University of Alberta

Enhanced Revegetation and Reclamation of Oil Sand Disturbed Land Using Mycorrhizae.

By

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ABSTRACT

This study examined the potential of using conifer seedlings *Picea glauca* and *Pinus banksiana* inoculated with ectomycorrhizal (ECM) fungi to improve revegetation success and plant establishment in reclaimed oil sands mining sites. Mycorrhizal inoculum potential of the reclamation soils was low with the maximum inoculum potential of 23% and 29%for ECM and arbuscular mycorrhizae, respectively. The response of seedlings in the field to ECM inoculation varied between plant species and measured parameters. A significant effect of ECM inoculation on height was observed in *P. banksiana* but not in *P. glauca*. The average survival rate for *P. glauca* seedlings inoculated with different species of ECM varied between 36% and 56%, whereas the control (uninoculated) seedlings had minimum and maximum survival rates of 22 and 41% respectively. Generally, it was construed that the re-introduction of mycorrhizal fungi during reclamation process is an important approach that should be further exploited.

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LIST OF ABBREVIATIONS

AMF – Arbuscular mycorrhiza fungi

AOSR – Athabasca oil sands region

BLAST – Basic local alignment sequence tool

CSS - Cyclic steam stimulation

CT – Composite tailing

CTAB – Cetyl trimethyl ammonium bromide

ECM – Ectomycorrhizal fungi

FMID – Field mycorrhization inoculum dependency

ITS – Internal transcribed sequence

LFH – Forest floor

LSD – Least square difference

MBSU – Molecular biology services unit

MPN – Most peobable number

NCBI – National Centre for Biotechnology Information

OSPM – Oil sand processed materials

PMM – Peat mineral mix

PVI – Plot volume index

RFLP – Restriction fragment length polymorphism

SAGD – Steam assisted gravity drainage

TS – Tailing sand

1 Literature Review

1.1 Introduction

The oil sands industry in Alberta disturbs massive areas of land, which need to be reclaimed. To achieve reclamation and revegetation success, several organic amending materials are used by mining industries to cap reconstructed soils, which contain oil sand processed materials (OSPM). However, the potential use of these materials in establishing a sustainable plant community is of concern and their quality can be reduced during manipulations, stockpiling and storage (Danielson et al., 1983; Malajczuck et al., 1994). Vegetation establishment in the oil sands region is often faced with challenges such as high sodicity and soil nutrient deficiencies, which cause plant succession and establishment to be either slow or even unsuccessful under the harsh environmental conditions that prevail in those areas.

Many plant species in forest habitats rely upon symbiotic mycorrhizal fungi to accomplish their nutrient requirements and particularly assist in establishing under harsh environmental conditions. Like all other fungi, mycorrhizal fungi are heterotrophs as they derive their organic compounds from other processes rather than photosynthesis, since they do not have photosynthetic pigments (Isaac, 1992). The photosynthates, which are organic carbon compounds are transferred from plant to the fungus, while soil macronutrients such as phosphorus, nitrogen and micronutrients such as copper and zinc are transferred from the fungus to the plant (Smith and Read, 2008). This was referred to as the fundamental concept of a trilogy symbiosis (plant-fungus-soil) as this process enhances better survival of mycorrhizal plants than those that cannot form a mycorrhizal association (Smith and Read, 2008; Fortin et al., 2009).

The exploitation of this trilogy symbiosis has not been greatly explored in reclamation of oil sand disturbed areas. Establishing the potential use of mycorrhizal fungi to enhance survival in reclamation processes is one of the most important goals in current mycorrhizal application research.

1.2 Oil Sands mining

Oil sand, otherwise known as tar sands, is crude bituminous sand that contains naturally occurring mixtures of clay and water. Each grain of sand is surrounded by a layer of water and a film of bitumen, hence, the sticky and viscous mixture that requires several stages of industrial processing to extract and convert into refined oil (Government of Alberta, 2009b). Oil sand exploration can be dated back to the

Paleolithic times (Bilkadi, 1984). In Canada, this crude oil was first mentioned in 1719 by fur traders who brought oil sand samples to the Hudson Bay post at Churchill. In 1788, Alexander Mackenzie wrote a detailed description of oil sands in the Athabasca region. Subsequently, several attempts were made to commercially develop the Athabasca oil sands (AOS). This nurtured the interest in oil sands till the 1920s when Dr. Karl Clark successfully pioneered experiments with hot water flotation process to extract oil from the sand (Syncrude, 2010). This led to the first sale of commercially produced bitumen in Edmonton by Robert Fitzsimmons in 1930.

Figure 1.1: Map showing Alberta's boreal forest and oil sand deposits (Government of Alberta, 2009b).

To date, the AOS located in the boreal forest region of Northern Alberta Canada (Figure 1.1) represents one of the largest deposits of oil in the world after Saudi Arabia (Figure 1.2) with an extracted bitumen volume of 1.7 trillion barrels (Fung and Macyk, 2000). The AOS comprises of three major commercial surface mining plants: Syncrude Canada Ltd (Syncrude), Suncor Energy Inc. (Suncor), Albian Sands Energy Inc. and a host of others. Syncrude which began operation in

1978, is the largest commercial plant and produces up to 129, 000 barrels per day (Humphries, 2008). While Suncor, which was the first established commercial plant (previously known as Great Canadian Oil Sands) in 1967 produced not less than 120,000 barrels per day (Humphries, 2008).

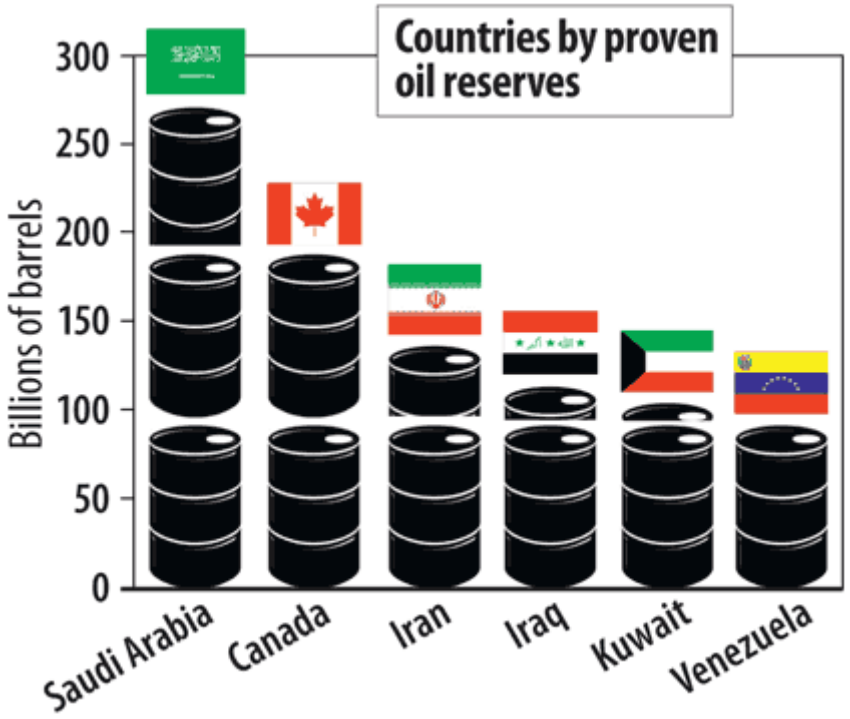

Figure 1.2: Diagram showing the ranking of oil producers and number of barrels produced. (http://www.newint.org/features/2010/04/01/keynote-tar-sands/)

In the Athabasca region, oil can be obtained from oil sand through two techniques. One way is using in situ methods for deposits that are usually 400 m below the surface. This method accounts for about 80% of oil sand recovery in the AOS region. This process involves steam injection through chambers using either cyclic steam stimulation (CSS) or steam assisted gravity drainage. Alternatively, surface mining is another technique that is used if there is a close proximity of ore layer to the surface. The method involves the removal of overburden layer within a maximum depth of 45metres. The removed topsoil and overburden is stockpiled close to the mining pit pending its use to back fill the pit to form a reconstructed landscape. Subsequently, the mined ore is transported with heavy hauler trucks to the extraction plant and the ore is then extracted using the hot water floatation process. Once the slurry is fed into a chamber, it is separated into sand, water and bituminous layer. The

oil is skimmed off, purified and upgraded leaving behind tailing materials that consist of particles of sand, clay and silt with a fraction of residual hydrocarbon (less than 5%). Over time, the tailings materials when discharged into dykes as tailing ponds separate into layers that are referred to as mature fine tailings or fine tailings (Government of Alberta, 2009b). Composite tailings (CT) on the other hand, involves the process of mixing coarse and fine tailings with a chemical coagulant (gypsum or alum) to form slurry that rapidly releases water when deposited, thereby reducing fine tailing fluid and allowing the formation of a soil matrix (Renault et al., 2003). These materials have reclamation limitations such as high erosion potential, low water holding capacity, high soluble sodium content, absence of organic matter and microbial activity. Hence, they pose a reclamation and revegetation challenge.

1.3 Oil Sands reclamation and revegetation

Revegetation and reclamation are processes that are implemented after land disturbance by natural or anthropogenic factors. Land reclamation is defined as the process of transforming any disturbed land to its previous land capacity state or better, taking into consideration stability and restoration of biological self sustaining processes (Quoreshi, 2008). One of such land disturbances is oil sand mining that is predominant in Northeastern Alberta (Figure 1.1). In this region oil producers are obliged to reclaim their operational land as set out within the *Land and Surface Conservation Act* 1973 and *Environmental Protection and Enhancement Act* 1992 (Government of Alberta, 1999).

In Alberta, oil sand mining has disturbed approximately 715 km^2 of land since operations began, which represents approximately 0.19% of Alberta's boreal forest. However, approximately 10% of the mining footprint has been or is being reclaimed (Government of Alberta, 2011) due to the large areas of land that are bound to be disturbed during the process. Between 2009 and 2010, the Government of Alberta established new definitions to better track the level of land disturbance and reclamation processes. This was to redefine the definition of reclamation used by oil industries. Syncrude, one of Alberta's largest oil producers defines a reclaimed land as "land that at a minimum, had been shaped, formed, capped with soil and ready for revegetation". This did not comply with government policies that demanded areas to be revegetated before being referred to as reclaimed. Nevertheless, Syncrude is the first company to be issued a reclamation certificate in March 2008 for a 104 ha piece of land known as Gateway hill located 53km North of Fort McMurray (Syncrude, 2009).

Oil sand reclamation is considered a long-term endeavour as it takes over 20 years to successfully restore a disturbed area to equivalent capability prior to disturbance (Government of Alberta, 2011). In early times, oil sand reclamation research paid attention to suitable soil capping or substrates remedies and adaptability of indigenous plants species to grow in tailing sands or OSPM. However, present reclamation research which still includes the latter but with improvements is aimed at reducing reclamation times while facilitating a better establishment of native plant communities, soil stability, microbial processes and nutrient cycles (Singh et al., 2002; MacKenzie and Naeth, 2009; Sheoran et al., 2010). Other research has also studied the reclamation of tailing wetlands to restore wetland functions such as invertebrates that are among the most basic links in a biotic food web (Cooper and Foote, 2003).

In terms of plants and soil amendments, Renault et al. (2003) studied the potential of using barley to reclaim freshly deposited saline CT amended with peat. It was observed that the amendment of CT with peat improved growth and survival of barley but did not prevent leaf injury. This was assumed to be due to sodium and chloride ion as well as nutrient deficiencies in CT. Barley is used in oil sands reclamation as a precursor for quick vegetation and erosion control. However, it has poor competition competence and is easily invaded by indigenous flora within a few years (Hardy BBT Ltd, 1990). Mcmillan (2005) studied the benefits of forest floor amendments (LFH) compared to peat mineral mix for oil sand reclamation. This was in terms of determining the net nitrogen mineralization and microbial biomass. It was reported that LFH had a decreased field potential compared to peat mineral mix. Nevertheless, under the right conditions (increased soil moisture) there is a likelihood of LFH to have a higher microbial biomass, respiration rates and net N mineralization than peat mineral mix.

Peat is a common wetland organic substrate that is used in oil sand reclamation and is categorized based on the decomposition stages of materials present. Fibrisols, mesisols and humisols are the three major groups of organic soils (Kroetsch et al., 2011), with fibrisol and mesisols being dominant in peat bog of the Athasbasca oil sand region (AOSR) (Turchenek and Lindsay, 1982; Oil sands vegetation committee, 1998). Hemstock (2008) studied the plant productivity in peat amendments and reported a low species richness and cover with fibric peat. This was assumed to be because of the acidic nature and reduced amount of decomposition of the material in the fibric peat. However, mesic and humic peat provided a favourable environment for early succession of plant species. Other studies have investigated the dewatering ability of plants in mine tailings (Silva, 1999); reclamation interactions of soil

chemical, physical and hydrologic parameters (Burger, 2005); revegetation of OSPM using Frankia-inoculated alders (Lefrançois, 2009); the use of LFH to improve the native plant community (Mackenzie and Naeth, 2009); and the influence of depth on plant growth in four overburden types used in reclamation (Danielson et al., 1983).

Generally, one important fact is whether the use of any soil amendment in reclamation could result in an established functional forest ecosystem. This was one of the questions tested by Rowland et al. (2009), when they compared the characteristics of reclamation materials (peat, peat mineral mix and overburden) to natural ecosystems, in addition to the time it takes for a remodelled ecosystem to be established. In this study, high pH of reclamation materials was observed compared to the natural areas, which was attributed to the high pH of salvaged materials in AOSR. In terms of plant diversity, reclamation materials were stable for a short time and began to decline when canopy closure at about age 31 and 35 progressed. However, it was concluded that treatments using repeated or one-time fertilized peat mineral mix and underlying subsoil over clean overburden were developing into functional forest soils with supporting ecosystems to mimic those of the natural boreal forest.

1.4 Application of mycorrhizal biotechnology in reclamation

Mycorrhizal fungi are key components in terrestrial ecosystem functioning and are explored biotechnologically to deal with factors such as global change, land disturbances, crop production and food web interactions (Cordell 1996; Rillig et al., 2002; Wardle and Van der Putten, 2002; Quoreshi et al., 2006). However, these ecosystem processes do not occur in isolation and may affect mycorrhizal symbioses (mycobiont or phytobiont) directly or indirectly (Rillig et al., 2002).

1.4.1 Mycorrhizas and benefits

Mycorrhizal associations are formed with a majority of higher plants and can be classified into several categories based on structural characteristics at different stages of their life cycle. Further distinctions are based on hyphal septation, intra-intercellular hyphal colonisation with the formation of a mantle and Hartig net as well as the plant or fungal taxa involved (Smith and Read, 2008). Arbuscular mycorrhiza (AM), ectomycorrhizas (ECM), ectendomycorrhiza, ericoid, arbutoid, monotropoid and orchid mycorrhizas are the seven major types of mycorrhizal fungi formed with their host plants (Brundrett et al., 1996; Smith and Read, 2008). These groups differ

broadly in nutrient exchange compartments formed within host roots and the plant species in which they occur (Molina et al., 1992). When structures form and penetrate the root cortical cells, they are referred to as endomycorrhizal fungi, when outside the root cell they are called ectomycorrhizal fungi (Brundrett et al, 1996; Friberg, 2001; Smith and Read, 2008).

Mycorrhizae have been studied for about 100 years. However, early studies focused on the descriptive morphology and type of association that fungi form with their host (mutualistic rather than pathogenic). It was not until the 1930s that it became established that the presence of mycorrhizas with any host was better than none (Danielson and Visser, 1988). In the 1950's, mycorrhizae became a concept that was refined into suggested practical and technological applications in land reclamation (Danielson and Visser, 1988). Nevertheless there were still some areas that required further advancement such as inoculation techniques, genetic studies of host/symbionts, screening of isolates for compatibility and response, and intensive inoculation programs (Navratil, 1988).

The main benefit of mycorrhizal fungi to their hosts is improved uptake of phosphorus (P). Phosphorus is one of the growth limiting factors to plants. Therefore, the presence of mycorrhizal fungi helps in reaching inaccessible P as well as to breakdown complex forms of P in soils (Smith and Read, 2008). Bending and Read (1997) reported the ability of ECM and ericoid fungi to breakdown phenolic compounds in soils that could interfere with nutrient uptake. In addition, mycorrhizas present other benefits to their phytobionts. They can facilitate uptake of water (Mushin and Zwiazek, 2002), increase drought resistance and reduce transplanting shock of young seedlings (Augé, 2001; Mridha, 2003). Some types of mycorrhizal fungi can also offer their host plant the ability to withstand high temperatures and salinity stress. Bunn et al. (2009) reported the ability of AM fungi to ameliorate temperature stress in thermophilic plants, given that the thermal and non-thermal AMF treatments behaved alike. Therefore, it was concluded that AMF possess a broad tolerance to high temperatures. Of practical importance to mine reclamation and nursery management, various mycorrhizal fungi protect young seedlings against damage from heavy metals/pollutants using a variety of mechanisms (Jentschke and Godbold, 2000) and increase host resistance to pathogens (Azcón-Aguilar and Barea, 1996). For example, ECM fungi were reported to facilitate the clean-up of soil contaminated with persistent organic pollutants (e.g. polyhalogenated biphenyls, polyaromatic hydrocarbon phenols and pesticides) by transforming these compounds

(Merharg and Cairney, 2000) perhaps through sequestration process. ECM species were also reported to possess enzymatic capacity to breakdown complex carbon materials (lignin or cellulose) as well as utilize such nutrients (Cullings and Courty, 2009; Fortin et al., 2009). This led to ECM being referred to as facultative saprotrophs (Fortin et al., 2009). However, it is still in question whether their lignocelluloses-decomposing enzymes are responsible for this saprophytic character or their patterns of gene expression that is based on root-fungus carbon balance (Baldrian, 2009). Nevertheless, the continued existence of trees from the families of Betulaceae, Pinacea, Fagaceae, Caesalpiniaceae, Dipterocarpaceae and Myrtaceae is partly due to ECM fungal association (Muchovej, 2004).

ECM, unlike their AM fungal counterparts, can be cultivated *in vitro* even though they generally have slow growth (Horton and Bruns, 2001). Their ability to utilize simple sugars (glucose, fructose and mannose) and dissacharides as carbon sources in the absence of their host has led to their experimental manipulations (Satyanarayana et al., 1996; Ohta, 1997). Kim et al., (2003) investigated with liquid cultures the growth response of three ECM fungi (*Paxillus involutus, Rhizopogon subcaerulescens* and *Suillus bovines*) to various concentrations of glucose substrates. They indicated an increase in fungal dry mass relative to the concentration of substrates. Studies by Ohta (1997) also successfully obtained large quantity of mycelia mass for sporocarp production by manipulating *in vitro* the nutrition requirement of *Hebeloma radicosum* and *Hebeloma* sp. without the host plant. The growth of ECM fungi *in vitro* is crucial for various research purposes such as inoculum production, understanding their physiology, biodiversity maintenance of species as well as their symbiotic relationship (Satyanarayana et al., 1996). However, it has been reported that repeated subcultures of ECM on media brings about a decline in their ability to successfully colonise plant roots. This problem can be largely dealt with by inoculation onto and re-isolation from a suitable host (Thompson et al., 1993).

Generally, the dependence of certain plants on their mycorrhizal counterpart has been demonstrated in different parts of the world (Plenchette et al., 1983; Gemma et al., 2002; Zangoro et al., 2007). According to Mikola (1973), certain exotic trees can only survive and thrive when accompanied by their associated ECM fungal symbionts. However, it has been reported that this mycorrhizal dependency varies between and within plant species (Zangoro et al., 2007; Plenchette and Fortin 2009). Cardoso Filho et al. (2008) demonstrated the dependency of mangaba tree (*Hancornia speciosa* Gomes- Apoynaceae) on mycorrhizal fungi (*Glomus etunicatum*

and *G. margarita*) regardless of soil P content or P addition. Thus, it would be of importance to exploit the mycorrhizal dependency of certain plant species. This can be achieved by producing planting stocks inoculated with site adapted fungi for use in disturbed sites that have low mycorrhizal inoculum potential.

1.4.2 Possible factors that can affect mycorrhizal establishment in reclamation

Mycorrhizal growth and association with host plants can be affected by various environmental and anthropogenic factors such as mining (Trappe et al., 2006; Tibbet and Cairney, 2007). In ECM, two key stages that impel root colonisation of their host plant are commonly affected. They include the mycelial growth and sporocarp formation (Hawley, 2008). Trappe et al. (2006) measured the effects of past and current anthropogenic disturbance to mycorrhizal sporocarp fruiting pattern and observed that fungal productivity was markedly reduced in the most disturbed microsites. Another study also reported the ability of repeated harvesting of forest residues to decrease ECM fungal association (Mahmood et al., 1999).

Seasonal moisture and temperature changes do affect fungal growth. However, contrasting results have been obtained given that the effect of these factors may be related to the fungal species or host plant used. Nevertheless, *in vitro* studies by Sharma et al. (2010), using *Cantherellus tropicalis* showed that temperatures above 35^{o}C reduced fungal mycelial biomass. Similarly, it was reported that for stipe elongation growth, a stage in sporocarp formation temperatures between 5^{o} and10^{o}C are of importance (Kawakami et al., 2004). On the contrary, Heinemeyer and Fitter (2004) reported an increased extraradical hyphae production of *Glomus mosseae* at temperatures between 20-25^{o}C in the two plant species *Plantago lanceolata* and *Holcus lanatus*. Rao et al. (1997) conducted a study to determine the distribution of ECM fungi in various ages of pine plantation. They observed maximum number of mycorrhizal sporocarps during the rainy season and revealed a positive significant correlation with soil moisture in all the plantations.

Besides the above-mentioned factors, soil pH and salinity are important factors that influence fungal growth. Experiments investigating these factors have mostly been carried out *in vitro*. However, results from such studies can help in determining the best functional parameters of the fungal species in nursery-plantation programs and for inoculum production. Culture experiments of ECM fungi show that they are mostly acidophilic and sensitive to a pH of 7 or greater (Hung and Trappe,

1983; Kernaghan et al., 2002; Sanmee et al., 2010). As such, they are usually maintained on solid media at a pH range from 4.0 to 6.2 (Sundari and Adholeya, 2003). Sanmee et al. (2010), studied *in vitro* the growth conditions of *Phelebopus portentosus* and the effect of temperature and pH on mycelia growth. They observed an optimum mycelium growth at 30^{o}C and pH of 4. Furthermore, they observed that at pH 2, the fungi grew but at a slow rate and failed to grow at pH greater than 7. Optimal pH ranges differ between and within species of ECM fungi as some species tend to be more tolerant to certain pH ranges (6.5 to 10) (Sundari and Adholeya, 2003). Even though the growth of ECM fungi on culture media could indicate an optimum pH range, their association with the host plants in any soil substrates could bring about changes in stipulated pH ranges (Erland et al., 1990). Therefore, *in vitro* growth experiments only assess and do not accurately depict *in vivo* processes; as such, interpretation should be with caution (Hung and Trappe, 1983; Erland et al., 1990).

