Effects of Ericoid Mycorrhizal Fungi on Growth and Salt Tolerance of Blueberry (*Vaccinium myrtilloides*), Lingonberry (*Vaccinium vitis-idaea*), and Labrador tea (*Rhododendron groenlandicum*): Implications for Oil Sands Reclamation

by

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Abstract

Oil sands mining can have negative environmental impacts in the boreal forest region of northern Alberta, Canada. The Government of Alberta requires the companies to reclaim disturbed lands after mine sites are closed. The initial steps in oil sands mining operations involve the removal of forest vegetation and the topsoil, which is commonly stockpiled for several years. Stockpiling can have negative impact on the reclaimed oil sands soils through decreasing the composition and abundance of soil microorganisms and changing soil physical and chemical properties. Moreover, the presence of high concentration of dissolved Na^+ and Cl^- in the soil can negatively affect plant growth in the reclamation areas. This thesis project consists of two research studies. In the first study, I examined the ericoid mycorrhizal fungi (ERM) colonization potential between fresh and one-year stockpiled topsoil from the oil sands reclamation site. I also examined effects of the topsoil on growth and physiological parameters in blueberry (*Vaccinium myrtilloides*), Labrador tea (*Rhododendron groenlandicum*) and lingonberry (*Vaccinium vitis-idaea*) plants. Using the DNA identification method, I found that ERM colonization potential of fresh topsoil is higher than that of the topsoil that was stockpiled for one year. My results also demonstrated that blueberry and Labrador tea plants growing in fresh topsoil had higher root and shoot dry weights and higher root colonization intensity compared to the stockpiled topsoil. Concentrations of leaf chlorophyll, P, and N in blueberry plants were also lower in the stockpiled topsoil compared to the fresh soils. Soil stockpiling did not affect net photosynthesis (Pn) and transpiration rates in any plant species. The results of this experiment indicate that the stockpiled soil was less suitable as a growth substrate compared with the fresh soils for the growth of some ericaceous plants.

In the second study, blueberry, Labrador tea and lingonberry plants were inoculated with *Oidiodendron maius* (Om) *and Meliniomyces variabilis* (Mv) ERM fungi and subjected to none (control) or 30 mM NaCl treatments for four weeks. In control blueberry and lingonberry, Pn was significantly higher in plants inoculated with *O. maius* compared with the non-inoculated plants. The 30 mM NaCl treatment decreased Pn and this decrease was greater in non-inoculated plants compared with those inoculated with both ERM fungi. In all three plant species, Pn of the non-inoculated plants subjected to NaCl treatment was reduced to negative values. Control blueberry plants that were inoculated with *O. maius* had higher total plant dry weights compared with non-inoculated plants. In Labrador tea, inoculation with *M. variabilis* resulted in the highest total plant dry weights. The inoculation of lingonberry plants with both species of ERM fungi prevented the total dry weight reduction by the NaCl treatment. In all three plant species, both inoculation treatments resulted in higher leaf chlorophyll concentrations compared with non-inoculated plants. The overall effect of ERM inoculation on NaCl tolerance was especially pronounced in lingonberry.

The results of both studies point to the importance of ERM for the revegetation of oil sands reclamation sites with some ericaceous plants.

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List of abbreviations

AMF	arbuscular mycorrhiza fungi
EMF	ectomycorrhizal fungi
ERM	ericoid mycorrhizal fungi
BLAST	basic local alignment sequence tool
CTAB	cetyltrimethyl ammonium bromide
ITS	internal transcribed sequence
LSD	least square difference
MBSU	molecular biology services unit
NCBI	National Centre for Biotechnology Information
Al	aluminium chemical element
Ca	calcium chemical element
Cl	chloride chemical element
cm	centimetre
CO_2	carbon dioxide
Cu	copper chemical element
DMSO	dimethyl sulfoxide
µS/cm	microsiemens per centimeter
E	transpiration rate
EC	electric conductivity
Fe	iron chemical element
K	potassium chemical element
B	boron chemical element
Hg	mercury chemical element
km_2	square kilometre
KOH	potassium hydroxide
m	meter
ANOVA	analysis of variance
mg	milligram

mg g^{-1}	milligram per gram
Mg	magnesium chemical element
mL	milliliter
mM	millimolar
Mn	manganese chemical elements
N	nitrogen chemical element
Na	sodium chemical element
NaCl	sodium chloride
PCR	polymerase chain reaction
nm	nanometer
NO_3^-	nitrate ion
P	phosphorus chemical element
pH	power of hydrogen
Pn	net photosynthesis rate
PO_4^{3-}	phosphate ion
PPFD	photosynthetic photon lux density
Zn	zinc chemical element
Ni	nickel chemical element
Co	cobalt chemical element
Cd	cadmium chemical element
Pb	Lead chemical element
w/v	weight/volume
NH_4^+	ammonium
NH_4^+-N	ammonium-nitrogen
$CuSO_4$	copper sulphate
$ZnSO_4$	zinc sulfate
H_2SO_4	Sulfuric acid

Chapter 1

1.1 General Introduction

The initial steps in oil sands mining operations involve the removal of forest vegetation and the soil, which is commonly stockpiled for one to ten years until all mining operation are completed. After finishing the oil sands mining operation, these stored soils are used to reclaim the disturbed areas and revegetate them with native plants. During stockpiling chemical and physical properties as well as microbial communities of the soil may be affected by different factors including the earthmoving equipment and anaerobic conditions (Strohmayer 1999). Since soil factors are crucial for plant growth, any changes in soil properties may have a significant impact on successful reestablishment of vegetation in reclaimed sites. The effect of stockpiling on soil mycorrhization potential has been identified among the key concerns for plant establishment and growth (Abdul-Kareem & McRae 1984; Strohmayer 1999).

Soil salinity is a common problem impacting plant growth and productivity across the world. Around 20% of cultivated and 33% of irrigated soils of the world are salt-affected (Shrivastava & Kumar 2015). Human activities including mining can discharge high salt contaminants in some areas, which may also increase and negatively affect growth of native plants. In oil sand mining areas, high concentrations of sodium, sulfate, bicarbonate, and chloride can be present in reclaimed areas (Renault et al. 1999).

Developing salt-tolerant plant species and increasing the adaptability of plants to saline environment have been an important research topic for many decades. However, until now, only a few major-determinant genetic traits of salt tolerance have been identified (Flowers 2004; Munns & Tester 2008; Schubert et al. 2009). Introducing soil microorganisms including mycorrhizal fungi

is an economical and environmentally friendly approach to improve salt tolerance in many plant species (Dodd & Pérez-Alfocea 2012).

Mycorrhizal fungi are able to alleviate salt stress in their host plant species. Arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (EMF) are able to prevent the excess uptake of Na^+ in plants under salinity condition (Muhsin & Zwiazek 2002; Hashem et al. 2015). However, a little is known about the effectiveness of the ericoid mycorrhizal fungi (ERM) in protecting the ericaceous plants against the effects of salt. Salt stress-induced changes in plants inoculated with AMF can be also reduced through the active role of these fungi in the antioxidant defense system of plants (Hashem et al. 2015).

Ericoid shrubs including *Vaccinium myrtilloides, Vaccinium vitis-idaea*, and *Rhododendron groenlandicum* can form symbioses with ERM (Read 1996; Smith & Read 2008). One of the most important benefits of ERM symbioses with ericaceous plants is increasing N uptake (Read & Stribley 1973). It has been reported that ERM may take up ammonium or nitrate or organic N compounds such as amino acids and peptides and turn them to the accessible form of N for their host plants (Mitchell & Gibson 2006).

In this thesis I examined the effects of topsoil (fresh vs. one-year stockpiled topsoil) from the boreal forest sites on three ericaceous plants species including *V.myrtilloides, V.vitis-idaea* and *R.groenlandicum* and also determined ericoid mycorrhizal colonization potential in these soil types. I also studied the effects of two ERM fungi *Oidiodendron maius* (Om) and *Meliniomyces variabilis* (Mv) on salt tolerance of the above ericaceous plant species, which are often used for oil sands reclamation. The objectives of the present study were to:

1) Determine whether ericoid mycorrhizal colonization potential of the topsoil can be affected by stockpiling storage.

2) Examine the effects of fresh topsoil and one-year stockpiled topsoil of boreal forest on growth and physiological responses (gas exchange, shoot and root dry weight, chlorophyll content, and plant leaf elements) three ericaceous plant species.
3) Evaluate the effects of two ERM on growth and physiological responses of three different boreal forest plant species.
4) Investigate whether ERM fungi increase salt tolerance in some ericaceous plants.

I tested the following hypotheses:

1) ERM colonization potential of the topsoil is decreased by stockpiling storage, probably due to the anaerobic conditions within the soil piles and/or mechanized handling during soil removing, stripping and stockpiling.
2) The growth of plants in fresh topsoil is higher than in the one-year stockpiled topsoil, likely due to the greater ERM fungi colonization of their roots.
3) Inoculation of ericaceous plants with ERM fungi improves plant salt tolerance, probably by increasing nutrient and water uptake in these plant species.

1.2 Literature Review

1.2.1 Biology of blueberry (Vaccinium myrtilloides)

Blueberry is a perennial, deciduous or evergreen shrub that can grow up to approximately one m in height (Camp 1945). This rhizomatous plant can produce an extensive network of roots and woody rhizomes (Shubat 1983). The diameter of subsidiary roots can vary from 0.1-0.5 mm (Barker et al. 1964). Roots and rhizomes of this species have a long and tapering structure and can penetrate the soil as deep as one m (Hall 1957). Ovoid, brown and rugose blueberry seeds are approximately 1 mm in length. This shrub has thin velvet elliptic leaves, small and short clusters of greenish white to pink flowers at branch tips (Vander & Hall 1981). In some plants, the tap root is present and can reach a diameter of up to 10 mm (Hall 1957; Smith 1962). Velvety pilose branches (Shubat 1983), green or brown color twigs (Vander 1988), and dirty brown or green bark (Vander & Hall 1981) are other characteristic morphological features. Flowers of blueberries appear in terminal or lateral racemes (Vander & Hall 1981). Blueberry can adapt to a wide range of climatic conditions (Vander & Hall 1981), from dry to sub humid and temperate climates (Vander & Hall 1981; Klinka et al. 1989), and is found in most of Canada (Vander & Hall 1981). Acidic and infertile soils, which are poor in essential elements, can provide favorable growth conditions for many *Vaccinium* species (Korcak 1988). Blueberry can grow on various soil types including well-drained coarse, or light-textured soils and is commonly found in fine sandy soils, loam, clay loam, till, and lacustrine deposits (Jameson 1961; Corns 1983; Maillette 1988). Optimal pH range for these plants is from 3.0 to 5.9 (Hancock & Draper 1989; Jeglum 1971). Germination of *V. myrtilloides* seeds occurs sporadically (Vander 1983).

1.2.2 Biology of lingonberry (Vaccinium vitis-idaea)

Lingonberry is one of the *Vaccinium* species that typically grow in dense rhizomatous colonies (Vander 1988). This plant species is a creeping and evergreen shrub with slender stems (Hall & Shay 1981) and can grow to 15 cm in height (Robuck 1985). This rhizomatous plant can form a network of fine, shallow, fibrous roots, often without a taproot (Fernqvist 1977; Hall & Shay 1981). The maximum depth of the root system of 28 cm has been reported for this plant (Holloway 1981; Strong & LaRoi 1986). Plants have thick obovate, oblong, or elliptic leaves (Hall & Shay 1981; Robuck 1985). In this plant species, single or groups of up to 15 flowers appear on terminal racemes (Robuck 1985). The size of bright to dark red and round fruits of this lingonberry can reach nearly 10 mm in diameter (Keeler 1969; Vander 1988). Also, the length of yellow and short-beaked seeds of about 1mm has been reported (Holloway 1981; Vander 1988). The average germination of fresh seeds is higher than the seeds stored for 12 to 16 months, and this value can decline from 76 percent in fresh seeds to less than 10 percent in the stored seeds (Hall & Shay 1981). It has been demonstrated that exposure to the temperatures of 0-5ºC for up to 5 months can improve the germination condition in this species (Lehmushovi 1975; Holloway 1981). Moreover, it has been reported that after exposure to the temperatures of 20-25ºC, the seeds of this plant can germinate in 3 weeks both under light and dark conditions (Fernqvist 1977; Holloway 1981). Climatic conditions would not be a big constraint to the growth of this lingonberry, because the plants can grow under various climatic regimes. For example, in Canada, lingonberry can be found in the northern forests with arctic air masses or in the harsh rock field and tussock communities affected by moist Pacific air masses and they generally can grow in the regions with long cold winter (Larsen 1971; Hall & Shay 1981). The favorable soil conditions for the growth of this lingonberry can vary from shallow, and poor mineral soil to drained peat (Kardell 1986), and from acidic sandy loams to loamy clays (Holloway 1981; Kuchko 1988). Also, favorable soil for this

plant species can be formed from some parent materials like sandstone, gneiss, granite, and glacial outwash sands and gravel (Hall & Shay 1981). Furthermore, plants can grow in a wide range of pH between 2.7 and 8.2, however, the best growth has been reported at pH 4 to 4.9. (Jeglum 1971; Hall & Shay 1981; Holloway 1981).

1.2.3 Biology of Labrador tea (Rhododendron groenlandicum)

Labrador tea is a branched, dwarf, decumbent and ascending shrub that can grow between 30 to 60 cm in height. The dark green and linear leaves of this species are covered by a dense, rust-colored fluffy layer referred to as 'felt' (Porsild 1953; Aiken et al. 2003). This plant has white and strongly aromatic flowers in umbrella-shaped clusters (Porsild 1953). Labrador tea grows across North America in different places such as bogs, wet shores or even rocky alpine slopes. (Kathleen & Walter 1990). Since Labrador tea requires acidic soil for optimum growth in these areas, it can be found in moss-lichen heathlands (Porsild 1953). The plant is believed to have some medicinal properties and its leaves have been used as a substitute for tea (Aiken et al. 2003; Karst 2010).

1.2.4 Oil sands mining

Oil sands mining activities in Northern Alberta disturb boreal forests, which must be restored. Mining companies are responsible for reclaiming the disturbed areas, which may contain various oil sands processed materials including composite tailings and tailings sands on top of which reclamation soil is added. However, it has been reported that manipulations, stockpiling, and storage of reclamation soils can reduce its quality (Danielson et al. 1983; Malajczuck et al. 1994), through reductions in mycorrhizal inoculation potential, changing in physical and chemical properties of the soil (Abdul-Kareem & McRae 1984). Oil sands deposit is a natural mixture of sand, water, clay, and bitumen. Although oil sands are present in various parts of the world, the largest oil sands deposits are in Canada and Venezuela (Government of Alberta 2009b). Athabasca, Cold Lake and Peace River oil sands in Alberta, Canada contain 1.7 to 2.5 trillion barrels of oil trapped in the complex oil sand mixture that can provide all Canada's petroleum needs for the next 475 years (Chastko 2004).

In the Athabasca region, there are two techniques to obtain oil from the oil sands. The first technique can be used for 80% of oil sands recovery in the Athabasca oil sands region, which are 400 m below ground surface. In this process, the steam is injected into the reservoirs under the ground surface through chambers, the steam then heats up the viscous oil and the heated oil is pumped to the surface. The second technique is used when the deposits are close to the surface and is referred to as surface mining. In this process, overburden layer within a maximum depth of 45 m is removed and stockpiled near to the mining pit. This stockpiled topsoil and overburden are later re-used to fill the pit during reclamation. Afterwards, the mined ore is transported to the extraction plant where it is crushed, and hot water is added to extract bitumen. Finally, the slurry

undergoes centrifugal separation to separate sand, water and bituminous layer (Government of Alberta 2009b).

1.2.5 Oil sands environment

Boreal forest of an area of 346,964 km^2 in the Northern part of Alberta has three important oil sands reserves (Athabasca, Cold Lake and Peace River) (Canadian Boreal Initiatives 2003). This large intact ecosystem is the home for a diverse flora and fauna. Additionally, many natural processes like flood mitigation, carbon sequestration, nutrient cycling, water storage, etc. can be supplied by these forests (Leatherdale 2008). Many evergreen and deciduous tree species including white spruce (*Picea glauca*), black spruce (*Picea mariana*), aspen (*Populus tremuloides*), balsam poplar (*Populus balsamifera*), jack pine (*Pinus banksiana*), balsam fir (*Abies balsamea*), and tamarack (*Larix laricina*) constitute the vegetation cover of these boreal forests (Rowe 1972). Oil sands mining activities severely disturb the lands which must be reclaimed and re-vegetated, and this process must be certified by the Alberta Environment and Sustainable Resource Development. To attain high land capability, some important indicators including plant communities, soil salinity, nutrient cycling, water holding capacity, and net primary production must be certified (Rowland 2008). Reconstructing materials such as removed topsoil and subsoil is the most important step that must be done at the beginning of reclamation process. Typically mining residuals, like the peat-mineral mix, tailing sand, topsoil, subsoil, overburden, lean oil sand and mine by-products are mixed together and used as reclamation materials (Rowland 2008; Hrudey et al. 2010). These materials must have some capabilities, such as supplying water and nutrients for plants, adequate stability against erosion, helping to develop the root system, and buffering environmental changes (Rowland 2008). Among these reclamation materials, peat-mineral mix is the most the most commonly used substrate in this process due to its high abundance in the boreal

forest in Alberta and its ability to improve organic carbon content, soil nutrients, and water holding capacity (Rowland 2008; Rowland et al. 2009).

1.2.6 Mycorrhizal fungi

Mycorrhiza is a mutualistic relationship between specialized fungi and root system of many plant species around the world, which both benefit from each other. There are two general broad groups of mycorrhizal associations, ectotrophic and endotrophic mycorrhizas. The former is ectomycorrhiza and the latter group is divided into arbuscular, ericoid and orchidaceous mycorrhizas (Smith & Read 1997). Ectomycorrhizal fungi tend to colonize the roots of woody plant species and are characterized by an enveloping sheath of hyphae that surrounds their host plant rootlets. Arbuscular mycorrhizal fungi penetrate inside the cell walls of their host plant roots and form arbuscules (used for nutrient transfer) and vesicles (recognized as sites of lipid storage) within the roots (Danielson 1985). Most forest plants require mycorrhizal fungi to provide their nutritional requirements and service them under harsh environmental conditions. Due to the lack of photosynthetic pigments, fungi are heterotrophs (Isaac 1992). Mycorrhizal fungi can obtain their organic carbon from the host plant species and transfer some macronutrients such as P and N as well as micronutrients, such as Cu and Zn, to the host plants (Smith & Read 2008). It has been demonstrated that the presence of mycorrhizas can increase survival various plant species (Smith & Read 2008; Fortin et al. 2009).

1.2.7 Biology of ericoid mycorrhiza

The roots of ericaceous plant family can host different ericoid mycorrhizal fungal species (Midgley et al. 2004). It has been reported that the roots of one ericaceous plant alone can shelter more than ten fungal species (Midgley et al. 2004). Ericoid mycorrhizal fungi (ERM) are the only fungi that can be found in the root system of ericaceous plants. This plant family is large, with

4500 species within 125 genera and can be found mostly in acidic soils and areas with harsh and infertile growing conditions including arctic, temperate, and tropical regions (Luteyn 2002). Ericoid fungi form intracellular hyphal coils in epidermal cells of the fine hair roots of their host plant species (Read 1984). Also, ERM fungi produce extracellular enzymes which are able to break down simple and complex structures of organic matters and lead to improve nutrient mobilization in their host plants (Read et al. 2004). Ericoid mycorrhiza fungi can provide plants with NO_3- (Kosola et al. 2007), organic N (Sokolovski et al. 2002), organic and inorganic P (Myers & Leake 1996), and Fe (Shaw et al. 1990). Furthermore, ERM could boost plant tolerance of harsh environmental conditions and reduce accumulation of Al (Yang & Goulart 2000), Mn (Hashem 1995), and organic acids (Leake & Read 1991).

The location of this type of mycorrhizal association is confined to areas with lower nutrients where are either peaty or sandy, such as humus heathlands in the northern hemisphere, Mediterranean woodlands, tropical forests, the dry sand plains of Australia, and old copper mining sites of Ireland and the UK (Read 1983). In the study conducted in a south-eastern Australian sclerophyll forest in the roots of Ericaceae family, a vast range of various fungi were identified by DNA fingerprinting and isolated (Bougoure & Cairney 2005). This study demonstrated that populations of ERM may be more diverse than had been previously thought (Bougoure & Cairney 2005). As mentioned above, the growth of ericaceous plant species may be enhanced by ERM fungi, however, the effect of other factors such as the nutrient content of the soil, amount of precipitation, host variety, and fungal partner should not be overlooked (Bizabani et al. 2016).

1.2.8 Root system and mycorrhizal colonization in ericaceous plants

Mycorrhizal associations enable plants to survive under harsh environmental conditions. Balloon-shaped, almost empty, epidermal cells in ericoid roots are essential to host the mycorrhizal fungi (Bonfante-Fasolo et al. 1981; Read 1983). Plant roots can be absorptive or conductive, and the roots within the same category have identical function and nature (McKenzie & Peterson 1995; Eissenstat & Achor 1999; McCully 1999; Hishi 2007; Guo et al. 2008). Moreover, many studies have indicated that among three main factors, including transport capacity, root aging and radial growth, there is a continuous connection, which leads to the increased number of xylem vessels (Steudle & Peterson 1998; Kumar et al. 2007). The root morphology of plants that have been colonized with ericoid mycorrhizal fungi may be changed due to the auxins production by ERM fungi (Berta et al. 1988). The first phase of connection between the ericoid mycorrhizal fungus and plant root to create an ericoid mycorrhizal association is through cellulose microfibrils in the cell wall. In ericoid mycorrhizae colonization, there are dense coils inside the cells of the outermost root layer and also external mycelium can be developed from the surface of plant hair roots. Both the lengths of these mycelium and the lifespan of ericoid mycorrhizal associations are very short, no more than 1 cm and 11 weeks respectively (Mitchell & Gibson 2006). Epidermal cells of hair roots of the ericaceous plant species include some fungi which produce dark and slow growing sterile and dematiaceous mycelia in agar cultures (Read 1996).

Some of mycorrhizal fungi can make zigzag chains of arthroconidia in culture (Burgeff 1961; Pearson & Read 1973) which belong to the genus *Scytalidium* (Dalpe et al. 1989). Another specific feature identified for some of these fungi which isolated from Ericaceae is that they can produce *apothecia* which has enabled them to be identified as the *ascomycete Hymenoscyphus ericae* (Read 1974). Comparisons between ribosomal DNA sequences of an isolate of *Vaccinium* identified two different types of fungi including *Scytalidium accinii* and *H. ericae* and suggested

that between there is an anamorph-teleomorph relationship between these two fungi (Egger & Sigler 1993).

