCOFE799B internal transcribed spacer 1	0	98.24%	98.70%	GU452515
7	Basidiomycota	784	Coltricia aff. confluens isolate Fire Survey 578 voucher TENN-F-072287 internal transcribed spacer 1	0	100.00%	96.50%	MN121008

	8	Basidiomycota	795	Coltricia confluens voucher GO-2009-008 18S ribosomal RNA gene	0	98.24%	97.30%	KC152083
	9	Basidiomycota	783	Coltricia confluens voucher GO-2009-483 18S ribosomal RNA gene	0	98.11%	97.10%	KC152084
	10	Basidiomycota	812	Coltricia perennis strain P. Salo 10282 internal transcribed spacer 1	0	99.87%	94.80%	MF319057
Laccaria bicolor	1	Basidiomycota	537	Laccaria bicolor strain ATCC MYA-4686 18S ribosomal RNA gene	0	100.00%	99.80%	KC881087
	2	Basidiomycota	537	Laccaria laccata voucher DAVFP 28143 18S ribosomal RNA gene	0	100.00%	99.80%	HQ650753

3	Basidiomycota	537	Laccaria bicolor isolate OUC97014 18S ribosomal RNA gene	0	100.00%	99.80%	DQ367906
4	Basidiomycota	537	Uncultured Laccaria clone 54E 18S ribosomal RNA gene	0	100.00%	99.60%	KP403076
5	Basidiomycota	537	Laccaria bicolor isolate AWW585 18S ribosomal RNA gene	0	100.00%	99.60%	JX504111
6	Basidiomycota	535	Laccaria bicolor isolate S238 18S ribosomal RNA gene, partial sequence	0	99.63%	99.60%	DQ179123
7	Basidiomycota	538	Laccaria bicolor isolate UBCOGTR0455s 18S ribosomal RNA gene	0	100.00%	99.30%	EU597085

	8	Basidiomycota	533	Laccaria cf. proxima KGP74 type OTU: KGP74 18S ribosomal RNA gene	0	99.26%	99.60%	DQ822818
	9	Basidiomycota	533	Laccaria laccata 18S ribosomal RNA gene, partial sequence	0	99.26%	99.40%	AF204814
	10	Basidiomycota	539	Laccaria bicolor strain CBS 594.89 small subunit ribosomal RNA gene	0	100.00%	99.10%	MH862188
Nectria mauritiicola	1	Ascomycota	616	Nectria mauritiicola 18S rRNA gene (partial)	0	93.05%	99.90%	AJ557830
	2	Ascomycota	616	Corallomycetella repens genomic DNA sequence contains 18S rRNA gene	0	93.05%	99.90%	LT576166

3	Ascomycota	616	Uncultured soil fungus clone LMRF_13 18S ribosomal RNA gene	0	93.05%	99.90%	EU826887
4	Ascomycota	616	Sarocladium kiliense strain 02499 internal transcribed spacer 1	0	93.05%	99.70%	KT878333
5	Ascomycota	616	Sarocladium kiliense strain 01915 internal transcribed spacer 1	0	93.05%	99.70%	KT878329
6	Ascomycota	616	Sarocladium kiliense strain 01914 internal transcribed spacer 1	0	93.05%	99.70%	KT878328
7	Ascomycota	616	Uncultured fungus clone D0-30d_22 18S ribosomal RNA gene, partial sequence	0	93.05%	99.70%	GU370762

	8	Ascomycota	616	Uncultured fungus clone D0-30a_32 18S ribosomal RNA gene	0	93.05%	99.70%	GU370755
	9	Ascomycota	616	Acremonium sp. 11665 DLW-2010 18S ribosomal RNA gene	0	93.05%	99.70%	GQ867783
	10	Ascomycota	613	Uncultured Nectriaceae 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA	0	92.60%	99.80%	FN689680
Phellinus tremulae	1	Basidiomycota	678	Phellinus tremulae isolate FP-135820-T 18S ribosomal RNA gene	0	90.16%	99.30%	KU139136
	2	Basidiomycota	678	Phellinus tremulae isolate A-17 18S ribosomal RNA gene	0	90.16%	99.00%	KU139137