Soil salinity is a cosmopolitan stress that can be natural or anthropogenically created (Bios et al., 2006b). This soil condition inhibits survival of plants and glycophytes as living cells are affected due to disturbance of their osmotic homeostasis (Dixon et al., 1993; Bios et al., 2006b). Extremely saline soils could adversely affect mycorrhizal propagules that facilitate colonisation of roots in the rhizosphere (Dixon et al., 1993). However, numerous studies have demonstrated an increased salt tolerance by certain ECM fungal species (Mushin and Zwiazek, 2002; Bois et al., 2006a; Calvo Polanco et al., 2008). Nevertheless, some ECM species still tend to show less tolerance to different sodium salts. This was demonstrated by Dixon et al. (1993) who observed *in vitro* the intolerance of the genera *Cenococcum* (Moug & Fr.) and *Thelephora* (Ehrh. ex Willd) to sodium citrate ($Na_3C_6H_5O_7$) and sodium sulphate (Na_2SO_4). In addition, they observed a significantly reduced plant dry weight of *Pinus taeda* seedlings colonised by *Laccaria laccata* (Scop. Cooke) and *Pisolithus tinctorius* (Pers. Coker & Couch) in 80mM of sodium chloride (NaCl) after 14 weeks.

1.4.3 Reclamation of disturbed lands using ectomycorrihzal fungi

The reclamation of disturbed sites caused by mining, construction and agriculture often takes place using exposed subsurface materials resulting from excavation. As such, the re-establishment of vegetation is difficult and not sustainable as these soil materials are low in fertility and biological activity (Classen and Zasoski, 1993; Bois et al., 2005). Therefore, the use of micro-organisms (especially mycorrhizal fungi) can aid in revegetation enhancement. The absence of mycorrhizal

association on plant root systems has been reported as one of the major reasons for plant establishment failure in disturbed sites with low inoculum potential (Requena et al., 2001; Cardoso and Kuyper, 2006). However, before ectomycorrhizal or microbial biotechnology is applied in reclamation programs, a schematic design for a successful reclamation and remediation of disturbed ecosystems should be considered (Figure 1.1).

Mycorrhizal fungi are naturally occurring in soils but their propagules and infectivity can be reduced substantially in disturbed ecosytems (Quoreshi, 2008). However, the recovery of mycorrhizae propagules can be facilitated by inoculation, pre-inoculation of desired plants and the use of soil amendments concentrated with propagules (Classen and Zasoski, 1993; Quoreshi, 2008;). The two primary reasons why mycorrhizae should be considered in revegetation strategies are firstly, they form an important component of natural soil ecosystems by interacting with soil microbes, fauna and plants, which make them an essential part of a complete soil environment. Secondly, they have numerous benefits to plants that could help improve revegetation (Brundrett et al., 1996; Smith and Read, 2008). Hence, there have been experimental methods that attempted the re-establishment of mycorrhizal fungi to promote a sustainable plant community (Skujins and Allen, 1986; Duñabeitia et al., 2004; Menkis et al., 2007).

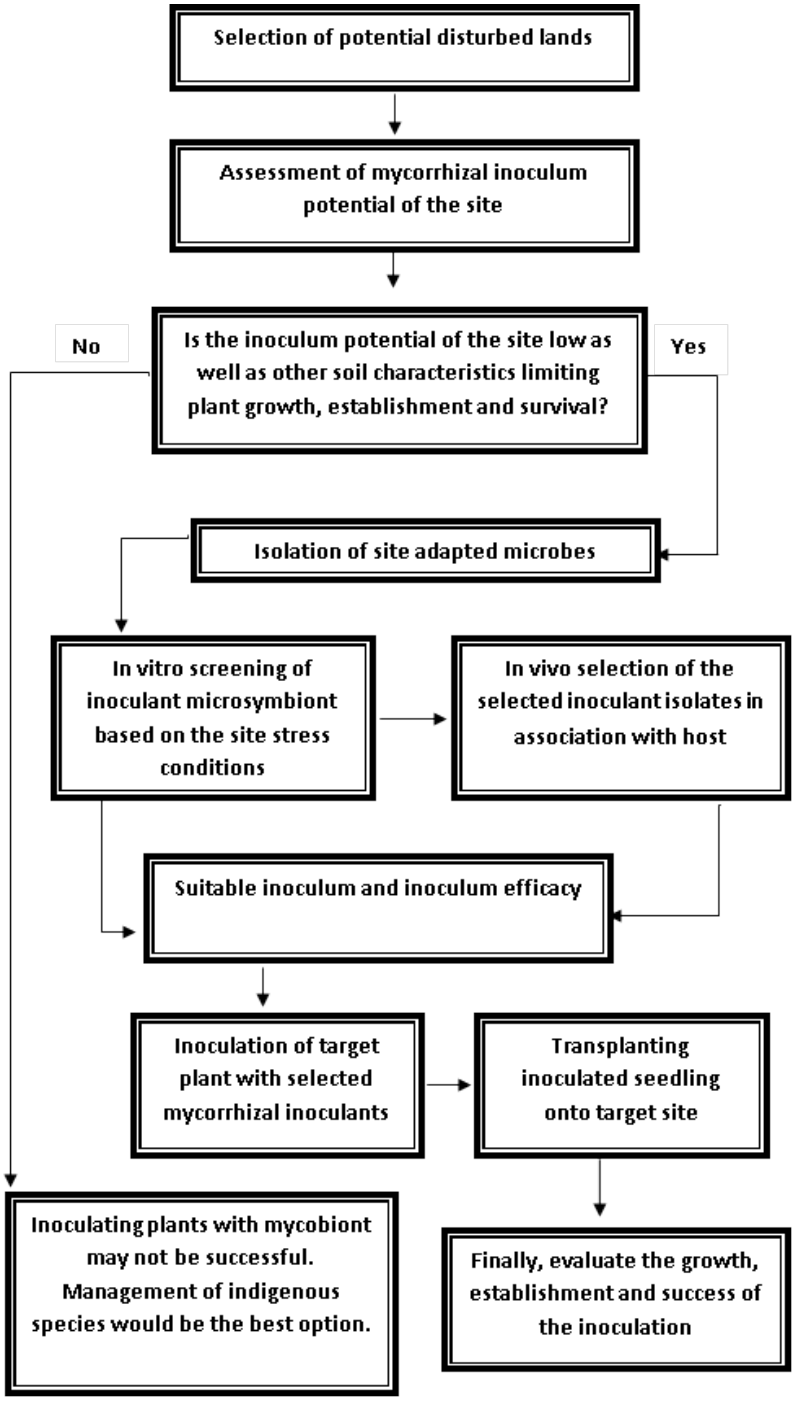

Figure 1.1: Suggested approaches for the successful use of mycorrhizal biotechnology in reclamation and remediation of disturbed ecosystem (modified from Quoreshi, 2008).

The intensive use of fertilizers and fungicides to increase seedling growth is a common nursery management practice that may inhibit mycorrhizal establishment (Linderman, 1994; Landis, 2002; Quoreshi, 2003). However, if used appropriately, especially fungicides, this could minimize the population of wild nursery mycorrhizal fungi and microbial antagonists that will compete with the beneficial fungi (Molina

and Trappe, 1984). Presently, there are several types of inocula that can be used to inoculate seedlings in the nursery, they include: (1) soil inoculum i.e. native topsoil, (2) liquid/ mycelia slurry, (3) spores and sporocarps, and (4) colonised roots. These inoculum sources have their advantages and disadvantages and as such, should be chosen based on the desired goal (Molina and Trappe, 1984). One of the inoculum sources that has received the most attention nowadays is the use of mycelia slurry from pure cultures that allow easy analyses of fungal beneficial characteristics. In addition, the use of pure cultures allows for a mixture of two or more fungal species to optimise synergistic characteristics in the field (Khasa et al., 2011; Quoreshi et al., 2007; Greer et al., 2011). In contrast, the use of colonised roots is less often considered because of the risks that root materials to harbour other pathogenic fungi in the root system and the likelihood of mycorrhizal colonisation to be uneven. Other ECM inoculum sources have been explored by Coughlan and Piché (2005) using transformed root organ cultures and chitosan beads for fungal mycelia encapsulation (Quoreshi, 2008).

The function of ECM biotechnology has been reported to not only rely on the performance of the inoculum but also on cost effectiveness which seems to be one of the setbacks of commercial nurseries adopting this method. Another reason may be the assumed cumbersome nature of inoculation prior to seeding (Chapman, 1991). Nevertheless, to end users (mining industries and reforestation professionals), the benefits of inoculation outweigh these setbacks given that seedlings may be able to surmount initial transplanting stress and be well established in the field (Rićon et al., 2006). Furthermore, these impediments can be prevailed over if different inoculation methods or inoculation timings are considered in nursery management schemes. Chapman (1991) studied the effect of two inoculation techniques for vegetative mycorrhizal inoculum in a commercial nursery. One method was by injecting slurries of vegetative (mycelia) inoculum into the plugs of container grown seedlings, while the other was by squirting inoculum on top of the plug. It was observed that both types of inoculation produced similar levels of colonisation. On the other hand, Koide et al. (1999) studied the best inoculation timing for commercial nurseries (at sowing, transplanting or both). It was observed that inoculation at sowing was as effective as at transplanting or when doing both and required less amount of inoculum. Therefore, if inoculation strategies are optimised to suit different nursery practices, it will be more cost-effective and prevent repeated plantings by end users to offset mortality in the field when seedlings are not inoculated (Cordell et al., 2002).

Due to possible low mycorrhizal inoculum levels in disturbed sites (Bois et al., 2005; Sanon et al., 2010), the restoration of land to its productive capacity can be

challenging (Roy et al., 2007). The transformation of a disturbed landscape to its productive capability involves reclamation with forest plants (mostly ECM plants) and other kinds of vegetation cover for quick restoration. Therefore, it is essential to consider the use of AM and ericoid mycorrhizae plants for revegatation (Quoreshi, 2008). Khasa et al. (2002) highlighted the potential of using selected ECM and AM fungi for inoculation trials to improve the establishment of introduced poplars on previously cleared agricultural or disturbed sites in the province of Alberta. One universal problem in mining sites is soil concentration of metals that affects plant growth in a variety of ecosystems (Jentschke and Godbold, 2000). These metals can arise as a result of acid rain breaking down soils and releasing heavy metals, dust containing metals, wash waters from contaminated soils or from atmospheric factors produced due to mining, smelting, burning of fossil fuels, industrial or agricultural activities and incineration of municipal waste (Gaur and Adholeya, 2004). Some metals such as Zn, Cu, Fe and Co only become toxic and cause metal stress when present in high concentrations (Orcutt and Nilsen, 1996). Heavy metals once present in plant tissues, hinder physiological process at all levels of metabolism. These processes include photosynthesis, ion and water exchange, respiration, cell division, cell homeostasis, stomatal function, plasmodesmatal blockage etc. Nevertheless, these processes are dependent on the concentration of metal ions and the sensitivity of plant species (Fodor, 2002). According to Wilkins (1991), if ECM fungi can facilitate the uptake of nutrients then they are much likely to uptake trace nutrients and non-essential metals. Therefore, he suggested the need to first analyze the toxicity of the metal to the fungus as metal amelioration strongly depends on the fungal species and the metal present. Ray et al. (2005) carried out such an analysis in which they tested the tolerance of 8 ECM fungi *in vitro* to Al, As, Cd, Cr, Ni and Pb. Six out of the eight species showed tolerance to the above metals in varying concentrations. Krupa and Kozdrój (2007) also reported a reduced translocation of Zn(II), Cd(II) or Pb(II) by *Scleroderma citrinum, Amanita muscaria* and *Lactarius rufus* from roots to shoots of pine seedlings. However, the pattern of metal accumulation was dependent on fungal species. Furthermore, phenolic acids (ferulic, o-coumaric, and ohydroxyphenylacetic acid), that are commonly found in Kalmia and inhibit growth of black spruce, were also reported to have no effect on the ECM fungus *Paxillus involutus* when grown *in vitro* at a 1mM concentrations (Zeng and Mallik, 2006). Ectomycorrhizal fungi no doubt ameliorate metal stress in their host, but the mechanism involved is still unclear (Jentschke and Godbold, 2000). Different mechanisms have been proposed such as increased production of chelating agents such as oxalic acid, ligand-exchange and dissolution of the metallophosphate

complex (Pedersen and Sylvia, 1996; Finlay, 2004). According to Jentschke and Godbold (2000), ECM fungi may alleviate metal stress through the following possible mechanisms: "1) a reduction of metal exposure by excretion of chelating substances, (2) extracellular sequestration (e.g. by mucilage, pH gradients in the rhizosphere), (3) modified uptake systems at the plasmalemma, or (4) intracellular detoxification".

Salinity and drought are environmental stresses that are prevalent in many disturbed lands (Naidu and Harwood, 1997). They are also of a predominant concern in arable lands and are often attributed to land clearing or irrigation (Munns, 2002; Bandou et al., 2006). These abiotic stresses are considered as major factors limiting plant growth and productivity, especially in oil sand mining that generates saline tailing sands and composite tailings as by-products that require reclamation. Salinity and drought stress are linked, as salinity reduces plant ability to uptake water as well as causing changes in metabolism similar to those caused by drought stress (Munns, 2002). Drought stress occurs when water available in the soil is reduced and whenever transpiration losses are greater than water uptake, while salinity stress occurs when there is a decrease in osmotic potential around the roots due to high solute content or the concentration of ions in toxic amounts (Hale and Orcutt, 1987). These stress factors bring about disruption of osmotic and ionic homeostasis, damage to structural and functional proteins, reduction in net photosynthesis as well as cell membrane injury in plants (Wang et al., 2003; Bois et al., 2006b). To overcome salinity stress in reclamation, possible approaches would be to find a cost-effective way to desalt water for irrigation purposes, desalinate soil by leaching, select salt-tolerant crop plants (halophytes), or to use biological processes such as mycorrhizal interactions (Hale and Orcutt, 1987; Munns, 2002; Bandou et al., 2006). Numerous studies have indicated the use of ECM and other mycorrhizal fungi to improve drought and salinity tolerance (Orcutt and Nilsen, 1996). According to Marjanovic and Nehls (2008), the first evidence of water uptake and transportation to plants by ECM fungi was demonstrated by Duddridge et al. in 1980. This opened up various research hypotheses as to the means by which ECM fungi achieve such benefits. Mushin and Zwiazek (2002a) were also interested in the method of water uptake by ECM fungi of which *Hebeloma crustuliniforme* and *Ulmus americana* seedlings were used. They observed that colonisation of the plant by ECM fungi increased the hydraulic conductance of roots, which were facilitated by decreasing water flow resistance of the apopolast rather than water channel-mediated transport.

Landhäusser et al. (2002) also studied the effect of the same ECM fungi on water relations of aspen and white spruce of which higher root hydraulic conductance was obtained in mycorrhizal colonised plants. Studies on water conductivity of ECM fungi have also been analyzed using other abiotic factors (temperature, nutrient, oxidation etc) that may cause plant stress or influence mycorrhizal benefits (Landhäusser et al., 2002; Muhsin and Zwiazek, 2002a; Marjanovic and Nehls, 2008; Alvarez et al., 2009a; Alvarez et al., 2009b). However, these studies still reported the potential of ECM to be of benefit in alleviating water stress. Likewise, ECM fungi have been reported to alleviate salinity stress by limiting uptake of sodium ions (Na+) to shoots and leaf tissues while increasing water conductivity (Bandou et al., 2006; Muhsin and Zwiazek, 2002b, Bois et al., 2006a). In the study conducted by Tang et al., (2009), three ECM fungi (*Suillus bovinus*, *Suillus luteus* and *Boletus luridus*) studied i*n vitro* were not affected by varying concentrations of NaCl, rather their growth on agar reduced NaCl concentration. This is similar to the results obtained by Bandou et al. (2006), who tested the capacity of an ECM fungus specifically associated with sea grape to alleviate saline stress. They concluded that once a symbiosis was established, the ECM fungus *Scleroderma bermudense* increased the host tolerance to salt stress. However, not all ECM fungi are resistant to saline stress as *Laccaria bicolor* was reported to be sensitive to NaCl (Bois et al., 2006b). In addition, there is a possibility that salt resistance by some ECM fungi at high concentrations of NaCl can increase photochemical stress and dehydration of their host (Bois et al., 2006a).

The use of ECM in ecological restoration and mine site reclamation is gaining popularity around the world (Sheoran et al., 2010). In Spain, ECM inoculation has been used to improve reforestation programmes because bare root seedlings are usually of poor quality and lack high mycorrhizal colonisation levels in root systems (Dunabeitia et al., 2004). Throughout the United States and several other countries, the successful use of ECM inoculation as a biological tool in revegetation of drastically disturbed sites has been reported (Cordell et al., 2002; Sheoran et al., 2010). A practical example of inoculation success on mined sites was in Ohio, mine land reclamation-abandoned mineland programs (AML) that used the ECM fungi *Pisolithus tinctorius* (PT) and tree species in reforestation. An average of 85% survival was recorded in PT inoculated tree plantings with 5% mortality compared to about 75% mortality in non-inoculated trees (Cordell et al., 2002). Similar reclamation success has also been recorded in the Rocky Mountains at high altitude and mineral sites in other parts of United States using mycorrhizal inoculated trees, shrubs and grasses (Sturges, 1997; Cordell et al., 2002; Cripps, 2003). Other studies

from around the globe have also reported success in reclamation of adverse sites by inoculating conifers and angiosperms with beneficial ECM fungi (Khasa et al., 2002; Glen et al., 2008; Quoreshi et al., 2008). Furthermore, advanced molecular tools such as microsatellite or simple sequence repeat (SSR) markers are being used to monitor the persistence of inoculated ECM fungi in the field (Jany et al., 2003; 2006), thereby, giving more room for studying the fungal ecological effects.

1.5 Ectomycorrhizal fungi and their eological importance

Forests are known worldwide to be critically important ecosystems as they contain biological diversity and serve ecological functions. Forest ecosystems are characterized predominantly by trees, and by the fauna, flora and ecological cycles (energy, water, carbon and nutrients) with which they are closely associated (Randolph et al., 2005). Forests are distributed in all regions capable of sustaining tree growth unless the environment has been altered by human activity or natural disaster. They have been reported to make up 82.2 to 91.5% of the Earth's terrestrial biomass with the tropical forest having the largest total biomass (Randolph et al., 2005). The latitudes 10° North and South of the Equator are mostly covered in tropical forest and the latitudes between 53^{o}N and 67^{o}N have boreal forest.

Mycorrhizal fungi are well known influences of plant diversity patterns in a variety of global ecosystems (van der Heijden *et al.*, 1998; Klironomos, 2000). The distributions of their associations in forest ecosystems can be generalized based on their global patterns. Boreal forests are mostly dominated by ectomycorrhizal trees, temperate forests contain both ecto and AM trees and tropical rain forests have mostly AM trees (Mcguire, 2008). Ectomycorrhizal fungal associations evolved more in recent times and despite their widespread distribution, they associate with only 3% of vascular plant families (Smith and Read, 2008). The majority of ECM fungi belong to the Ascomycota and Basidiomycota phyla and their mutualism has been thought to have saprophytic lineages (Hibbett *et al.*, 2000). This group of fungi form mutualistic symbioses with many tree species and are considered as vital organisms in nutrient and carbon cycles in forest ecosystems. They differ from other mycorrhizal fungi as they form a thick sheath or mantle around the lateral roots of the host as well as forming other structures such as Hartig net, intercellular hyphae (rhizomorphs, sclerotia) and sexual reproductive bodies. All these structures are ecologically relevant and play important functions during the life cycle of the fungi (Peterson et al., 2004; Fortin et al., 2009). However, an appreciation of their ecological role is

hampered by a lack of understanding of their soil-borne mycelial systems (Korkama et al., 2006; Anderson and Cairney, 2007). Nevertheless, the fungal mycelium plays other ecological roles such as contributing up to one-third of total soil microbial biomass and production of dissolved organic carbon with host plants (Hogberg and Hogberg, 2002; Wallander *et al*, 2001). In this regard, more emphasis is therefore placed on the ecological role (carbon and or nitrogen transport) of common mycorrhizal networks and the decomposition capacity of ECM mycelium in forest ecosystem.

To appreciate the ecological role of ECM fungi in forest ecosystems, an understanding of the mode of colonisation that subsequently leads to the spread of fungal mycelium is necessary. ECM colonisation of a plant root is triggered by the release of root exudates such as water soluble and volatile exudates into the rhizosphere; this stimulates germination of the propagules and subsequent growth of the hyphae of the fungus, after which a mycelial mass aggregate is formed around the root outer surface **(**Bécard and Piché, 1989; Koske and Gemma, 1992). This phenomenon is followed by the hyphae exerting pressure on the cortical cell walls of the root, and sometimes, a localized enzymic degradation (through enzymes such as pectinases and cellulases) of the cell wall is involved (Garcia-Garrido et al., 1992; Bonfante and Perotto, 1995). The hypha then grows between the cortical cells, pushing apart the cells to establish a network called Hartig net. This formation rarely penetrates the cortical cells and will not extend beyond the endodermis (Isaac, 1992).

In forest ecosystems, the mode of colonisation that subsequently leads to the spread of fungal mycelium is the pinnacle of their ecological significance (Korkama et al., 2006; Anderson and Cairney, 2007). EM fungal mycelia can comprise up to 80% of the total fungal biomass and 30% of the microbial mass (Wallander et al., 2001; Hogberg and Hogberg, 2002; Wallander, 2006) and are regarded as vital drivers of forest ecosystem processes. The mycelial biomass of ECM fungi varies with soil depth and tree species but may be influenced by the distribution of tree roots in the various soil profiles (Wallander et al., 2004). Considering that ECM fungi are said to be host specific, it is yet to be elucidated if the mycorrhizal mycelium formed below ground facilitates growth of other plants or distributes resources among plant irrespective of the plant species. This led to the study by van der Heijden et al. (2009) who tested the ability of mycorrhizal networks to facilitate growth of small seedlings that establish between or near larger plants. They also addressed issues such as plant species benefit from mycorrhizal networks, size and age of plants as beneficial factors, possible host dependent barriers and impact of mycorrhizal networks on

plant-plant interaction and community dynamics. This study observed that mycorrhizal networks promoted seedling growth in 48% of the cases (for 21 seedling species), which ECM benefited plants in the majority of the cases and thus concluded that the supporting effects of mycorrhizal fungal networks depend on seedling species identity, mycorrhizal identity, plant species combinations and study system. Furthermore, the ECM mycelium can extend to considerable distances and persist for several years in forest soils (Nehls, 2008). This is attributed to the ability of ECM fungi to spread over areas with sizes up to several square metres. The mycelium remains functionally interconnected, forming specialized long distance hyphae that explore the surrounding soil. When the hyphae recognize an emerging fine root of a plant partner, they grow towards it (Martin et al., 2001) and colonise the root surface, (often) forming a sheath or mantle of hyphae, which encloses the root and isolates it from the surrounding soil (Nehls, 2008). In an attempt to understand ECM mycelial distribution and dynamics, Anderson and Cairney (2007) reviewed the literature on fungal fruiting bodies and root tips as well as microcosm analyses. However, it remains that current knowledge using the above subjects as determinants could give only limited insight to the soil borne mycelial distribution or community.