Some evidence has been obtained from light (Reed 1989; Hutton et al. 1994) and electron (Allen et al. 1989) microscope studies showing that the hair roots of family *Epacridaceae* have fungal isolates which could immediately and easily make typical root ericoid mycorrhiza in the hair roots of Ericaceae, and vice versa (Reed 1989). Other studies based on pectic zymogram have shown that fungal isolates of some of Western Australian epacrids did not produce banding patterns which perfectly match those of ericaceous isolates. (Hutton et al. 1994). Some studies have suggested that *Oidiodendron* spp. are other fungal isolates that could be obtained from mycorrhizal hair roots of ericaceous species. (Burgeff 1961; Couture et al. 1983; Dalpe 1986; Douglas et al. 1989; Xiao & Berch 1992).

1.2.9 Phosphorus , nitrogen and other mineral nutrients in ericoid mycorrhizal associations

Some mycorrhizal fungi such as *H.ericae* have a grate significance in sandy heathland soils of warmer regions (Read 1996). The existence of this type of fungus in such soils can be led to many improvements in plants growth. For instance, this fungus can assimilate NO_3^-, NH_4^+ (Bajwa & Read 1986), and PO_4^{-3} (Pearson & Read 1975) ions.

Nitrification is one of the important steps in the N cycle and can happen in any type of soil but may be faster or slower depending on the soil type (Hannon 1956), and because of the very low amount of NO_3^-, the ability of ericaceous (Havill et al. 1974) and epacridaceous (Stewart et al. 1993) plants themselves to access nitrate is very low. Therefore, access to the NO_3^- ion may be largely dependent upon mycorrhizal colonization.

Many studies have reported the role of ericoid mycorrhizal fungi in N uptake. For instance, according to (Bajwa et al. 1985), protease is secreted by ERM fungi which help their host plants

to obtain N from protein in meal-based fertilizers. Other studies which have been carried out to examine the role of ERM fungi in N assimilation, suggested that these fungi allow ericaceous plants to draw out their required N from organic pools (Yang et al. 2002; Walker et al. 2010). The slow decomposition rates in areas with acidic soils reduce the amount of inorganic N sources and limit the access of plants to N (Straker 1996). As mentioned above, ERM can form associations with ericaceous plants species such as blueberry, lingonberry, and Labrador tea, and provide various sources of N for these plants. In the study conducted with lingonberry plants, both mycorrhizal and non-mycorrhizal cranberries were grown in the soil amended with ^{15}N ammonium sulphate. The results of this study demonstrated that non-mycorrhizal plants utilized NH_4^+-N, but mycorrhizal plants had access to other N sources within the soil as well as NH_4^+-N. Moreover, the results of the study by (Stribley & Read 1974) indicated that ericoid mycorrhizal fungi provided accessible organic N for their host plants. Also, in this experiment both mycorrhizal and non-mycorrhizal lingonberry plants were grown in sand amended with nutrients containing a range of amino acids. The data obtained in this study have shown that mycorrhizal plants had higher growth rates and total N contents than non-mycorrhizal plants (Stribley & Read 1980). Another N source for ericoid mycorrhizal fungi is a range of proteins (Bajwa & Read 1985) as well as chitin (Leake & Read 1990b; Kerley 1993). The presence of carbon in the growth medium of fungi can affect the assimilation of glutamine, NH_4^+, or NO_3^- by ERM fungi (Grelet et al. 2005). Some ERM are able to release enzymes in order to gain their essential N by degrading complex N compounds. For instance, active polyphenol oxidases can be produced by ERM to detach organic N from recalcitrant compounds (Bending & Read 1996). Moreover, extra-cellular proteinase enzyme carboxyl (acid) proteinase can be secreted by ERM fungi to break down proteins to amino acids (Leake & Read 1989). According to Lake and Read’s investigations, the above enzyme is active

at very low pH values, even lower than the pH of acidic soils (Leake & Read 1990a). Soil/litter interface is a part of soil that ericaceous plants are able to penetrate their mycorrhizal roots. As a result of H^+-releasing processes in this interface, pH value of this region is mostly lower than that of bulk soil (Read et al. 1989). A study conducted to examine proteinase activity in mycorrhizal fungi (Leake & Read 1990a) reported that the function of some enzymes such as proteinase can be adjusted by a strategy known as 'Noah's Ark'. According to this strategy, low amounts of proteinase enzyme can be released by mycorrhizal fungi (Leake & Read 1990c), however, an abundant release of proteinase could occur when end products known as 'reporter' molecules are identified by *H. ericae*, and when fungi access appropriate substrate in the soil. (Leake & Read 1990c). Ericoid mycorrhizal fungi are capable of taking up end products which, in this case, are amino acids, without removing an amino group. These forms of N would be transferred easily from the fungi to their host plants (Mitchell & Gibson 2006). On the other hand, in acidic soils, the P sources including inorganic phosphate, phosphomonoesters, phytates, and nucleic acids either are in low amounts or are less available to plants (Cosgrove 1967; Griffiths & Caldwell 1992), but ERM enable their host plants to access these P sources (Mitchell & Read 1981). For example, phosphodiesters are among the most important and valuable sources of soil P, however, their concentration is low in many soils especially in acidic ones (Griffiths & Caldwell 1992). According to the study conducted by (Leake & Miles 1996), ERM fungi can produce and release both extra-cellular and wall-bound phosphodiesterase enzymes to use phosphodiester DNA.

In addition to N and P assimilation in ericoid mycorrhizal associations, other vital elements can be up taken up and transferred by some fungi to their host plants (Mitchell & Gibson 2006). For instance, *H.ericae* is an ERM, which is able to create a strong affinity with Fe at all concentration levels (Mitchell & Gibson 2006). Therefore, this fungus can assist its host plant in

absorbing Fe due to this trait (Shaw & Read 1989). Schuler and Haselwandter (1988) declared that this characteristic of ERM could be due to the release of Fe-specific siderophores. Moreover, some investigators have suggested that *H.ericae* fungus can have more affinities with other essential and non-essential elements (Bradley et al. 1981, 1982; Denny & Ridge 1995) including Zn, Cu (Gibson & Mitchell 2005a), Al, and Mn (Marschner 1995). Burt et al. (1986) reported that one reason for the ability of ERM to uptake metals can be due to environmental conditions where they are found, because the majority of these fungi can survive in acidic soils containing high levels of Fe, Al and Mn (Marschner 1995).

Research has been conducted to evaluate the influence of two different fertilizers (organic and inorganic) on the nutrient uptake of blueberries inoculated with three different isolates of ericoid mycorrhizal fungi (Scagel 2005). The results demonstrated that total levels of all nutrients in plants inoculated with *O. griseum* and *P. ericae* isolates were higher in comparison to non-inoculated plants when they were supplied with inorganic fertilizer. In plants inoculated with *O. griseum* and *P. ericae* isolates and fertilized with different amounts of organic fertilizers, the uptake of all nutrients except Ca, Zn, and fluorine was enhanced (Scagel 2005). The effect of inoculation of blueberry plants with *H. ericae* on their nutrient uptake varied with fertilizer types. Inorganic fertilizers acted as a barrier in the uptake of N, K, Mn and B in plants inoculated with *H. ericae* and in organic fertilizers, the total level of N, K, Mn and B was higher in mycorrhizal, compared with nonmycorrhizal, plants. In contrast, plants inoculated with *H. ericae* absorbed more Ca and Zn from the inorganic compared with organic fertilizers (Scagel 2005).

1.2.10 Ericoid mycorrhizas and heavy metal resistance

Toxic metal pollution due to geochemical and man-made activities is a big problem in today's world (Leyval et al. 1997). Although the concentrations of toxic metals from the geochemical origin can be high and generate some environmental concerns (Jeng & Bergseth 1992), human activities including burning of fossil fuels, mining and smelting of metalliferous ores, municipal wastes, fertilizers, pesticides, sewage sludge amendments, and use of pigments and batteries pose more threat to the environment and human health (Adriano 1986) . Leyval et al. (1997) suggested that the more correct name for these toxic metals should be trace, rather than heavy metals, because the former represents the concentration in soil (<0.1%) and biological materials (100 mg kg^{-1}). High soluble concentrations of metals such as Al, Fe, and Mn in the soil indicates high acidity status of the soil (Cairney & Meharg 2003). Although metals such as Fe, Zn, Cu, Mn, Ni, Co (Marschner 1995) are essential for plant growth, other metals including Cd, Pb, and Hg have no beneficial impact on plant growth (Leyval et al. 1997).

As mentioned above, elevated concentrations of heavy metal can have detrimental effects on all organisms including soil micro-organisms. In metal-contaminated places, mycorrhizal associations become especially important for plant survival (Meharg & Cairney 2000). It has been suggested that physiological processes in ERM can adapt to toxic and metal-contaminated environments. Moreover, these fungi are able to transfer their Cu resistance to their host ericaceous plants, which allows these plant species to survive in copper-contaminated sites (Bradley et al. 1981). Some of the important processes contributing to metal tolerance in ERM include the production of enzymes that are resistant to metal inactivation, proteins which are able to detoxify potentially toxic metals, and increased resistance of cell membranes to reactive oxygen species (Meharg & Cairney 2000).

Mycorrhizal fungi are the best way to make a connection between soil and the roots of different plants and, therefore, can play a key role in transferring toxic and heavy metals to plants (Leyval et al. 1997). However, mycorrhizal fungi can also protect their host plants against heavy metals through either metal “avoidance” or “tolerance” processes. The avoidance processes can happen when fungi reduce the amount of metal uptake or increase efflux, and either create complexes with metals outside of the cells or release organic acids to help with metal avoidance. Organic acids such as oxalic, succinic and citric acid were reported to remove Cu and Zn from contaminated soils (Kim et al. 2013). The tolerance processes occur when fungi can tolerate the presence of high metal levels and make some metal-chelating compounds through the synthesis of ligands such as metallothioneins, polyphosphates, and/or compartmentation within vacuoles (Turnau et al. 1996b).

On the one hand, the vast majority of research indicates that the existence of mycorrhizal colonization can assist in enhancing the nutrient absorption of ericaceous plants in environments with the lack of nutrients. On the other hand, this quality can lead to some physiological impairment for plants growing in soils with high levels of toxic elements (Read 1982). Although, increased levels of metals such as Al and Cu can be toxic for non-ericaceous plants, ericaceous plants are the predominant species in soils containing high levels of metals (Read 1982). Several studies revealed that some plants including *Calluna* sp. (Marrs & Bannister 1978; Oxbrow & Moffatt 1979), *Vaccinium* sp., and *Gaultheria* sp. (Freedman & Hutchinson 1980) could be found as the sole plant species in soils contaminated by copper- and zinc-mining activities in Europe and in soils contaminated by smelting in Canada. Furthermore, several studies showed that ectomycorrhizal colonization may have a fundamental role in providing resistance to heavy metals (Read 1982). For instance, Bradley et al. (1981, 1982) conducted a study with *Calluna vulgaris*,

Vaccinium macrocarpon, and *Rhododendron ponticum* to examine the effect of ectomycorrhizal colonization on heavy metal resistance. Plants were planted in sand with different concentrations of $CuSO_4$ and $ZnSO_4$. The results demonstrated that the non-mycorrhizal plants in all three species at all treatment levels did not grow or show very little growth and high plant mortality was observed at the higher concentration treatments. In mycorrhizal plants, a decrease in growth occurred only at the highest treatment concentrations. Moreover, after measuring the amounts of Cu and Zn in plant shoots, it was concluded that the ratio of metal concentration was much lower in mycorrhizal compared with non-mycorrhizal plants (Read 1982). Depending on the type of host plant and ERM, various mechanisms can be utilized to protect plants in toxic soils (Mitchell & Gibson 2006). One of the proposed hypotheses is that this resistance process may be due to an exclusion mechanism operating at the mycorrhizal root surface, which protects both the plant and the fungus (Mitchell & Gibson 2006). Mucilaginous slime produced by fungal hyphae at the mycorrhizal root surface can play a role in the exclusion mechanism to limit the movement of metal ions (Denny & Ridge 1995). Furthermore, some of ERM fungi are able to protect their host plants against organic arsenic compounds (Mitchell & Gibson 2006). The study conducted to examine the mechanism of arsenate resistance in one of the ERM demonstrated that *C.vulgaris* plants were able to survive on arsenate sites when colonized by *H. ericae* (Sharples et al. 2001). Due to some similarities between arsenate and phosphate, fungus can carry this toxic element across the plasma membrane through the phosphate co-transporter system (Meharg & Macnair 1992). In addition, this arsenate-resistant ERM fungus, has an ability to transform the accumulated and stored arsenate to arsenite, which can be removed from the fungal mycelium without any interference with phosphate transfer (Sharples et al. 2000a.b, 2001).

1.2.11 Effects of ericoid mycorrhizal inoculation on plant growth

Mycorrhizal fungi are able to increase plant growth through associating their hyphae with their host plant roots by providing nutrients for root tissues. Increased surface area of the root by mycorrhizal fungi can cause higher nutrient uptake. Mycorrhizal fungi are considered as biofertilizers and are able to provide required nutrients for the host plants (Hodge et al. 2010; Miransari 2010; Smith & Smith 2011; Nadeem et al. 2014; Vafadar et al. 2014). The symbiosis of ERM with the roots of ericaceous plants can facilitate the exchange of nutrients (Rice & Currah 2006) and protect their host plants against heavy metal toxicity (Daghino et al. 2016).

Nitrogen uptake in plants can occur through either nitrate or ammonium. Lingonberry (*V.macrocarpon*) is not able to absorb NO_3^- as a sole source of N in hydroponic culture (Rosen et al. 1990; Smith 1993), as this plant has adapted to acid soils (pH from 4 to 5) and in this range of pH, the nitrification is minimal (Paul & Clark 1989). (Kosola et al. 2007) found that cranberries inoculated with the fungus *Rhizoscyphus ericae* can absorb NO_3- compared to non-inoculated cranberries. In another study, blueberry (*V. corymbosum* L.) cultivars inoculated with ERM had higher N concentrations and growth rates compared with non-inoculated ones (Scagel 2005). Also, ERM were able to enhance N uptake in *Rhododendron fortunei* plants (Yin et al. 2010).

Several studies reported that the growth of plants was reduced in autoclaved peat which prevented mycorrhizal development (Bain 1937; Burgeff 1961). However, it is not certain if this effect can be only attributed to mycorrhizal fungi, since other organisms in the peat including saprophytic fungi may be also able to alleviate the toxicity of soil compounds such as heavy metals. According to Stribley et al. (1975), the most appropriate sterilization treatment for soils is gamma irradiation which makes soils completely free of living organisms including bacteria, fungi, algae, nematodes, fungal endophytes etc., and does not change soil chemistry.

Chapter 2

Study 1: Evaluation of Ericoid mycorrhizal fungi (ERM) colonization potential in fresh and stockpiled topsoil from the oil sands reclamation site

2.1 Introduction

During oil sands mining operations, the topsoil (A horizon) is removed and stockpiled in small or large piles for the duration of the mining project. The subsoil layer (B horizon), which is also removed and stored separately. When the mining operation is complete, overburden material is reapplied and then topsoil is spread over the overburden material before site revegetation (Strohmayer 1999).

Many physical and chemical changes can occur in the stockpiled soils as a result of mechanized handling during stripping and stockpiling the soil and during storage. These changes can include increased bulk density, decreased microbial activity, reduced nutrient cycling, and decreased water holding capacity (Strohmayer 1999). Anaerobic conditions increase with the increasing depth of piles over time and are more severe in fine textured soils (Abul-Kareem & McRae 1984). One of the most important adverse effects of prolonged soil stockpiling is a reduction in microbial diversity, which can lead to an unbalanced soil ecosystem (Fresquez & Aldon 1984).

Since the beneficial soil microorganisms such as N fixing bacteria and mycorrhizal fungi are essential for plant survival and growth (Van der Heijden et al. 2008), a reduction in microbial diversity of the soil may be detrimental to revegetation efforts. Soil fungal communities, especially mycorrhizal fungi, can assist their host plant species with nutrient and water uptake (Guiñazú et al. 2010), improve resistance to abiotic stress (Selvakumar et al. 2012), and increase photosynthetic capacity (Xie et al. 2009). Ericoid mycorrhizal (ERM) fungi specifically form symbiotic

associations with roots of plants in the family Ericaceae (Perotto et al. 2012). These mycorrhizal fungi can improve N uptake in ericaceous plants (Bucking & Kafle 2015), also protect their host plants against heavy metal toxicity (Daghino et al. 2016). Therefore, it is important to understand how stockpiling can affect the microbial compositions of the soil.

The Ericaceae have a nearly worldwide distribution and can be found as understory plants in the boreal forests (Stevens 2004). The growth of these plants in reclamation sites is severely impacted by the soil factors, however they can successfully colonize poor nutrient and acidic soils with ERM fungi (Bradley et al. 1982).

In this study, I compared the diversity of ericoid mycorrhizal fungi of the fresh topsoil collected from the boreal forest site near Fort McMurray, Alberta, Canada with the soil that was stockpiled for one year. Three ericaceous plant species including blueberry (*Vaccinium myrtilloides*), Labrador tea (*Rhododendron groenlandicum*), and lingonberry (*Vaccinium vitis-idaea*) were grown in both types of soil to examine the effects of stockpiling on growth and physiological parameters. I hypothesised that fresh topsoil would have a greater ericoid mycorrhizal colonization potential compared to the one-year stockpiled topsoil, which would benefit plant growth.

2.2 Materials and methods

2.2.1 Soil substrates

The studied substrates included topsoil (LFH, approximately top 30 cm from the soil surface) of the boreal forest site near the oil sands mining areas of the Canadian Natural Resources Limited (CNRL) Horizon lease, north of Fort McMurray, Alberta, Canada (56° 43' 35.94" N, 111° 22' 44.1732" W). The soil was collected in the late spring of 2016 and stockpiled for one year on

the site (one-year stockpiled soil). In the spring of 2017, LFH soil from the same area was collected as the fresh soil. After delivering to the University of Alberta, all large debris were removed from both types of soils, and a half of the one-year stockpiled topsoil was autoclaved at 121°C for 1 h in order to remove all mycorrhizal fungi and contamination. In total, three soil types were used in this study including the non-autoclaved fresh topsoil, non-autoclaved one-year stockpiled topsoil, and autoclaved one-year stockpiled topsoil.

2.2.2 Seedling preparation

Seeds of blueberry (*V.myrtilloides*), lingonberry (*V.vitis-idaea*) and Labrador tea (*R.groenlandicum*) plants were collected from the boreal forest sites in the vicinity of the oil sands mining areas near Fort McMurray, Alberta, in the summer of 2015. The seeds were stored in airtight plastic bottles at 4°C. The seeds were surface-sterilized with sodium hypochlorite 20% for 2 min, followed by ethanol 70% for 5 min, and rinsed with autoclaved distilled water. The sterilized seeds were placed on the solid Murashige and Skoog medium (MS) (pH 5.7) (Murashige & Skoog 1962) in Petri dishes to germinate. The Petri dishes were sealed with parafilm, kept at 4°C for 48 h, and then transferred to the growth chamber until germination. Environmental conditions in the growth chamber were 22/18°C (day/night) temperature and 16-h photoperiod.

2.2.3 Experimental setup

The germinants were transferred from Petri dishes to nine Styrofoam blocks with 45 cells (each cell 450 ml in volume, three blocks for each soil treatment). Fifteen plants of each species were planted in each container (three replicates per treatment). All plants were watered (water pH adjusted to 5.5 with H_2SO_4) every day and fertilized with N_{20}-P_{20}-K_{20} commercial fertilizer every two weeks. The seedlings were grown for seven months before harvesting. The experiment was conducted in the greenhouse at the University of Alberta. Environmental conditions in the

greenhouse were maintained at 24/19°C (day/night) temperature, 65±10% relative humidity (RH) and 16-h photoperiod with natural light supplemented by the full-spectrum Philips fluorescent bulbs. (Philips high output, F96T8/TL835/HO, Markham, ON, Canada).

2.2.4 Shoot and root dry weights

Shoot and root dry weights were determined for nine seedlings from each species and each soil treatment. After harvesting all plants, shoots and roots were separated and dried in an oven at 65°C for 72 h.

2.2.5 Leaf chlorophyll concentrations

Leaf chlorophyll-a and chlorophyll-b concentrations were determined in fully-expanded leaves in in the mid-part of the shoot in nine randomly selected seedlings from each treatment and each species. The leaves were dried in a freeze-drier (LABCONCO FREEZE DRY LYPH.LOCK 4.5, Kansas, USA) for 72 h and then immediately ground with a Thomas Wiley Mini-Mill (Thomas Scientific, NJ, USA). Chlorophyll was extracted from 10 mg dry weight of grinded leaf samples with 8 ml dimethyl sulfoxide (DMSO) at 65°C for 22 h. After filtering, chlorophyll concentrations were measured in the DMSO extracts with a spectrophotometer (Ultrospec, Pharmacia LKB, Uppsala, Sweden), at 648 nm and 665 nm for chlorophyll-a and chlorophyll-b concentrations, respectively. The Arnon's equation (Sestak et al. 1971) was used to calculate the total chlorophyll concentration.

2.2.6 Net photosynthesis and transpiration

The measurements of gas exchange were carried out in nine seedlings of each plant species and soil type after seven months of growth. Net photosynthesis (Pn) and transpiration (E) rates were measured in the upper, fully developed leaves using the infrared gas analyzer (LI-6400, LI-

COR, Lincoln, Nebraska USA) at 400 μmol m^{-2} s^{-1} PPFD. For the blueberry and Labrador tea, one leaf of each plant was inserted in the leaf chamber, while, for cranberries, between four to ten leaves were inserted in the chamber. Following the measurements, the leaves were detached, and the leaf areas were determined following computer scanning using the Sigmascan Pro 5.0 computer software (Systat Software, San Jose, CA).

2.2.7 Leaf elemental concentrations

Quantitative elemental analyses were carried out on the remaining leaves of the studied plants. Nine plants of each species from each soil treatment were used to measure leaf elemental concentrations. The leaves were oven-dried at 65°C for three days and ground with the Thomas Wiley Mini-Mill (Thomas Scientific, NJ, USA) grinder. Extraction of Ca, K, Mg, and P concentrations were determined by the Inductivity Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) (iCAP6300, Thermo Fisher Corp, United Kingdom). Total N was analyzed by the dry combustion method using the Flash 2000 Organic Elemental Analyzer (Thermo Fisher Scientific Inc., Bremen, Germany 2016). All measurements were conducted in the Natural Resources Analytical Laboratory of the University of Alberta, Canada).

2.2.8 Elemental concentrations in soil samples

Concentrations of Ca, K, Mg, P, Na, and N were determined in five soil samples from each treatment (fresh topsoil, autoclaved one-year stockpiled topsoil, and non-autoclaved one-year stockpiled topsoil) as described above for the leaves.

2.2.9 Soil pH and electrical conductivity (EC)

Five soil samples of each treatment were air-dried in aluminium trays at the room temperature for two weeks. Soil pH and EC were determined in saturated pastes in the Natural

Resources Analytical Laboratory. Soils were brought to their water saturation level by gradual addition of water and then vacuum filtered.

