The Role of Nutrient and Carbon Reserve Status of Aspen Seedlings in Root-Soil Interactions

by

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Abstract

The boreal forest is one of the largest forest ecosystems in the world, covering 14.7 million km^2 globally. The Canadian boreal forest has a wealth of natural resources, including coal, timber, and oil; as resource exploration and exploitation has expanded, anthropogenic disturbance in the boreal forest has increased as well. After resource extraction, Alberta regulations mandate that disturbed land be restored to ecosystems of 'equivalent productive capability'. Given the severity of such landscape-level disturbances, restoration can be a challenge. Towards improving boreal forest restoration, I examined the influence of aspen seedling nutrient and carbon reserve factors on the community development of important belowground mutualists, ectomycorrhizal fungi (EMF), in a reclamation context. Specifically, I examined (1) the ectomycorrhizal community composition on two aspen seedling (*Populus tremuloides* Michx.) feed types (high and standard), which had differing tissue carbon storage and nutrient levels, planted into two types of cover soils, a reclaimed forest floor and a peat-mineral soil mix. I also investigated (2) the relationship between carbon storage and the release of carbon compounds from the roots when exposed to environmental stressors common in the boreal forest; these compounds (root exudates) are important for the development of the ectomycorrhizal symbiosis. I found that (1) the abundance of EMF was influenced by feed type but not cover soil type, with high feed seedlings having increased colonization by EMF than standard feed seedlings. However, cover soil type influenced the abundance of one EMF species at the site. I also found that (2) exudation was determined by the concentration of C reserves when seedlings were exposed to the same environmental conditions; when exposed to different conditions, factors specific to each stress had a greater influence on exudation. Based on these results, I propose that seedlings should undergo nutrient loading prior to outplanting to improve EMF recovery and that the mechanism behind increased EMF colonization of high feed seedlings may be related to exudation.

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List of Abbreviations

ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
C	Carbon
DM	dry mass
ECM	Ectomycorrhizal
EMF	Ectomycorrhizal fungi
FFM	Forest floor material
K	Potassium
LMW	Low molecular weight
N	Nitrogen
NL	Normalized label
NSC	Non-structural carbohydrate
OTU	Operational taxonomic unit
P	Phosphorous
PAR	Photosynthetic active radiation
PCR	Polymerase chain reaction
PPM	Peat mineral mix
RSR	Root to shoot ratio
S	Sulfur
TOC	Total organic carbon
VPDB	Vienna Pee Dee Belemnite

Chapter 1: General Introduction

1.1 Anthropogenic disturbance and reclamation in the boreal forest

The boreal forest is one of the largest biomes in the world, comprising 14.7 million km^2 in a near-continuous belt across most of North America, Europe, and Asia (Bonan and Shugart 1989). The boreal forest dominates the landscape in northern Canada, encompassing 3.5 million km^2 as part of the larger boreal zone, which also includes lakes, rivers, heathlands, wetlands, alpine areas, and grasslands of the high northern latitudes (Brandt 2009). The Canadian boreal forest is dominated by cold-tolerant coniferous tree-species such as pines (*Pinus*), spruce (*Picea*), and fir (*Abies*), as well as deciduous trees such as aspen (*Populus*) or birch (*Betula*). Winters in the region are characteristically long and cold, with up to six months spent below freezing; summers are typically short and warm, with the growing season averaging only 50-100 days. The soils in the Canadian boreal forest originated from primarily glacial or post-glacial deposits; soils present on fine or medium textured parent materials are Gray Luvisols, whereas soils present on coarse-textured parent materials are Brunisols or Podzols (Macdonald et al. 2012). As a disturbance-driven ecosystem, the boreal forest experiences natural disturbances such as wildfires, disease, and insect outbreaks, which interact and contribute to landscape heterogeneity (Brandt et al. 2013).

Disturbance due to anthropogenic activity in the boreal forest has typically been limited due to the severity of the winters and difficulty of the terrain (Kurz et al. 2008). However, the development of natural resource exploitation such as timber harvesting, coal mining, and oil extraction has dramatically increased anthropogenic disturbances in Canada's boreal zone. The timber industry alone disturbs approximately 9,700 km^2 per year, 0.28% of Canada's boreal

forest (Masek et al. 2011). There are currently 99 active metal and mineral mines as well as nine coal mines in the Canadian boreal zone, as well as at least 1,300 former mines and mining exploration sites, not all of which have been reclaimed (Brandt et al. 2013). The world's third largest reserve of oil is located in Alberta, Canada, underlying 142,000 km^2 of a total 381,000 km^2 of Alberta's boreal forest. As of 2012, 844 km^2 of boreal forest has been disturbed due to oil sands mining activities. Annual disturbance of boreal forest in Alberta for oil sands mining is expanding, with an estimated 38 km^2 disturbed annually in 2012, increasing to 68 km^2 disturbed annually by 2022 (Grant et al. 2013).

Alberta environmental law requires that land disturbed for mining be restored to ecosystems of equivalent land capability according to the Land Surface Conservation and Reclamation Act of 1973 and the Environmental Protection and Enhancement Act of 1992 (Government of Alberta 2013). Simply put, the goal of boreal forest restoration is to create ecosystems comprised of native tree and understory plant species that are both self-sustaining and resilient to disturbances. Surface mining is a particularly severe disturbance, as it requires the removal of all vegetation, organic soil, and inorganic subsoil layers in order to gain access to the resource. After the deposits have been mined, sites undergo reclamation to reshape the landform and restore the disturbed land to its previous productivity (Alberta Environment and Natural Resources 2013). In brief, the reclamation process begins with subsoil being returned to a mined site to form the landscape. As subsoil is low in organic material and prone to erosion, it is capped with a layer of organic cover soil (referred to as the capping material), often consisting of salvaged upland forest floor (including the L, F, H, and A horizons) or lowland peat material. The type and depth of capping materials placed varies according to the availability of materials as well as site characteristics; however, a depth of 10-100 cm is typically placed. Once a site has

been capped, vegetation is planted at the site, usually in the form of tree seedlings, with species chosen according to the target ecosite and end land use (Alberta Environment 2010).

Tree seedlings planted into or naturally established on reclaimed mine sites may be exposed to a diverse array of harsh conditions which can negatively impact their growth, cause seedling death, and ultimately delay stand development. Soils can impose a variety of stressors on the seedlings, as soils used in reclamation are often less suitable for the establishment of vegetation than undisturbed forest soils (Bussler et al. 1984). Soils in reclaimed areas often have low nutrient availability, which can inhibit the growth of vegetation as well as the development of the soil microbial community (Andersen et al. 1989). Other possible factors may be altered in soils of highly disturbed areas, including water availability, salinity, pH, and compaction, which may also contribute to seedling stress in reclaimed sites (Dimitriu et al. 2010). Moreover, biotic factors such as competition or herbivory can also hinder seedling growth and development (Elmarsdottir et al. 2003; Rizza 2007).

Amendments such as fertilization or the planting of a cover crop can be applied to the soils to mitigate some of the stress to which seedlings are exposed; however, such amendments are expensive to broadcast over large areas and are often ineffective (Casselman et al. 2006). A promising treatment for improving the ability of seedlings to withstand environmental stressors is through nutrient loading prior to being planted in disturbed areas (Schott et al. 2013). Nutrient loading is the process of providing seedlings with nutrients above what is needed for growth but below levels where they become toxic. This has been shown to increase initial seedling performance on reclamation sites (Hu et al. 2014; Schott et al. submitted). However, the effects of nutrient loading upon the interaction between seedlings and their surroundings (particularly soil biota) remain unclear.

1.2 Mycorrhizal fungi

Restoring functional interactions between above (vegetation) and belowground (soil biota) components promotes the creation of self-sustaining forest ecosystems following severe landscape level disturbances such as mining (Macdonald et al. 2012). A mycorrhiza is a symbiosis between a plant and a fungus functionally connecting roots with soils, and is necessary for the growth and survival of most boreal plant species (Smith and Read 2008). The plant provides photosynthetically-derived carbon to the fungus for supporting metabolic activities including growth; in exchange, the fungus transfers nutrients (N and P), acquired by emanating hyphae throughout the surrounding soil, back to the plant. There are two types of mycorrhiza commonly found in the boreal zone, arbuscular mycorrhizas and ectomycorrhizas. Arbuscular mycorrhizas are common in boreal herb and a few tree species; ectomycorrhizas are more common in boreal tree species, including members of the Pinaceae family as well as the Salicaceae family, including trembling aspen (*Populus tremuloides*). In the boreal forest, relationships with ectomycorrhizal fungi (EMF) for nutrient acquisition by the host are particularly important because low rates of decomposition due to cooler temperatures leads to nutrient limitations, particularly of nitrogen (Read et al. 2004). In addition to foraging for nutrients, ectomycorrhizal fungi provide other benefits to their plant hosts. Ectomycorrhizal fungi protect their hosts from a variety of pathogens and root-borne diseases (Newsham et al. 1995) and provide resistance to drought (Shi et al. 2002), herbivory (Gehring et al. 1997), acidity, excess soil contaminants (such as aluminum) (Lux and Cumming 2001), and salinity (Onwuchekwa et al. 2014) . The ectomycorrhizal community may be an important component in boreal forest restoration efforts, as the presence of ECM fungi has been shown to mitigate stress

and to improve tree seedling survival and growth following landscape-level disturbances (Amaranthus and Perry 1987; Bauman et al. 2011; Sousa et al. 2014).

The diversity of the ectomycorrhizal community is an important factor in stress mitigation for the host. The community of EMF typically present on a single mature tree host is diverse; Bahram et al. (2011) found over one hundred species of EMF on an individual host tree. The diversity of ectomycorrhizal fungal species colonizing a host is linked with benefits to plant host performance. Ectomycorrhizal fungal species differ in their abilities to take up nutrients such as phosphorous (Dighton et al. 1993) or ammonium (Jongbloed et al. 1991) due to differential expression of degradative enzymes (Baxter and Dighton 2005); thus, EMF vary in their capabilities to utilize organic nutrients, which suggests that diversity of the ectomycorrhizal community may increase growth and nutrition in the plant host (Reddy and Natarajan 1997; Baxter and Dighton 2001; Diagne et al. 2013). However, ectomycorrhizal fungal diversity in areas following large-scale disturbances is often lower than undisturbed areas (Iordache et al. 2009). Furthermore, common reclamation practices such as the stockpiling of soils can further inhibit the ectomycorrhizal community by decreasing the fungal inoculum potential (Bois et al. 2005).

Fungal abundance and diversity in reclaimed areas is dependent upon a combination of biotic and abiotic site and soil characteristics. Ectomycorrhizal fungi are sensitive to soil characteristics, such as pH, salinity, and nutrient and water availability, which will lead to spatial heterogeneity in the mycorrhizal community due to differences in fungal physiology (Miller and Jastrow 2000; Jones et al. 2012). For example, *Cenococcum geophilum*, an EMF with an abundance of melanin, a polymer found in fungal cell walls that resists desiccation, is more resilient in areas which experience frequent drought stress than fungi with less melanin

(Fernandez and Koide 2013). In reclamation soils, both the soil characteristics as well as the plant community supported by the soil prior to salvaging will influence the fungal propagule bank of the reclamation soil and thus the EMF community composition (Schalamuk and Cabello 2009; O'Brien et al. 2011). The identity of the host will also affect the development of the mycorrhizal community, as many EMF exhibit strong preference for certain host species (Ishida et al. 2007, Hankin et al. in press). Plant hosts are able to preferentially allocate carbon to fungal symbionts which are more mutualistic, altering the mycorrhizal community for the benefit of the plant (Bever et al. 2009; Werner and Kiers 2015). Plants are also able to limit the establishment of symbioses when conditions for the symbiosis are unfavorable (Connell et al. 1996). A mechanism whereby a plant is able to exert control over ectomycorrhizal fungi is through the release of carbon-based compounds known as root exudates (Hartmann et al. 2009).

1.3 Root exudates

Plants release exudates into the rhizosphere, the region of soil directly encompassing the plant root, which is a hotspot of mycorrhizal and microbial activity (Hirsch et al. 2003). Root exudates are predominantly comprised of low molecular weight carbon compounds, such as sugars, organic acids, and amino acids (Uren 2007). The release of some exudate compounds utilizes ATP and is mediated by the plant; however, the principal mechanism by which exudation occurs is diffusion of low molecular weight compounds across the cell membrane due to electrochemical gradients (Farrar et al. 2003). Root exudates are integral in nearly all aspects of the mycorrhizal symbiosis, including the germination of fungal spores (Martin et al. 2007), the arbitration of the establishment of the symbiosis and formation of fungal structures (Miller and Oldroyd 2012), and the release of sugars to the mycorrhizal fungi, which is the basis for the

exchange of resources between host and symbiont (Jones et al. 2009). As can be seen in Appendix Figure 1, the release of exudate compounds, and consequently the outcome of biological interactions, is mediated by a combination of interacting exogenous and endogenous factors (Groleau-renaud et al. 1998; Jones et al. 2004).

Plants in the boreal forest are exposed to a variety of abiotic stressors, such as cold soil temperatures, light limitations, or periods of water limitation or excess; these stressors will alter the quantity and composition of exudates, though the effect is dependent on the magnitude and type of stress (Neumann and Romheld 2007). Soil temperature can affect passive exudation processes, as changes in temperature disrupt the permeability of cell membranes, leading to an increase in carbon exuded to the rhizosphere (Rovira 1959; Campos et al. 2003). Changes in light intensity such as those from competitive shading will also modify exudation, with decreases in the exudation of carbon compounds corresponding to decreases in light intensity (Hodge et al. 1997). Water availability will also influence exudation; drought can cause disruptions in membrane permeability or damage to the root cells from increased soil mechanical impedance, which leads to an increase in the amount of carbon compounds lost to the rhizosphere (Reid 1974; Reid and Mexal 1977).

Exudation processes are also affected by both soil nutrient availability and plant tissue nutrient levels. High availability of soil nutrients (such as nitrogen or phosphorous) has been shown to inhibit the exudation of carbon compounds, whereas low soil nutrient availability stimulates exudation (Ratnayake et al. 1978; Lu et al. 1999). The nutrient levels within host tissues also have a significant effect upon exudation, similar to that of soil nutrient availability: root exudation is stimulated when tissue nutrient levels are low, in part because plants increase the release of compounds which increase nutrient uptake (Wojtaszek et al. 1993; Schilling et al.

1998). Low tissue nutrient levels may also increase exudation to stimulate mycorrhizal establishment, which will in turn increase nutrient uptake (Grayston et al. 1996). Elevated tissue nutrient levels have been shown to inhibit the establishment of the symbiosis, though the mechanisms behind the inhibition are unknown (Wallenda and Kottke 1998).

The carbon status of the host plant also affects exudation. As the carbon status of the host is influenced by abiotic and biotic stressors, stressful environmental conditions may indirectly affect exudation through modifications to the carbon status of the plant. As recently-acquired photosynthates comprise a significant percentage of exudates, disruptions in carbon uptake, such as from shading or herbivory, will affect the amount of carbon exuded to the rhizosphere (Holland et al. 1996; Heinemeyer et al. 2006). This is further supported by the fact that exudation is severely inhibited when trees are girdled, thus preventing photoassimilates from reaching the roots (Hogberg et al. 2001; Schaefer et al. 2009). The amount of non-structural carbohydrates (NSC) stored in root tissues may also affect exudation. Non-structural carbohydrates are starches and sugars which have not been allocated to growth, cell respiration, defense, reproduction, or other carbon sinks, which is stored within plant cells and can be used for future growth or other plant functions (Chapin et al. 1990). As the passive diffusion of low molecular weight compounds (especially sugar) across the cell membrane is the main mechanism of exudation (Uren 2007), increased concentrations of stored carbon compounds should lead to an increase in diffusion into the rhizosphere. Furthermore, the amount of carbon stored in the roots may also influence root exudation, as root NSC reserve status has been shown to influence rhizosphere respiration (Kuzyakov and Domanski 2002; Xu et al. 2008).

1.4 Objectives

The first objective of this thesis was to determine the importance of aspen seedling nutrition, non-structural carbohydrate (NSC) reserves, and the cover soil type into which seedlings were planted on the development of the ectomycorrhizal community after reclamation. In Chapter 2, results are presented from a field study examining the effects two seedling stock types, nutrient loaded and standard greenhouse aspen seedlings, and two reclamation cover soils, peat-mineral mix (PMM) and reclaimed forest floor material (FFM), on the amount of colonization of ectomycorrhizal fungi (EMF) on aspen roots two years after being planted on a reclamation site. This was done using morphotyping followed by molecular techniques to identify the ectomycorrhizal fungal species on aspen roots and examining rates of ectomycorrhizal colonization on seedlings according to seedling nutrition and soil type.

The second objective was to investigate the influence of root NSC reserve status in determining the exudation of organic carbon when NSC reserves have been modified by different types of stress. In Chapter 3, results are presented from a growth chamber study examining the effects of three environmental stressors common in the boreal forest (cold soils, shading, and drought stress) on aspen seedling root NSC reserves and exudation. Morphological characteristics (height, root collar diameter, leaf area) and carbon reserve characteristics (non-structural sugar and starch concentrations) were compared with exudation rates across treatments. The influence of NSC reserves on the amount of carbon found in exudates was also examined.

