Development of a new green technology for the revegetation of abandoned gold mine tailings using specific symbionts associated with *Picea glauca*

Mémoire

Martin Beaudoin Nadeau

Maîtrise en agroforesterie
Maître ès sciences (M.Sc.)

Québec, Canada

Résumé

Le rôle et l'importance des micro-organismes telluriques (PGPR et champignons ECM) à favoriser la santé, la croissance et la nutrition de *Picea glauca* ont été étudiés sur stériles et résidus miniers fins riches en quartz-biotite de la mine d'or Sigma-Lamaque située dans la région de l'Abitibi au Canada. L'étude a été divisée en trois composantes. Premièrement, la structure des communautés de champignons ECM associés aux racines de *Picea glauca* a été analysée sur quatre sites différents à proximité du site minier. Deuxièmement, une expérience en laboratoire a été effectuée afin de sélectionner *in vitro* des champignons ECM prometteurs qui démontrent une excellente croissance sur résidus miniers. Troisièmement, une expérience en serre impliquant la croissance de semis de *Picea glauca* sur stériles et résidus miniers fins a été réalisée et la performance de différents traitements de champignons ECM et PGPR a été évaluée. Les résultats suggèrent que les champignons ECM et PGPR adaptés aux conditions du site jouent un rôle très important au niveau de la santé et de la croissance de *Picea glauca* sur résidus miniers riches en quartz-biotite.

Abstract

The role and importance of soil microorganisms (PGPR and ECM fungi) in promoting the health, growth, and nutrition of *Picea glauca* were investigated on biotite-quartz-rich waste rocks and fine tailings of Sigma-Lamaque gold mine located in the Abitibi region of Canada. The study was divided into three components. Firstly, the community structure of ECM fungi associated with *Picea glauca* was analyzed on four locations near the mining site. Secondly, a laboratory experiment was conducted in order to *in vitro* select promising ECM fungi that were growing well on mine tailings. Thirdly, a glasshouse experiment involving the growth of *Picea glauca* seedlings on waste rocks and fine tailings was conducted and the performance of different treatments of ECM fungi and PGPR was evaluated. Results suggested that site-adapted ECM fungi and PGPR play a very important role in the health and growth of *Picea glauca* on biotite-quartz-rich waste rocks and fine tailings.

Table of contents

List of tables

List of figures

List of abbreviations and acronyms

Abbreviations	Definitions
AC	*Azotobacter chroococcum*
ADP	Adenosine diphosphate
AM	Arbuscular mycorrhizae
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CEC	Cation exchange capacity
CEF	Centre for Forest Research
CF	*Cadophora finlandia*
CFU	Colony forming unit
CG	*Cenococcum geophilum*
CTAB	Cetyl trimethylammonium bromide, an antiseptic agent
DNA	Deoxyribonucleic acid
ECM	Ectomycorrhizae
EDTA	Ethylenediaminetetraacetic acid
FE	Forest edge
FT	Fine tailings
FV/FM	Photochemical efficiency
HC	*Hebeloma crustuliniforme*
HPLC	High-performance liquid chromatography
ITS	Internal transcribed spacer
LA	*Lactarius aurantiosordidus*
LB	*Laccaria bicolor*
MRNF	Ministère des ressources naturelles et de la faune (QC)
LMMOAs	Low molecular mass organic acids
MNM	Modified Melin-Norkrans medium
MS	Mining site
NF	Natural forest
NO	No ECM fungi or No PGPR
NPK	Nitrogen-Phosphorous-Potassium

Abbreviations	Definitions
NSERC	Natural Science and Engineering Research Council of Canada
PCA	Principal Component analysis
PCR	Polymerase Chain Reaction
PGPR	Plant growth promoting rhizobacteria
PI	*Paxillus involutus*
PP	*Pseudomonas putida*
PPSRTC	Politique de protection des sols et de réhabilitation des terrains contaminés
R	Pearson correlation coefficient
R^2	Coefficient of determination
RCB	Randomized complete block
RFLP	Restriction fragment length polymorphism
RR	*Rhizobium radiobacter*
RuBP	Ribulose-1,5-bisphosphate
SAS	Statistical Analysis System
SSFA	Specific surface foliar area
ST	*Suillus tomentosus*
TN	Trecesson nursery
TS	*Tricholoma scalpturatum*
WR	Waste rocks

To all human beings who have been negatively affected by ecological degradation,
I dedicate this research work

Acknowledgements

I would like to begin by thanking the love of my life, Stéphanie Bisson, who has always encouraged me during the whole process of completing this master's degree even if I was working 12 hours a day and seven days a week on a regular basis. I would also like to thank my parents, Robert Nadeau and Solange Beaudoin, who have given me the opportunity to return and stay at the family house for two years so that I can start my professional career without any debts. Furthermore, I would like to show my appreciation to the Natural Sciences and Engineering Research Council of Canada (NSERC) for its financial support and for nominating me as a recipient of the prestigious Alexander Graham-Bell Scholarship. Likewise, I would like to express gratitude to my research director, Dr Damase Khasa, who has believed in and given me the opportunity to fulfill this ambitious research project, and who has financially supported me throughout the completion of my graduate studies. Finally, I would like to say thanks to all my colleagues at the Centre for Forest Research (CEF) and at the Institute of Integrative and Systems Biology from Université Laval, who have contributed in some way to the completion of this venture.

Foreword

In this research project, Dr. Damase Khasa has provided the scientific direction and support for the completion of all three studies. The study on the ectomycorrhizal fungal community structure (chapter 1) and the glasshouse experiment (chapter 3) were planned by Martin Beaudoin Nadeau and Damase Khasa. Martin did all the field, laboratory, and glasshouse work with the help of assistants and wrote the two manuscripts. The manuscript associated with the ectomycorrhizal fungal community study has been prepared for submission in the scientific journal ‘’New Phytologist’’. On the other hand, the manuscript related to the glasshouse experiment has been prepared for submission in the scientific journal ‘’Tree Physiology’’.

The *in vitro* selection study (chapter 2) was planned by Martin Beaudoin Nadeau, Aida Azaiez, and Damase Khasa. Martin carried out the solid medium experiment while Aida executed the liquid medium experiment. The manuscript was written by Martin and Aida. They are both considered co-authors who have equally contributed to the completion of this study. Martin wrote the sections on introduction, fungal isolation method, solid medium experiment, some results and discussion. Aida wrote the abstract, the conclusion, and the liquid medium experiment, some results and discussion. The manuscript has been prepared for submission in the scientific journal ‘’Mycorrhiza’’.

1. General introduction

Natural resource exploitation is a very important industrial activity in Canada. It promotes economic growth and provides access to essential and fundamental resources needed for the wellbeing of human civilization. However, this industrial activity often disturbs and destroys natural ecosystems as we can see on open mining sites where the soil becomes very rocky, poor, without organic matter and often contaminated by heavy metals [46]. Today in Canada, the mining companies have the responsibility to revegetate the abandoned mine tailings after exploitation, which is considered to be very challenging due to poor soil conditions in which not too many plant species can grow [46]. In the province of Quebec, the mining act since 1995 requires that all mining companies must perform, in accordance with the management and restoration plan approved by the minister, all work necessary for land restoration as soon as the mining activities have been completed. Practices currently used in Quebec in order to restore mining sites and revegetate mine tailings come from the agricultural sector where the soils are prepared mechanically, hydro-seeded with grasses and/or legumes, and fertilized for several years in order to improve soil conditions before planting trees [60]. The big problem with this technique is that grasses and legumes tend to compete strongly against tree seedlings once they are planted in the field. Furthermore, these practices are very expensive; therefore, it is very important for our society to find cheaper methods for the rehabilitation of abandoned mine tailings.

The use of alders (*Alnus incana* ssp. *rugosa* and *Alnus viridis* ssp. *crispa*) and commercially valuable tree species (*Picea glauca* and *Pinus banksiana*) were proven to be very effective in the revegetation of degraded land near large hydroelectric dams in Quebec [43] and on oil sand tailings in Alberta [34,37]. Alder trees form root nodules in symbiotic relationship with actinomycetes of the genus *Frankia*, which gives them the ability to fix atmospheric nitrogen thereby increasing its availability in soil for other plants [48]. Alder trees also form a symbiotic root relationship with ecto- and endomycorrhizal fungi [48]. On the other hand, white spruce (*Picea glauca*) and jack pine (*Pinus banksiana*) form a symbiotic root relationship with ectomycorrhizal fungi [9]. Mycorrhizal fungi secrete organic acids such as oxalic acids that increase the mineralization process of rock tailings and mobilize important mineral elements (P, K, Ca, N, Mg, Mn, Zn, Cu, Al, and Fe), which are generally

unavailable to plants [33]. In addition, these mycorrhizal fungi produce enzymes that give them the ability to use the organic form of nutrients, amino acids, proteins, peptides, amino sugars, and nucleic acids, which are usually not available to plants, and thereafter, transfer them to plant roots [33]. For these reasons, the use of ecological restoration techniques involving tree root inoculation with mycorrhizal fungi is very promising [40,41], but, for now, we have very little knowledge about the role and utility of these fungi in the revegetation of gold mine rock tailings.

1.1 Literature review

1.1.1 Principles of ecological restoration and bioremediation

1.1.1.1 Ecological restoration

Ecological restoration is the practice of renewing and restoring, within a short period of time, degraded, damaged, or destroyed natural ecosystems and habitats that were altered by industrial human intervention and action [26]. This practice is an intentional activity used to promote and accelerate the recovery of altered ecosystems with respect to its health, integrity, and sustainability [26]. It includes a wide range of projects such as erosion control, reforestation, use of genetically local native species, removal of non-native species and weeds, revegetation of disturbed land, reintroduction of native species, and habitat improvement for certain species [26]. Restoration activities are thought to be complementary to conservation efforts because it is assumed that environmental degradation and population decline are somewhat reversible processes where human intervention can be used to promote ecosystem recovery of habitat, biodiversity, and services [16].

Ecological restoration involves two important ecological concepts: disturbance and succession. Disturbances, which are a change in environmental conditions, tend to alter species composition and functioning of natural ecosystems and often reduce available ecosystem services (human benefits supplied by natural ecosystems) [26]. Succession is the process by which the species of a community changes over time [26]. Following a disturbance, an ecosystem generally progresses, over few generations, from simple organization of a very few species to a more complex community with many interdependent species [26]. Late-successional stages tend to generate a wider range of ecosystem services

than early-successional stages [26]. In many ecosystems, communities tend to recover quite easily and quickly following mild to moderate natural and anthropogenic disturbances and restoration efforts are usually not needed in these situations [26]. However, an ecosystem that experiences more severe disturbances with physical and chemical alteration of the environment often require intensive restoration efforts to recreate environmental conditions that favour ecosystem succession and recovery [26]. Like Choi [16] mentioned, ecological restoration is essential for the survival and wellbeing of both humans and nature in the industrialized society we are living. This research project will focus on the use of genetically local native species for the revegetation of land disturbed by industrial mining activities in the boreal forest of Canada.

1.1.1.2 Bioremediation

One of the most important concepts in ecological restoration of ecosystems altered by mining is bioremediation. Tailings left after mineral extraction often contain high concentration of heavy metals. These heavy metals in high concentration are known to cause soil contamination and degradation and tend to negate plant growth and survival. Bioremediation is the use of living organisms to manage and restore contaminated soils [61]. These living organisms can restore contaminated soils by degrading, stabilizing, extracting or transforming polluting substances in soil [61]. According to the U.S. Environmental Protection Agency (EPA), bioremediation is the use of microbes (bacteria, yeast, fungi), algae and plants to break down or degrade toxic chemical compounds that have accumulated in the environment into less toxic or non-toxic substances.

Phytoremediation is one of the bioremediation technique, which can be applied for the treatment of contaminated soils, sludges, sediments and ground waters through contaminant removal, degradation or stabilization and which includes technically different operation procedures, such as phytostabilisation, phytodegradation, phytoextraction, and rhizofiltration [50]. Phytostabilisation aims at establishing a plant cover by the use of tolerant plants that help to reduce the mobility and toxicity of organic or inorganic pollutants through adsorption or precipitation and, at the same time, may increase soil fertility and improve plant establishment [61]. Phytostabilisation reduces contaminant transfer to other ecosystem compartments and in the food chain [61]. Some plant species have the capacity to convert

harmful substances into useful sources of nutrients or take up harmful organic contaminants and degrade/transform them into neutral ones [50]. This phenomenon is called phytodegradation and the plants usually chelate the contaminants in the soil in inactive forms using secreted organic compounds [50,61]. Phytoextraction refers to the removal of contaminants in soil and the storage of these contaminants in plant tissues by species that have the ability to hyperaccumulate metals/metalloids in their shoots and roots [61]. These plants can sequester contaminants in their cell walls or vacuoles away from the sensitive cell cytoplasm where most metabolic processes occur [50]. They can also store the pollutants in their tissues after transporting them into specialized cells and cell compartments [50]. Phytoremediation provides a wide range of advantages over other mechanical and chemical restoration practices: the restoration costs are way cheaper, plants require little care, plants absorb CO_2 instead of producing it, plants do not require the consumption of a huge quantity of fossil fuels, and plants have the potential to yield a wide variety of commercially valuable products such as biomass and timbers [50].

Only a small portion of plant species possess the tolerance and resistance characteristics needed to be used in phytoremediation [35]. Genetic variation within a plant species can also play a very important role in the ability of plant to grow in harsh contaminated environments [35]. Some genes in plants are known to control the resistance and tolerance of plants to contaminated soils [35]. Therefore, some individuals and varieties within a species have better growth and survival success in polluted soils than others. Plant breeding and selection for heavy metal tolerance may be a promising field for the restoration of abandoned mining sites. When we look at the restoration of ecosystems located in the temperate or boreal regions, not too many local native species of trees can be considered for their use in revegetation of mine tailings due to low species richness. The tolerance or resistance of temperate or boreal plant species to the harsh soil conditions of the mine tailings can be improved with the association between commercially valuable tree species and their microsymbionts (mycorrhizal and/or nitrogen-fixing symbionts). Phytobial remediation is based on the use of plants and their associated microorganisms to remove, stabilise, or detoxify pollutants. Mycorrhizoremediation, is a phytobial remediation technology in which plants are associated with their mycorrhizal fungal symbionts to restore and revegetate disturbed land [30]. Mycorrhizal fungi can immobilise metals/metalloids in soil by taking up

the elements and accumulating them in their biomass via intracellular sequestration, by precipitating them, or by absorbing them onto their chitin cell walls [61]. They can also efficiently explore the soil micro-pores that are not accessible to plant roots due to their relatively small size, impede soil contaminant transport through increased soil hydrophobicity, and protect plant roots from direct interaction with the pollutant via the formation of ectomycorrhizal sheath [61]. This study will focus on the role and utility of mycorrhizal fungi in the revegetation of gold mine tailings located in the Canadian boreal forest.

1.1.2 Traditional use of agricultural practices, trees, and organic amendments

In the past, most mine tailing restoration efforts have focused on agricultural practices such as fertilization, soil mechanical improvement, hydro-seeding of herbaceous species and use of organic amendments. Warman [57] studied the success of different herbaceous species and fertilizers in the revegetation of lead-zinc mine tailings in Nova Scotia, Canada. In a potting experiment and in the field, the growth of 12 different grass and legume species (alfalfa (*Medicago sativa*), buckwheat (*Fagopyrum esculentum*), couchgrass (*Agropyron repens*), ladino clover (*Trifolium repens*), meadow fescue (*Festuca elatior*), orchard grass (*Dactylis glomerata*), red clover (*Trifolium pratense*), reed canary grass (*Phalaris arundinacea*), annual ryegrass (*Lolium multiflorum*), sweet clover (*Melitotus offinalis*), timothy (*Phleum pratense*), and yellow foxtail (*Setaria glauca*)) in mine tailings was evaluated with or without fertilizers [57]. It was found that the use of fertilizers every year was essential (high level of N-P-K) for plant growth because none of the 12 species was able to mature without fertilization [57]. Hydro-seeding was very efficient with fertilizers and was also a lot cheaper than transplanting herb seedlings [57,60]. Alfalfa, couchgrass, and red clover were the three most successfully introduced species capable of revegetating the tailing site [57,60].

Furthermore, Renault *et al.* [45,46] studied seed germination and seedling survival of different herbaceous species planted on gold mine tailings of central Manitoba (AU) Minesite. In greenhouse, seeds of Indian mustard (*Brassica juncea*), white mustard (*Sinapis alba*), slender wheatgrass (*Agropyron trachycaulum*), altai wildrye (*Elymus angustus*), reed canary grass, creeping foxtail (*Alopecurus arundinaceus*), streambank wheatgrass

(*Agropyron riparium*), and tall fescue (*Festuca alatior*) were seeded on tailings without peat, mixed with peat, and mixed with peat and sand (1:1) [45,46]. Indian mustard, white mustard, tall fescue seeds had the highest germination rates, but survival after three months was low [46]. Addition of peat to the tailings increased significantly germination rates and greatly improved seedling survival rate [45]. Tall fescue and reed canary grass were the two species that produced the highest biomass when grown on tailings with peat [45]. In the field, a peat layer of 5 cm was added on the tailing surface and red-osier dogwood (*Cornus stononifera*), yellow willow (*Salix lutea*), white spruce (*Picea glauca*), jack pine (*Pinus banksiana*), tamarack (*Larix laricina*), and bog birch (*Betula glandulosa*) were planted on site [45]. Seedling survival rate was relatively low in treatments without peat [46]. Most tree seedlings were able to survive in treatments with peat [45]. Tamarack and white spruce had the highest survival rate followed by bog birch and jack pine [46]. In the field again, seeds of the same herbaceous species used in greenhouse experiment were hydro-planted on tailings with and without peat [46]. Wheatgrass species had the highest survival rate followed by tall fescue and altai wildrye on all treatments, but it was quite low [46]. Indian mustard and white mustard were able to survive on tailings with peat [45].

Giasson *et al.* [23] evaluated the impact of arbuscular mycorrhizal fungi (AMF) on the extraction of different heavy metals (As, Cd, Zn, Se, Pb) in contaminated soil similar to the ones found on many mining sites. A grass mixture of *Festuca rubra* (35%), *F. eliator* (35%), *Agropyron repens* (25%), and *Trifolium repens* (5%) with five different treatments of arbuscular mycorrhizal fungi (*Rhizophagus irregularis Funneliformis mosseae, Claroideoglomus etunicatum, Gigaspora gigantean*, and no AMF) was grown on heavy metal contaminated soil in greenhouse in order to identify the best AMF for metal extraction [23]. This type of grass mixture is currently used to revegetate mine tailings in Eastern Canada [23]. No fertilizers were added during the experiment. It was found that 30 to 70 % of grass roots in the mycorrhizal treatments were colonized [23]. There was variation in metal translocation to plants among AM fungi [23]. Plants inoculated with *R. irregularis* had the highest extraction [23]. The four species of AM fungi significantly increased plant survival, growth, and metal extraction [23].

Soil organic matter plays an important role in plant growth and nutrition. Microorganisms use it as a carbon source and food [10]. As they break down the organic matter through decomposition, they release excess nutrients (N, P, K, etc.) into soil in forms available to plants [10]. This process of decomposition is called mineralization and is considered the heart of ecosystem nutrient cycling [10]. Soil organic matter is composed of humic and non-humic substances. 5 to 25 % of the organic matter is made of non-humic substances. These substances are nutritional carbohydrates that are easily decomposed by microorganisms [56]. They tend to promote aggregate formation and better soil structure because of their capacity to bind to inorganic soil particles [10]. Humic substances, on the other hand, are decay resistant by-products of the decomposition of organic matter [56]. They have variable electromagnetic charges [56]. Their negative charges from the carboxyl groups (-COOH) ease the storage of positively charged nutrient cations, which helps to retain nutrients available to plants at all-time improving fertilizer efficiency [10,56]. These negative charges can also promote the storage of considerable quantities of positively charged toxic pollutants contributing to the reduction of plant toxicity caused by excess trace metals [10,56]. Furthermore, the addition of organic matter is beneficial to plants because it tends to increase soil water retention and infiltration capacity, buffer soil pH, protect soil against erosion, reduce evaporation and negate plant desiccation from the soil surface, supply nutrients for plant uptake and soil biological activity, and ameliorate soil microbial diversity. The application of organic amendments is often utilized in order to improve soil organic matter content.

Many different organic amendments have been incorporated on mine tailings in order to improve soil conditions and increase success rate of restoration activities. Some organic and inorganic amendments that have been used in the past include sewage and paper sludge, compost [19,57], peat [45], lime [44], limestone, and sawdust [46]. Organic amendments are known to improve plant survival on tailings through the incorporation of a layer of organic matter that can furnish important nutrients to plant, improve rooting medium, facilitate vegetation establishment, and ameliorate fertility, humidity and temperature of soil surface [19]. Coninck and Karam [19] tested the growth and survival of maize (Zea mays) on tailings, amended with compost (made of peat moss and shrimp wastes) and a chelating solution (EDTA), in the Gaspé Copper mine in Quebec. The addition of compost significantly

increased both shoot and root biomass of maize [19]. Chelators also had a positive effect on plant biomass but not as much as the compost treatment [19]. The height of maize plant was lower in tailings treated with chelators alone compared to tailings amended with both compost and chelators [19] suggesting that the use of both organic amendments together was beneficial to plant growth.

In the mining industry, it exists many different mine closure methods. Reid *et al.* [44] outlined the tailing site management and closure methods that were developed for a copper zinc underground mine located in Quebec (Canada). Two options are usually considered during mine operation: tailing may be sent to a disposal area or used for backfilling of the mine [44]. The first option is generally chosen due to its cheaper cost [44]. Three methods are currently used for the mine closure phase [44]. In the first one, tailings are kept submerged in water to limit oxygen contact with heavy metals and sulphidic minerals. This method is not environmentally acceptable because it does not fix the problem of contamination. In the second option, the top one metre of tailings is desulphurized in order to decrease environmental contamination and the tailing surface is then stabilized with 30 cm of granular soil [44]. This method is better but it is very expensive to add granular soil over the whole tailing area. In the last and more environmentally friendly method, the tailing disposal site is reclaimed using three layers of geological materials: a supporting layer of waste rock from the mine, a low permeability layer of silt, and a protective layer of organic soil and grass seeding in order to limit erosion [44]. This method is the most interesting of the three, but it is very expensive to find and transport all the required substrates over the tailings. It is our responsibility to find a cheaper phytotechnology for the restoration of these disturbed ecosystems.

1.1.3 Sigma-Lamaque mine and the Abitibi-Temiscamingue's boreal forest

Sigma-lamaque complex is a gold mine located in the city of Val d'Or in the Abitibi region of the province of Quebec, Canada. Exploitation of this mining site started in 1935. Since then, mineral extraction has been realized in both above- and underground forming an open pit, a pile of waste rocks, and a basin full of fine grinded tailings. The day mineral exploitation will be completed, 150 ha of waste rocks and fine tailings will have to be restored and revegetated.

Tailings of the Sigma-Lamaque mine in Val d'Or is mainly composed of biotite [55]. Biotite from gold deposits in Val d'Or is associated with pyrite and pyrrhotite, and tends to be rich in Fe [55]. Biotite is a phyllosilicate mineral within the mica group with the chemical formula $K(Mg,Fe)_3AlSi_3O_{10}(F,OH)_2$. Iron, magnesium, aluminium, silicon, oxygen, and hydrogen form silicate sheets that are weakly bound together by potassium ions. The mineral pyrite is an iron sulfide with the formula FeS_2. On the other hand, the mineral pyrrhotite is an unusual iron sulfide with a variable iron content: $Fe(1-x)S$ ($x = 0$ to 0.2). They are all very rich in iron. In Sigma-Lamaque mine, aluminum content in the biotite tends to be constant among sampled sites while iron and magnesium are highly variable [55]. Sigma-Lamaque mine contains two types of biotite: hydrothermal and metamorphic [55]. The two types of biotite show almost the same chemical composition [55]. However, hydrothermal biotite is a little richer in Al and Na, and poorer in Ti and K than metamorphic biotite [55].