2.2.10 Microscopy

To examine the root colonization intensity, six seedlings per treatment were randomly selected from the same three plant species. After harvesting the plants, their roots were excised, wrapped in aluminum foil and kept on ice, until they were delivered to the laboratory. The root tips were approximately 20 cm long and fixed in (formaldehyde (10 ml): glacial acetic acid (5 ml): ethanol (50 ml): distilled water (35 ml). Prior to microscopy, the roots were rinsed twice with distilled water and then cleared in potassium hydroxide 2.5% KOH at 60°C for 1 h. After removing from the oven, the roots were washed twice with distilled water and commercial vinegar. The root samples were stained in the ink (5%) plus commercial vinegar at 60°C for 20 min. The stained roots were rinsed with distilled water, cut in to 12 fragments (each approximately 1-cm long), mounted on microscope slides and observed under the light microscope. All root pieces were rated from 0 to 5 (Fig.2.1) according to the extent of colonization by ericoid mycorrhizal fungi (Trouvelot et al. 1986). Finally, the intensity of mycorrhizal colonization in the root system (M) was calculated using the following equation:

$$M\% = (95n5+70n4+30n3+5n2+n1)/ (n\ total)$$

where *n5=number of root fragments rated 5, n4=number of root fragments rated 4, n3=number of root fragments rated 3, n2=number of root fragments rated 2, n1=number of root fragments rated , n total=total number of root fragments*

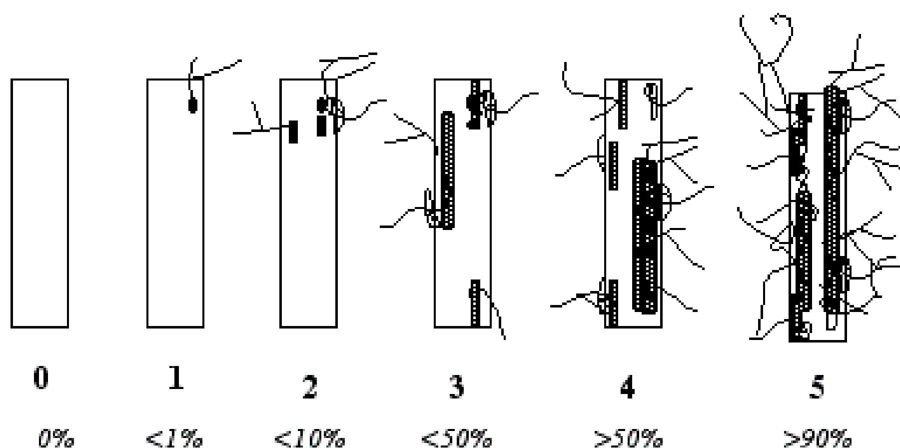


Figure 2.1. Scoring mycorrhizal colonization method to quantify ericoid mycorrhizal fungi in the roots of ericaceous plant species. In rate number 5 more than 90 % of root fragment, in rate number 4 between 50 to 90 % of root fragment, in rate number 3 between 10 to 50 % of root fragment, in rate number 2 between 1 to 10 % of root fragment, in rate number 1 lower than 1 % of root fragment colonized by ERM fungi.

2.2.11 Extraction of genomic DNA and PCR amplification

Six root samples per treatment from all three plant species were selected to extract genomic DNA. After harvesting plants in the greenhouse, the roots were excised, wrapped in aluminum foil and kept on ice before delivering to the laboratory. The roots were kept in the freezer at -80°C before DNA extraction. For DNA extraction, the roots were flash frozen in liquid N and ground in an autoclaved porcelain mortar. Approximately 150 mg of each root sample was placed in a 2 ml micro-centrifuge tube. After thawing in 1 ml lysis buffer (2% w/v cetyltrimethylammonium bromide (CTAB) (Saghai-Maroof et al. 1984), 1.42 M NaCl, 20 mM EDTA (0.5 M), 100 mM Tris-HCL (pH 8.0), 2% PVP-40, 1% v/v 2-mercaptoethanol), the samples were incubated in a water bath at 60°C for 1 h. About 1 mL of chloroform:isoamyl alcohol (24:1 v/v) was added to each sample followed by centrifugation at 14,000 rpm (16,700 g) for 10 min. The supernatants

were transferred to 2 mL centrifuge tubes and again purified with 500 µl of chloroform:isoamyl alcohol (24:1 v/v). After centrifugation, DNA was precipitated with isopropanol at -20ºC , for 15 min) and the pellet was rinsed with 70% ethanol and centrifuged. DNA was suspended in 30 µl of 1XTE buffer and its concentration determined with the Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA). For PCR amplification, all extracted DNA samples were diluted to 100 ng/µL. One µL of diluted DNA sample was combined with 24 µL of Master Mix buffer. Master Mix buffer contains all the components for PCR mix to occur; including internal transcribed spacer (ITS1 and ITS4) (1.25 µL), Taq DNA polymerase, dNTPs, MgCl2, 10x pcr Reaction and Nuclease-Free water) the individual building blocks of DNA (nucleotides, or dNTP's), a special buffer to maintain optimum pH, salts, and $MgCl_2$. Amplifications were performed with an initial denaturation at 95°C for 2 min, followed by 30 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min, with the final extension of 72°C for 10 min. All PCR products were visualized on a 1% agarose gel. Successfully amplified PCR products were purified using the Wizard@ SV Gel and PCR Clean-up System kit following the manufacturer's protocol (Promega, Madison, USA). The positive bands were excised and were subjected to sequencing using an IST1. The manufacturer's BigDye terminator cycle sequencing reagent, and an AB13730 sequencer (Applied Biosystem) were used for sequencing experiments.

2.2.12 Molecular identification

Six plants from each experimental group were included in the analyses. Roots of each experimental plant were pulverized, and DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Allen et al. 2003). Subsequent PCR reactions were performed using fungal specific ITS1F (Allen et al. 1993) and ITS4 (White et al. 1990) primers. Thermocycling conditions were as follows: 95ºC for 2 min; 33 cycles of 95 ºC for 30 sec., 56 ºC for 30 sec, 72 ºC for 1 min, and final elongation for 10 min at 72 ºC. PCR products were purified using the GeneJET PCR Purification Kit (Thermofisher, Crawley, UK), and cloned into the pCR2.1-TOPO vector (Invitrogen, Paisley, UK). From each experimental group, 25-30 white colonies were selected and stored. Colonies were amplified by PCR using M13 sequencing primers. Sequencing of 20-30 clones per experimental group was performed using the Sanger DNA Sequencing (MBSU, University of Alberta). Molecular identification of isolated fungi was conducted using BLASTn searches of the ITS region at the NCBI website (http://www.ncbi.nlm.nih.gov).

2.2.13 Species diversity and rarefaction curves

Species diversity curve and rarefaction curves (expected number of species) were generated using nonlinear regression (Hyperbola, single rectangular (y=ax/b+x) in SigmaPlot 11.0 (Systat Software Inc., Chicago, IL, USA). The cumulative number of fungal species (y-axis) is plotted as a function of the cumulative number of samples (upper x-axis), pooled in random order.

2.2.14 Statistical analysis

All data were analyzed using SAS GLM model (Version 9.2, SAS Institute Inc., Cary, NC) to determine statistically significant ($p \leq 0.05$) differences between treatments. For this experiment one-way ANOVA was used with soil type as the main factor. The data that did not meet the ANOVA assumptions of normality of distribution and homogeneity of variance were transformed

with a log10 function. Comparisons between different treatment means were conducted using protected Fisher's LSD test. A representative table of the ANOVA results is shown in the Appendix section.

2.3 Results

2.3.1 Root and shoot dry weights

Blueberry plants in fresh topsoil had about two-fold higher root and shoot dry weights compared with the plants growing in the non-autoclaved soil stockpiled for one year (Fig.2.2A, B). Shoot dry weights and shoot:root dry weight ratios of blueberry plants in the autoclaved topsoil that was stockpiled for one year were lower compared with the plants growing in fresh topsoil (Fig.2.2B,C). However, root dry weights of blueberries in the autoclaved one-year stockpiled topsoil were significantly higher compared with the non-autoclaved stockpiled topsoil and were similar to those of plants growing in fresh topsoil (Fig.2.2A).

In Labrador tea plants, root and shoot dry weights were significantly higher in fresh and one-year stockpiled topsoil treatments in comparison to the non-autoclaved stockpiled topsoil treatment (Fig.2.2A,B). There was no effect of the soil type on shoot: root dry weight rations in Labrador tea (Fig.2.2C).

Total root dry weights of lingonberry plants in the autoclaved stockpiled topsoil treatment were higher compared with the other two soil types (Fig.2.2A). However, shoot dry weights (Fig.2.2B) and shoot:root ratios (Fig.2.2C) were higher in the non-autoclaved stockpiled soil compared with fresh topsoil.

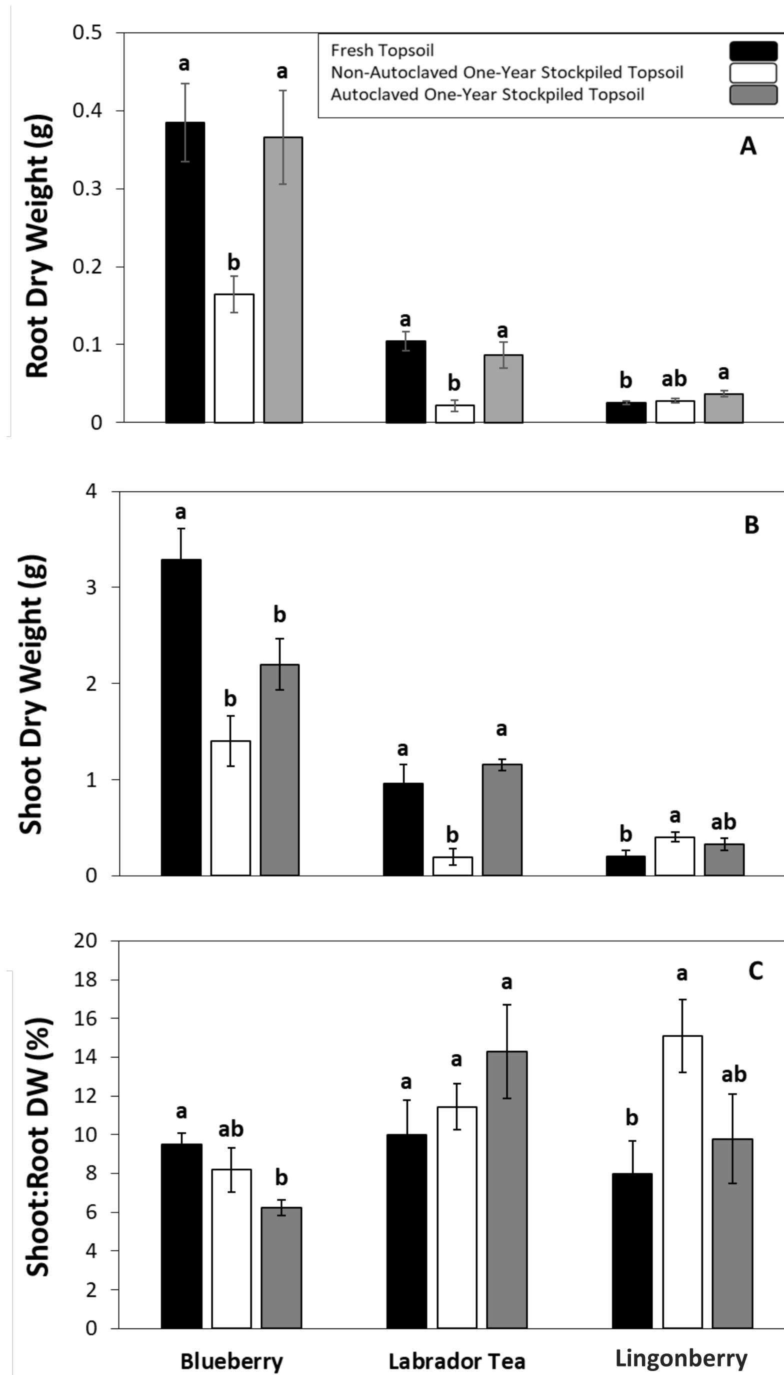


Figure 2.2. Root dry weights (A), shoot dry weights (B), and shoot:root dry weight ratios (C) in blueberry (*Vaccinium myrtilloides*), Labrador tea (*Rhododendron groenlandicum*) and lingonberry (*Vaccinium vitis-idaea*) plants growing in the fresh topsoil, autoclaved one-year stockpiled topsoil, and non-autoclaved one-year stockpiled topsoil from the oil sands reclamation areas. Different letters above the bars indicate significant differences (α = 0.05) between treatments within each plant species according to protected Fisher's LSD test. Means (n = 9) ± SE are shown.

2.3.2 Chlorophyll concentration

Blueberries in fresh topsoil had higher leaf total chlorophyll, chlorophyll-a, and chlorophyll-b concentrations compared with those blueberry plants growing in non-autoclaved stockpiled topsoil (Fig.2.3A,B,C). For blueberry plants there was no significant difference in leaf chlorophyll concentrations between the autoclaved stockpiled soil and the other soil types (Fig.2.3A,B,C). Leaf total chlorophyll-a, and chlorophyll-b concentrations in both Labrador tea and lingonberry plants were not affected by the soil type (Fig.2.3B,C).

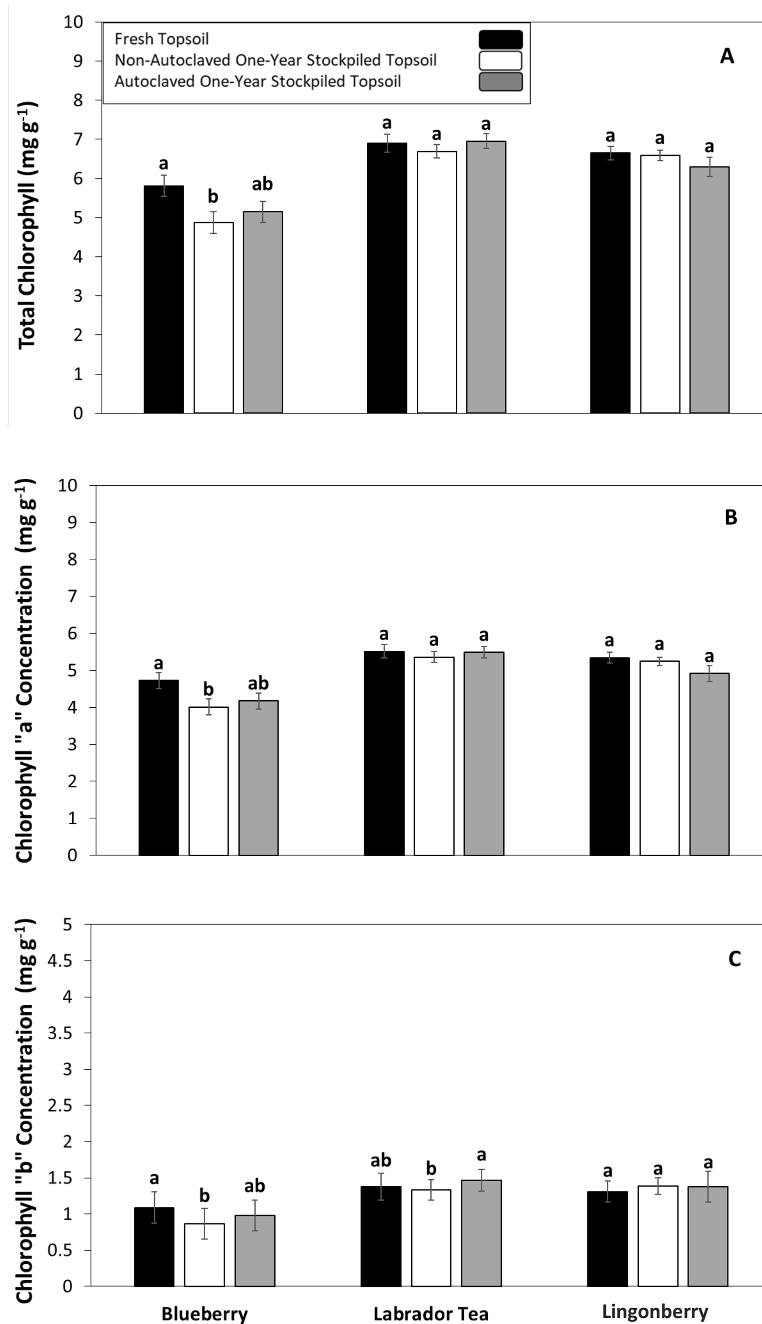


Figure 2.3. Total leaf chlorophyll (A), chlorophyll "a" (B) and chlorophyll "b" (C) concentrations of blueberry (*Vaccinium myrtilloides*), Labrador tea (*Rhododendron groenlandicum*) and lingonberry (*Vaccinium vitis-idaea*) growing in fresh topsoil, autoclaved one-year stockpiled topsoil and non-autoclaved one-year stockpiled topsoil from oil sands reclamation areas. Different letters above the bars indicate significant differences (α = 0.05) between treatments within each plant species according to protected Fisher's LSD test. Means (n = 9) ± SE are shown.

2.3.3 Net Photosynthesis (Pn) and transpiration (E)

There were no differences in Pn (Fig.2.4A) and E (Fig.2.4B) between the three different soil treatments in any of the examined plant species.

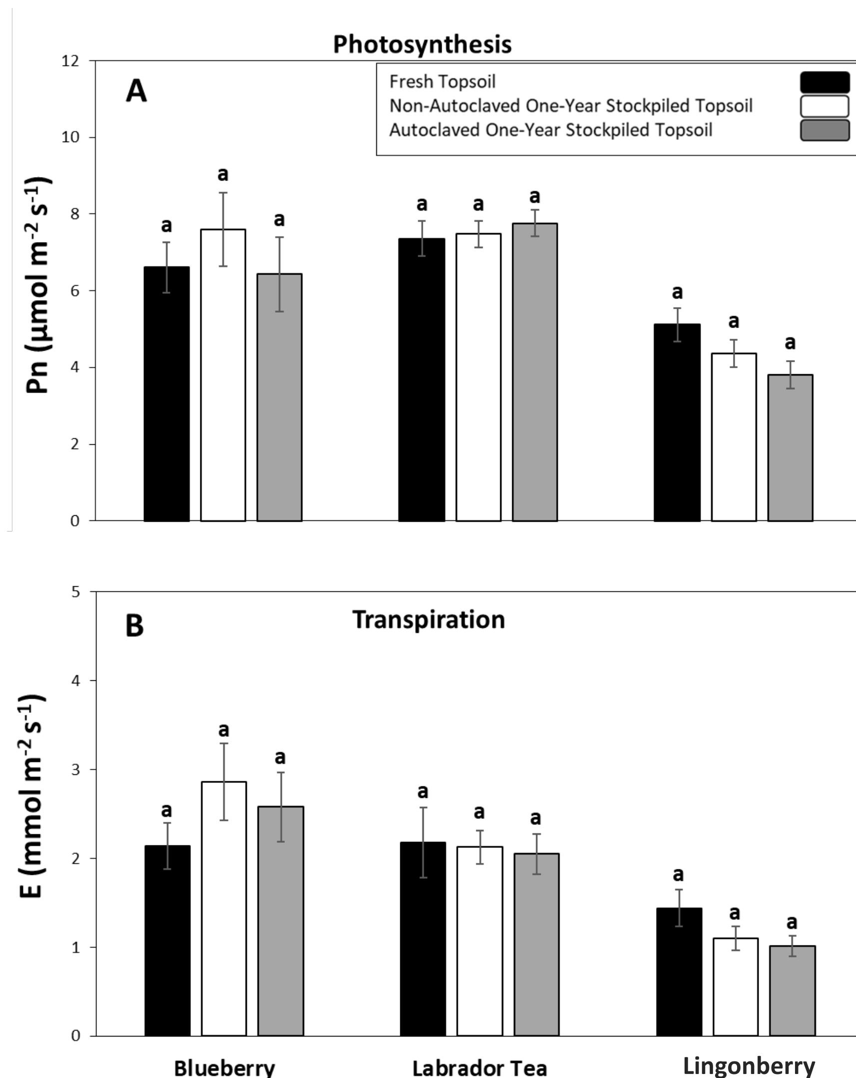


Figure 2.4. Net photosynthesis (Pn, A) and transpiration (E), B) rates in blueberry (*Vaccinium myrtilloides*), Labrador tea (*Rhododendron groenlandicum*) and lingonberry (*Vaccinium vitis-idaea*) in plants growing in fresh topsoil, autoclaved one-year stockpiled topsoil and non-autoclaved one-year stockpiled topsoil from reclamation areas. Different letters above the bars indicate significant differences (α=0.05) between treatments within each plant species according to protected Fisher's LSD test. Means (n=9) ± SE are shown.

2.3.4 Elemental analysis of leaf tissues

Leaf Ca concentrations of blueberry plants in the autoclaved stockpiled topsoil were significantly higher compared with the blueberry plants in other two soil types (Fig. 2.5A). In Labrador tea, there were no differences in leaf Ca concentrations among the three soil types (Fig. 2.5A). In lingonberry, plants in the autoclaved and non-autoclaved stockpiled topsoil had higher leaf Ca concentrations compared with the plants in fresh topsoil (Fig.2.5A).

Leaf K concentrations were similar in plants growing in the three different soil types in all three species (Fig.2.5B).

The soil type had no significant effects on leaf Mg concentrations in blueberry plants species (Fig.2.6A).

Leaf P concentrations of blueberries in fresh topsoil were significantly higher compared with plants in the non-autoclaved and autoclaved stockpiled soil (Fig.2.6B). In Labrador tea and lingonberry, no significant differences were observed in leaf P concentrations among the different soil types (Fig.2.6B).

Blueberry plants growing in fresh topsoil had higher N compared with the non-autoclaved and autoclaved stockpiled topsoil (Fig.2.7). Soil type had no effect on leaf N concentrations in Labrador tea and lingonberry plants (Fig.2.7).