In Chapter 4, the findings of the two studies are summarized and integrated, exploring the implications of the results on current boreal forest restoration practices and detailing possible limitations of the research as well as areas for future research needs.

Chapter 2: The role of seedling nutrient status on development of ectomycorrhizal fungal communities in two soil types following surface mining disturbance

2.1 Introduction

Restoring functional interactions between vegetation and soils is an important element to create self-sustaining ecosystems following landscape level disturbances (Macdonald et al. 2012). In the boreal forest, trees develop intimate associations with ectomycorrhizal (ECM) fungi; these symbioses affect tree survival and growth. Ectomycorrhizal hyphae extending from colonized roots are physical linkages that functionally connect tree roots to soils, where hosts supply the fungi with photosynthetically-derived sugars and the fungi provide water and soil-derived nutrients to their hosts. Communities of ECM fungi are highly diverse on micro-spatial scales, with multiple species often within centimeters of each other (Bruns 1995). Though the relationship between mycorrhizal species diversity and host plant productivity is often context-dependent (Jonsson et al. 2001), high mycorrhizal diversity has been demonstrated to increase nutrient uptake and seedling growth (Baxter and Dighton 2001; Velmala et al. 2014). During severe soil disturbances such as surface mining, vegetation, soils, and parent geological material are stripped to access resource deposits. Following mining, ECM associations must re-establish with planted seedlings. However, depending on the type and severity of disturbance, the diversity and abundance of the ECM fungal community is often much lower than in undisturbed areas (Read 1991; Kipfer et al. 2011). Restoring ECM fungal communities can be a challenge in heavily disturbed soils, which generally have a low ectomycorrhizal inoculum potential (Bois et al. 2005, Hankin et al. in press).

Seedling establishment is the first step towards the restoration of vegetation on heavily disturbed sites. On these sites seedlings are often exposed to stress such as poor nutrient

availability, drought, or mineral toxicity. Elevated nutrient (nitrogen, phosphorous, and potassium) and non-structural carbon reserves (sugar and starch) in the tissues of planted seedlings can increase seedling growth and nutrient acquisition in stressful conditions like those found in disturbed areas (Quoreshi and Timmer 2000a; Landhäusser et al. 2012, Schott et al. submitted). Although the higher reserves in seedlings are often only temporary, the improved growth performance persists beyond the presence of elevated tissue nutrient levels (Schott et al. submitted). What underlies this longer-term response is not clear; however, it could be driven by belowground interactions such as those formed between plants and mycorrhizal fungi. The effect of seedling nutrient and carbon reserve status on the establishment of mycorrhizal symbionts in disturbed sites has received little attention. Prior studies have shown that soil nutrient availability influences the relationship between mycorrhizal fungi and their host (Johnson et al. 1992; Johnson 1993); however, host nutrient and carbon reserve status may also influence the outcome of mycorrhizal interactions (Nylund 1988). For instance, Quoreshi and Timmer (1998, 2000b) found that nutrient loading (i.e., artificially increasing nutrient reserves) black spruce seedlings (*Picea mariana* [Mill.] BSP.) stimulated mycorrhizal formation during inoculation. Since a balance exists between internal plant carbon and nutrients which governs mycorrhizal symbioses (Johnson 2010), alterations to the nutrient levels in plant tissues may influence the development of the ECM symbiosis. High amounts of nutrients in the roots, particularly N, can inhibit the development of ECM fungi (Richards and Wilson 1963). As ECM fungi rely predominantly on their host for carbon, variation in carbon reserves in seedling roots may also influence the abundance of ECM fungi.

In addition to seedling physiology, soil characteristics may also influence the abundance and species composition of ECM fungi occurring at disturbed sites. Soils commonly used in

restoration of surface mines in the boreal forests are materials salvaged prior to mining: forest floor material (FFM), which is composed of the leaf litter layer plus a portion of the underlying mineral from upland forest sites, and peat material, which is salvaged from lowland peatlands and is often mixed with underlying mineral subsoil resulting in a peat-mineral mix (PMM). As can be expected these two materials differ greatly in soil structure and chemistry, nutrient availability, and their plant and fungal propagule bank, which reflects the plant and fungal communities present at the sites prior to salvage (McMillan et al. 2007; Dimitriu et al. 2010; Schott et al. submitted). Salvaged soils may differ in the ECM propagules they retain in addition to acting as different habitats suitable for certain ECM species, dependent upon fungal physiology, and thus lead to the development of dissimilarly structured ECM fungal communities.

The objective of this study is to characterize the respective influence of host internal plant nutritional status, and soil type on the establishment of ECM fungal communities in highly disturbed areas. Specifically, we assessed the influence of *Populus tremuloides* seedling nutrient and carbon reserve status on the early development of an ECM fungal community two years after planting and how the early ECM community was influenced by soil type (FFM and PMM). We hypothesized that seedlings with initially lower nutrient reserves would have greater ECM fungal abundance and species richness compared to seedlings with higher nutrient reserves because a lower nutrient status ought to lead to a greater necessity to develop and facilitate the establishment of the ECM symbiosis (Johnson et al. 1997). As *P. tremuloides* is an upland tree species, we expected a more abundant and diverse ECM community when seedlings were planted in FFM than in PPM.

2.2 Methods

2.2.1 Site description

The research area is located in the Central Mixedwood subregion of the Boreal Forest Natural Region (Natural Regions Committee 2006). Uplands of this region are typically dominated by white spruce (*Picea glauca* (Moench) Voss) and trembling aspen (*P. tremuloides* Michx.). The low lying areas are wetlands dominated by black spruce (*Picea mariana* (Mill.) Britton, Sterns & Poggenb.). The Central Mixedwood subregion endures long, cold winters and short, warm summers. Average daily temperatures range from -18.8 °C in January to 16.8 °C in July (Environment Canada 2013). Average annual rainfall and snow is 342 mm and 156 mm, respectively. The growing season (May to September) of our sample collection (2012) was near average with 350 mm of precipitation and a 12.7 °C average temperature (Environment Canada 2013).

2.2.2 Soil salvage and placement

The field site was located at a reclaimed overburden dump (>100 ha) 26 km north of Fort McMurray, Alberta, Canada. Over six months beginning August 2008 and prior to building the overburden structure, the surface soils were stripped and stockpiled by soil type (forest floor material and peat-mineral mix). Upland forest floor material (FFM) was comprised of a mixture of the top 30 cm and included the organic L, F, H, and the mineral A and a portion of the B soil horizons of a Gray Luvisol occurring under stands of white birch (*Betula papyrifera*), balsam poplar (*Populus balsamifera*), and/or trembling aspen (*Populus tremuloides*). Peat-mineral mix (PMM) was salvaged from lowland sites where the surface material was stripped to a depth of

roughly 30 cm, which included the transition/peat layer and mineral soil beneath it and was monitored to ensure a mix of 60:40 (volume:volume) peat to mineral soil. Mineral soils in this region are typically Gray Luvisols with the characteristic eluvial and Bt horizons. In 2009, after the soils had been stripped and stockpiled on-site for at least six months, the dump was filled with overburden material (sodic and/or saline) and the site was sloped with a height to volume ratio of six. In 2010, the construction of the overburden dump finished and 1 m of subsoil (low-sodic soil salvaged from the C-horizon from a depth of 60 cm to 300 cm) was placed across the surface. Capping soil was placed at a depth of 50 cm; placement commenced in August 2010 and was finished in June 2011. The study site was 1.5 ha in size; the salvaged and stockpiled FFM and PMM soil types were placed in alternating 20 m wide and 65 m long strips. A pair of FFM and PMM strips (40 m × 65 m) was considered an experimental block (n=5).

2.2.3 Tree seedling production and planting

Aspen seedlings were grown commercially from an open-pollinated seed source collected in the Fort McMurray (Alberta, Canada) region. Seedling growing conditions during nursery production are described in more detail in Schott et al. (2013). Briefly, seeds were sown into Styroblock containers (5-12A, 220 ml, Beaver Plastic, Edmonton, Alberta) and grown for a single growing season at Smoky Lake Nursery (Smoky Lake, Alberta, Canada). Once seedlings had reached an average height of 35 cm, they were assigned to two treatments: *high feed* and standard. Standard seedlings were grown under typical seedling production conditions and fertilized with a mixture of macro and chelated micronutrients (78 ppm N, 77 ppm P, 161 ppm K, 46 ppm S) while the high feed seedlings received double the amount of the same fertilizer (i.e., 156 ppm N, 154 ppm P, 322 ppm K, 92 ppm S, including chelated micronutrients).

Fertilization continued at these concentrations until early fall after which all fertilization ceased. Dormancy and hardening was induced by leaving seedlings outside and seedlings were lifted and stored frozen (-3ºC) once day temperatures were below freezing. Roots of seedlings were not examined for ectomycorrhizas prior to planting. Aspen seedlings were planted in early spring of 2011 in alternating rows of high feed and standard seedlings within each capping treatment and block. Seedlings were regularly spaced at 1.3 m (5,917 stems/ha). At the time of planting, high feed seedlings were the same size as standard seedlings; however, high feed seedlings had higher concentrations of root macronutrients (N and P) as well as higher root non-structural starches (Table 2-1). Seedling root:shoot ratios were similar between stock types. Total N was determined by the dumas combustion method using the Costech Model EA 4010 Elemental Analyzer (Costech International Strumatzione, Florence, Italy, 2003). Total P was determined by nitric acid digestion then colourimetry using the SmartChem Discrete Wet Chemistry Analyzer (Westco Scientific Ltd., Brookfield, CT, USA, 2007). Nonstructural carbohydrates were analyzed using a phenol-sulfuric acid assay for total sugar concentration and enzyme digestion for total starch concentration according to Chow and Landhäusser (2004)

2.2.4 Mycorrhizal root collection and identification

In 2012, after two growing seasons, a root section approximately 30-40 cm long was collected from five seedlings of each treatment combination in each block (total 100 seedlings). These sections were clearly outside of the initial root plug and would have been roots grown subsequent to planting. Prior to collection, roots were traced to their stem to ensure correct feed type was collected. Root samples were stored at -20°C until further processing. After thawing, adhering soil and debris was gently removed by lightly rinsing roots with tap water over a 0.4

mm sieve. The root systems were then cut into 1 cm fragments and mixed in water; a number of segments were then randomly selected such that between 150 and 200 root tips were examined under a dissecting microscope. Root tips were classified as either non-mycorrhizal or mycorrhizal, with mycorrhizal root tips being further classified into unique morphotypes based on presence of hyphae, mantle color and texture, and root tip thickness and shape (Tedersoo et al. 2003). Four representative samples of each morphotype per root sample were collected and separately placed into 0.2 µL microcentrifuge tubes for DNA-based identification (see below). The amount of ectomycorrhizal colonization was determined by dividing the number of living mycorrhizal root tips by the total number of living mycorrhizal and nonmycorrhizal root tips found in a subsample. Following examination, root systems were dried at 100° C for one hour to halt enzymatic breakdown of root carbohydrates, followed by at least 48 hours of drying at 70° C. Fine roots (<1mm) were separated from the root system and first ground using a mortar and pestle, followed by grinding for fifteen minutes in a micro-ball mill (MRC International, Holon, Israel) to ensure uniform particle size. The fine roots were then analyzed for total nitrogen (N), total phosphorous (P), and nonstructural carbohydrate (NSC) concentration; the methods for analyzing total N, P, and NSC concentrations were the same as those described in section 2.2.3. The leaves were also analyzed for foliar nitrogen, phosphorous, and potassium (K). Foliar N and P were determined using acid digestion then colourimetry using the SmartChem Discrete Wet Chemistry Analyzer (Westco Scientific Ltd., Brookfield, CT, USA, 2007). Foliar K was determined by atomic absorption using a SpectrAA 880 Atomic Absorption Spectrometer (Varian Australia Pty Ltd, Mulgrave, Victoria, AUS, 2002.)

2.2.5 Molecular confirmation of ectomycorrhizal morphotypes

Genomic DNA from mycorrhizal root tips was extracted using Sigma Extraction Buffer and Neutralization Solution B (Sigma Aldrich, Gillingham, Dorset, UK) according to the manufacturer's protocol. To confirm morphotype identities, twenty seedlings were selected at random, from which all extractions from each morphotype present were amplified using a nested polymerase chain reaction (PCR) using the fungal-specific nested primer combinations of NSA3/NLC2 and NSI1/NLB4 (Martin and Rygiewicz 2005). Reactions using the outer primers consisted of 8 µL of RedTAQ (Sigma, Gillingham, Dorset, UK), 5.4 µL autoclaved MilliQ water, and 0.8 µL each of 10 µM NSA3 and 10 µM NLC2 combined with 1 µL of template DNA in a 16 µL reaction. Amplifications were performed with an initial denaturation at 95 °C for five minutes, followed by 30 cycles of 95 °C for 90 seconds, 67 °C for 60 seconds, and 72 °C for 90 seconds, with a final extension of 72 °C for ten minutes. Reactions using the inner primers consisted of 8 µL of RedTAQ (Sigma, Gillingham, Dorset, UK), 5.4 µL autoclaved MilliQ water, and 0.8 µL each of 10 µM NSI1 and 10 µM NLB4 combined with 1 µL of PCR product from the other reaction in a 16 µL reaction. Amplifications were performed with an initial denaturation at 95 °C for five minutes, followed by 27 cycles of 95 °C for 90 seconds, 55 °C for 60 seconds, and 72 °C for 90 seconds, with a final extension of 72 °C for ten minutes. Success of the reaction was determined by gel electrophoresis using 1% agarose gel and GelRed gel stain (Biotium Inc., Hayward, California, USA) and successful PCR products were purified with ExoSAP-IT (USB, Cleveland, Ohio, USA). Bi-directional sequencing was conducted with BIGDYE v3.1 (Applied Biosystems, Foster City, California, USA) using the NLB4 and NSI1 primers, with 10 µl reactions containing 2 µl autoclaved milli-q water, 1.5 µl 5x buffer sequencing buffer, 1 µl BIGDYE, 0.5 µl of 10µM primer, and 5 µl of PCR product. The

resulting products were precipitated according to the manufacturer's instructions for EDTA/ethanol. Bi-directional sequences were analyzed on an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Sequences were edited with Geneious software (Biomatters, Auckland, New Zealand). Nucleotides were converted to Ns if they had phred scores below 15. The ends of sequences were trimmed using an error probability of 3%. Any sequences with more than 10% Ns remaining or total length less than 300 base pairs were disregarded from further analysis. Sequences were aligned using a multiple alignment into operational taxonomic units (OTUs). Consensus sequences were queried against Genbank, using nBLAST. Sequences derived from nBLAST were examined for chimeras or other technical errors and erroneous sequences were discarded, according to Nilsson et al. (2012). Sequences with ≥97% sequence similarity were considered to be reasonable approximations of fungal species. Sequences of all fungal species were then submitted to Genbank; accession numbers are listed in Table 2-2.

2.2.6 Statistical analyses

All non-normal colonization data were transformed using the arcsine transformation prior to analysis. Initial (2011) characteristics of high and standard feed seedlings were compared using t-tests. All 2012 data were analyzed as a randomized block split-plot design, with capping material as the whole-plot factor and feed type as the split-plot factor; block (n=5) and the interactions of block with feed and soil type were set as random factors. Analysis of variance (ANOVA) was used to determine the effects of soil and feed type and their interaction on 2012 seedling growth (height, stem diameter) and nutritional characteristics (root starch, root sugar, leaf total nitrogen, leaf total phosphorous, and leaf total potassium). Due to inconsistencies in

matching OTUs to ECM fungal species (see Results), relative abundance was examined for only one species, *Cenococcum geophilum*, which was easily recognized due to its distinct radiate mantle morphology (LoBuglio 1999). To determine relative abundance, the total number of colonized tips of *Cenococcum geophilum* was divided by the total number of all colonized tips found on the seedling. ANOVAs were used to test for the effects of soil type, feed type, and their interaction on total ECM colonization, and relative colonization of *Cenococcum geophilum*. All statistics were run using SAS 9.2 (SAS Institute Inc., Cary, North Carolina, U.S.).

2.3 Results

2.3.1 Seedling growth and nutrient reserve status after two growing seasons in the field

Total seedling height was affected by feed type, ($F_{(1,4)}$=11.6; p=0.03), but not by soil; high feed seedlings grew taller than standard feed seedlings (Table 2-3). Similarly, seedling root collar diameter was also greater in high feed than in standard seedlings, ($F_{(1,4)}$=36.12; p<0.004). Despite having different tissue nutrient concentrations when planted, feed type did not significantly influence either current foliar or root nutrient concentrations (Table 2-3). Current root non-structural carbohydrate (NSC) concentrations, including sugar, starch, and total NSC, were also unaffected by feed type (Table 2-3). Soil type did not affect seedling foliar or root nutrient concentrations (Table 2-3). Total concentrations of fine root NSC was unaffected by soil type; however, seedlings grown in PMM had marginally higher concentrations of fine root sugars, ($F_{(1,4)}$=7.09; p=0.056), than seedlings grown in FFM, though concentrations of root starch was unaffected by soil type (Table 2-3).