Rhizopogon pseudoroseolus	1	Basidiomycota	440	Rhizopogon pseudoroseolus isolate 29_DZR2_ITS4B small subunit ribosomal RNA gene	0	100.00%	99.30%	MN737853
	2	Basidiomycota	439	Uncultured fungus isolate OTU10 small subunit ribosomal RNA gene	0	100.00%	99.30%	MH411697
	3	Basidiomycota	440	Rhizopogon pseudoroseolus strain PBM4128 small subunit ribosomal RNA gene	0	100.00%	99.30%	MG773823
	4	Basidiomycota	440	Rhizopogon pseudoroseolus strain RAS253 small subunit ribosomal RNA gene	0	100.00%	99.30%	MG773821

5	Basidiomycota	440	Rhizopogon pseudoroseolus strain WMH0013 (TENN) small subunit ribosomal RNA gene	0	100.00%	99.30%	MF773618
6	Basidiomycota	439	Uncultured fungus clone DA10179H11 18S ribosomal RNA gene	0	100.00%	99.30%	KM596885
7	Basidiomycota	439	Uncultured fungus clone DA10179E1 18S ribosomal RNA gene	0	100.00%	99.30%	KM596879
8	Basidiomycota	440	Uncultured fungus clone 4 18S ribosomal RNA gene, partial sequence	0	100.00%	99.30%	KJ938033

	9	Basidiomycota	439	Rhizopogon pseudoroseolus voucher K98C31T213 18S ribosomal RNA gene	0	100.00%	99.30%	GQ267483
	10	Basidiomycota	437	Uncultured fungus clone 001C8 18S ribosomal RNA gene	0	99.54%	99.30%	KM596882
Suillus brevipes	1	Basidiomycota	735	Suillus sp. RT-2012 isolate FFP350 18S ribosomal RNA gene	0	97.96%	99.60%	JQ711787
	2	Basidiomycota	735	Suillus luteus isolate FFP1046 18S ribosomal RNA gene	0	97.96%	99.30%	JQ711923
	3	Basidiomycota	735	Suillus brevipes voucher SMI330 18S ribosomal RNA gene	0	97.96%	99.30%	FJ845440

4	Basidiomycota	723	Uncultured Suillus clone LTSP_EUKA_P6A22 18S ribosomal RNA	0	96.33%	99.90%	FJ554247
5	Basidiomycota	723	Uncultured Suillus clone LTSP_EUKA_P4L18 18S ribosomal RNA	0	96.33%	99.90%	FJ553861
6	Basidiomycota	723	Uncultured Suillus clone LTSP_EUKA_P4I14 18S ribosomal RNA	0	96.33%	99.90%	FJ553799
7	Basidiomycota	723	Uncultured Suillus clone LTSP_EUKA_P2E19 18S ribosomal RNA	0	96.33%	99.90%	FJ553096
8	Basidiomycota	723	Uncultured Suillus clone LTSP_EUKA_P2A14 18S ribosomal RNA	0	96.33%	99.90%	FJ553013

	9	Basidiomycota	735	Suillus brevipes voucher MQ18R031-QFB30114 small subunit ribosomal RNA gene	0	96.87%	99.60%	MN992275
	10	Basidiomycota	723	Uncultured Suillus clone LTSP_EUKA_P6H07 18S ribosomal RNA	0	96.33%	99.70%	FJ554330
Suillus tomentosus	1	Basidiomycota	471	Suillus tomentosus voucher TRTC156486 internal transcribed spacer 1	0	100.00%	99.60%	JN021100
	2	Basidiomycota	471	Uncultured fungus clone 340D small subunit ribosomal RNA gene	0	100.00%	99.20%	MT812274