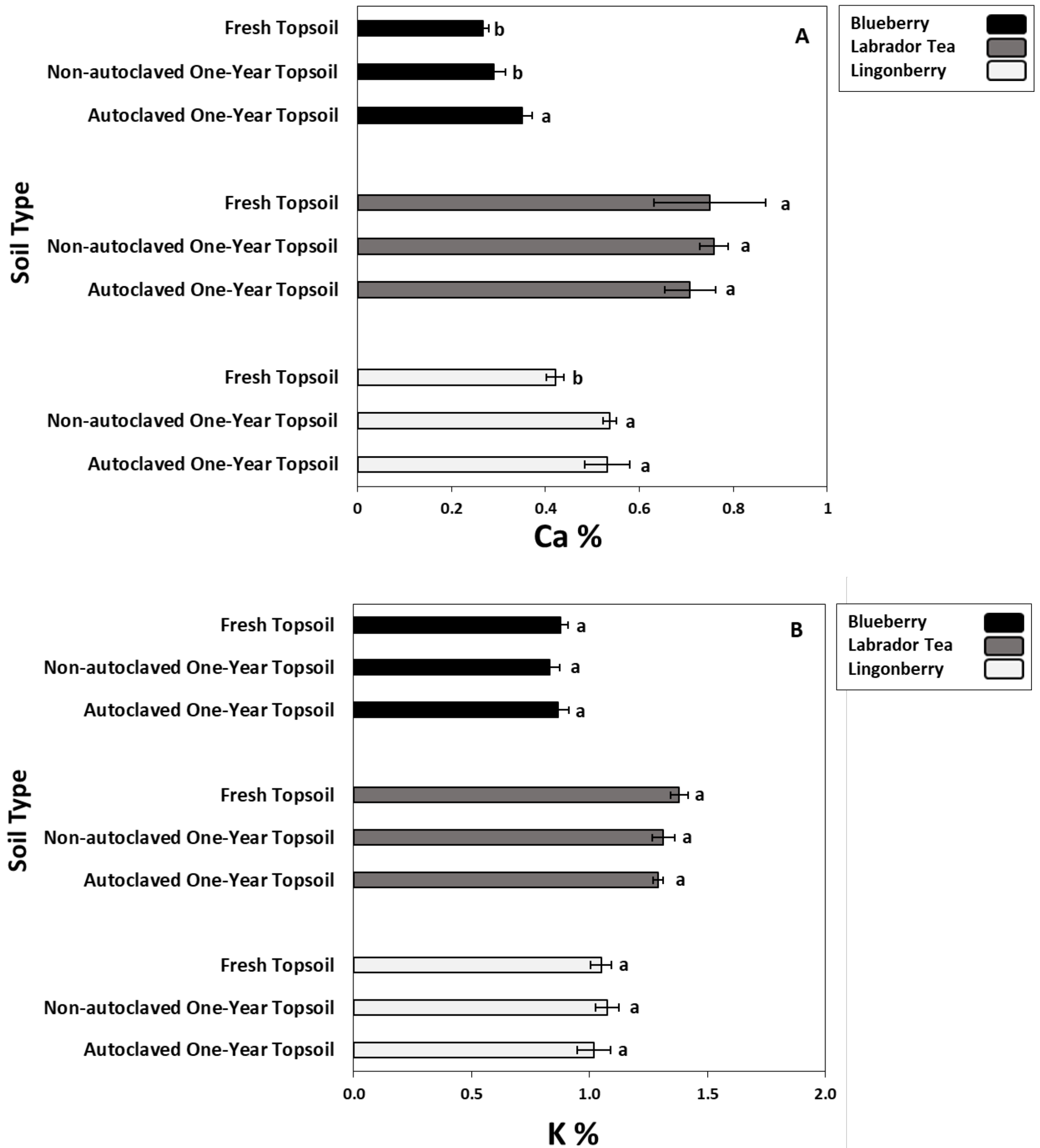
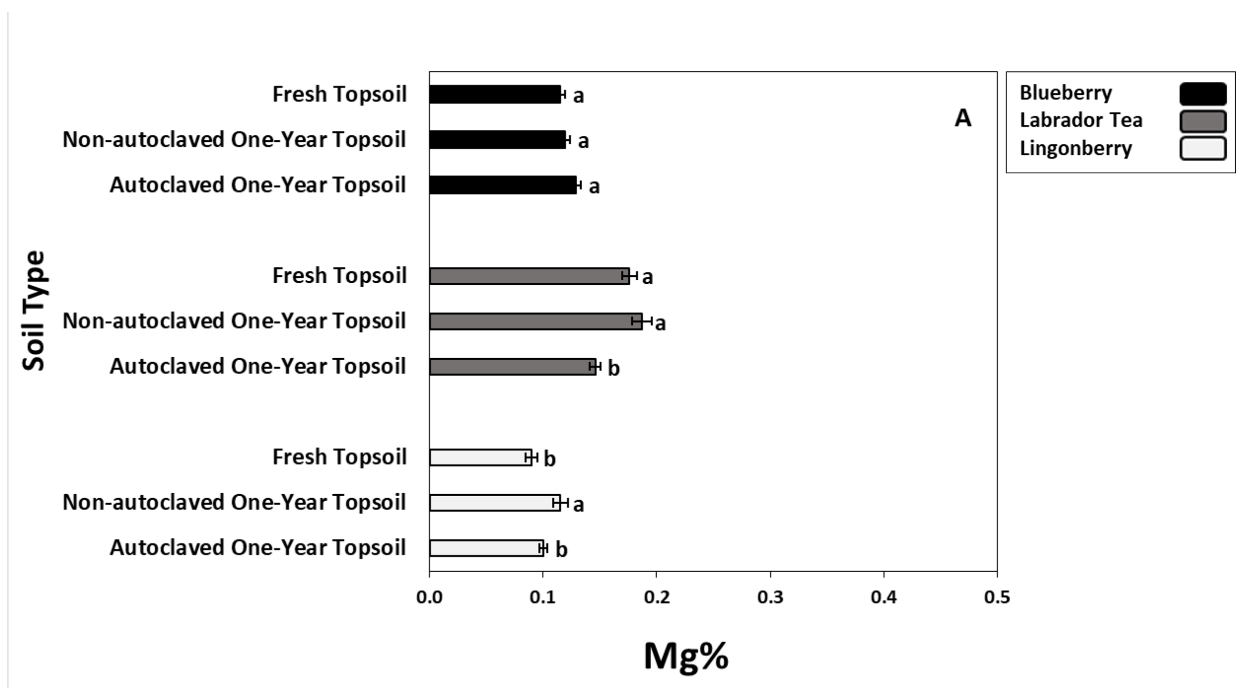


Figure 2.5. Leaf calcium (Ca, A) and Potassium (K, B) concentrations of blueberry (*Vaccinium myrtilloides*), Labrador tea (*Rhododendron groenlandicum*) and lingonberry (*Vaccinium vitis-idaea*) plants growing in fresh topsoil, autoclaved stockpiled topsoil and non-autoclaved stockpiled topsoil from oil sands reclamation areas. Different letters above the bars indicate significant differences (α = 0.05) between treatments within each plant species according to protected Fisher's LSD test. Means (n = 9) ± SE are shown.



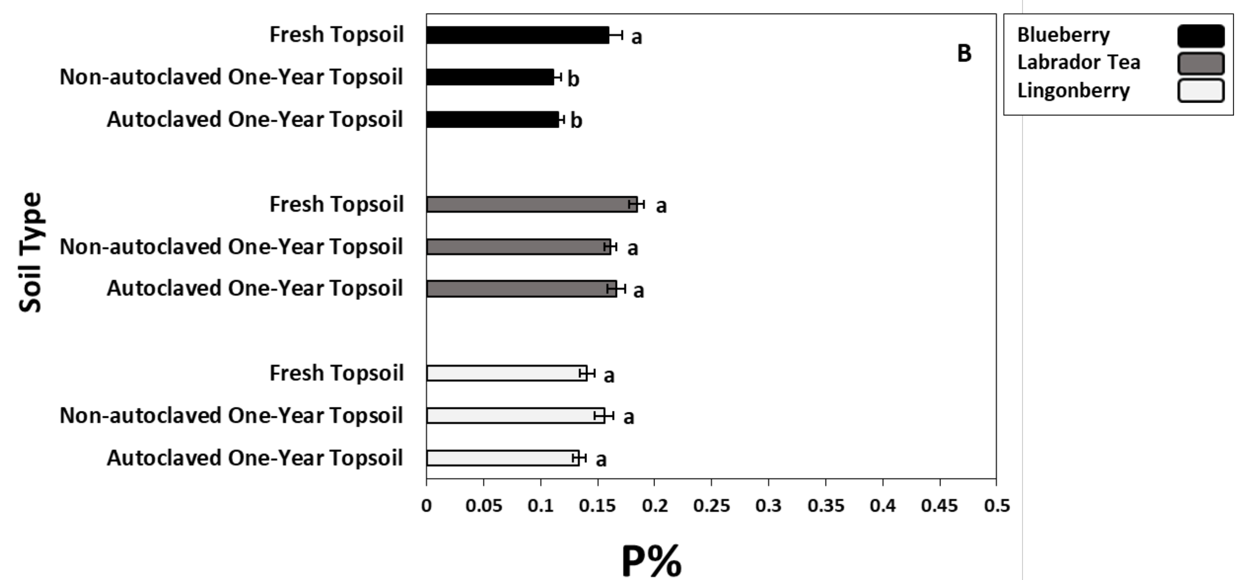


Figure 2.6. Leaf magnesium (Mg, A) and Phosphorus (P), B) concentrations of blueberry (*Vaccinium myrtilloides*), Labrador tea (*Rhododendron groenlandicum*) and lingonberry (*Vaccinium vitis-idaea*) plants growing in fresh topsoil, autoclaved stockpiled topsoil and non-autoclaved stockpiled topsoil from oil sands reclamation areas. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species according to protected Fisher's LSD test. Means (n = 9) ± SE are shown.

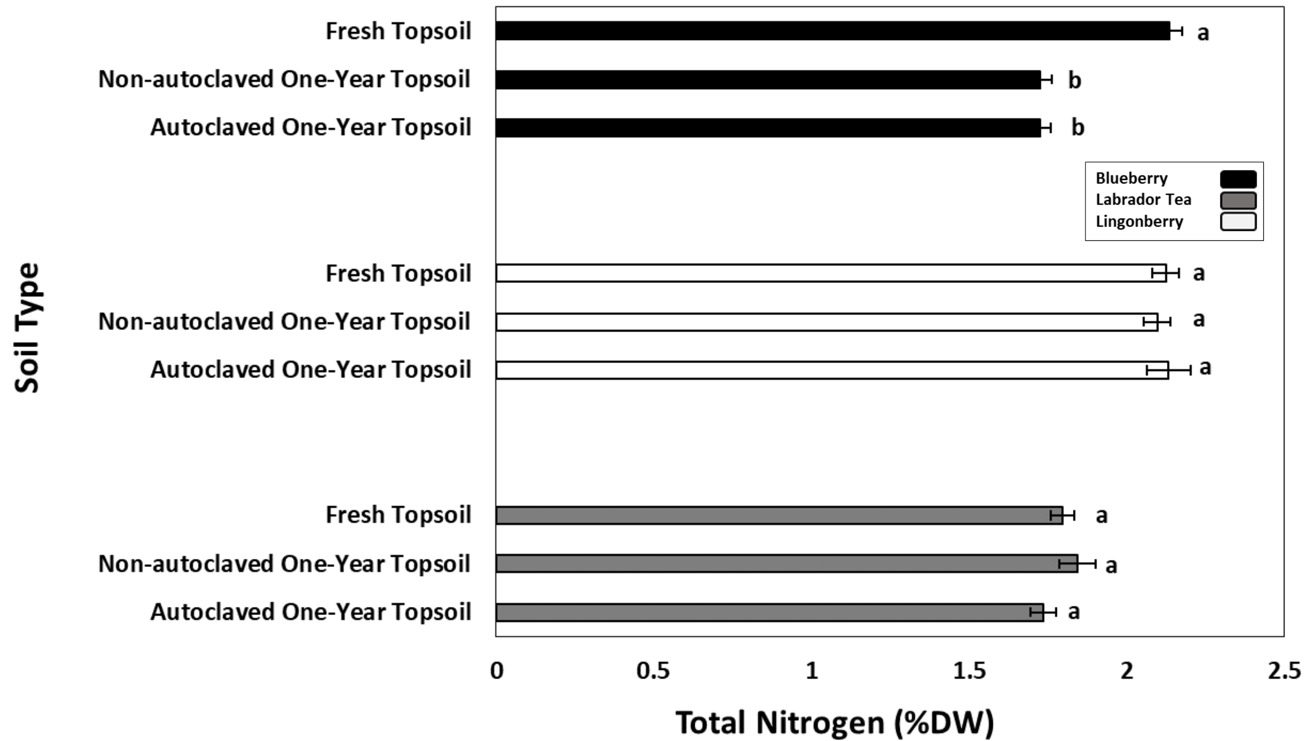


Figure 2.7. Total Nitrogen (TN) concentrations of blueberry (*Vaccinium myrtilloides*), Labrador tea (*Rhododendron groenlandicum*) and lingonberry (*Vaccinium vitis-idaea*) plants growing in fresh topsoil, autoclaved stockpiled topsoil and non-autoclaved stockpiled topsoil from oil sands reclamation areas. Different letters above the bars indicate significant differences (α = 0.05) between treatments within each plant species according to protected Fisher's LSD test. Means (n = 9) ± SE are shown.

2.3.5 Soil analyses

2.3.5.1 Soil pH and Electrical Conductivity

Increasing pH was observed in non-autoclaved stockpiled topsoil. pH from 5.7 in fresh soil raised to 6.5 in stockpiled topsoil. However, the pH of one-year stockpiled topsoil decreased to 5.7 after autoclaving. Fresh topsoil had the highest EC among all soil types (Table 2.1).

Table 2.3. pH, and electrical conductivity (EC) in the fresh topsoil, non-autoclaved stockpiled topsoil and autoclaved stockpiled topsoil (n= 5).

Soil Type	pH	EC(µS/cm)
Fresh Topsoil	5.7 b ±0.05	1146 a ±42.8
Non-autoclaved One-year Stockpiled Topsoil	6.5 a ±0.02	619.2 b ±21.5
Autoclaved One-year Stockpiled Topsoil	5.7 b ±0.1	571.4 b ±27.8

2.3.5.2 Soil Elements

No significance difference was observed in total nitrogen (TN) among different soil types. The highest calcium (Ca) and phosphorus (P) concentrations were observed in non-autoclaved one-year stockpiled topsoil. Also, the highest potassium (K), magnesium (Mg) and sodium (Na) were reported for autoclaved one-year stockpiled topsoil (Table 2.2).

Table 2.4. Elemental concentrations in the fresh topsoil, non-autoclaved stockpiled topsoil and autoclaved stockpiled topsoil (n=5).

Soil Elements	-- Soil Type --		
	Non-Auto One-Year Topsoil	Autoclaved One-year Topsoil	Fresh Topsoil
TN (W/W%)	0.499 a ±0.08	0.519 a ±0.05	0.314 a ±0.08
Ca	12882.5 a ±1446.7	8111.3 b ± 1503.1	3474.6 c±291.4
P	748.5 a ± 101.5	510.7 b ± 61.6	377.1 b ± 34.3
K	332.5 b ± 8.7	1196.5 a ± 288.1	217.6 b ± 9.06
Mg	1004.9 b ± 95.2	2669.9 a ± 570.04	468.9 b ± 25.2
Na	63.7 b ±3.2	121.4 a ± 21.8	32.9 b ±1.5

2.3.6 Root fungal colonization

Root colonization by ericoid mycorrhizal (ERM) fungi in blueberry plants was significantly higher in fresh topsoil (~ 47%) compared with the non-autoclaved one-year stockpiled topsoil (~ 21%) (Fig.2.8A). There was no significant difference root colonization intensity for blueberry plants between autoclaved and non-autoclaved soil types.

Root ERM colonization of Labrador tea plants was higher in fresh topsoil than in the non-autoclaved one-year stockpiled topsoil (~ 45% and ~ 30%, respectively) (Fig.2.8B). Similarly to blueberry plants, there was no significant difference root colonization intensity in Labrador tea between autoclaved and non-autoclaved soil types.

No significant differences in ERM colonization were found for all three soil types in lingonberry plants (Fig.2.8C).

In the present study, epidermal cells of the fine hair roots of all plant species colonized by ERM fungal coils. Hyphal coils were present inside many epidermal cells of plant roots growing in fresh topsoil, but for those plants growing in stockpiled topsoil less root epidermal cells were occupied by hyphal coils. Unlike those plant roots were in stockpiled topsoil, the external side of hair plant roots growing in fresh topsoil surrounded by high loose hyphal networks (Fig.2.9).

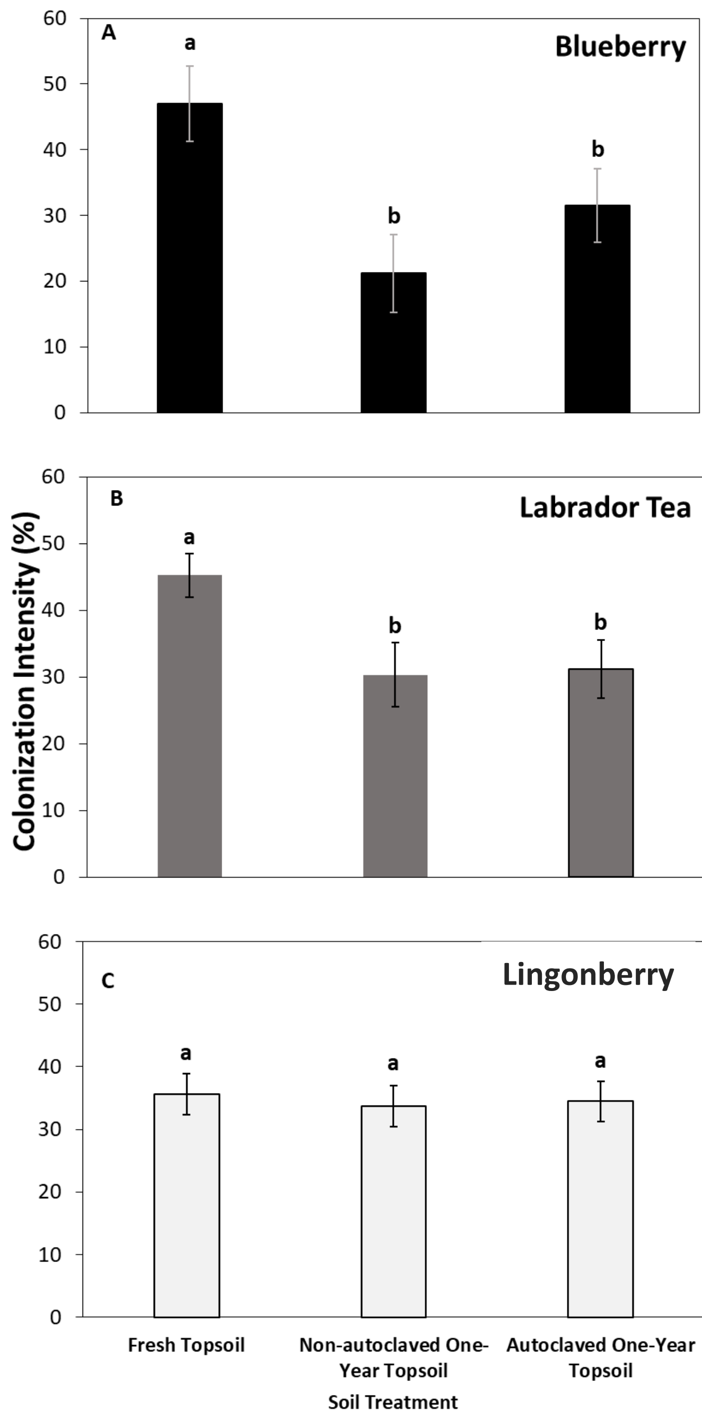


Figure 2.8. Percentage colonization of roots by ericoid mycorrhizal (ERM) fungi in blueberry (*Vaccinium myrtilloides*), Labrador tea (*Rhododendron groenlandicum*) and lingonberry (*Vaccinium vitis-idaea*) plants growing in fresh topsoil, autoclaved stockpiled topsoil and non-autoclaved stockpiled topsoil. Means (n = 6) with the same letter are not significantly different as determined by the protected Fisher's LSD test.

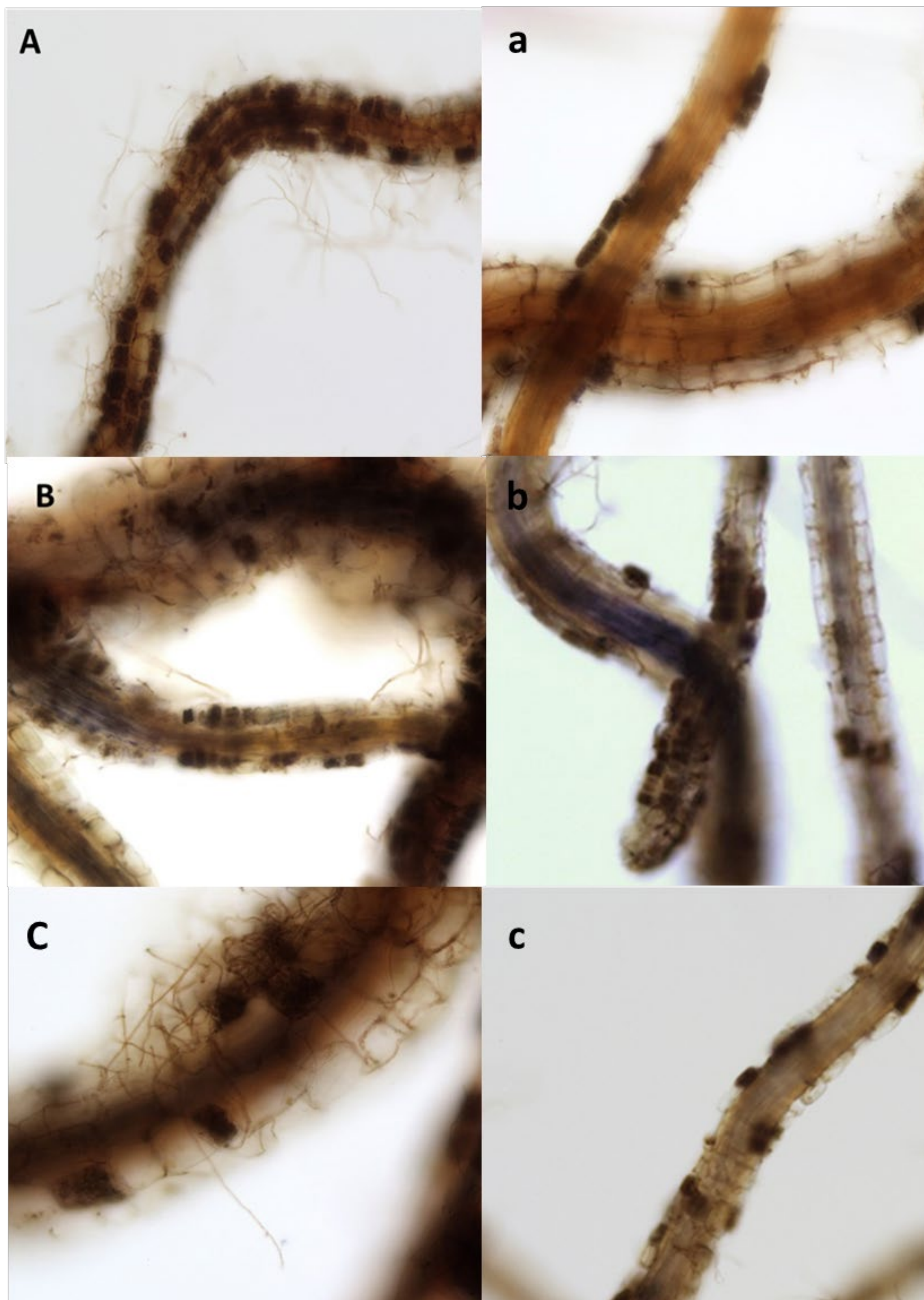


Figure 2.9. Intracellular hyphal coils of ericoid mycorrhizal fungi in epidermal cells of stained roots of Blueberry (*Vaccinium myrtilloides*) in fresh (A) and non-autoclaved stockpiled topsoil (a) , Labrador Tea (*Rhododendron groenlandicum*) in fresh (B) and stockpiled topsoil (b) and Lingonberry (*Vaccinium vitis-idaea*) in fresh (C) and stockpiled topsoil (c).