2.3.2 Soil and seedling feed type: ectomycorrhizal community composition

A total of three OTUs were assigned from two observed morphotypes: *Hebeloma leucosarx*, *Thelephora terrestris*, and *Meliniomyces bicolor* (Table 2-2). Of the 75 root tips analyzed for the presence of fungal DNA, 56% of consensus sequences obtained were discarded due to insufficient sequence quality or non-mycorrhizal species identity. For the first morphotype, consensus sequences from 14 root tips remained, of which 64% were colonized by *H. leucosarx* and 36% were colonized by *T. terrestris*. For the second morphotype, consensus sequences from 19 root tips remained, of which 74% were colonized by *M. bicolor* and 26% were colonized by *T. terrestris*.

Total colonization of root tips by ECM fungi was affected by seedling feed type, ($F_{(1,4)}$=16.5; p=0.015), but not by soil type, ($F_{(1,4)}$=0.1; p=0.77) (Fig. 2-1). High feed seedlings had a greater percentage of root tips colonized by ECM fungi (24.8 ±3.91% SE) than standard seedlings (15.1 ±3.91% SE). While feed type had a significant effect on colonization, only root collar diameter of the measured current growth and nutritional characteristics (height, diameter, root sugar, root starch, leaf nitrogen, leaf phosphorous, and leaf potassium), was positively correlated with colonization, (r^2 (98)=0.198, p=0.047).

While only feed type affected the total colonization by ECM fungi, relative colonization by *C. geophilum* was affected by soil type ($F_{(1,4)}$=11.57; p=0.027, Fig. 2-2). Seedlings grown in FFM had a greater percent of root tips colonized by *C. geophilum* (5.6 ±1.52% SE) than seedlings grown in PMM (0.64 ±0.33%). The relative colonization by the other OTUs was unable to be determined due to uncertainty concerning fungal identity, with one fungal OTU (*T. terrestris*) being attributed to both morphotypes.

2.4 Discussion

2.4.1 Seedling nutrient reserve status and ectomycorrhizal colonization

Seedlings planted with initially higher nutrient reserves had greater colonization by ECM fungi than standard feed seedlings. This finding is contrary to the accepted relationship between mycorrhizal development and nutrient supply: as nutrient availability (predominantly N and P) increases in soils, ECM colonization tends to decrease (Nilsson et al. 2005; Hoeksema et al. 2010). However, our results highlight the importance of differentiating between soil nutrient availability and plant nutrient reserves contained in their tissues (Dixon et al. 1981). Soil type had no significant influence on total ECM colonization, possibly because N and P availability as measured by plant root simulator probes (Schott et al. submitted) did not significantly differ between FFM and PPM. Specifically, total nitrogen availability (nitrate and ammonium) was 40±11 (SE) μg 10 cm^{-2} 91 $days^{-1}$ in the FFM and 41 ± 23 μg 10 cm^{-2} 91 $days^{-1}$ in the PMM; phosphorous availability was 4.1 ± 1.1 μg 10 cm^{-2} 91 $days^{-1}$ in the FFM and 2.2 ± 0.2 μg 10 cm^{-2} 91 $days^{-1}$ in the PMM (Schott et al. submitted). In the absence of differences in soil nutrient availability, ECM colonization was likely increased by initially elevated plant nutrient reserves, though the underlying mechanisms are unclear. The increased colonization was not related to current plant nutrient reserves: after two growing seasons in the field, there were no differences in seedling nutrient or carbohydrate concentrations between high feed and standard seedlings. Rather than current conditions, we propose that the increased colonization of high feed seedlings was the result of the initial elevated nutrient and starch reserves, a legacy effect which might have allowed high feed seedlings to facilitate colonization by ECM fungi. In order to investigate these mechanisms, we would need data on initial colonization of the seedlings by ECM fungi as well as more detailed seedling physiology throughout both growing seasons, including data on

carbon acquisition, root turnover, and nutrient reserves. This research indicates that seedling quality (e.g. nutrient or carbon reserve status) at the time of planting can play a significant role in determining the abundance of mycorrhizal associations present on seedlings, which may prove to be a driver for the continued improved growth performance found in these seedlings (Schott et al. submitted).

2.4.2 Ectomycorrhizal fungal community characteristics

We classified three operational taxonomic units present on seedlings: *Hebeloma leucosarx, Meliniomyces bicolor,* and *Thelephora terrestris,* as well as a fourth species, identified as *Cenococcum geophilum* due to its distinct radiate mantle morphology. The presence of *C. geophilum*, *H. leucosarx*, and *T. terrestris* at our site is to be expected, as they often colonize roots of trees establishing on recently disturbed sites. For instance, *C. geophilum* has been found on sites recently disturbed by fire (Visser 1995), clearcut logging (Ingleby et al. 1998), glacial activity (Mühlmann and Peintner 2008), and in soil following surface mining (Bois et al. 2005). Members of the genus *Hebeloma* have been found on sites recently disturbed by wildfire (Visser 1995), deforestation (Obase et al. 2007), and surface mining (Hankin et. al., in press). *Thelephora terrestris* has been found on sites after fire (LeDuc et al. 2013), deforestation (Obase et al. 2007), and surface mining (Onwuchekwa et al. 2014). Little autecological information is available on *M. bicolor*; this study reports the first observed instance of *M. bicolor* occupying a site recently disturbed by mining activities, though it has been observed following fire disturbance (Bent et al. 2011; Sousa et al. 2014).

The abundance of *C. geophilum* was mediated by soil type; *C. geophilum* was more prevalent in FFM than PMM. Fungal physiology may explain some of the observed variation in

the relative abundance of *C. geophilum*. The forest floor soil type has a lower water holding capacity than PPM (Schott et al. submitted); *C. geophilum* is recognized as more drought tolerant, as it has an abundance of melanin, a class of polymer which contributes to the tolerance of drought stress, allowing the fungi to persist where other species may be inhibited (Cripps 2001; Fernandez and Koide 2013). Early successional species of ECM fungi such as *C. geophilum* are typically generalists with low host specificity (Dickie et al. 2013), which may explain its greater sensitivity to environmental factors such as water or nutrient availability relative to host characteristics (Dickie 2007).

Recently disturbed sites typically harbor fewer species of ECM fungi than intact forests (Nara et al. 2003); correspondingly, the number of fungal OTUs found at our site was low. Overall, species richness at the site was less than half of that found in other areas of similar strip mining disturbances (Dimitriu et al. 2010; Bauman et al. 2011). There are several possible explanations underlying the discrepancy. The soil types were stockpiled for two years prior to placement on the site; stockpiling for as little as six months can reduce the ability of fungi to colonize potential hosts due to propagule (both spore and hyphal) death (Persson and Funke 1988). The age of other comparable mine areas is five to twenty years compared with two years at our site; time since disturbance has been shown to increase ECM fungal abundance and richness after clearcutting and wild fire (Twieg et al. 2007). A single ECM host was planted at the site which likely recovers fewer ECM fungal species than sites planted with mixed species, as many ECM fungi display host specificity (Ishida et al. 2007). A study examining the fungal community in similar reclamation soils after a single growing season found similarly low rates of colonization, though more species were recovered, possibly due to the fact that cover soils were directly placed onto the reclamation site and not stockpiled and/or the presence of three potential

ECM hosts (*P. tremuloides*, *Picea glauca* and *Pinus banksiana*) compared to one at our site (Hankin et. al., in press). Though the overall fungal diversity at the site was low, subsequent establishment of vegetation, spore dispersal, and soil development may promote future ECM species diversity (Fujiyoshi et al. 2010; Dickie et al. 2013).

2.4.3 Summary

We examined the effect of the nutritional status of planted aspen seedlings on the abundance of ECM fungi and its dependence on the soil type in which the seedlings were growing. We hypothesized that fungal abundance, measured by colonization level, would be less for seedlings planted with higher nutrient reserves (high feed), as seedlings would need to invest less into the symbiosis than seedlings with lower nutrient status. Contrary to our predictions, high feed aspen seedlings had greater current total colonization than standard feed seedlings. Total colonization of roots did not differ between soil types, nor was there an interaction between soil and feed type. A total of four species, one identified through distinct morphology and three ECM fungal OTUs, were found recurring across a relative large area (1.3 ha), three of which are commonly found in highly disturbed sites. While total colonization was influenced by only seedling feed type, the relative abundance of *C. geophilim* was influenced by soil type. The results of this study suggest that initial seedling quality (e.g., nutrient or carbon reserve status) can play a significant role in structuring the developing ECM fungal community.

Tables

Table 2- 1: Initial *Populus tremuloides* seedling characteristics by feed type (n=10). Feed types are nutrient loaded (High) and unloaded (Standard). Means are presented ± 1 SE. Significant *p*-values are bolded (α=0.05).

	Feed		
	High	**Standard**	***p***
Seedling height (cm)	43.5 (2.43)	41.0 (2.43)	0.47
Root collar diameter (mm)	4.71 (0.191)	4.92 (0.191)	0.44
Root:Shoot ratio	3.58 (0.162)	3.58 (0.162)	0.98
Shoot total NSC (% dry wt.)	12.3 (0.50)	13.7 (0.55)	0.08
Root total NSC (% dry wt.)	29.9 (0.66)	30.0 (0.66)	0.9
Root sugar (% dry wt.)	16.2 (0.46)	18.8 (0.46)	**0.001**
Root starch (% dry wt.)	13.7 (0.62)	11.2 (0.62)	**0.01**
Root nitrogen (%)	2.46 (0.081)	1.41 (0.081)	**<0.001**
Root phosphorous (ug g^{-1})	3513.0 (67.23)	2697.2 (67.23)	**<0.001**

Table 2- 2: Operational taxonomic units of ectomycorrhizal fungi identified across two stock types of *Populus tremuloides* seedlings grown in a reclaimed site in northern boreal forest (Alberta, Canada).

Genbank Accession	Query/hit length	Reference	Identities (%)	Score	Closest Genbank match
KM115028	931/738	AY948191	99	1301	*Hebeloma leucosarx*
KM115029	812/1058	AY394885	98	1200	*Meliniomyces bicolor*
KM115030	882/877	JQ712012	98	1473	*Thelephora terrestris*

Table 2- 3: *Populus tremuloides* seedling (n=5) characteristics by treatment. Feed types are nutrient loaded (High) and unloaded (Standard); soil types are forest floor material (FFM) and peat-mineral mix (PMM). Mean are presented ± 1SE. The interaction between soil type and feed type was not significant (p>0.05) for all variables.

	Feed			**Soil type**		
	High	**Standard**	***p***	**FFM**	**PMM**	***p***
Seedling height (cm)	87.0 (5.74)	77.2 (4.33)	**0.027**	80.5 (5.52)	83.6 (5.06)	0.41
Root collar diameter (mm)	11.4 (0.63)	9.5 (0.44)	**0.004**	10.1 (0.54)	10.8 (0.69)	0.45
Root sugar (% dry wt.)	5.1 (0.51)	4.8 (0.35)	0.33	4.6 (0.47)	5.3 (0.38)	0.056
Root starch (% dry wt.)	7.8 (1.29)	9.3 (1.37)	0.12	8.7 (1.17)	8.4 (1.50)	0.54
Root total NSC (% dry wt.)	12.9 (0.65)	14.0 (0.64)	0.25	13.3 (0.57)	13.7 (0.71)	0.48
Root nitrogen (%)	0.70 (0.068)	0.70 (0.049)	0.99	0.63 (0.044)	0.77 (0.064)	0.14
Root phosphorous (%)	0.10 (0.011)	0.10 (0.009)	0.64	0.10 (0.007)	0.10 (0.012)	0.72
Foliar nitrogen (%)	2.0 (0.07)	2.0 (0.09)	0.41	1.90 (0.071)	2.09 (0.078)	0.10
Foliar phosphorous (%)	0.16 (0.012)	0.16 (0.012)	0.98	0.16 (0.010)	0.17 (0.014)	0.22
Foliar potassium (%)	0.81 (0.074)	0.76 (0.042)	0.39	0.78 (0.070)	0.78 (0.049)	0.91

Figures

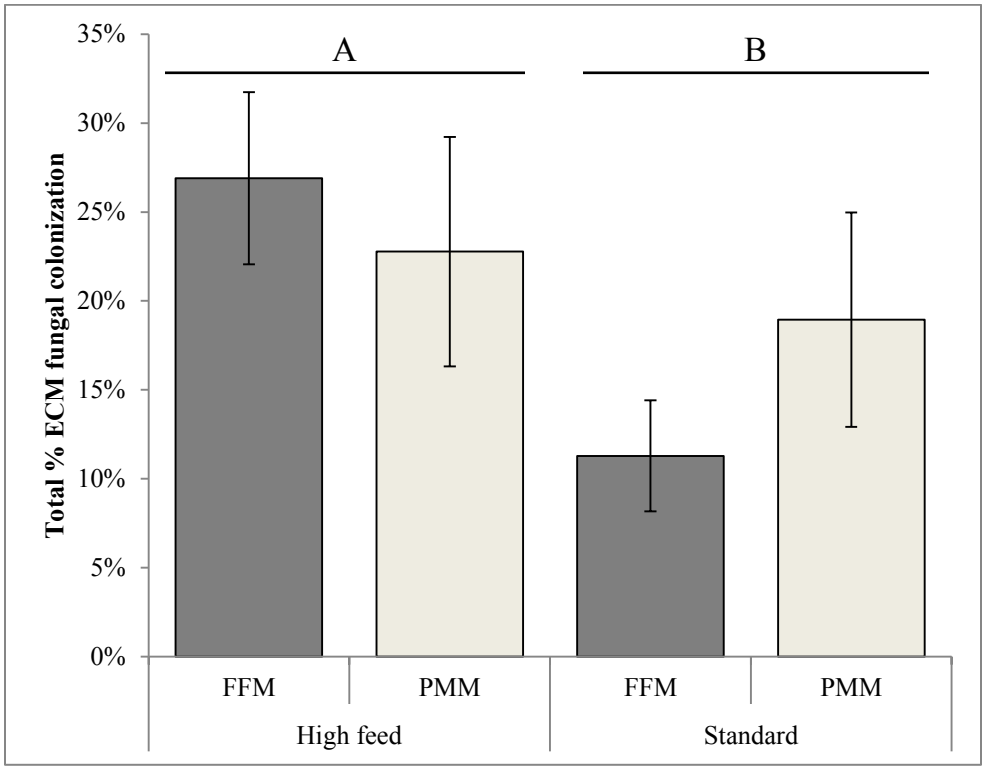

Figure 2- 1: Total ectomycorrhizal colonization of high feed and standard aspen (*Populus tremuloides*) seedlings growing in two soil types, forest floor material (FFM) and a peat-mineral mix (PMM) (n=5). Significance is denoted on the graph using letters. High feed seedlings had more colonization than control seedlings (p=0.015), neither soil type nor the interaction between the two had a significant effect.

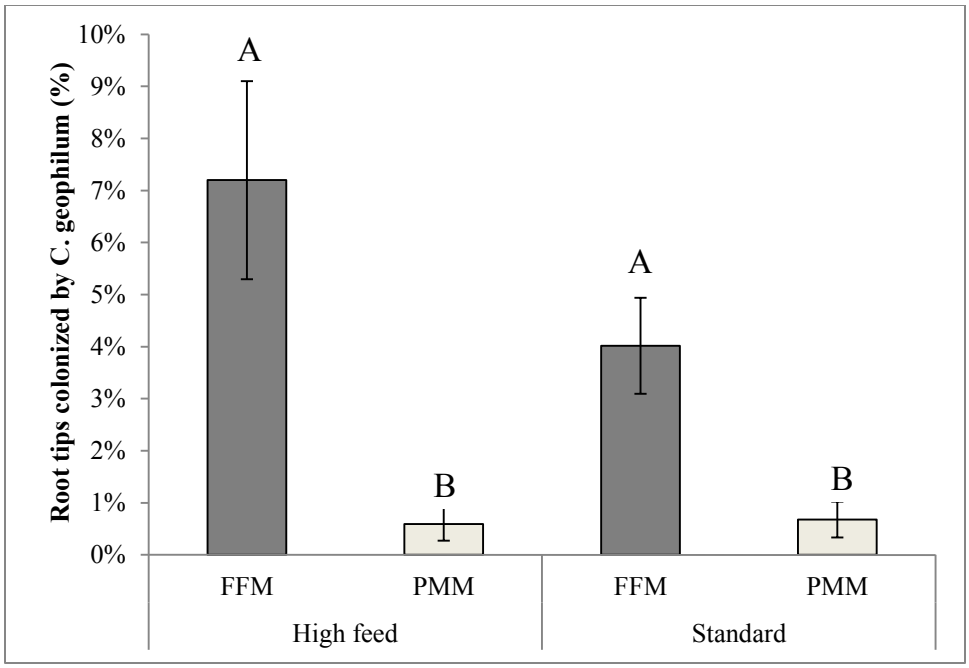

Figure 2- 2: Relative colonization of *Cenococcum geophilum* on the roots of high feed and standard aspen (*Populus tremuloides*) seedlings planted in two soil types, forest floor material (FFM) and a peat-mineral mix (PMM) (n=5). Colonization by *C. geophilum* was higher in FFM than PPM soils (p=0.027) but was unaffected by seedling feed type. Significance is denoted on the graph using letters.