ECM fungi acquire most of their carbon from their host plant (Smith and Read, 2008), but in the absence of a host they can utilize other forms of carbon (Satyanarayana et al., 1996; Ohta, 1997). This carbon is used in maintaining the existing fungal biomass present in the mycorrhizal root tips and mycelial network in the soil (Söderström, 1992). Based on the assumption presented in a review by Söderström (1992), there is increased carbon demand in boreal forest soils. Therefore, the export of carbon to the soil via a mycorrhizal mycelial network might be of considerable significance. The ecological importance of such a system of carbon transport may enhance plant-to-plant linkages or improve mobilization of nutrients (organic and recalcitrant sources) in competition and cooperation with soil saprotrophs (Anderson and Cairney, 2007). More so, it is likely that decreased saprophytic activity in forest soils is not limited by inorganic nutrient availability but rather by energy deficiency (soil C) (Söderström, 1992). Studies have reported systems where nutrients such N and C were transferred between ECM seedlings through mycorrhizal networks (Finlay and Read, 1986; Amaranthus and Perry, 1994; Read et al., 2004; Philip and Simard, 2008). In a study by Simard et al. (1997), the authors used reciprocal isotope labelling in the field to demonstrate bidirectional carbon transfer between the ECM tree species *Betula papyrifera* and *Pseudotsuga menziiesii*. The average amount of received isotope that was retranslocated from roots to foliage was 13% for *P. menziiesii* and 45% for *B. papyrifera*. They suggested that

the exchange was primarily possible through a direct hyphae pathway. Similarly, Wu et al. (2001) reported the ability of ^{14}C labeled photosynthetic product to move between *Pinus densiflora* seedlings linked by ECM mycelia. However, both studies highlighted the possibility of one plant species having a higher net carbon gain than the other. Nitrogen is the major limiting element in terrestrial ecosystems and is present in soil in multiple forms as ammonium, nitrate or organic N (Aerts, 2002). The inorganic form is formed by mineralization, nitrification process or external inputs. In forest ecosystems, plant litter and its breakdown products are the main organic N sources that are made available by internal cycling. The amount of N in litter has been estimated to vary between 10-170 kg N ha^{-1} $year^{-1}$ and initially consists of soluble cell contents or solid cell wall components. The cell content contains a wide variety of compounds from inorganic ammonium, amino acids, peptides to proteins and possible secondary metabolites like tannins and phenolics. Wood litter, on the other hand, is rich in lignin, cellulose and hemicelluloses (Aerts, 2002). Many soil animals, bacteria and fungi are involved in the breakdown of plant litter that leads to the release of nutrients (Dighton, 1991). These nutrients are then available for uptake by plants and microorganisms. In this regard, ECM fungi have the dual ability to decompose plant litter and translocate broken-down products. This functional role was demonstrated by Entry et al. (1991) who measured the microbial biomass, needle decomposition rates, nutrient release from needles and exchangeable soil nutrients in ECM mat soils of Douglas fir forest. Their data suggest that the ECM fungus *Hysterangium setchelli* provided an improved microenvironment for organic matter decomposition that resulted in the faster release of N, P, K and Mg as well as the removal/translocation of the nutrients from the soil solution. The uptake and removal of N and P from soil solution by mycorrhizal mycelium was further confirmed by Brandes et al. (1998) using the ECM fungus *Paxillus involutus* and Norway spruce seedlings. The ECM fungus increased the nutrient concentration with a 73% and 76% of hyphal contribution to the total N and P uptake, respectively.

Studies by Mcguire (2008) showed the ability of ECM fungi to degrade litter and wood debris in mixed and monodominant forest. However, ECM fungi had the potential to reduce to rate of decomposition by suppressing the activity of other saprophytic fungi and bacteria. This suggests the multitrophic interaction that ECM fungi have with trees, soil microbial community and the microbes associated with the mycelium (Korkama et al., 2006). The possibility of ECM fungi to decompose plant litter and wood debris that is rich in N may be attributed to the production of catalytic enzymes and ectoenzymes such as proteases (Nygren et al., 2007; Fortin et al., 2009). This uncertainty led to the study of Cullings and Courty (2009) that performed a

controlled experiment in natural setting to determine the response of ECM fungi to added litter in terms of proliferation and expression of enzymes that could break down litter. Their present and previous analysis demonstrated that ECM fungi used litter as a nutrient source and when assays of cellulase, phosphatase and laccase activities of ECM root in litter were conducted, significant increases in all enzyme activities was observed. These enzymes degrade cellulose, P sources and lignin, respectively (Kunamneni et al., 2007). In addition, their saprophytic nature has been reported to play a part in the decomposition of organic matter (Read and Perez-Moreno, 2003; Nygren et al., 2007).

1.6 Mycorrhizal research applications in Canada

The benefits of ectomycorrhizal fungi in forest ecosystems have fostered research in plant physiological parameters. This is because the presence of mycorrhizal fungi during plant stress situations may bring about a difference between survival and death of the plant (Hale and Orcutt, 1987). In an undisturbed terrestrial ecosystem, mycorrhizal fungal propagules that are capable of colonizing host plants are typically high. By contrast, areas disturbed by mining and or agriculture tend to have lower active fungal propagules present in the soil (Parent and Moutoglis, 2009). Therefore, amelioration of this concern has been sought through artificial application of mycorrhizal fungi.

The development and use of commercial mycorrhizal inocula have grown considerably over the last three decades as they are seen as natural means of improving plant production. In Canada, mycorrhizal research and field applications of mycorrhizal biotechnologies have advanced through national and international partnership among research groups (Parent and Moutoglis, 2009). These groups have made significant breakthroughs ranging from cultivation of arbuscular mycorrhizal *in vitro* and in pot cultures (Bécard and Piché, 1992; Fortin et al., 2002; Dalpé and Monreal, 2004) to the use of mycorrhizal inocula in agriculture, forestry, horticulture, and reclamation (Alarcón et al., 2007; Bois and Coughlan, 2009; Larsen et al., 2007; Quoreshi et al., 2009).

In Alberta, Canada, the industrial application of mycorrhizal fungi has been largely for reclamation purposes, which commenced in the late 1990's (Parent and Moutoglis, 2009). The use of mycorrhizal fungi in revegetation and improved reclamation is of fundamental importance that is becoming widely accepted

(Quoreshi et al., 2006). However, in Canada the use of inoculation methods in plantation forestry is still at its infancy. This could be attributed to the unavailability of high-quality inocula, lack of technological expertise, or a convenient method of application in commercial nursery settings (Quoreshi et al., 2006). More recently, large-scale applications of mycorrhizal inoculation in agriculture are underway (Anon 2011). Furthermore, mycorrhizal fungal inocula are regulated under the *Fertilizers Act* and the Canadian Government mandates the Canadian Food Inspection Agency to regulate all products referred to as plant enhancers. As such, product registration requires series of experiments and approval processes that are costly and time-consuming. These and all other governmental procedures have led to the minimal implementation of this emerging sustainable biotechnological tool in Canada and other western countries (Parent and Moutoglis, 2009).

1.7 Motivation for the study

The study was motivated by the present need to enhance revegetation in oil sand disturbed lands using environmentally-friendly technology that would not only have a long term beneficial effect on establishing a sustainable plant community, but that would also reduce the rate of plant mortality in reclamation sites.

1.8 Objectives and thesis structure

The primary objectives of this study were to:

i) Assess the natural mycorrhizal inoculum potential in various reclamation materials currently used for reclamation practices.

ii) Identify and characterize the natural mycorrhizal strains that are present in reclamation materials.

iii) Evaluate the potential benefits of nursery-inoculated white spruce and jack pine seedlings with selected ECM fungal species on growth performance, survival, and persistence of introduced fungi after planting on Suncor reclamation sites.

This thesis is composed of two studies. The first study in Chapter 2 addresses the first and second objective. This study was designed due to the necessity to conduct a mycorrhizal inoculum potential bioassay prior to field application studies. This was to help determine if the mycorrhizal establishment was a result of natural ECM propagules present in amending materials or if re-installation of these

organisms through nursery inoculation is indeed necessary. Specifically, the study objective was to evaluate the arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) inoculum potential of amendment materials used as cover soils for overburden and tailing sands structures on reclaimed lands; and to identify ECM species present using morphological and molecular typing.

The study in Chapter 3 addresses the third objective while attempting to also identify ECM fungi on the root system of plant species based on fungal morphology and DNA analyses. In general, the aim of the study was to assess the effect of nursery-inoculated seedlings with mycorrhizal fungi in improving reclamation success. This was to be achieved using different fungal species singly and in combination. Therefore, in this study, the following hypotheses were tested: (i) The amending materials used in the current reclamation practices are devoid of mycorhhizal propagules; ii) Seedlings inoculated with a combination of fungal species will perform better than single inoculated seedlings; (iii) Inoculation with combined fungal species will at least allow persistence of one strain of fungus following resident fungal competition; and (iv) Response of inoculated seedlings to various fungal species may be influenced by the host plant species.

The final chapter of this thesis (Chapter 4) summarizes the result obtained from both studies and highlights the limitations encountered in the study and provides suggestions for future work.

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2 Mycorrhizal inoculum status of reclamation soil substrates from the Athabasca Oil Sands region

2.1 Introduction

The Athabasca Oil Sands Region (AOSR) is the largest bitumen deposit in Alberta Canada from which 1.7 trillion barrels of oil can be recovered (Fung and Macyk, 2000). The recovery of bituminous sand using surface mining is a common process in the oil sands and entails the extraction of oil sand deposits that are close to the surface. This extraction process results in creation of large areas of disturbed lands that will require reclamation (Fung and Macyk, 2000; Rowland et al., 2009). During mining, the topsoil, deep overburden and organic material (muskeg) that sits above the oil sand deposit are removed. These materials are stored and later used as amendments to reconstruct reclamation areas prior to revegetation (Bois et al., 2005; Ghose, 2004a).

In oil sands mining areas, vegetation establishment is required as a method of reclamation to return reclaimed land to productive ecosystems that could provide a variety of benefits. This is in accordance with *Land Surface Conservation and Reclamation Act* 1973 and the *Environmental Protection and Enhancement Act* 1992 (Government of Alberta, 1999). Tailings sand (TS) and composite tailing (CT) sands are the major types of mine wastes that require reclamation in the oil sands region (Fung and Macyk, 2000). They contain tailing slurry as well as a significant quantity of water, residual bitumen, and coarse-grained and fine-grained materials (Bois et al., 2005). Disturbed land following open pit mining are given high priority because nutrient cycles are disconnected, may have high alkalinity and salinity as well as may contain low organic matter and may lack necessary biological activity or moisture holding capacity (Fung and Macyk, 2000; Quoreshi et al., 2006; Zwiazek et al., 1998).

The re-establishment of a self-sustainable ecosystem is a major challenge in the reclamation of land disturbed by oil sands mining (Li et al., 1998). This is because in such ecosystems the soil must be physically stable to support plant growth, be chemically and biologically active to buffer environmental effects as well as maintain sufficient nutrient within the root zone for plant growth (Monenco Consultant, 1983). Several organic amending materials used by mining industries over the past 30 years to cap reconstructed soils include forest floor (LFH), peat-mineral mix, fresh peat, stock piled peat, sub or top soil and overburden (Rowland et al., 2009). However, the quality of these materials to be used in

establishing a sustainable plant community can be reduced during manipulations, or when stockpiled for longer time period (Bois *et al*., 2005; Ghose, 2004b; Ghose, 2001).

Studies have shown that mycorrhizal symbiosis may be a vital determinant of plant diversity, productivity and ecosystem variability (van der Heijden *et al*., 1998; Hartnett & Wilson, 1999; Klironomos *et al*., 2000). This has been attributed to the enhanced mineral nutrition, resistance toward pathogens and buffering environmental stress that they confer to their host in exchange for photosynthates (Smith and Read, 2008), thus indicating these components may have a potential to facilitate a successful reclamation process. Mycorrhizal mycelial networks are the most dynamic and functionally diverse components of the symbiosis but the destruction of mycorrhizal fungal networks in soil systems is the vital event of soil disturbance. Therefore, there is a need for comprehensive understanding of inoculum potential of available materials to assess the potential re-introduction of mycorrhizas to accelerate ecosystem development. In addition, since the establishment of new roots as well as the persistence of mycorrhizal fungi in ecosystems is dependent on the distribution and infectivity of propagules, it is pertinent that these infective propagules are present during root growth activity to allow rapid colonisation of the root system (Brundrett and Kendrick, 1990; Bowen, 1987).

Several studies have reported the presence of some levels of mycorrhizal fungi in different amending materials used previously to reconstruct soil from oil sand processed materials (OSPM) (Danielson et al., 1983, 1984; Danielson and Visser, 1989). Recent changes in reclamation policies and practices allow the oil sand industry in Alberta to use various types of capping materials, such as fresh and stored LFH materials, peat-mineral mix, and peat alone (BCG report, 2010). These capping materials may provide an alternative source of indigenous mycorrhizal fungal propagules. However, the inoculum potential of these materials varies as a result of hauling and handling processes (Danielson et al., 1983; Malajczuck et al., 1994). Substrate inoculum potentials may be defined as the percentage colonisation by mycorrhizal fungi of each bioassay plant and represent development of active propagules in a specific substrate under greenhouse conditions (Brundrett et al., 1996). Investigation of mycorrhizal fungi in soils has been constrained by the difficulty in identifying them by using morphotypes alone and separating them from non-mycorrhizal soil fungi. However, recent advances in use of molecular technology provide greater precision for ecological studies.

Historically, reclamation efforts in the AOSR use peat and overburden to improve plant growth performance (BCG Engineering Inc., 2010). These amending materials were considered to be more effective in improving moisture retention, nutrient availability and soil organic matter in tailings sand (Naeth and Wilkinson, 2004). To date, mining operators use and are urged by Alberta Environment to salvage surface soils and LFH as soil amendments

in place of peat or peat mineral mixes (BCG Engineering Inc., 2010). The use of LFH as an amendment has been reported successful because it contains greater quantity and diversity of propagules and woody materials (Mackenzie and Naeth, 2010). However, this success is not sustainable because LFH is not readily available in large quantities to measure up to mining foot print and long term moisture retention is uncertain. On the other hand, the use of surface soil as an amendment once salvaged has been given little or no attention. This may foster the establishment of a sustainable plant ecosystem using natural processes, which in the long run will favour mycorrhizal associations.

Since soil placement of amending materials is conducted in AOSR prior to tree planting as part of the reclamation process, it is necessary to conduct a mycorrhizal inoculum potential bioassay prior to field application studies. This can determine if the mycorrhizal establishment is as a result of natural ECM propagules present in amending materials or if re-installation of these organisms through nursery inoculation (Quoreshi, 2008) is indeed necessary. The main objective of this study was to evaluate the arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) inoculum potential of amendment materials used as cover soils for overburden and tailing sands structures on reclaimed lands. An additional objective was to identify ECM species present using morphological and molecular characterisation.

2.2 Materials and Methods

2.2.1 Soil Substrate

The studied substrates included peat-mineral mix (PMM), overburdened soil (OVB), tailing sands (TSA), top soil (TPS) and intact forest soil (IFS) collected around the mining area of Suncor Energy Inc., Fort McMurray. Soil cores of overburdened substrates were randomly collected from a pile of soil material freshly excavated from a depth of 20–30 cm, while the peat mineral mix was taken from freshly spread material. Tailing sands were collected straight from recent tailing piles and topsoil material from stockpile of subsoil using an 8 x 20 cm (diameter x height) core borer and a shovel. Soil cores were collected from areas located within 56^{o}59’N and 11^{o}27’W and were put in surface sterilized pots on site.

2.2.2 Seedling Preparation

Soil cores were planted with red clover (*Trifolium pratense* L. and white spruce (*Picea glauca* (Moench) Voss) to evaluate the presence of AM and ECM propagules. Seeds were surface- sterilized for 2 min in 1% sodium hypochlorite rinsed six times with distilled water and sown in Sunshine mix (a potting soil) to pre-germinate. The Sunshine mix (Sun Gro Horticulture Canada Ltd) consists of coarse grade Canadian Sphagnum peat moss, coco fiber,

coarse grade perlite, coarse grade vermiculite, screened pumice, dolomitic limestone, an organic fertilizer and an organic wetting agent. This potting soil was autoclaved (121°C, 45 min) twice with a 24 h interval between each treatment before seeds were sown. To maximize plant survival, about 200 seedlings per plant species were grown in sterilized Styrofoam containers for 3 weeks before transplanting to the cores. To check for contamination by greenhouse mycorrhizal fungi, the same sterile mix was used as a control.

2.2.3 Bioassay Setup

The bioassay was prepared using pot size of 14 x 20 cm (diameter x height) in a completely randomized design. Pots were sterilized with 1% sodium hypochlorite prior to being filled with cores of substrates (10 replicates per substrate type). Each core was planted with one red clover and one white spruce seedling (Bois et al., 2005). In total, 120 cores were collected [two core per pot x (5 substrates+ control) x (10 replicates)], while 120 seedlings of plant species were sown [(2 per pot x (5 substrates + control) x 10 replicates unless otherwise stated]. All plants were watered as required and seedlings grown for 8-12 weeks before harvesting. Seedlings were grown under the following greenhouse conditions: photoperiod of 16hrs, 25/22^{o}C day/night temperature and 16± 10% relative humidity.

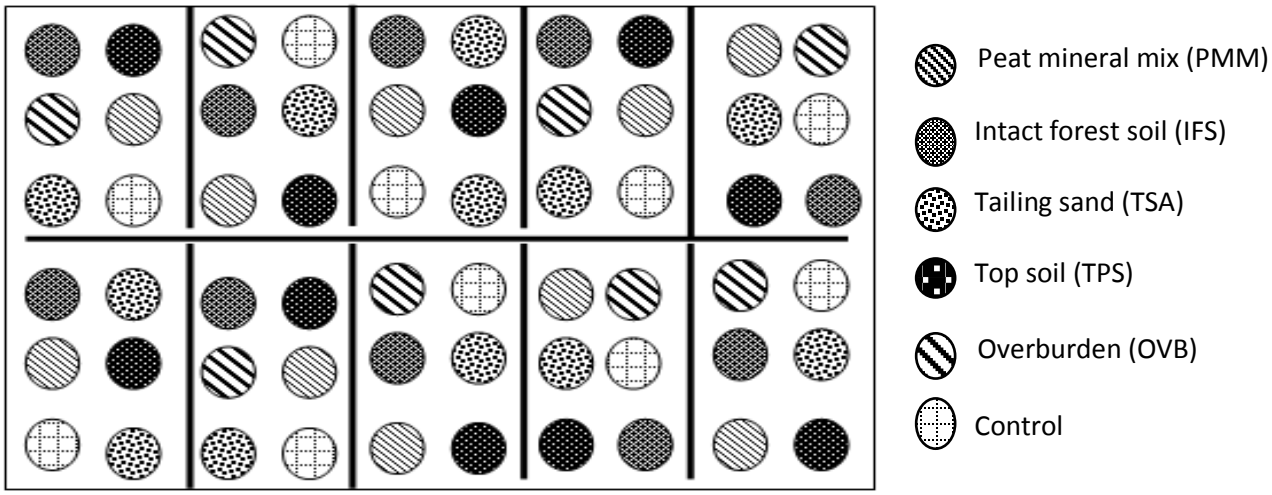

Figure 2.1 Illustration of the pot layout of soil cores in the greenhouse. Different patterns within a section indicate six different soil substrates. Lines in between sections are the space between pots.

At the end of the experiment, plants were harvested and the root systems were washed. Dry weights of shoot and root were obtained for plant biomass. AM fungi within roots of red clover were stained using the method described by Gaur and Varma (2007). The stained plant roots were examined for the presence or absence of AM fungal colonisation

(hyphal coils, arbuscules or vesicles) under a microscope and expressed as percentage colonisation using a modified Line Intersect Method (McGonigle et al., 1990). To ascertain the presence of a hartig net and possible colonisation of white spruce roots by endophytes, roots were stained using a modified version of Brundrett et al. (1996). A modification was in the time used to stain roots which was usually left for 5 hrs or overnight. The percentage of root tips colonised by ECM was calculated as the number of ECM root tips divided by the total number of root tips and multiplying by 100 (Gehring and Whitham, 1994).

2.2.4 Morphological description and molecular identification of ectomycorrhizal fungi

Morphotyping

Mycorrhizal morphotypes was conducted immediately after harvesting using an identification manual (Agerer, 1999; Goodman et al., 1996-2000) and characteristics such as branching patterns, colour, tip shape, texture, emanating hyphae and outer mantle were used in morphological identification. Subsequently, root tips and segments in given substrates were obtained for further analyses by selecting at least 2 and at most 8 samples of root tips or root segment and stored in cetyl trimethylammonium bromide (CTAB) buffer (Henrion et al., 1994) at -20^{o}C prior to DNA extraction. Root segments in this study consist of one or more root tips. A total of 36 root segment/root tips from white spruce were obtained.

DNA extraction and amplification

Fresh root samples (1g) were crushed with a micropestle and total DNA was extracted using Sigma Extract-N-Amp Tissue Kit following the manufacturer's protocol (Sigma, USA). Polymerase chain reaction (PCR) amplification was carried out using an alternate ITS1F and NLB4 fungal-specific primer combination coined from Martin and Rygiewicz (2005). An aliquot of 1.5 µL of extracted DNA was combined with 5 µL of Extract-N-Amp PCR (Sigma) solution in a 10 µL reaction. Amplifications were performed with an initial denaturation at 95°C for 4 minutes, followed by 40 cycles of 95°C for 1 minute, 55 °C for 1 minute and 72°C for 1 minute, with a final extension of 72°C for 10 minutes. All PCR products were visualized on a 1% agarose gel. Successfully amplified PCR products were purified using ExoSAP-IT (USB, Cleveland, OH, USA). Cycle sequencing was carried out in one direction using the Big Dye Terminator Sequencing Mix (v. 3.1, Applied Biosystems, Inc.), with the same PCR primers at a final concentration of 0.1 µM. The resulting products were precipitated following the manufacturer's instructions for EDTA/ethanol. Due to a high degree of bias and uncertainty in restriction fragment length

polymorphic (RFLP) patterns (data not shown), samples were analysed after purification using DNA-sequencing of ITS region. Sequencing reactions contained 1 µl of Big Dye Reaction Pre-Mix, 1.5 µl Big Dye 5X Sequencing Buffer, 6 µl of nanopure water and 1 µl of cleaned PCR product in addition to primer (one direction-forward primer). Reactions were dried and sent to the MBSU unit Biological Sciences Department University of Alberta, Edmonton, Canada, where sequencing reactions were purified and analysed on an Applied Bioscience 3730 capillary genetic analyzer. DNA sequences were checked against the sequence database of National Centre for Biotechnology Information (NCBI) online standard Basic Local Alignment Search Tool (BLAST) program to assign a taxonomic name to each morphotype. The significant level of similarities with ITS sequences in the Genbank database was determined by noting the percentage identity and the expectation value (E-value). The E-value displays the equivalent or similarity of a number of alignment score to the raw alignments score that are expected to occur in a database; the lower the E-value the more significant the score (Wheeler *et al*., 2006). An accepted value of >95% and >98% was taken in this study as belonging to the same genus and species, respectively.