In 2010, Group Roche Inc. hired the company COREM for analyzing the tailing element composition of Sigma-Lamaque mine (unpublished document). The samples showed higher sulfur content (0.48 to 0.51%) than the standard (0.3%) set by Quebec policies on soil protection and rehabilitation of contaminated land (PPSRTC). However, it is not considered a potential acid generator. Arsenic (As) presented concentrations (8 to 9 mg/kg) higher than the PPSRTC standard of 5 mg/kg. Total cyanides had also concentrations (3.7 to 6.3 mg/kg) higher than the PPSRTC standard of 2 mg/kg. Nevertheless, the mine tailings are considered to have low risk of contamination. Metal concentration of Al (5500 to 6100 mg/kg), Ca (21000 to 23000 mg/kg), Fe (14000 to 16000 mg/kg), and Mg (4000 to 4500 mg/kg) was found to be quite high. Furthermore, tailings also contain inorganic P (0 to 560 mg/kg), K (86 to 100 mg/kg), and other mineral elements important for plant growth in low concentrations such as Zn, Mn, Cu, Mo, and Na. Sources of nitrogen (NO_3^-, NO_2^-, or NH_4^+) were absent. No sources of phosphate were identified. The pH of tailings was relatively alkaline with values varying between 8.55 and 8.68. The only important element not present in waste rocks and fine tailings is nitrogen. All the other elements could potentially be transferred to plants by microbial weathering and absorption as we will see in the following sections.

1.1.3.1 Vegetation zone and bioclimatic domain

Sigma-Lamaque mine is located in the Balsam fir – white birch bioclimatic domain. This domain occupies the southern portion of the boreal forest [47]. Forest stands are mainly composed of balsam fir (*Abies balsamea*), white spruce (*Picea glauca*), and white birch (*Betula papyrifera*) on mesic sites [47]. Black spruce (*Picea mariana*), jack pine (*Pinus banksiana*) and tamarack (*Larix laricina*) are often found on less favourable sites with trembling aspen (*Populus tremuloides*) [47]. Forest dynamics are controlled primarily by the spruce budworm due to the high abundance of balsam fir in the forest stands [47]. However, forest fire is also an important factor and tends to form pure stands of jack pine [47].

1.1.3.2 **White spruce** *as a candidate for ecological restoration*

Picea glauca (Moench) Voss, known as white spruce, is one of the most commercially valuable and planted tree species in Quebec boreal forest [54]. It is used primarily for pulpwood and as lumber for general construction [32,36]. White spruce has a broad native distribution across Canada from Newfoundland to Yukon and grows from sea level to about 1520 m of altitude [32,36,54]. It has been described as a plastic species because of its ability to repopulate rocky areas at the end of glaciation [36]. It grows under highly variable conditions, including extreme climates and soil conditions [36]. Therefore, it has a substantial potential to be used in revegetation of mine tailings. White spruce grows on a wide variety of soils (glacial, lacustrine, marine, and alluvial origin) and rock formations (granites, gneisses, sedimentaries, slates, schists, shales, and conglomerates) [32,36]. It can thrive on both acid and alkaline soils but tends to reach its best growth at pH between 4.7 and 7.0 [32,36]. This species tolerates relatively well low fertility conditions, but other conifer species such as jack pine and tamarack are usually more tolerant [36]. White spruce seeds show dormancy that can be overcome by stratification (usually three weeks at 4°C) or prechilling [36]. Optimal germination temperatures range from 10 to 24°C [36]. Optimal conditions for seedling growth in greenhouse are alternating temperature of 25°C/20°C (day/night) with light intensity of 400 lux (5.56 uE m^{-2} s^{-1}), 16 hours/day [36]. A photoperiod less than 14 hours causes growth cessation while 16 hours procure continuous free growth [36]. After the first growing season, natural growth may be between 10 and 20 mm tall with

a root system of 20 to 100 mm long depending on site conditions [36]. White spruce is highly sensitive to transplanting shock [36]. This species has shallow roots and around 85% of the root biomass is usually found within the first 30 cm of the soil [32]. Only a few species of fungi forming mycorrhizas have been found on white spruce [36]. White spruce is relatively tolerant to shade; thereby well suited to be planted with shade intolerant tree species such as poplars and alders [36]. Slow initial root growth makes young seedling particularly susceptible to frost in the first three years of life [28,36]. White spruce populations are highly variable genetically over its range. The variation pattern is clinal and generally follows latitudinal gradients [36]. Soil-related adaptive variation has been demonstrated in the past [36]. Seedling plantation must adhere to seed zoning and seed transfer rules due to the strong adaptive affinity of white spruce to local environment [36]. Transfer of provenances more than 3° latitude may be very detrimental to seedling growth and survival (provenance not adapted to local conditions) [36]. Large difference exists in intraspecific genetic variation among individual trees [36], which makes the species a great candidate for genetic improvement through tree selection and breeding programs.

1.1.3.3 Other boreal tree species potentially useful in ecological restoration of mining sites

1.1.3.3.1 Jack pine

Pinus banksiana Lamb., known as jack pine, is a medium-sized coniferous tree [12]. It is a commercially valuable species found in the province of Quebec and an important source of pulpwood, lumber, and round timber [9]. Jack pine is an early-successional species and invades quite easily areas where mineral soil has been exposed by major disturbances such as fires [12]. For this reason, it has a tremendous potential to be used in the revegetation of mining sites. Jack pine is usually found on sandy soils of the spodosol and entisol soil orders [12]. It can also grow on loamy soils, on thin soils over granites and metamorphic rocks of the Canadian Shield, on limestones, and on peat moss [12]. However, it does not grow naturally where the soil surface is alkaline. It can grow on calcareous soils (pH ≈ 8.2) in association with alkaliphilic ectomycorrhizal fungi [12]. Jack pine seed usually germinates within 15 to 60 days under favourable conditions, but some seeds require more than 100 days to germinate [12]. Optimal conditions for survival and seedling establishment are supplied by mineral soil and burned seedbeds where competition from other vegetation is not severe

and water table is high [12]. Jack pine is one of the most shade-intolerant trees in its native range. Therefore, it may be better to plant it in pure stand and avoid mixing it with alders or white spruce seedlings. Under forest conditions, seedling growth is slow during the first three years (15-25 cm), and increases rapidly the subsequent years (30-90 cm after four years) [12]. Jack pine frequently forms a taproot as a seedling and maintains it through maturity [12]. The seedling root system under natural conditions usually penetrates to a depth of 13 to 25 cm in soil in the first growing season [12]. The roots may penetrate more than 2.7 m on deep in well-drained soils [12]. Severe drought may kill many seedlings particularly on coarse soils [12]. Jack pine seedlings are susceptible to various rust fungi that can cause growth loss and mortality [12]. The wide genetic variation of Jack pine suggests that it has a large effective breeding population [12]. Most provenance tests demonstrated that trees from provenances close to the planting sites grow usually better than those from geographically distant provenances [12].

1.1.3.3.2 Speckled alder and Green alder

Speckled alder (*Alnus incana* ssp. *rugosa* (Du Roi) J. Clausen) is a tall shrub (3-8 m tall) found all around Canada, mainly in the boreal forest [27]. It usually grows along streams, rivers, and wetland; however, it does not necessarily require moist soil and can grow on dry shallow stony sites. Speckled alder is often mixed with black spruce (*Picea mariana*) and white cedar (*Thuja occidentalis*). It is shade intolerant and grows very quickly even on poor soils. On the other hand, green alder (*Alnus viridis* ssp. *crispa* (Ait.) Turill) tends to grow on dry upland sites with jack pine [27]. It is the most widespread tall shrub (3-12 m tall) found in the Canadian boreal spruce-fir forest [27]. Green alder tolerates shade and drought better than any of the other alder species [27]. It also grows well on poor soils [27]. Green alder is known for colonizing avalanche chutes in mountains where larger trees that compete with it are killed regularly by avalanche damage. It survives the avalanches through its ability to regenerate from roots and broken stumps [27]. Therefore, it may be a very good candidate for the restoration of open mining sites with steep slope.

Both alder species are often used in the revegetation of non-fertile soils, which they enrich by means of nitrogen fixing actinomycetes from the genus *Frankia* found in their root nodules [38]. The actinomycetes *Frankia* obtains most of its carbon source directly from the

alder [48]. In exchange, alder trees acquire 70 to 100% of its nitrogen from the *Frankia* symbionts [48]. They have shallow root system and form vigorous stump and root suckers when cut or damaged [27]. They can also form a symbiotic relationship with both ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) fungi leading to the development of a symbiosis between four different organisms: Alder-*Frankia*-ECM-AM [48]. Alder species tend to develop a closer and more frequent association with ECM fungi compared to AM fungi [48]. However, this association is only formed with a small number of ECM fungal species (high specificity, mainly species of the *Alpova* genus) [38]. These mycorrhizal fungi play an important role in the enhancement of alder nutrition (essentially P and N) [48]. Therefore, mycorrhizal fungi can help alders to acquire phosphorous needed for nitrogen fixation [48]. The use of alders has been a success story for the rehabilitation of many disturbed sites in the past due to its rapid growth, easy establishment, huge litter production, and excellent nitrogen fixation capacity that makes it available to other plants [38,48]. For these reasons, green alder and speckled alder have a tremendous potential to be used for the revegetation of gold mine tailings.

1.1.4 Woody plant nutrient uptake in mineral soils

Mineral nutrients are known to play many roles in woody plants: elements in plant tissues, regulators of osmotic potential, important constituents of buffer systems, activators of enzyme activities, and regulators of membrane permeability [31]. Nutrients can only be absorbed by tree roots as ions dissolved in water [51]. There are two mechanisms involved in plant uptake of mobile ions: (1) movement of ions with mass flow of water absorbed by roots and (2) diffusion of ions in soil solution to regions of depletion near the rhizosphere [51,59]. The acquisition of immobile ions by roots is accomplished through the growth of roots into new soil surfaces, which leads to the interception of exchangeable ions that were adsorbed by organic and mineral colloids [51,5]. The predominating mechanism of plant uptake is determined by the mobility of ions in soil solution and the rate at which they are taken by woody plants [59]. Macronutrients needed in large quantities for plant growth include N, P, K, Ca, Mg, and S [59]. For their part, micronutrients (Fe, Zn, Cu, Mn, B, Cl, and Mo) are only needed in small quantities [57]. Their ionic forms (cations or anions) in soil consist of NH_4^+, NO_3^-, $H_2PO_4^-$, HPO_4^{2-}, K^+, Ca^{2+}, Mg^{2+}, SO_4^{2-}, Fe^{3+}, Zn^{2+}, Cu^{2+}, Mn^{2+},

$H_2BO_3^-$, HBO_3^{2-}, BO_3^{3-}, Cl^-, and MoO_4^{2-} [56]. Calcium is usually an immobile ion and needs to be intercepted by fine roots in order to be absorbed [59]. Magnesium, sulfur, and iron ions are generally taken by roots through mass flow [59]. Plant demand for N, P, and K are often greater than delivery by mass flow [59]. Therefore, diffusion is the dominant mechanism in this case [59]. Phosphate ions are more immobile than potassium and nitrogen ions in most soils, which may limit greatly its supply to plant root by diffusion [59]. Phosphorous uptake is often increased by successful mycorrhizal formation within root tips, especially in soils with low P content [51]. Ion uptake is controlled within the membrane of root cells, requires energy obtained from photosynthesis, and intensifies as the concentration of ions increases in the soil solution until it reaches saturation [59]. Nutrient requirements vary considerably among species [59].

Fine root tips are the main plant tissue involved in nutrient and water absorption [51]. Moisture in soil plays an important role in nutrient uptake by root tips. Mass flow of mobile ions to root tips is proportional to the rate of plant transpiration [51]. Diffusion of ions from soil solution to the surface of root tips increases as the number of pores filled with water augments in soil [51]. Lack of water in soil reduces both the rate of plant transpiration and the number of pores filled with water, which triggers the reduction of both the rates of diffusion and mass flow to roots [51]. Therefore, under low soil moisture, plant nutrient acquisition and growth is limited by slower rates of both nutrient transport and uptake in soil [51]. When water is widely available in soil, woody plants tend to grow faster with a dense root system containing a high number of fine root tips, which maximize nutrient uptake [51]. Roots have the ability to modify their rhizosphere by secreting enzymes and protons in response to nutrient deficiency [51]. These secretions increase nutrient availability in soil, especially immobile ions, thereby, enhancing plant nutrient uptake [51].

The availability of one nutrient element in soil can affect the ability of plants to uptake other elements by changing the accessibility of the other elements in soil and interacting with them during the uptake process [51]. Some ions are known to have greater affinity to binding sites than others [51]. Uptake of ions with low affinity can be suppressed considerably by ions that have higher affinity to binding sites. For example, uptake of Mg^{2+}, which has low affinity to binding sites, by roots is often greatly suppressed by the presence of excess Ca^{2+}

and K^+ in soil [51]. Furthermore, the uptake of one specific element can affect the uptake of other elements through its effects on plant growth, on mycorrhizal development, and on root morphology, permeability, and distribution [51]. For example, if nitrogen uptake is enhanced and leads to an increase in plant growth, uptake of other elements will also be favoured if they are available in enough quantity in soil [51]. However, under this circumstance, the concentration of the other elements may decrease considerably in plant tissues due to faster growth caused by the improved N supply [51].

Nutrient availability to plants is determined by soil conditions, climate, and genetic variability within and among species [56]. In soil, nutrient availability depends mainly on soil mineral composition, texture, organic matter, cation exchange capacity, and pH [56]. In forest ecosystems, more than 80 % of the Ca, Mg, K, and P input come from the weathering of parent material [59]. Mineral soil is mainly composed of oxides, carbonates, and primary and secondary silicates [56]. Fe and Al oxides affect the availability of P and certain trace elements such as Cu, Zn, Mo, and B in soil [56]. In the presence of Fe^{2+} and Al^{3+}, there is an ionic exchange (non-specific adsorption) between oxides and P ions and then phosphorous molecules become largely transformed in insoluble inorganic complexes causing a P deficiency in plant foliage [56]. Carbonates weather quite easily and liberate great quantities of Ca^{2+} and Mg^{2+} in soil [56]. Apatite is the main source of inorganic phosphorous in mineral soil [59]. Generally, nutrient availability in forest ecosystems is greatly influenced and even dependent on the presence of primary and secondary sillicates [56]. Quartz is the simplest silicate mineral; it is very resistant to chemical weathering, and has no effect on nutrient availability because it does not contain any important nutrient element in its matrix [56,59]. On the other hand, other primary silicates contain various cationic elements (Al^{3+}, Ca^{2+}, Na^+, K^+, Mg^{2+}, etc.) and trace metals (Fe, Mn, etc.) substituted in their crystal lattice [56]. They are less stable and more prone to chemical weathering [56]. They include micas (biotite, etc.), feldspars, olivine, pyroxene, amphibole, and hornblende [56,59]. They are an important source of Ca, Mg, K and Fe for plant nutrition [56]. Weathering releases ions available to plants and this process forms secondary minerals [59]. Unlike primary silicates, secondary silicates (montmorillonite, vermiculite, and kaolinite) are not a direct source of nutrients to plants, but instead they play a major role in nutrient storage through the adsorption of positively charged ions that bind to negatively charged mineral colloids [56].

The rate at which nutrients are released from primary minerals is considerably affected by soil texture as it dictates the amount of surface area exposed to weathering [56]. For example, feldspars minerals have little values as nutrient source when they are found in the form of sand and gravel, but they become significant contributors of K and Ca ions in the form of clay [56]. Soil organic matter also significantly contributes to nutrient availability to plants (See section 1.1.2 for more information) [56]. Higher cation exchange capacity (CEC) increases nutrient availability to plants [56]. CEC represents the quantity of exchangeable cations present in soil; it is related to the quantity of negative electrical charge displayed by soil mineral and organic constituents and depends on the amount of clay minerals and organic matter found in soil [56]. CEC increases as the pH rises because the concentration of H^+ in soil decreases leaving more adsorption sites for exchangeable cations [56]. The rate of cation adsorption mainly depends on the nature of the cation and the concentration of the different cations in soil solution [56]. At equal concentrations, the rate of cation adsorption is: $Al^{3+} > Ca^{2+} > Mg^{2+} > K^+ = NH_4^+ > Na^+ = H^+$ [53]. For anions at equal concentrations, the rate of absorption on positively charged colloids is: $PO_4^{-3} > SO_4^{-2} > Cl^- > NO_3^-$ [59].

Nutrient availability may be affected by a change in pH (Figure 1.1). Changing the pH alters the balance between soluble and insoluble form of mineral salts in soil [51]. Furthermore, it may release some new ions in soil solution triggering a transformation in the solubility of other ions [51]. This phenomenon is caused by the competition for membrane binding sites between H^+ and other cations at low pH and between OH^- and other anions at high pH [51]. When a pH change suppress cation or anion uptake by plants, uptake of ions with opposite charge is enhanced [51]. Low pH has a negative effect on the activity of soil heterotrophic microorganisms, which are known for degrading organic matter and releasing nutrients for reuse by plants [56]. Furthermore, low pH inhibits the activity of nitrifying bacteria reducing the production of NO_3^- [56]. Nitrogen ions dominate in the form of NO_3^- at pH > 5.5 and NH_4^+ at pH < 5.5 [53]. The availability of K, Ca, and Mg decreases with decreasing pH due to the loss of effective CEC [56]. At pH > 8.5, Ca and Mg ions are replaced from exchange sites by Na^+ and lost in soil by leaching [56]. The availability of P also decreases with decreasing pH because Fe and Al ions react with $H_2PO_4^-$ to form compounds of very low solubility [56]. P ions are in the form of $H_2PO_4^-$ at pH between 3 and 6, HPO_4^{2-}

at pH > 6, and something $CaH_2PO_4^+$ at neutral pH [56]. Fe, Mn, Zn, and Cu ions are more available in soil solution for plant uptake at pH < 5.5 [56].

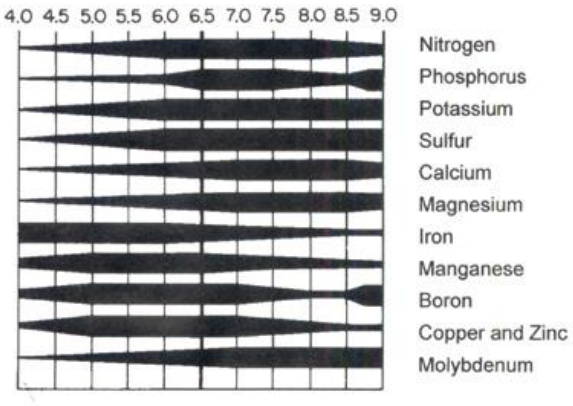

Figure 1.1: The effect of pH on nutrient availability (larger line means higher nutrient availability) (Source: http://www.ext.colostate.edu/mg/gardennotes/images/222-2.jpg).

In root cells, nutrients move with water between apoplast, symplast, and cytoplasm until it reaches the endodermal layer [56]. The endodermal layer gives roots control over the rate of assimilation and the type of materials that enters the vascular system [56]. Thus, it regulates the transport of materials from roots to shoot controlling plant tissue nutrient composition and it protects these plant tissues against the entry of excess of toxic elements [56]. Excessive nutrient uptake of even the most essential nutrient elements can be detrimental to plant growth and survival [31]. Uptake of trace metals such as Cu, Fe, and Zn in small amount is important for plant growth, but excess is very toxic to plant tissues [31]. This is the reason why plants need to control their nutrient uptake in root cells. Mineral deficiency is another problem that can limit woody plant growth and it often occurs in soils with very high and low pH or in rocky soils containing very high or low concentrations of certain elements [31]. One common symptom of mineral deficiency or toxicity to plants is the loss of healthy green foliage caused by the breakdown of chlorophyll and the interference with their synthesis [31]. Tree species differ significantly in their ability to absorb nutrient elements and their capacity to tolerate excess or limited supply of these elements [31].

Nutrient translocation in plant tissues is highly associated with the osmotic movement of water [56]. Sometimes, under low salt roots, ions can still be transported to plant tissues

without the entry of water, but it is usually not an important source of nutrients to plants [56]. Plants accumulate ions in their root cells where they are transported to xylem cells and then toward shoot tissues [56]. In the xylem, solutes containing nutrient ions and water move upward by mass flow and then the ions and amino acids are unloaded by xylem parenchyma cells in specific plant tissues [56]. This unloading process requires energy from photosynthesis [56]. In leaves and needles, water is removed by transpiration, which tends to concentrate solutes [56]. Once ions reach their destination, they are utilized by cells for growth and development [56].

The rate at which nutrients are taken up by roots is closely related to root surface and root geometry [56]. It depends greatly on total fine root biomass and morphology, the ability of root transport system to accumulate nutrients rapidly in poor soils, and translocation efficiency within plant cells [56]. Many trees have the ability to grow in nutrient limited ecosystems and the selection of individuals with roots capable of more effective nutrient uptake and efficient use of accumulated nutrients will be very beneficial for the revegetation of mine rock tailings. Tree genetic improvement programs could be developed in order to select tree varieties with bigger root system, more effective symbiotic associations, greater tolerance to extreme conditions, faster and more selective rates of nutrient accumulation, more effective distribution and reutilization of nutrients, and finally improved translocation system [56].

1.1.5 Ecology of ectomycorrhizal fungi and their role in plant nutrition

Ectomycorrhizal (ECM) fungi evolved 225 million years ago [18]. It has been estimated that 7750 species of fungi (of the subdivisions Basidiomycotina, Ascomycotina and Zygomycotina) form ECM symbioses. However, based on estimates of knowns and unknowns in macromycte diversity, a final estimate of ECM species richness would likely be between 20000 and 25000 [49]. ECM fungi have been well adapted to rocky substrates for a very long time and have permitted the colonization of trees on new environments such as mountain bedrocks [18]. Associated with bacteria, they have developed the ability to alter minerals in order to acquire essential elements (P, K, Ca, Mg, Fe, S, Mn, Mo, Cu, Zn, B and Cl) for their development [18]. Elements such as P, Ca, and K tend to be rarely found under ionic form in soil [18]. ECM fungi associated with bacteria are able to extract these important

elements from the rock and transform them into soluble forms improving the availability of these elements in soil for plant nutrition [18]. Although ECM plant partners (phytobionts) represent only about 8000 species or about 5% of vascular plants (mostly in the families Pinaceae, Betulaceae, Fagaceae, Dipterocarpaceae, Salicaceae and Myrtaceae), these species are of global importance because of their disproportionate occupancy and domination of terrestrial ecosystems in boreal, temperate and subtropical forests [52]. The symbiosis Tree-ECM fungus is very complex. A single plant may associate with many different ECM fungal species while a single fungus may connect with many plants at the same time [1]. Most ECM fungal species can form a symbiotic association with a broad range of tree species [18]. They may even form mycorrhizas with both angiosperm and gymnosperm species [18]. This finding suggests that it might be possible to inoculate an ECM fungal strain on a different tree species than the one in which it was isolated from. ECM fungi can live months to years within root tips [1].