Chapter 3: Stress, non-structural carbohydrate reserves, and seedling root exudation of *Populus tremuloides*

3.1 Introduction

Plants release a broad spectrum of inorganic and organic exudates through their roots, which perform a variety of important functions in ecosystems. Exudates can function as chemoattractants for beneficial microbes such as mycorrhizal fungi towards the plant rhizosphere (Kuzyakov et al. 2006), as mediators for the acquisition of mineral soil nutrients (Dakora and Phillips 2002), as well as altering the chemical and physical environment surrounding the root (Walker et al. 2003). In particular, organic exudates can function as a supply of easily accessible carbon for microbial growth and respiration (Grayston et al. 1996), as the majority of organic root exudates are comprised of low molecular weight (LMW) compounds, including sugars, amino acids, and organic acids (Uren 2007). The release of some exudates is an active process, utilizing ATP and therefore is mediated by the plant; however, the predominant mechanism by which exudation occurs is via passive diffusion of LMW compounds across the cell membrane as a consequence of electrochemical gradient potentials (Dennis et al. 2010).

As can be seen in Appendix figure 1, the release of exudates from the plant is determined by a combination of interacting endogenous and exogenous factors, of which many have been identified (Jones et al. 2004). Deficiencies in essential nutrients or minerals such as nitrogen, phosphorous, potassium, or iron, affect both the quantity and composition of compounds found in root exudates; however, any changes in exudation are dependent upon the context of the nutrient deficiency in the plant (i.e. roots will increase exudation of carboxylates when experiencing P deficiency but will decrease carboxylate exudation when experiencing nitrate deficiency) (Neumann and Romheld 2007). Soil contaminants, such as soluble aluminum or

sodium chloride, have been shown to alter the composition of exudates, such as increasing the release of carboxylates in the case of aluminum toxicity to form stable Al-carboxylate complexes, thereby decreasing aluminum's toxicity (Jones 1998; Marin et al. 2009). Low soil moisture can increase the mechanical impedance of soils, which in turn has been shown to increase exudation rates of roots, due at least in part to damage to root cells (Reid and Mexal 1977; Boeuf-Tremblay et al. 1995); in contrast, high levels of soil moisture or flooding can also enhance the exudation of sugars and amino acids (Smucker and Erickson 1987). Extreme soil temperatures (either high or low) can lead to an increase in exudation due to compromised cell membranes (Rovira 1959); however, exudation processes dependent on metabolic energy will decrease at low soil temperatures (Marschner et al. 1986). The majority of carbon released in root exudates appears to be derived from newly assimilated carbon, therefore any decrease in light availability should decrease the amount of carbon released due to reduced photosynthetic activity (Hodge et al. 1997). Furthermore, when the flux of newly assimilated carbon from the leaves to the roots is terminated (i.e., due to stem girdling), root exudation is strongly inhibited (Hogberg et al. 2001; Nguyen 2003; Schaefer et al. 2009).

In addition to the newly assimilated carbon, carbon reserves stored as non-structural carbohydrate (NSC) in various organs are also an available carbon source. The amount of NSC reserves stored in roots may also influence root exudation, as root reserve status has been shown to influence rhizosphere respiration (Xu et al. 2008). Non-structural carbohydrate reserves are water soluble sugars and starches that have not been allocated to other sinks, including growth, respiration, reproduction and defense. Non-structural carbohydrate reserves are formed either through direct investment into storage at the expense of growth (e.g. during environmental stress) or through accumulation during periods when the carbon supply exceeds demand (Chapin

et al. 1990). As the most abundant compounds in root exudates are soluble sugars, their release will be dependent upon the concentration gradients between roots and soil (Uren 2007). Altered source-sink gradients of soluble carbon between roots and soils driven by the root reserve status will likely affect exudation, since the dominant mechanism underlying the exudation of organic carbon is diffusion.

The amount of NSC reserves a plant stores is mediated by environmental and physiological drivers that affect resource availability (e.g. nutrients, light, and water) and environmental conditions such as soil temperature (Ericsson et al. 1996; Niinemets 2010). When light-limited, plants must draw on carbon reserves when growing to offset decreased total carbon assimilation, thus causing a decline in NSC reserves (Myers and Kitajima 2007). Under drought stress, meristematic growth declines quicker than carbon assimilation, leading initially to a surplus of available carbon and an increase in NSC reserves (Galvez et al. 2011), though prolonged drought can lead to a decline in NSC reserves and ultimately to carbon starvation and potential root death (Galvez et al. 2013). When plant roots are under cold temperature stress, growth can be restricted similarly to drought; however, carbon supplied by photosynthesis can still exceed the carbon demand for respiration and growth leading to a carbon surplus and an increase in carbon allocated to reserves (Karst and Landhäusser 2013; Hoch 2015).

The objective of this study is to examine and compare how the root NSC reserve status, when mediated by different types of stress, affects the root exudation of organic carbon. Specifically, we altered seedling NSC reserves by exposing aspen (*Populus tremuloides* Michx.) seedlings, a common boreal pioneer tree species, to drought, cold temperature, and shade stress, and then examined the relationship between NSC reserve status and root mass-specific exudation rates of organic carbon. We hypothesize that root NSC reserve status and the type of stress will

influence the exudation rate of organic carbon. We predict that stressed seedlings will differ in carbon reserve status as a result of the type of stress and that seedlings with higher root NSC reserves will have higher exudation rates of organic carbon than those with decreased root NSC reserves, due at least in part to greater disparity between sugar concentrations between root cell and rhizosphere.

3.2 Methods

3.2.1 Aspen seedlings

Seed collected from open-pollinated trees originating from the Fort McMurray region (56.72° N, 111.38° W), Alberta, Canada were sown into cavities (3.8 cm by 3.8 cm by 6.4 cm deep, total volume of 92.4 cm^3) filled with peat amended with perlite. Trays were placed into a growth chamber subject to a 16 hour diurnal photoperiod using fluorescent lamps; on average, photosynthetic active radiation (PAR) was 350 μmol m^{-2} s^{-1} during the photoperiod. Air temperature was maintained at 21°C. Once germinated, seedlings were thinned to three germinants per cavity and given a 0.1% solution of a 10-52-10 NPK fertilizer (Agrium Inc., Calgary, Alberta, Canada) twice a week for the first two weeks of growth and 0.1% solution of a 20-20-20 NPK fertilizer (Agrium Inc., Calgary, Alberta, Canada) once a week thereafter until transplanting. Seedlings were watered once a week in addition to the fertilizer application. After 35 days, the seedlings (with peat plugs intact) were transplanted into pots made from lengths of PVC pipe (10 cm diameter × 12 cm length) fitted with stainless steel mesh (mesh size: 150 microns, The Mesh Company, Dorset, UK) at the bottom and filled with fine sand (approximate particle size 250 microns, SIL Industrial Minerals, Edmonton, AB). Care was taken to protect roots from excessive air exposure and damage during the transplanting process. Once

transplanted, seedlings were fertilized once a week with 50 ml of the 0.1% solution of a 20-20-20 N-P-K soluble fertilizer containing chelated micronutrients and watered daily keeping soil close to field capacity using approximately 150 ml of water; light and temperature conditions remained the same. Seedlings were thinned to one seedling per pot and allowed to grow for an additional 42 days in the sand, until the average seedling height was 15 cm. The most uniform 72 seedlings were selected and randomly assigned to one of four stress treatments.

3.2.2 Stress treatments

To manipulate carbon reserve status, seedlings were exposed to different stress treatments. There were a total of four treatments, three of which were designed to simulate different environmental stresses and the fourth was an unstressed *control*. The stress treatments were *shade*, *drought*, and *cold* soil temperature. Stress treatments began 77 days after germination and lasted for 42 days. *Control* seedlings were kept at the same pre-treatment growing conditions. *Shade* seedlings were placed under shade cloth which limited PAR to 50 $\mu mol\ m^{-2}\ s^{-1}$ while the water and fertilizer regimes remained the same as the *control*. *Drought* seedlings were watered with 15 ml of water per day; light regime remained the same as that of the control treatment. Fertilizer was reduced from 50 ml to 15 ml of the 0.1% solution due to decreased water supplied to *drought* seedlings. Seedlings in the cold soil treatment had their root systems cooled to 4 °C, by placing the PVC pots into a temperature controlled water bath. These PVC pots were sealed at the bottom (creating a false bottom) using a modified design of Landhäusser et al. (2001), allowing drainage of the soil. The soil in which *cold* seedlings were grown was kept at field capacity, fertilizer was reduced from 50 ml to 15 ml similarly as for

drought (due to decreased water uptake at cold soil temperatures), and the light regime remained the same as *control* seedlings.

3.2.3 Acclimation and isotope labeling

After 42 days of stress treatment, all seedlings were returned to pre-treatment conditions (i.e., given 150 ml water each day, 50 ml 20-20-20 NPK fertilizer once a week, and 350 µmol m^{-2} s^{-1} of PAR) and remained in these conditions for the remainder of the study (11 days). Any seedlings exhibiting damage or dieback were removed from the sample group at this time. Seedlings were allowed to acclimate to the pre-treatment conditions for three days, during which 21 seedlings, six from each of the *shade* and *control* treatments, five seedlings from the *drought* treatment, and four from the *cold* soil treatment, were destructively sampled. Seedlings were separated into leaves, shoots, coarse roots (diameter > 1mm), and fine roots (diameter < 1mm), and dried at 70 °C for at 48 hours (see below). This sampling was done to determine the baseline conditions and the effects of the stress treatments on seedling morphology and carbon reserve status.

During the morning of the fourth day since returning to pre-treatment conditions, the remaining 44 seedlings were pulse-labeled with $^{13}CO_2$ according to Norris et al. (2012) to examine the allocation of the most recent photosynthates to roots and exudates for seedlings under the different stress treatments. In brief, seedlings were covered with a mylar bag (40 cm x 20 cm) designed with a short section of tubing sticking out the top; the bag was sealed to the base of the stem. Fifty ml of 99% $^{13}CO_2$ (Sigma-Aldrich, Ontario, Canada) was injected into the bag through the protruding tube using a syringe. The tube was sealed and the seedlings were left for thirty minutes, after which the bags were removed.

After labeling, the seedlings were removed from the sand and were gently washed to remove any adhering sand particles from the roots protruding from the peat. No attempt was made to remove the peat plugs from roots as this would have caused extensive damage to the root system. The root systems of each seedling were then dipped in an antibacterial mixture (50 ppm penicillin and 50 ppm streptomycin) to reduce rhizosphere bacterial activity. Cleaned seedlings were then transplanted into glass beads, which had approximately the same dimensions as the sand (particle size 250 microns, SIL Industrial Minerals, Edmonton, AB) but less molecular adsorption of organic acids (Phillips et al. 2009). At the same time, ten peat plugs without seedlings and associated roots (hereafter 'blank peat cores') were also transplanted into glass beads, to obtain an approximation of dissolved C originating from the peat core rather than the root exudates. Seedlings were given three days to overcome transplant shock, after which the seedlings were again pulse-labeled with $^{13}CO_2$, according to the same protocol as stated above, to ensure that a sufficient amount of labelled carbon was present in the roots and exudates at the time of sampling. Immediately following labeling, photosynthesis was measured on the newest fully expanded leaf of each seedling using an LI6400 Portable Photosynthesis System (LiCor, Nebraska, U.S.) to obtain carbon assimilation rates.

3.2.4 Exudate collection

One hour after photosynthesis was measured, roots of all seedlings were flushed with 250 ml of distilled water, approximately two times the holding capacity of each core, to expel any previously accumulated exudates or fertilizer; seedlings were then left to release exudates for nine hours, the time period chosen for a static bathing solution culture according to Phillips et al. (2009). After the exudation period, the cores were again flushed with 250 ml of distilled water

but the runoff (hereafter 'exudate solution') was collected. The exudate solutions were immediately frozen at -40 °C until further analysis. Once exudates were collected, seedlings were destructively sampled and separated into respective components: leaves, stems, and four root components, coarse and fine (< 1 mm) roots inside the peat core and coarse and fine roots outside the peat core. Any exudates released inside the peat core were assumed to be absorbed by the peat, thus root components were split between those inside and outside the peat core and only those outside were used in subsequent exudation rate calculations. Root to shoot ratios (RSR) were calculated using total root mass and stem mass. Root volumes were measured according to Sattelmacher (1987) and used to calculate root densities for both roots inside and outside the peat cores. Leaf area was measured using an LI-3100C Leaf Area Meter (LiCor, Nebraska, U.S.). Tissue components were then dried at 70 °C for at least 48 hours, weighed, and ground using a Thomas Wiley Mini-Mill (Thomas Scientific, New Jersey, U.S.). The solutions were thawed and exudation was measured by total organic carbon (TOC) analysis using a TOC-V CHS/CSN Model Total Organic Carbon Analyzer (Shimadzu Corporation, Kyoto, Japan). The leachate from the blank peat cores was also analyzed for total organic carbon using the same method; the average TOC found in the empty peat cores was then subtracted from the TOC found in the exudate solutions. Nonstructural carbohydrate concentrations of the fine roots were analyzed using a phenol-sulfuric acid assay to determine total sugar concentration and enzyme digestion analysis to determine total starch concentrations according to Chow and Landhäusser (2004). The fine roots were then analyzed for nitrogen (N), phosphorous (P), and potassium (K) concentrations. Nitrogen concentration was determined by the dumas combustion method using the Costech Model EA 4010 Elemental Analyzer (Costech International Strumatzione, Florence, Italy, 2003). Phosphorous and potassium concentrations were determined by nitric acid digestion

then colourimetry using the SmartChem Discrete Wet Chemistry Analyzer (Westco Scientific Ltd., Brookfield, CT, USA, 2007). Mass-specific exudation rates were calculated by dividing the total organic carbon in the exudate solution by the exudation period (nine hours) and the mass of the fine roots outside the peat core (see Table 3- 1). The exudate residue was obtained by drying a subsample of the exudate solution (30 ml) for at least 48 hours at 50°C. Enrichment of fine root tissue and exudate residue by ^{13}C were analyzed using continuous flow isotope ratio mass spectrometry (ThermoFinnigan Delta^{+} Advantage Continuous Flow Isotope Ratio Mass Spectromoeter, Thermo Finnigan Corp, Germany, 2003) performed by the Natural Resources Analytical Laboratory at the University of Alberta. One unlabeled seedling per treatment was randomly selected from those destructively sampled at the beginning of the acclimation period, prior to exudate collection, to determine background ^{13}C levels in roots. The reference material used for ^{13}C analysis was the Vienna Pee Dee Belemnite (VPDB).

3.2.5 Data analysis

Seedling characteristics both prior to the acclimation period and following exudate collection, including height, leaf mass, stem mass, root mass and density, leaf area, area-based leaf carbon assimilation, stomatal conductance, and fine root non-structural carbohydrates, were compared across treatments using analyses of variance (ANOVAs). All post-hoc comparisons were made using least-squares means with a Bonferroni adjustment for multiple comparisons to maintain family-wise type I error at 0.05. Seedling characteristics were also compared between sampling periods (prior to the acclimation period and following exudate collection) using a t-test. Differences in mass-specific carbon exudation rates among treatments were compared using an ANOVA and post-hoc comparisons were made using least-squares means test. Two-way

analyses of covariance (ANCOVA) were used to determine the effects of stress treatments on mass-specific carbon exudation rates, with fine root sugar concentrations, foliar nutrients (nitrogen, phosphorous, or potassium), leaf area-based photosynthetic rate, or whole seedling carbon assimilation rate as covariates.

As the raw isotopic data was expressed in per mille (‰) $\delta^{13}C$ vs VPDB, it needed to be converted into a percentage of ^{13}C. The ratio of $^{13}C/^{12}C$ in each sample was calculated by multiplying the $\delta^{13}C$ value by the VPDB standard (0.0020672) and dividing by 1000, a modification of the equation found in Werner and Brand (2001); the percentage of ^{13}C then calculated by using the $^{13}C/^{12}C$ ratio. To test how seedlings allocated newly acquired carbon between fine roots and exudates, the ^{13}C atom percent excess, hereafter referred to as the percentage of ^{13}C surplus (that is, the percentage of ^{13}C atoms in excess of background ^{13}C levels) for fine roots and exudates was calculated by subtracting the background percent ^{13}C in the fine roots of unlabeled seedlings, one seedling per treatment, from the percent ^{13}C in fine roots of labeled seedlings in the corresponding treatment. Since we did not collect exudates from unlabeled seedlings, the calculation for surplus ^{13}C in the exudates was done using the background ^{13}C percentage of fine roots, assuming that there was little or no fractionation of ^{13}C as exudates leave fine roots. The mass of surplus ^{13}C atoms in fine roots and exudates was then calculated by multiplying the surplus ^{13}C percentage by the mass of fine roots or exudates. The proportion of surplus ^{13}C atoms in fine roots to surplus ^{13}C atoms in exudates was then examined among treatments using an ANOVA and post-hoc comparisons. The proportion of surplus ^{13}C atoms to ^{12}C atoms in exudates was also examined using linear regression. The All statistics were performed using SAS 9.2 (SAS Institute Inc., Cary, North Carolina, U.S.).

3.3 Results

3.3.1 Seedling morphology and physiology in response to stress

Seedlings grown under the four treatments differed in morphological (Table 3-1) and reserve (Table 3-2) measurements. However, the majority of the measured seedling variables did not differ significantly between sampling periods of pre and post exudate collection within each stress treatment (Table 3-1 and 3-2), Only specific leaf area of *shade* seedlings ($t(_{14})$=5.04,p<0.001, Table 3-1) and fine root sugar concentrations of *control* seedlings decreased significantly ($t(_{16})$=3.29,p=0.004, Table 3-2) between the two measurement times.