	3	Basidiomycota	472	Uncultured fungus clone otu2_ns_esb2015 small subunit ribosomal RNA gene	0	100.00%	99.20%	MG754914
Wilcoxina mikolae	1	Ascomycota	635	Uncultured fungus clone 6 18S ribosomal RNA gene, partial sequence	0	95.20%	100.00%	KJ938035
	2	Ascomycota	626	Uncultured Wilcoxina clone P1_Contig_0295 18S ribosomal RNA gene	0	93.85%	100.00%	JN704821
	3	Ascomycota	635	Uncultured fungus isolate OTU4 small subunit ribosomal RNA gene	0	95.20%	99.10%	MH411691

4	Ascomycota	636	Wilcoxina mikolae voucher K04C38T193 18S ribosomal RNA gene	0	95.20%	99.40%	GQ267499
5	Ascomycota	635	Uncultured fungus clone EB18 18S ribosomal RNA gene	0	95.20%	99.40%	GQ205367
6	Ascomycota	642	Uncultured fungus clone otu26_ns_esb2015 small subunit ribosomal RNA gene	0	95.20%	98.90%	MG754938
7	Ascomycota	627	Uncultured Wilcoxina clone R3389 18S ribosomal RNA gene	0	93.85%	99.40%	FJ786638
8	Ascomycota	624	Uncultured Wilcoxina clone OT-65 18S ribosomal RNA gene	0	93.55%	99.40%	FJ013051

9	Ascomycota	611	Uncultured Wilcoxina clone 1214-717 18S ribosomal RNA gene	0	91.60%	100.00%	HM146893
10	Ascomycota	617	Uncultured Wilcoxina clone 3D 18S ribosomal RNA gene, partial sequence	0	92.50%	99.50%	KU245941

Table S4.4 Two-way ANOVA table showing the response of jack pine (*Pinus banksiana*) shoot biomass (ln transformed) to agar plate type (Plate type) and the inoculum type (Inoc type). Sums of squares [SS], degrees of freedom [df], *F*-statistic, and *P*-value are shown).

Term	df	SS	*F*	*P*
Plate type	3	5.99	7.433	**0.0001**
Inoc type	2	5.97	11.116	**<0.0001**
Plate type × Inoc type	6	0.72	0.448	0.846
Error	153	41.08		

Table S4.5 Three-way mixed ANOVA table showing the response of jack pine (*Pinus banksiana*) root length to the inoculum type (Inoc type), Agar plate type (Plate type), and Time. Degrees of freedom of the numerator [DFn], Degrees of freedom of the numerator [DFd], *F*-statistic, and *P*-value are shown, *P-value* of the test of sphericity [W p value], *P*-value of the term after the Greenhouse-Geisser correction has been applied p [GG].

Term	DFn	DFd	F statistic	p value	W p value	p [GG]
Inoc type	2	153	7.332	**<0.0001**	-	-
Plate type	3	153	77.436	**<0.0001**	-	-
Time	4	612	355.24	**<0.0001**	**<0.0001**	**<0.0001**
Inoc type × Plate type	6	153	4.305	**<0.0001**	-	-
Inoc type × Time	8	612	8.472	**<0.0001**	**<0.0001**	**<0.0001**
Plate type × Time	12	612	58.342	**<0.0001**	**<0.0001**	**<0.0001**
Inoc type × Plate type × Time	24	612	7.065	**<0.0001**	**<0.0001**	**<0.0001**

Table S4.6 Three-way mixed ANOVA table showing the response of *Cenococcum geophillum* surface area to the presence of jack pine (*Pinus banksiana*) (Pine), Agar plate type (Plate type), and Time. Degrees of freedom of the numerator [DFn], Degrees of freedom of the numerator [DFd], *F*-statistic, and *P*-value are shown, *P-value* of the test of sphericity [W p value], *P*-value of the term after the Greenhouse-Geisser correction has been applied p [GG].