These ECM fungi do not colonize cortical root cells but rather form an intercellular interface, consisting of highly branched hyphae forming a latticework between epidermal and cortical root cells, known as the Hartig net [18]. Furthermore, they form an extracellular layer of mycelium called fungal mantle (sheath), which surrounds the tips of fine roots [18]. Transfer of materials between fungus and plant take place in the Hartig Net [33]. The mantle protects fine roots from pathogens and toxic environments and all materials must go through the fungus first before reaching the root cells [18]. The association plant-ECM is a mutualistic relationship because ECM fungi obtain most of its carbon from their host plant in exchange to soluble nutrients and minerals [52]. ECM mycelia growing in soil are smaller than tree fine roots, so they can reach areas and micro-pores where roots cannot grow [52]. Therefore, ECM fungi have access to a bigger water and nutrient pool (better absorbing surface) in soil than plants. When low P and N concentrations limit plant photosynthesis, there is an excess of carbon in plants [52]. At this moment, fungal hyphae explore soil for both P and N and transport them into plant roots in exchange for excess plant C (energy as simple sugars and amino acids) [52]. Photosynthetic rate depends mainly on N (for RuBP carboxylate), P (for ATP and ADP), Fe and Mg (for chlorophyll), water (to keep stomata open in order to fix CO_2), and internal CO_2 content [52]. ECM fungi can create a C sink in plants by increasing P and N uptake which enhances photosynthesis [52]. ECM fungi can also improve plant water

uptake opening stomata in the process, which increases again photosynthesis [52]. ECM mycelia play an important role in water absorption and transport to plants in soil [52]. Some species form rhizomorphs (hyphal aggregates) that can transport water and nutrients to roots over long distances in soil [1,22]. These species are usually more effective in transporting water to their host [1]. ECM fungi can reduce the negative effects of drought on plants by furnishing water, not accessible to roots, to plants [1].

Furthermore, ECM fungi can acquire K and P in saline soil and transfer them to plants in order to balance high levels of Na [1,52]. They can also improve the Ca:Mg ratio in bedrock [1,52]. This means that ECM fungi can render available to plants the most limited elements in soil, which may improve the overall plant growth and survival. It has been proven that roots colonized by ECM fungi are a lot more efficient in taking up P, N, Zn, Cu, Ni, S, Mn, B, Fe, Ca, and K from soil than roots without their symbionts, especially in low fertility soil [1,52]. External hyphae contribute up to 25% of N, 80% of P, and 10% of K absorbed by plants [1,52]. ECM fungi influence plant nutrient uptake through enzyme production and mineral alteration [22]. On the basis of enzyme production, ECM fungi mobilize nutrients from organic N and P, amino acids, peptides, proteins, amino sugars, chitin, and nucleic acids that were unavailable to plants [22]. Most of the P content in forest soils is only found in organic forms such as phytates, nucleic acids, and phospholipids [1,52]. ECM fungi produce a wide variety of enzymes called phosphatases that degrade and transform organic P into inorganic forms available to plants [1,52]. When N sources are absorbed by the fungus, it is rarely transported in the same form to host plant [1,52]. NO^{-3} is transported directly to plants, but NH_4^+ cannot be transported within the fungal tissue because it is toxic. For that reason, NH_4^+ is transformed into glutamine and this amino acid can be transferred through fungal tissues to plant roots [1,52]. Certain ECM species, such as *Thelephora americana* and *Pisolithus tinctorius*, are specialized in acquiring NH_4^+ by scavenging large areas in soil while others, such as *Hebeloma crustuliniforme* are adept at acquiring organic N [1]. Proteins and amino acids containing N can also be taken up by ECM fungi, transformed into non-toxic forms and then finally transferred to the plant for nutrition [18].

Soil disturbances and vegetation succession may change ECM fungal community structure in forest ecosystems [18]. Several ECM fungal species prefer to grow in the organic

layer while others thrive mostly in the mineral layer of the soil [18]. On the other hand, certain species are generalists and can grow in a wide variety of soil [18]. Clearcutting and stand replacing fire tend to change ECM fungal community through selection pressure favouring species more adapted to early-successional stages [18]. Allen *et al*. [2] have studied the AM fungal communities in both early- and late-successional forest stands in Mexico and their potential use in the restoration of forest stands disturbed by high intensity fire. Early-successional AM fungal species were dominated by small-spored *Glomus* spp. while late-successional species were composed mainly of large-spored Gigasporaceae [2]. Early-successional AM fungal inoculum gave the highest height growth on all tree species tested, even for the late-seral tree species [2]. In some cases, late-successional inoculum produced smaller trees than the treatment without inoculum [2]. This study suggests that early-successional AM fungi should be used for ecological restoration activities even for late-seral tree species because they are more adapted to the extreme conditions of degraded ecosystems and may induce better plant growth. ECM fungi may act in similar ways and form different early- and late-successional communities. It would be interesting to test survival and growth of tree seedlings inoculated with ECM fungi, which came from different early- versus late-successional ecosystems, on mine tailings.

1.1.6 Role of ectomycorrhizal fungi in weathering

Fungi have the ability to penetrate solid materials through physical and chemical mechanisms [25]. Their hyphal tips tend to produce organic anions and protons when growing which help to break down weak spots in solid rock [25]. The production of organic anions has substantial carbon costs. Because mycorrhizal fungi can receive high amount of carbon from the host plant, they are the most important living organism involved in weathering [25]. Fungal hyphae usually grow following scratches, ridges, and grooves, and penetrating cracks, pores, and tunnels that were formed in rocks during previous abiotic weathering [25]. The expansion and contraction of fungal hyphae during wetting, drying, freezing, and thawing periods can accelerate physical weathering of rocks [25].

Fungi produce two different groups of chemical weathering agents: proton-based and ligand-based agents [25]. Proton-based agents (carbonic acids) are produced right alongside the fungal hyphal tips and ECM mantle [25]. Ligand-based agents take into account organic

anions, siderophores, and other polyphenolic acids [25]. Low molecular weight organic acids belong to both groups and are the most important agents involved in biological weathering [25,33]. They include oxalic, citric, and malic acids [29,33]. These acids tend to bind easily to metal cations (such as Al^{3+}, Fe^{3+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, and Cu^{2+}) reducing free cation activity in the soil solution which leads to a decrease in the saturation state and promotes further mineral dissolution and weathering [29]. More than 50% of the respiration in soil is done by mycorrhizal fungi [29]. The dissolution of respiratory CO_2 in water generates carbonic acids, which leads to a decrease in pH and thus increases mineral solubilisation and weathering [29,33]. Uptake of ammonium by ECM fungi also leads to soil acidification due to excess cation uptake that causes an efflux of H+ [29]. Weathering caused by the decrease in pH increases cation exchange capacity and concentrations of soluble and exchangeable K^+, Ca^{2+}, and Mg^{2+} in soil improving plant nutrition [29]. Weathering performance varies greatly among ECM fungal species because some species produce more organic acids than others [29]. Nutrient release has been positively correlated to oxalic acid concentration in soil [29]. Therefore, ECM fungal species that produce a lot of oxalic acids might be more efficient in transferring nutrients to plant roots than others.

Recent works have demonstrated that ECM fungi have the capacity to alter minerals such as apatite, feldspath, biotite, and hornblende in order to obtain essential minerals for their development [6,53,58]. ECM fungi associated with plants are able to increase weathering of minerals composed of silicate [5]. In this process, P, K, Ca, Mg, and Fe are extracted by the ECM fungi from the minerals under soil nutrient limitation [5]. Weathering rates depend mainly on pH and the production of organic acids by soil microorganisms tends to decrease pH in the rhizosphere [5]. Biotite is an important source of K, Mg, and Fe for plants [5]. Reducing pH under 7 increases the dissolution rate of biotite [5]. The release of K^+ in biotite increases rapidly with decreasing pH [5]. The dissolution of the octahedral layer (Mg_2^+ and Fe_2^+) occurs at pH between 3 and 7 and tends to also increase with decreasing pH [5]. The tetrahedral layer (Al_3^+ and Si_4^+) does not dissolve much with pH above 4 [5]. Therefore, important nutrient elements become available to plants as the pH decreases in soil containing biotite. Sugar promotes fungal production of organic acids [5]. Balogh-Brunstad *et al*. [5] studied biotite weathering by the ECM fungal species *Suillus tomentosus*. Fungal treatments caused a rapid increase in acidity and pH dropped between 2 and 3 units in four

weeks [5]. Fungal activity significantly increased by almost 3-fold Mg_2^+ and Fe_2^+ release rates in the biotite [5]. Channels were formed in biotite in the fungal treatments while no channel was formed in non-fungal treatments [5].

1.1.7 Ectomycorrhizal fungal community response to heavy metal in soil

Weathering by ECM fungi often triggers the formation of secondary minerals [25]. Secondary minerals include metal oxalates, iron and aluminum oxides, and carbonates [25]. These minerals play a very important role in the immobilization of excess calcium and toxic heavy metals [25]. As stated earlier, nutrients and water must go through the ECM fungus first before entering root cells [18]. This phenomenon can be quite useful in soil that contains high toxicity of certain elements, because the ectomycorrhizal fungal mantle can protect plant roots against metal toxicity [18,20]. ECM fungi can absorb toxic elements in soil and sequester them in their cell walls or vacuoles, which reduces considerably the toxicity of heavy metals to plants [30]. However, heavy metals in high concentration can also decrease ECM fungal development (growth and survival) in soil. *Suillus luteus* growth on inoculated jack pine and white spruce seedlings was significantly reduced by the addition of heavy metals in soil [30]. ECM fungal formation on white spruce roots was almost completely eliminated with high levels of Pb, Ni, Cd, and Cu in soil [30]. ECM fungal formation on jack pine was significantly reduced by concentrations of Ni and Cd above 10 ppm [30]. Yet, root and shoot biomass of ECM-inoculated white spruce and jack pine seedlings was relatively greater than non-inoculated seedlings [30]. It is also important to not forget that heavy metal tolerance can vary greatly among ECM fungal species and strains [30]. There is high intraspecific variation in the sensitivity of ECM fungi to toxic metals such as Al, Zn, and Cd [13,17]. Some strains are more adapted and tolerant to high concentration of toxic elements than others [13,17]. For that reason, it is very important to identify ECM fungal species and strains that are adapted to toxic conditions of soils in which revegetation activities have to be carried out.

1.1.8 Ectomycorrhizal diversity in boreal forest

The boreal forest is known to have very high ECM fungal species diversity. Buée *et al.* [11] have identified between 600 and 1000 different uncultured fungi (between 249 and 408 taxonomic groups) in each of the forest soil samples they studied by pyrosequencing

analyses. ECM fungal species from the orders Boletales, Agaricales, Thelephorales, Russulales, Cantharellales, and Sebacinales were the most abundant species in the analyzed boreal forest soil samples [11]. Species from genera such as *Cortinarius, Tomentella, Thelephora, Russula, Lactarius, Clavulina, Descolea*, and *Laccaria* were present in all communities studied and they may be considered as generalists because of their capacity to colonize most boreal ecosystems [11]. Gagné *et al.* [22] found that ECM fungal species richness in different clearcut plantations in Alberta was considerably similar (11 to 13 species found on tree roots on each site). The community had relatively high diversity, but again the variability among sites was quite low because many species were found in all plantations [22]. Kernaghan *et al.* [29] discovered that fertilizers may decrease considerably ECM fungal species diversity on seedling roots in nurseries. It may also decrease root colonization by ECM fungal species such as *Amphinema byssoides* that are known to improve seedling growth and health [29]. In a study conducted in the Laurentian mountain forests of Quebec, Villeneuve *et al.* [57] demonstrated that the highest ECM fungal dependency and diversity was found in the Balsam fir - white birch forest, but difference was not significant showing that ECM community richness in Quebec Laurentian forests does not vary much among stands. ECM fungal community was dominated by species belonging mainly to the families Cortinariaceae and Russulaceae [57]. Diversity tends to decrease because of disturbances [57]. Mature forests contain usually both generalist and specialist species, while disturbed ecosystems are mainly dominated by generalists such as *Paxillus involutus* and *Laccaria lacata* [57]. For the ecological restoration of disturbed gold mine tailings, it will be important to identify ECM generalist fungal species that are adapted to the disturbed site.

ECM fungi not only have high species diversity, but they can also show considerably high genetic diversity within species. This high genetic diversity is often displayed by intraspecific physiological variation among ECM fungal strains [13,32]. Different strains of the same species can have different temperature and pH optimums for growth [13]. Furthermore, some strains may produce more mycelia and rhizomorphs than others [13]. Lamhamedi and Fortin [32] found that mycelial development (biomass) varied greatly among *Pisolithus* sp. strains. The authors suggested that mycelial production was under genetic control [32]. ECM fungal strains of the same species may also exhibit variations in their ability to colonize host plant roots. It was demonstrated that some dikaryons of *Laccaria*

bicolor have a better capacity to form Hartig net between root cortex cells and mantle sheaths around fine roots than others [13]. It is believed that ECM fungal colonization is controlled by the production of hormones and the activity of enzymes, which varies greatly within ECM fungal species and is also probably under genetic control [13]. Strains may vary in their ability to stimulate host plant growth [13]. Furthermore, they may differ in their ability to improve the availability of nutrient in soil and protect the host plant against water stress [13]. Mycelial strands are the main channels for the translocation of water and nutrients to host plants [32]. Nutrient and water uptake tends to be greater in ECM fungal strains that produce mycelial strands in large quantities [28]. Furthermore, NH_4^+ absorption and organic acid production abilities can vary significantly among ECM fungal strains [13]. Therefore, some ECM fungal strains may be more efficient in altering minerals in soil than others because they can release higher content of organic acids. For all these reasons, it may be important to identify and use strains that are adapted to abiotic and biotic conditions of the restoration site in order to maximize inoculation benefits for plant growth and survival.

1.1.9 Use of trees and their symbionts in mine reclamation: previous studies in the Canadian boreal forest

The extraction of bitumen from tar sands involves the removal of the organic layer (made of muskeg peat), and the deep geological overburden layer (first mineral soil layer) [7]. Many studies have been conducted in Alberta on the potential use of trees and their symbionts for the restoration of disturbed tar (oil) sand tailings. These oil sand tailings are formed by the extraction of the bitumen from the tar sand and tend to create saline-alkaline sites with high content of sodium, sulfate, and calcium [28]. Kernaghan *et al.* [28] studied the growth of many different ECM fungal species native to Canadian boreal forest in water released by the tailings and in medium with different levels of alkalinity, under aseptic laboratory conditions, in order to identify promising fungi for the reclamation of oil sand tailings. It was found that the ECM fungal species *Suillus brevipes, Rhizopogon rubescens*, *Paxillus involutus*, and *Amphinema byssoides* were very sensitive to alkaline soils and were not able to growth with the presence of water released by oil sand tailings [28]. On the other hand, different strains and species of *Laccaria* and *Hebeloma* in addition to *Wilcoxina mikolae* were more tolerant to alkaline treatments and had better overall growth in the presence of tailing-released water than the other species [28]. *Laccaria* strains displayed the

highest mycelial growth and the authors suggested that these species may be excellent candidates for use in rehabilitation of degraded saline-alkaline sites [28]. These *Laccaria* strains were collected from roots of white spruce, tamarack, Douglas-fir, jack pine, lodgepole pine, and balsam fir trees [28]. *Hebeloma* strains came from roots of white spruce, lodgepole pine, and Norway spruce trees [28]. It would be interesting to study to use of *Laccaria* and *Hebeloma* species for the revegetation of gold mine tailings in the boreal forest of Quebec.

Bois *et al.* [7] investigated the mycorrhizal status of pure reclamation materials and revegetated tailing sands from the Canadian oil sand industry. This study is the first crucial step in the use of microbial inoculants in the real world [39]. The authors found that the composite tailing sands material was deprived of active mycorrhizal propagules while all other materials showed some level of inoculum potential (tailing sands, deep overburden, muskeg peat, three reclaimed sites) [7]. Arbuscular mycorrhizal fungi were observed on roots of clover and poplar. Pine roots were also colonized by vesicle forming hyphae of an unidentified fine endophyte and by dark septate fungi [7]. Ectomycorrhizas were observed on both pine and poplar [7]. Using morpho-molecular analyses, six ectomycorrhizal (ECM) fungi were identified to the genus or species level: *Laccaria* sp., *Thelephora americana* Lloyd, *Wilcoxina* sp. (E-strain), *Tuber* sp. (I-type), a Sebacinoid, and a Pezizales species. Fungi of the genus *Laccaria* and *Wilcoxina* were the most frequently observed ECM species on the roots of jack pine and hybrid poplar [7]. The researchers indicated that planting grass species such as barley many years before tree planting may favour the development of AM fungi over ECM fungi and explain why the ECM status was low on conifer species of the reclaimed sites [7]. Because of poor status of ECM on disturbed sites, the study suggested the need to inoculate tree seedlings with suitable mycorrhizal fungi before outplanting in order to improve their survival and growth.

Furthermore, Bois *et al.* [8] compared *in vitro* growth development of two salt-resistant ECM fungal strains (*Laccaria bicolor* and *Hebeloma crustuliniforme*) identified by Kernaghan *et al.* [19] with three ECM fungal species (*Suillus tomentosus*, *Hymenoscyphus* sp., and *Phialocephala* sp.) that were isolated from Syncrude's oil sand tailings in Alberta, Canada. This experiment was done on modified Melin-Nokrans medium (MMN) containing different concentrations of NaCl [8]. The authors found that the two Ascomycota species

(*Hymenoscyphus* sp. and *Phialocephala* sp.) were more resistant to NaCl treatments than the other three species known as basidiomycetes [8]. *L. bicolor* had the highest decrease in growth and biomass yield of the three basidiomycota species under increasing NaCl concentration [8]. *H. crustuliniforme* was the most resistant of the three basidiomycota species to water stress while *S. tomentosus* showed the best biomass yield under all NaCl treatments [8]. This study suggests that native strains of ECM fungi growing naturally on oil sand tailings may be better adapted and resistant to the extreme conditions and yield better growth on disturbed oil sand tailings than exotic species from natural forests.

In another research, Bois *et al.* [9] looked at the effects of ECM fungal inoculation on white spruce and jack pine seedlings grown in salt-affected oil sand tailings in greenhouse. The authors inoculated the seedlings with three different ECM fungal strains (*Hebeloma crustuliniforme*, *Laccaria bicolor*, and *Suillus tomentosus*) that were selected for their tolerance to high salinity in soil [9]. It was found that inoculation improved considerably the growth and decreased stress caused by salinity in both white spruce and jack pine seedlings [9]. The highest biomass production was encountered on white spruce seedlings inoculated by *S. tomentosus* [9]. These seedlings were also the least affected by saline treatments [9]. Thus, it was suggested that white spruce seedlings inoculated by *S. tomentosus* may be the most suited treatment for the revegetation of saline oil sand tailings in Alberta [9]. Once again, better growth results were obtained from ECM fungi that came directly from the disturbed sites. In treatments where salinity was not too high (< 200mM NaCl), high biomass production was also obtained with trees inoculated by *L. bicolor* [9]. Other results showed that white spruce seedlings inoculated with *H. crustuliniforme* were not affected negatively and biomass yield was not reduced significantly by salinity treatments compared to the controls [9]. It was also discovered that sodium accumulation was higher in jack pine seedlings compared to white spruce indicating better salinity tolerance in white spruce seedlings [9].

Onwuchekwa *et al.* [37] examined the effect of white spruce and jack pine seedling inoculation with ECM fungi on their growth and survival on oil sand tailings, in a field trial. Seedlings were inoculated with *Hebeloma crustuliniforme*, *Suillus tomentosus*, and/or *Laccaria bicolor* [37]. The inoculation increased the growth rate of both tree species, but this

increase was greater on jack pine [37]. White spruce and jack pine seedlings inoculated with *H. crustuliniforme* showed the greatest increase in height growth rate compared to controls [37]. Inoculation did not affect the survival of jack pine seedlings [37]. On the other hand, white spruce seedlings inoculated with *S. tomentosus* only and with treatments of mixed ECM fungal species (HC+ST, HC+LB, ST+LB, and HC+ST+LB) had a greater significant survival than controls [37]. These results demonstrated very well the importance of ectomycorrhizal association on white spruce and jack pine for seedling survival and growth on oil sand tailings.

Lefrançois *et al.* [34] evaluated the effect of growing *Frankia*-inoculated alders on soil quality of oil sand tailings in Alberta. Biomass and nitrogen content of alder seedlings were measured to assess the impact of *Frankia* inoculum on alder growth and health [34]. The plantation of *Frankia*-inoculated alders on the oil sand tailings improved considerably soil quality after two years through the increase of both organic matter content (leaf litter) and cation exchange capacity in soil [34]. Furthermore, growth of alders with its *Frankia* symbiont led to a decrease in soil pH [34]; thereby, reducing the negative effect of salinity on plants (less Na^+ content in soil). The process of decreasing pH is thought to be linked to the nitrification activity associated with nitrogen fixation by the actinomycetes *Frankia* [34]. Improvement of soil conditions by alders and its *Frankia* symbiont demonstrated that this symbiotic interaction can create a better environment for plant growth in extreme site conditions of degraded ecosystems through nitrogen fixation (nitrogen is usually not present on these degraded land) and fast accumulation of organic matter (leaf litter) on soil surface. *Frankia*-inoculated alders also had a positive impact on native microbial community activity [34]. This study suggests that alders associated with its *Frankia* symbiont are able to grow and perform well without the addition of fertilizer in harsh and nutrient-limiting environment. They have a tremendous potential in the restoration and rehabilitation of temperate degraded ecosystems. Quoreshi [39] has proposed a flow chart for the use of microbial (fungal and bacterial) inoculants in the real world (Figure 1.2).

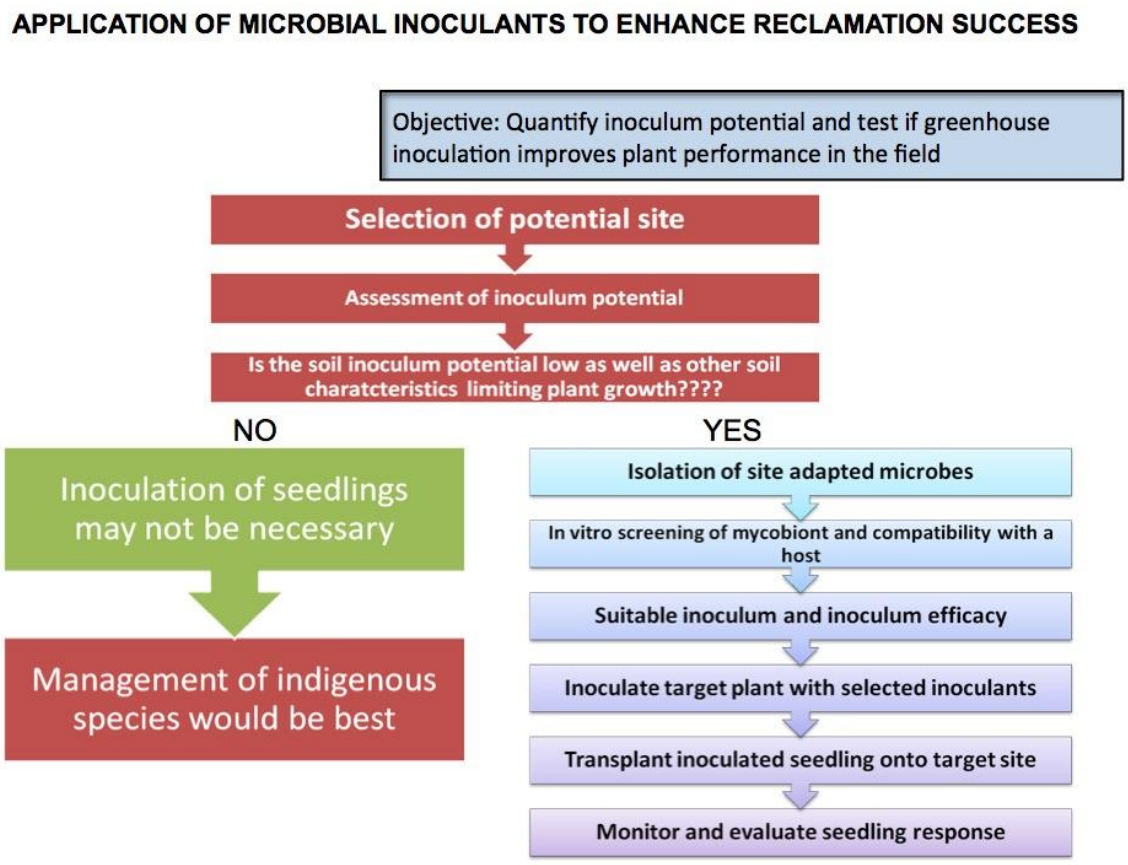

Figure 1.2 Flow chart for the use of microbial inoculants in the real world.