After exudate collection (post), seedlings grown under the four stress treatments differed in mass and height (Table 3-1 and Appendix Table 1). *Control* seedlings were significantly larger than those in the other treatments, having at least double the root mass, triple the shoot mass, and 65% more leaf mass than seedlings from any other treatment (Table 3-1). *Drought* seedlings had the lowest aboveground (stem and leaf) mass, resulting in the highest (5.2) root to shoot ratio (RSR), while *cold* seedlings had the lowest root mass and RSR (1.6) (Table 3-1). *Shade* seedlings were proportioned similarly to *control* seedlings (both RSR 3.1), but had lower total mass compared with *control* seedlings. Seedlings from all treatments differed in height ($F(_{3,33})$=8.79; p<0.001, Table 3-1), with *control* seedlings being the tallest and *drought* seedlings the shortest. Leaf area was highest for both *control* and *shade* seedlings and lowest for *drought* seedlings (Table 3-1).

After the exudate collection (after recovery period) seedlings differed in carbon assimilation, with *control* seedlings having the highest photosynthetic rate per leaf area compared with those from the other three treatments ($F(_{3,33})$=5.04; p=0.006, Table 3-1). However, stomatal conductance was not affected by the treatments ($F(_{3,33})$=0.86; p=0.47, Table

3-1). Seedlings from all treatments also differed in root mass, both for fine roots outside the peat core ($F_{(3,33)}$=36.8; p<0.0001) as well as total root mass, which includes both fine and coarse roots both inside and outside of the peat core ($F_{(3,33)}$=31.5; p<0.001, Table 3-1). Root density did not differ significantly among treatments ($F_{(3,32)}$=2.63; p=0.07). The concentration of foliar nitrogen was different among treatments (F(3,33)=9.51; p<0.001, Table 3-1), with *shade* seedlings having higher foliar N than seedlings from the other stress treatments. Neither foliar phosphorous (F(3,33)=0.33; p=0.81, Table 3-1) nor foliar potassium (F(3,33)=2.69; p=0.06, table 3-1) differed among treatments.

The concentrations of non-structural carbohydrates (NSC) in the fine roots was different among treatments ($F_{(3,33)}$=4.23; p=0.012, Table 3-2). Of the non-structural carbohydrates, concentrations of sugars in the fine roots differed among treatments ($F_{(3,33)}$=8.22; p<0.001), with *cold* seedlings having the highest and *drought* and *shade* seedlings having the lowest concentrations thereof (Table 3-2). The concentrations of starch in the fine roots followed a similar trend as sugars and was highly correlated ($r^2_{(34)}$=0.76, p<0.001) but did not significantly differ among stress treatments ($F_{(3,33)}$=2.32; p=0.09).

3.3.2 Stress and exudation of total organic carbon

At the individual seedling level, the total amount of organic carbon released from roots did not differ among treatments ($F_{(3,33)}$=0.48; p=0.7); the total amount of organic carbon released by seedlings together with peat cores was 4.64 mg (± 1.16 s.d.) and the amount released by blank peat cores was 2.33 mg (± 0.99 s.d.). However, mass-specific exudation (total organic carbon in the exudates divided by the mass of the fine roots outside the peat core) varied among treatments ($F_{(3,33)}$=9.97; p<0.001, Fig. 3-1), with *cold* and *shade* seedlings exuding organic

carbon at a higher rate than *control* seedlings. While there was no relationship between fine root sugar concentrations and the mass-specific exudation rate of organic carbon among all four treatments ($F_{(1,3)}$=0.637; p=0.51, Fig. 3-2), there was a positive linear relationship between mass-specific exudation and fine root sugar concentrations when examining within single stress treatments (Fig. 3-3). The analysis of covariance (ANCOVA) of mass-specific exudation, with stress treatment as the main effect and fine root sugar concentration as the covariate, was significant for both the stress treatment ($F_{(3,32)}$=10.31; p<0.001) and the concentrations of sugar in fine roots ($F_{(1,32)}$=6.51; p=0.016, Table 3-3). However, the interaction between mass-specific exudation rate and fine root sugar concentrations was not significant ($F_{(3,29)}$=1.22; p=0.32) indicating that the increase in mass-specific exudation in response to fine root sugar concentrations was similar for seedlings in all four stress treatments. The ANCOVA of mass-specific exudation, with stress treatment as the main effect and adjusted for differences in foliar nutrients (nitrogen, phosphorous, or potassium), was significant for stress treatment ($F_{(3,32)}$=9.64, p<0.001; $F_{(3,32)}$=10.14, p<0.001; and $F_{(3,32)}$=8.78, p<0.001 for each nutrient, respectively), but was not significant for the covariates nitrogen ($F_{(3,32)}$=0.05; p=0.83), phosphorous ($F_{(3,32)}$=2.64; p=0.11) and potassium ($F_{(3,32)}$=0.15; p=0.70) (Table 3-4). The ANCOVA of mass-specific exudation, using leaf area-based photosynthetic rate or whole seedling carbon assimilation rate as covariates, was significant for stress treatment ($F_{(3,32)}$=5.53; p<0.004 and $F_{(3,32)}$=5.86; p<0.003, respectively), but also not significant for either covariate (photosynthetic rate ($F_{(3,32)}$=1.47; p=0.23; rate of total carbon assimilation ($F_{(3,32)}$=2.74; p=0.11) (Table 3-5).

3.3.3 Allocation of newly acquired carbon to roots and exudates (^{13}C labelling)

There were no differences in $\delta^{13}C$ versus VPDB among treatments (Table 3-6). However, the ratio of surplus ^{13}C atoms allocated to fine roots versus exudates differed significantly among treatments ($F_{(3, 29)}$= 9.49, p<0.001, Fig. 3-4). *Control* seedlings had significantly more new ^{13}C atoms allocated to fine roots than to exudates compared with *cold* (t(29)=5.06,p<0.001), *shade* (t(29)=3.68;p<0.001) and marginally more than *drought* seedlings (t(29)=2.82, p=0.009) (Fig. 3-4). *Drought* seedlings had marginally more ^{13}C atoms allocated to fine roots than to exudates compared to *cold* seedlings (t(29)=2.24, p=0.03, Fig. 3-4). There was no overall relationship between fine root sugar concentrations and the ratio of ^{13}C labeled to unlabeled carbon in the exudates across all treatments ($F_{(1,3)}$=0.08; p=0.81, Fig. 3-5).

3.4 Discussion

3.4.1 Differences in carbon storage and exudation rates among stress treatments

The release of exudates is mediated by plant physiological and environmental factors, including carbon status, nutrition, and stress (Neumann and Romheld 2007). We exposed aspen seedlings to stress to manipulate root non-structural carbohydrate (NSC) reserves, and as a possible consequence, changes in the exudation of organic carbon. Drought, cold soil temperature, and shade significantly altered seedling physiology and morphology relative to unstressed (*control*) seedlings (Tables 3-1 and 3-2). Importantly, the concentrations of NSC reserves varied across treatments, with *cold* seedlings having more and *drought* and *shade* having less NSC reserves than *control* seedlings. Mass-specific exudation rates of organic carbon

also varied among the stress treatments, with *cold* and *shade* seedlings having greater exudation rates than *drought* or *control* (Fig. 3-1).

We predicted that exudation rates of organic carbon would increase as fine root NSC reserve concentrations increased. There was no relationship between root NSC reserve status and exudation of organic carbon when examined across treatments (Fig. 3-2), however within a treatment, this relationship was apparent. Within treatments, exudation rates of organic carbon were linearly dependent upon the concentration of sugars in the fine roots; furthermore, the relationship was similar across all treatments, with greater sugar concentrations leading to similarly increased exudation rates of organic carbon (Fig. 3-3). This supports our hypothesis that root NSC reserve status influences the exudation rates of organic carbon. The relationship is likely due to a simple variation in the diffusion coefficient between root tissue and soil, which causes an increased movement of sugars across the cell membrane into the rhizosphere (Carvalhais et al. 2011). Further, *control* seedlings had proportionally more ^{13}C contained in their roots than in their exudates compared with *cold* and *shade* seedlings, indicating that the amount of newly acquired carbon exuded relative to the amount allocated to root tissue was higher in *cold* and *shade* seedlings than *control* seedlings (Fig. 3-4). Thus, allocation priorities of newly acquired carbon differed with the type of stress. Newly acquired carbon in *control* seedlings may have been preferentially directed to root C sinks such as NSC reserves, growth or respiration (Epron et al. 2012). *Cold* and *shade* seedlings in the other hand may have been less able to utilize the newly acquired carbon for these functions, resulting in more carbon released to the rhizosphere (McKay 1992). When differences in allocation of newly acquired carbon is taken together with the fact that the relationship between NSC reserves and exudation is evident within

but not across treatments, it suggests that other factors specific to individual stress types are superseding the influence of NSC reserves on exudation.

3.4.2 The influence of cold soil temperatures on NSC storage and exudation

In seedlings exposed to low soil temperature stress, we saw a sharp decline in structural root growth but an increase in NSC reserves, likely a response of reduced root growth (Andersen and Rygiewicz 1991; Landhäusser et al. 2001) which could be a scenario of carbon surplus where rates of photosynthesis exceed carbon demand for root respiration and growth (Karst and Landhäusser 2013; Hoch 2015). The total root mass in the *cold* seedlings was the lowest of all stress treatments, while the concentrations of NSC reserves in the fine roots was highest among treatments. Increased concentrations of solutes in root cells is also a common response for plants exposed to cold stress, as it provides protection against freezing (Mahajan and Tuteja 2005).

The mass-specific exudation rates of organic carbon for *cold* seedlings were the highest of all treatments. Alterations in permeability occur in the cell membranes of plant roots under cold stress (McKay 1992). Thus, the increased exudation in *cold* seedlings may have been a response to irreversible changes in permeability (e.g. the aggregation of lipids upon rewarming) or the damage of membrane components which can occur at low temperatures (Campos et al. 2003). This is also supported by the ratio of labelled and unlabeled carbon in the exudates compared to the sugar concentrations in the fine root (Fig. 3-5). A positive trend would be present if the cold soils treatment was excluded: as the concentration of sugar stored in roots increases, the amount of newly acquired carbon exuded relative to older carbon would also increase. However, the proportion of newly acquired carbon exuded by *cold* seedlings falls well below the predicted, meaning that seedlings may be exuding a greater proportion of older carbon, which is consistent

with increased C being lost due to compromised membrane integrity and less so with a surplus of recent photosynthates that exceed carbon demand within the root.

3.4.3 The influence of shade on NSC storage and exudation

In seedlings exposed to shade, we expected decreased total biomass and NSC reserves compared with seedlings in the control treatment (Marshall 1986; Piper et al. 2009). As expected, the total biomass of *shade* seedlings was significantly reduced, despite having a similar height, root:shoot ratio, and total leaf area compared to *control* seedlings. Concentrations of NSC reserves were lower compared to *control* seedlings, likely due to seedlings drawing on NSC reserves to support growth while under conditions that limit carbon assimilation (Myers and Kitajima 2007).

Even with lower carbon assimilation and NSC reserves relative to the *control*, mass-specific exudation rates of organic carbon were high for *shade* seedlings. Shading has been shown to affect the composition of root exudates, but the underlying mechanism, as well as its effect on total C exudation, are unknown (Rovira 1959). The observed increase in exudation rates in *shade* seedlings may be the result of poorly constructed cells and membranes. Reich et al. (1998) found that seedlings grown under shade produced more fragile root structures, as less carbon was invested into their production; if *shade* seedlings invested less carbon into structural growth, leakage of carbon compounds to the rhizosphere may increase. This is supported by the low ^{13}C isotope ratio between fine roots and exudates for *shade* seedlings, which suggests that more of the newly acquired carbon leaked to the rhizosphere than what was incorporated into roots (Fig. 3-4). Morphological changes that took place during the week between treatment and exudate collection may also have increased exudation. The sudden increase in photosynthetic active

radiation (50 μmol m^{-2} s^{-1} while being treated to 350 μmol m^{-2} s^{-1} during the acclimation week) caused shaded seedlings to increase carbon allocation to leaf mass over the course of the week, leading to a significant decline in specific leaf area and a thus potential increase in carbon assimilation (Table 3-1). If the new light regime created a surplus of C supplied to the roots, the rate at which C is released to the rhizosphere would increase (Thornton et al. 2004).

3.4.4 The influence of drought on NSC storage and exudation

In seedlings exposed to drought, we expected to see an increase in root:shoot ratio and NSC reserve concentrations relative to unstressed seedlings (Ericsson et al. 1996; Galvez et al. 2011). *Drought* seedlings had increased root:shoot ratios compared with the other treatments; however, the concentration of NSC reserves in the fine roots was lower than *cold* or *control* seedlings. This was likely due to the severity of the drought treatment, which led to the consumption of NSC reserves to meet C demands (such as osmotic transport) not met by reduced photosynthetic assimilation under such intense water stress (Galvez et al. 2011).

The mass-specific exudation rates of organic carbon for seedlings in the drought treatment were significantly lower than the other two stress treatments and similar to *control* seedlings. Mild drought stress has been found to increase exudation in plants, which was attributed to increased membrane permeability (Reid and Mexal 1977). However, when under severe drought stress, root exudation was found to decrease; the author suggested that significant changes occur in the plant when exposed to severe drought stress, though the exact changes are unknown (Reid 1974). Osmotic adjustments may be a mechanism limiting exudation rates in *drought* seedlings; seedlings would have been able to maintain elevated osmotic potential by decreasing the rate at which sugars are released into the rhizosphere by closing protein channels in the cell membrane ,

allowing increased water uptake and transfer to aboveground organs (Landsberg and Fowkes 1978; Javot and Maurel 2002).

3.4.5 Other factors influencing exudation

Several other factors may have affected exudation rates of organic carbon among the treatments, namely potential nutrient deficiency (Neumann and Romheld 2007), rates of carbon assimilation (Hill et al. 2006), and the absence of microbial communities (Lynch 2007). Seedlings in different treatments were given different amounts of fertilizer throughout the study, an artifact of treatment methodology. For example, *control* seedlings had more than three times the fertilization than *drought* and *cold* seedlings during stress treatment, a result of the fertilizer being applied during watering (see methods section for further details). However, neither nitrogen, phosphorous, nor potassium had a significant influence on exudation rates (Table 3-4). Rates of carbon assimilation also varied significantly between treatments; however, neither leaf area-specific rate of photosynthesis nor whole-seedling carbon assimilation rate influenced exudation rates (Table 3-5). An antimicrobial treatment was applied to the roots during transplanting, as the presence of microbes will decrease the amount of C recovered due to consumption. However, as the presence of microbes (ubiquitous in natural environments) will affect the quantity and composition of exudates, the exudation rates found in this study are likely not comparable to exudation rates found in seedlings in a natural setting (Meharg and Killham 1995).

3.4.6 Conclusions

We examined the relationship between carbon reserve status and exudation of organic carbon in aspen seedlings after exposure to three environmental stressors: shading, cold soils, and drought. The carbon reserve status varied across treatments, with cold seedlings having higher and drought and shade seedlings having lower concentrations of NSC reserves than unstressed (control) seedlings. Mass-specific exudation rates also varied significantly across treatments, with rates for shade and cold seedlings being higher than for drought or control seedlings. When examined within treatments, the exudation rate was dependent on NSC reserves. Furthermore, the relationship was similar for all treatments, where an increase in fine root sugar concentrations resulted in similar increases in root exudation. However, the relationship between exudation and NSC reserves disappeared when examined across treatments. Thus, fine root NSC reserves determined the exudation rates of carbon compounds in seedlings when exposed to similar conditions; however, the influence of NSC reserve concentrations was superseded by the type of stress impacting the plant which likely influenced changes at a cellular level, such as membrane permeability.

One important distinction between this and other studies examining exudation and stress (see Reid 1974; Reid and Mexal 1977; Marin et al. 2009) is that exudates were not collected while the seedlings were under stress; rather, exudates were collected one week after seedlings were removed from the stressful conditions and returned to control conditions (see methods). As a result variation in exudation rates might not be directly derived from the physical effects of the stress itself but might represent a legacy effect of the particular stress on seedling physiology. Thus, exudation rates of organic carbon in plants may be affected by past environmental conditions as well as current conditions. Exposure to even brief periods of stress has the potential

to have an enduring impact on the microbial community through alterations in plant morphology: as exudation regulates of plant-microbe interactions which occur in the rhizosphere, alterations to the exudation of organic carbon, such as those derived from varying levels of NSC reserves, may affect the composition of the microbial community (Dakora and Phillips 2002; Dimkpa et al. 2009). In turn, alterations in the microbial community will affect nutrient transfer and stress tolerance in the host, which will play a role in determining plant community diversity (Butler et al. 2003; Singh et al. 2004). Further study is needed examining the effects of carbon storage on the mechanisms regulating exudation in order to better understand the influence of stress on the exudation of organic carbon and its potential impacts on the rhizosphere and the development of the plant community.