Term	DFn	DFd	F statistic	p value	W p value	p [GG]
Pine	1	108	48.719	**<0.0001**	-	-
Plate type	3	108	319.545	**<0.0001**	-	-
Time	4	432	6236.736	**<0.0001**	**<0.0001**	**<0.0001**
Pine × Plate type	3	108	6.915	**<0.0001**	-	-
Pine × Time	4	432	36.286	**<0.0001**	**<0.0001**	**<0.0001**
Plate type × Time	12	432	224.064	**<0.0001**	**<0.0001**	**<0.0001**
Pine × Plate type × Time	12	432	12.146	**<0.0001**	**<0.0001**	**<0.0001**

Table S4.7 Three-way mixed ANOVA table showing the response of *Suillus tomentosus* surface area to the presence of jack pine (*Pinus banksiana*) (Pine), Agar plate type (Plate type), and Time. Degrees of freedom of the numerator [DFn], Degrees of freedom of the numerator [DFd], *F*-statistic, and *P*-value are shown, *P-value* of the test of sphericity [W p value], *P*-value of the term after the Greenhouse-Geisser correction has been applied p [GG].

Term	DFn	DFd	F statistic	p value	W p value	p [GG]
Pine	1	101	0.0.294	0.589	-	-
Plate type	3	101	8.788	**<0.0001**	-	-
Time	4	404	1938.033	**<0.0001**	**<0.0001**	**<0.0001**
Pine × Plate type	3	101	5.076	**0.005**	-	-
Pine × Time	4	404	1.525	0.194	**<0.0001**	0.222
Plate type × Time	12	404	5.415	**<0.0001**	**<0.0001**	**<0.0001**
Pine × Plate type × Time	12	404	5.251	**<0.0001**	**<0.0001**	**0.001**

Table S4.8 Three-way mixed ANOVA table showing the response of fungal surface area by fungal species (Fungal species), Agar plate type (Plate type), and Time. Degrees of freedom of the numerator [DFn], Degrees of freedom of the numerator [DFd], *F*-statistic, and *P*-value are shown, *P-value* of the test of sphericity [W p value], *P*-value of the term after the Huynh-Feldt correction has been applied p [HF].

Term	DFn	DFd	F statistic	p value	W p value	p [HF]
Plate type	1	340	403.939	**<0.0001**	-	-
Fungal species	8	340	518.465	**<0.0001**	-	-
Time	2	680	5112.304	**<0.0001**	**0.001**	**<0.0001**
Plate type × Fungal species	8	340	74.698	**<0.0001**	-	-
Plate type × Time	2	680	231.607	**<0.0001**	**0.001**	**<0.0001**
Fungal species × Time	16	680	280.194	**<0.0001**	**0.001**	**<0.0001**
Plate type × Fungal species × Time	16	680	46.932	**<0.0001**	**0.001**	**<0.0001**

Table S4.9 Two-way ANOVA table showing the response of fungal surface area (box-cox transformed 0.57) to agar plate type (Plate type) and fungal species (Fungal species) on day 40. Degrees of freedom [df], Sums of squares [SS], *F*-statistic, and *P*-value are shown).

Term	df	SS	*F*	*P*
Plate type	1	271	9.996	**0.002**
Fungal species	3	4525	55.565	**<0.0001**
Plate type × Fungal species	3	1845	22.653	**<0.0001**
Error	150	4072		

Table S4.10 Two-way ANOVA table showing the response of fungal melanin (Ln transformed) to agar plate type (Plate type) and fungal species (Fungal species). Degrees of freedom [df], Sums of squares [SS], *F*-statistic, and *P*-value are shown).

Term	df	SS	F	P
Plate type	1	3.79	79.431	<0.0001
Fungal species	8	30.99	81.22	<0.0001
Plate type × Fungal species	8	2.69	7.059	<0.0001
Error	54	2.58		

Figure S4.1 Examples of various combinations of jack pine (*Pinus banksiana*) microcosm plates. Examples include (A) Pine seedling with no fungi grown on agar mixed with lean oil sand with a total concentration of 1.5% hydrocarbons. (B) Pine seedling with no fungi grown agar with glass plate. (C) Pine seedling on agar plate inoculated with *Cenococcum geophillum.* (D) Pine seedling on an agar mixed with lean oil sand with a total concentration of 0.5% hydrocarbons inoculated with *Suillus tomentosus*.