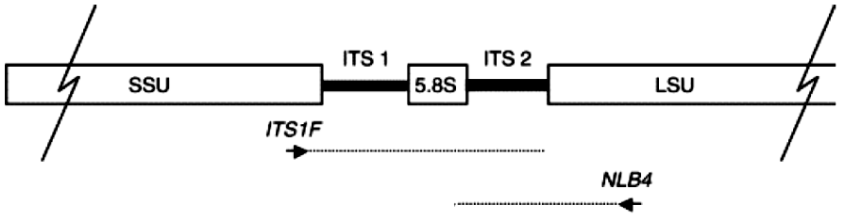

Figure 2.2 Primer amplification map indicating location of primers used in this study (Martin and Rygiewicz, 1999). Primer ITS1F was used to amplify ITS1 and 5.8s region and NLB4 was used to amplify ITS2. NLB4 has a wide range and lie outside primer site targeted by ITS1F.

2.2.5 Data Analysis

Data from the greenhouse bioassay, which was arranged in a completely randomized design was analysed using a one-way analysis of variance (ANOVA). Seedling within substrate type was treated as an experimental unit. Shoot, root, biomass and mycorrhizal percentage colonisation were analysed. Prior to analysis, logarithm transformations were done for white spruce biomass and arcsine square root transformation for mycorrhizal colonisation percentage when the raw data failed a normality test, while the raw data were used for red clover biomass as no transformation was necessary. The Bartlett test was used to ensure homogeneity of variances between samples (Zar, 1999). Means were compared using Fisher's Least Significant Difference (LSD) when a significant F-test value at $p<0.05$ was obtained. These statistical analyses were conducted using SAS 9.2 software.

2.3 Results

2.3.1 Seedling growth

Chemical soil parameters (Table 1) were measured to determine the possible influence on plant growth. There was no significant correlation between seedling biomass and soil parameters for both plant species (data not shown). Red clover biomass displayed significant differences ($p<0.05$) between substrates (Table 1). The substrate IFS supported the highest clover growth than others with OVB ranking the lowest. There was no significant difference between white spruce seedlings grown in any of the substrates.

Table 2.1: Chemical properties and plant growth responses on various substrates. Means followed by the same letter are not significantly different.

Substrate	pH (H_20)	EC (dS/cm)	Red clover Dry weight (g/plant)	White spruce Dry weight (mg/plant)
CTRL	7.36	1.44	0.67^a ±0.25	26.89^a ±4.33
PMM	7.66	0.92	1.87^b ±0.25	22.48^a ±4.33
OVB	7.99	0.88	0.19^a ±0.25	27.78^a ±4.33
IFS	7.03	0.79	2.13^b ±0.25	21.55^a ±4.33
TPS	7.67	0.36	1.87^b ±0.25	28.70^a ±4.33
TSA	7.55	0.40	1.10^a ±0.25	25.08^a ±4.33

**CTRL – potting soil, PMM – peat mineral mix, OVB – overburden soil, IFS – intact forest soil, TPS – topsoil, TSA – tailing sand.

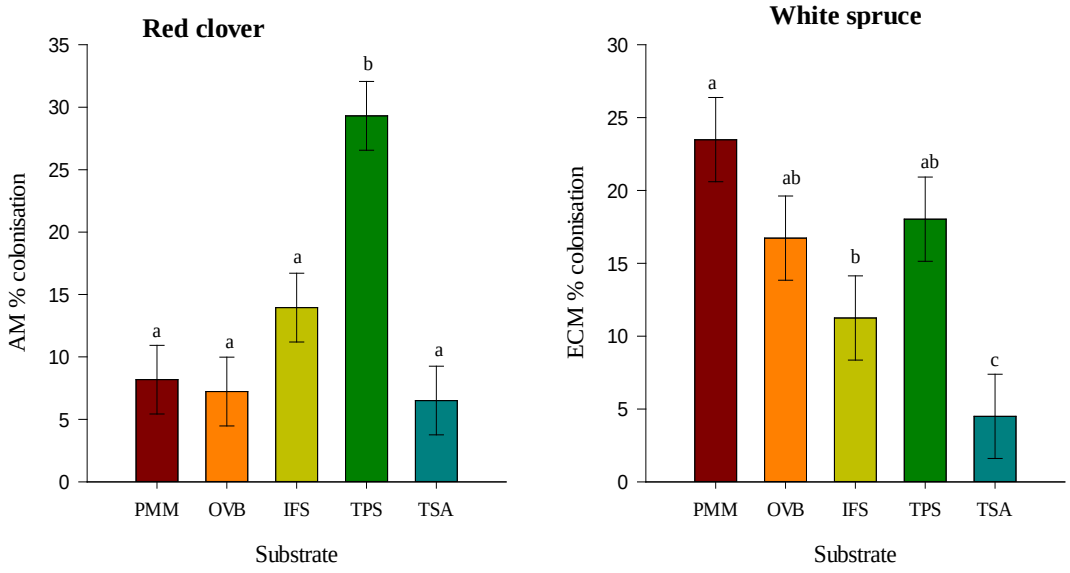

Figure 2.3: Percentage colonisation by arbuscular mycorrhizal (AM) fungi and ectomycorrhizal (ECM) fungi on red clover and white spruce seedlings, respectively, grown on peat mineral mix (PMM), overburden (OVB), intact forest soil (IFS), topsoil (TPS) and tailing sand (TSA). Means (AM, n=10; ECM n= 8) with the same letter are not significantly different from each other at 5% level Fisher's LSD.

2.3.2 Morphotyping and mycorrhizal status

There were no ECM structures in the control with white spruce seedlings. Based on morphological features, at most 8 different ECM morphotypes were observed from all soil types. Morphotype occurrence was about 20% with the majority of the white spruce roots being non-mycorrhizal. The Arum-type colonisation (Smith and Read, 2008) was mostly observed in red clover endomycorhizal roots. Arbuscules were occasionally observed in red clover root but were not well developed; however, higher numbers of vesicles than arbuscules were observed in the root cortex. The presence of a Hartig net in white spruce after staining was detected in a few samples of OVB, TPS and TSA substrate but was not well developed. Majority of the samples had septate runner hyphae that are characteristic of ectomycorrhizas. Amongst substrates determined for AM propagules, PMM, OVB and TSA had an inoculum potential of less than 10%. Topsoil had the highest inoculum potential of approximately 29% (Fig.1A). For ECM, the highest and least inoculum potential was observed in PMM with approximately 23% and TSA 5% respectively (Fig 1B).

For the molecular analyses, out of the 36 samples amplified by PCR, 17 samples that had distinct bands were finally sequenced (Table 2.3). Generally, 9 taxonomic groups closely related to NCBI database sequences were obtained. Five out of 9 were identified to species level, which includes species of Ascomycota division, *Pezizales* sp., *Wilcoxina* sp., and

Olpidium brassicae. The other 4 taxonomic groups were identified to order and family level with 2 uncultured fungal species and 1 unknown similar sequence (Table 3).

Table 2.2: Description of *Picea glauca* ectomycorrhizal morphotype found on substrates.

Morphotype Name	Morphotype description	Substrate	Frequency	% occurrence
Russula like	White tip, unbranched, straight to club shaped, smooth to wart texture with no emanating hyphae	TSA	4	8.3
Cenoccocum geophilum	Pale brown, club shaped smooth to felty, unbranched tip with occasional presence of black cystidia-like cap that is shiny.	IFS, TPS	6	12.5
Thelephora terrestis	Yellow to golden brown tip, club shaped with black cystidia like cap. Monopodial pinnate, smooth to felty	IFS	2	4.2
Lactarius like	Pale brown tip wth white apex, monopodial pinnate, felty to smooth, club shaped to bent with occasional straight tip with matte lustre	PMM, TPS, TSA	8	16.7
Unidentified	Unbranched to monopodial pinnate pale brown to dark brown tip. Straight to bent smooth and matte lustre	OVB, TPS, PMM,	4	8.3
Tomentella like	Dark brown unbranched to slightly dichotomous tip, straight, felty to stingy, matte lustre with little emanating hyphae	OVB, PMM, IFS, IFS	6	12.5
Amphinema byssoides	Monopodial pinnate, pale brown with creamish apex. Club shaped grainy to stringy with matte lustre	OVB, TPS	5	10.4
Suillus like	Pale brown to golden brown tip, felty to cottony, monopodial pinnate, straight to bent tip shape.	TPS	2	4.2
Non-mycorrhizal	Non-mycorrhizal	CTRL,TSA, OVB	11	22.9

***Percentage frequency of occurrence of each morphotype was determined by no. of seedlings with morphotype/total no. of seedlings (48) x 100.

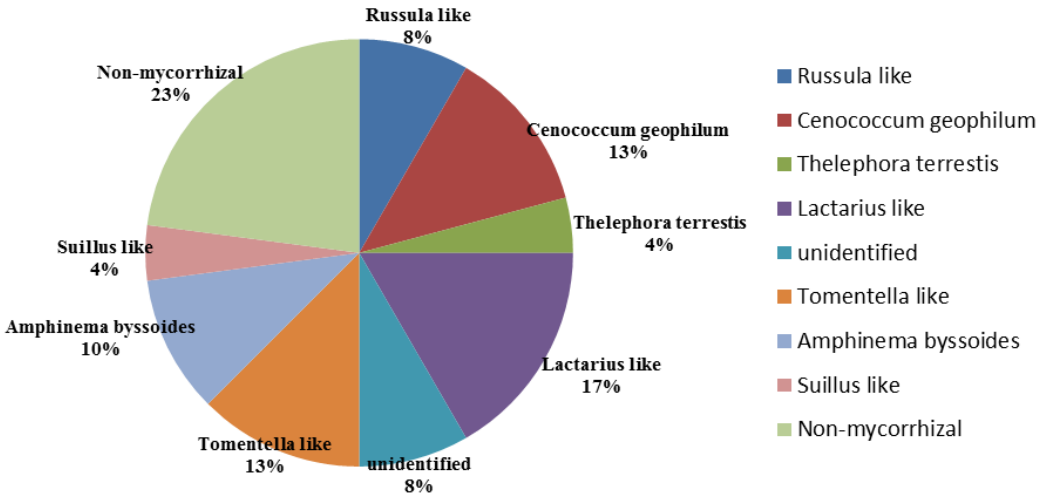

Figure 2.4: Frequency distribution of identified morphotypes in the five studied substrates.

Table 2.3: Summary of sequence affinity as analysed by DNA sequencing

Operational taxonomic unit	Closest match in Genebank	Closest match Accession No.	E value	% Similarity
TPS1,TPS3,OVB7	Pyronemataceae	EU649088	0.0	97
TPS7	Helotiales type	FJ197203	3e-39	97
IFS2	Helotiales	FM997933	0.0	99
TSA1	*Plectosphaerella sp.*	FJ430715	0.0	99
TSA7	*Olpidium brassicae*	AB205207	0.0	99
TSA8, OVB8,IFS3A	Pleosporales	EF027385	0.0	97
PMM4	Thelephoraceae	AJ893343	0.0	96
PMM2	*Ascomycota sp.*	HM141067	0.0	100
OVB3	*Pezizales sp.*	HQ649910	3e-172	89
OVB5	*Wilcoxina sp.*	GU181901	0.0	87
IFS4	Uncultured fungus clone	EU517040	0.0	99
TPS5	Uncultured fungus clone	EU517037	0.0	99
IFS5	Uncultured fungus clone	GU174331	0.0	95
IFS1A	Unidentified	-		-

*** PMM – peat mineral mix, OVB – overburden soil, IFS – intact forest soil, TPS – topsoil, TSA – tailing sand.

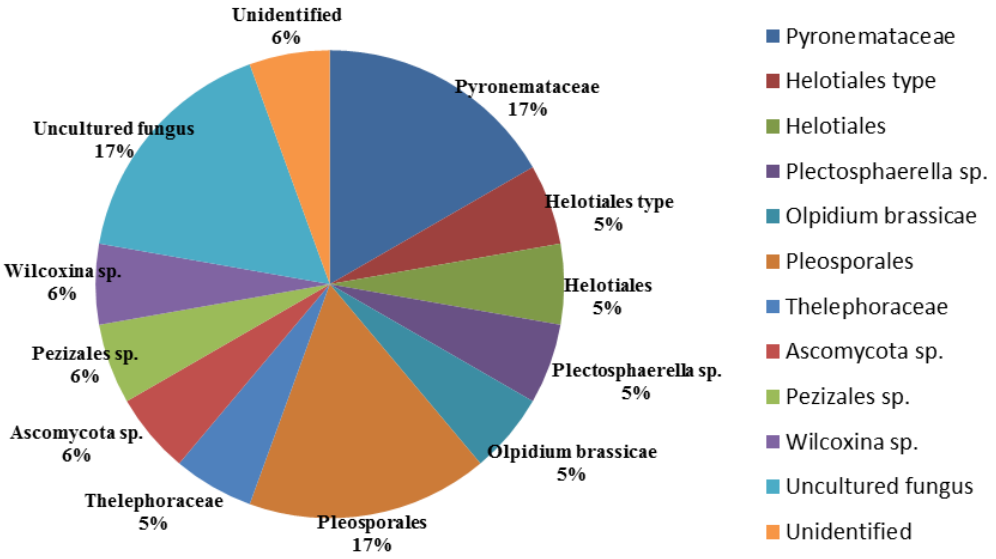

Figure 2.5: Frequency distribution of identified operational taxonomic units (OTUs) in the five studied substrates.

2.4 Discussion

During oil sands reclamation, different soil materials are used to achieve a suitable growth medium for selected plant species. Mycorrhizal propagules are naturally occurring in soil but their absence in plant root systems is a major cause for a lack of vegetation establishment and growth, especially when soil has a low inoculum potential (Sanon et al., 2010). Several authors have reported the low presence of mycorrhizal propagules in TSA (Zak et al., 1982; Zak and Parkinson, 1983; Bois et al., 2005), which consistent with results obtained in this study (Figure 1A and 1B). Overall, the majority of the mycorrhizal colonisation in TSA of this study was with AM fungi in the cortical root cells of red clover. This can be attributed to mycorrhizal spores that could have probably been dispersed through dispersal vectors as TSA cores were collected from alleged new landfill site.

Peat mineral mix consists predominantly of peat and other decaying organic mineral soils obtained from diverse sources. This substrate exhibited higher ECM than AM inoculum potential in accordance with Danielson et al. (1984). This substrate is commonly used for growing coniferous seedlings and has been reported to favour more ECM species than AM fungi (Marschner and Dell, 1994; Bois et al., 2005; Repáč, 2007). This could be due to the capability of ECM and ericoid mycorrhizal fungi to produce ectoenzymes such as acid proteases that help in the breakdown and absorption of complex nitrogen sources (Marshner and Dell, 1994). Nygren and Rosling (2009) also showed the production of this degradative enzyme when they tested 16 ECM species on media containing orthosphosphate, phytic acid or apatite. It was expected that IFS would have a higher number of AM propagules (Fig. 1A).

However, dominating plant species may have played a role in the reduction of AM propagules as substrate was collected from mature mixed conifer stand with the presence of few herbaceous species. The high biomass of red clover in IFS is indicative that increasing plant biodiversity may bring about AMF richness (Friberg, 2001). In the present study, TPS showed considerable amount of both AM and ECM compared to others and should be a more acceptable substrate given that it favours both types of mycorrhizal species. According to Ghose (2004a), TPS is economical and mostly condensed with propagules, plant propagates, organic substance and soil microorganisms. Nevertheless, if the stripping of TPS is not timed, handled and stored properly the soil could become sterile.

ECM morphotyping and molecular typing provide complementary and sometimes contradictory results (Wurzburger et al., 2001). It has become a routine to generate RFLP patterns while studying the nuclear rDNA ITS ribotypes of ectomycorrhiza even though more often than not discrimination between banding patterns has been reported (Burke et al., 2005). In the present study both methods produced 8-9 morphotypes from different taxa (Table 3) similar to previous studies (Wurzburger et al., 2001; Sakakibara et al., 2002; Bois et al., 2005; Burke et al., 2005). Sequences showed 95-100% homology with the exception of OVB3 and OVB5 with a homology of 89 and 87%, respectively (Table 3).

In this study, the most frequently recorded taxa were the *Pleosporales* sp., Helotiales type and a species that corresponds to the Pyronemataceae (Table 3). These groups were found in TPS, OVB, IFS and sparingly in TSA and were reported to be isolated in disturbed to contaminated areas (Bidartondo et al., 2001; Vralstad et al., 2002). Stefani and Bérubé (2006) also reported the presence of *Lophodermium piceae* in Quebec on white spruce, which corresponds to the Helotiales order. This order consists mainly of species that are root endophytes and sometimes form ericoid mycorrhizas with host plants (Kjøller et al., 2010; Walker et al., 2011). In most cases, species in this order are non-host specific and have been reported to form parasitic, saprophytic and mutualistic associations under certain conditions (Jumpponen and Trappe, 1998; Kjøller et al., 2009). One of the common endophytic species in the order Helotiales that is globally distributed is *Phialocephala fortinii*. Although this species was not observed in this study, several authors reported its presence in the roots of *Picea abies* from the temperate zone as well as a potential mycorrhizal relationship they form with their plant host (Haselwandter and Read, 1980; Ahlich and Sieber, 1996; Ahlich et al., 1998; Grünig et al., 2002; Jumpponen et al., 1998). Fernando and Currah (1996) were the first to present anatomical data showing that *P. fortinii* forms ectomycorrhiza with *Salix glauca* in an axenic resynthesis experiment. Several authors have also reported mycorrhizal formation by *P. fortinii* and Scots pine (*Pinus sylvestris*) from molecular field data (Jonsson et al., 1999; Heinonsalo and Sen 2007).

Olpidium brassicae is a saprophyte that was obtained (Table 3) from the roots of *P. glauca*. So far, no reports have indicated the presence of this fungus on selected hosts. However, it has been reported by Tewari and Bains (1983) that *O. brassicae* is commonly found in Canada in roots of *T. pratense* that is commonly used for horse diet. This was confirmed in the present study as stained roots of *T. pratense* had zoosporangia of *O. brassicae*. These zoosporangia could be mistaken for AMF vesicles or spores (Tarbell and Koske, 2007). However, supplementary PCR analysis with primer specific to AMF did confirm AMF presence in roots (data not shown). Therefore, the presence of *O. brassicae* on *P.glauca* is an indication of their lack in host specificity even though this species are suggested to be found mainly in edible crop roots such as cabbage and raddish (koganezawa et al., 2005).

All substrates seemed to have high fungal diversity as rarely were the same fungal taxa obtained from roots taken from the same substrate. However, one major setback in inoculum potential studies is the strong difference between mycorrhizal development in the field and greenhouse. Greenhouse bait experiments over estimate the rate of mycorrhizal colonisation and underestimate number of species of ECM that maybe present in the field (Danielson and Visser, 1989). However, results from the molecular analyses portray qualitatively the kind of mycorrhizal taxa present for ectomycorrhizal fungi. Furthermore, the mycorrhizal percentage colonisation of soil substrates in this study may portray infectivity but its effectivity as an inoculum is still questionable. According to Henkel et al., (1989) mycorrhizal infectivity is a function of effectivity; however, further ways to determine soil mycorrhizal effectivity as an inoculum would be using the most propable number (MPN) technique on a gram of soil (Porter, 1979; Woomer, 1994).

In conclusion, mycorrhizal inoculum potential appears to be affected by the slightest soil disturbance as all substrates generally had low level of mycorrhizal percentage colonisation (< 35%). This was consistent irrespective of the origin of sample substrates. Similar conclusions can be drawn from studies by Danielson and Visser (1989), whereby the percentage colonisation of jack pine roots by ECM fungi in an undisturbed muskeg peat compared to stockpiled muskeg peat was about 75% and 3%, respectively. Since mining is an on-going process, in Alberta, ecological reclamation of disturbed sites through re-installation of mycorrhizal propagules should be promoted for a successful revegetation. Furthermore, salvaged TPS appears to be the best substrate recommended for operational application in reclamation mining areas. This is on the basis that soil removed during the mining process is readily available, has more activity that is microbial and only needs proper preservation to protect the shelf life. A feasible procedure of management that takes into account a foreseeable stock piling of TPS has been outlined by Ghose (2001). Furthermore, the use of TPS will decrease dependency on peat soil and the expanse of peat land reclamation

generated because of peat harvesting. Finally, it is pertinent to understand that no matter the physical benefits of these substrates in reclamation, once transferred, a disturbance to the amount of mycorrhizal propagules has occurred and therefore, may still require mycorrhizal inoculation to boost plant benefits.

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3 Ectomycorrhizal biotechnology: An application to enhanced revegetation and reclamation of oil sands disturbed lands.

3.1 Introduction

Oil sands are unconventional crude oil yet reported in subsoil of Canada, Russia, the United States, Venezuela and some countries in the Middle East. The largest reserves of obtainable oil sands are in Canada and Venezuela. These two countries have oil sands reserves that are approximately equal to the world's total reserves of conventional crude oil. In Canada, the majority of the oil sands are located in three major deposits in Alberta. These are the Athabasca oil sands, and the Cold Lake and Peace River deposits. The Alberta oil sands account for at least 85% of the world's reserves of natural bitumen, with the Athabasca oil sands being the largest bitumen deposit that can be obtained through surface mining (Government of Alberta, 2008; 2009). Other extraction methods that are currently in use are *in situ* methods such as cyclic steam stimulation (CSS) and steam assisted gravity drainage (SAGD). The extraction of oil sands through surface mining is the most commonly used method but depends on the depth of the ore to be mined and, as such, it has an environmental impact on the land. In the Athabasca region in north-eastern Alberta, surface mining is conducted by clear-cutting of thousands of hectares of boreal forests to obtain the ore. After oil sands processing, large amounts of tailing sands are produced as a by-product. To back-fill mining pits, the tailing sands are further used with the addition of available mining substrate (muskeg peat, forest floor (LFH) etc) to create a reconstructed soil that needs to be suitable for growth of native plant species (Fung and Macyk, 2000). In Alberta, this mining process has affected about 602 square kilometres of land (Government of Alberta, 2008; 2009).

In Alberta, reclamation of lands disturbed by oil sands mining is faced with many challenges. These include harsh growing conditions due to oil sand processed materials (OSPM), slow seedling growth leading to long-term reclamation periods, low soil organic matter content, competition with non-native species, high salt content, poor soil structure, poor seedling survival and decreased viability of propagules (Danielson et al., 1983; Bois et al., 2006). As such, oil companies are required to return reconstructed/reclaimed land to a boreal forest, grassland or wetland as the case may be, as well as to satisfy legislative conditions of licensing to commensurate the enormous lands disturbed. Hence, there is a need to enhance seedling survival and growth that will lead to successful reforestation.