Tables

Table 3- 1: Morphological and physiological characteristics of *Populus tremuloides* seedlings grown in four treatments, cold soils (n=7), shade (n=8), drought (n=8), and control (n=10), collected prior to the acclimation period (pre) and after exudate collection (post). All values are presented as mean (standard error). Capital letters preceding the mean in superscript denote significant differences between treatments within the same sampling period; lowercase letters following the standard error in subscript denote significant difference between sampling periods.

		Cold soil	Shade	Drought	Control
Height (cm)	Pre	23.5 (4.19)	26.6 (1.83)	17.8 (3.19)	32.3 (4.23)
	Post	^{B}24.2 (1.51)	^{AB}28.1 (3.28)	^{B}19.3 (1.46)	^{A}35.8 (2.16)
Leaf area (cm^2)	Pre	^{AB}286 (76.3)	^{A}417 (45.0)	^{B}162 (32.3)	^{A}537 (91.3)
	Post	^{BC}313 (42.7)	^{AB}518 (91.0)	^{C}214 (27.6)	^{A}572 (29.4)
Photosynthesis ($\mu mol\ m^{-2}\ s^{-1}$)	Pre	N/A	N/A	N/A	N/A
	Post	^{B}3.22 (0.346)	^{B}3.31 (0.665)	^{AB}4.42 (0.596)	^{A}5.82 (0.517)
Stomatal conductance ($mmol\ m^{-2}\ s^{-1}$)	Pre	N/A	N/A	N/A	N/A
	Post	^{A}27.9 (2.60)	^{A}68.2 (29.40)	^{A}53.0 (10.07)	^{A}66.7 (13.57)
Leaf mass (g)	Pre	^{AB}2.15 (0.566)	^{B}1.57 (0.160)	^{B}1.02 (0.186)	^{A}3.63 (0.483)
	Post	^{B}2.66 (0.326)	^{BC}2.60 (0.388)	^{C}1.53 (0.225)	^{A}4.38 (0.176)
Specific Leaf area($cm^2\ g\ leaf^{-1}$)	Pre	^{B}142 (20.8)	^{A}265 (8.5)$_{a}$	^{B}159 (13.5)	^{B}145 (5.6)
	Post	^{C}116 (4.0)	^{A}193 (9.6)$_{b}$	^{B}145 (6.6)	^{BC}131 (3.8)
Stem mass (g)	Pre	^{B}0.91 (0.264)	^{B}0.54 (0.052)	^{B}0.61 (0.140)	^{A}2.51 (0.297)
	Post	^{B}1.07 (0.123)	^{B}0.99 (0.170)	^{B}0.85 (0.087)	^{A}3.12 (0.184)
Fine root mass, outside peat core (g)	Pre	N/A	N/A	N/A	N/A
	Post	^{C}0.69 (0.135)	^{C}1.32 (0.201)	^{B}2.19 (0.220)	^{A}4.57 (0.385)

Total root mass (g)	Pre	^{B}1.14 (0.368)	^{B}1.29 (0.254)	^{B}2.73 (0.394)	^{A}6.46 (0.630)
	Post	^{C}1.62 (0.222)	^{C}2.40 (0.271)	^{B}4.19 (0.330)	^{A}9.46 (0.961)
Root to Shoot ratio	Pre	^{B}1.24 (0.085)	^{B}2.36 (0.311)	^{A}5.12 (0.842)	^{B}2.65 (0.249)
	Post	^{C}1.55 (0.187)	^{ABC}3.08 (0.629)	^{A}5.18 (0.463)	^{B}3.11 (0.358)
Root Density (g cm^{-3})	Pre	N/A	N/A	N/A	N/A
	Post	0.13 (0.007)	0.15 (0.015)	0.18 (0.013)	0.14 (0.008)
Foliar nitrogen (% dry mass)	Pre	N/A	N/A	N/A	N/A
	Post	^{B}1.80 (0.098)	^{A}2.47 (0.082)	^{B}2.07 (0.092)	^{B}2.12 (0.075)
Foliar phosphorous (% dry mass)	Pre	N/A	N/A	N/A	N/A
	Post	0.24 (0.027)	0.27 (0.022)	0.27 (0.025)	0.25 (0.020)
Foliar potassium (% dry mass)	Pre	N/A	N/A	N/A	N/A
	Post	0.82 (0.105)	0.92 (0.088)	0.64 (0.098)	0.62 (0.080)

Table 3- 2: Nonstructural carbohydrate (NSC) reserves of *Populus tremuloides* grown in four treatments, cold soils (n=4), shade (n=6), drought (n=5), and control (n=6), collected prior to the acclimation period (pre) and after exudate collection (post). All values are presented as mean (standard error). Capital letters preceding the mean in superscript denote significant differences between treatments within the same sampling period; lowercase letters following the standard error in subscript denote significant difference between sampling periods.

		Cold soil	Shade	Drought	Control
Total fine root sugar (g)	Pre	$^{B}0.109\ (0.047)$	$^{B}0.049\ (0.0056)$	$^{B}0.162\ (0.0457)$	$^{A}0.471\ (0.0565)$
	Post	$^{B}0.049\ (0.0062)$	$^{B}0.054\ (0.0099)$	$^{B}0.084\ (0.0135)$	$^{A}0.312\ (0.0448)$
Total fine root starch (g)	Pre	$^{A}0.065\ (0.0251)$	$^{A}0.035\ (0.0059)$	$^{A}0.178\ (0.0493)$	$^{A}0.533\ (0.1466)$
	Post	$^{B}0.052\ (0.0104)$	$^{B}0.060\ (0.0119)$	$^{B}0.133\ (0.0306)$	$^{A}0.438\ (0.0985)$
Total fine root NSC (g)	Pre	$^{AB}0.17\ (0.071)$	$^{B}0.084\ (0.011)$	$^{AB}0.34\ (0.095)$	$^{A}1.00\ (0.197)$
	Post	$^{B}0.101\ (0.0141)$	$^{B}0.114\ (0.0212)$	$^{B}0.217\ (0.0423)$	$^{A}0.750\ (0.1406)$
Fine root sugar (% dry mass)	Pre	$^{AB}8.48\ (1.59)$	$^{B}4.13\ (0.450)$	$^{AB}5.66\ (0.916)$	$^{A}7.28\ (0.415)_{a}$
	Post	$^{A}7.13\ (1.106)$	$^{AB}3.79\ (0.508)$	$^{B}3.11\ (0.349)$	$^{A}5.30\ (0.369)_{b}$
Fine root starch (% dry mass)	Pre	$^{AB}5.00\ (1.183)$	$^{B}2.85\ (0.441)$	$^{A}6.24\ (1.017)$	$^{A}7.51\ (1.56)$
	Post	8.05 (2.226)	4.05 (0.723)	4.82 (0.791)	6.76 (0.920)
Fine root total NSC (% dry mass)	Pre	$^{A}13.5\ (2.60)$	$^{B}6.97\ (0.755)$	$^{AB}11.9\ (1.923)$	$^{A}14.8\ (1.658)$
	Post	$^{A}15.2\ (3.23)$	$^{A}7.8\ (1.20)$	$^{A}7.9\ (1.11)$	$^{A}12.1\ (1.13)$

Table 3- 3: ANOVA table for the general linear model testing the effect of stress treatment and fine root sugar concentrations ('Sugar') on mass-specific exudation.

Source of variation	Sum of squares	*df*	Mean square	*F*	*p*
Stress treatment	0.298674	3	0.0996	10.31	<0.001
Sugar	0.062821	1	0.0628	6.51	0.016
Residual	0.30893705	32			
Total	0.67043205	36			

Table 3- 4: ANOVA table for the general linear model testing the effect of stress treatment and A.) foliar nitrogen (% dry mass), B.) foliar phosphorous (% dry mass), or C.) foliar potassium (% dry mass) on mass-specific exudation. The interaction term for each model (main effect x covariate) was not significant and was dropped from the analysis.

A.)

Source of variation	Sum of squares	*df*	Mean square	*F*	*p*
Stress treatment	0.336	3	0.112	9.64	<0.001
Foliar N	0.001	1	0.001	0.05	0.828
Residual	0.709	32			
Total	1.045	36			

B.)

Source of variation	Sum of squares	*df*	Mean square	*F*	*p*
Stress treatment	0.327	3	0.109	10.14	<0.001
Foliar P	0.028	1	0.028	2.64	0.114
Residual	0.709	32			
Total	1.063	36			

C.)

Source of variation	Sum of squares	*df*	Mean square	*F*	*p*
Stress treatment	0.305	3	0.102	8.78	<0.001
Foliar K	0.002	1	0.002	0.15	0.701
Residual	0.709	32			
Total	1.016	36			

Table 3- 5: ANOVA table for the general linear model testing the effect of stress treatment and A.) photosynthetic rate (μmol m^{-2} s^{-1}) or B.) total carbon assimilation rate (μmol s^{-1})on mass-specific exudation. The interaction term for each model (main effect x covariate) was not significant and was dropped from the analysis.

A.)

Source of variation	Sum of squares	*df*	Mean square	*F*	*p*
Stress treatment	0.18424	3	0.0614	5.53	0.004
Photosynthesis	0.0163	1	0.0163	1.47	0.235
Residual	0.20053	32			
Total	0.67043	36			

B.)

Source of variation	Sum of squares	*df*	Mean square	*F*	*p*
Stress treatment	0.18806	3	0.0627	5.86	0.003
Carbon assimilation	0.02931	1	0.0293	2.74	0.108
Residual	0.21736	32			
Total	0.67043	36			

Table 3- 6: Isotopic signatures ($\delta^{13}C$) of the fine roots and exudates in *Populus tremuloides* seedlings grown in four treatments, cold soils (n=4), shade (n=6), drought (n=5), and control (n=6). All $\delta^{13}C$ results are expressed relative to the VPDB (Vienna Pee Dee belemnite) standard. There were no significant differences in isotopic signatures in either fine roots ($F_{(3, 32)}$=1.87; p=0.16) or exudates ($F_{(3, 36)}$=1.82; p=0.16). All values are presented as mean (standard error).

	Cold soil	**Shade**	**Drought**	**Control**
Fine root $\delta^{13}C$ (‰)	136 (31.0)	159 (21.1)	98 (11.6)	135 (7.9)
Exudate $\delta^{13}C$ (‰)	20 (5.1)	21 (9.9)	17 (4.7)	39 (8.0)

Figures

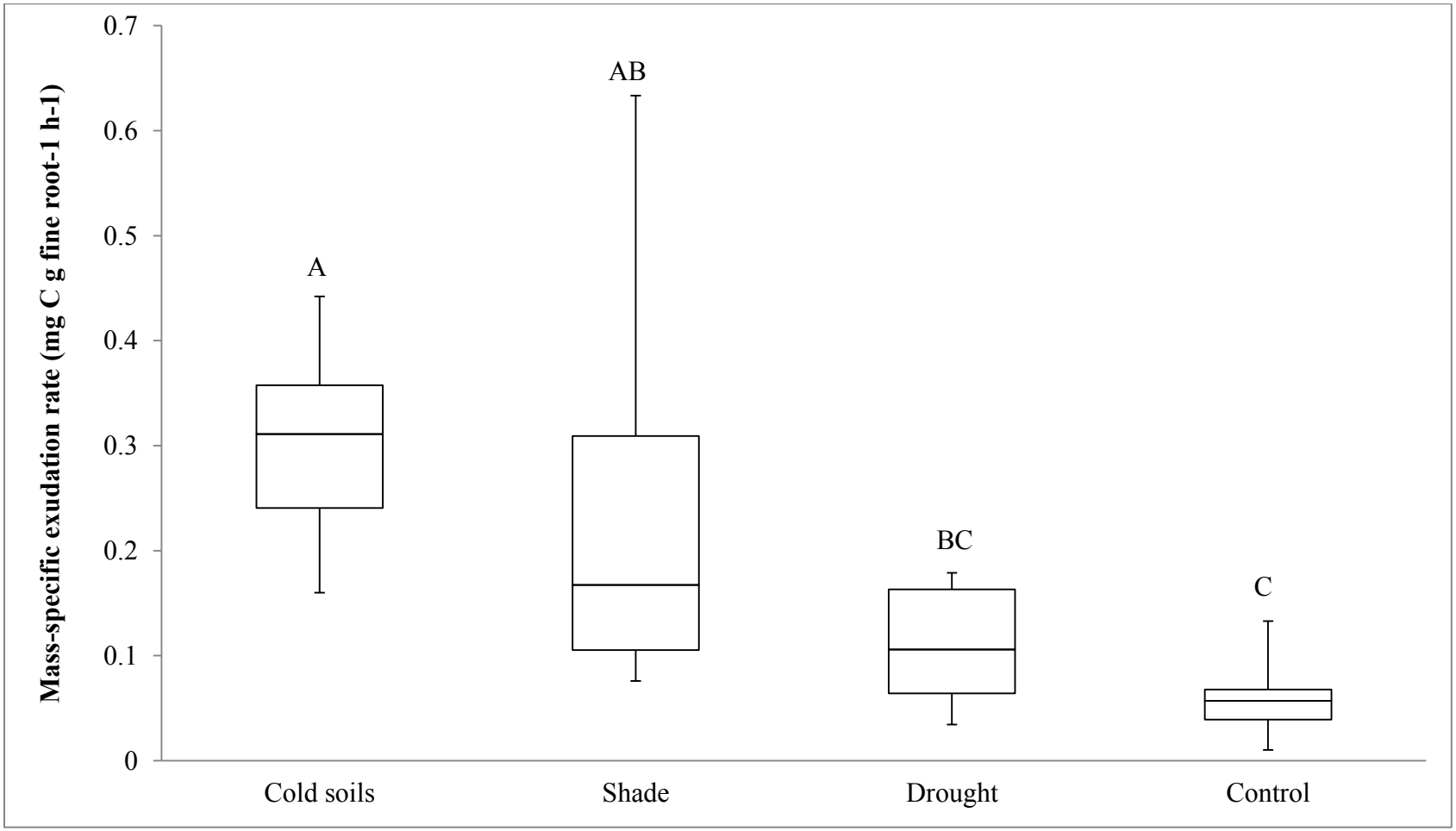

Figure 3- 1: Mass-specific exudation rates of *Populus tremuloides* seedlings grown in four treatments: cold soils (n=7), shade (n=8), drought (n=8), and control (n=10). The line in the box indicates the median value, the upper and lower and upper edge of the box are the 75th and 25th percentile, and the error bars are the maximum and minimum values. Significant differences between treatment means are denoted by lettering above the bars (α=0.008).

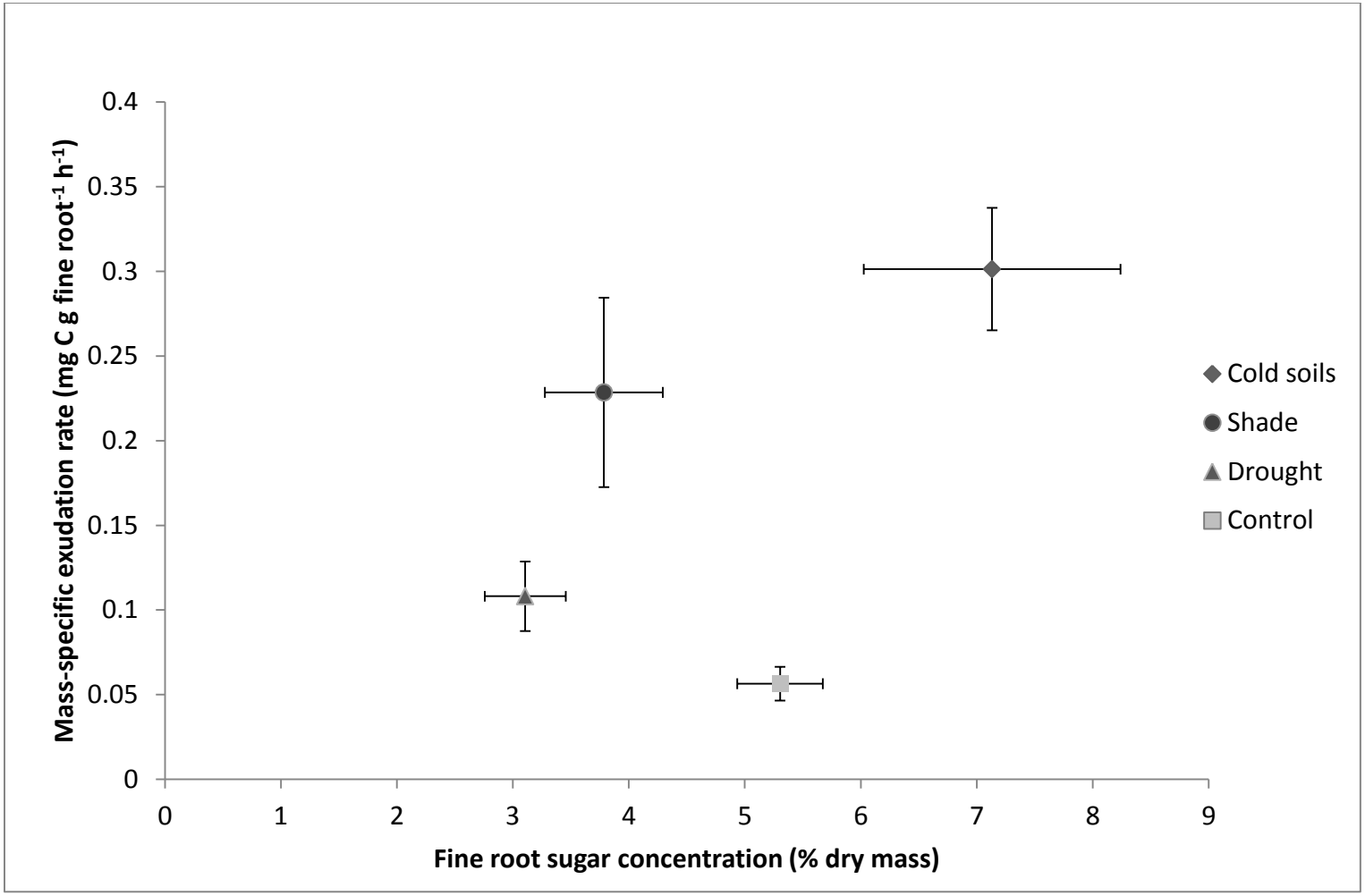

Figure 3- 2: Mass-specific exudation rates by fine root sugar concentrations of *Populus tremuloides* roots grown in four treatments: cold soils (n=7), shade (n=8), drought (n=8), and control (n=10). Vertical lines are the standard error of the mean for mass-specific exudation rate; horizontal lines are the standard error of the mean for fine root sugar concentration.