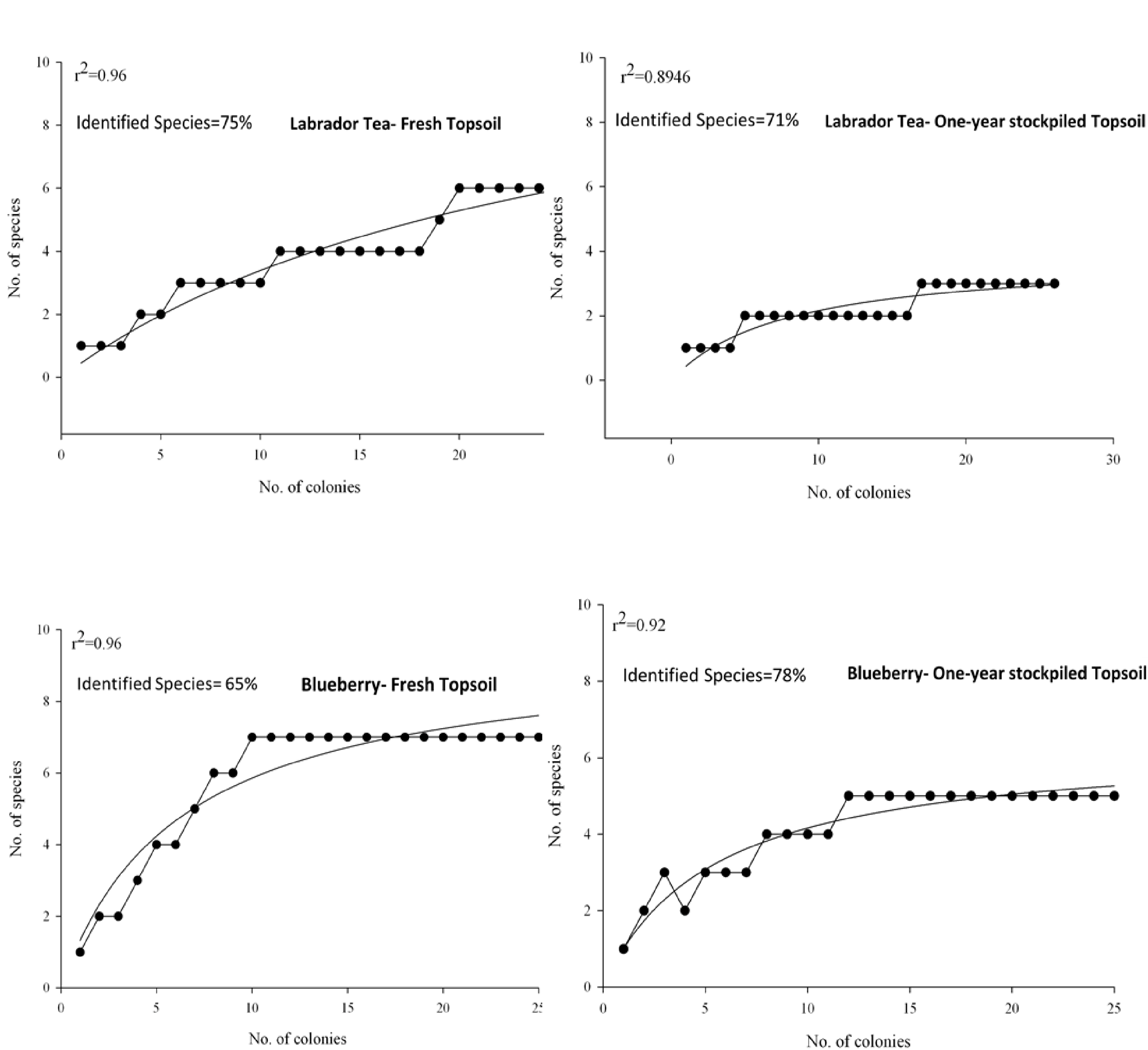


Figure 2.10. Species richness (dotted lines) and the rarefaction-species-accumulation curve (solid lines) for fungi identified in the roots of six seedlings from Labrador tea (*Rhododendron groenlandicum*) and blueberry (*Vaccinium myrtilloides*) growing in fresh Topsoil and one-year stockpiled Topsoil .

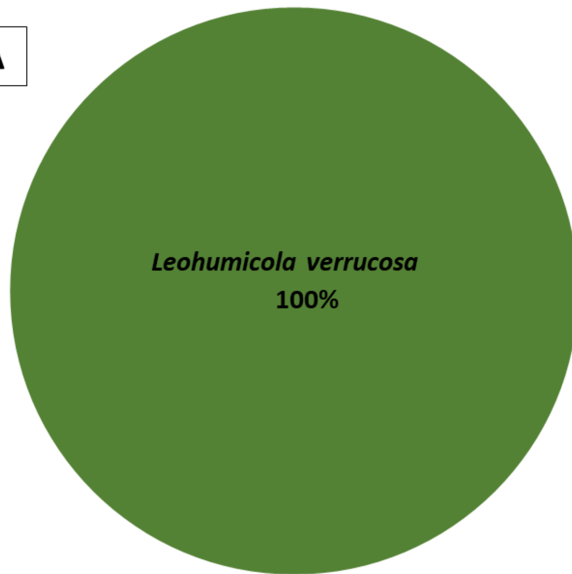
2.3.7 DNA identification of ERM

Through molecular cloning, out of the 25 selected colonies from each Labrador roots, I obtained one ERM fungus from plants growing in the stockpiled autoclaved soil (Table 2.3), six potential ERM fungi from plants growing in fresh soil (Fig.2.10A, Table 2.3), and three in the stockpiled non-autoclaved soil (Fig.2.10B, Table 2.3). For blueberry plant roots, there was one ERM fungus in the autoclaved stockpiled soil (Table 2.3), seven ERM fungi in the roots of plants growing in fresh topsoil (Fig.2.10C, Table 2.3), and five in the non-autoclaved stockpiled topsoil (Fig.2.10D, Table 2.3).

The roots of six blueberry and Labrador tea plants from the autoclaved soil were taken for molecular analyses. According to the NCBI database sequences, in the 25 sequenced colonies in each plant species the only identified fungal species was *Leohumicola verrucosa* from the Ascomycota division (Fig.2.11). Also, to identify of fungal species in plant roots of the other soil treatments, six blueberry and Labrador tea plants were randomly selected from both fresh and non-autoclaved one-year stockpiled topsoil. Molecular analyses showed that *L.verrucosa* and *Serendipita herbamans* fungi from the *Ascomycota* and *Basidiomycota* divisions, respectively, were present in both plant species in both soil treatments (Fig.2.12 and 2.13). *Clavaria citrinorubra* was recorded in both Labrador tea and blueberry plants grown in one-year stockpiled topsoil (Fig.2.12 A and 2.13 A). *Pezoloma ericae* was observed in roots of plants growing in fresh topsoil (Fig.2.12 B and 2.13 B). Other fungi, including *Phacidium grevilleae*, *Mycosymbioces mycenaphila*, *Acremonium dichromosporum*, *Micarea soralifera*, and *Xenopolyscytalum pinea* from the Ascomycota division, were found in the roots of both ericaceous plant species growing in both fresh and non-autoclaved stockpiled topsoil treatments (Fig.2.12 and 2.13).

Table 2.5. Molecular identification of fungal species isolated from roots of Labrador tea (*Rhododendron groenlandicum*) and blueberry (*Vaccinium myrtilloides*), were grown in fresh topsoil, non-autoclaved stockpiled topsoil and autoclaved one-year stockpiled topsoil. Maximum identity was determined using BLASTn searches of nuclear ribosomal internal transcribed spacer (ITS) region.

Plant species	Type of soil	Close related species	Number of bp (Maximum identity)	Reference
Labrador Tea	Fresh	*Serendipita herbamans*	84%	Vohník et al (2016)
		Leohumicola verrucosa	99%	Hambleton et al (2005)
		Phacidium grevilleae	85%	Present study
		Cadophora finlandica	93%	Gorfer et al (2009)
		Pezoloma ericae	99%	Midgley et al (2017)
		Mycosymbioces mycenaphila	89%	Present study
	Autoclaved stockpiled	*Leohumicola verrucosa*	99%	Hambleton et al (2005)
	Non-autoclaved Stockpiled	*Clavaria citrinorubra*	86%	Englander and Hull (1980), Mueller et al. (1986)
		Serendipita herbamans	84%	Vohník et al (2016)
		Leohumicola verrucosa	99%	Hambleton et al (2005)
Blueberry	Fresh	*Pezoloma ericae*	99%	Midgley et al (2017)
		Acephala applanata	98%	Lukešová et al (2015)
		Cadophora finlandica	93%	Gorfer et al (2009)
		Acremonium dichromosporum	97%	Present study
		Serendipita herbamans	84%	Vohník et al (2016)
		Leohumicola verrucosa	99%	Hambleton et al (2005)
		Micarea soralifera	93%	Present study
	Autoclaved Stockpiled	*Leohumicola verrucosa*	99%	Hambleton et al (2005)
	Non-autoclaved Stockpiled	*Clavaria citrinorubra*	86%	Englander and Hull (1980), Mueller et al. (1986)
		Xenopolyscytalum pinea	93%	Present study
		Serendipita herbamans	84%	Vohník et al (2016)
		Leohumicola verrucosa	99%	Hambleton et al (2005)
		Cadophora finlandica	93%	Gorfer et al (2009)



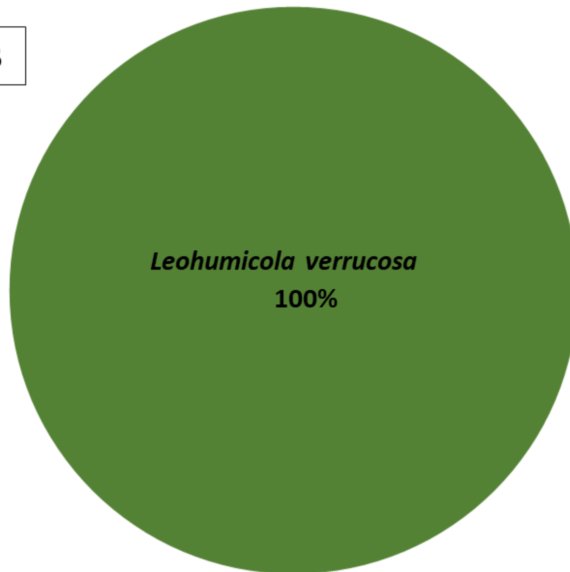
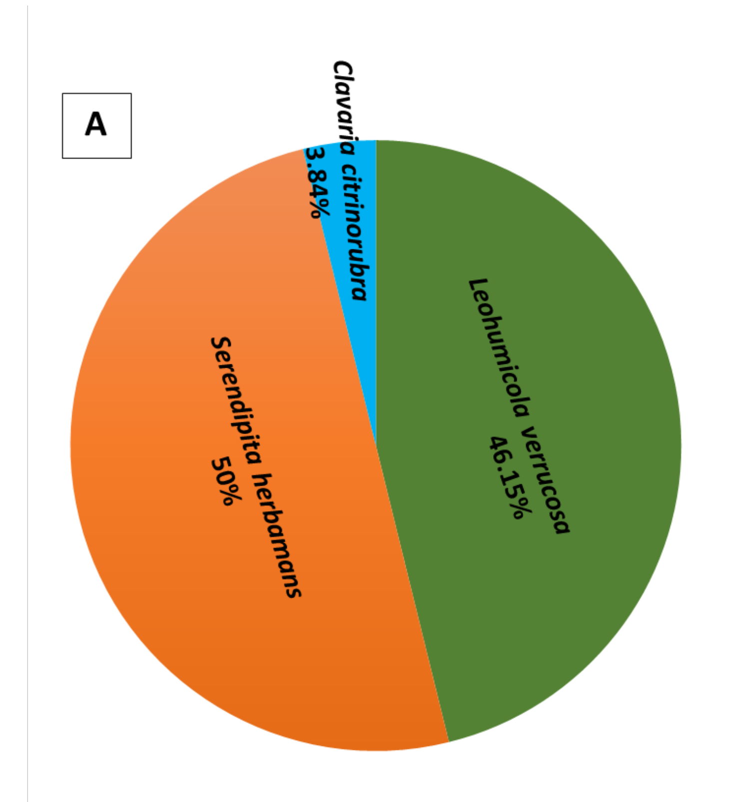


Figure 2.11. Frequency distribution of *Leohumicola verrucosa* in the roots of Labrador tea (*Rhododendron groenlandicum*) (A) and blueberry (*Vaccinium myrtilloides*) (B) plants grown in autoclaved one-year stockpiled topsoil.



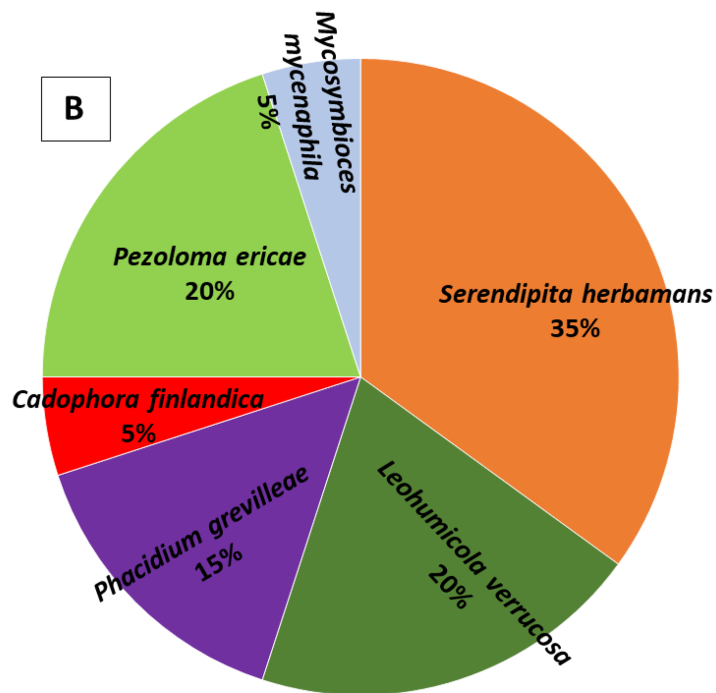


Figure 2.12. Frequency distribution of various fungal species in the roots of Labrador tea (*Rhododendron groenlandicum*) plants grown in non-autoclaved one-year stockpiled topsoil (A) and fresh topsoil (B).

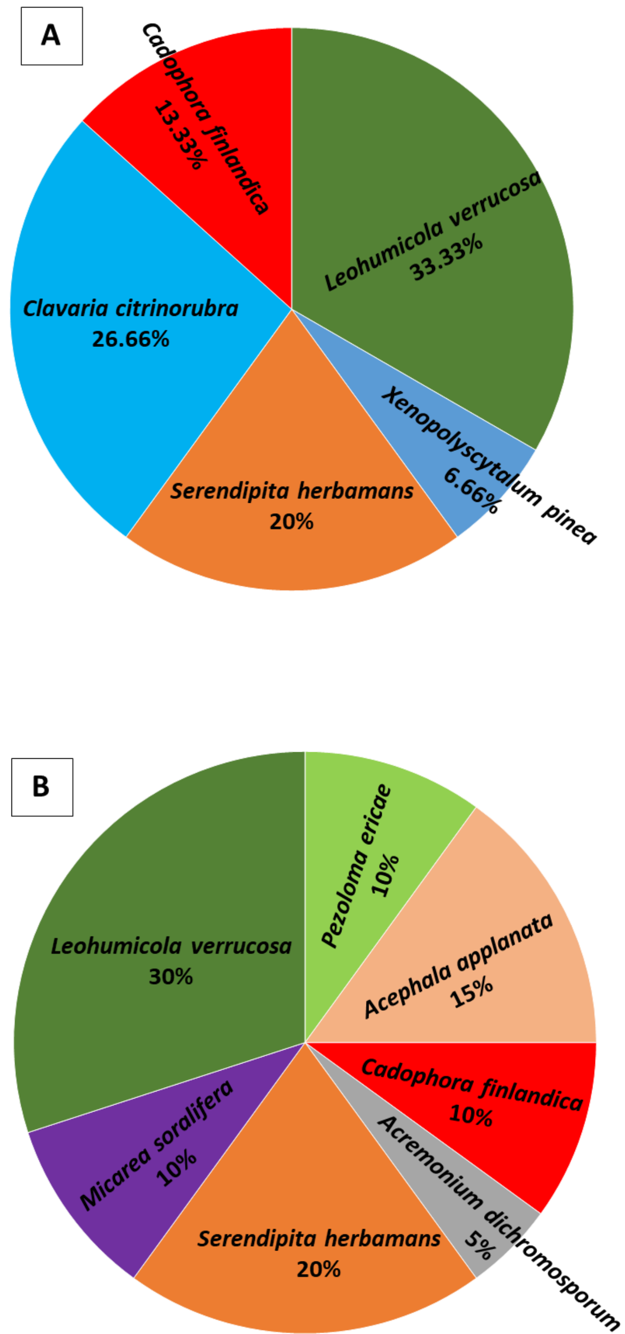


Figure 2.13. Frequency distribution of various fungal species in the roots of blueberry (*Vaccinium myrtilloides*) plants grown in non-autoclaved one-year stockpiled topsoil (A) and fresh topsoil (B).

2.4 Discussion

In the present study, seedlings from three ericaceous plant species were examined to compare their physiological responses when grown in fresh soil and in the soil that was stockpiled for one-year. After seven months of growth, blueberry and Labrador tea plants had higher shoot and root dry weights in fresh topsoil compared with one-year stockpiled topsoil. Similar findings were reported for the legume species (*Accacia saligna*), which showed reduced growth in sandy soils from Western Australia that were stockpiled for one, two, three, five, and ten years under controlled-environment conditions (Birnbaum et al. 2017). Interestingly, the biomass reduction of *A.saligna* increased with increasing the duration of stockpiling (Birnbaum et al. 2017).

Possible factors that may contribute to this effect on plant growth include changes in soil physical and chemical properties and in microbial communities including beneficial mycorrhizal fungi and root endophytes as well as fungal pathogens (Gustafson & Casper 2006).

The pH increase in one-year stockpiled topsoil may resulted in plant growth reduction in this soil type, as ericaceous plant species require acidic soil to grow better (Morley et al. 1990). Changing in the chemical properties of the soil that was stockpiled for one year can be resulted from the mixing of soil horizons during removal and stockpiling (Wick et al. 2008).

In the present study, one-year stockpiled topsoil was autoclaved to eliminate soil microorganisms. The dry weights of blueberry and Labrador tea plants were higher in the autoclaved one-year stockpiled topsoil compared with the non-autoclaved stockpiled soil and had similar values to those in plants growing in the fresh topsoil. Autoclaving may change chemical and physical characteristics of soil (Trevors 1996; McNamara et al. 2003; Berns et al. 2008) and reduce soil pH (Darbar & Lakzian 2007). It is plausible that these changes in soil properties could

have contributed to the observed effects. In the present study, the pH of one-year stockpiled topsoil after autoclaving decreased from 6.6 to 5.7, which is near the optimum soil pH for ericaceous plants (4.4-5.5) (Morley et al. 1990). However, it is also possible that autoclaving alleviated potential effects of harmful microorganisms, which could hamper plant growth in the stockpiled soil.

In contrast to blueberries and Labrador tea, lingonberry plants in the non-autoclaved one-year stockpiled topsoil had higher shoot to root dry weight ratios compared to the fresh topsoil. Cranberries growing in fresh topsoil were partly shaded by blueberry and Labrador tea plants that grew faster and were larger than lingonberry plants. Since blueberry and Labrador tea plants in the non-autoclaved one-year stockpiled topsoil were relatively small, they did not shade the lingonberry plants in this soil treatment.

The higher root and shoot dry weights of blueberry and Labrador tea plants were accompanied by higher P and N concentrations in the leaf tissues in the fresh topsoil treatment compared to the stockpiled topsoil. The higher levels of P and N in plants could be among the reasons for the increased plant growth (Oskarsson et al. 2006; Razaq et al. 2017).

Similarly, to N and P, total chlorophyll concentrations in blueberry plants were higher in fresh topsoil than in the remaining two soil treatments. Higher N and P tissue levels can enhance stromal and thylakoid proteins which can increase photosynthetic pigment concentrations in plant leaves (Cooke et al. 2005; Bojovic & Stojanovic 2006; Filho et al. 2011).

According to Cairney and Ashford (2002) and Perotto et al. (2002), in ericoid mycorrhizae, fungal hyphae can penetrate individual cells and create hyphal coils. Ericoid mycorrhizae usually

do not form a hyphal mantle. Therefore, the presence of ericoid mycorrhizal colonization can only be verified using microscopy aided by staining of fungal hyphae as carried out in the present study.

Abdul-Kareem and McRae (1984) demonstrated that P, K and Mg values in clayey stockpile from Tonbridge region in London increased compared to the undisturbed soil. In the present study, electrical conductivity of the fresh topsoil was significantly higher than that of stockpiled topsoil, but the levels of the examined essential elements, including P and Ca, in (non-autoclaved) one-year stockpiled topsoil treatment were significantly higher than those in the fresh topsoil. No significant differences were found in other nutrients including N, K, Mg and Na between the fresh topsoil and non-autoclaved one-year stockpiled topsoil, suggesting that these mineral nutrients remained unchanged after one-year of stockpiling. In experiment by Ross and Cairns (1981), no significant differences were detected between mineral N levels in the ten-year-old stockpiled soil and adjacent undisturbed soil. It has been suggested that pedogenesis could restore soil chemical properties during these ten years. In another study by Birnbaum et al. (2017), there were no significant differences in chemical properties between the different soil stockpiles and references undisturbed sites. The pH of stockpiled topsoil in my study was higher than that of the fresh topsoil, which could have resulted from the build-up of ammonium (Abdul-Kareem & McRae 1984). It has been reported that the mineral nutrient uptake by plants, especially P, can be reduced by increased salinity levels (high EC) (Poss et al. 1985; Munns 1993; Grattan & Grieve 1999) .However, despite the higher salinity of the fresh topsoil (1146 µS/cm) compared with the stockpiled topsoil (619.2 µS/cm), blueberries in the fresh topsoil accumulated more P and N in their shoot tissues compared with the stockpiled topsoil. Increased P uptake by plant tissues under salinity conditions has been reported in some studies. Various factors can affect P accumulation in plant tissues, including the plant type, or even cultivar, and plant growth conditions (Grattan &

Grieve 1994). High levels of P and N in the leaves of blueberries in fresh topsoil could be due to the mycorrhizal fungi. The colonization intensity in blueberry and Labrador tea roots in fresh topsoil were higher than in the roots of plants growing in the stockpiled topsoil. Therefore, it is plausible that mycorrhizal fungi enhanced P and N uptake in these plants. Phosphorus acquisition in mycorrhizal plants can be increased due to the external fungal hyphae which can obtain P even from sparingly soluble P sources (Bolan et al. 1987). The effectiveness of external hyphae in P absorption can be attributed to the production of ectoenzymes, such as acid phosphatases and phytase, in the external hyphae of ecto- (Hilger & Krause 1989; Ho 1989) and endomycorrhizal fungi (Straker & Mitchell 1986). Plants colonized by the ectomycorrhizal and ericoid mycorrhizal fungi can access N sources which are not accessible to non-mycorrhizal plants (Bajwa & Read 1985; Abuzinadah & Read 1989; Finlay et al. 1992). These mycorrhizal fungi are able to produce extracellular acid proteinases which can mobilize N from organic matters (Leake & Read 1990; Zhu et al. 1990; Maijala et al. 1991). Furthermore, inflow of N into root tips of mycorrhizal plants can be increased (Hogberg 1989) due to the existence of fungal hyphae over nutrient depletion zone or because of a relatively continuous release of N from the fungal tissues (Nye & Tinker 1977; Harley & Smith 1983).