1.1.10 Role of free-living soil bacteria in plant growth and nutrient

Soil bacteria have been used in agricultural practices for many decades and they have tremendously helped enhancing plant productivity worldwide [24]. Free-living soil bacteria that are beneficial to plant growth are commonly referred to as plant growth promoting rhizobacteria (PGPR) [24]. These PGPR can promote plant growth by synthesizing particular compounds, such as hormones, beneficial to plants, easing nutrient uptake by plants, and preventing plant diseases [15,24]. Free-living N-fixing bacteria such as *Azotobacter* and *Azospirillum* species have showed the ability to increase yield of many crops (rice, cotton, wheat, etc.) [24]. It is thought that this increase in yield is associated with enhanced root development thanks to the increase in biological N_2 fixation and water and mineral uptake [24]. Other free-living diazotrophic bacteria sometimes capable of N_2 fixation include species of the genus *Acetobacter, Bulkholderia, Enterobacter, Citrobacter*, and *Pseudomonas* [24].

Phosphorous is one of the three most essential macronutrients for plant growth and development [24]. Bacteria from the genus of *Pseudomonas, Bacillus, Rhizobium, Azotobacter, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aerobacter, Flavobacterium,* and *Erwinia* are known to have the capacity to solubilize insoluble inorganic phosphate (tricalcium phosphate, dicalcium phosphate, hydroxul apatite, and rock phosphate) and render it available to plants [24]. PGPR have been used in bioremediation in the past in order to remove complex contaminants and heavy metals from iron, copper, silver, and uranium mines [24]. Important bacterial genus used in bioremediation includes *Bacillus, Pseudomonas, Methanobacteria,* and *Deinococcus* [24]. PGPR involved in plant pathogen suppression belongs especially to the genus *Bacillus* and *Pseudomonas* [24]. These bacterial strains have the ability to produce antibiotic and induce systemic resistance to diseases in plant host [24]. For example, *Pseudomonas* strains suppress fungal pathogens by producing antifungal metabolites and by sequestering iron in the rhizosphere rendering it unavailable to other organisms [24]. For all these reasons, PGPR play a very important role in plant health, growth, and nutrition.

Weathering of soil minerals is a very important nutrient source for trees in temperate and boreal forest ecosystems [14]. Calvarulo *et al.* [14] wanted to determine the contribution of tree roots and specific PGPR to mineral weathering. Scots pine seedlings (*Pinus sylvestris*) were grown with or without PGPR in a substrate containing quartz and biotite [14]. The three bacterial strains (*Bulkholderia glathei*) used in this experiment were isolated from the rhizosphere of an oak tree (*Quercus petreae)* symbiotically associated with the ECM fungal species *Scleroderma citrinum* [14]. The authors found that pine root inoculation with *B. glathei* significantly increase biotite weathering by 40% for Mg and 50% for K [14]. Furthermore, two of the three bacterial strains had a positive effect on pine growth and root size and this phenomenon was caused by improved plant nutrition [14]. Weathering rate of biotite was highly variable among *B. glathei* strains [14]. This study suggests that certain PGPR species have developed great abilities to weather soil mineral. Furthermore, some strains within the same species may be more effective in weathering and favouring plant growth and nutrition than others.

The effect of PGPR on coniferous tree growth in temperate forests has been fairly investigated in the last four decades. Many studies have demonstrated that tree seedling height and biomass were increased by the inoculation of *Arthrobacter spp*. and *Agrobacterium spp.* (for *Pinus sylvestris*), *Pseudomonas putida* (for *Pinus banksiana*), *Bacillus polymyxa* (for *Pinus contorta*), *Arthrobacter citreus* and *Pseudomonas fluorescens* (for *Picea mariana* and *Picea glauca*), *Pseudomonas putida*, *Hydrogenophaga pseudoflava*, *Bacillus polymyxa*, and *Staphylococcus hominis* (for *Picea glauca x engelmanii*) in laboratory, greenhouse and/or field trials [15]. In these studies, inoculated pine and spruce biomass was increased, on average, from 32 to 49 % after one growing season on reforested site [15]. Furthermore, certain PGPR strains (*Pseudomonas* and *Bacillus* species) have the ability to enhance considerably conifer root colonization by ECM fungi [15]. The inoculation of many bacterial strains from the genus of *Bacillus* and *Paenibacillus* on Loblolly pine (*Pinus taeda*) and Slash pine (*Pinus elliotti*) had mixed effect on seedling growth and biomass under greenhouse conditions [21]. Some strains improved seedling growth and biomass while others did completely the opposite [21]. These findings suggest that the effects of PGPR inoculation on tree growth are species-specific. In another study, many *Bacillus* and *Paenibacillus* strains were found thriving in high abundance in root tissues of lodgepole pine (*Pinus contorta* var. *latifolia*) and western red cedar (*Thuja plicata*) [3]. These two tree species are known for their ability to grow in soils severely limited in nitrogen sources [3]. Three *Paenibacillus* strains were showing abilities to fix atmospheric nitrogen [3]. Furthermore, in growth chamber experiments, inoculated pine seedlings were receiving 30 to 66% of their foliar N from bacterial N fixation [4]. Similar results were obtained with diazotrophic PGPR thriving inside *Suillus tomentosus/Pinus contorta* tuberculate ectomycorrhizae [42]. These findings suggest that some diazotrophic PGPR may favour coniferous tree growth and nutrition in N-poor soils through atmospheric N fixation.

1.2 Research objectives and hypotheses

The general objective of this research project was to develop a new green technology for the revegetation of abandoned gold mine tailings in the Canadian Abitibi boreal forest using specific ectomycorrhizal (ECM) fungi and plant growth promoting rhizobacteria (PGPR) in symbiotic relationship with white spruce seedlings (*Picea glauca* (Moench)

Voss), a commercially valuable coniferous tree species native to Canada. This tree species was chosen because some scarce healthy individuals were found to naturally regenerate on the mining site. The project was divided into three studies. The first study was an ECM fungal community study (chapter 1). Here, we aimed to investigate the structure of the ECM fungal community associated with roots of white spruce seedlings on four different locations – Trecesson nursery, mining site, forest edge, and natural forest – near Sigma-Lamaque gold mine in the Abitibi region of Canada. The second study was a laboratory experiment (chapter 2). In this study, we intended to evaluate, under axenic conditions, the *in vitro* growth of different ECM fungal species, isolated from the Sigma-Lamaque mining site or natural forest stands, on mine tailings and to select the most promising ones for further research. The third study was a glasshouse experiment (chapter 3). In this experiment, we aimed to *in vivo* study the role and importance of specific symbiotic microorganisms – ECM fungi and PGPR – in promoting the health, growth, and nutrition of white spruce seedlings on waste rocks and fine tailings of Sigma-Lamaque gold mine and to select the most efficient tree-symbiont combinations that could be used for field testing and large-scale reforestation program of Sigma-Lamaque rock tailings.

In this research, the following hypotheses were set up:

- ECM fungal community composition differs among the four sampled locations.
- Some ECM fungal strains associated with white spruce grow better than others on biotite-quartz rich mine tailings.
- Root inoculation of white spruce with specific symbiotic microorganisms – ECM fungi and PGPR – improves seedling health, growth, and nutrition on biotite-quartz rich mine tailings.

1.3 References

[1] Allen, M.F., W. Swenson, J.I. Querejeta, L.M. Egerton-Warburton, and K.K. Treseder. (2003). Ecology of mycorrhizae: A conceptual framework for complex interactions among plants and fungi. *Annual Review of Phytopathology. 41*: 271-303.

[2] Allen, E.B., M.F. Allen, L. Egerton-Warburton, L. Corkidi, and A. Gomez-Pompa. (2003). Impacts of early- and late-seral mycorrhizae during restoration in seasonal tropical forest, Mexico. *Ecological Applications 13*(6): 1701-1717.

[3] Bal, A., R. Anand, O. Berge, and C.P. Chanway. (2012). Isolation and identification of diazotrophic bacteria from internal tissues of *Pinus contorta* and *Thuja plicata*. *Canadian Journal of Forest Research 42*: 807-813.

[4] Bal, A. & C.P. Chanway. (2012). Evidence of nitrogen fixation in lodgepole pine inoculated with diazotrophic *Paenibacillus polymyxa*. *Botany 90*: 891-896.

[5] Balogh-Brunstad, Z., C.K. Keller, J.T. Dickinson, F. Stevens. C.Y. Li, and B.T. Bormann. (2008). Biotite weathering and nutrient uptake by ectomycorrhizal fungus, *Suillus tomentosus*, in liquid-culture experiments. *Geochimica et Cosmochimica Acta 72*: 2601-2618.

[6] Berner, C., T. Johansson, and H. Wallander. (2012). Long-term effect of apatite on ectomycorrhizal growth and community structure. *Mycorrhiza.* DOI: 10.1007/s00572-012-0438-y

[7] Bois, G., Y. Piché, M.Y.P. Fung, and D.P. Khasa. (2005). Mycorrhizal inoculum potentials of pure reclamation materials and revegetated tailing sands from the Canadian oil san industry. *Mycorrhiza 15*: 149-158.

[8] Bois, G., A. Bertrand, Y. Piché, M. Fung, and D. P. Khasa. (2006). Growth, compatible solute and salt accumulation of five mycorrhizal fungal species grown over a range of NaCl concentrations. *Mycorrhiza 16*: 99-109.

[9] Bois, G., F.J. Bigras, A. Bertrand, Y. Piché, M.Y.P. Fung, and D. Khasa. (2006). Ectomycorrhizal fungi affect the physiological responses of *Picea glauca* and *Pinus banksiana* seedlings exposed to an NaCl gradient. *Tree Physiology 26*: 1185-1196.

[10] Bot, A. & J. Benites. (2005). The importance of soil organic matter: key to drought-resistant soil and sustained food production. *Food and Agriculture Organization of the United Nations,* Rome, Italy.

[11] Buée, M., M. Reich, C. Murat, E. Morin, R.H. Nilsson, S. Uroz, and F. Martin. (2009). 454 pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. *New Phytologist 184*: 449-456.

[12] Burns, R.M. 1990. *Pinus banksiana* Lamb. Silvics of North America. Volume 1 Conifers. USDS, USA. Retrieved October 27th 2012.

[13] Cairney, J.W.G. (1999). Intraspecific physiological variation: implications for understanding functional diversity in ectomycorrhizal fungi. *Mycorrhiza 9*: 125-135.

[14] Calvaruso, C., M. Turpault, and P. Frey-Klett. (2006). Root-associated bacteria contribute to mineral weathering and to mineral nutrition in trees: a budgeting analysis. *Applied Environmental Microbiology 72*(2): 1258-1266.

[15] Chanway, C.P. (1997). Inoculation of tree roots with plant growth promoting soil bacteria: an emerging technology for reforestation. *Forest Science 43*(1): 99-112.

[16] Choi, Y.D. (2007). Restoration ecology to the future: a call for new paradigm. *Restoration Ecology 15*(2): 351-353.

[17] Colpaert, J.V., P. Vandenkoornhuyse, K. Adriaensen, and J. Vangronsveld. (2000). Genetic variation and heavy metal tolerance in the ectomycorrhizal basidiomycete *Suillus luteus. New Phytologist 147*: 367-379.

[18] Dahlberg, A. (2001). Community ecology of ectomycorhizal fungi: an advancing interdisciplinary field. *New Phytologist 150*: 555-562.

[19] De Coninck, A.S. & A. Karam. (2008). Impact of organic amendments on aerial biomass production, and phyto availability and fractionation of copper in a slightly alkaline copper mine tailing. *International Journal of Mining, Reclamation, and Environment 22*(4): 247-264.

[20] Dixon, R.K., C.A. Buschena. (1988). Response of ectomycorrhizal *Pinus banksiana* and *Picea glauca* to heavy metals in soil. *Plant and Soil 105*: 265-271.

[21] Enebak, S.A., G. Wei, and J.W. Kloepper. (1997). Effects of plant growth-promoting rhizobacteria on loblolly and slash pine seedlings. *Forest Science 44* (1): 139-144.

[22] Gagné, A, J. Jany, J. Bousquet, and D.P. Khasa. (2006). Ectomycorrhizal fungal communities of nursery-inoculated seedlings outplanted on clear-cut sites in northern Alberta. *Canadian Journal of Botany 36*: 1684-1694.

[23] Giasson, P., A. Jaouich, P. Cayer, S. Gagné, P. Moutoglis, and L. Massicotte. (2006). Enhanced phytoremediation: a study of mycorrhizoremediation of heavy metal-contaminated soil. *Remediation.* DOI: 10.1002.rem

[24] Hayat, R., S. Ali, U. Amara, R. Khalid, and I. Ahmed. (2010). Soil beneficial bacteria and their role in plant growth promotion: a review. *Annual Microbiology 60*: 579-598.

[25] Hoffland, E., T.W. Kuyper, H. Wallander, C. Plassard, A.A. Gorbushina, K. Haselwandter, S. Holmstrom, R. Landeweert, U.S. Lundstrom, A. Rosling, R. Sen, M.M. Smits, P.A.W. Van Hees, and N. Van Breemen. (2004). The role of fungi in weathering. *Frontiers in Ecology and the Environment 2*(5): 258-264.

[26] Jackson, L.L., N. Lopoukhine, and D. Hillyard. (1995). Ecological restoration: a definition and comments. *Restoration Ecology 3*: 71-75.

[27] Karolewski, P., J. Oleksyn, M.J. Giertych, R. Zytkowiak, P.B. Reich, and M.G. Tjoelker. (2008). Primary and secondary host plant differ in leaf-level photosynthetic response to herbivory: evidence from *Alnus* and *Betula* grazed by the alder beetle, *Agelastica alni. New Phytologist 140*(2): 239-249.

[28] Kernaghan, G., B. Hambling, M. Fung, and D. Khasa. (2002). *In vitro* selection of boreal ectomycorrhizal fungi for use in reclamation of saline-alkaline habitats. *Restoration Ecology 10*(1): 43-51.

[29] Kernaghan, G., L. Sigler, and D. Khasa. (2003). Mycorrhizal and root endophytic fungi of containerized *Picea glauca* seedling assessed by rDNA sequence analysis. *Mycrobial ecology 45*(2): 128-136.

[30] Khan, A.G. (2006). Mycorhizoremediation – an enhanced form of phytoremediation. *Journal of Zhejiang University SIENCE B 7*(7): 503-514.

[31] Kozlowski, T.T., P.J. Kramer, and S.G. Pallardy. (1991). The physiological ecology of woody plants. San Diego, California, USA. *Academic Press.*

[32] Lamhamedi, M.S. & J.A. Fortin. (1991). Genetic variations of ectomycorrhizal fungi: extrametrical phase of *Pisolithus sp.*. *Canadian Journal of Botany 69*: 1927-1934.

[33] Landeweert, R., E. Hoffland, R.D. Finlay, T.W. Kuyper, and N. Van Bremen. (2001). Linking plants to rocks: ectomycorrhizal fungi mobilize nutrients from minerals. *TRENDS in Ecology & Evolution 16*(5): 248-254.

[34] Lefrançois, E., A. Quoreshi, D. Khasa, M. Fung, L.G. Whyte, S. Roy, and C.W. Greer. (2010). Field performance of alder-*Frankia* symbionts for the reclamation of oil sands sites. *Applied Soil Ecology 46*: 183-191.

[35] Marmiroli, N. (2007). Genetic variability and genetic engineering in phytoremediation. *Advanced Science and Technology for Biological Decontamination of Sites Affected by Chemical Agents*: 89-108.

[36] Nienstaedt, H. & J.C. Zasada (1990). *Picea glauca* (Moench) Voss. Silvics of North America, Volume 1:Conifers. United States Forest Service, USA. Retrieved October 27th 2012.

[37] Onwuchekwa, N.E., J.J. Zwiazek, A. Quoreshi, and D.P. Khasa. (2014). Growth of mycorrhizal jack pine (*Pinus banksiana*) and white spruce (*Picea glauca*) seedlings planted in oil sands reclaimed areas. *Mycorrhiza*: DOI 10.1007/s00572-014-0555-x.

[38] Quoreshi, A.M., S. Roy, C.W. Greer, and J. Beaudin. (2007). Inoculation of green alder (*Alnus crispa*) with *Frankia*-ectomycorrhizal fungal inoculant under commercial nursery production conditions. *Native Plants Journal 8*(3): 271-281.

[39] Quoreshi**,** A**.**M**.** (2008)**.** The Use of Mycorrhizal Biotechnology in Restoration of Disturbed Ecosystem pp. 303-320. In: Siddiqui, ZA; Akhtar, MS; Futai K (2008**)** Mycorrhizae : Sustainable agriculture and forestry. *Springer*. Ottawa, Canada. 362 pp.

[40] Quoreshi, A.M. & D.P. Khasa. (2008). Effectiveness of mycorrhizal inoculation in the nursery on root colonization, growth, and nutrient uptake of aspen and balsam poplar. *Biomass and Bioenergy 32*: 381-391.

[41] Quoreshi, A.M., Y. Piché, and D.P. Khasa. (2008). Field performance of conifer and hardwood species 5 years after nursery inoculation in the Canadian prairie provinces. *New Forests 35*: 235-253.

[42] Paul, L.P., W.K. Chapman, and C.P. Chanway. (2013). Diazotrophic bacteria reside inside *Suillus tomentosus/Pinus contorta* tuberculate ectomycorrhizae. *Botany 91*: 48-52.

[43] Pregent, G., C. Camiré, J.A. Fortin, P. Arsenault, and J.G. Brouillette. (1987). Growth and nutritional status of green alder, jack pine, and willow in relation to site parameters of borrow pits in James Bay territory, Quebec. *Reclamation and Revegetation Research 6*: 33-48.

[44] Reid, C., V. Bécaert, M. Aubertin, R.K. Rosenbaum, and L. Deschênes. (2009). Life cycle assessment of mine tailings management in Canada. *Journal of Cleaner Production 17*: 471-479.

[45] Renault, S., E. sailerova, and M.A.F. Fedikow. (2001). Phytoremediation of mine tailings and bio-ore production: results from a study on plant survival at the central Manitoba (AU) minesite. *Trade and Mines, Manitoba Geological Survey*: 138-149.

[46] Renault, S., E. sailerova, and M.A.F. Fedikow. (2002). Phytoremediation of mine tailings and bio-ore production: progress report on seed germination, survival and metal uptake of seedlings planted at central Manitoba (AU) minesite. *Trade and Mines, Manitoba Geological Survey*: 255-265.

[47] Ressources Naturelles du Québec. (2012). Vegetation zones and bioclimatic domains in Québec. *Retrieved* October 30th 2012 from www.mrn.gouv.qc.ca/english/publications/forests/publications/zone-a.pdf

[48] Roy, S., D.P. Khasa, and C.W. Greer. (2007). Combining alders, frankiae, and mycorrhizae for the revegetation and remediation of contaminated ecosystems. *Canadian Journal of Botany 85*: 237-251.

[49] Rinaldi, A.C., O. Comandini, and T.W. Kuyper. (2008). Ectomycorrhizal fungal diversity: separating the wheat from the chaff. *Fungal Diversity 33*(1): 1-45.

[50] Saier, M.H. & J.T. Trevors. (2010). Phytoremediation. *Water Air Soil Pollution 205*(1): S61-S63.

[51] Smith, W.K. & T.M. Hinckley. (1995). Resource physiology of conifers: acquisition, allocation, and utilization. *Academic Press,* San Diego, California, USA.

[52] Smith, S.E. & D.J. Read. (2008). Mycorrhizal symbiosis, 3rd ed. *Academic Press*, London, UK.

[53] Smits, M.M., S. Bonneville, L.G. Benning, S.A. Banwart, and J.R. Leake. (2012). Plant-driven weathering of apatite – the role of an ectomycorrhizal fungus. *Geobiology 10*(5): 445-456.

[54] Sutton, R.F. (1973). Histoire naturelle de l'Épinette blanche (*Picea glauca* (Moench) Voss). *Ministère de l'Environnement, Service Canadien des Forêts*, Publication # 1250f, Ottawa, Canada.

[55] Taner, M.F., P. Trudel, and G. Perrault. (1986). Géochimie de la biotite associée à certains gisements d'or de Val d'Or, Malartic et Chibougamau, Québec. *Canadian Mineralogist 24*: 761-774.

[56] Van den Driessche, R. (1991). Mineral nutrition of conifer seedlings. *CRC Press*, Boca Raton, Florida, USA.

[57] Villeneuve, N., M.M. Grandtner, and J.A. Fortin. (1989). Frequency and diversity of ectomycorrhizal and saprophytic macrofungi in the Laurentide mountains of Quebec. *Canadian Journal of Botany 67*: 2616-2629.

[58] Wallander, H. & D. Hagerberg. (2004). Do ectomycorrhizal fungi have significant role in weathering of minerals in forest soils? *Symbiosis 37*: 249-252.

[59] Waring, R.H. & W.H. Schlesinger. (1985). Forest ecosystems: concepts and management. *Academic Press,* Orlando, Florida, USA.

[60] Warman, P.R. (1988). The Gays river mine tailing revegetation study. *Landscape and Urban Planning 16*: 283-288.

[61] Wenzel, W.W. (2009). Rhizosphere processes and management in plant-assisted bioremediation (phytoremediation) of soils. *Plant and Soil 321*: 385-408.

2. Chapter 1: Edaphic selection pressures as drivers of contrasting white spruce ectomycorrhizal fungal diversity and community structure in the Canadian boreal forest of Abitibi-Temiscamingue region

Martin Beaudoin Nadeau[1] and Damase P. Khasa[1]

1. Centre for Forest Research and Institute of Integrative and Systems Biology, Université Laval, Quebec city, QC, Canada, G1V0A6.