Mycorrhizal fungi have been widely reported to be beneficial to many plants in natural and disturbed ecosystems due to their symbiotic benefits (Brundrett, 1991; Sturges, 1997, Auge, 2001; Jentschke and Goldbold, 2000). Studies have also reported the ability of mycorrhizal species to improve salt stress, water conductance and reduce accumulation of Na in shoots of plant species in axenic cultures (Kernaghan et al., 2002; Chen et al., 2001; Mushin and Zwiazek, 2002; Bois et al., 2006; Dixon et al., 1993). This has led to their exploitation with various host plants in enhancing land reclamation and revegetation efforts on various sites (Quoreshi et al., 2008). In reclamation, seedling establishment is a critical phase and depends on the capacity of seedlings to capture resources quickly (Duñabeitia et al., 2004) in order to withstand or survive climatic and nutritional stress factors (Perry et al., 1987). According to Amaranthus and Perry (1987), seedlings on disturbed sites with certain growth limiting factors may have only a brief favourable period of growth, which is essential for seedling survival. However, one factor that could determine if this "window" is sufficient is the presence and competition offered by a colonised mycorrhizal root system (Danielson and Visser, 1989). Subsequently, mycorrhizal fungi are important mycobionts in terrestrial ecosystems; therefore the amount of propagules found after a soil is "disturbed" should be of paramount importance (Smith and Read, 2008). This will support rapid colonisation prior to root growth activity since roots may have a limited period of susceptibility to nutrient (Brundrett and Kendrick, 1990; Brundrett, 1991).

Presently, the inoculation of seedlings with selected mycorrhizas prior to out planting in the field to enhance reclamation or revegetation is gaining popularity because of the potential of establishing selected fungi in roots (Smith and Read, 2008). Reclamation is defined as the process of transforming any disturbed land to its previous land capacity state or better, while revegetation is the process of rebuilding the soil of an eroded land by establishing a vegetation cover. The most widely used mycorrhizal fungi for inoculation studies are the ectomycorrhizas because of their ability to be manipulated and grown in the laboratory, thereby making it easier to study their physiology, biochemical and genetic behaviour (Marmeisse et al., 1999). Coniferous trees commonly used during reforestation are known to form ectomycorrhizal associations (ECM) with a variety of ascomycetes and basidiomycetes (Molina et al., 1992). These seedlings grown in nurseries are often containerised seedlings that are only colonised to a minimal extent by mycorrhizal fungi (Menkis et al., 2007, Duñabeitia et al., 2004). It has been reported that inoculating nursery trees with ECM fungi may facilitate initial nursery growth as common artificial mixes such as sphagnum and vermiculite that have a long history in horticulture, lack mycorrhizal propagules and so must be added to produce mycorrhizal seedlings (Trappe, 1977). The

advantages of ECM inoculation are an increased survival and rooting of seedlings when out planted in reclamation sites.

Field performance of out-planted seedlings may be affected by the presence of mycorrhizal fungi. However, major determination factors such as the inoculum potential of the site, early establishment by resident fungi, or lack of mycorrhizal propagules in the soil can bring about this influence (Trappe, 1977). Inoculation of seedlings with mycorrhizal fungi prior to out-planting is one approach to improve revegetation successes and several authors have reported positive effects on growth and survival of nursery mycorrhizal inoculated seedlings prior to out-planting on disturbed sites (Ortega et al., 2004; Parlade et al., 2004; Ringe and Graves, 1990). In contrast, other authors have recorded no effect of inoculation on seedling growth (Maestre et al., 2002; Teste et al., 2004; Castellano and Trappe, 1991). However, incongruence in reported results seem to be due to certain factors which are not limited to colonisation rate at time of planting, persistence of introduced fungus, host-seedling combinations, efficiency of native species present and site adaptability of inoculated fungus (Quoreshi et al., 2008). Ringe and Graves (1990) using loblolly pine observed no effect of ECM inoculation after the first growth season but by the 9th growth season, a difference in height compared to non-inoculated seedlings was observed. Menkis et al., (2007), who studied the afforestation of farmland, inoculated conifer seedlings with three ECM fungi and reported that the inoculated seedlings had a significantly higher survival and growth compared to non-inoculated controls. The authors concluded that the inoculation of nursery seedlings, though minimal, might be responsible for the survival of seedlings in harsh conditions during the first few weeks of out-planting. Hence, mycorrhizal application to enhance field performance of seedlings is worth considering.

In the Canadian oil sands areas, the application of mycorrhizal technology has a great potential in the restoration of disturbed lands (Quoreshi et al. 2006; Quoreshi, 2008), leading to the alleviation of reclamation challenges. High salt content is characteristic of oil sand processed materials and plants find it difficult to withstand physiological drought due to high ionic content (Franklin et al., 2002). Therefore, inoculation of seedlings with a salt-tolerant ECM could increase growth and reduce the effect of salt stress (Bois et al., 2006). Furthermore, studies have shown that the basidiomycete *Hebeloma crustiliniforme* (Bull. Ex St. Amans.) Quel., has the potential to reduce Na uptake by maintaining high root transpiration rates and root hydraulic conductivity (Mushin and Zwiazek, 2002; Kernaghan et al., 2002; Menkis et al., 2007). Other species of basidiomycetes including *Suillus tomentosus* (Kauff.) Sing., Snell and Dick) and *Laccaria bicolor* (Maire) P.D. Orton, are known to improve host biomass, are drought tolerant, respond positively to osmotic stress and have

characteristic nutrient accumulation properties (Bois et al., 2006a; Selosse et al., 2000). These are beneficial plant survival properties that non-inoculated seedlings would not benefit from when grown in OSPM.

In spite of previous out-planting studies with ECM fungi, the use of ECM fungal inoculants in oil sand disturbed lands to enhance reclamation and revegetation is still at an infancy stage. This study focuses on three mycorrhizal fungal species that have been observed *in situ* to have valuable characteristics to plants (Bois et al., 2006a, 2006b; Mushin and Zwiazek, 2002; Calvo-polanco et al., 2008, 2009). In addition, mycorrhizal benefits of three ECM fungal species, namely *H. crustuliniforme*, *S. tomentosus* and *L. biolor*, singly and in combination, relating to improving survival and growth in OSPM have rarely been conducted in field trials.

The aim of this study was to assess the effect of nursery-inoculated seedlings with mycorrhizal fungi on a reclamation site. Specific objectives were: to (*i*) evaluate the benefit of nursery inoculation on growth and survival of outplanted seedlings; (*ii*) evaluate the effectiveness of three selected ECM species alone and in combination on the establishment of conifers planted on reclamation sites; and (iii) evaluate the persistence of introduced ECM strains on seedlings after two seasons of growth.

3.2 Materials and Methods

3.2.1 Study site and seedling establishment

The study was conducted at Suncor Energy Inc. mine area 25 km North FortMcmurray in Alberta, Canada. The study area, which was created in June 2009, is called Mine Dump 5 (MD5) by the company as a landform description and is located at 56^{o}58′ 55″ N, 111^{o} 19′ 97″W of the mine area. The study site MD5 was approximately 0.3 ha and was reconstructed by directly placing upland soil on the benches (clean overburdened) and peat mineral mix in a 60:40 depth respectively. The mean annual temperature of the area is about 1.22^{o}C with an annual mean historical precipitation of 455.7 mm and rainfall of 3.24 mm annually. This area was prepared for reclamation and as such was plain with some unlevelled contours and soil amendments that had vegetative roots. The physical and chemical characteristic of the site is summarized in Table 3.1.

Inoculum production and seedling inoculation

The liquid inoculum used in this study was prepared by Symbiotech Inc. The liquid inocula were grown from the three selected fungi on Glucose yeast malt extract agar (see Appendix III) using a shake flask that was continuously agitated. Before using the liquid inoculum the mycelial suspension were homogenized with a Waring blender. The suspensions were diluted with water before inoculation to obtain desired concentration of propagules/ml in the mycelial slurry.To inoculate seedlings, a 1.5 L stock mycelial slurry solution was diluted with water to produce 3.9 L of liquid inoculum with a minimum final concentration of 5×10^3 viable propagules/ml or 0.46 mg dry mycelia/ml. The diluted mycelial slurry was mixed into the growing medium (peat: vermiculite) prior to sowing seeds and then the mixture was used to fill the styroblocks cavity. Each container cavity (98 ml) received 10 ml of diluted liquid mycelial slurry. The seeds were sown on each cavity containing the media. The species used were Jack pine (*Pinus banksiana* Lamb.) seed lot no: syn-95-10-4-90 and white spruce (*Picea glauca* (Moench) Voss) seed lots no: syn-16-92-11-4-05. These plants were inoculated with three different ECM species and a consortium of the three-ECM fungi and grown under commercial nursery environment at the Bonnyville Forest Alberta (540 16' N 1100 44' W). The seedlings were grown in commercial nursery, for no less than 6 months and hardened prior to out planting in spring 2009.

Table 3.1: Soil physical and chemical properties of MD5 reclamation site.

Soil properties	**Concentration**
Mg	583.35
Na	36.20
K	102.10
P	0.81
Ca (%)	0.65
Total N (%)	6.65
Organic S (%)	0.06
Organic C (%)	5.60
pH (H_2O)	7.99
Electrical conductivity ($\mu s/cm^{-1}$)	499.5
Microbial biomass C	83.86
%Silt	35.00
%Clay	14.00
%Sand	51.00

Mg, Na, K and P concentrations are in mg kg^{-1}.

3.2.2 Study design and treatments

The experimental design consisted of a randomised complete block design of dimension 56x30 m for each plant species with four replicates blocks (Figure 3.1). This study was carried out to test three different ectomycorrhizal species and a combination of the three-ectomycorrhizal fungi. The tested ECM fungi were *Hebeloma crustuliniforme* (HC), *Laccaria bicolor* (LB) and *Suillus tomentosus* (ST). Eight inoculation treatments were established for both jack pine and white spruce plots as the following: HC, LB, ST, HCLB, HCST, STLB, HCLBST, and non-inoculated control (CTRL). Each treatment block was planted with 30 seedlings each with a distance of 1 m and block distance of 2 m. A total of 960 seedlings per plant species were planted on each experimental plot to give a grand total of 1920 seedlings planted on a 6720 m^2 study area.

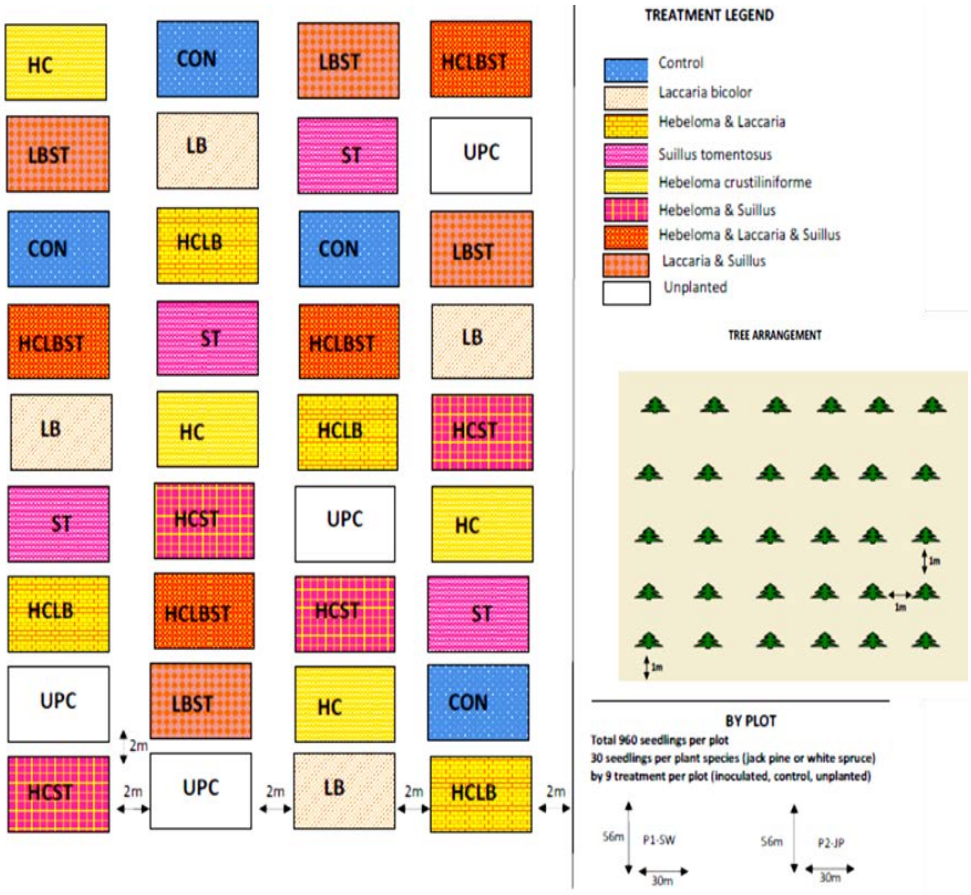

Figure 3.1: Experimental field block layout of treatments at MD 5 Suncor reclamation site. Abbreviations are as follows: CON (Control-uninoculated); LB (*Laccaria bicolor*); HC (*H*ebeloma *crustuliniforme*); ST (Suillus tomentosus); HCLB (*Hebeloma and Laccaria*); HCST (*Hebeloma* and *Suillus*); HCLBST (*Hebeloma, Laccaria* and *Suillus*); LBST (*Laccaria* and *Suillus*); UPC (unplanted).

3.2.3 Data collection and statistical analyses

Measurements were taken in September 2009 and 2010 with one destructive sampling in 2010. Five randomly selected seedlings from each block were assessed for growth measurements (n= 20) that were further averaged and pooled. Seedlings were measured for shoot height and stem diameter at the root collar as well as biomass (oven-dried at 72^{o}C for 24 h). Obtained data were used to calculate the stem volume index [(root collar diameter)^2 x total shoot height] and plot volume index (PVI). The PVI was calculated by multiplying stem volume index and the number of surviving plants per treatment (Marx et al., 1991). To detect early differences in height, relative growth was used to determine annual gains of stem length measured as height (Pera et al., 1999). This was calculated using the formula $\mathbf{(h_f\text{-}h_i)/h_i}$, where h_f is the height at the end of one growing season (e.g. 2010) and h_i is the height at the beginning of the period considered (e.g. before out-planting). The seedling survival was used as a response variable to evaluate the response to mycorrhizal inoculation (Maestre et al., 2002) and was expressed as a percentage. To determine the degree of mycorrhizal inoculum effect on biomass, field mycorrhizal inoculum dependency (FMID) was calculated as the difference between the biomass of inoculated and non-inoculated (control) plants and expressed as a percentage of the biomass of inoculated plants (Zangaro et al., 2000; Plenchette et al., 1983). All growth data were analysed by a one way ANOVA PROC MIXED model to determine the significance of inoculation. Following a significant treatment effect, protected LSD multiple comparisons were done in order to specify which treatments differed from the others. Some contrasts were also done to specify the block effect and to compare the single treatments (HC, LB, ST) with the double treatments (HCLB, HCST, LBST) or with the triple treatments (HCLBST). Furthermore, for the survival data, a binomial randomized complete block ANOVA was adjusted to the data. This parametric model had a better fit to the data than the usual Gaussian model. All data were subjected to analysis of variance (ANOVA) where necessary using SAS 9.2 software.

3.2.4 Morphological and molecular typing

Morphology description

The sampled roots were washed under running water on a 2 mm mesh sieve and the fine lateral roots incised into 5-7-cm long segments. The segments were spread in water in a glass dish and examined under the stereomicroscope. ECMs were grouped using branching patterns, colour, tip shape, texture, emanating hyphae and outer mantle (Agerer, 1999;

Goodman et al., 1996-2000). Subsequently, root tips and segments in the given treatments were obtained for further analyses and stored in cetyl trimethylammonium bromide (CTAB) buffer (Henrion et al., 1994) at 20^{o}C, prior to DNA extraction. In addition, representative root samples were stained with trypan blue to confirm the presence of the Hartig net using a modified version of Brundrett et al. (1996). A modification was in the time used to clear, stain and destain roots which was usually 5 hrs for clearing roots and over night to stain and destain roots. The percentage of root tips colonised by ECM was calculated as the number of ECM root tips divided by the total number of root tips and multiplying by 100 (Gehring and Whitham, 1994).

DNA extraction and amplification

Two to five representative root tips of each morphotype were selected for DNA extraction for each sampled plot. Total genomic DNA was extracted using Sigma Extract-N-Amp Tissue Kit following the manufacturer's protocol (Sigma, USA). To assess the ECM fungal diversity and fungal persistence of three inoculated fungi, a total of 150 DNA extracts from root tips were sampled in 2010. The internal transcribed spacer (ITS) of the nuclear ribosomal DNA (rDNA) was amplified using ITS1F and ITS4 (Table 3.2) primers (Gardes and Bruns 1993; White et al., 1990). During polymerase chain reaction (PCR), amplification was conducted using Extract-N-Amp PCR (Sigma) solution in a 10 µL reaction with the PCR primers at a final concentration of 0.1 µM. Amplifications were performed in a vapo-protect thermocycler (Eppendorf) with an initial denaturation cycle at 95°C for 2 min, followed by 15 cycles of 95°C for 2 min, 55 °C for 2 min and 72°C for 2 min; then 25 cycles of 94°C for 35 s, 53 °C for 55 s and 72°C for 3 min, with a final extension of 72°C for 5 min. All PCR products were visualized on a 1% agarose gel. Successful PCR products were purified using ExoSAP-IT (USB, Cleveland, OH, USA) and, when necessary, by gel incision following the manufacturer's protocol (Qiagen). Cycle sequencing was carried out in one direction using the Big Dye Terminator Sequencing Mix (v. 3.1, Applied Biosystems, Inc.), with the same PCR forward primer at a final concentration of 0.1 µM. The resulting products were precipitated using EDTA/ethanol following the manufacturer's instructions.

Persistence of inoculated species and primer design

As three introduced species were basidiomycetous, primers were designed online specifically for each fungus to reduce further ambiguity after amplifications and to avoid unnecessary sequencing. Therefore, DNA extraction–PCR was conducted from fungal plate cultures following the same procedure as above but with the use of NSI1 and NLB4 primer

pair. The PCR products were purified and sequenced in both forward and reverse directions to confirm identity and similarity to the NCBI database. Having obtained 99% identity to isolates with accession number EU379675, FJ845417, FJ845541, the sequences were then used to design primers for each organism. The sequences were aligned with Bioedit software (Hall, 1999) while primers were designed using the NCBI primer-blast tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers were synthesised by Integrated DNA Technologies (IDT) considering melting temperature, non-complementarity of the 3^1ends, the G+C (between 40 - 60%) contents, non-occurrence of secondary structures, absence of cross hybridization and the length (18 -28 nucleotides) of the oligonucleotides (van Tuinen *et al*, 1998).

Table 3.2: List of PCR primers used in amplification.

Primers	Sequence (5'--------3')	Melting temperature (^{o}C)	Expected product size (~bp)
ITS1F	CTTGGTCATTTAGAGGAAGTAA	55	550-750
ITS4	TCCTCCGCTTATTGATATGC	53	
NSI1	GATTGAATGGCTTAGTGAGG	50.8	550-800
NLB4	GGATTCTCACCCTCTATGAC	51.7	
HCF*	TGGTTGTTGCTGGCTCTTTCGAGG	61.7	400-480
HCR*	TGCAGATGTCCACGGCGTAGAT	60.7	
LBF*	CGGATTTGAGGATCGCCGTGCTGT	63.5	500-520
LBR*	TTGTACACGGTCCAGCGCGGAT	63.3	
STF*	CGTGCACGCCCTCTTTCTCGA	62.1	350-430
STR*	AACAGGTCTCCGGCAGCCTC	62.1	

*Designed primers

Primer specificity and efficiency

Designed primers were tested on pure cultures of *H. crusuliniforme*, *L.bicolor* and *S. tomentosus* to confirm their efficiency. The PCR products were separated electrophoretically and visualised on Sybersafe stained 1% agarose gel to determine the sizes of the product. The primers were tested for specificity on a variety of fungal pure culture obtained from University of Alberta Microfungus Collection and Herbarium (UAMH) that included *Amphinema byssoides*, *Cenoccum geophilum*, *Hebeloma longicaudum*, *Lactarius affinis*, *Paxillus involuntus*, *Tricholoma flavovirens, and Wilcoxinia mikloae*. A touch-down PCR in a final volume of 10µl with appropriate primers (0.1 µM) was optimised to enhance their specificity (Hecker and Roux, 1996). The PCR protocol consisted of 95°C for 2 min, 10 cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 1 min; 10 cycles of 94°C for 1min, 63°C

for 1 min, 72°C for 1 min; 15 cycles of 94°C for 35 sec, 60°C for 1 min, 72°C for 2 min and a final elongation of 72°C. PCR products were separated electrophoretically on 1% agarose gel and stained using a Sybersafe DNA stain (1µl/10ml gel). Hence, for the ECM persistence study, PCR products obtained using IT1F and ITS4 primers from the ECM diversity reaction were used as templates (i.e. Nested PCR).

3.3 Results

Height, stem volume and plot volume index

No significant difference was observed in height between treatments at the time of planting for both plant species (data not shown). Nevertheless, the second year showed significant ($P < 0.05$) growth differences (Figure 3.2). In Jack pine, the overall effect of treatment (inoculation) on plant height was significant ($P = 0.023$). Differences were observed between inoculation treatments and the control of which treatments HCLB, HC, HCST, LBST, HCLBST and LB had not less than 7% increase in height. Treatments with HCLB and LB had the highest and lowest percentage increment in height (approximately 18% and 8%, respectively) (Figure 3.3). In white spruce, significant differences in height were observed between treatments HC, HCLB and HCLBST when compared to the controls, but there was no overall effect ($P= 0.37$) of inoculation treatment on plant height (Table 3.3).

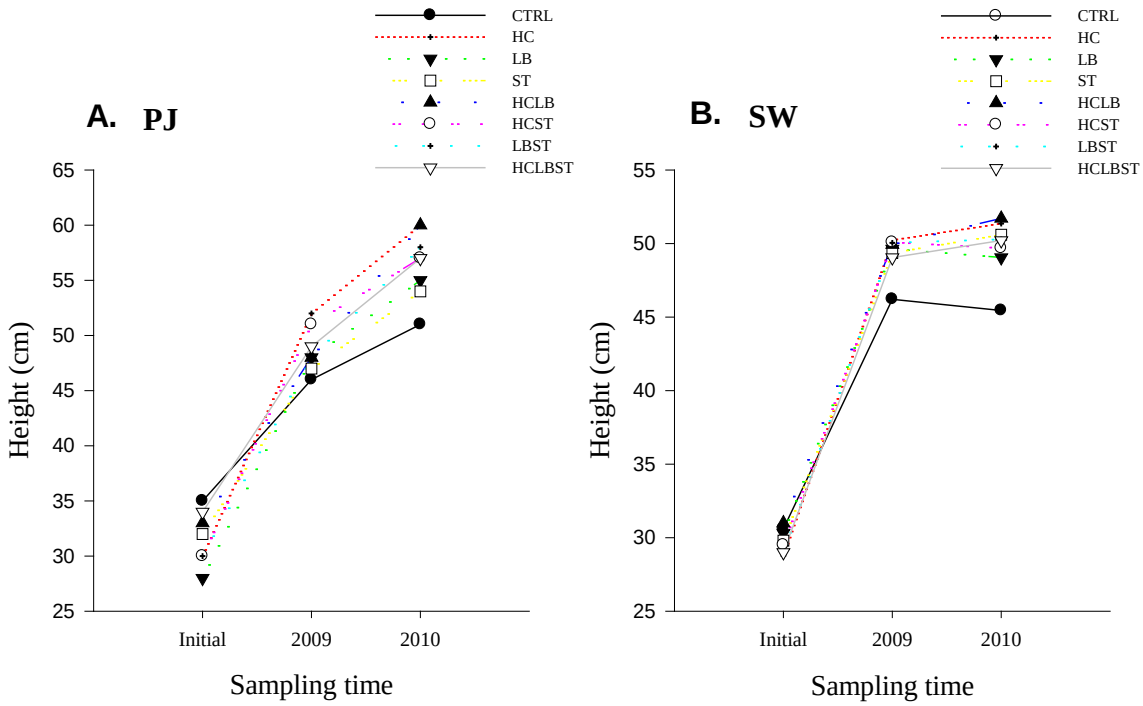

Figure 3.2: Height progression of inoculated and non-inoculated jack pine (PJ) and white spruce (SW) seedlings out planted at MD5. Treatments are CTRL (Control-uninoculated); LB (*Laccaria bicolor*); HC (*H*ebeloma *crustuliniforme*); ST (Suillus tomentosus); HCLB (*Hebeloma and Laccaria*); HCST (*Hebeloma* and *Suillus*); LBST (*Laccaria* and *Suillus*); HCLBST (*Hebeloma, Laccaria* and *Suillus*).