In roots of lingonberry growing in the three types of soil, colonization intensity was similar. and it was lower compared with blueberry and Labrador tea roots in the fresh topsoil. The roots of blueberry and Labrador tea plants in the fresh topsoil treatment had higher ERM colonization intensity compared with the plants in one-year stockpiled topsoil. These results support previously reported findings, which demonstrated that the topsoil storage can reduce arbuscular mycorrhizal colonization of plant roots in *A.saligna* during the first four years of storage (Birnbaum et al. 2017). The colonization of corn roots with arbuscular mycorrhizal fungi was also reported to decline after

three years of stockpiling storage (Gould & Liberta 1981). Soil microorganisms including mycorrhizal fungi require carbon to survive. Soil microbial communities can be re-established in older stockpiled topsoil (five and ten-years) due to the higher access to carbon. Because of the existence of dense vegetative cover on older stockpile soils, decomposition of plant residue will be higher, therefore, more carbon will be accessible for different soil microorganisms including mycorrhizal fungi (Birnbaum et al. 2017).

It was expected that fresh topsoil would have more ERM fungal species than one-year stockpiled topsoil, because soil stockpiling has been previously reported to reduce mycorrhizal diversity (Strohmayer 1999). In the study, which was conducted in south-west Australian jarrah forest, reduced ericoid endophyte infectivity was reported in forest topsoil following bauxite mining. This reduction could be due to disruption of the hyphal network, absence of host plants or increased competition from soil micro-organisms antagonistic to the endophytes (Hutton et al. 1996).

In the present study, molecular analyses demonstrated that ERM richness in fresh topsoil was higher than that in the one-year topsoil. In my study hair roots of blueberry and Labrador tea plants grown in fresh and one-year stockpiled topsoil, collected from the natural forest sites around the oil sands mining areas, had different fungal species. Most frequently recorded taxa were *L.verrucosa* and *S.herbamans* (Fig 2.11 and 2.12). These fungi were found in the hair roots of both examined plant species and in both fresh and non-autoclaved one-year topsoil. It has been reported that the roots of individual Ericaceous plants are typically colonized by several ERM fungal taxa simultaneously (Midgley et al. 2004; Bougoure & Cairney 2005).

Generally, *Sebacinales* spp. have been reported as common ERM fungi (Selosse et al. 2007) in *Vaccinium* roots. The *Sebacinales* spp. can form ericoid mycorrhizae and other types of

mycorrhizae such as orchid mycorrhizae, ecto- and ectendomycorrhizae with wide range of plants (Vohník et al. 2016). *Serendipita herbamans* is a fungal species in *Serendipitaceae* family from *Sebacinales* order. According to the microscopic and molecular results of several studies, *Serendipitaceae* spp. can be found in ericaceous plant roots. (Berch et al. 2002; Allen et al. 2003; Selosse et al. 2007; Ishida & Nordin 2010; Wurzburger et al. 2011; Setaro et al. 2013; Bruzone et al. 2015). *Serendipitaceae* has been also reported to be a fungal endophyte (Selosse et al. 2009; Weiss et al. 2011), which can grow in living plant tissues and not form any mycorrhizae (Wilson 1995; Rodriguez et al. 2009). My analysis of ITS sequences revealed the existence of *L.verrucosa* species in the roots of blueberry and Labrador tea plants grown in all soil types even in the plant roots grown in the autoclaved one-year topsoil. This species was isolated from heated soil of the commercial lowbush blueberry field in Nova Scotia, Canada. *Leohumicola verrucosa* has been also isolated from the unheated soil in Puerto Rico and from ericoid roots in Alberta, which shows that this fungus can have a broad distribution (Hambleton et al. 2005). In the present study, other frequent species of fungi in Labrador tea hair roots of fresh topsoil and blueberry roots of the non-autoclaved stockpiled topsoil were, respectively, *P.ericae* and *C.citrinorubra*. *Pezoloma ericae* is one of the prominent ERM fungi that has been reported from South America (Bruzone et al. 2016), South Africa (Kohout & Tedersoo 2017), Australia (Midgley et al. 2017), Europe, and North America in a wide range of plant species (Pearson & Read 1973b; Hambleton & Currah 1997; Sharples et al. 2000a; Bougoure et al. 2007; Kjøller et al. 2010; Walker et al. 2011; Wurzburger et al. 2011; Hazard et al. 2014). Much of our understanding of ericoid mycorrhizae came from *P. ericae* (Pearson & Read 1973a; Perotto et al. 2002; Smith & Read 2008). Fungal species in the *Clavaria* group are saprophytes, however, some of them are able to form ericoid mycorrhizae with ericaceous plant roots (Englander & Hull 1980; Mueller et al. 1986). Fungi of the genus *Clavaria*

can be found in wide range of areas in the boreal, temperate and tropical regions (Olariaga et al. 2015).

Acephala applanate species from Ascomycota division was found in blueberry roots in the fresh topsoil treatment. In the central European forest, *A.applanate* was able to create structures resembling ericoid mycorrhizae and Dark Septate Endophytes in blueberry roots (Lukešová et al. 2015).

There is no earlier evidence for *Phacidium grevilleae*, *Mycosymbioces mycenaphila*, *Acremonium dichromosporum*, *Micarea soralifera* and *Xenopolyscytalum pinea* from Ascomycota division to form ericoid mycorrhizae. This study is the first report demonstrating that the above species may be present in the roots of ericaceous plants.

Some of the common ERM fungi species that are globally distributed in Ericaceae roots are *Rhizoscyphus ericae*, *Oidiodendron maius*, and a group of unidentified *Helotiales* isolates. Although these species were not observed in this study, several authors reported their presence in the roots of *C. vulgaris* ((Perotto et al. 1996; Sharples et al. 2000a), and *Gaultheria shallon* (Monreal et al. 1999).

In conclusion, in the controlled-environment study, blueberry and Labrador tea plants showed greater growth in fresh topsoil compared with non-autoclaved one-year stockpiled topsoil. However, cranberries had lower shoot and dry weights in fresh topsoil compared to non-autoclaved one-year stockpiled topsoil. Cranberries in fresh topsoil were shaded by the larger blueberry and Labrador tea plants. High shoot and root dry weights, leaf chlorophyll concentration, P and N in blueberry plants in fresh topsoil were likely possible due to the greater mycorrhizal fungi colonization of their roots. The results also confirm that high degree of ericoid mycorrhizal fungi

colonization in plant roots can result in an increased uptake of N and P in blueberries. Some plants in the autoclaved one-year stockpiled topsoil showed high dry weights, which is probably due to the presence of the heat-resistant ericoid mycorrhizal fungus.

Chapter 3

Study 2: Effects of ericoid mycorrhizal fungi on salt tolerance of ericaceous plant species

3.1 Introduction

Elevated soil salinity is among the major concerns in oil sands reclamation areas. Oil sands mining in northeastern Alberta, Canada, especially in the Athabasca River area, has become one of the most economically important industrial activities in this country (Audet et al. 2005). Mining disturbs vast areas of the northern boreal ecosystems, which need to be restored after mine closure. In the process of oil sands mining, bitumen is separated from the substrate through recycled hot water extraction, which leads to increased salinity levels. Soil salinity in oil sands reclamation areas is among the most challenging issues for vegetation establishment (Howat 2000). The uptake of Na^+ by plants can be increased by the presence of Cl^- (Renault et al. 2001) and both Na and Cl ions together can be extremely harmful for plants (Martin & Koebner 1995).

Ericaceous plants including blueberry (*Vaccinium myrtilloides*), Labrador tea (*Rhododendron groenlandicum*), and lingonberry (*Vaccinium vitis-idaea*) are found in many northern boreal forest sites. These plant species are able to form symbiotic associations with ericoid mycorrhizal fungi (ERM). This mycorrhizal association is characterized by the formation of extracellular hyphae inside the epidermal cells of the fine hair roots of ericaceous plants and by a loose hyphal network around the plant roots (Read 1984).

Several studies have reported alleviation of the effects of salt stress on plants by the ectomycorrhizal and arbuscular mycorrhizal fungi (Evelin et al. 2009). Ectomycorrhizal fungi have been demonstrated to decrease Na uptake to shoots and leaf tissues (Muhsin & Zwiazek 2002b; Bois et al. 2006a). Several studies have also shown that arbuscular mycorrhizal fungi improve salt tolerance in their host plants by increasing water absorption capacity and adjusting the osmotic

balance and composition of carbohydrates (Rosendahl & Rosendahl 1991; Al-Karaki & Clark 1998; Ruiz-Lozano & Azco´n 2000; Ruiz-Lozano 2003). However, potential significance of ERM fungi in improving salt tolerance of ericaceous plants is unknown.

Meliniomyces variabilis can form ericoid mycorrhiza with the shrubs in the Ericaceae family, including *Vaccinium*, *Calluna*, and *Empetrum* which dominating understory layer of boreal forests (Hambleton & Sigler 2005). It has been investigated that this fungus has a world-wide distribution and has been detected molecularly and cultured from both ectomycorrhizal and ericoid mycorrhizal roots in many countries such as Canada, New Zealand, Norway, Finland, Germany, Czech Republic, Great Britain, and Ireland (Hambleton & Sigler 2005). Its ericoid mycorrhizal ability has beneficial effects on host plant growth and mutual transfer of carbon and nitrogen, with its host *in vitro* (Grelet et al. 2009).

Oidiodendron maius has a symbiotic relationship with the roots of ericaceous plants, and facilitate the exchange of nutrients between host plant roots and the soil (Rice & Currah 2006). Also, this fungus can protect it host plant under stress conditions such as heavy metal toxicity (Daghino et al. 2016).

The present study was conducted to gain better understanding of the effects of two ERM fungi including *O.maius* and *M.vaiabilis* on growth and physiology responses of ericaceous plants under NaCl stress. Theses two ERM fungi were isolated from the roots of ericaceous plants collected from Canadian boreal forest sites, and used as mycorrhiza inoculation in this experiment.

A controlled-environment study was carried out with blueberry *(V.myrtilloides*), Labrador tea *(R.groenlandicum*), and lingonberry (*V.vitis-idaea*) plants, which are used for oil sands reclamation. The following hypotheses were tested: 1. Plants of these three ericaceous plants vary

in their sensitivity to salt stress, and 2. Negative effects of high salinity on ericaceous plants can be alleviated by with the presence of ERM fungi on plant roots.

3.2 Material and Methods

3.2.1 Plant material and growth conditions

Seeds of blueberry, lingonberry and Labrador tea were surface-sterilized with sodium hypochlorite 20% for 2 min, followed by ethanol 70% for 5 min, and rinsed with autoclaved distilled water. Petri dishes with solid Murashige and Skoog medium (pH 5.7) (Murashige and Skoog 1962) were used to germinate sterilized seeds. The seeds were germinated, and seedlings grown in a controlled-environment growth room under the 16 h photoperiod, 23/18°C (day/night) temperature, and 65 ± 10% relative humidity. The germinants were transferred from Petri dishes to sterilized plug trays with 150 cm^3 cells filled with the autoclaved potting mix and grown in these containers for five months before being transplanted to the bigger pots (500 cm^3) containing the autoclaved potting mix. The seedlings were provided with water that was adjusted to pH 5.5 by H_2SO_4 when required and with N_{20}-P_{20}-K_{20} commercial fertilizer every week.

3.2.2 Preparation of fungal inoculum

Fungal cultures of *O.maius* and *M.vaiabilis* were grown on potato dextrose agar solid medium in Petri dishes. They were kept in a dark place at room temperature (approximately 23°C) for four months. The cultures were then transferred to a flask containing liquid Melin Norkrans medium and continuously shaking for two months. The fungal cultures were homogenized with a Waring blender and autoclaved distilled water was added to mycelium slurry to dilute it to the final concentration of 0.42 mg dry mycelia/ml. The seedlings were inoculated with the mycelial slurry twice (Calvo-Polanco et al. 2009). The first inoculation was carried out with six-month-old

seedlings that were still inside the plug trays, and each tray cavity (150 ml) received four ml of the slurry. The second inoculation was conducted one month after the first inoculation immediately after transplanting the three-month-old seedlings to the 500 cm^3pots. This time, 10 ml of the fungal slurry added to each pot. Twenty seedlings of each plant species were inoculated with *O.maius* and another 20 were inoculated with *M.variabilis*. The remaining 20 seedlings of each species were not inoculated and served as mycorrhizal control.

3.2.3 Treatments

Sodium chloride (30 mM NaCl) solution was applied to the plants one month after the second inoculation. Ten seedlings of each mycorrhizal and non-mycorrhizal group from each species were treated with 30 mM NaCl and another 10 with 0 mM NaCl (NaCl control). Ten pots of each plant group was placed in a tray (25 x 250 cm) with 0 or 30 mM NaCl for two days. After two days, the pots were removed and drained for two days and this treatment was repeated for four weeks. The trays were flushed to prevent salt accumulation every three days.

3.2.4 Shoot and root dry weights

Shoot and root dry weights of five seedlings for each of the three-plant species and treatment were determined. After harvesting the plants, their shoots and roots were separated, placed in paper bags and dried in an oven at 65°C for 72 h.

3.2.5 Leaf chlorophyll concentrations

Chlorophyll-a and chlorophyll-b concentrations were measured in fully expanded leaves of six plants from each species and treatment. The leaves were excised and placed in a freeze-drier for 72 hours and then immediately ground with a Thomas Wiley Mini-Mill (Thomas Scientific, NJ, USA). Approximately 10 mg dry weight of each ground leaf sample was combined with 8 ml

dimethyl sulfoxide and chlorophyll was extracted at 65°C for 22 h. After filtering, chlorophyll concentrations were measured in dimethyl sulfoxide extracts with a spectrophotometer (Ultrospec, Pharmacia LKB, Uppsala, Sweden), at 648 nm and 665 nm for chlorophyll-a and chlorophyll-b concentrations, respectively. The total chlorophyll "a" and "b" concentrations were determined from the Arnon's equation (Sestak et al. 1971).

3.2.6 Gas exchange

After four weeks of NaCl treatments, six seedlings were selected from each species and treatment to measure the net photosynthesis (Pn) and transpiration (E) rates. The uppermost fully developed leaves were selected for the measurements with an infrared gas analyzer (LI-6400, LI-COR, Lincoln, Nebraska USA) at 400 µmol m^{-2} s^{-1} PPFD, four weeks after the onset of photoperiod. Due to the small size of lingonberry leaves, five to ten leaves were inserted in the leaf chamber. Following the measurements, the leaves were detached and scanned, and the leaf areas were determined using the Sigmascan Pro 5.0 computer software (Systat Software, San Jose, CA).

3.2.7 Root fungal colonization

Five seedlings from each species and treatment were selected to examine fungal colonization on roots. In total 90 root samples were harvested and covered with aluminum foil and kept on ice, until they were delivered to the laboratory. The root tips were approximately 10 cm in length and were fixed in (formaldehyde (10 ml): glacial acetic acid (5 ml): ethanol (50 ml): distilled water (35 ml) Prior to microscopy, the roots were rinsed twice with distilled water, and then cleared in potassium hydroxide 2.5% KOH at 60°C for 30 min. After removing from the oven, the roots were washed twice with distilled water and commercial vinegar. The root samples were stained with ink (5%) in commercial vinegar at 60°C for 10 minutes. The stained roots were rinsed with

distilled water, cut in to 12 equal segments, mounted on microscope slides and observed under the light microscope. All root pieces were rated from 0 to 5 according to the extent of colonization with ericoid mycorrhizal fungi (Trouvelot et al. 1986). Finally, the intensity of mycorrhizal colonization in the root system (M) was calculated using the following equation:

$M\% = (95n5+70n4+30n3+5n2+n1)/ (n\ total)$

where n5 = number of fragments rated 5

n4 = number of fragments rated 4

n3=number of fragments rated 3

n2=number of fragments rated 2

n1=number of fragments rated 1

n total=total number of root fragments

3.2.8 Statistical analysis

All data were analyzed using SAS GLM model (Version 9.2, SAS Institute Inc., Cary, NC) to determine statistically significant ($p \leq 0.05$) differences between treatments. In this study, a two-way ANOVA was used with ERM inoculation and NaCl treatments as the main factors. The data that did not meet the ANOVA assumptions of normality of distribution and homogeneity of variance were transformed with a log10 function. Comparisons between different treatment means were conducted using protected Fisher's LSD test. A representative table of the ANOVA results is shown in the Appendix section.

3.3 Results

3.3.1 Root fungal colonization

Root fungal colonization of blueberry seedlings inoculated with *O.maius* was ~62% compared with ~60.4% in seedlings inoculated with *M.variabilis* (Table 3.1). Non-inoculated seedlings had between about 17% and 30% of roots colonized by the unknown fungi depending on the species (Table 3.1). Treatment with NaCl significantly reduced root colonization in all plant species (Table 3.1).

Table 3.1. Percentages of root mycorrhizal colonization intensity in non-inoculated seedlings of blueberry (*Vaccinium myrtilloides*), Labrador tea (*Rhododendron groenlandicum*), and lingonberry (*Vaccinium vitis-idaea*) and in seedlings inoculated with *Oidiodendron maius* and *Meliniomyces variabilis* subjected to 0 and 30 mM NaCl. Different letters after means indicate significant differences (α = 0.05) between treatments within each plant species according to protected Fisher's LSD test. The values are means ± SE of six seedlings per treatment.

	Oidiodendron maius	*Meliniomyces variabilis*	Non-Inoculated
		Blueberry	
0 mM NaCl	62.8 a ±2.6	60.4 ab ±5.8	17.6 c ±1.7
30 mM NaCl	51.6 b ±2.03	53.3 ab ±5.4	12.1 c ±1.3
		Labrador Tea	
0 mM NaCl	69.7 a ±3.1	70.8 a ±2.06	30.4 c ±2.1
30 mM NaCl	59.7 b ±2.4	61.3 b ±3.4	19.6 d ±3.3
		Lingonberry	
0 mM NaCl	71.7 a ±3.9	69.9 ab ±2.2	21.1 c ±3.08
30 mM NaCl	69.7 ab ±4.04	61.5 b ±2.2	12.9 c ±2.2

3.3.2 Gas Exchange

In control (not treated with NaCl) blueberry seedlings, net photosynthesis (Pn) was significantly higher in seedlings inoculated with *O.maius* compared with the non-inoculated plants (Fig.3.1A). Net photosynthesis decreased as a result of the 30 mM NaCl treatment and this decrease was greater in non-inoculated blueberries compared with those inoculated with ERM fungi (Fig.3.1A). There were no significant differences in the net photosynthesis between blueberry seedlings inoculated with *O. maius* and *M.variabilis* treated with 30 mM NaCl (Fig.3.1A).

There was no significant inoculation effect on Pn in Labrador tea plants (Fig.3.1B). However, when treated with 30 mM NaCl, plants inoculated with *O. maius* and *M. variabilis* had higher net photosynthesis than NaCl-treated non-inoculated Labrador tea plants (Fig.3.1B).

In lingonberry that was not treated with NaCl, Pn was higher in cranberries inoculated with the ERM fungi compared with the non-inoculated control cranberries (Fig.3.1C). Similarly, to blueberry and Labrador tea, in non-inoculated cranberries subjected to NaCl treatment, Pn was reduced to negative values (Fig.3.1C). In plants inoculated with *O. maius* and *M. variabilis* and treated with 30 mM NaCl, Pn did not significantly decrease compared with the untreated control (Fig.3.1C).

In control (not treated with NaCl) blueberry seedlings, transpiration rates (€) were significantly higher in seedlings inoculated with ERM fungi compared with the non-inoculated plants (Fig.3.2A). Transpiration rates decreased as a result of the 30 mM NaCl treatment and this decrease was greater in non-inoculated blueberries compared with those inoculated with ERM fungi (Fig.3.2A). There were no significant differences in the transpiration rates between blueberry seedlings inoculated with *O. maius* and *M.variabilis* treated with 30 mM NaCl (Fig.3.2A).

In Labrador tea that was not treated with NaCl, transpiration was higher in seedlings inoculated with *O. maius* compared with the non-inoculated control Labrador tea . When plants subjected to 30 mM NaCl treatment, Labrador tea seedlings inoculated with *M.variabilis* had higher transpiration rates compared with other two mycorrhizal inoculation (Fig.3.2B).

In lingonberry that was not treated with NaCl, transpiration was higher in cranberries inoculated with the ERM fungi compared with the non-inoculated control cranberries (Fig.3.2C). Non-inoculated cranberries subjected to NaCl treatment had the lowest value of transpiration (Fig.3.2C).