Keywords: Boreal forest, Community structure, Diversity, Ecological gradient, Ectomycorrhiza, *Picea glauca,* Soil chemical fertility

Article prepared for submission in the scientific journal ‘’New phytologist’’

2.1 Résumé

- Nous connaissons très peu les effets des pressions sélectives édaphiques sur la structure et la diversité des communautés de champignons ectomycorhiziens (ECM) associées symbiotiquement aux racines de l'épinette blanche (*Picea glauca*) en forêts boréales du Canada. Notre hypothèse principale était que la composition et la diversité des communautés diffère entre les quatres sites échantillonnés – pépinière, site minier, bordure de forêt et forêt naturelle – près de la mine d'or Sigma-Lamaque dans la région de l'Abitibi.
- La structure et la diversité des communautés ectomycorhiziennes ont été étudiées sur les quatre sites à travers des analyses morpho-moléculaires et phylogénétiques en relation avec les propriétés chimiques du sol rhizosphérique.
- 41 espèces de champignons ECM ont été identifiées. Le site minier avait une composition d'espèces significativement différente des trois autres sites. Les résultats ont démontré que le pH du sol et le pourcentage de racines colonisées par les champignons ECM augmentaient tandis que les concentrations de P, N, Fe, C et de cations échangeables (K, Mg, Ca et Na) diminuaient suivant le gradient écologique : pépinière → forêt naturelle → bordure de forêt → site minier.
- Contrairement à la préférence des sols acides par des champignons ectomycorhiziennes, quelques-uns écologiquement adaptés à des pH élevés, à une faible fertilité chimique du sol et à une faible teneur en matière organique colonisent les racines d'épinettes blanches sur le site minier à substrat non-acidogénique, permettant une régénération naturelle des semis d'épinette blanche. D'autres sont adaptés à un régime de fertigation élevé en pépinière commerciale. Cette étude montre clairement la différence contrastée de la structure et de la diversité ectomycorhiziennes suite aux pressions sélectives édaphiques.

2.2 Summary

- Little is known about the edaphic selection pressures as drivers of contrasting white spruce ectomycorrhizal fungal diversity and community structure in the Canadian boreal forest. We hypothesized that community composition and diversity differ among the four sites sampled – nursery, mining site, forest edge, and natural forest.

- Ectomycorrhizal fungal diversity and community structure was studied at the four locations through morpho-molecular and phylogenetic analyses in relationships with rhizospheric soil chemical properties.
- 41 different ECM fungal species were identified. Mining site had a significantly different ECM fungal species composition than the surrounding environments. Soil pH and percentage of roots colonized by ECM fungi increased while soil P, N, Fe, C, K, Mg, Al, Ca, and Na content declined across the ecological gradient: nursery → natural forest → forest edge → mining site.
- Contrary to the preference of acid soils by ECM fungi, a few ecologically adapted to high pH, poor soil chemical fertility, and low organic matter content colonize white spruce roots on the non-acidogenic mining site, allowing natural regeneration of white spruce seedlings. Other ECM fungi are adapted to high fertigation level of commercial nursery. This study clearly shows the contrasting difference in white spruce ectomycorrhizal fungal diversity and community structure driven by the edaphic selection pressures.

2.3 Introduction

Ectomycorrhizal (ECM) fungi have been an important component of temperate forest ecosystems since they first evolved more than 200 million years ago (Tedersoo *et al.*, 2010). In the boreal forest, they are symbiotically associated with fine roots of conifer tree species such as white spruce (*Picea glauca*), black spruce (*Picea mariana*), jack pine (*Pinus banksiana*), and balsam fir (*Abies balsamea*) but also with fine roots of various deciduous species such as trembling aspen (*Populus tremuloides*), green alder (*Alnus viridis*), speckled alder (*Alnus incana*), paper birch (*Betula papyrifera*), and willow (*Salix spp.*) (Burns, 1990; Dixon and Buschena, 1988; Gagné *et al.*, 2006; Kernaghan *et al.*, 2003; Nienstaedt and Zasada, 1990; Quoreshi *et al.*, 2007; Quoreshi and Khasa, 2008; Quoreshi *et al.*, 2008; Roy *et al.*, 2007). These symbiotic microorganisms supply nutrient elements and water to plants in exchange for carbon sources essential for their development (Allen *et al.*, 2003a; Allen *et al.*, 2003b; Dahlberg, 2001; Smith and Read, 2008). It has been proven that ECM fungi can utilize important soil elements (P, N, Mg, Ca, K, Zn, Cu, Ni, S, Mn, B, and Fe) that are generally unavailable to plants under organic and insoluble forms by producing specific

organic acids and enzymes (Berner *et al.*, 2012; Smits *et al.*, 2012; Wallander and Hagerberg, 2004; Landeweert *et al.*, 2001). Therefore, ECM fungi play a huge role in tree nutrition especially in low fertility soils of the boreal forest where most soil elements are stored in mineral rocks and organic matter (Balogh-Brunstad *et al.*, 2008; Hoffland *et al.*, 2004; Landeweert *et al.*, 2001). Contrary to arbuscular mycorrhizal fungi that mostly colonized plant roots in nutrient-rich soils with near-neutral pH, ECM fungi generally favour soils with low pH and low mineralization rates (Toljander *et al.*, 2006).

Boreal forests are well known for having high ECM fungal species diversity (Buée *et al.*, 2009; Gagné *et al.*, 2006; Kernaghan *et al.*, 2003; Villeneuve *et al.*, 1989). Like plants, ECM fungal communities go through species succession over time until forest ecosystems reach climax (Keizer and Arnolds, 1994). Species diversity decreases considerably following disturbance events, which return forest stands to early-successional stages (Dahlberg, 2001). At the beginning, the ecosystem is colonized by pioneering species adapted to site conditions (Keizer and Arnolds, 1994). Early-successional forest stands usually have low ECM fungal species richness and are mainly composed of generalist species (Dahlberg, 2001). In late-successional stages, both generalist and specialist species are present in the forest stands and diversity augments greatly due to increasing site and soil complexity (Dahlberg, 2001). ECM fungi also generally display high genetic diversity within species (Cairney, 1999). This high genetic diversity generates intraspecific physiological variation among strains (Cairney, 1999; Colpaert *et al.*, 2000; Lamhamedi and Fortin, 1991). Mycelium growth, plant colonization, enzyme production, plant growth stimulation, pH and temperature optimum for mycelium growth, tolerance to harsh conditions such as heavy metal toxicity and drought, nutrient uptake, and organic acid secretion by ECM fungi may differ greatly among strains of the same species (Cairney, 1999; Colpaert *et al.*, 2000; Lamhamedi and Fortin, 1991; Smith and Read, 2008). Therefore, some species and strains may be better adapted than others to abiotic stressors.

ECM fungal species diversity and composition influence considerably the growth and nutrient uptake of host trees; thereby, affecting ecosystem productivity (Baxter and Dighton, 2001; Jonsson *et al.*, 2001). ECM diversity at the local scale is controlled by disturbances, nutrient partioning, competition and interactions with other microorganisms (Bruns, 1995).

Many abiotic and biotic factors such as soil chemistry, microclimate and stand age are known to play an important role in determining the distributions of ECM fungal species and the composition of their communities (Debellis *et al.*, 2006; Korkama *et al.*, 2006). Moreover, ECM fungal community structure in the boreal forest has often been strongly correlated with soil properties such as extractable ammonium, base saturation, and soil pH (Hogberg *et al.*, 2006; Toljander *et al.*, 2006).

White spruce is one of the most commercially valuable, important, and widespread tree species of the Canadian boreal forest (Nienstaedt and Zasada, 1990; Sutton, 1973). We have little knowledge of the structure of the ECM fungal community associated with white spruce across an ecological gradient of different ecosystems and habitats and how abiotic stress can shape the ECM fungal community and diversity on different niches. Studying the structure and diversity of white spruce ECM fungal community in disturbed versus natural forest stands is a key first step towards understanding symbiotic modulation that occurs both temporally and spatially allowing plants to adapt to changing environmental conditions. In this study, we have identified the ECM fungal community associated with white spruce in nursery, mining site, forest edge, and natural forest near Sigma-Lamaque mine in the Canadian Abibiti region. We hypothesized that white spruce ECM fungal community and diversity differs among sites. We also predicted that soil chemical fertility may play an important role in ECM fungal species composition.

2.4 Materials and methods

2.4.1 Study area

The Sigma-Lamaque mine is an open pit gold mine situated in the municipality of Val d'Or, Quebec, Canada. It is located in the balsam fir – white birch bioclimatic zone (MRNF, 2012). This zone occupies the southern part of the Canadian boreal forest. Mature forest stands are mainly composed of balsam fir, white spruce, and white birch on mesic sites (MRNF, 2012). Black spruce, tamarack (*Larix laricina*), trembling poplar, and jack pine dominate on less favourable hydric or xeric sites (MRNF, 2012). Forest dynamics is controlled by two types of natural disturbances: (1) outbreak of eastern spruce budworm

(*Choristoneura fumiferana*) that feeds principally on highly abundant balsam fir and (2) forest fires which favour the formation of pure stands of jack pine (MRNF, 2012).

2.4.2 Sampling of ECM fungal communities

Four sites near Sigma-Lamaque mine were sampled for their white spruce ectomycorrhizal fungal communities (Appendix I and II). Trecesson nursery located 100 km from the mining site in Amos represented the first site. This nursery furnishes most of the tree seedlings planted after timber harvest in the Abitibi region. The second site was regarded as the waste rock pile and fine tailings of the mining site that will be restored and revegetated after mine closure. The third site was represented by the forest edge surrounding the mining site. The fourth site was a natural mature forest stand next to Sigma-Lamaque mine. Within each site, five specific locations were randomly selected on a map and geographic coordinates were recorded on a GPS Garwin 60CSx (Garmin International Inc., Olathe, KS, USA). In the field, fine roots of two white spruce trees at least 5 m apart were sampled in each location for a total of 10 trees sampled per site following ECM sampling techniques described by Gagné *et al.* (2006). Fine roots were carefully collected from two opposite directions (north – south) starting at the base of the tree. Roots with surrounding bulk soil were stored in plastic bags at 4°C for up to six days.

2.4.3 Identification of ECM fungi

2.4.3.1 Morphotyping

Fine roots were washed gently with tap water in a 2 mm mesh sieve at Université Laval. Roots were stored in tap water in a 150-ml plastic tube until further handling. 100 ECM root tips of each sample were randomly chosen and characterized based on their color, texture, form, size and presence of hyphae and rhizomorphs under a stereomicroscope following morphotyping techniques used by Wurzburger *et al.* (2001). The percentage of mycorrhization for each morphotype was calculated by comparing the number of root tips represented by a specific morphotype with the total number of root tips. Five ECM root tips of each morphotype were collected and placed into 1.5 ml tubes containing a CTAB solution and stored at -20°C in a freezer as described by Quoreshi *et al.* (2008) until further molecular analyses.

2.4.3.2 DNA extraction and sequencing

Fungal DNA extraction, PCR amplification, and DNA sequencing were performed following the method employed by Gagné *et al.* (2006). Total genomic DNA was extracted using DNeasy® plant mini kit (Qiagen, Mississauga, Ontario). The internal transcribed spacer (ITS) of the nuclear ribosomal DNA (rDNA) was amplified using the primers ITS-1F and ITS-4. PCR amplifications were carried out using a PTS-225 thermocycler (MJ Research, Waltham, Massachusetts). PCR amplifications were accomplished through initial denaturation at 95°C for 2.5 minutes, 30 cycles of denaturation at 95°C for 30 seconds, DNA extension at 72°C for 3 minutes, and a final extension at 72°C for 10 minutes. The products were seen on 2% agarose gels with 0.5% SynergelTM (Diversified Biotech, Boston, Massachusetts) stained with ethidium bromide. For DNA sequencing, partial ITS sequence for each type of RFLP pattern was determined. Using the amplification primers ITS-1F and a sequenase GC-rich kit (Applied Biosystems, Cleveland, Ohio), direct sequencing of forward DNA strand was conducted with a dideoxynucleotide chain termination procedure. Sequencing was performed using the ABI 3100 genetic analyser (PE Applied Biosystems, Foster City, California, USA). Bioedit v7.2.5 software (Ibis Biosciences, Carlsbad, California, USA) was used to edit raw sequences. Finally, sequences were submitted to BLASTn against the GenBank (http://www.ncbi.nlm.nih.gov) search engine in order to identify ECM fungal taxa: species (≥ 98% homology), genus or family (< 98% homology).

2.4.4 Bulk soil analyses

Three samples of rhizospheric bulk soil were randomly selected in each of the four sites for chemical analyzes. Before analyses, soil samples were sieved (2 mm sieved), finely grounded, and oven-dried. Total organic C was quantified following the method developed by Yeomans and Bremner (1988). Total N was determined following the Quickchem method (Lachat Instruments Division, Milwaukee, WI, United States) (Bouyoucos, 1962). Available P in soil was calculated using the Bray 2 method (Bray and Kurtz, 1945). Exchangeable K, Ca, Mg, Na, Fe, Mn, and Al were measured following techniques developed by Arnacher *et al.* (1990). Finally, pH was determined in a saturated paste extract and electrical conductivity in 1:2 water solution.

2.4.5 Numerical analyses

2.4.5.1 Phylogenetic analyses

Closely related taxa (≥ 98% sequence similarities) were grouped together using the Bioedit v7.2.5 software (Ibis Biosciences, Carlsbad, California, USA) and considered the same species. Phylogenetic analyses were performed using the Mega 5 software developed by Tamura *et al.* (2011). Phylogenetic trees were constructed using the maximum parsimony tree application and the bootstrap method. GenBank taxa showing the most similar BLASTn results and utilized for ECM fungal identification were included in the first phylogenetic tree. All our ECM fungal sequences were submitted to the GenBank (http://www.ncbi.nlm.nih.gov) search engine for public consultation. Genetic distance represented the genetic divergence among species and was calculated for each site by the Mega 5 software (Tamura *et al.,* 2011). Smaller genetic distance means that individuals in the same population have more similar genes and are more closely related genetically compared to higher genetic distance.

2.4.5.2 Diversity and colonization analyses

For each of the four sites, ECM fungal species richness was measured by calculating the number of species found in the community. Shannon-wiener and Simpson's diversity indices were calculated using species relative abundance results for each site following the method used by Wright *et al.* (2009). Because there was no replicate for each site sampled, only total diversity of each site, not means, was considered in our analyses. Species relative abundance was demonstrated as the number of root tips colonized by each fungal species divided by the total number of root tips sampled per site multiplying by 100. Species relative frequency was calculated for each sampling site by dividing the number of samples in which a species was found by the total number of samples.

Mean percentage of roots colonized by ECM fungi was calculated for each site by averaging the percentage of ECM root tips from the 10 samples. No data transformation was necessary in order to meet ANOVA assumptions. Significant different means at $P \leq 0.05$ were statistically determined based on one-way ANOVA using Tukey tests (SAS 9.3 software, SAS Institute, North Carolina, USA).

2.4.5.3 Multivariate analyses

All multivariate analyses were executed with the PC-ORD 6 software (MJM Software Design, Gleneden Beach, Oregon, USA). A PerMANOVA analysis including pairwise comparisons was performed in order to evaluate if any of the sampling sites differ significantly in their ECM fungal species composition. Additionally, a two-way cluster analysis was performed for identifying which groups of ECM fungi tend to thrive together in same habitat and which sites have similar species composition. Species relative abundance data was serving as the starting point for these analyses. Species with horizontal line above the 75% of information remaining are considered to be significantly in the same ecological group as recommended by PC-ORD 6. Furthermore, in order to recognize similar linear patterns among soil chemical (pH, % of N and C, C/N ratio, P, Ca, K, Mg, Fe, Na, Mn, and AlOH) and mycorrhization variables across the ecological gradient, a principal component analysis (PCA) was carried out with our four sampling sites. Significant differences and linear patterns were set at $P \leq 0.05$ for all multivariate analyses.

2.5 Results

2.5.1 ECM fungal community and phylogenetic trees

In total, 111 morphotypes were described. Through molecular analyses, 90 of them (81.1%) were successfully identified uncovering a total of 41 different ECM fungal species associated with white spruce tree roots including 27 basidiomycota and 14 ascomycota within the four sampling sites (Fig. 2.1). The main phylogenetic tree shows the evolutionary relationship among the 41 identified ECM fungal species (in bold with their respective GenBank number) (Fig. 2.1). Species within each of the four sampling sites are well distributed throughout the phylogenetic tree (see colored squares in Fig. 2.1). 13, 4, 18, and 16 ECM fungal species were found respectively on the mining site, Trecesson nursery, forest edge, and natural forest (Fig. 2.2). Genetic distance among ECM fungal species was the highest for the mining site (0.967) followed by the forest edge (0.757), then the natural forest (0.45), and finally the Trecesson nursery (0.236) (Fig. 2.2). As a result, genetic similarities among species across the ecological gradient were at the lowest for the

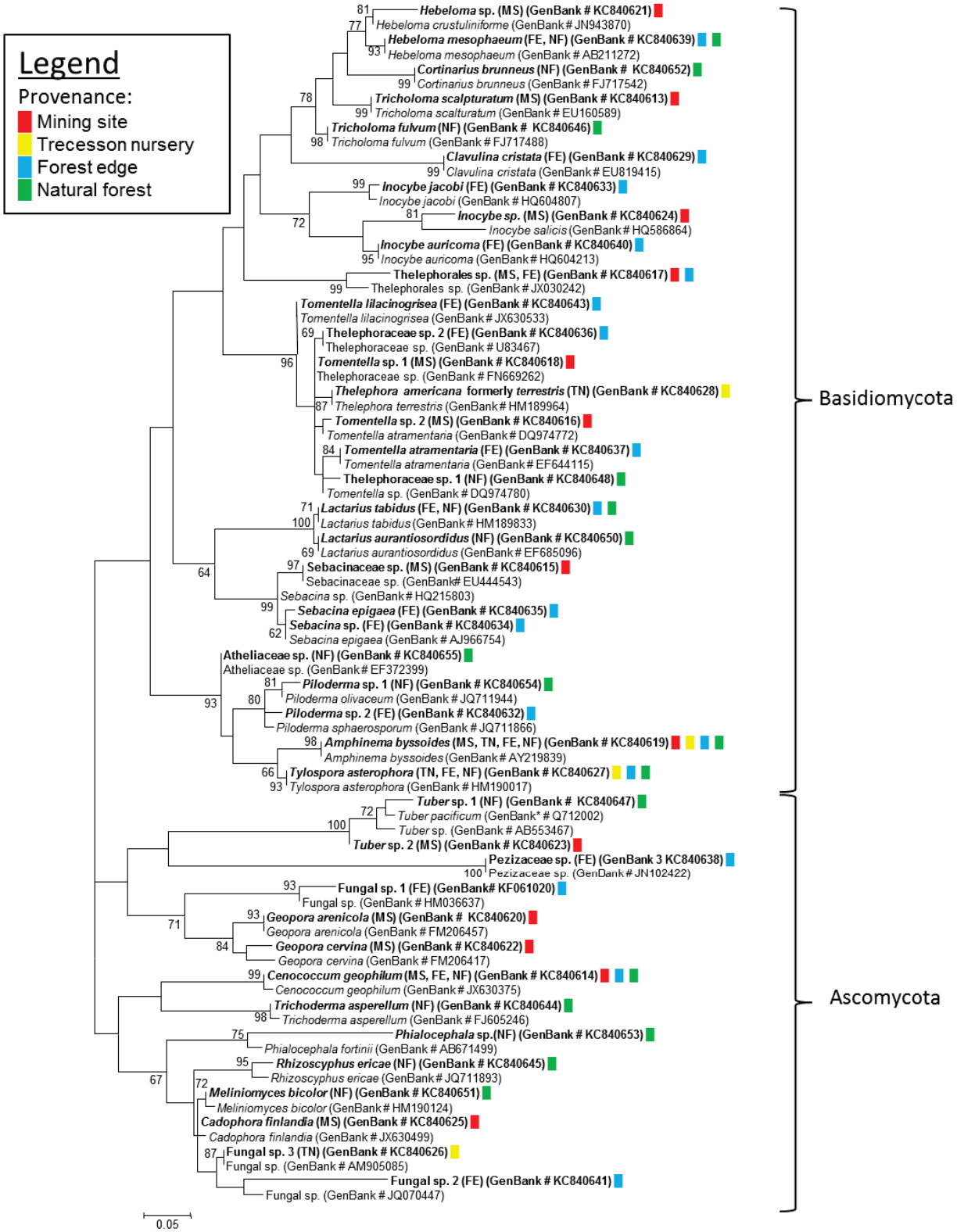

Figure 2.1: Phylogenetic tree containing all ECM fungal taxa (in bold) that have been identified on the four sites (mining site (MS) in red, Trecesson nursery (TN) in yellow, forest edge (FE) in turquoise, and natural forest (NF) in green) with their corresponding GenBank accession number – Taxa not in bold refer to the most similar blastn results and GenBank taxa used for ECM fungal species identification.

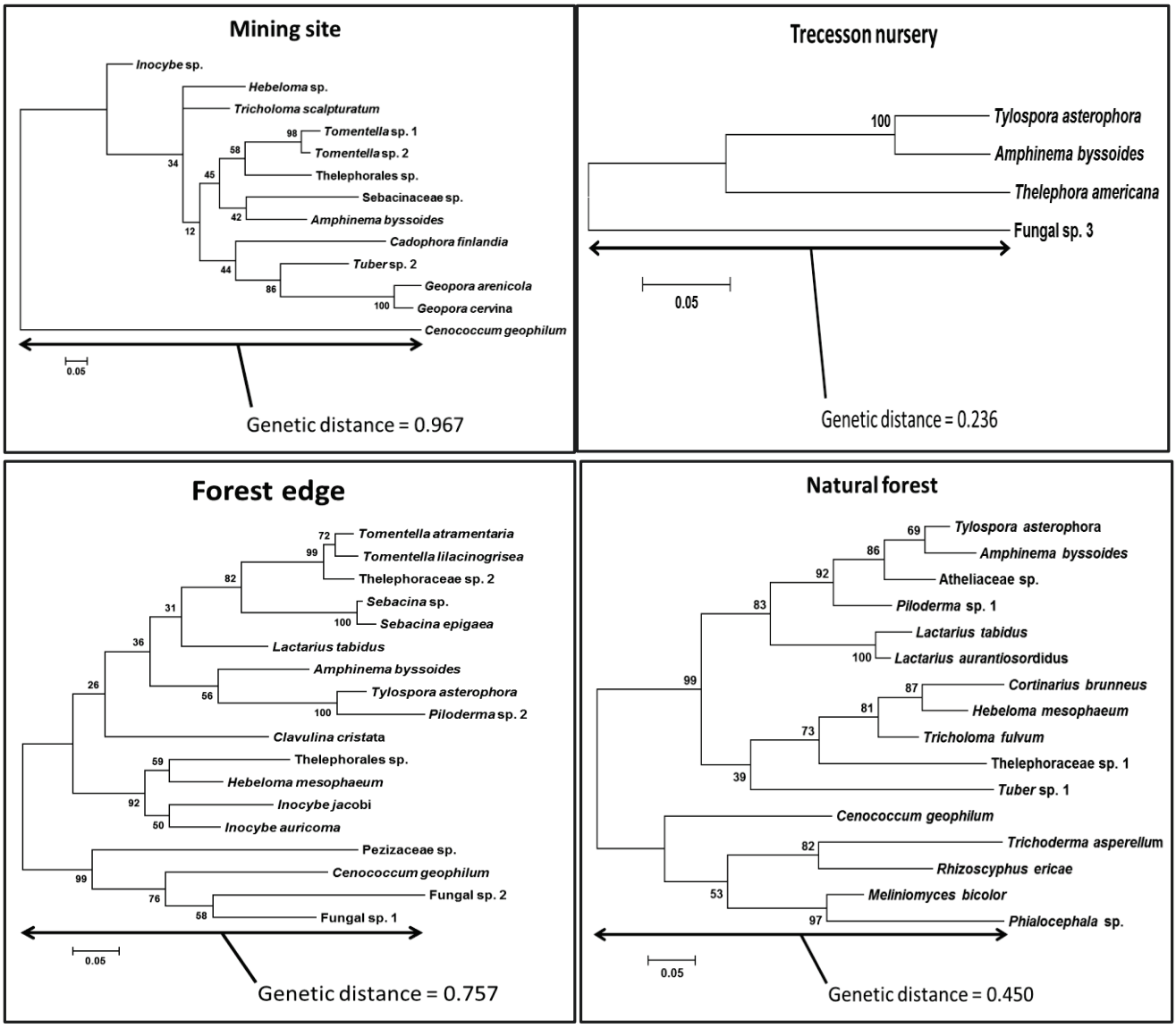

Figure 2.2: Phylogenetic trees showing the ECM fungal community of each site and the genetic distance among species within site.

mining site, increasing from the forest edge to the natural forest, and finally at the highest for the nursery.