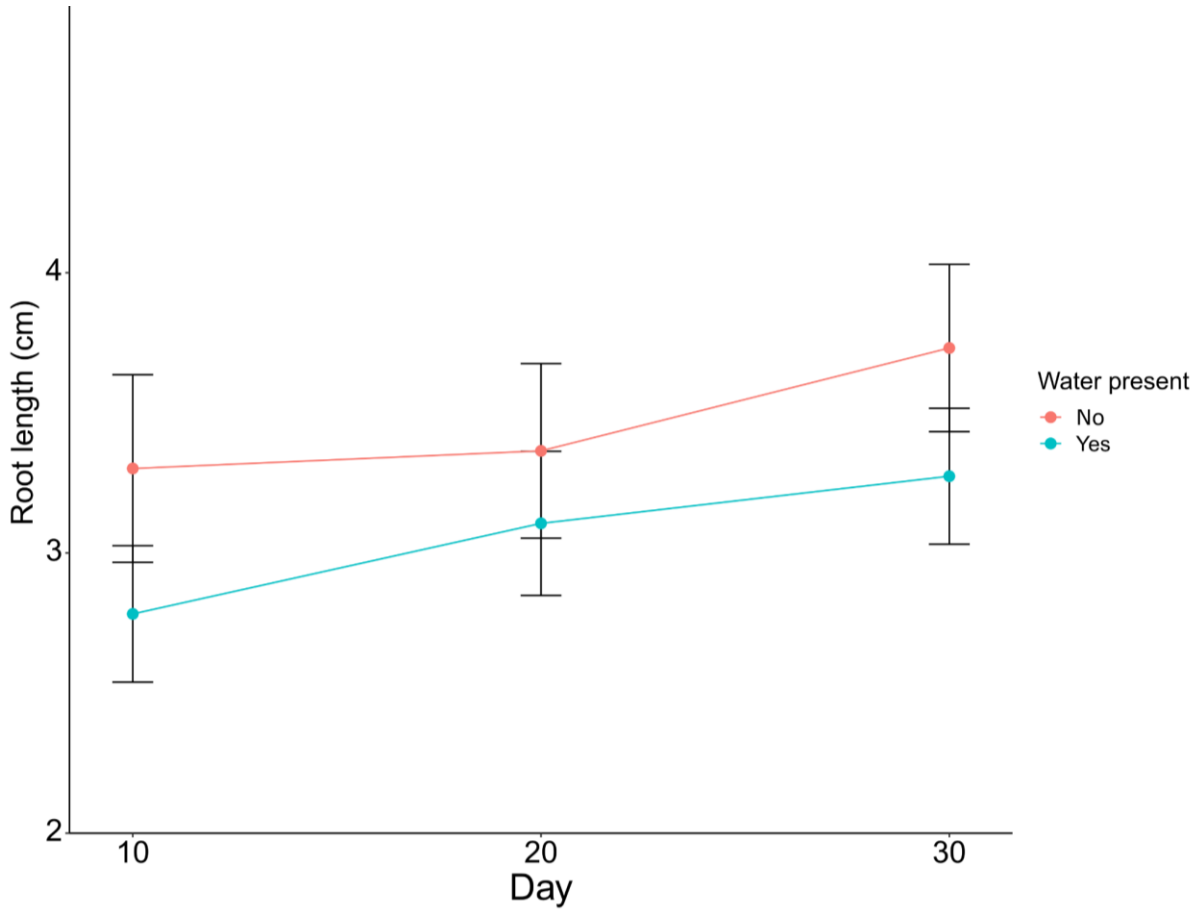

Figure S4.2 Mean root length (± 95% CI) of jack pine (*Pinus banksiana*) seedlings grown without (pink) or with (blue) standing water at the bottom of the microcosm plate. (n = 15).