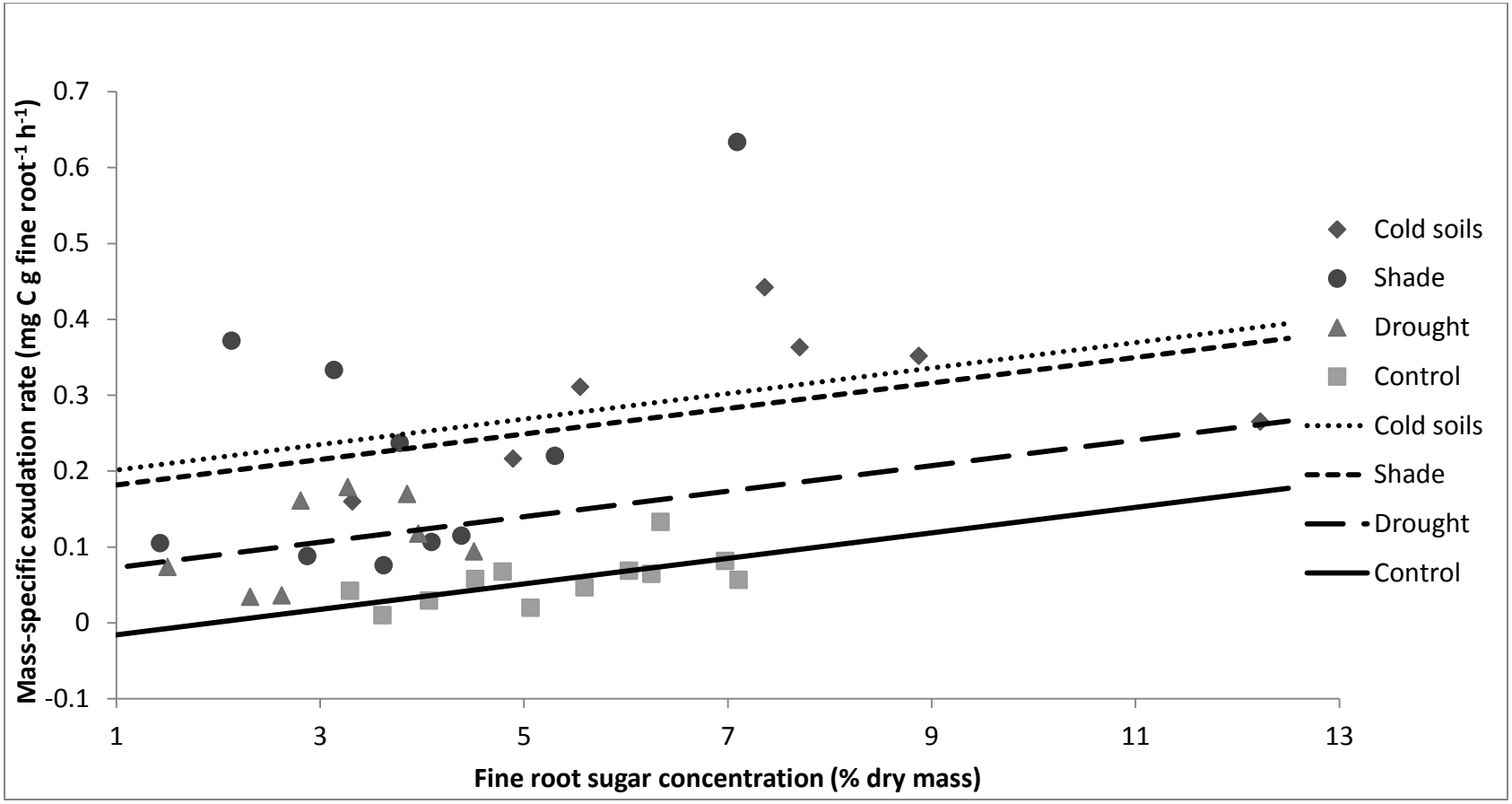

Figure 3- 3: Mass-specific exudation rates of *Populus tremuloides* roots grown in four treatments: cold soils (n=7), shade (n=8), drought (n=8), and control (n=10). There was no effect of the interaction between treatment and sugar concentrations on exudation rates (p=0.32), so the variable was dropped from the model and the common slope was used. Lines represent the relationship between exudation and the concentration of sugars in the fine roots for each treatment. The model has an r^2 value of 0.56 and the effects of both treatment ($F_{(3,32)}$=10.31; p<0.001) and sugar ($F_{(1, 32)}$=6.51; p=0.016) were significant. The intercept of the relationship between fine root sugar and exudation is significantly lower in seedlings grown in the control treatment than seedlings grown in cold soils (p<0.001) or shade (p<0.001).

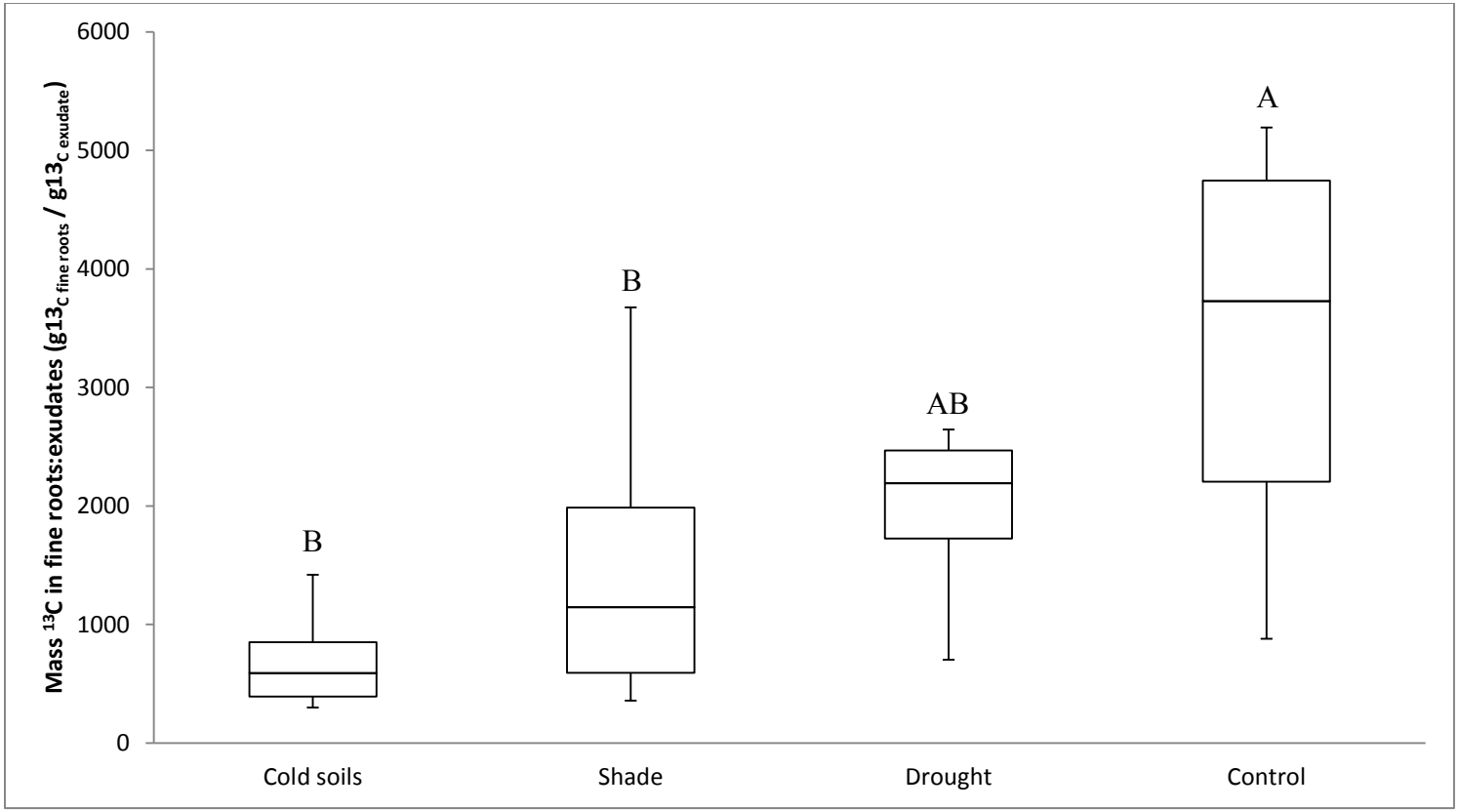

Figure 3- 4: The ratio of surplus ^{13}C in the fine roots (see materials and methods) to surplus ^{13}C in the exudates of *Populus tremuloides* seedlings grown in four treatments: cold soils (n=7), shade (n=8), drought (n=8), and control (n=10). The line in the box indicates the median value, the upper and lower and upper edge of the box are the 75th and 25th percentile, and the error bars are the maximum and minimum values. The ratio was significantly affected by treatment ($F_{(3,29)}$=5.49; p<0.001). Seedlings in the control treatment had a significantly higher ratio of surplus ^{13}C in roots:exudates than seedlings grown in colds soil (p<0.001) or shade (p=0.04), as well as marginally more than seedlings grown in drought (p=0.07); seedlings grown in drought also had a significantly higher ratio than seedlings grown in cold soils (p=0.004). This effect was observed one week after removal from the stress treatments. Different letters denote significant differences between treatments (α=0.008).

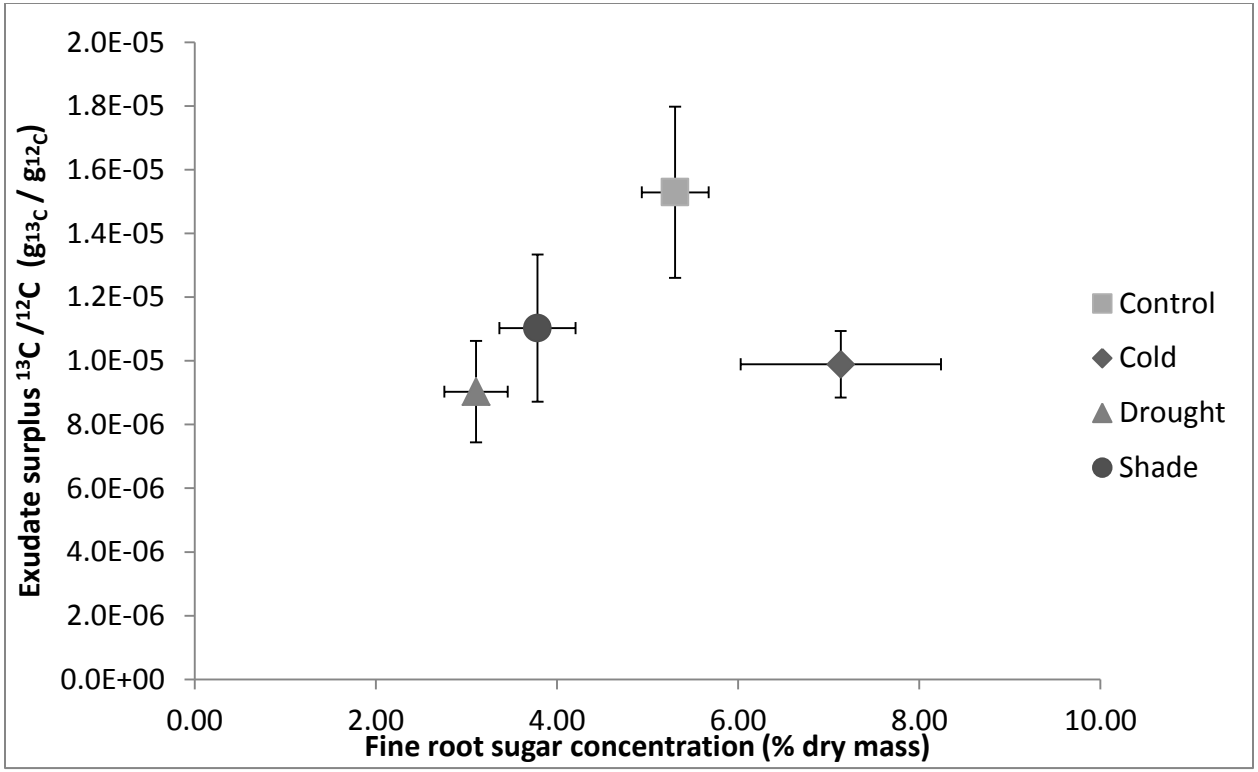

Figure 3- 5: The ratio of surplus ^{13}C labelled carbon to ^{12}C unlabelled carbon in the exudates by fine root sugar concentrations of *Populus tremuloides* roots grown in four treatments: cold soils (n=7), shade (n=8), drought (n=8), and control (n=10). The surplus ^{13}C label is ^{13}C recovered in excess of atmospheric ^{13}C. Vertical lines are the standard error of the mean for the isotopic carbon ratio; horizontal lines are the standard error of the mean for fine root sugar concentration.

Chapter 4: General Discussion and Conclusions

4.1 Research summary

Restoration of forests in the boreal biome following landscape-level disturbances such as surface mining requires an ecological approach to understanding the relationship between vegetation and soils (Macdonald et al. 2012). A common tree species used in boreal forest restoration is trembling aspen (*Populus tremuloides* Michx.), an early successional species which is fast growing and exhibits an indeterminate growth pattern. An important feature in the survival and growth of aspen is the development of relationships with ectomycorrhizal fungi (EMF). Ectomycorrhizal fungi are soil fungi which form symbiotic associations with tree roots, linking trees to the soil; a pivotal role that may be a good indication of ecological function (Itoo and Reshi 2013). In this relationship between EMF and trees, the host provides a supply of carbon in exchange for a wide array of benefits derived from the presence of the fungi, including increased nutrient and water uptake, increased resistance to disease and pathogens, and stress tolerance (Smith and Read 2008). In addition to supplying carbon for fungal growth and development, plant roots release other carbon compounds which influence the chemoattraction, establishment, and development of EMF (Martin et al. 2007). The release of these compounds, known as root exudation, is determined by interacting biotic and abiotic factors between plants and soil, including soil nutrient availability, soil physical and chemical properties, environmental conditions, root carbon (C) supply, and plant morphology (Jones et al. 2004). The establishment of EMF after landscape-level disturbances is influenced by both plant traits (species identity, physiological status), and soil characteristics (pH, salinity, water holding capacity, etc.) as well as the availability of ectomycorrhizal propagules (Martin et al. 2007). Operational forest restoration practices include altering seedling nutrition prior to planting and using various cover

soils, which likely impact interactions between outplanted tree seedlings and EMF (Quoreshi and Timmer 1998; Bois et al. 2005); however, the repercussions of these practices are largely unknown.

The objective of my thesis was to determine the influence of aspen internal plant nutrition and carbon reserves on interactions with EMF in the context of boreal forest restoration. Towards this objective, I performed a field study on a recently reclaimed mine site in which I examined the influence of aspen nutrition and the type of cover soil into which aspen seedlings were planted on the ectomycorrhizal community. I then proceeded with a growth chamber experiment examining the effects of aspen root non-structural carbohydrate reserves on root exudation of organic compounds.

In the field study, I examined the ectomycorrhizal community present on the roots of aspen seedlings two growing seasons following planting in a reclaimed area that was previously surface-mined. Seedlings were planted into two different salvaged cover soils which differed in initial vegetative community as well as physical soil characteristics. The first cover soil was a salvaged upland forest floor material (FFM) which consisted of the L, F, H, and A soil horizons from soil whose plant community prior to salvaging included aspen. The second cover soil was a lowland peat-mineral mix (PMM), a mixture of inorganic mineral soil and peat, whose plant community prior to salvaging did not include aspen. Half of the seedlings which were planted had tissue nutrient and carbon levels altered in an effort to improve outplanting success (Schott et al. 2013). Seedlings were categorized according to tissue nutrient levels: high feed and standard feed seedlings: high feed seedlings were supplied with twice the fertilizer given to normal feed seedlings in the greenhouse prior to outplanting. This treatment increased the tissue nutrient reserves (nitrogen, phosphorous, potassium, and micronutrients) of the high feed

seedlings relative to standard feed seedlings, and altered the concentrations of non-structural carbohydrate (NSC) reserves. I examined the roots of aspen for ectomycorrhizal fungal abundance and species diversity for each feed type and cover soil material. I predicted that seedlings with initially lower nutrient reserves would have greater ectomycorrhizal fungal abundance and species richness compared to seedlings with higher nutrient reserves, as lower nutrient reserves should lead to a greater necessity for and facilitation of the establishment and development of the ectomycorrhizal symbiosis (Richards and Wilson 1963; Johnson et al. 1997). As *P. tremuloides* is an upland boreal forest species, I also predicted that seedlings grown in the upland soil material, FFM, would have higher colonization than those grown in PMM, as the legacy of the plant community in FFM includes aspen.