2.5.2 Species diversity

2.5.2.1 Relative abundance and frequency

Amphinema byssoides was the most frequent ECM fungal species symbiotically associated with white spruce at the mining site followed by *Tricholoma scalpturatum* and *Tomentella* sp.2 (Fig. 2.3a). *Thelephora americana* was the most common species thriving

on white spruce roots in the nursery ecosystem (Fig. 2.3a). At the forest edge, *Hebeloma mesophaeum* and *Tomentella lilacinogrisea* were the two most frequent species followed by *Amphinema byssoides* and Fungal sp.1 (Fig. 2.3a). Finally, *Lactarius tabidus* was the most common ECM fungal species thriving on white spruce roots in the natural forest followed by *Tricholoma fulvum* (Fig. 2.3a). All other species were seen in low frequency (0.1) within their corresponding ecosystem (Fig. 2.3a).

Furthermore, *Amphinema byssoides* was also the most abundant (14.3%) ECM fungal species associated with white spruce at the mining site (Fig. 2.3b). The second most abundant species on site was *Inocybe* sp. (7.2%) and all the other species tended to have a relative abundance smaller than 5% (Fig. 2.3b). At the Trecesson nursery, *Thelephora americana* (2.8%) and *Amphinema byssoides* (2.3%) had the highest relative abundance (Fig. 2.3b). The other species had very low abundance smaller than 1% (Fig. 2.3b). At the forest edge, *Tomentella lilacinogrisea* was the most abundant species (10.9%) followed by *Hebeloma mesophaeum*, *Clavulina cristata*, Thelephoraceae sp.2, and Fungal sp.1 with, more or less, 5% relative abundance (Fig. 2.3b). The other species all had relative abundance less than 4% (Fig. 2.3b). In the natural forest, the most abundant species associated with white spruce root tips was *Lactarius tabidus* (7%) followed by *Tricholoma fulvum* and *Lactarius aurantiosordidus* (± 4%) (Fig. 2.3b). The other species had relative abundance smaller than 4% (Fig. 2.3b).

2.5.2.2 Percentage of colonized roots, richness and diversity index

White spruce roots at the mining site, the forest edge, and the natural forest were significantly much more colonized by ECM fungi (at least 7 times) than those at the Trecesson nursery (Fig. 2.4a). The biggest difference was between the mining site and the nursery where white spruce roots at the mining site displayed an average of 69.2% roots colonized by ECM fungi, 10 times higher than the 6.9% average of those at the nursery (Fig. 2.4a). Adversely, there were no significant differences in the percentage of white spruce root tips colonized by ECM fungi between the mining site, the forest edge, and the natural forest (Fig. 2.4a).

With an ecological gradient that goes from the mining site to the forest edge, natural forest, and finally Treceson nursery, total site species richness (4 to 18), Shannon-

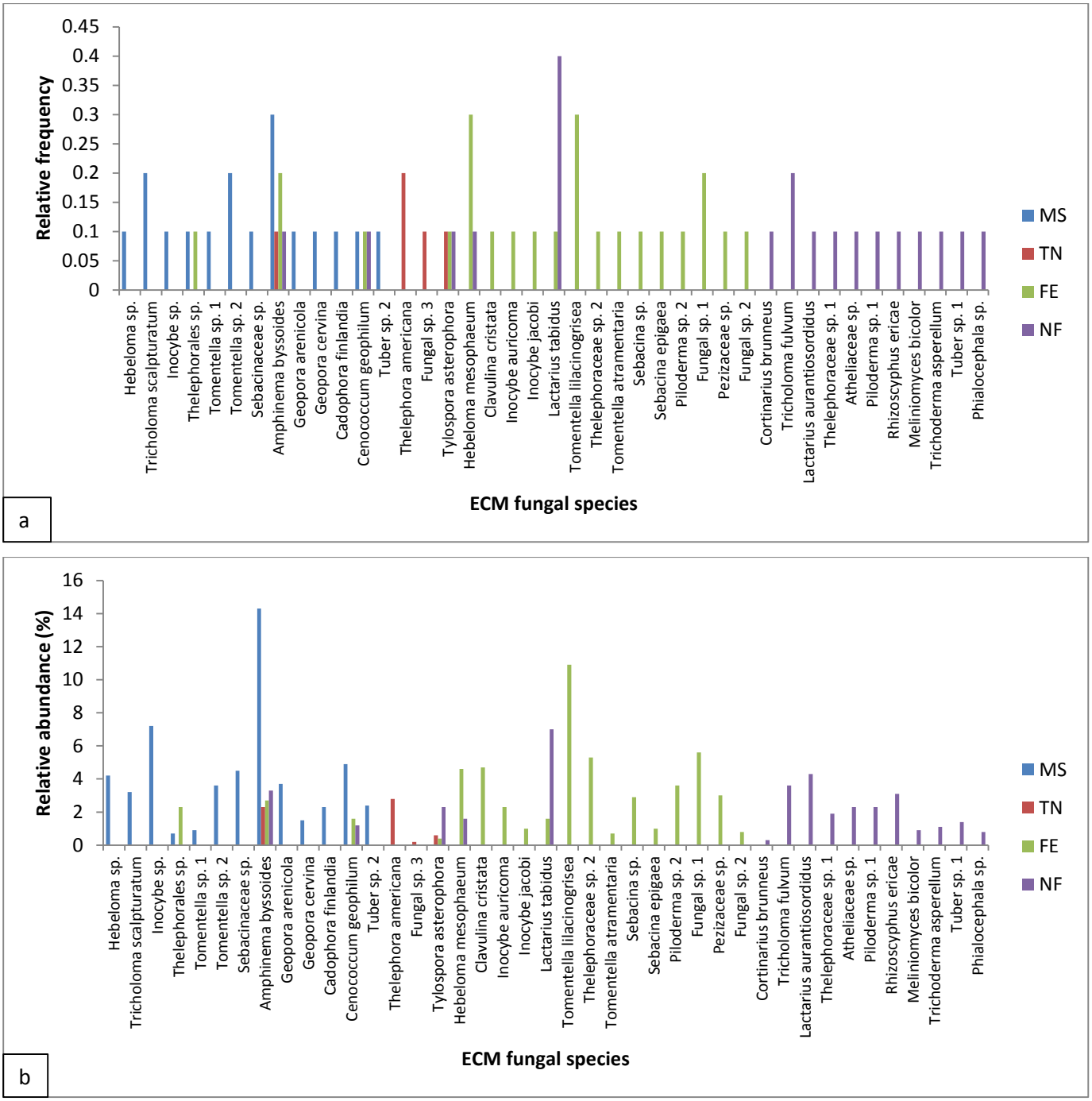

Figure 2.3: (a) Relative frequency and (b) Relative abundance of all identified ECM fungal species within each sampling site (mining site (MS), Trecesson nursery (TN), forest edge (FE), and natural forest (NF)).

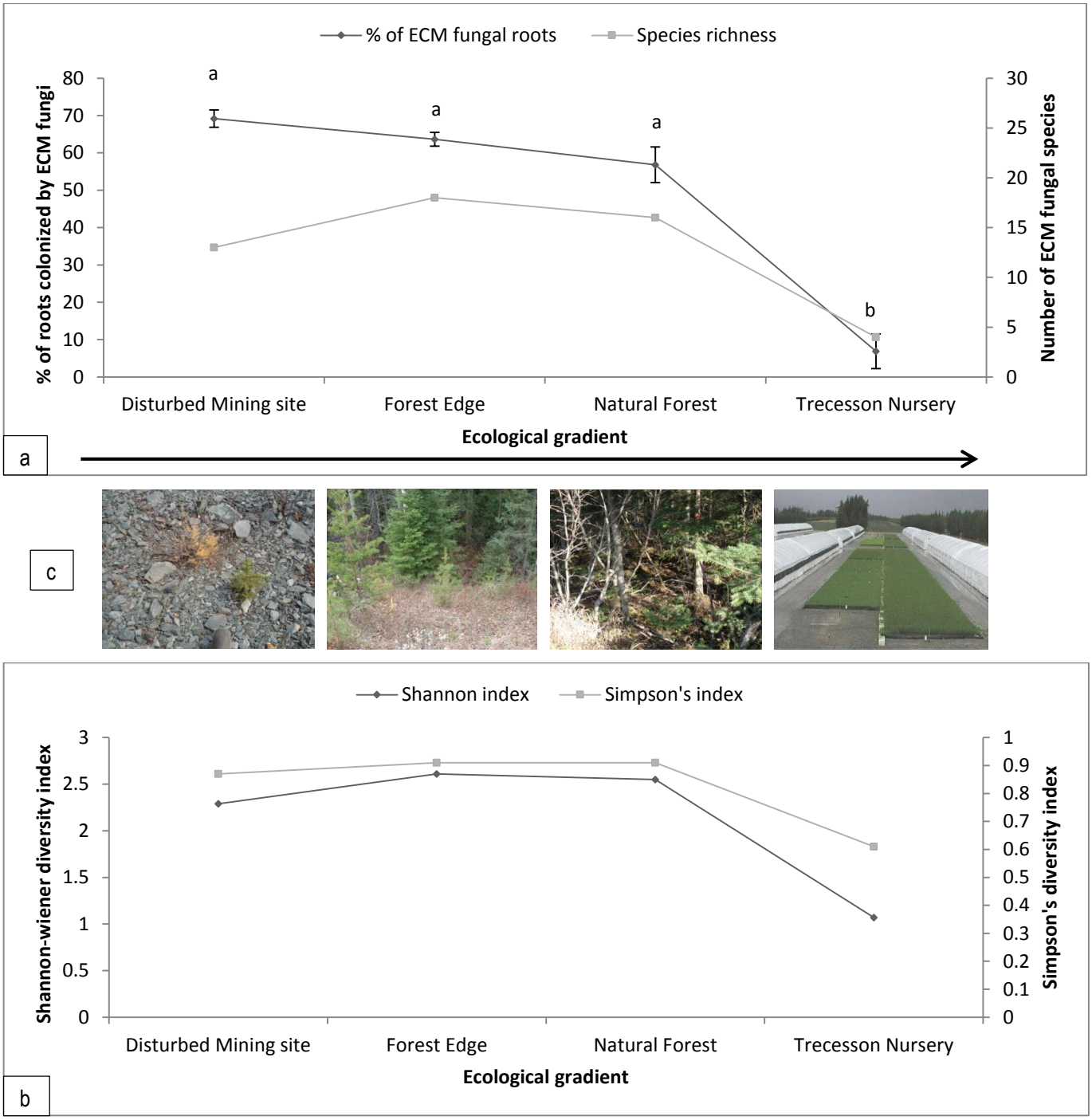

Figure 2.4: (a) Percentage of roots colonized by ECM fungi (Means ± SE with same letters are not significantly different at α = 0.05, Tukey test) and total site species richness, (b) Shannon-wiener diversity index and Simpson's index across the ecological gradient of the four sampling sites (Trecesson nursery, disturbed mining site, forest edge, and natural forest) including (c) representative illustrations of each ecosystem.

wiener (1.07 to 2.61) and Simpson's diversity (0.61 to 0.91) index were all following a similar pattern (Fig. 2.4a; Appendix II). Total site ECM fungal species richness and diversity indices were increasing from the mining site to its highest point at the forest edge and then decreasing from the forest edge, passing by the natural forest, to the nursery (Fig. 2.4b). Therefore, in this study, forest edge was showing the highest ECM fungal species richness and diversity.

2.5.3 Shaping groups with similarities

PerMANOVA results suggested that ECM fungal species composition differed between at least two of the four sampling sites ($P = 0.0004$). Through PerMANOVA pairwise comparison analyses, we demonstrated that species composition was significantly different between all sites (adjust *P*-values of 0.0154 (MS vs TN), 0.0424 (MS vs FE), 0.0150 (MS vs NF), 0.0082 (TN vs FE), and 0.0056 (TN vs NF)) except between forest edge and natural forest (adjust *P*-value of 0.1204). Two-way cluster analysis permitted to further confirm these findings. Species composition of forest edge and natural forest was significantly similar (> 75% information remaining) (Fig. 2.5). Species composition of mining site was significantly different to nursery, forest edge, and natural forest (< 75% information remaining) (Fig. 2.5). Species composition of Trecesson nursery was also significantly different to forest edge and natural forest (Fig. 2.5). However, it is important to mention here that nursery species composition was much more alike to forest edge and natural forest (50% information remaining) than mining site (0% information remaining) (Fig. 2.5).

In the two-way cluster dendrogram, four different groups of ECM fungal species were recognized to thrive together in similar habitats (> 75% information remaining) (Fig. 2.5). In this study, *Hebeloma* sp., Sebacinaceae sp., *Tricholoma scalpturatum, Tomentella* sp. 2, *Geopora arenicola, Inocybe* sp., *Cenococcum geophilum, Tomentella* sp. 1, *Geopora cervina, Cadophora finlandia, and Tuber* sp. 2 were a group of ECM fungal species that tended to inhabit white spruce roots of the mining site (Fig. 2.5). Moreover, Thelephorales sp., *Inocybe auricoma, Sebacina* sp., Pezizaceae sp., *Piloderma* sp. 2, *Hebeloma mesophaeum*, *Clavulina cristata*, Thelephoraceae sp. 2, Fungal sp. 1, and *Tomentella lilacinogrisea* tended to reside together within white spruce rhizosphere in the forest edge

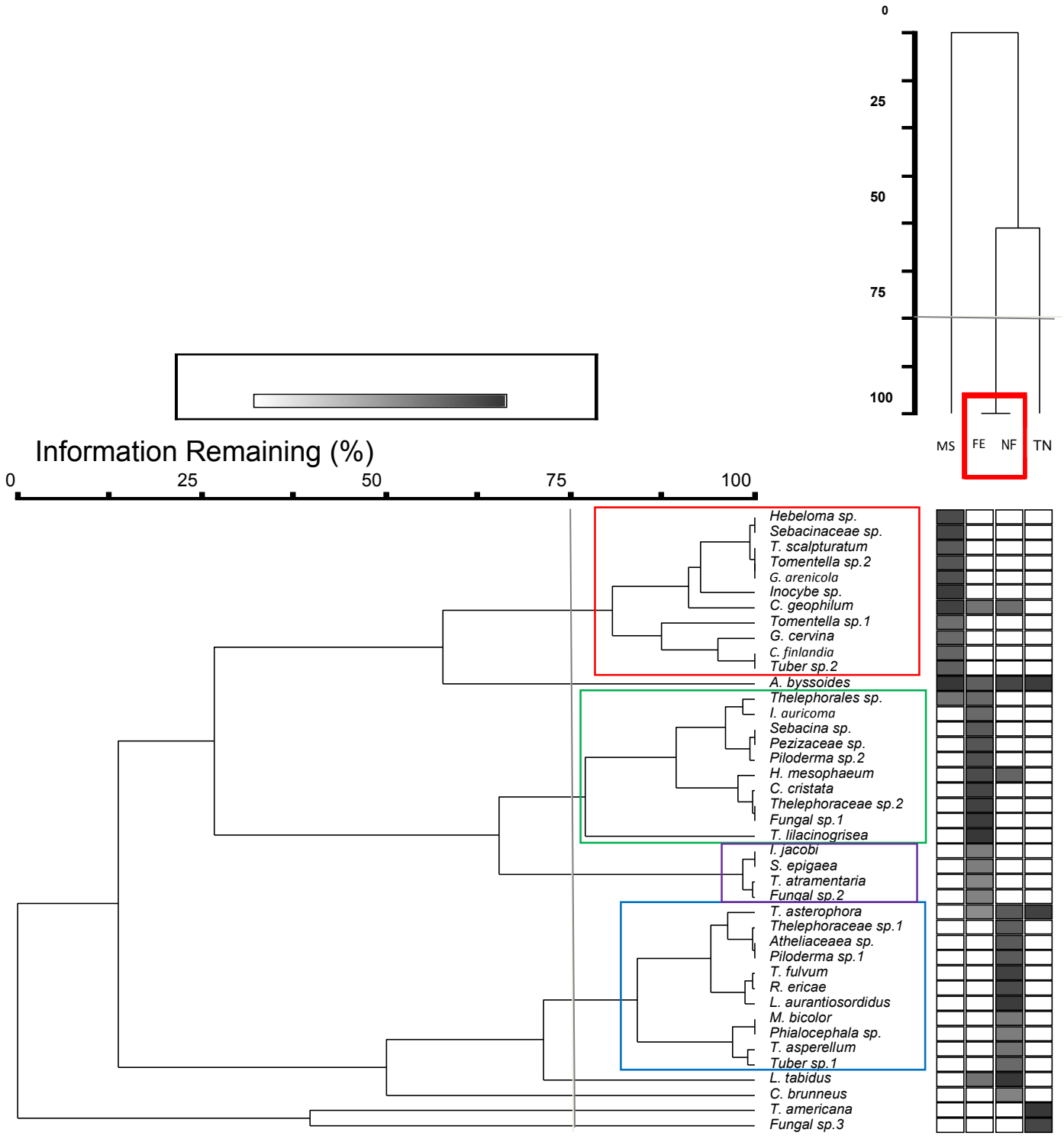

Figure 2.5: Two-way cluster analysis (α = 0.05) showing groups of species thriving in same habitats (small coloured rectangles) (darker grey squares mean higher abundance; Big red rectangle = no difference between site species composition).

ecosystem (Fig. 2.5). A second group of species composed of *Inocybe Jacobi*, *Sebacina epigaea*, *Tomentella atramentaria*, and Fungal sp. 2 tended to live and colonized white spruce roots, in lower abundance, in the forest edge ecosystem (Fig. 2.5). Finally, the fourth group of ECM fungal species which included *Tylospora asterophora*, Thelephoraceae sp. 1, Atheliaceae sp., *Piloderma* sp. 1, *Tricholoma fulvum*, *Rhizocyphus ericae*, *Lactarius aurantiosordidus*, *Meliniomyces bicolor, Phialocephala* sp., *Trichoderma asperellum*, and *Tuber* sp. 1 tended to form a closely related community within the white spruce rhizosphere of the natural forest (Fig. 2.5). *Amphinema byssoides* was encountered in all four sampling sites across the ecological gradient (Fig. 2.5). Nevertheless, this ECM fungal species was more closely associated with white spruce seedlings naturally regenerating on the mining site (Fig. 2.5). *Lactarius tabidus* and *Cortinarius brunneus* were more closely affiliated with the natural forest, but they did not form any closely related groups (Fig. 2.5). On the other hand, *Thelephora americana* and Fungal sp. 3 were important ECM fungal species that tended to inhabit the rhizosphere of white spruce seedlings at the Trecesson nursery (Fig. 2.5).

2.5.4 Looking for linear patterns

The principal component analysis (PCA) allowed identifying linear patterns across the ecological gradient within soil chemical and mycorrhization data. This reality was supported by 78.555 % of the variance explained by the Axis 1 and a very small *P*-value (0.0010) reflected by the randomization test. Therefore, the Axis 1 was significantly showing linear patterns (Fig. 2.6). On the contrary, the Axis 2 and 3 were not significant for linear patterns with *P*-values of 1.0000 and 0.9990, respectively, and thus they were not considered in this multivariate analysis. Recommendations from the different stopping rules did not vary considerably, so the assumption of data homogeneity was met. Looking at the PCA diagram from mining site, forest edge, natural forest to finally Trecesson nursery, we realize that soil pH and percentage of root tips colonized by ECM fungi decreased across the ecological gradient while soil K, Mg, P, N, C, Fe, Ca, Na, and AlOH content and C/N ratio tended to increase (Fig. 2.6).

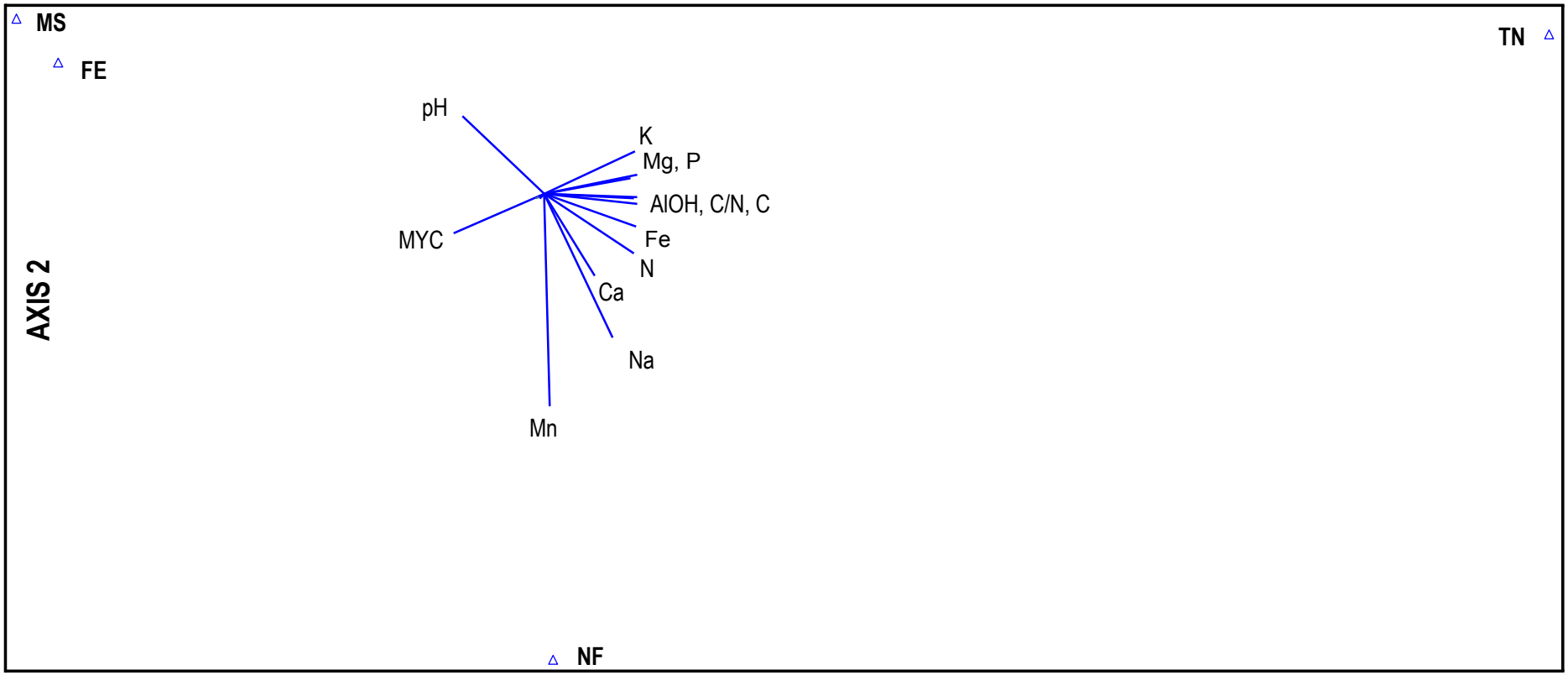

Figure 2.6: Principal component analysis (α = 0.05) showing linear patterns of a wide range of variables across the ecological gradient (Sampling sites: MS = mining site, FE =forest edge, NF = naturel forest, and TN = Trecesson nursery) (only the axis 1 is significant; closer to center means smaller values) (MYC = % of roots colonized by ECM fungi).