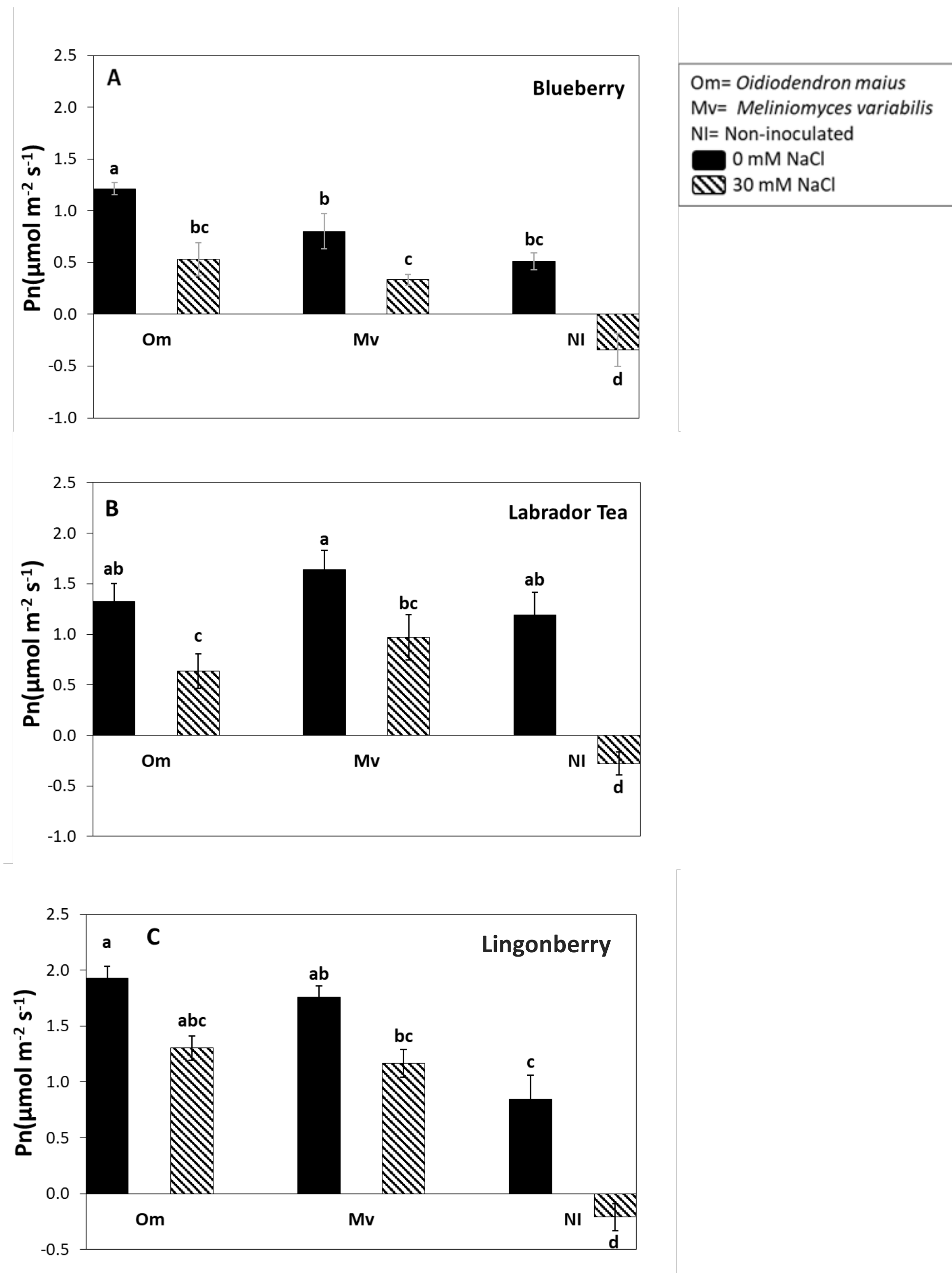


Figure 3. 1. Net photosynthesis (*Pn*) rates in non-inoculated (NI) blueberry (*Vaccinium myrtilloides*) (A), Labrador tea (*Rhododendron groenlandicum*) (B) and lingonberry (*Vaccinium vitis-idaea*) (C) and in plants inoculated with *Oidiodendron maius* (Om) and *Meliniomyces variabilis* (Mv*)*. The plants were subjected to 0 (control) and 30 mM NaCl treatments for four weeks. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species according to protected Fisher's LSD test. Means. Means ($n = 6$) ± SE are shown.

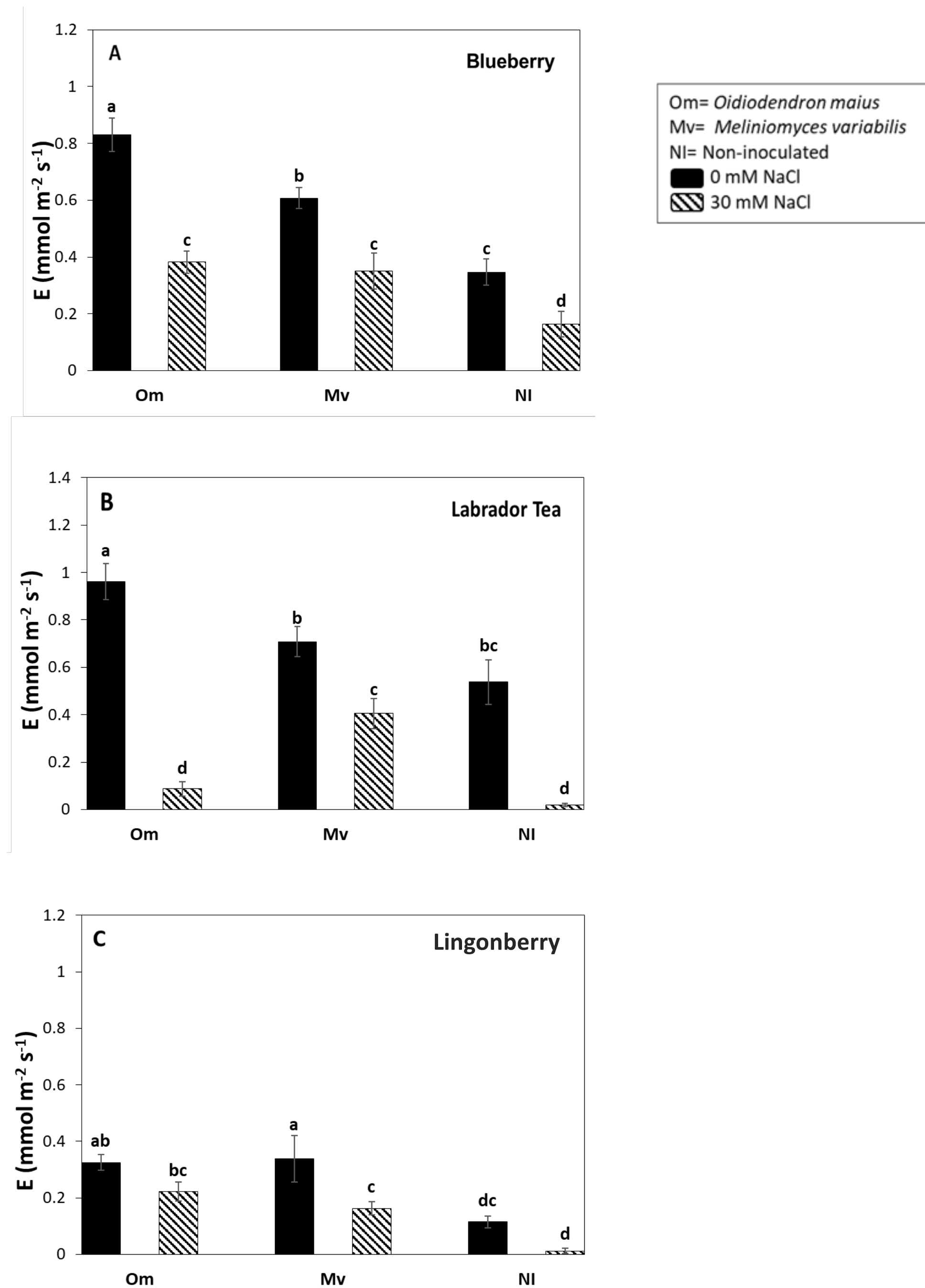


Figure 3. 2. Transpiration (*E*) rates in non-inoculated (NI) blueberry (*Vaccinium myrtilloides*) (A), Labrador tea (*Rhododendron groenlandicum*) (B) and lingonberry (*Vaccinium vitis-idaea*) (C) and in plants inoculated with *Oidiodendron maius* (Om) and *Meliniomyces variabilis* (Mv*)*. The plants were subjected to 0 (control) and 30 mM NaCl treatments for four weeks. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species according to protected Fisher's LSD test. Means. Means ($n = 6$) ± SE are shown

3.3.3 Shoot and root dry weights

There was no effect of inoculation on root dry weights in blueberry plants that were not treated with NaCl (Fig.3.3A). The treatment with 30 mM NaCl reduced root dry weights in non-inoculated plants and in plants inoculated with *O.maius* and did not affect root dry weights in plants inoculated with *M.variabilis* (Fig.3.3A). In Labrador tea, plants that were not treated with NaCl and inoculated with *M.variabilis* had higher root dry weights compared with untreated plants inoculated with *O.maius* and with non-inoculated plants (Fig.3.3B). NaCl treatment reduced root dry weights in plants of all three inoculation treatments (Fig.3.3B).

In lingonberry, inoculation with *O.maius* and *M.variabilis* resulted in higher root dry weights (Fig.3.3C). Treatment with NaCl reduced root dry weights in non-inoculated plants but had no effect on root dry weights in plants inoculated with both ERM fungi.

In blueberry, there was no effect of inoculation on shoot dry weights in plants that were not treated with NaCl (Fig.3.4A). The 30 mM NaCl treatment reduced shoot dry weight in inoculated plants with *O.maius* and did not affect shoot dry weights of inoculated plants with *M.variabilis* and non-inoculated plants (Fig.3.4B). In Labrador tea, NaCl-untreated plants that were inoculated with *M.variabilis* had higher shoot dry weights compared with untreated non-inoculated plants (Fig.3.4B). NaCl treatment reduced shoot dry weights in plants inoculated with *M.variabilis* and did not affect shoot dry weights of inoculated plants with *O.maius* and non-inoculated plants (Fig.3.4B). For lingonberry plants, Inoculation with *O.maius* and *M.variabilis* resulted in higher shoot dry weights (Fig.3.4C). Treatment with NaCl had no effect on shoot dry weights in all non-inoculated and inoculated plants with both ERM fungi (Fig.3.4C).

There was no significant difference in shoot: root ratios between inoculated and non-inoculated plants that were treated with 30 mM and 0 mM NaCl in all three-plant species (Fig.3.5).

Blueberry plants that were not treated with NaCl and were inoculated with *O.maius* had higher total plant dry weights compared with non-inoculated plants. There were no significant difference between total dry weights of inoculated plants with *M.variabilis* and non-inoculated plants (Fig.3.6.A).

In Labrador tea, inoculation with *M.variabilis* resulted in the highest total plant dry weights (Fig.3.6B). A reduced total dry weight was observed in Labrador tea plants inoculated with *M.variabilis* and no significant differences observed in total dry weights of plants inoculated with *O.maius* and non-inoculated plants (Fig.3.6B).

In lingonberry, *O.maius* inoculation resulted in a significant increase in the total plant dry weights compared with the other inoculation treatments (Fig.3.6C). The inoculation of plants with both ERM fungi was effective in preventing a total dry weight reduction by the NaCl treatment (Fig.3.6C).

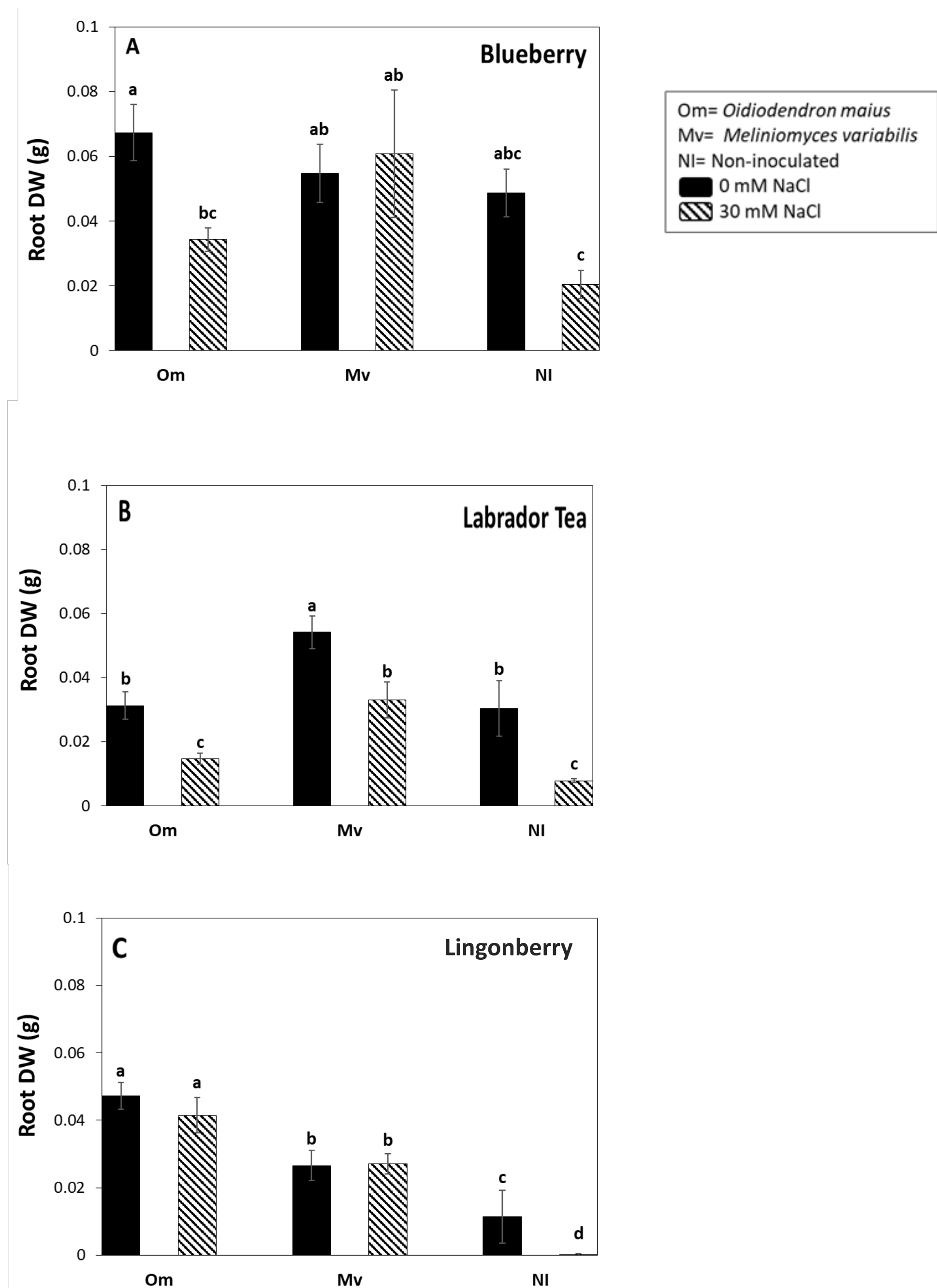


Figure 3.3. Root dry weights (g) in non-inoculated (NI) blueberry (*Vaccinium myrtilloides*) (A), Labrador tea (*Rhododendron groenlandicum*) (B) and lingonberry (*Vaccinium vitis-idaea*) (C) and in plants inoculated with *Oidiodendron maius* (Om) and *Meliniomyces variabilis* (Mv). The plants were subjected to 0 (control) and 30 mM NaCl treatments for four weeks. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species according to protected Fisher's LSD test. Means. Means ($n = 6$) ± SE are shown.

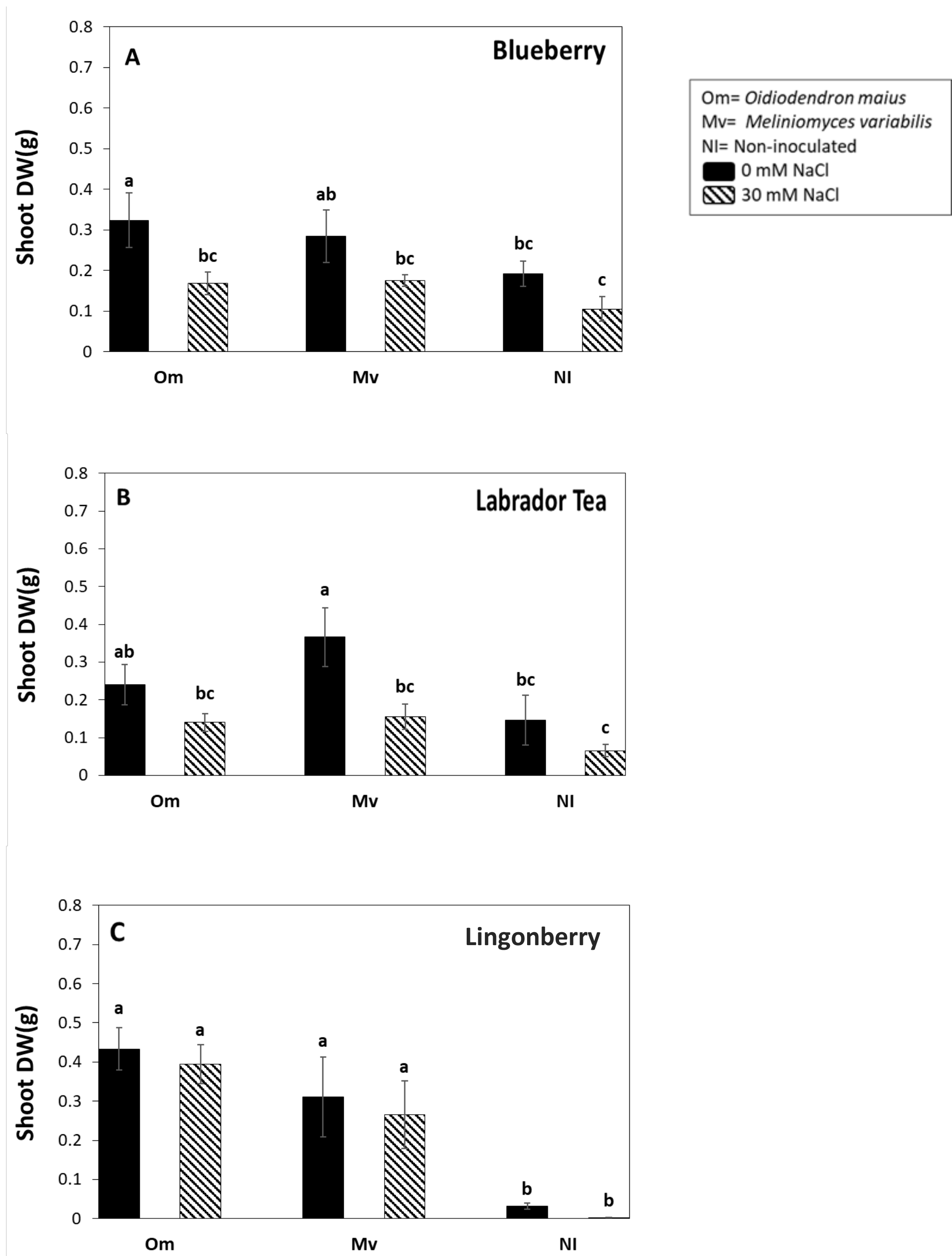


Figure 3.4. Shoot dry weights (g) in non-inoculated (NI) blueberry (*Vaccinium myrtilloides*) (A), Labrador tea (*Rhododendron groenlandicum*) (B) and lingonberry (*Vaccinium vitis-idaea*) (C) and in plants inoculated with *Oidiodendron maius* (Om) and *Meliniomyces variabilis* (Mv). The plants were subjected to 0 (control) and 30 mM NaCl treatments for four weeks. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species according to protected Fisher's LSD test. Means. Means ($n = 6$) ± SE are shown.

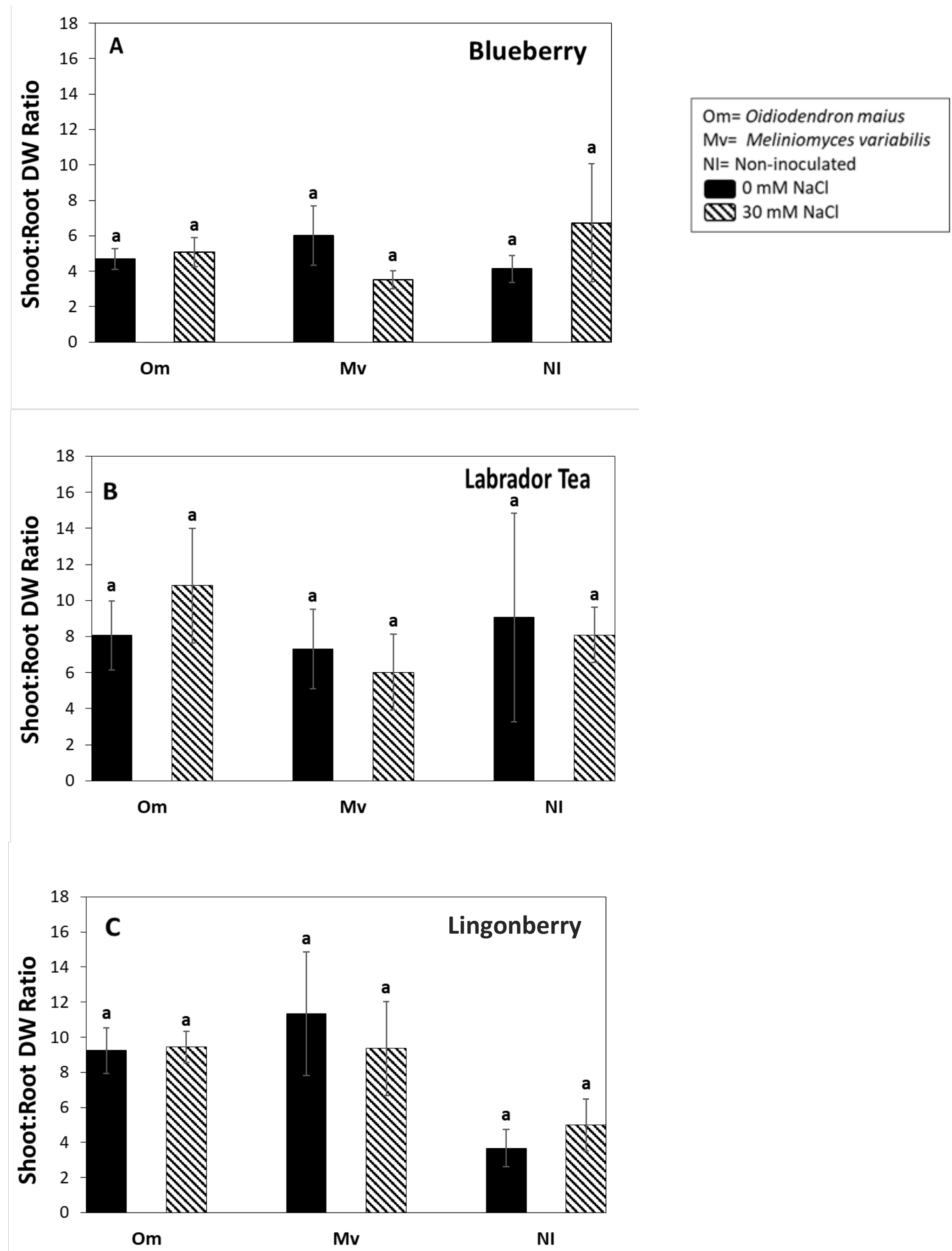


Figure 3.5. Shoot: Root dry weight Ratios in non-inoculated (NI) blueberry (*Vaccinium myrtilloides*) (A), Labrador tea (*Rhododendron groenlandicum*) (B) and lingonberry (*Vaccinium vitis-idaea*) (C) and in plants inoculated with *Oidiodendron maius* (Om) and *Meliniomyces variabilis* (Mv). The plants were subjected to 0 (control) and 30 mM NaCl treatments for four weeks. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species according to protected Fisher's LSD test. Means. Means ($n = 6$) ± SE are shown.