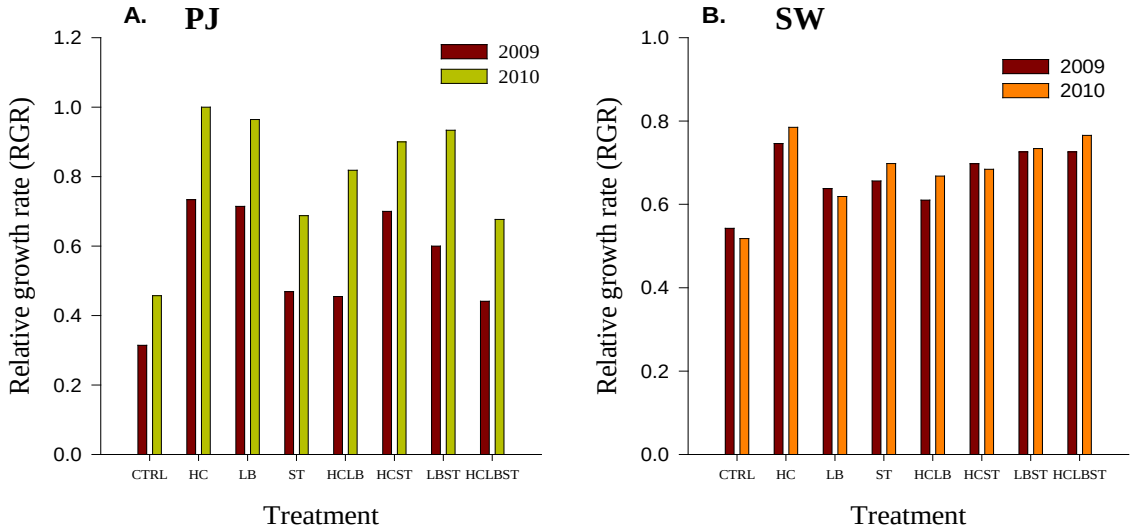

Figure 3.3: Relative annual growth of inoculated and non-inoculated jack pine (PJ) and white spruce (SW) seedlings after two growth seasons. Treatments are CTRL (Control-uninoculated); LB (*Laccaria bicolor*); HC (*H*ebeloma *crustuliniforme*); ST (Suillus tomentosus); HCLB (*Hebeloma and Laccaria*); HCST (*Hebeloma* and *Suillus*); LBST (*Laccaria* and *Suillus*); HCLBST (*Hebeloma, Laccaria* and *Suillus*).

In jack pine, the PVI increased with all inoculated seedlings mainly with treatments HC, LB, ST and HCST, but the differences were not statistically significant (P= 0.32). On the other hand, the stem volume of seedlings inoculated with HC, LB and HCST increased by about 63, 40 and 41% respectively, compared with the controls (Figure 3.4A). In white spruce, seedlings inoculated with HCLB showed a greater significant increase in PVI of up to 124% when compared to the control. However, there was a minimal increase in stem volume of treatments that were not significantly different from the control (Figure 3.4B). In the treatment groups, the PVI of single treatments increased by about 57% compared to the control for jack pine and the volume by 45%, while in white spruce, the double inoculation treatment resulted in about 83% increase in PVI and 10% increase in volume (Figure 3.6B and C).

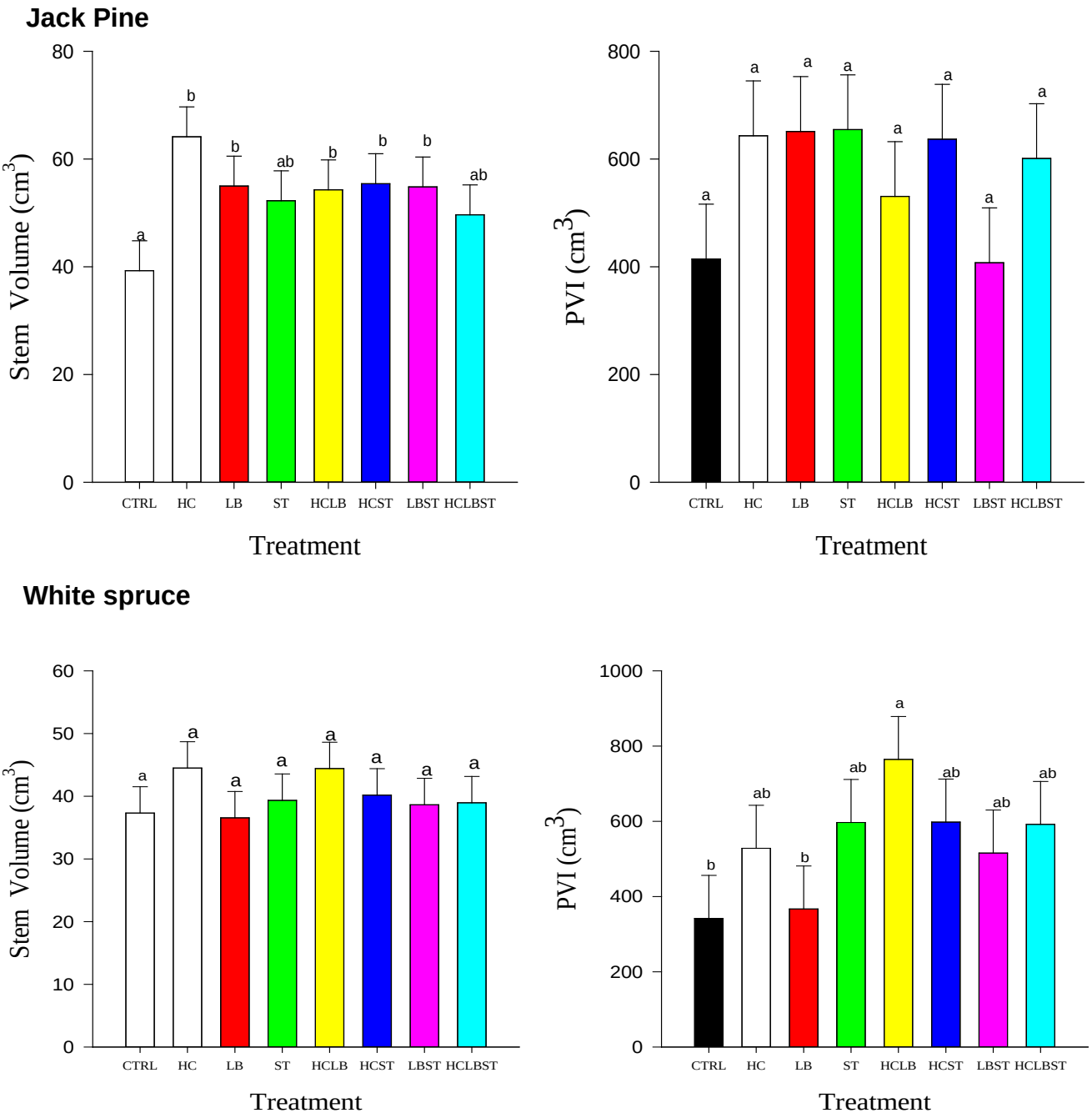

Figure 3.4: Effect of nursery mycorrhizal inoculation on stem volume and plot volume index (PVI) of out-planted jack pine and white spruce seedlings at second growth season (2010) in MD 5, Suncor AB. Vertical bars represent ± standard error of the mean. Same letters are not significantly different from each other at $p < 0.05$ (Fisher LSD). Values are means of 20 replicates. Treatments are CTRL (Control-uninoculated); LB (*Laccaria bicolor*); HC (*H*ebeloma *crustuliniforme*); ST (Suillus tomentosus); HCLB (*Hebeloma and Laccaria*); HCST (*Hebeloma* and *Suillus*); LBST (*Laccaria* and *Suillus*); HCLBST (*Hebeloma, Laccaria* and *Suillus*).

Survival rate

In general, the survival rate of seedlings varied between plant species. In jack pine, there was no overall significant effect of inoculation on seedling survival. However, significant survival differences (P= 0.024, 0.041) between LBST, ST and LB treatments were observed but these were not different from the control (Figure 3.5). The survival of seedlings in white spruce was influenced ($P < 0.05$) by inoculation with ECM fungi. The average survival rate for white spruce seedlings inoculated with different strains of ECM varied between 36% and 56%, whereas the control (uninoculated) had minimum and maximum survival rates of 22 and 41% respectively. In inoculation treatment groups, inoculated jack pine seedlings had similar survival rates as the control. In white spruce, double and triple-inoculated seedlings, respectively, were significantly higher (P= 0.003, 0.012) than the control. Single inoculated seedlings had a lower % survival and so differed (P= 0.039) from double-inoculated treatments (Figure 3.6D). Jack pine had the highest FMID of 25.6% and the lowest FMID of 13.6% was observed in treatments HCLB and HCLBST, respectively. In white spruce, the lowest recorded FMID was negative 7.48% and the highest 19.9% in treatments LB and HCLBST, respectively (Table 3.3).

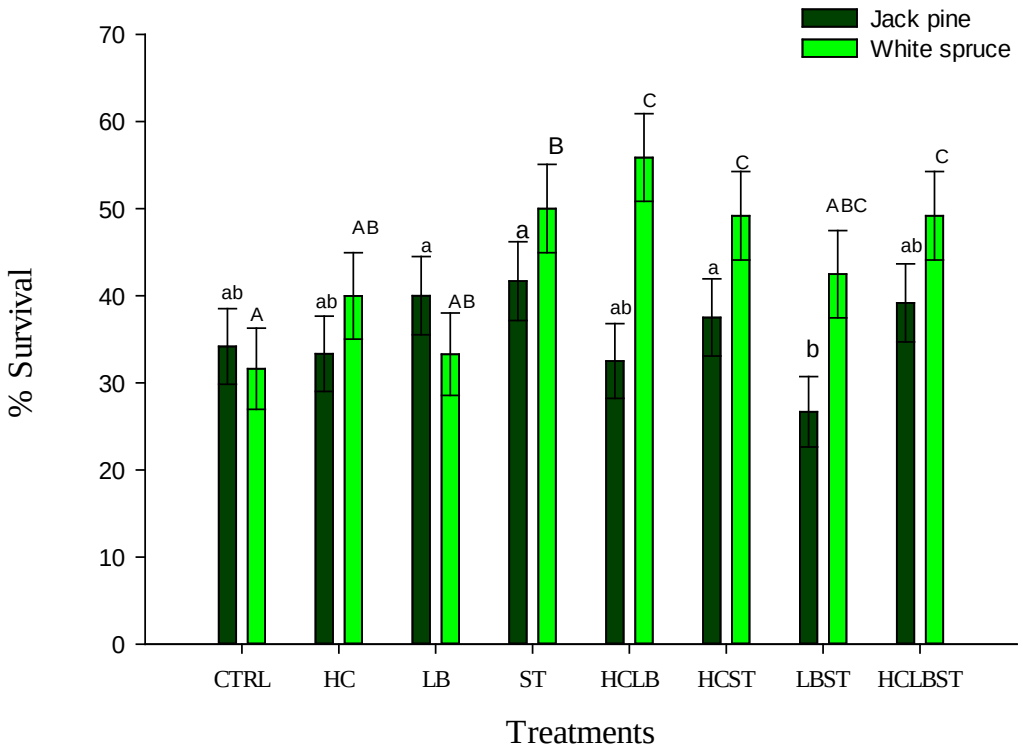

Figure 3.5: Survival rate of out-planted jack pine (PJ) and white spruce (SW) seedlings in MD 5 reclamation site at Suncor. Vertical bars represent ± standard error of the mean. Same letters are not significantly different from each other at $p < 0.05$ (Fisher LSD) between plant species. Values are means of 20 replicates. Treatments are CTRL (Control-uninoculated); LB (*Laccaria bicolor*); HC (*H*ebeloma *crustuliniforme*); ST (Suillus tomentosus); HCLB (*Hebeloma and Laccaria*); HCST (*Hebeloma* and *Suillus*); LBST (*Laccaria* and *Suillus*); HCLBST (*Hebeloma, Laccaria* and *Suillus*).

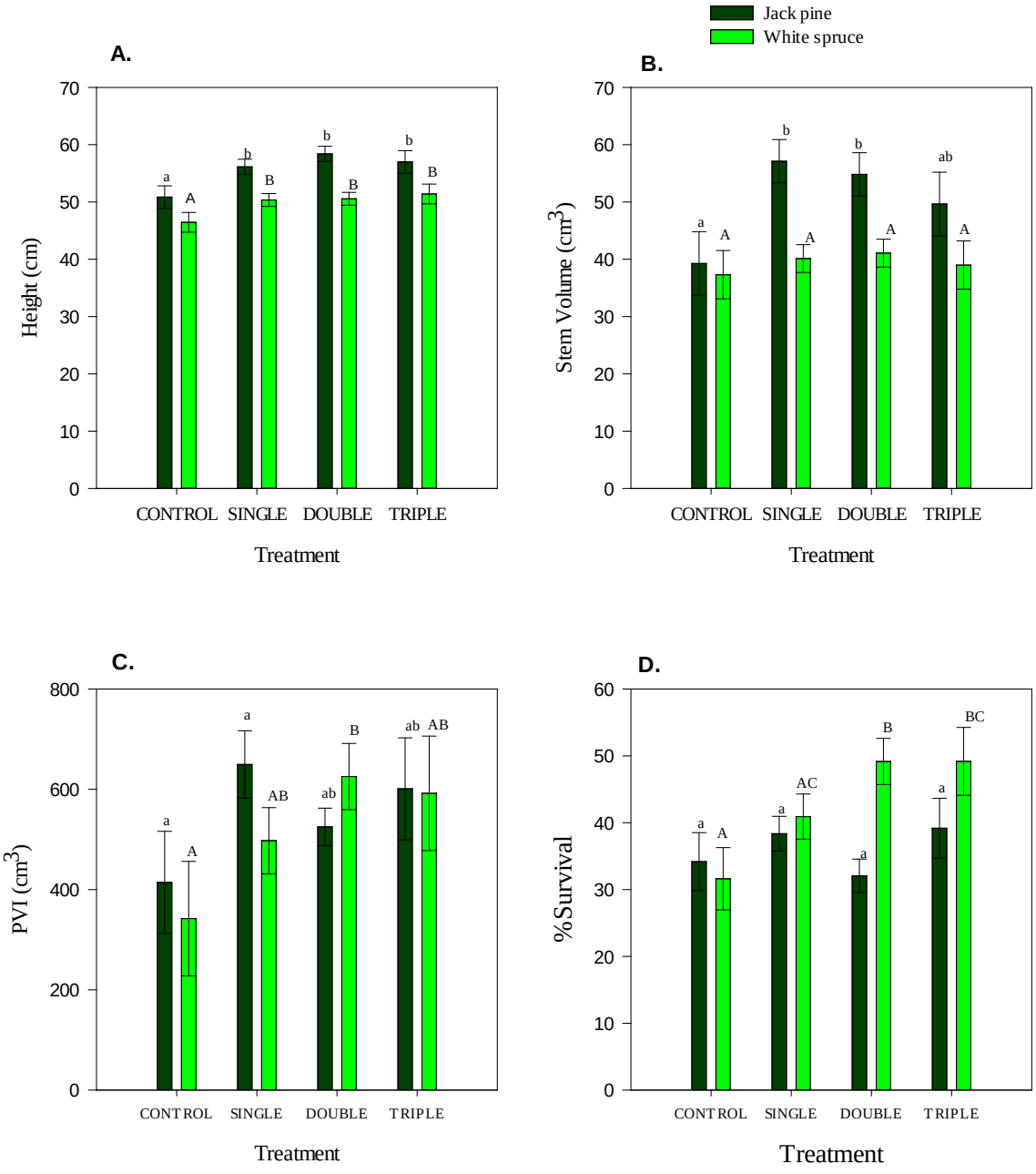

Figure 3.6: Combined effect of nursery mycorrhizal inoculation on seedling traits of out planted jack pine (PJ) and white spruce (SW) seedlings at second growth season (2010) in MD 5, Suncor AB. Vertical bars represent ± standard error of the mean. Same letters are not significantly different from each other at $p < 0.05$ (contrasts) between plant species. Single T consists of *Hebeloma crustuliniforme* (HC), *Laccaria bicolor* (LB) and Suillus *tomentosus* (ST). Double T consists of *Hebeloma* and Laccaria (HCLB*)*, *Hebeloma* and *Suillus* (HCST), *Laccaria* and *Suillus* (LBST). Triple T is *Hebeloma*, *Laccaria* and *Suillus* (HCLBST) and uninoculated (CTRL).

Morphology and molecular typing

Mycorrhizal fungi were observed on the root tips of both plant species. A gross morphology of 17 morphotypes (See appendix A) was distinguished. The identification is tentative since phenotypic variations of fungi were observed, possibly due to differences at maturity or on young roots. In stained roots of both plant species, the presence of mantle and Hartig net were observed. Roots were also observed to have septate runner hyphae, zoospores from endophytes, and occasional vesicle-like structures. A total of 70 successful amplifications out of 150 tested roots were obtained using general fungal primers (ITS1F and ITS4). Multiple PCR fragments which barred further sequencing accounted for 15% of amplified samples. However, with specifically designed primers this problem was surmounted. It should also be noted that only samples with high DNA concentration were further sequenced to ascertain species identity (27 samples). ITS sequencing produced a total of 8 taxa: one to the family level, 2 to genus level and 5 were identified to the species level (Table 3.4). Five samples could not match any known sequence in GenBank.

Table 3.3: Seedling field performance of out planted seedling (Second growth season).

Treatment	Height	Biomass	Root:Shoot	FMID	Colonisation (%)
			Jack pine		
CTRL	50.85a	102.25a	0.310a	0.00	21.5a
HC	59.45b	136.65b	0.329a	25.17	64.1b
LB	55.05ab	135.83b	0.318a	24.72	76.3c
ST	53.88ac	134.70b	0.336a	24.09	61.8b
HCLB	59.85b	137.35b	0.337a	25.55	87.7d
HCST	57.00b	136.05b	0.295a	24.84	67.5b
LBST	58.35b	132.07b	0.310a	22.57	68.7b
HCLBST	56.98b	118.32ab	0.303a	13.58	75.8b
			White spruce		
CTRL	46.45a	107.30ab	0.286a	0.00	20.1a
HC	51.35bc	117.87ab	0.298a	11.53	68.6b
LB	49.05ac	101.20a	0.239a	-7.48	67.7b
ST	50.60ac	109.47ab	0.256a	2.47	52.2c
HCLB	51.70bc	122.54ab	0.303a	16.14	76.6d
HCST	49.67ac	117.10ab	0.287a	10.77	64.5b
LBST	50.28ac	110.15ab	0.275a	3.30	59.5e
HCLBST	51.4bc	127.77b	0.2412a	19.88	63.7be

**Field mycorrhizal inoculation dependency (FMID) = biomasss of inoculated – biomass of uninoculated /biomass of inoculated X100. Treatments are CTRL (Control-uninoculated); LB (*Laccaria bicolor*); HC (*H*ebeloma *crustuliniforme*); ST (Suillus tomentosus); HCLB (*Hebeloma and Laccaria*); HCST (*Hebeloma* and *Suillus*); LBST (*Laccaria* and *Suillus*); HCLBST (*Hebeloma*, *Laccaria* and *Suillus*).

Table 3.4 Fungal taxa identified by PCR sequencing.

Fungal taxa	Numbers of sequences that matched	Maximum %homology	Corresponding E value
Thelephora terrestris	7	99	0.0
Amphinema byssoides	9	99	0.0
Sebacina sp.	1	90	0.0
Suillus tomentosus	2	84	3e-151
Wilcoxina sp.	1	99	0.0
Laccaria bicolor	2	99	0.0
Uncultured ectomycorrhizal fungi	1	99	0.0
Unidentified	3	-	-
Pyronemataceae	1	92	0.0
Olpidium brassicae	1	86	0.0

3.4 Discussion

Out planting performance

In revegetation establishment, the success of nursery-inoculated seedlings with mycorrhizal fungi can be assessed by evaluating seedling performance when out- planted. In this study, seedling performance was determined using height, biomass, and stem volume/PVI, and survival parameters (Marx, 1980). The result of this study indicates that inoculation of nursery seedlings with mycorrhizal fungi stimulated tree height. However, this increase in height varied between the two plant species tested as indicated by the different growth values obtained between species after two growing seasons. Furthermore, the ability of plants to exhibit different growth rates could be attributed to individual plant responses to changes in root physiology. These changes were reported to be influenced by ECM inoculation, site characteristics and, perhaps, transplanting shock (Mridha, 2003; Quoreshi et al., 2008). Browning and Whitney (1992) reported a fast growth response in jack pine and delayed response in black spruce (*Picea mariana* (Mill.) B.S.P) until the end of the second growing season. They attributed this to the ability of jack pine seedlings to increase mass when changes in root physiology occur, which is congruent with biomass data from the present study that showed jack pine having a 15% maximum increase compared to white spruce. Generally, the height of jack pine and white spruce differed in their responses to inoculation with single ECM species or in combination. Conversely, jack pine showed more differences between fungal species and control compared to white spruce that only differed

between the inoculated and uninoculated treatments. Indeed, this finding is in accordance with other studies and actually supports the suggestion of Quoreshi et al. (2008) who attributed this difference to specific plant-fungus combinations and (or) site characteristics.