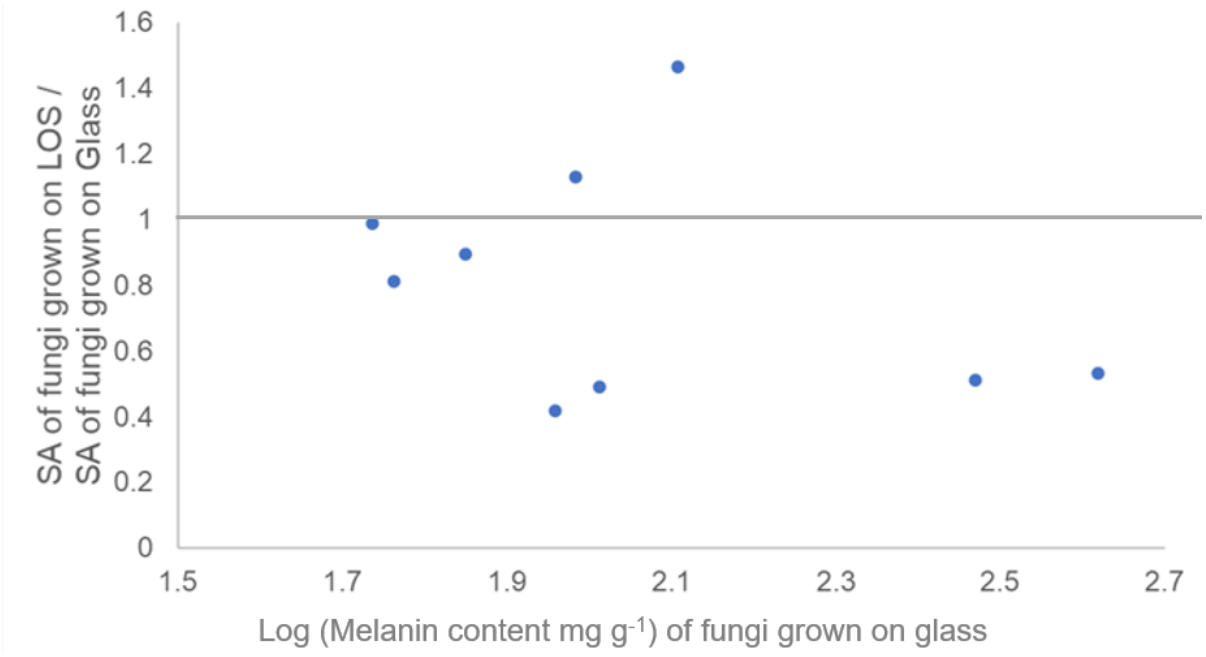

Figure S4.3 Scatter plot of fungal surface area ratio (fungi grown on plates containing lean oil sand (1.5%) over fungi grown on glass plates) vs log melanin concentration of fungi grown on glass plates. Each data point represents one fungal species.

Calculation of hydrocarbon concentration for microcosm plates

The sterilized lean oil sand was ground with a mortar and pestle and passed through a 1 mm sieve. This material had a bulk density of 1 g ml^{-1}. The concentration of hydrocarbons in this material was 127,333 mg kg^{-1}or mg l^{-1}. Using the $C_1V_1 = C_2V_2$ calculation with a final hydrocarbon concentration of 15,000 mg/l and a final volume of 50 mL, the amount of LOS needed to be added to each microcosm plate to have a final hydrocarbon concentration of 1.5% and 0.5% was calculated to be 5.89 grams and 1.96 grams of LOS, respectively per plate.

Testing root growth with free standing water

Previous research showed that LOS is a hydrophobic material (Neil and Si, 2019). As such, to determine if the hydrophobicity of the LOS pushed water out of the agar, I grew *Pinus banksiana* seedlings in 15 microcosms filled with MMN agar. Two and a half millilitres of sterile DI H_2O were placed into each microcosm. When stood vertically, this created a pool of water at the bottom of each microcosm. These microcosms were grown in the same growth cabinet under the same conditions for 30 days days. As before, to assess the growth rate of pine roots, plates were scanned every 10 days and measured using ImageJ (see Methods in Chapter four).

I compared the growth of pine roots grown on agar with or without free standing water on days 10-30. To evaluate how root length responded to the presence of water, I ran a two-way mixed ANOVA. Time was the within factor and the presence of water was the between factor.