2.6 Discussion

2.6.1 Genetic divergence within communities

In this study, 81.1% of morphotypes were successfully sequenced and identified through biomolecular analyses. These results follow the trend of other studies that have obtained an identification success rate between 75% and 83% (Rosling *et al*., 2003; Twieg *et al*.,2007). 41 different ECM fungal taxa were encountered on white spruce roots within our four sampling sites and each taxon was considered as an individual species. Using similar sampling techniques, Dahlberg *et al.* (1997) identified only 25 ECM fungal taxa associated with Norway spruce (*Picea abies*) root tips. Furthermore, Rosling *et al.* (2003) found only 22 ECM fungal taxa on roots of Norway spruce and Scots pine (*Pinus sylvestris*). A smaller number of taxa was obtained in these two previous studies probably because trees were sampled only in natural forest stands whereas in this study, we also sampled in nursery, forest edge, and disturbed ecosystems. However, when we compared species diversity in natural forests, 18 ECM fungal taxa were identified in this study, a number slightly smaller compared to the previous authors (Dahlberg *et al.*, 1997; Rosling *et al.*, 2003).

Within our 41 ECM fungal species, 27 (66%) and 14 (34%) belonged to the phyla Basidiomycota and Ascomycota, respectively. Our results agree with O'Brian *et al.* (2005) who suggested that the majority of ECM fungal species recovered in rhizopheric soil belong to the two phyla Basidiomycota and Ascomycota. Dahlberg *et al.* (1997) also demonstrated that a small number (20%) of ECM fungal species associated with Norway spruce were ascomyceta. Our four sites had a greater number of fungi associated with white spruce belonging to the phylum Basidiomycota (62%, 75%, 78%, and 63% for the mining site, nursery, forest edge, and natural forest, respectively) than to the phylum Ascomycota. These findings partially agree with Gehring *et al.* (1998) who found that 85% of ECM fungi associated to pinyon pines (*Pinus monophylla*) at sandy-loam sites were Basidiomycota members whereas 52% of them at cinder sites were Ascomycota members, suggesting that this proportion Basidiomycota versus Ascomycota is site-specific. This variation in the proportion of ECM fungi belonging to Basidiomycota versus Ascomycota among sites may also be host-specific.

ECM fungal species located in each of the four sites were well distributed across the whole phylogenetic tree. Genetic divergence among species was at the highest on the mining site. These results were quite surprising. One would think that genetic divergence among ECM fungal species for the mining site would be considerably low compared to other sites due to extreme site conditions in which only a small group of fungi (order, family or genus) would have successfully evolved to thrive in. However, it is not the case here. The ECM fungal adaptation to mining site may be greatly controlled by high genetic variability within species, which allows some individual strains adapted to site conditions to colonize the highly disturbed ecosystems. This hypothesis agrees with results obtained by Colpaert *et al.* (2000) and Adriaensen *et al.* (2005) where the tolerance of the ECM fungus *Suillus luteus* to soils with high concentrations of Zn, Cd, and Cu was much higher for strains isolated from the polluted habitats than those isolated from non-polluted soils.

2.6.2 ECM fungal species diversity

Amphinema byssoides was the most frequent and abundant ECM fungal species colonizing white spruce roots on the mining site. Furthermore, this ECM fungal species was found on all sites. For that reason, *A. byssoides* is considered to be a generalist species thriving in a wide range of habitat in this experiment. Hunt (1991) has suggested that *A. byssoides* is an early-successional fungal species. This statement agrees with our findings because *A. byssoides* was much more abundant and frequent in the early-successional disturbed mining site ecosystem compared to the forest edge and the natural forest. *Tricholoma scalpturatum* and *Inocybe sp.* had a relatively high frequency and abundance, respectively on the mining site. *T. scalpturatum* has also been known for colonizing roots of a wide variety of deciduous and coniferous species including European aspen (*Populus tremula*) on disturbed mining sites contaminated by lead, zinc, and cadmium (Krpata *et al.*, 2008). *Cadophora finlandia* was present on roots of white spruce naturally regenerating on the waste rocks. Results obtained by Rosling *et al.* (2003) showed *C. finlandia* as an ECM fungal species colonizing mainly mineral soils. This species may be well adapted to mineral rocky soils.

Thelephora americana was the most frequent and abundant ECM fungal species colonizing white spruce seedlings at the nursery. Kernaghan *et al.* (2003) and Quoreshi *et al.*

(2008) have also found *T. americana* colonizing white spruce seedlings at the nursery. Then, Twieg *et al.* (2007) discovered that *T. americana* was not present on Douglas-fir (*Pseudotsuga menziesii*) seedlings of older plantations but only on those that were newly planted on clearcut sites. This ECM fungal species may be well adapted to high fertilization regimes and it would explain why it is mostly encountered on nursery seedlings and not in natural ecosystems. *Hebeloma mesophaeum* and *Tomentella lilacinogrisea* were the two most frequent and adundant ECM fungal species colonizing white spruce roots at the forest edge. There is currently little knowledge about ECM fungal communities colonizing tree roots in forest edge ecosystems. As far as we know, this study is the first of its kind.

Lactarius tabidus was the most frequent and abundant ECM fungal species colonizing white spruce roots in the natural forest followed by *Tricholoma fulvum* and *Lactarius aurantiosordidus*. Wright *et al.* (2009) found similar results with two different species from the genus *Lactarius* being in the top five most frequent and abundant ECM fungal species associated with western hemlock (*Tsuga heterophylla*) in natural forest stands. On the other hand, many studies have identified *Cenococcum geophilum* and species of the genus *Piloderma* as being the most frequent and abundant ECM fungal species associated with coniferous species in soils of temperate and boreal forests (Bahram *et al.*, 2012; Dahlberg *et al.*, 1997; Rosling *et al.*, 2003). Moreover, *Russula decolorans* and *Tylospora asterophora* were referred by Toljander *et al.* (2006) as the most abundant species associated with Scots pine and Norway spruce in the boreal forest. In this study, the ECM fungal communities were mainly composed of few abundant and frequent species colonizing the majority of white spruce fine roots and a large number of sparse and infrequent species. The findings corroborate those by Toljander *et al.* (2006) and Horton & Bruns (2001).

Within the 41 ECM fungal species identified in this study, 13, 4, 18, and 16 of them inhabited the mining site, the nursery, the forest edge, and the natural forest, respectively. The number of species associated with white spruce seedlings was much smaller in the nursery compared to the other three sites. Seedlings in nursery are amended regularly with fertilizers containing high concentration of N. Lilleskov *et al.* (2002) found that ECM fungal species richness decreased tremendously on white spruce roots with increasing N content in soils. As much as 30 taxa were identified on sites with low N content while only nine were

encountered in high N content sites (Lilleskov *et al.*, 2002). Nilsson & Wallander (2003) and Lilleskov *et al.* (2001) also demonstrated that N fertilization and deposition reduces considerably ECM fungal species richness. Overall, species diversity was increasing from the nursery, the mining site, the natural forest to the forest edge. ECM fungal diversities for conifers are usually lower in early-successional ecosystems such as clearcuts compared to late-successional forest stands (Twieg *et al.*, 2007) which agrees with our diversity results. The forest edge and the natural forest displayed a Shannon-wiener diversity index of 2.55 and 2.61, respectively. These results were very similar to those obtained by Kranabetter *et al.* (2009) where the Shannon-wiener index for ECM fungi associated with white spruce in the boreal forest was around 2.36 on average.

2.6.3 Difference in species composition among sites

In this study, we found no difference in ECM fungal species composition on white spruce roots between the forest edge and the natural forest. On the opposite, Trecesson nursery had a species composition different than the forest edge and the natural forest. However, the biggest difference in species composition associated with white spruce was between the mining site and the other three sites: nursery, forest edge, and natural forest. These findings agree with Twieg *et al.* (2007) who showed that ECM fungal species compositions were similar in older forest stands but differed when young stands were compared to the old ones. Stand age seems to explain a big part of the variation among ECM fungal communities (Twieg *et al.*, 2007). Wright *et al.* (2009) demonstrated that ECM fungal species composition differed considerably between N-P-fertilized trees and those that were not. Therefore, fertilization is probably the reason why species composition at the nursery was unlike the other three sites. Yet, what could explain the difference in ECM fungal species composition between the mining site and the surrounding forest edge and natural forest?

After major soil disturbance events such as glaciations or mining activities, soil is generally devoid of its microbial community (Jumpponen *et al.*, 2005). In these primary successional ecosystems, the vast majority of ECM fungal propagules colonize the newly-disturbed ecosystem through aerial spore dispersal and transport which allow establishing a dormant spore bank in these rocky soils (Jumpponen *et al.*, 2005). Every ECM fungal species expresses its own optimal set of environmental conditions in which it can grow more

effectively (Jumpponen *et al.*, 2005). Furthermore, niches vary greatly among ECM fungal species (Jumpponen *et al.*, 2005). Species colonizing the mining site are probably present in the natural forest and forest edge in low abundance and frequency because site conditions are not optimal for them. At one point, spores may be transported by wind or animals to the mine tailings. Environmental pressures of the mining site allow the selection of the most adapted ECM fungal species and strains, which later colonize newly regenerating seedlings (Jumpponen *et al.*, 2005). This colonization hypothesis is the most likely reason why the ECM fungal community associated with white spruce on the mining site is significantly different than communities of the surrounding environments.

2.6.4 ECM fungal communities and soil chemical properties

In this study, exchangeable K, Mg, P, Fe, Ca, Na and Al concentrations, C/N ratio, N and C contents decreased in soil from the nursery, natural forest, forest edge to the mining site while the pH and the percentage of white spruce roots colonized by ECM fungi tended to increase following the same ecological gradient. The first group of ECM fungi inhabiting the roots of white spruce trees on the mining site were adapted to poor mineral soil chemical fertility, very low organic matter content (low C content), and high pH. *A. byssoides* was well adapted to those extreme mining site conditions but also to all other site conditions due to its presence on all sites. The widespread distribution of *A. byssoides* across our ecological gradient confirms the wide ecological amplitude of the taxon. The second and third groups that were residing in the white spruce rhizospheric soil of the forest edge ecosystem were adapted to similar but not as poor soil chemical fertility, high pH, and low organic matter content compared to those of the mining site. Here, we suspect that the forest edge and the mining site had relatively similar soil chemical properties due to transport of tailing fine particles by wind erosion from the mining site to the surrounding forest edge causing a change in soil chemical properties at the soil surface which would normally be much more similar to the natural forest. For their part, the fourth group of ECM fungi colonizing white spruce roots of the natural forest were adapted to richer soil chemical fertility, higher organic matter content, and lower pH. *T. americana* and Fungal sp.3 encountered on white spruce roots at the nursery were adapted to even much richer soil chemical fertility, higher organic matter content and lower pH which mimics nursery conditions with regular fertilization

regimes. As a result, soil chemical properties played an important role in ECM fungal species composition of the four sampling sites. Toljander *et al.* (2006) also found that ECM fungal community structure is strongly related to soil properties such as extractable NH_4^+, base saturation, pH, and C/N ratio, with extractable NH_4^+ being the most powerful determinant of ECM fungal community. Bahram *et al.* (2012) also demonstrated at the local scale that soil nutrient content was one of the key factors controlling ECM fungal community composition. Furthermore, other studies have showed a strong relationship between soil edaphic characteristics and ECM fungal community composition (Kranabetter *et al.*, 2009; Gehring *et al.*, 1998). Kennedy (2010) has suggested that competition among species impacts considerably the interactions between them and has a big influence on ECM fungal community structure. ECM fungal competitive efficiency is dependent to environmental conditions such as soil pH, temperature, and nutrient content (Kennedy, 2010). As a result, species with the highest abundance and frequency may be better adapted to site conditions enabling them to outcompete others.

In this study, the percentage of white spruce roots colonized by ECM fungi was increasing as soil chemical fertility and organic matter content was decreasing and as the pH was rising. Total fungal biomass tends to be higher in soils with low nutrient availability and low tree productivity (Nilsson *et al.*, 2005). In the past, field trials have clearly demonstrated the importance of mycorrhizal colonization for the survival and growth of tree seedlings in primary successional ecosystems (Jumpponen *et al.*, 2005). It may explain why perfectly healthy naturally regenerating white spruce seedlings on the mining site have such a high ECM fungal colonization rate on their fine roots.

2.6.5 Edaphic selection pressures – drivers of ECM fungal community structure

It is well known that ECM fungi are, in general, acidophilic symbiotic microorganisms that prefer to thrive in poor acid soils with high recalcitrant litter content (Toljander *et al.*, 2006). However, in this study, we identified few ECM fungi ecologically adapted to poor alkaline soils with low organic matter content. These microsymbionts successfully colonized the root system of young white spruce seedlings on the non-acidogenic mine tailings of Sigma-Lamaque gold mine, allowing scarce natural regeneration. On the other hand, other ECM fungi are adapted to rich acid soils of commercial nursery. In

congruence with our results, Kernaghan *et al.* (2003) also recognized *Thelephora americana* and *Amphinema byssoides* as ECM fungi adapted to high fertigation level of nursery. Edaphic selection pressures after fungal spore dispersal lead to the selection of the most site-adapted ECM fungi through species survival and competition dynamics (Jumpponen *et al.*, 2005). As a result, edaphic selection pressures are important factors controlling ECM fungal community structure, composition, and diversity. There is a symbiotic modulation that occurs both temporally and spatially in the disturbed versus natural environment allowing plants to adapt to changing environmental conditions.

2.7 Conclusion

The ECM fungal community composition of the mining site was significantly different compared to the surrounding environments: Trecesson nursery, forest edge, and natural forest. To thrive on white spruce roots of the mining site, ECM fungi had to be adapted to high pH, poor soil chemical fertility, and low organic matter content. ECM fungal adaptation to mining site seems to be more related with high genetic variability within species than phylogenetic group adaptation. White spruce seedlings naturally regenerating on the mining site are highly colonized by ECM fungi. This study clearly shows the contrasting difference in white spruce ectomycorrhizal fungal diversity and community structure driven by edaphic selection pressures.

2.8 Acknowledgements

The authors thank the Natural Sciences and Engineering Research Council of Canada (NSERC) for their financial support. Furthermore, the authors show their gratitude to André Gagné and Marie-Ève Beaulieu for their precious help and advices throughout the realization of this study. Then, the authors offer their appreciation to Mathieu Boudreau for his assistance in the laboratory, Alain Brousseau for fulfilling soil chemical analyses, and Mark Mazerolle for his statistical guidance. Last but not least, we are grateful to Dr Suzanne Simard (University of British Columbia) and Line Lapointe (Université Laval) for their careful review that helped improve the quality of the manuscript.

2.9 References

Adriaensen K, Vralstad T, Noben J-P, Vangronsveld J, Colpaert JV. 2005. Copper-adapted *Suillus luteus*, a symbiosis solution for pines colonizing Cu mine spoils. *Applied Environmental Microbiology* **71**: 7279-7284.

Allen MF, Swenson W, Querejeta JI, Egerton-Warburton LM, Treseder KK.. 2003a. Ecology of mycorrhizae: A conceptual framework for complex interactions among plants and fungi. *Annual Review of Phytopathology* **41**: 271-303.

Allen EB, Allen MF, Egerton-Warburton LM, Corkidi L, Gomez-Pompa A. 2003b. Impacts of early- and late-seral mycorrhizae during restoration in seasonal tropical forest, Mexico. *Ecological Applications* **13**: 1701-1717.

Amacher MC, Henderson RE, Breithaupt MP, Seale CL, La Bauve JM. 1990. Unbuffered and buffered salt methods for exchangeable cations and effective cation exchange capacity. *Soil Science Society of America Journal* **54**: 1036-1042.

Bahram M, Polme S, Koljalg U, Zarre S, Tederson L. 2012. Regional and local patterns of ectomycorrhizal fungal diversity and community structure along an altitudinal gradient in the Hyrcanian forests of northern Iran. *New Phytologist* **193**: 465-473.

Balogh-Brunstad Z, Keller CK, Dickinson JT, Stevens F, Li CY, Bormann BT. 2008. Biotite weathering and nutrient uptake by ectomycorrhizal fungus, *Suillus tomentosus*, in liquid-culture experiments. *Geochimica et Cosmochimica Acta* **72**: 2601-2618.

Baxter JW, Dighton J. 2001. Ectomycorrhizal diversity alters growth and nutrient acquisition of grey birch (*Betula populifolia*) seedlings in host-symbiont culture conditions. *New Phytologist* **152**: 139-149.

Berner C, Johansson T, Wallander H. 2012. Long-term effect of apatite on ectomycorrhizal growth and community structure. *Mycorrhiza.* doi: 10.1007/s00572-012-0438-y

Bouyoucos GV. 1962. Hydrometer method improved for making particle-size analysis of soils. *Agronomy Journal* **54**: 464-465.

Bray RL. Kurtz LT. 1945. Determination of total organic and available forms of phosphorus in soils. *Soil Science* **59**: 39-45.

Bruns TD. 1995. Thoughts on the processes that maintain local species diversity of ectomycorrhizal fungi. *Plant and Soil* **170**: 63-73.

Buée M, Reich M, Murat C, Morin E, Nilsson RH, Uroz S, Martin F. 2009. 454 pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. *New Phytologist* **184**: 449-456.

Burns RM. 1990. *Pinus banksiana* Lamb. Silvics of North America. Volume 1 Conifers. USDS. Retrieved October 27th 2012.

Cairney JWG. 1999. Intraspecific physiological variation: implications for understanding functional diversity in ectomycorrhizal fungi. *Mycorrhiza* **9**: 125-135.

Colpaert JV, Vandenkoornhuyse P, Adriaensen K, Vangronsveld J. 2000. Genetic variation and heavy metal tolerance in the ectomycorrhizal basidiomycete *Suillus luteus. New Phytologist* **147**: 367-379.

Dahlberg A, Jonsson L, Nylund J. 1997. Species diversity and distribution of biomass above and belowground among ectomycorrhizal fungi in an old-growth Norway spruce forest in south Sweden. *Canadian Journal of Botany* **75**: 1323-1335.

Dahlberg A. 2001. Community ecology of ectomycorhizal fungi: an advancing interdisciplinary field. *New Phytologist* **150**: 555-562.

DeBellis T, Kernaghan G, Bradley R, Widden P. 2006. Relationships between stand composition and ectomycorrhizal community structure in boreal mixed-wood forests. *Microbial Ecology* **52**: 114-126.

Dixon RK, Buschena CA. 1988. Response of ectomycorrhizal *Pinus banksiana* and *Picea glauca* to heavy metals in soil. *Plant and Soil* **105**: 265-271.

Gagné A, Jany J, Bousquet J, Khasa DP. 2006. Ectomycorrhizal fungal communities of nursery-inoculated seedlings outplanted on clear-cut sites in northern Alberta. *Canadian Journal of Botany* **36**: 1684-1694.

Gehring CA, Theimer TC, Whitham TG, Keim P. 1998. Ectomycorrhizal fungal community structure of Pinyon pines growing in two environmental extremes. *Ecology* **79**: 1562-1572.

Hoffland E, Kuyper TW, Wallander H, Plassard C, Gorbushina AA, Haselwandter K, Holmstrom S, Landeweert R, Lundstrom US, Rosling A, Sen R, Smits MM, Van Hees PAW, Van Breemen N. 2004. The role of fungi in weathering. *Frontiers in Ecology and the Environment* **2**: 258-264.

Hogberg MN, Hogberg P, Myrold DD. 2006. Is microbial community composition in boreal forest soils determined by pH, C-to-N ratio, the trees, or all three? *Oecologia* **150**: 590-601.

Horton TR, Bruns TD. 2001. The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. *Molecular Ecology* **10**: 1855-1871.

Hunt GA. 1991. *Ectomycorrhizal fungi in British Columbia container nurseries*. FRDA Handbook no. 009. Victoria, BC, Canada: Forestry Canada.

Jonsson L, Nilsson M, Wardel DA, Zackrisson O. 2001. Context dependant effects of ectomycorrhizal species richness on tree seedlings productivity. *Oikos* **93**: 353-364.

Jumpponen A, Egerton-Warburton LM. 2005. Mycorrhizal fungi in successional environments: a community assembly model incorporating host plant, environmental, and biotic filters. In: Dighton J, White JF, Oudemans P, eds. *The fungal community: its organization and role in the ecosystem*. Boca Raton, Florida, USA: Taylor & Francis Group LLC, CRC Press, pp. 139-168.

Korkama T, Pakkanen A, Pennanen T. 2006. Ectomycorrhizal community structure varies among Norway spruce (*Picea abies*) clones. *New Phytologist* **171**: 815-824.

Keizer PJ, Arnolds E. 1994. Succession of ectomycorrhizal fungi in roadside verges planted with common oak (*Quercus robur* L.) in Drenthe, The Netherlands. *Mycorrhiza* **4**: 147-159.

Kennedy P. 2010. Ectomycorrhizal fungi and interspecific competition: species interactions, community structure, coexistence mechanisms, and future research directions. *New Phytologist* **187**: 895-910.

Kernaghan G, Sigler L, Khasa D. 2003. Mycorrhizal and root endophytic fungi of containerized *Picea glauca* seedling assessed by rDNA sequence analysis. *Mycrobial ecology* **45**: 128-136.

Kranabetter JM, Durall DM, Mackenzie WH. 2009. Diversity and species distribution of ectomycorrhizal fungi along productivity gradients of a southern boreal forest. *Mycorrhiza* **19**: 99-111.

Krpata D, Peintrer U, Lancer I, Fitz WJ, Schweiger P. 2008. Ectomycorrhizal communities associated with *Populus tremula* growing on a heavy metal contaminated site. *Mycological Research* **112**: 1069-1079.

Lamhamedi MS, Fortin JA. 1991. Genetic variations of ectomycorrhizal fungi: extrametrical phase of *Pisolithus sp.*. *Canadian Journal of Botany* **69**: 1927-1934.

Landeweert R, Hoffland E, Finlay RD, Kuyper TW, Van Bremen N. 2001. Linking plants to rocks: ectomycorrhizal fungi mobilize nutrients from minerals. *TRENDS in Ecology & Evolution* **16**: 248-254.

Lilleskov EA, Fahey TJ, Lovett GM. 2001. Ectomycorrhizal fungal aboveground community change over an atmospheric nitrogen deposition gradient. *Ecological Applications* **11**: 397-410.

Lilleskov EA, Fahey TJ, Horton TR, Lovett GM. 2002. Belowground ectomycorrhizal fungal community change over a nitrogen deposition gradient in Alaska. *Ecology* **83**: 104-115.

MRNF. 2012. Vegetation zones and bioclimatic domains in Québec. *Retrieved* October 30th 2012 from www.mrn.gouv.qc.ca/english/publications/forests/publications/zone-a.pdf

Nienstaedt H, Zasada JC. 1990. *Picea glauca* (Moench) Voss. Silvics of North America, Volume 1:Conifers. United States Forest Service, USA. Retrieved October 27th 2012.

Nilsson LO, Wallander H. 2003. Production of external mycelium by ectomycorrhizal fungi in a Norway spruce forest was reduced in response to nitrogen fertilization. *New phytologist* **158**: 409-416.

Nilsson LO, Giesler R, Baath E, Wallander H. 2005. Growth and biomass of mycorrhizal mycelia in coniferous forests along short natural nutrient gradients. *New Phytologist* **165**: 613-622.