Mycorrhizal colonisation under field conditions is known to be governed by ecological factors such as soil characteristics, tree species and fungi present (Hedlund and Gormsen, 2002). In this study, the colonisation of control seedlings that were not inoculated with indigenous mycorrhizal fungi eliminates proper field comparisons as found by Grossnickle and Reid (1982). Contradictory findings (Ortega et al., 2004, Parlade et al., 2004; Teste et al., 2004, Maestre et al., 2002) were reported on the effect of ECM inoculation and its ability to improve plant growth and (or) survival. However, the use of an index such as relative field mycorrhizal dependency proposed by Plenchette et al. (1983) could help to explain these contrasting findings. This index was numerically defined as "the mycorrhizal dependency (MD) by expressing the biomass of a mycorrhizal plant as a percentage of the biomass of a non-mycorrhizal plant at a given level of soil fertility" (Plenchette et al., 1983, Menge et al., 1978). However, the term "non-mycorrhizal" is practicably feasible in a controlled environment. Studies that made use of this index were conducted in a greenhouse or in fumigated soils under certain phosphorus (P) levels and mycorrhizal inoculation (Bá et al., 1999; Adjoud et al., 1996; Plenchette et al., 1983). Nevertheless, the application of this index in a field study is considerable (Plenchette et al., 1983) because the P concentrations of soils are not parameters used for computing index value and mycorrhizal dependency is also affected by species of mycorrhizal fungi (Graham and Syvertsen, 1985). However, as long as soil P of the site is initially determined, this index may still prove useful. To focus on the necessity of assessing the effect of ECM inoculation during out-planting, this study suggests that for field purposes the term "field mycorrhizal inoculation dependency" (FMID). This would be defined as the degree to which a plant is dependent on inoculation of seedlings in the nursery with mycorrhizal fungi to increase growth and survival when out planted. Gemma et al., (2002) also deemed it fit to modify the terminology to accommodate certain soil fertility characteristics that differ in land use. In our experiment, FMID differed slightly between fungal treatments in both plant species. A value of 25.55% corresponding to a dependency value in PJ indicates the possibility that improved seedling traits in this plant species resulted from mycorrhizal inoculation. In contrast, the low mycorrhizal inoculum dependency value obtained, especially in treatment LB (negative 7.48%), for SW could be as a result of the fungal species used. This is because there is the likelihood of spruce seedlings to behave differently when inoculated with a spruce loving mycorrhizal fungus like *Amphinema byssoides* (Quoreshi et al., 2009).

ECM fungal species have been used widely in inoculation as a single treatment in various mycorrhizal studies that have focused on growth response, seedling establishment, mycorrhizal dependency, reclamation or changes in root physiology (Duñabeitia et al., 2004; Duponnois et al., 2005; Bois et al., 2006; Selosse et al., 2000). Common routinely used ECM species belong to the genera *Laccaria, Paxillus, Hebeloma, Suillus, Pisolithus, Scleroderma* or *Rhizopogon* (Gagné et al., 2006). Species from these genera have been less exploited in ECM mycorrhizal studies in terms of using a combination of two or more fungal species as a single treatment. This may be due to the debate as to the efficiency of this combination. However, this study explored this technique in order to evaluate the possible economic advantage of ECM inoculation while maximising fungal properties (Ortega et al., 2004). Furthermore, the combination of fungal species may result in synergistic plant fitness response (Bois et al., 2006a). Our results of ECM group combination showed a significant effect of the different forms (single, double or triple) of inoculations on plant growth, PVI and stem volume when compared to the control. This finding helps to identify where possible treatment differences may occur and gives an overall outlook on mycorrhizal combinations. It was expected that the combined treatments would give a higher growth response than the single fungal treatments. Nevertheless, there were inconsistencies in the results depending on the measured parameter and plant species. Therefore, it was impossible to draw firm conclusions from the data, even though this grouping was assumed solely for the study purpose.

Survival rate

In oil sands reclamation sites the major anticipated plant stresses are drought and salinity because of the presence of salts and the low water retention capacity of tailing sands. Although after stabilization with reclamation substrates these conditions are reduced, they are not entirely eliminated and still affect plant survival. Survival rates are important for determining plant success after transplanting to the field. The survival rates in the present study differed between plant species, as no overall significant effect of inoculation was observed for jack pine. The treatment combinations differed especially in those that had ECM fungi LBST, LB or ST in various treatments. This observation would require further analyses because it may be an indication of fungal dominance or site climatic conditions favouring a type of fungal species. In contrast, white spruce survival rate was significantly influenced by ECM inoculation, with HCLB, HCST and HCLBST having the greatest survival, which might be attributed to ECM inoculation in accordance with Var et al. (2011), who studied the survival successes of mycorrhizal and non-mycorrhizal *Cotoneaster franchetti* Bois under

different climate and various growing media. They observed that the total mortality of mycorrhizal *C. franchetti* plants was 7.69%, while a mortality of 26.92% was recorded for the control group. Differences in survival rates between the two plant species could also be due to weed competition (Benayas et al., 2005) and water logged conditions (Turjaman et al., 2007). Jack pine had a higher weed level compared to the white spruce while some site depressions became water logged in our study area (data not shown).

Stem volume and Plot volume index

Overall, ECM inoculation improved stem volume and PVI of jack pine and white spruce. These indices take into account the quantity of seedlings without or with a number of surviving plants as a measured value (Marx et al., 1985). In jack pine PVI, there was no significant difference between ECM treatments and control, while a difference was observed in stem volume. However, the opposite was true for white spruce. Of all the treatments, HCLB was consistently indicating a difference in either stem volume or PVI when compared to controls. A number of studies have reported increased PVI of mycorrhizal inoculated symbionts with similar plant and fungal as species used in the present study. Sometimes the differences were observed only in the species and ecological conditions. Gagné et al. (2006), who studied the ECM fungal communities of nursery-inoculated seedlings out-planted on clear-cut sites, reported increases in PVI of ST, LB and *Hebeloma longicaudum*-inoculated white spruce. Similar results were also reported by Quoreshi et al., (2008) in their studies on plant performance of conifers (white spruce, black spruce and lodgepole pine) and angiosperm species. In the present study, an increase in PVI in inoculated treatments was pointed towards percentage survival rate with the exception of HCST and HCLBST. These plant establishment abilities were analysed for a relationship that showed a significant ($P= 0.0149$, $P< 0.001$) correlation for jack pine and white spruce, respectively (data not shown). This indicates that a difference between treatments in PVI is greatly influenced by the survival rate that ideally reflects seedling response to the above or below-ground environmental factors.

ECM morphology and Persistence of inoculated fungal species

Ectomycorrhizal fungi unlike the AM fungi, merely colonise about 3% of seed plants all of which are woody species, trees and shrubs (Molina et al. 1992). In spite of this miniature fraction, the significance of ECM association worldwide is greatly amplified globally by the large area covered by these plants, and their economic value as the source of timber (Smith and Read, 2008). In the present study, the ECM found colonising the root tips of seedlings was analysed morphologically and molecularly two years after out planting, to determine qualitatively ECM species present and the persistence of inoculated fungi. The number of ECM fungal morphotype detected on both plant species was 17. This is in accordance with previous studies that described range of ECM fungal morphotypes to vary between 5- 27 in various plant species including pine, spruce, oak, and poplar birch (Glen et al., 2008; Visser, 1995; Wurzburger, 2001, Duñabeitia et al., 2004). Different ECM fungi have been reported to colonise host root tips at various plant stages, leading to the categories "early"-, "multi"- and "late"-stage fungi (Visser, 1995). The fungal species detected in this study using morphology and molecular means belong to the early- and multi-stage fungal species. This means that they were found on young and mature root tips of the host plants. Furthermore, *Amphinema byssoides* and *Thelephora terrestis* were the most frequent fungal species present on site. However, one Ascomycetous mycorrhizal fungus, *Wilcoxina sp.*, and a pathogenic fungus *Olpidium brassicae* belonging to the class *Chytridiomycetes* were also detected. The present results are congruent with those of the previous studies reporting the dominance of *A. byssoides, C. geophilum and Thelephora sp.* on seedlings inoculated prior to out-planting (Duñabeitia et al., 2004; Quoreshi et al., 2008; Gagne et al., 2006). This suggests the aggressive colonising nature of these fungal species and their possible exploitations; even though this dominance could be influenced by the site and plant species used. However, it is still uncertain as to whether these fungal species are field or nursery root colonisers (Khasa et al., 2001; Kernaghan et al., 2003; Flykt et al., 2008).

Determining the persistence of fungal species using single sequence repeat primers (SSR) that amplify microsatellite loci region (Jany et al. 2006) is a technique that is now popular as opposed to amplified or restriction fragment length polymorphism (AFLP or RFLP). This technique is more informative and is mostly essential if unique genetic identity or genetic diversity between strains are desired (Akter et al., 2008). This was not the case with this study and so other molecular means of determining persistence were used. The primer ITS1F and ITS4 are general in nature and it is difficult to differentiate among fungal samples

because they amplify region from most fungi. In this study, the use of these primers was to determine the type of fungi present on root tips and segments of plants. However, fungal species that were closely related produced double bands or false positive bands, which became a problem when further use of the PCR product was needed. This problem was eliminated by purification using gel excision that was performed for a few samples. The ITS-RFLP technique is less expensive than sequencing but this method rarely gives unique patterns for each species (Gardes et al, 1991, Aviram et al, 2002), leading to false RFLP results. Hence, this method was not considered. The design of specific primers to determine presence of inoculated fungal strains was conducted to overcome co-amplification of related species and to detect the persistence of inoculated species because it was relatively fast, inexpensive and eliminates intraspecific variability, more so when we had a combination of fungal species as one treatment. Therefore, once primer specificity was proven, further sequencing of samples was not necessary.

Mycorrhizal inoculation before out-planting is known to improve plant establishment, survival and growth. However, the extent of these benefits to plants is dependent on the initial colonisation and persistence of inoculated strains (Grove et al., 1993). Amongst the three major fungal species inocula (either singly or in combination), *H. crustuliniforme* was the only species that was not detected on the root tips of plants. *L. bicolor* and *S. tomentosus* persisted after two years of planting, but their occurrence was rather erratic. The colonisation of the host root system of inoculated and control seedlings after planting by resident ECM fungi is an expected phenomenon, and only a few studies have reported outstanding competitive success with introduced fungal strains (Duñabeitia et al., 2004; Selosse et al., 2000). A number of studies have reported the persistence or disappearance of inoculated mycorrhizal symbionts that are similar to plant species used in this study but under dissimilar ecological conditions. Indeed, Menkis et al., (2007) studied the refforestation of an abandoned farmland with conifers (*Pinus sylvestris*, *Picea abies*) inoculated with *C. geophilum*, *Piceirhiza bicolorata* and *H. crustuliniforme*. Out of these three fungi, only *H. crustuliniforme* disappeared completely from both plant species after two growing seasons and failed to improve seedling survival and growth. Bledsoe et al. (1982) also reported the replacement of *H. crustuliniforme* by resident fungi in out-planted mycorrhizal inoculated Douglas-fir. Similarly, *H. crustuliniforme* was not detected in our study, but contrary to the former study, it was observed to improve seedling height and volume, as there was a significant difference between treatments with this fungus and controls. In addition, HC had a higher RGR than the control in both sampling seasons. This suggests that the ability of fungal species to persist

after one season of growth may be dependent on the soil characteristics, fungal strain, and ability to withstand competition with resident fungi.

However, the absence of any inoculated mycorrhizal symbiont in the field does not exclude the possibility that they played and initial role in reducing transplanting shock leading to the survival of seedlings. Conversely, *L. biocolor* inoculated on black spruce (Buschena et al. 1992), jack pine (Browning and Whitney, 1992) and Douglas-fir (Selosse et al. 2000) were shown to persist several years after nursery inoculation. *S. tomentosus* is one that has not often been reported to persist after inoculation. Khasa et al. (2001) attempted to re-isolate inoculated mycorrhizal fungi from feeder roots of five conifers and amongst six other tested beneficial ECM fungi; *S. tomentosus* was the only fungus not recovered in black and white spruce seedlings. Gagné et al. (2006) also reported the absence of *S. tomentosus* five years after introduction. This is contrary to what was observed in this study. However, commonalities between studies exist in terms of these organisms improving survival of the host plants. Generally, it would be difficult to ascertain which fungal species improved seedling survival and growth because of the combinations of treatments and possible fungal stimulation or inhibition. Nevertheless, HCLB would be regarded as the best fungal treatment in terms of height and PVI in both plant species.

In summary, the above results suggest that ECM nursery inoculation of conifers should be considered to improve out-planting performance in oil sands disturbed land. However, this study also suggests the need to conduct further experiments to confirm these findings and data need to be collected annually over a longer period of time. In addition, the use of different reconstructed sites in the oil sand region to determine the competency of these fungal species or inoculum needs to be determined.

3.5 Literature cited

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4 Synthesis

In the Athabasca oil sands region (AOSR), reclamation following oil sands mining has focused on the use of organic treatments such as forest floor (LFH) materials from upland forests, peat mineral mix and overburden soil to improve soil quality and fertility and, thus, plant establishment and productivity. However, little attention has been given to the use of topsoil and the potential benefit of mycorrhizal biotechnology in long-term plant establishment. In this thesis, it was hypothesized that the inoculation of nursery trees with mycorrhizal fungi prior to out planting could facilitate an enhanced reclamation in AOSR.

This study used two approaches to investigate the overall objective, which was to evaluate the field performance and benefits of ECM seedlings (jack pine and white spruce) when used in reclamation compared to un-inoculated seedlings. The first approach was to determine the mycorrhizal inoculum potential of reclamation materials. This bioassay was conducted *in vivo* and involved the use of morphological typing to assess mycorrhizal colonisation and molecular typing to determine the actual ECM species present on plant root tips. In the inoculum potential bioassay, AM and ECM fungi were present in red clover and white spruce roots, respectively, of all reclamation materials. For AM fungal assessment, topsoil (TPS) had the highest percentage colonisation, while peat mineral mix (PMM) had the highest ECM percentage colonisation. With percentage colonisation of less than 30%, results indicate that both ECM and AM fungal inoculum potential of soils are low (Danielson and Visser, 1989). The second approach determined the field performance of inoculated seedlings compared to controls by comparing various seedling parameters including relative growth rate (RGR), plot volume index (PVI) and field mycorrhizal inoculation dependency (FMID). In general, the inoculation effect on survival rates differed in plant species. Jack pine seedling survival was not influenced by ECM inoculation, which was contrary to the increase in survival observed for white spruce seedlings. There was an overall increase in stem volume and plot volume index (PVI) of ECM inoculated jack pine and white spruce seedlings when compared with controls. The entire data suggest that pre-inoculation of seedlings with selected ECM fungal species improve their growth and establishment in the reclamation sites compared to non-inoculated plants. However, it is important to note that more studies will be needed to examine the effects of various resident fungi that may be present in the nursery environment or on seeds, which can spontaneously colonise non-inoculated seedlings. The presence of these resident fungi is often a major consideration in mycorrhizal field studies. However, it has been construed that introduced mycorrhizal fungi provide greater benefits in some cases than indigenous mycorrhizal fungi (Brundrett, 1991).

Microbial biotechnology for the reclamation and remediation of disturbed lands is now well appreciated by the scientific community, especially when man-made disturbances ultimately destruct mycorrhizal networks and other microbial activities in the soil systems (Sanon et al., 2010). In land reclamation, the establishment of vegetation cover is usually of greater importance than the below ground processes. However, what is not essentially understood is how below ground biological processes greatly influences plant establishment and sustainability. Mycorrhizal fungi are important drivers in microbial ecology and have their propagules concentrated within 20 cm below soil surface (topsoil), likewise other major microbial functional groups (Mummey et al., 2002). Hence, the activity of soil microbes is becoming a necessary indicator of ecosystem recovery following surface mine reclamation (Mummey et al., 2002). Although emphasis on mycorrhizal fungi may appear overrated when looked from a reclamation point of view, other groups of microorganisms such as plant growth promoting rhizobacteria (PGPR), mycorrhiza helper bacteria and N-fixing bacteria are now being considered inoculants in reclamation processes (Ahn et al., 2008; Hrynkiewicz and Baum, 2011). For example, the use of PGPR 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). The re-introduction of mycorrhizal propagules through artificial means is an option to be considered in improving reclamation success, since mycorrhizal fungi may not only confer growth and survival benefits but may also increase plant diversity and soil quality in disturbed lands (van der Heijden et al., 1998; Requena et al., 2001). According to Isbel et al. (2011), high plant diversity is needed to maintain ecosystem processes, of which the soil microbial community plays an essential role. Without groups of organisms such as mycorrhizal fungi, saprophytes, N fixing bacteria, etc., there will be an imbalance in soil nutrient cycles. In addition, managing the soil community, which is rarely considered during reclamation may have long term benefits for the environment. For example, if fungal and bacterial components of soil are relatively balanced, this can reduce fertilizer or chemical inputs because these organisms will help maintain the processes of nutrient mineralization and decomposition at sustainable levels (Requena et al., 2001).

In mining, the removal of topsoil to obtain mineral ore is a common process, but less attention is given to its re-use during landscape reconstruction. Topsoil is rich in organic matter/microbial activity, has less salt and high nutrient levels (Schwenke et al., 1999). However, proper preservation of this material is what determines its efficacy when re-used in reclamation (Ghose, 2001). In this study, topsoil indicated higher mycorrhizal percentage colonisation and this may be an indication of its potential use as an amendment compared to the peat mineral mixes and overburden soil. Although laboratory experiments seldom mimic the complex environmental conditions in the field, it is possible that the mycorrhizal percentage colonisation of amendments to reach 30% may be influenced by several factors such as moisture and temperature in the greenhouse. In essence, it is difficult in field studies to attribute the

success of plant establishment and survival to a particular cause without acknowledging possible climatic influence. According to Bellgard and Williams (2011), climatic and edaphic factors such as temperature extremes, soil pH and topography, soil nutrient levels and moisture are drivers of a global climate change. These factors were said to impact soil rhizosphere conditions directly by bringing about a change in resource availability and mycorrhizal fungi distribution, while an indirect impact is to change ground allocation of C to roots or cause changes in plant species distribution. These impacts have been suggested to bring about possible competition/invasion by exotic plants or agents such herbivores (Bellgard and Williams, 2011). Hence, if not favourable (suitable host) they may result in low inoculum potential of mycorrhizal propagules. However, possible variations in tolerance to edaphic and climatic factors (e.g. drought, soil toxicity, extreme temperatures etc.) often occur between and within species of mycorrhizal fungi and these variations may signify their ability to adapt to specific site conditions (Brundrett, 1991).

4.1 Reclamation application and significance

Results from this study indicate that nursery inoculation of trees with mycorrhizal fungi prior to out-planting offers a viable means of rebuilding the forest ecosystem comparable to a pre-disturbed land. Inoculation prior to out planting has the potential of reducing the constant mortality rate encountered in AOSR during tree planting in oil sand processed materials as well as to lessen human hours and resources (e.g., equipment, plant propagules, cost inputs) channeled into the reclamation process. Furthermore, since topsoil removed from above the mined ore is not readily planted with cover crops due to project timing, the use of inoculum should act as a complement. However, one of the limitations encountered with pre-inoculation of seedlings prior to out planting is the possible displacement by resident mycorrhizal or pathogenic fungi. Another area of concern is the response of seedlings to inoculation, given that some seedlings may have their own mycorrhizas without inoculation prior to planting or some may acquire mycorrhizas after planting (Zwiazek, J. Per. Com.). Nevertheless, there is a greater chance for these inoculated fungi to stimulate the function of other soil microbes and play a role in early plant establishment before being displaced.

This research has immediate benefits to Suncor's reclamation program responsible for forest renewal, because the results would lead to improved reforestation practices using mycorrhizal inoculated stock with better growth and survival potential in the disturbed sites. This study also revealed information on the status of mycorrhizal populations, in particular amending materials that could help the industry to improve ECM inoculation in nursery. In addition, these results would help in the development of a

process for transferring an important mycorrhizal biotechnology to the reclamation industry and commercial forest nurseries.

4.2 Project limitations/Future research

One of the limitations of this study was the lack of replication of the reclaimed sites to ascertain inoculum effect or mycorrhizal influence. Two experimental sites were proposed for this study. However, only MD5 was properly constructed as the other proposed site (POND 1) was heavily flooded and resulted in a 90% death rate of planted seedlings. The experimental sites were already established and as such could not be modified. Ideally, two or more sites within the same area would have been a better test of the benefit of ECM inoculation in oil sand disturbed lands. Therefore, future studies will be required to confirm the results.

While the results of this study show the potentials of ECM technology in reclamation of oil sand disturbed lands, there are a few areas of interest for further research. If research into the use of microorganisms to improve soil quality and reclamation is conducted, one area of the study may involve determining the interactions between ECM and other fungal and (or) bacterial counterparts e.g. actinomycetes, plant growth promoting bacteria or mycorrhiza helper bacteria. These could be explored in terms of the following:

- Carrying out fungal-fungal *in vitro* synthesis to determine the stimulatory or inhibitory properties of each fungus and in combinations.
- Conduct *in situ* remediation procedures on field sites that would lessen resident soil fungus or weed competition while allowing mycorrhizal dependency of inoculated plants to be determined.
- Determine sensitive chemical contents in OSPM such as PAHs and if these organisms have catalytic properties that could facilitate detection and design of biosensors.
- Determine the influence of mycorrhizal technology on soil organic matter content towards improving soil quality.

4.3 Literature cited

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Appendices

Appendix I: Morphotype descriptions from field seedlings and morphotype picture plate

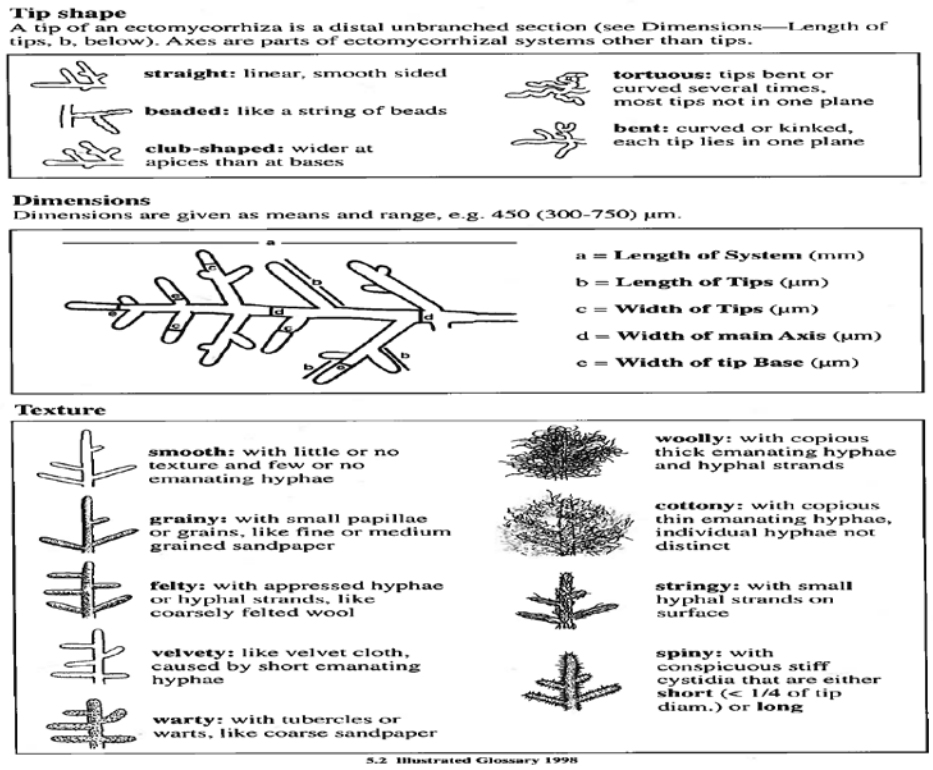
Appendix II: Primer nucleotide sequence alignments and primer specificity on agarose gel

Appendix III: Culture Media

Appendix IV: Picture Plates

Appendix I: Morphotype descriptions from field seedlings

The section contains the characteristics of morphotypes encountered on jack pine and white spruce seedlings in chapter 3 of this thesis. The procedure used to characterize morphotypes was that outlined by Goodman et al., (1996). Thus for terms used in this description refer to glossary section of Goodman et al., (1996).