The results of the field study suggest that the seedling nutrient and carbon status at the time of planting will affect colonization by EMF more than the cover soil into which they are planted; however, both feed and soil type may have an effect on the abundance of individual species. I found that high feed seedlings had greater colonization than standard feed seedlings. Neither total colonization nor species richness of EMF was affected by cover soil type. I found four EMF species at the site: *Cenococcum geophilum*, *Hebeloma leucosarx*, *Meliniomyces bicolor*, and *Thelephora terrestris*. Both total colonization and species richness across the site were low, which may be attributable to the young age of the site, proximity to propagule sources, and the fact that the soils were stockpiled prior to placement on site (Bois et al. 2005). When examining the colonization of one of the species, *C. geophilum*, among treatments, cover soil type influenced relative colonization, with more roots of seedlings grown in FFM being colonized by *C. geophilum* than those grown in PMM. Feed type determined the extent of EMF colonization on aspen seedlings; however, the differences between feed types in seedling

nutrition which existed at the time of planting had disappeared after two growing season. Thus, higher colonization of high feed seedlings was a legacy effect of the nutrient loading of seedlings. The specific mechanism responsible for the increased colonization was unclear but differences in initial seedling nutrient or carbon reserve status between feed types may have affected the exudation of carbon compounds and subsequent development of the EMF community.

In the second study I tested how aspen root non-structural carbohydrates (NSC) reserve status influenced the exudation of organic compounds. I exposed aspen seedlings to three environmental stressors known to alter root NSC reserves: cold soil temperatures (*cold*), decreased water availability (*drought*), and limited light availability (*shade*). The NSC reserve status and exudation was compared to a fourth, unstressed treatment (*control*). I predicted that NSC reserves will be modified by the type of stress treatment and that seedlings with higher root NSC reserves will have higher exudation rates of organic carbon than those with decreased root NSC reserves, due to greater disparity between sugar concentrations between root cell and rhizosphere.

The results suggest that NSC reserves determined exudation rates of organic carbon in seedlings exposed to similar environmental conditions, but the influence of NSC reserves on exudation was less than the influence of each stress type. Fine root NSC reserves were altered by stress treatment: *cold* seedlings had higher NSC concentrations than *control* seedlings, while *drought* and *shade* seedlings had lower NSC concentrations than *control* seedlings. Exudation rates of organic carbon also varied among treatments; mass-specific exudation rates of organic carbon were higher in *cold* and *shade* seedlings than in *drought* or *control* seedlings. There was no relationship between NSC reserves and exudation of organic carbon when examined across

all four treatments. When examining the relationship between carbon storage and exudation within each treatment, mass-specific exudation rates of organic carbon were linearly dependent on the concentration of NSC reserves, particularly sugar, present in the fine roots. Moreover, the relationship was similar across all treatments, with higher sugar concentrations resulting in higher exudation rates of organic carbon. Differences in the proportion of newly acquired carbon allocated to roots relative to exudates suggests that carbon allocation priorities differed among treatments. Taken together with the fact that there was no relationship between carbon storage and the exudation of organic compounds when examined among treatments, it suggests that the influence of other factors specific to each stress type, such as disruptions in membrane permeability in seedlings exposed to cold, was greater than NSC reserves in determining exudation rates.

4.2 Research synthesis and implications for forest restoration

The nutrient loading of aspen seedlings may improve seedling growth and development in areas following landscape-level disturbance due to increased access to EMF (van der Heijden et al. 1998; Onwuchekwa et al. 2014). The first experiment demonstrated that the nutrient loading of aspen seedlings had left a legacy of increased EMF colonization on the roots of high feed seedlings. Seedlings with more root tips colonized by EMF may have better nutrient acquisition (Read and Perez-Moreno 2003), increased resistance to pathogens (Newsham et al. 1995), higher stress tolerance (Andersen and Rygiewicz 1991), and enhanced growth and survival (Gebhardt et al. 2007) compared to seedlings with fewer colonized root tips. This may give nutrient loaded seedlings an advantage over non-loaded seedlings. However, despite the fact that colonization was higher on high feed than standard feed seedlings, it was still low when

compared to roots from undisturbed areas, which may limit the benefits of enhanced symbiosis for high feed seedlings (Dickie 2007). Moreover, only one sampling of the EMF community is likely not sufficient for drawing conclusions regarding the influence that the ectomycorrhizal community will have on the development of the forest community.

The nutrient loading of aspen seedlings may also improve the recovery of the EMF community following landscape-level disturbances. The host nutritional quality influenced the abundance of root tips colonized by EMF; specifically, nutrient loaded seedlings had higher colonization than those which were not loaded. While EMF abundance was increased by the nutrient loading of the host, increasing the diversity of host species planted into disturbed areas has been shown to increase the diversity of EMF species recovered, due to many EMF exhibiting strong host specificity (Massicotte et al. 1999; Ishida et al. 2007, Hankin et al. in press). Therefore, this research suggests that the planting of nutrient loaded seedlings in combination with a diversity of ectomycorrhizal hosts into highly disturbed sties will best improve the recovery of EMF.

The exudation of organic carbon may have differed between feed types and influenced EMF establishment. Seedling feed types in the first experiment differed in initial concentrations fine root NSC reserves, with sugar concentrations being lower and starch concentrations being higher in high feed than standard feed seedlings, though there was no difference between feed types in the total concentration of NSC reserves. From the second experiment, the exudation rate of organic carbon in seedlings was linearly dependent on fine root sugar concentrations when seedlings had been exposed to the same environmental conditions. Thus, standard feed seedlings may have had increased exudation of carbon compounds resulting from elevated fine root sugar concentrations. However, as carbon compounds in root exudates are important for the attraction

and establishment of EMF (Broeckling et al. 2008), this suggests that exudation of carbon compounds would have been higher for high feed seedlings. In the second experiment, when seedlings had different morphological characteristics and carbon allocation priorities resulting from exposure to different stressors, the influence of other treatment-specific factors, not root sugar concentrations, determined the exudation rates of organic carbon. In the first experiment, while seedlings of both feed types were exposed to the same light, temperature, and precipitation conditions, seedling morphology and carbon allocation varied between feed types, evidenced by high feed seedlings allocating more carbon to above- and below-ground growth than standard feed seedlings (see Schott 2013). Thus, the influence of factors relating to differences in seedling morphology or carbon allocation between feed types may have exceeded the influence of root sugar concentrations in determining the exudation of organic carbon.

The growth chamber experiment is the first study directly identifying a link between root C storage and exudation of organic carbon. The results suggest that abiotic factors which affect plant carbon storage processes will also indirectly influence the amount of organic carbon released to the rhizosphere. Alterations in the exudation of organic carbon may have ramifications for the plant through changes to the microbial community, as the growth and development of not only mycorrhizal fungi but all rhizosphere biota, including pathogenic bacteria and fungi, have the potential to be influenced by organic carbon availability (Lynch 2007). However, further study is needed at the individual plant level to determine the relationship between C storage and exudation in more ecologically relevant conditions before its effects on microbial populations can be determined.

4.3 Experimental limitations and future research suggestions

In the first research chapter, no data on the ectomycorrhizal community was collected at either the time of planting or after the first season of growth. This limits the conclusions which can be drawn as it cannot take temporal changes in the EMF community into account (Dickie et al. 2013). Repeated site sampling would provide further information on the effects of cover soil on the development of the EMF community over time. Moreover, without data on EMF species present on the seedlings prior to planting, it is difficult to determine whether fungi were recruited at the site itself (either from viable propagules in the soil or through dispersal onto the site) or the fungi were imported from the greenhouse. In future studies, it is important to ensure that EMF species present prior to outplanting are identified.

Difficulties resulting from finding multiple operational taxonomic units (OTUs) matching individual fungal morphotypes prevented analysis of the relative EMF community composition according to feed and soil type, with the exception of root tips colonized by *Cenococcum geophilum*, which is easily identifiable due to its distinct radiate mantle morphology (LoBuglio 1999). The abundance of the other three ectomycorrhizal OTUs found at the site, *Hebeloma leucosarx, Meliniomyces bicolor*, and *Thelephora terrestris*, is unable to be compared due to two fungal OTUs being attributed to each morphotype. The findings presented here could be strengthened by examining the effects of seedling nutrition and cover soil type on relative EMF species abundance following reclamation.

In the second research chapter, the presence of the peat core during exudate collection increased the amount of organic carbon in the exudate solution, as carbon from the core would be flushed from the pots along with exudates (Freeman et al. 2001). The additional C derived from peat cores was accounted for by the inclusion of a blank (no seedling present) peat core;

however, the total organic carbon (TOC) found in blank peat cores will likely be higher than the total TOC derived from the peat cores containing seedlings, as seedling roots may prevent some erosion of organic material during exudate collection. However, the use of peat cores was required since attempts to germinate aspen in the sand were unsuccessful.

Transplant shock, which likely occurred when root systems were exposed to air, washed, and treated with an antimicrobial solution during transplant, may have also affected exudation rates (Norby et al. 1987). However, as the sand has greater adsorption of low molecular weight molecules, transplanting into glass beads was necessary to prevent underestimation of exudation rates (Phillips et al. 2009). Current methods to collect exudates without the need to transplant seedlings are limited. For example, hydroponic systems circumvent the need to transplant but remove the mechanical impedance to root growth provided by the soil, creating unnaturally structured root systems (Oburger et al. 2014). In addition, several mechanisms have been designed to collect exudates under more natural conditions (Phillips et al. 2008; Karlsson et al. 2012); however, they were too expensive and time-intensive for such a large-scale experiments.

Seedlings subjected to stress treatments were supplied with differing amounts of fertilizer, an artifact of the experimental design. *Control* and *shade* seedlings received 0.5 grams of 20-20-20 NPK fertilizer twice a week, while *drought* and *cold* seedlings received 0.15 grams twice a week, as the fertilizer was applied during watering. Thus, the nutrient status of seedlings varied significantly at the time of exudate collection. While the nutrient levels of plant macro nutrients (NPK) did not have a direct influence on exudation rates, variations in nutrient levels of other plant nutrients which were not measured (sulfur, micronutrients) may be contributing to observed variation in exudation rates (Neumann and Romheld 2007).

As this is the first study to examine the effects of plant carbon storage on exudation, the interactions between stored C and other factors affecting exudation processes (see chapter 3) should be further examined to determine the importance of root C storage for exudation processes in natural rhizosphere conditions. New sampling techniques allowing for the collection of exudates without the removal of the soil microbes as well as exudate measurements on fully grown trees can improve the accuracy and relevance to exudation under field conditions (Phillips et al. 2008; Shi et al. 2012). As methodologies for examining root exudation are constantly improving, there is significant potential for future research to increase understanding of the regulation of mechanisms controlling exudation.

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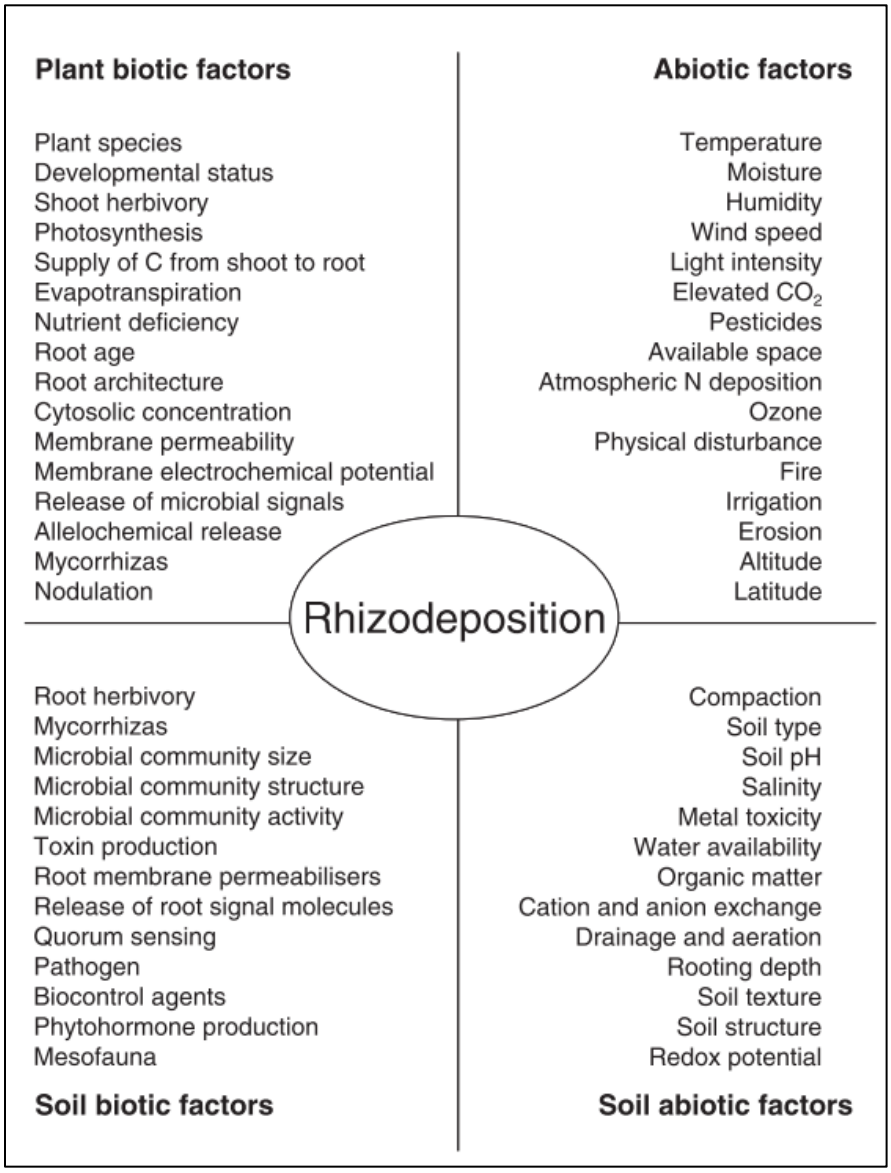
Appendices



Appendix figure 1: A list of the abiotic and biotic soil, plant, and environmental factors affecting the release of organic compounds into the rhizosphere, taken from Jones et al. (2004)**.**

Appendix table 1: One-way ANOVA of *F* and *p*-values on the effect of stress treatment on morphological, physiological, and root carbon reserve characteristics of *Populus tremuloides* seedlings sampled prior to the acclimation period ("Pre") and after exudate collection ("Post"). Degrees of freedom (df) are presented as numerator, denominator.

Characteristic		*df*	*F*	*p*
Height	Pre	3,17	3.20	0.049
	Post	3,33	8.79	<0.001
Leaf area	Pre	3,17	6.00	0.006
	Post	3,33	8.83	<0.001
Photosynthesis	Pre	N/A	N/A	N/A
	Post	3,33	5.04	0.006
Stomatal conductance	Pre	N/A	N/A	N/A
	Post	3,33	0.86	0.47
Leaf mass	Pre	3,17	9.91	<0.001
	Post	3,33	18.7	<0.001
Specific leaf area	Pre	3,17	27.6	<0.001
	Post	3,33	25.6	<0.001
Stem mass	Pre	3,17	21.5	<0.001
	Post	3,33	50.8	<0.001
Fine root mass, outside core	Pre	N/A	N/A	N/A
	Post	3,33	36.8	<0.001
Total root mass	Pre	3,17	31.6	<0.001
	Post	3,33	31.5	<0.001
Root to shoot ratio	Pre	3,17	11.2	<0.001
	Post	3,33	8.43	<0.001
Root density	Pre	N/A	N/A	N/A
	Post	3,32	2.63	0.067
Foliar nitrogen	Pre	N/A	N/A	N/A
	Post	3,33	9.51	<0.001
Foliar phosphorous	Pre	N/A	N/A	N/A
	Post	3,33	0.33	0.81
Foliar potassium	Pre	N/A	N/A	N/A
	Post	3,33	2.69	0.06

Total fine root sugar	Pre	3,17	21.1	<0.001
	Post	3,33	19.66	<0.001
Total fine root starch	Pre	3,17	7.26	0.002
	Post	3,33	8.68	<0.001
Total fine root NSC	Pre	3,17	12.0	<0.001
	Post	3,33	12.2	<0.001
Fine root sugar	Pre	3,17	5.33	0.009
	Post	3,33	8.22	<0.001
Fine root starch	Pre	3,17	3.41	0.041
	Post	3,33	2.32	0.09
Fine root NSC	Pre	3,17	4.54	0.016
	Post	3,33	4.23	0.012