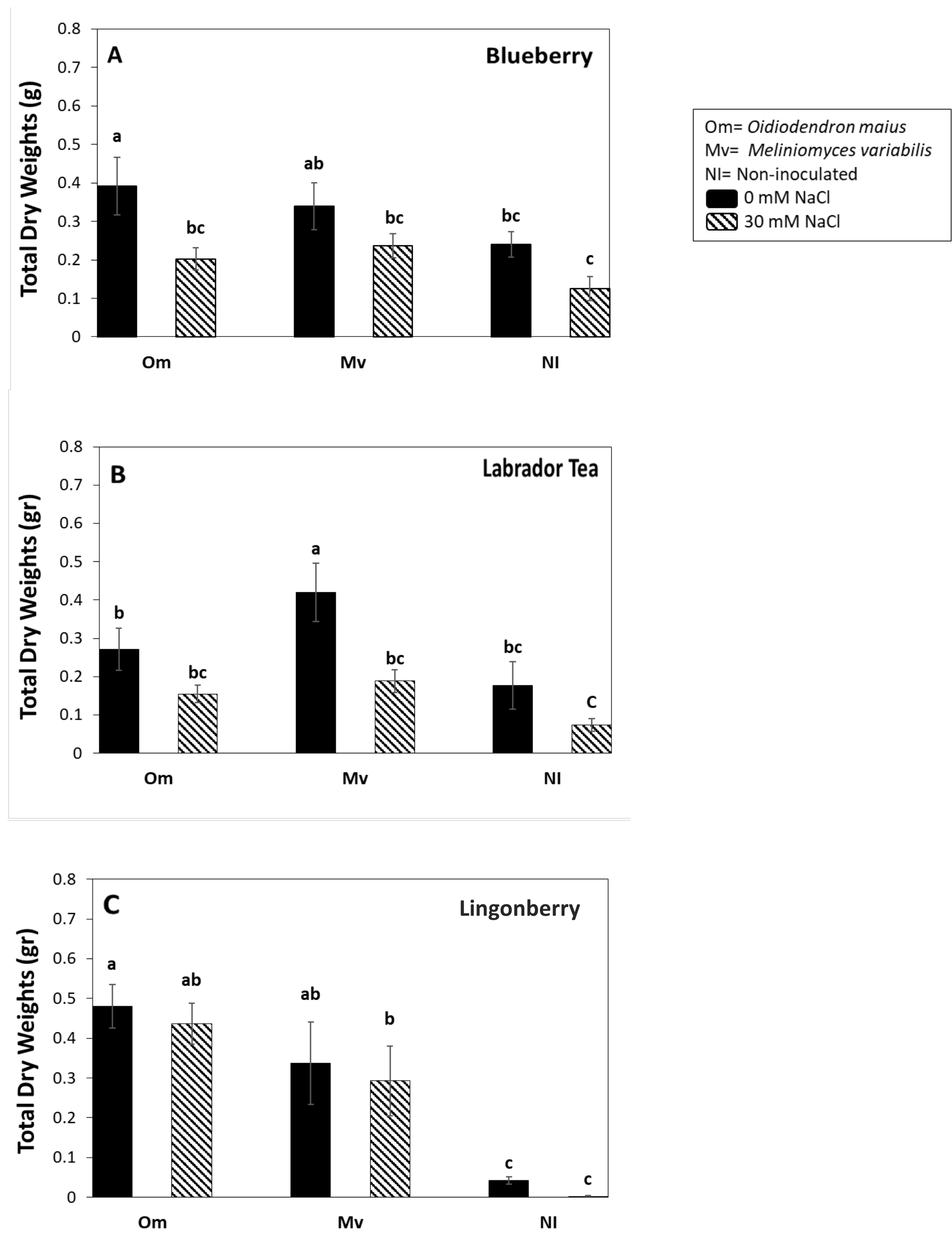


Figure 3.6. Total dry weights (g) in non-inoculated (NI) blueberry (*Vaccinium myrtilloides*) (A), Labrador tea (*Rhododendron groenlandicum*) (B) and lingonberry (*Vaccinium vitis-idaea*) (C) and in plants inoculated with *Oidiodendron maius* (Om) and *Meliniomyces variabilis* (Mv). The plants were subjected to 0 (control) and 30 mM NaCl treatments for four weeks. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species according to protected Fisher's LSD test. Means. Means ($n = 6$) ± SE are shown.

3.3.4 Leaf chlorophyll concentrations

In blueberry, leaf chlorophyll concentrations were higher in plants inoculated with the ericoid mycorrhizal fungi compared to non-inoculated plants (Fig.3.7). However, when subjected to 30 mM NaCl treatment, chlorophyll concentrations were similar in the inoculated and non-inoculated plants. In Labrador tea and lingonberry, both inoculation treatments resulted in higher leaf chlorophyll levels compared with non-inoculated plants both in control plants untreated with NaCl and following 30 mM NaCl treatment.

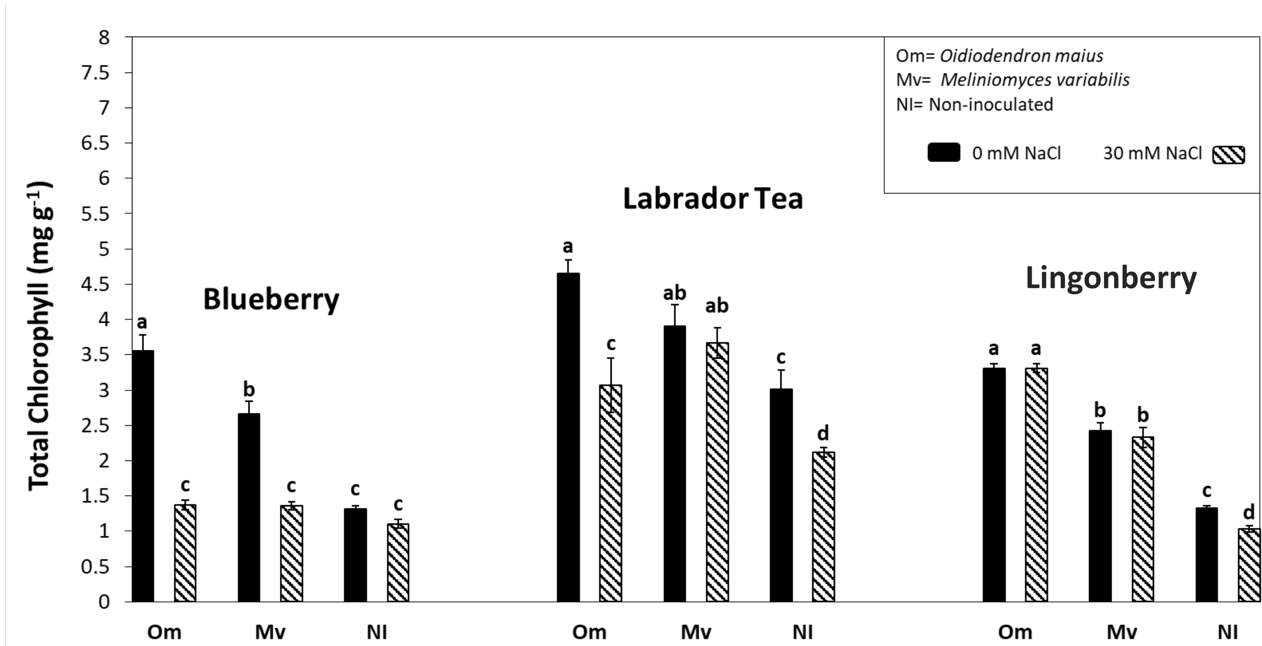


Figure 3.7. Total chlorophyll concentrations (mg g^{-1}) in non-inoculated (NI) blueberry (*Vaccinium myrtilloides*) Labrador tea (*Rhododendron groenlandicum*), and lingonberry (*Vaccinium vitis-idaea*), and in plants inoculated with *Oidiodendron maius* (Om) and *Meliniomyces variabilis* (Mv). The plants were subjected to 0 (control) and 30 mM NaCl treatments for four weeks. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species according to protected Fisher's LSD test. Means. Means ($n = 6$) ± SE are shown.

3.4 Discussion

In the present study, physiological responses of blueberry, Labrador tea and lingonberry were examined in plants inoculated with two ERM fungi to 30 mM NaCl application. Microscopic observations revealed that the colonization intensity of non-inoculated plant roots was much lower compared with the inoculated plants. It was reported by that non-inoculated blueberry plants can become naturally colonized in a nursery with sporadic ERM spores, but the colonization rate was very low (<15%)(Scagel et al. 2005b). Colonization of *Rhododendron* spp. and lowbush blueberry with ERM fungi was also observed in nurseries (Moore-Parkhurst & Englander 1981).

By inoculating all three-plant species by two species of ERM fungi, I increased the intensity of ERM colonization in all three plant roots to about 60-70 %. Different root colonization intensities by ERM in ericaceous plants were reported in different studies including 30% in blueberry roots in nursery (Scagel 2005), 50% in blueberry roots in field production (Scagel & Yang 2005), and >90% in ericaceous plant species naturally established close to bogs (Scagel 2002).

The results of my study demonstrated that inoculation with *O.maius* and *M.variabilis* increased salt tolerance in the examined ericaceous plants. Root colonization by *O.maius* increased total dry weights of non-treated blueberry and lingonberry plants and colonization with *M.variabilis* enhanced total dry weights of non-treated Labrador tea and non-treated and NaCl-treated lingonberry plants. The biomass of plants is negatively influenced by salt stress due to osmotic effects of salt as well as direct ionic toxicity and reduced nutrient uptake (Evelin et al. 2009). Different plant species vary in their effectiveness in reducing Na^+ and Cl^- transport from roots to the shoots, as one of the salt tolerance mechanisms (Yeo et al. 1977; Greenway & Munns 1980). However, some studies reported that highbush blueberries (*Vaccinium corymbosum*)

accumulated both Na^+ and Cl^- in their leaves and they could not exclude these ions from their leaves (Ballinger 1962; Morley et al. 1992).

Several studies that were conducted to examine the effects of mycorrhizal fungi on plant growth under salinity stress focused on vescicular-arbuscular- and ectomycorrhizal associations. Arbuscular mycorrhizal fungi inoculation can enhance plant growth under salt stress conditions compared with non-inoculated plants (Al-Karaki 2000; Cantrell & Linderman 2001; Muhsin & Zwiazek 2002; Giri et al. 2003; Sannazzaro et al. 2007; Zuccarini & Okurowska 2008). Some studies attributed this effect to nutrition acquisition, especially P (Plenchette & Duponnis 2005; Sharifi et al. 2007) while other studies reported decreased root and shoot uptake on Na^+ in mycorrhizal plants (Chandrasekaran et al. 2014).

In the present study, chlorophyll concentrations in plants inoculated with *O. maius* and *M.variabilis* fungi were higher compared with non-inoculated plants in all three-plant species that were not treated with NaCl. Labrador tea and lingonberry colonized with *O. maius* and *M. variabilis* had also higher chlorophyll concentrations under salt stress conditions compared with the non-mycorrhizal plants. It has been reported that the synthesis of photosynthetic pigments can be reduced due to suppression of specific enzymes that are responsible for the synthesis of chlorophyll pigments under salinity stress (Murkute et al. 2006). Furthermore, since Mg is essential for chlorophyll synthesis, the adverse effect of Na on Mg absorption can reduce the chlorophyll concentrations (Sheng et al. 2008). Numerous studies have reported high chlorophyll contents in the leaves of mycorrhizal plants (Giri & Mukerji 2004; Sannazzaro et al. 2006; Zuccarini 2007; Colla et al. 2008; Sheng et al. 2008). Negative effects of salt on leaf chlorophyll contents in plants can be alleviated by mycorrhizal fungi (Giri et al. 2003). This suggests that the adverse effects of Na^+ on Mg^{2+} absorption can be alleviated by mycorrhizae (Giri et al. 2003). In

general, mycorrhizal fungi are capable of increasing photosynthetic capacity of their host plants under salinity stress, which can help them reduce or counterbalance salt stress (Zuccarini 2007). Salt stress upsets water balance, which can reduce photosynthesis (Flexas et al. 2004). It has been documented that the stomata and mesophyll cells of plant leaves reduce CO_2 diffusion in the leaves under salt stress which can decrease photosynthetic rate (Flexas et al. 2004). On the other hand, Augé (2000) reported that increased CO_2 assimilation capacity in mycorrhizal plants can occur due to the increased stomatal opening, which may improve CO2 uptake for plant photosynthesis. In the present study, four weeks after the application of NaCl treatment, a significant reduction in net photosynthetic rate of both inoculated and non-inoculated blueberry and Labrador tea plants was observed. However, inoculated plants had a significantly higher net photosynthesis than non-inoculated plants when subjected to the NaCl treatment. After four weeks of NaCl treatment, no significant reduction was observed in the net photosynthesis of inoculated cranberries, but net photosynthesis was drastically reduced in non-inoculated cranberries exposed to NaCl. Wu et al. (2006) reported a reduced gas exchange in both inoculated and non-inoculated citrus plants under salt stress that most of the effect could be probably due to the stomatal conductance.

In conclusion, the results of the study demonstrated improvement in growth, physiological parameters, and salt tolerance in the three species of ericaceous plants inoculated with *O. maius* and *M. variabilis*. The best results were obtained for inoculated lingonberry plants, which showed the greatest improvement in salt tolerance after four weeks of 30 mM NaCl treatments.

3.5 Synthesis

The first study used two approaches to investigate its overall objective, which was to evaluate the effects of fresh and one-year stockpiled topsoil on three ericaceous plant species (blueberry, lingonberry and Labrador tea). The first approach was to evaluate the growth and physiological responses of blueberry, lingonberry and Labrador tea seedlings in fresh topsoil compared to one-year stockpiled topsoil. In general, blueberries grown in fresh topsoil showed better performance compared to those in stockpiled topsoil. The second approach was to determine the ERM inoculum potential of topsoil (fresh and one-year stockpiled topsoil). This bioassay involved the use of morphotyping to assess mycorrhizal colonisation and molecular typing to determine the actual ERM species present in plant roots. ERM fungi were present in blueberry and Labrador tea roots of all soil types. The results showed that the roots of plants in fresh topsoil had the highest percentage colonization. Moreover, ERM fungal diversity in the roots of plants grown in fresh topsoil was higher compared to stockpiled topsoil.

In the second study of this thesis, the overall objective was to evaluate the effects of ERM fungi effects on the salt tolerance of ericaceous plants. The inoculation effect on physiological parameters varied in different plant species. There was an overall increase in total dry weight, gas exchange and chlorophyll of cranberries inoculated with ERM fungi when compared with non-inoculated plants under no salt treatment. The gas exchange data for all plant species demonstrated that inoculation of seedlings with selected ERM fungi increased photosynthesis and transpiration rates under salt stress. In lingonberry seedlings, there was a positive effect of inoculation on all examined parameters in salt-treated plants.

The results demonstrated that introduced mycorrhizal fungi can have significant effects on growth and salt tolerance of their host plant species. However, mores studies should be carried out

to examine the presence of different resident fungi on seeds, which can spontaneously colonize plants. Since man-made disturbances, including oil sands mining activities, have an effect on soil mycorrhizal communities, the re-establishment of mycorrhizal networks may be one of the necessary steps for successful reforestation and rehabilitation of degraded lands (Sanon et al. 2010). Utilization of soil microsymbionts including mycorrhizal fungi in the process of land reclamation should be considered in improving reclamation success, as these fungi are able to increase both plant growth and improve soil quality in disturbed lands (Requena et al. 2001). In addition to mycorrhizal fungi, other groups of soil microorganisms, including plant growth promoting rhizobacteria, mycorrhiza helper bacteria, and N-fixing bacteria, are being utilized as inoculants in reclamation processes (Hrynkiewicz & Baum 2011). For example, the use of plant growth promoting rhizobacteria to enhance revegetation of mine tailings and minimise the need for compost amendment was explored and yielded increases in plant growth (Grandlic et al. 2008).

3.6 Reclamation application and significance

This thesis research benefits land reclamation programs responsible for boreal forest renewal, by demonstrating the importance of ERM fungi for growth and salt tolerance of blueberry, lingonberry, and Labrador tea plants. The results of my studies demonstrate that higher percentage of mycorrhizal colonization and greater diversity of ericoid mycorrhizal fungi in ericaceous plants growing in the fresh topsoil compared with the one-year stockpiled topsoil. This effect on mycorrhizal colonization of roots was likely the principal reason for lower growth rates of plants in the stockpiled soil. Since the stockpiling of soil may be necessary during oil sands operations, inoculation of ericaceous plants with mycorrhizal fungi should be considered prior to planting on reclamation sites. Reclamation plants are faced with numerous environmental stresses including salinity. Inoculation of ericaceous plants with mycorrhizal fungi species offers a viable

means for the restoration of forest understory plants and has the potential to enhance plant survival and growth.

3.7 Suggestions for future research

While the results of the first study in this thesis indicate that ERM colonization potential of topsoil can be affected by stockpiling, future research should be carried out to examine the effects of longer-term storage (more than one year) on the ERM inoculation potential. Furthermore, I listed below several important topics for further research concerning the effects of stockpiling on soil quality for land reclamation application.

1. Examine the effects of the physical properties of the topsoil, such as bulk density, on growth potential of both microorganisms and plants in land reclamation soils.
2. Determine the effects of mycorrhization on soil organic matter and its consequences for improving soil quality.
3. Identify mycorrhizal network of the topsoil by extraction fungal DNA directly from the soil.

While the results of the second study in this thesis show the potential benefits of ERM technology in reclamation of oil sand disturbed lands, there are also several areas that warrant further research. Among those, determining the interactions between ERM and other fungal and bacterial counterparts e.g. actinomycetes, plant growth promoting bacteria or mycorrhiza helper bacteria, could improve our knowledge concerning reclamation soil quality for revegetation of oil sands sites with ericaceous plants.

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Chapter 4

Appendix

Table 5.1. ANOVA table showing the effect of different soil types on root dry weight, shoot dry weight and shoot:root dry weight for blueberry, Labrador Tea and lingonberry.

	Root dry weight		Shoot dry weight		Shoot:Root dry weight	
Blueberry	F=6.67	P=0.0050	F=10.98	P= 0.0004	F=4.60	P=0.0204
Labrador Tea	F=11.92	P=0.0003	F=16.03	P <.0001	F=1.37	P=0.2730
Lingonberry	F=3.85	P=0.0356	F=3.15	P= 0.0612	F=3.27	P=0.0556

Table 5.2. ANOVA table showing the effect of different soil types on Total Chlorophyll, Chlorophyll-a and Chlorophyll-b concentration for blueberry, Labrador Tea and lingonberry.

	Total Chlorophyll		Chlorophyll-a		Chlorophyll-b	
Blueberry	F=3.23	P=0.0520	F=2.99	P= 0.0591	F=3.52	P=0.0458
Labrador Tea	F=0.47	P=0.6306	F=0.27	P=0.7623	F=2.61	P=0.0510
Lingonberry	F=1.01	P=0.3784	F=1.91	P= 0.1698	F=0.64	P=0.5359

Table 5. 3. ANOVA table showing the effect of different soil types on Photosynthesis and Transpiration of blueberry, Labrador Tea and lingonberry.

	Photosynthesis		Transpiration	
Blueberry	F=0.31	P=0.7398	F=0.89	P= 0.4232
Labrador Tea	F=0.21	P=0.8093	F=0.05	P=0.9533
Lingonberry	F=2.61	P=0.0946	F=2.30	P=0.1217

Table 5.4. ANOVA table showing the effect of different soil types on Calcium (Ca), Potassium (K), Magnesium (Mg), Phosphorus (P), and Nitrogen (N) of blueberry, Labrador Tea and lingonberry.

	Ca	K	Mg	P	N
Blueberry	F=4.36	F=0.36	F=2.80	F=6.58	F=40.03
	P=0.0243	P= 0.7047	P=0.0806	P=0.0053	P<.0001
Labrador Tea	F=0.12	F=1.61	F=9.96	F=3.33	F=0.13
	P=0.8872	P=0.2212	P=0.0007	P=0.0530	P=0.8778
Lingonberry	F=4.59	F=0.17	F=7.58	F=2.85	F=1.43
	P=0.0205	P= 0.8486	P=0.0028	P=0.0777	P=0.2593

Table 5. 5. ANOVA table for elemental concentrations : Calcium (Ca), Potassium (K), Magnesium (Mg), Phosphorus (P), Nitrogen (N), and Sodium (Na) in different soil types (Fresh Topsoil, Autoclaved One-Year Topsoil, and Non-Autoclaved One-Year Topsoil).

	Ca	K	Mg	P	N	Na
Soil Types	F=14.91	F=10.29	F=11.77	F=6.92	F=2.39	F=12.27
	P=0.0006	P= 0.0025	P=0.0015	P=0.0100	P=0.1335	P=0.0013

Table 5.6. ANOVA table for Photosynthesis and Transpiration and total chlorophyll of inoculated and non-inoculated blueberry, Labrador tea, and lingonberry under salt treatments (0 mM & 30 mM NaCl).

	Photosynthesis		Transpiration		Total Chlorophyll	
Blueberry	F=17.44	P<.0001	F=23.17	P<.0001	F=58.33	P<.0001
Labrador Tea	F=12.87	P<.0001	F=33.37	P<.0001	F=13.36	P<.0001
Lingonberry	F=9.47	P<.0001	F=9.88	P<.0001	F=122.40	P<.0001

Table 5.7. ANOVA table for Root dry weight, Shoot dry weight, Shoot:Root dry weight, and Total dry weight of inoculated and non-inoculated blueberry, Labrador tea, and lingonberry under salt treatments (0 mM & 30 mM NaCl).

	Root dry weight	Shoot dry weight	Shoot:Root dry weight	Total dry weight
Blueberry	F=2.93	F=3.39	F=0.54	F=4.15
	P=0.0333	P=0.0185	P=0.7447	P=0.0074
Labrador Tea	F=10.18	F=4.30	F=0.27	F=6.00
	P<.0001	P=0.0062	P=0.9260	P=0.0010
Lingonberry	F=70.51	F=8.60	F=0.39	F=10.05
	P<.0001	P<.0001	P=0.8534	P<.0001

Sequences of identified species:

>*Cadophora finlandia*

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TAAGCGGAGGA

> Serendipita herbamans

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> Leohumicola verrucosa

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GAGGA

>Pezoloma ericae

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> Mycosymbioces mycenaphila

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> Acephala applanata

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>Acremonium dichromosporum

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TTNNCNNNNN

> Micarea soralifera

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> Clavaria citrinorubra

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CGCANGTTCACCTACNGAAACCTTGTTACGACTTTTACTTCCTCTAAATGACC
AANAAGGNCGAATTCCAGCACACTGGCGGCCGTTACTANTGGATCCNANCTC
GGTACCNAGCTTGGCGTANCNNNNNNNNTTTTNNNNNNNNGGGGGNNNNN

> Xenopolyscytalum pinea

NNNNNNNNNNNNNNNNNNNNNNNGNNGCCCTCTAGATGCATGCTCGAGCGGCCGC
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