Goodman, D.M., Durall, D.M., Trofymow, J.A., and Berch, S.M. (1996–2000). A manual of concise descriptions of North American ectomycorrhizae: including microscopic and molecular characterization. Mycologue Publications, Sidney, B.C.

Morphotype 1:
Encountered on: White spruce and Jack pine
Morphology (dissection microscope)
Branching and shape: Monopodial pinnate to dichotomous systems with straight to bent tips.
Colour and Texture: Dark to light brown, felty
Lustre: matte
Emanating elements: none observed.
Probably: Tomentella-like sp.

Morphotype 2:
Encountered on: Jack pine
Morphology (dissection microscope)
Branching and shape: Irregular to collaroid.
Colour and Texture: Straight or bent tips with felty to stringy texture
Lustre: matte
Emanating elements: Thick emanating hyphae around the tip.

Morphotype 3:
Ecountered on: Jack pine
Morphology (dissection microscope)
Branching and shape: dichotomous systems with occasional bent tips.
Colour and Texture: Orange to light brown, grainy to felty.
Lustre: matte or reflective
Emanating elements: none observed.

Morphotype 4:
Encountered on: White spruce
Morphology (dissection microscope):
Branching and shape: unbranched to straight
Colour and Texture: Grainy to felty
Lustre: Matte
Emanating elements: None observed

Morphotype 5:
Encountered on: White spruce and Jack pine
Morphology (dissection microscope)
Branching and shape: Irregualar to tortuous
Colour and Texture: Dark to light brown
Lustre: Reflective
Emanating elements: None observed

Morphotype 6:
Encountered on: white spruce and Jack pine
Morphology (dissection microscope)

Branching and shape: Unbranched to dichotomous
Colour and Texture: Dark brown, felty to grainy
Lustre: Matte to shiny
Emanating elements: cystidia like cap with black emanating hyphae
Probably: Cenoccocum geophilium

Morphotype 7:
Encountered on: Jack pine
Morphology (dissection microscope)
Branching and shape: Dichotomous to collaroid, club-shaped to bent
Colour and Texture: Dark to light brown, felty to smooth.
Lustre: Matte to reflective
Emanating elements: little emanating hyphae observed.

Morphotype 8:
Encountered on: Jack pine
Morphology (dissection microscope)
Branching and shape: Dichotomous, Straight to bent
Colour and Texture: light to dark brown, felty to grainy
Lustre: matte
Emanating elements: Emanating hyphae present

Morphotype 9:
Encountered on: White spruce and Jack pine
Morphology (dissection microscope)
Non-mycorrhizal

Morphotype 10:
Encountered on: White spruce and jack pine
Morphology (dissection microscope)
Branching and shape: Irregular, straight to club-shaped
Colour and Texture: Golden to dark brown, felty to stringy
Lustre: Matte to shiny
Emanating elements: None observed.

Morphotype 11:
Encountered on: White spruce and Jack pine
Morphology (dissection microscope)
Branching and shape: Irregular, club-shaped to bent
Colour and Texture: Yellowish to dark brown, cottony to felty
Lustre: Matte
Emanating elements: None observed.

Morphotype 12:

Encountered on: White spruce
Morphology (dissection microscope)
Branching and shape: monopodial pinnate to unbranched, club-shaped to straight
Colour and Texture: Golden brown to white, felty to stringy
Lustre: Matte to shiny
Emanating elements: None observed.

Morphotype 13:
Encountered on: White spruce and Jack pine
Morphology (dissection microscope)
Branching and shape: Irregular, straight to bent
Colour and Texture: dark brown to white
Lustre: Matte
Emanating elements: none observed.

Morphotype 14:
Encountered on: White spruce
Morphology (dissection microscope)
Branching and shape: Irregular to monopodial pinnate, bent
Colour and Texture: Dark brown, felty
Lustre: Matte
Emanating elements: None observed.

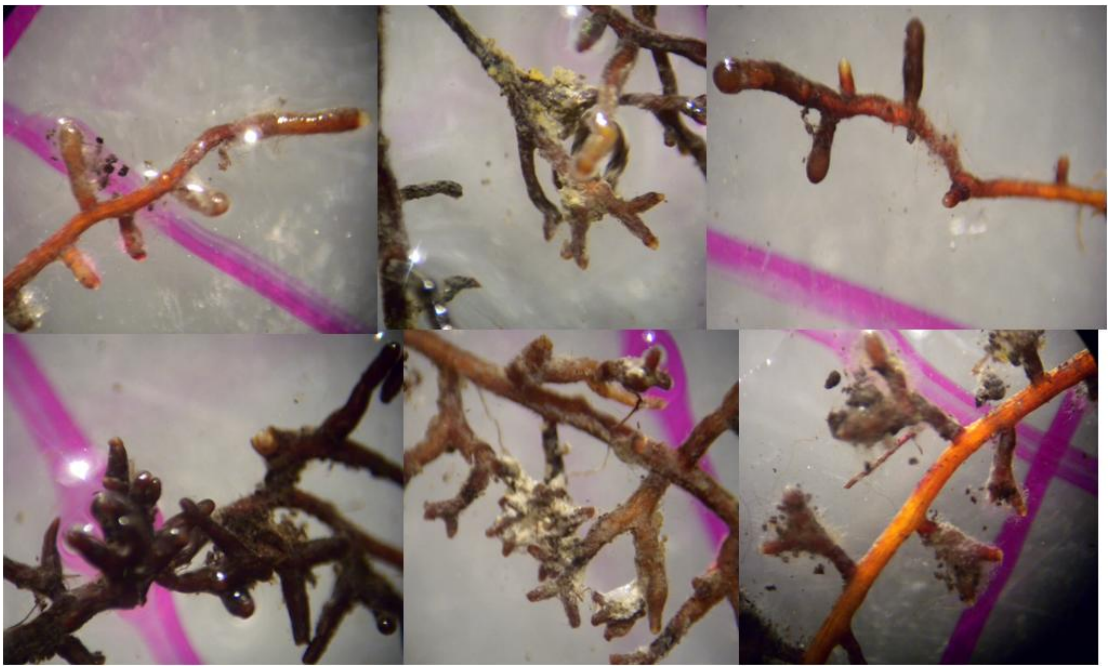
Morphotype 15:
Encountered on: White spruce and Jack pine
Morphology (dissection microscope)
Branching and shape: Unbranched to dichotomous
Colour and Texture: brown to yellow, grany to wolly
Lustre: Matte
Emanating elements: None observed.

Morphotype 16:
Encountered on: White spruce
Morphology (dissection microscope)
Branching and shape: Unbranched, straight to bent
Colour and Texture: Brown to whitish tip, wolly to stringy
Lustre: Reflective to matte
Emanating elements: None observed.

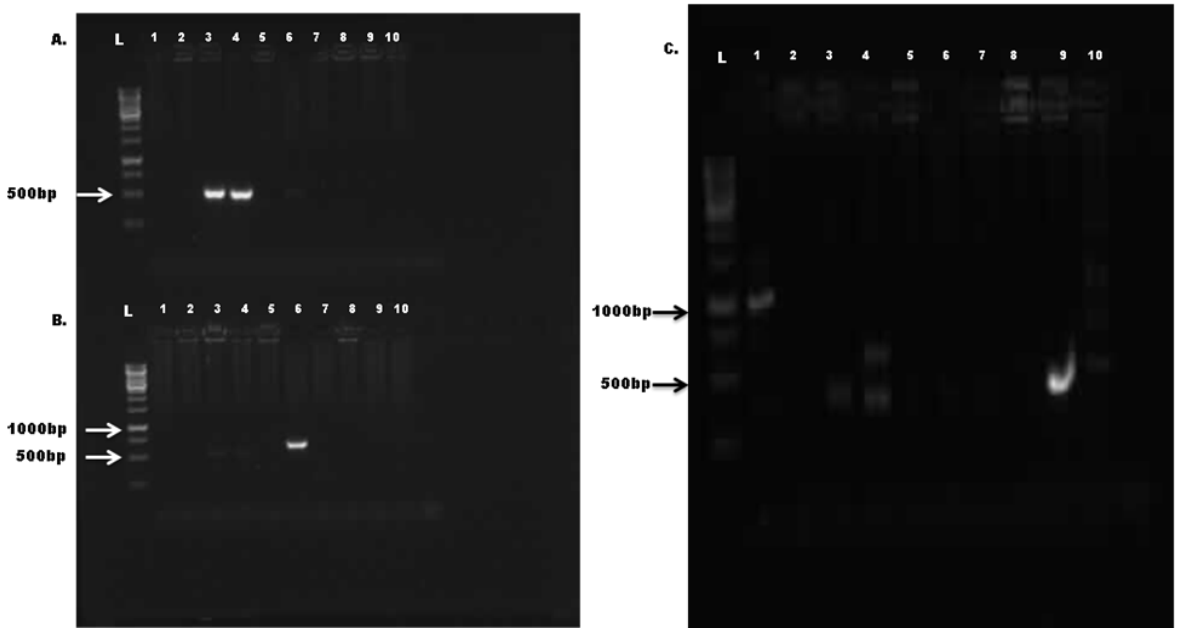
Morphotype 17:
Encountered on: Jack pine
Morphology (dissection microscope)
Branching and shape: Dichotomous to monopodial pinnate, straight to bent
Colour and Texture: Light brown to golden brown, felty
Lustre: Matte
Emanating elements: Few emanating hyphae observed.

MORPHOTYPE PLATE 1

MORPHOTYPE PLATE 2



Appendix II: primer nucleotide sequence alignments and specificity on agarose gel



A.2.1. Syber safe stained agarose gel showing primer specificity. Gels A, B and C are designed primers of *Hebeloma Crustuliniforme*, *Laccaria bicolor* and *Suillus tomentosus* respectively. Lanes L- 1kb DNA ladder, Lane 1- *Amphinema byssoides*, Lane 2- *Cenoccum geophilum*, Lane 3- *Hebeloma Crustuliniforme*, Lane 4- *Hebeloma longicaudum*, Lane 5- *Lactarius affinis*, Lane 6- *Laccaria bicolor*, Lane 7- *Paxillus involuntus*, Lane 8- *Tricholoma flavovirens*, Lane 9- *Suillus tomentosus*, Lane 10- *Wilcoxinia mikloae*

A.2.2. Designed primer sequence aligment

<*Hebeloma Crusliniforme*>

CCTGCGGAAGGATCATTATTGAATGAACTTGGTGTGGTTGTTGCTGGCTCTTTCGAGGGCATGTGCACGC
CCACCATCCTCATCTCTCCACCTGTGCACCTCTTGTAGACTTGGATTGACTTTCCGAGGCAACTCGGTCGG
GAGGATTGCCGGCGCCNGCTGGCTTTCTTTGCATTTCCAGGTCTACGTTTTTCATATACCCCATTGTATGT
AACAGAATGTAACATTGGGCACTTGTGCCTATAAACTTTATACAACTTTCAGCAACGGATCTCTTGGCTCT
CGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAT
CTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAAATTCTCAACC
TTTATCAACTTTTGTTGATAATGGCTTGGATATGGGGGTTTATTTTGCTGGCTTCTTTACAGATGGTCAGCT
CCCCTTAAATGCATTAGCCGGTGCCCCGCGTGGACTGTCTATTGGTGTGATAATTATCTACGCCGTGGACA
TCTGCAATCATATGGGTTTGCACTGCTTCCAACTGTCTGTTGAATCAGACAATACATGACAATTTGACCTC
AAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACAAGGATT
CCCCTAGTAACTGCGAGTGAAGCGGGAAAAGCTCAAAT

<*Laccaria bicolor*>

TTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATTGAATAAA
CCTGATGTGACTGTTAGCTGGCTTTTCGAAGCATGTGCTCGTCCATCATCTTTATCTCTCCACCTGTGCAC
ATTTTGTAGTCTTGGATACCTCTCGAGGAAACTCGGATTTGAGGATCGCCGTGCTGTACAAGTCGGCTTTT
CTTTCATTTCCAAGACTATGTTTTTATATACACCAAAGTATGTTTATAGAATGTCATCAATGGGAACTTGT
TTCCTATAAAATTATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAA
TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGG
TATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAAATTCTCAACCTTCCAACTTTTATTAGCTTGGTTAGG
CTTGGATGTGGGGCTTGCGGGCTTCATCACTGAGGTCGGCTCTCCTTAAATGCATTAGTGGAACTTTTGTG
GACCATCTATTGGTGTGATAATTATCTACGCCGTGGGTGTGAAGCAGCTTTATGAAGTTCTGCTTCTAACC
GTCCATTGACTTGGACAATTTTGACAATTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCAT
ATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGCTC
AAATTTAAAATCTGGCAGTCTTTGGCTGTCCGAGTTGTAATCTAGAGAAGTATTATCCGCGCTGGACCGT
GTACAANTCTCCTGGAATGGA

<*Suillus tomentosus*>

GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAAAGAAATAATCTC
GAGGGCCGATGGAAAGGAGAGAGGGTTGTAGCTGGCGTAAGCACGTGCACGCCCTCTTTCTCGACCTAG
GTCCTTATGGGCGCGGGGCGACCCGCGTCTTCATAAGCCCCTTCGTGTAGAAAGTCTATGAATGTTTTTAT
TATCATCGACTCGCGACTTCTAGGAGACGCGATTCTTTGAGACAAAAGTTATTACAACTTTCAGCAATGG
ATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAATCGCGATATGTAATGTGAATTGCAGATCTACAG
TGAATCATCGAATCTTTGAACGCACCTTGCGCTTATCGGTGTTCCGATGAGCATGCCTGTTTGAGCGTCAT
TAAATTCTCAACCCCTCTCGATTTGCTTCGAGAGGGTGCTTGGATTGTGGAGGCTGCCGGAGACCTGTTTT
TTCAGGACTCGGGCTCCTCTGAAATGTATTGGCTTGCGGTCGACTTTCGACTGTGCATGACAAGGCCTTTG
GCGTGATAATGATCGCCGCTCGCCGAAGTGCACGAACGAATGGTCTCGTGCCTCTAATCAGTCGATGTCT
TTTCGAAGGCGTCTTCCTTATTGACGTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATAT
CAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACTGCGAGTGAACCGGGAAAAGCTCA
AATTTTGAATCTGGCGGTCCTTCGGGCCGTCCG

Appendix III: Culture Media

Glucose Yeast Malt Extract (GYME) Medium for fungal culture:

Composition:

1. Glucose – 10g
2. Yeast extract - 2.5 g
3. Malt extract – 3.5g
4. KH_2PO_4 – 2g (Potassium phosphate monobasic)
5. $MgSO_4$, $7H_2O$ – 0.5g

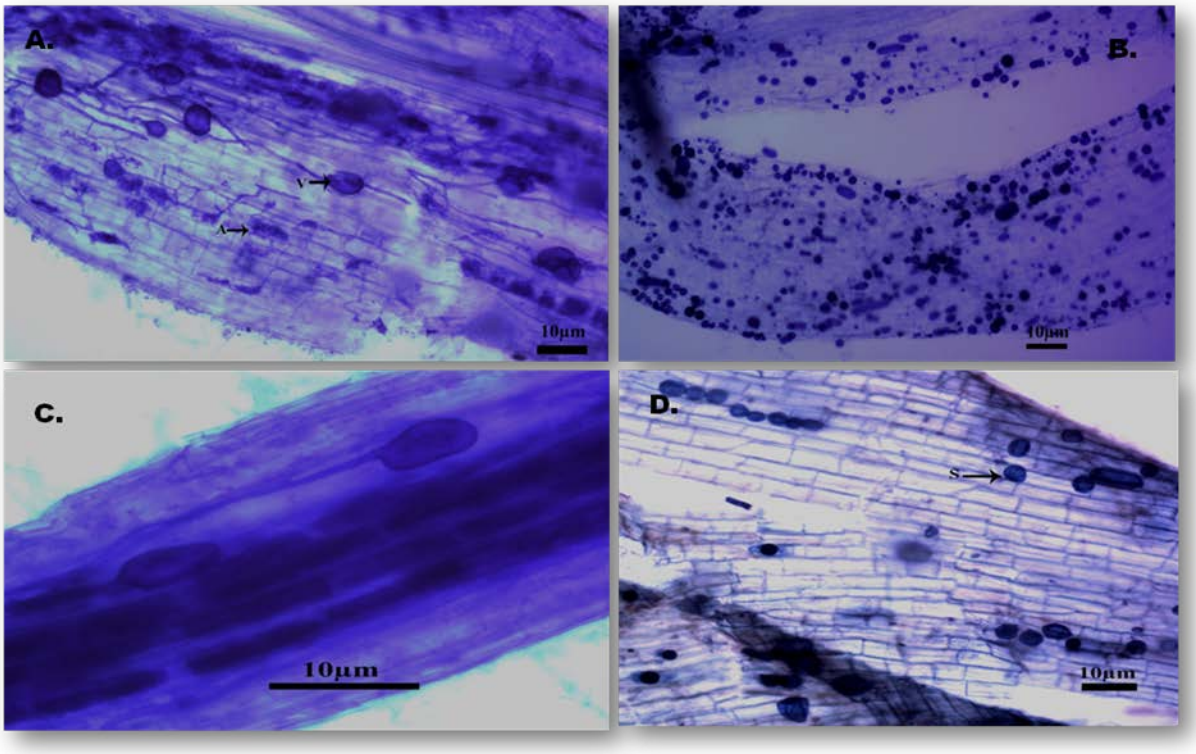
Add into one litre (1L) distilled water, adjust pH at 5.5. In case of agar media, add 15g agar/L

Appendix IV: Picture Plates

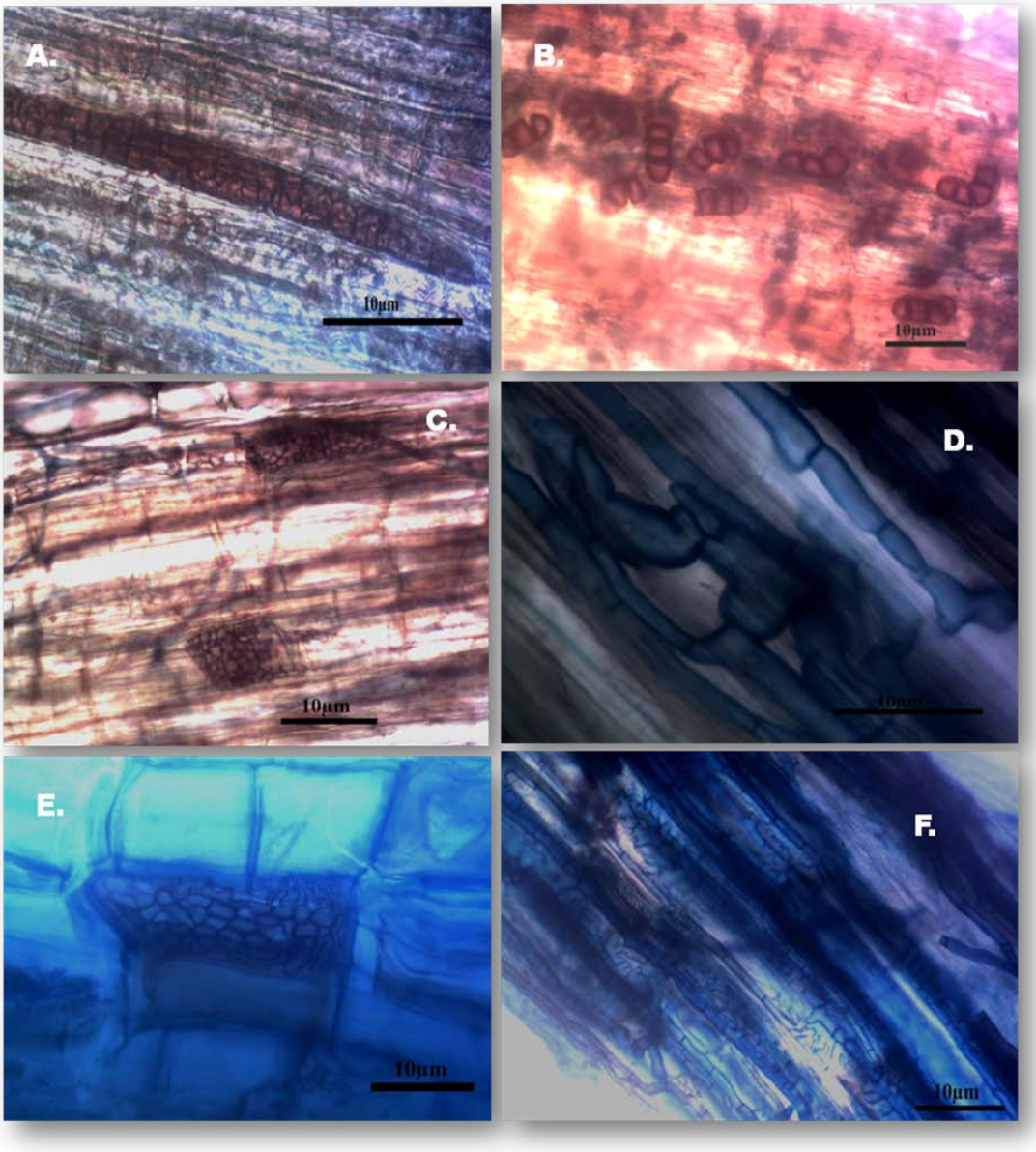
A.4.1. Inoculum potential bioassay setup. Picture A and B. Represents red clover and white spruce grown from sterilised seeds.C and D are the soil cores collected from the reclamation area.

A.4.2: Destructed samples of white spruce seedling from reclamation site. A is the control seedlings with combined fungal inoculated treatment. B is control seedlings with single inoculated seedlings.

A.4.3: Destructed samples of jack pine seedling from reclamation site. A is the control seedlings with combined fungal inoculated treatment. B is control seedlings with single inoculated seedlings.



A.4.4: Trypan blue stained roots of red clover obtained from inoculum potential bioassay. A. Shows well developed arbuscules (A) and vesicles (V). B and C shows sporadic resting spores of a pathogen (perhaps *Olpidium brassicae*). C. Shows a truly form vesicle that is known to be formed by AM fungi.



A.4.5: Trypan blue stained roots of jack pine and white spruce seedlings obtained from the reclamation site in 2010. Figure 4.5A and B. shows pathogenic structures of a root pathogen obtained from control PJ samples; 4.5Cand E shows hartig net with broad and infrequently ramified lobes obtained from PJ on treatments HCST and LB respectively; 4.5D shows and ectomycorrhizal septate hyphae with clamps obtained from PJ-LB and 4.5F shows an inner mantle with an interlocking but irregular net synenchyma obtained from SW-ST.