O'Brian HE, Parrent JL, Jackson JA, Moncalvo JM, Vilgalys R. 2005. Fungal community analysis by large-scale sequencing of environmental samples. *Applied Environmental Microbiology* **71**: 5544-5550.

Quoreshi AM, Roy S, Greer CW, Beaudin J. 2007. Inoculation of green alder (*Alnus crispa*) with *Frankia*-ectomycorrhizal fungal inoculant under commercial nursery production conditions. *Native Plants Journal* **8**: 271-281.

Quoreshi AM, Khasa DP. 2008. Effectiveness of mycorrhizal inoculation in the nursery on root colonization, growth, and nutrient uptake of aspen and balsam poplar. *Biomass and Bioenergy* **32**: 381-391.

Quoreshi AM, Piché Y, Khasa DP. 2008. Field performance of conifer and hardwood species 5 years after nursery inoculation in the Canadian prairie provinces. *New Forests* **35**: 235-253.

Rosling A, Landeweert R, Lindahl BD, Larsson K-H, Kuyper TW, Taylor AFS, Finlay RD. 2003. Vertical distribution of ectomycorrhizal fungal taxa in a podzol soil profile. *New Phytologist* 159: 775-783.

Roy S, Khasa DP, Greer CW. 2007. Combining alders, frankiae, and mycorrhizae for the revegetation and remediation of contaminated ecosystems. *Canadian Journal of Botany* **85**: 237-251.

Smith SE, Read DJ. 2008. Mycorrhizal symbiosis. 3rd ed. Academic Press, London, UK.

Smits MM, Bonneville S, Benning LG, Banwart SA, Leake JR. 2012. Plant-driven weathering of apatite – the role of an ectomycorrhizal fungus. *Geobiology* **10**: 445-456.

Sutton RF. 1973. Histoire naturelle de l'Épinette blanche (*Picea glauca* (Moench) Voss). *Ministère de l'Environnement, Service Canadien des Forêts*, Publication # 1250f, Ottawa, Canada.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution* **28**: 2731-2739.

Tedersoo L, May TW, Smith ME. 2010. Ectomycorrhizal lifestyle in fungi: global diversity, distribution, and evolution of phylogenetic lineages. *Mycorrhiza* **20**: 217-263.

Toljander JF, Eberhardt U, Toljander YK, Paul LR, Taylor AFS. 2006. Species composition of an ectomycorrhizal fungal community along a local nutrient gradient in a boreal forest. *New Phytologist* **170**: 873-884.

Twieg BD, Durall DM, Simard SW. 2007. Ectomycorrhizal fungal succession in mixed temperate forests. *New Phytologist* **176**: 437-447.

Villeneuve N, Grandtner MM, Fortin JA. 1989. Frequency and diversity of ectomycorrhizal and saprophytic macrofungi in the Laurentide mountains of Quebec. *Canadian Journal of Botany* **67**: 2616-2629.

Wallander H, Hagerberg D. 2004. Do ectomycorrhizal fungi have significant role in weathering of minerals in forest soils? *Symbiosis* **37**: 249-252.

Wurzburger N, Bidartondo MI, Bledsoe CS. 2001. Characterization of *Pinus* ectomycorrhizas from mixed conifer and pygmy forests using morphotyping and molecular methods. *Canadian Journal of Botany* **79**: 1211-1216.

Wright SHA, Berch SM, Berbee ML. 2009. The effect of fertilization on the belowground diversity and community composition of ECM fungi associated with western hemlock (*Tsuga heterophylla*). *Mycorrhiza* **19**: 267-276.

Yeomans JC, Bremner JM. 1988. A rapid and precise method for routine determination of organic carbon in soil. *Commun Soil Science and Plant Analasis* **19**: 1467-1476.

3. Chapter 2: *In vitro* selection of ecologically adapted ectomycorrhizal fungi through production of fungal biomass and metabolites for use in reclamation of gold mine tailings

Aida Azaiez[1*], Martin Beaudoin Nadeau[1*], Annick Bertrand[2], and Damase P. Khasa[1]

1. Centre for Forest Research and Institute of Integrative and Systems Biology, Université Laval, Quebec city, QC, Canada, G1V 0A6.
2. Soil and Crops Research and Development Centre, Agriculture and Agrifood, 2560, Hochelaga Blvd, Québec, QC, Canada, G1V 0A6.

* These authors have contributed equally to the completion of this study and to the writing of this manuscript (See Foreword for more information).

Keywords: Ectomycorrhizal (ECM) fungi, Ergosterol, *In vitro* culture, Low molecular mass organic acids (LMMOAs), Mine tailings.

Article prepared for submission in the scientific journal ‘’Mycorrhiza’’

3.1 Résumé

L'altération biologique des minéraux joue un rôle important dans les environnements pauvres en nutriments tels que les déblais et résidus miniers. Les champignons ectomycorhiziens (ECM) sont en mesure d'améliorer l'altération des minéraux en rendant certains minéraux et nutriments disponibles aux plantes. Dans cette étude, six champignons ECM (*Cadophora finlandia, Cenococcum geophilum, Hebeloma crustuliniforme, Lactarius aurantiosordidus, Paxillus involutus* et *Tricholoma scalpturatum*) ont été testés pour leur tolérance aux résidus miniers riches en biotite et en quartz. Des méthodes sur milieux solides et liquides ont été utilisées pour faire la sélection *in vitro* de champignons ECM avec une excellente capacité de croître sur les résidus miniers. Les champignons ECM prometteurs et tolérants aux résidus miniers ont été choisis en fonction de leur croissance radiale de mycélium et de leur production en métabolites (teneur en ergostérol, et en acides organiques de faible masse moléculaire). Nous avons constaté une forte corrélation entre la teneur en ergostérol fongique et la croissance radiale du mycélium suivant la méthode sur milieux solides. Cependant, le procédé en milieu liquide était plus approprié pour comparer la synthèse fongique de l'ergostérol entre différentes espèces et souches de champignons. En plus, ce procédé permettait la mesure de la production d'acides organiques par les champignons. Nous avons constaté que les acides organiques tels que les acides citrique, malonique et succinique ont été produits par les champignons ECM afin de solubiliser les résidus miniers pour leur croissance et nutrition. Enfin, nous avons conclu que les champignons ECM natifs de la mine - *C. finlandia* et *T. scalpturatum* - sont les espèces à l'étude les plus tolérantes aux résidus miniers et elles pourraient potentiellement améliorer le taux de survie, la santé et la croissance de semis d'épinette blanche plantés sur les déblais et résidus miniers.

3.2 Abstract

Mineral weathering plays an important role in poor-nutrient environments such as mine spoils and tailings. Ectomycorrhizal (ECM) fungi are able to enhance mineral weathering through different mechanisms; thereby, increasing the availability of minerals and nutrient to plants. Six ECM fungi (*Cadophora finlandia*, *Cenococcum geophilum*, *Hebeloma crustuliniforme*, *Lactarius aurantiosordidus*, *Paxillus involutus* and *Tricholoma*

scalpturatum) were tested in the present study for their tolerance to biotite-quartz rich mine tailings. Either solid- or liquid-media methods were used for *in vitro* selection of ECM fungi for their ability to grow on mine tailings. Potential ECM fungi tolerant to mine tailings were selected based on their mycelial radial growth and metabolite production (ergosterol and low molecular mass organic acids, LMMOAs). We found a strong correlation between fungal ergosterol content and mycelial radial growth in the solid-medium method. However, the liquid-medium method was suggested to be more appropriate for ergosterol synthesis. In addition, it permitted the measurement of organic acid production. We found that LMMOAs such as citric, malonic and succinic acids were exuded by ECM fungi in order to solubilize mine tailings for their own growth and nutrition. Finally, we concluded that the mine-native ECM fungi *C. finlandia* and *T. scalpturatum* are the most tolerant species to mine tailings and, through their action of increasing mineral availability to plants, they could potentially improve the survival rate, growth and health of white spruce seedlings planted on mine spoils and tailings.

3.3 Introduction

Biological weathering plays an important role in rendering available minerals and nutrients to plants in soil. Recent studies have demonstrated the ability of ectomycorrhizal (ECM) fungi to alter and increase weathering of silicate minerals such as apatite, feldspath, and hornblende (Wallander and Hagerberg 2004; Berner *et al.* 2012; Smits *et al.* 2012). In the process, essential minerals and nutrients released from the rock substrate are partly used by ECM fungi for their development and then transferred to tree roots for nutrition in exchange of photosynthates (Dahlberg 2001, Smith and Read 2008). Ectomycorrhizal weathering is controlled by four different mechanisms. Firstly, expansion and contraction of fungal hyphae during wetting, drying, freezing, and thawing periods accelerate physical weathering of minerals (Hoffland *et al.* 2004). Secondly, ECM fungi produce large quantities of low molecular mass organic acids (LMMOAs, particularly oxalic, citric, and malic acids) which bind easily to cations (Al^{3+}, Fe^{3+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, and Cu^{2+}) lowering free cation activity in soil solution (Drever 1997; Landeweert *et al.* 2001). This process leads to a decrease in saturation of cations within the soil solution favouring further mineral dissolution and weathering (Drever 1997; Hoffland *et al.* 2004). Thirdly, mycorrhizal fungi

account for more than 50 % of respiration in soil (Landeweert *et al.* 2001; Hoffland *et al.* 2004). Water dissolution of respiratory CO_2 in soil generates molecules of carbonic acids, which trigger a decrease of pH in soil thus promoting mineral dissolution and weathering (Taylor *et al.* 2009). Fourthly, NH_4^+ uptake by ECM fungi acidifies the soil solution through excessive release of H^+ promoting further mineral dissolution and weathering (Hoffland *et al.* 2004; Littke *et al.* 1983).

Weathering performance differs widely among ECM fungal species and strains because some have the ability to produce more organic acids than others (Cairney 1999). The measurement of organic acid production by ECM fungal tissues is a strong indicator of the capacity of a species or strain to alter and acquire minerals for their nutrition and development. In rocky soils, elements in very high concentrations may become toxic to ECM fungi and affect negatively their growth and survival (Khan 2006). Studies have shown inter- and intra-specific differences in adaptation and tolerance to high concentration of toxic elements (Cairney 1999; Colpaert *et al.* 2000). For the revegetation of disturbed mine spoils and tailings using trees and their symbionts, it is crucial to identify ECM fungi that are tolerant to tailings, highly performant in weathering, and fast growing.

Fungal metabolite such as ergosterol is a compound that is produced only by fungal membrane tissues; it has been widely used for the estimation of ECM fungal biomass in soil (Olsson 1999). The measurement of fungal ergosterol content is a promising technique for estimating ECM fungal growth and the extent of fungal membranes (Ruzicka *et al.* 2000). However, certain researchers argued that different fungal species synthesize different quantities of sterol, so it may be difficult to compare ergosterol content among species (Ruzicka *et al.* 2000). For these reasons, an approach combining ergosterol content and other techniques for measuring the *in vitro* growth of ECM fungi is more appropriate.

Sigma-Lamaque mine in the Abitibi region of Quebec in Canada is an open pit gold mine in which waste rocks and fine tailings are mainly composed of biotite (Taner *et al.* 1986). Biotite of the gold deposit is associated with pyrite and pyrrhotite rich in Fe (Taner *et al.* 1986). This type of tailings is mainly composed of Fe, Ca, Al, and Mg in high concentrations but also with other important mineral elements such as P, K, Zn, Mn, Cu, Mo, and Na in low concentrations (Taner *et al.* 1986). Balogh-Brunstad *et al.* (2008) demonstrated

that ECM fungi have the ability to increase weathering of biotite by producing organic acids and reducing soil pH. For instance, soil pH of biotite dropped by almost three units after four weeks of incubation with *Suillus tomentosus* (Balogh-Brunstad *et al.* 2008). The activity of *S. tomentosus* almost tripled the release rate of Mg^{2+} and Fe^{2+} (Balogh-Brunstad *et al.* 2008) and, in this process, H^+ ions from the organic acids, produced by ECM fungi, replace cations such as K^+, Mg^{2+} and Fe^{2+} in the biotite interlayers (Boyle *et al.* 1974). The biotite dissolution rate increased considerably with pH under 7 (Balogh-Brunstad *et al.* 2008).

In this study, we explored different *in vitro* techniques for selecting ECM fungal species ecologically adapted to biotite-quartz rich gold mine tailings. Our principal objectives were (1) to identify the most suitable methods for selecting *in vitro* highly performant ECM fungi and (2) to determine if some species isolated from fine roots of healthy white spruce (*Picea glauca*) host regenerating on mine tailings show better growth, production of organic acids, and tolerance to mine tailing conditions than others. We hypothesized that the measurements of ergosterol and organic acid contents are appropriate methods for selecting highly performant ECM fungi under axenic conditions. It was also hypothesized that native ectomycorrhizal fungal species are more tolerant and grow better in mine tailings than exotic species.

3.4 Materials and methods

3.4.1 ECM fungal isolation techniques and identification

Fine roots were collected from naturally regenerating white spruce seedlings at the Sigma-Lamaque gold mine and the surrounding natural forest near Val d'Or in the Abitibi region, Quebec, Canada (coordinates: 48°06'20.79''N – 77°45'03.47''W) (Nadeau and Khasa 2015, Chapter 1). Roots were washed gently with tap water in order to remove soil particles and were stored in a 150-ml plastic tube filled with water until further manipulation at Université Laval. ECM root tips were characterized based on their color, texture, form, size and presence of hyphae and rhizomorphs (Wurzburger *et al.* 2001).

Five root tips of each morphotype were sterilized by washing them in solutions of TWEEN 20 (0.2%) for 30 sec, tap H_2O for 30 sec, H_2O_2 for 30 sec, and finally sterilized H_2O for 1 min. Sterilized root tips were transferred into modified Melin Norkrans media (MNM)

with Chlortetracycline (30 mg/L 100X) and Benomyl (1 mg/L 100X) under aseptic conditions. The solid culture medium (pH = 5.5) contained some agar (10 g/l), glucose (10 g/l), malt extract (3 g/l), KH_2PO_4 (0.5 g/l), $(NH_4)_2HPO_4$ (0.25 g/l), $MgSO_4 \cdot 7H_2O$ (0.075 g/l), $CaCl_2 \cdot H_2O$ (0.067 g/l), NaCl (0.025 g/l), $FeCl_3 \cdot 6H_2O$ (0.001 g/l), and thiamine (100 ug/l). Contamination was verified and discarded every two days to avoid contaminating other root tips. The *in vitro* cultures were put in an incubator at 23.2°C. Cultures were observed regularly under a stereomicroscope. Cultures revealing fungal growth similar to ECM fungi were kept for further analyses while others were discarded.

After two months of growth on MNM media, potential ECM fungi were transferred on a potato dextrose agar (PDA) medium with cellophane. Three weeks later, fungal tissues growing directly above the cellophane were collected with a sterilized scalpel and transferred into an Eppendorf 1.5 ml tube for biomolecular analyses. The subsequent steps for ECM fungal DNA sequencing and species identification (Fungal DNA extraction, PCR amplification, DNA sequencing and identification through blastn using the GenBank search engine) were performed following the techniques described by Nadeau and Khasa (2015, Chapter 1).

3.4.2 *In vitro* ECM fungal growth in gold mine tailings using solid medium

Four ECM fungal strains were successfully isolated: *Tricholoma scalpturatum* MBN0213 and *Cadophora finlandia* MBN0213 from the mining site and *Lactarius aurantiosordidus* MBN0213 and *Cenococcum geophilum* MBN0213 from the natural forest. Two other species were chosen to be part of the experiment: *Hebeloma crustuliniforme* UAMH5247 and *Paxillus involutus* UAMH8235. These two strains were also isolated from natural forest many years ago and are known for their fast growing capacity and ability to considerably improve white spruce seedling growth (Onwuchekwa et al. 2014; Khasa et al. 2001; Bois et al. 2006b) and for their mineral weathering efficiency (Courty et al 2010; Lapeyrie et al 1991). All strains are available in the microbial collections of Centre for Forest Research (CEF, www.cef-cfr.ca) at Université Laval.

For each experimental unit, one plug (38 mm^3) of fungal tissues was added and grown above cellophane with or without 12 g of sterilized fine tailings (grains < 1 mm of diameter) in petri dish containing 22 ml of solid modified MNM medium during a period of 8 weeks at

23.4°C under axenic conditions. Normal MNM medium had a pH of 5.5 and contained agar (12 g/l), glucose (10 g/l), malt extract (3 g/l), $(NH_4)_2HPO_4$ (0.25 g/l), $MgSO_4 \cdot 7H_2O$ (0.075 g/l), $CaCl_2 \cdot H_2O$ (0.067 g/l), NaCl (0.025 g/l), $FeCl_3$ (1%) (1.5 ml), and thiamine (100 ug/l). For the poor MNM medium, the following modifications were done. The pH was increased to 8, similar to mine tailings. Agar was replaced by agarose for higher purity. Furthermore, P concentration was reduced by two third of the initial concentration and sources of Mg, K, Fe, and Ca were completely removed from the solution. The poor medium contained agarose (12 g/l), glucose (10 g/l), $(NH_4)_2HPO_4$ (0.25 g/l), and thiamine (25 µg/l).

The experimental design used for this experiment was a completely randomized design (CRD): two crossed fixed factors. The first factor was represented by ECM fungal species with six levels: *Tricholoma scalpturatum* (T.s.), *Cadophora finlandia* (C.f.), *Hebeloma crustuliniforme* (H.c.), *Paxillus involutus* (P.i.), *Lactarius aurantiosordidus* (L.a.), and *Cenococcum geophilum* (C.g.). The second factor was represented by the growing media with three different levels: poor MNM medium with fine tailings, poor MNM medium without fine tailings, and normal MNM medium without fine tailings. One petri dish containing poor or normal MNM medium with or without fine tailings was regarded as one experimental unit. Experimental units without fine tailings were considered as controls. There were 18 treatments and five replicates per treatment for a total of 90 experimental units.

After eight weeks of incubation, fungal radial growth was measured in four different opposite directions (0°, 90°, 180°, and 270°) starting at the centre of the petri dish where the plugs were deposited at the beginning of the experiment. Mean radial growth was calculated by averaging the four measurements. Then, fungal tissues were collected from the cellophane with and without tailings and lyophilized during 48 hours. Dry biomass of fungal tissues without tailings was measured by weighing the samples on an analytical balance with precision ± 0.00005 g. For us, it was very important to grow ECM fungi directly in contact with the fine tailings in order to mimic as much as possible the growing conditions of ECM fungi in its natural mining soil environment. For that reason, this experiment was limited in regards to the measurement of dry biomass in treatments with tailings. This limitation came from the fact that tailings weighted more than 1000 times higher than the fungal tissues at

the end of the experiment contributing to inaccurate assessment of ECM fungal dry biomass. Therefore, dry biomass of treatments with tailings was not assessed. Finally, ECM fungal ergosterol content was determined following the method developed by Gong *et al.* (2001). Four grams of acid-washed glass beads (2 g of 212-300 µm diameter and 2 g of 710-1180 µm diameter) purchased from Sigma-Aldrich Co. (St-Louis, MO, USA) and 20 ml of methanol were added to samples for optimal ergosterol extraction. The samples were vortexed for 15 sec and sonicated during 60 min at 320 rpm in an orbital HT-TR225 shaker (INFORS AG, Bottmingen, Switzerland). Samples were allowed to precipitate for 15 min. Then, 1.2 ml of supernatant was transferred into a 1.5 ml Eppendorf microtube and centrifugated for 3 min at 13 000 rpm. Subsequently, 1 ml of supernatant was collected without disturbing the residues and re-centrifugated. Then, 200 ul of the solution was transferred into a HPLC microtube. Ergosterol quantification was performed by a HPLC analysis system controlled by WATERS Empower software (WATERS, Milford, MA, USA) with a Model 515 pump, a Model 717^{plus} autosampler, and a Model 2487 UV detector. Ergosterol was separated on a C18 reverse-phase column (Nova Pak) and detected on a detector set at 282 nm. Methanol was used as the mobile phase at a flow rate of 1 ml min^{-1}. Under such conditions, the retention time of ergosterol was 14.2 min. Ergosterol purchased from Sigma-Aldrich Co. (St-Louis, MO, USA) (purity $\geq$ 95%) served as solution for preparing standards and identifying total ergosterol content of the different samples. Because of the impossibility to accurately divide the mixture of solid medium with tailings containing fungi, all the content was used to measure total ergosterol production by the ECM fungi. The production of organic acids by the ECM fungi was thus not measured because all the material has been used for ergosterol assessment.

Two-way analyses of variance (strains*media) was performed (PROC GLM) using the SAS 9.3 software (SAS Institute Inc. 2012, North Carolina, USA) in order to identify any differences in mean total ergosterol content, dry biomass, and radial growth between treatments. LOG and square root transformations were needed in order to meet the assumptions of normality and homoscedasticity for ergosterol content and radial growth, respectively. Standard errors and means were calculated and are presented in the figures. Significance for all analyses was set at P-value ≤ 0.05. Relationships between ergosterol content, dry biomass, and radial growth were investigated through linear regression analyses

(PROC REG, SAS 9.3 software, SAS Institute Inc., North Carolina, USA). *P*-value and Pearson correlation coefficients (r) were utilized for identifying significant correlations between the three variables.

3.4.3 *In vitro* ECM fungal growth in gold mine tailings using liquid medium

Tailings were milled into different particle sizes of <3 to >1.5, <1.5 to >0.5, <0.5 to >0.1, and <0.1 mm that were identified as particle sizes A, B, C and D respectively. Poor liquid MNM medium (same nutrient concentrations as the poor solid MNM medium with tailings described above) was used for fungal growth. Each of the 125 ml flasks used contained 1.5 g of mine tailings per 40 ml of MNM. Potassium hydrogen phthalate (50 mM) was added to maintain pH at 5.5 (otherwise pH reached 8 due to the alkalinity of mine tailings and fungi did not grow in the liquid solution). Four plugs, cut with a 6-mm cork borer, of actively growing mycelia were added to the flasks. Controls without tailings containing a solution with complete composition of MNM were included in the experience (NM medium). In order to test the medium effects, another control without ECM fungi containing poor MNM and tailings of different particle sizes was added. There were four replicates for each treatment. The flasks were incubated for 8 weeks at 23.4°C and shaken at 100 rpm.

The experimental design was a completely randomized design with two crossed fixed factors: ECM fungi and particles sizes. The first factor was composed of a control (no fungus) and three fungal species having great radial growth on poor solid medium with tailings (*Cadophora finlandia* MBN0213, *Tricholoma scalpturatum* MBN0213 and *Hebeloma crustuliniforme* UAMH5247). The second factor was the four particle sizes of mine tailings (A, B, C and D) as well as a control, giving a total of 20 treatments, which were replicated 4 times and assigned at random to experimental units.

Fungi and mine tailings were separated from growth media by filtration (filter paper Whatman, 11 µm). Fungi+tailings were lyophilized before ergosterol assessment. Ergosterol was quantified by a HPLC analytical system as described above. The concentration of organic acids produced by the fungi in the growth media was measured. The collected media were homogenized by vortexing and centrifuged for 1 min at 16000 rpm and the supernatant frozen at -80°C prior to HPLC analysis. 1 ml of the collected media was centrifuged for 3

min at 13000 rpm and a volume of 200 µl of the supernatant was transferred into vials for HPLC analyses. Organic acids were analyzed by HPLC as described in Adeleke *et al.* (2010). Organic acids were separated on a Bio-Rad HPX-87H column (Bio-Rad, Hercules, CA, USA) eluted isocratically at 40°C at a flow rate of 0.6 ml/min with 0.008 N sulfuric acid and detected on a dual absorbance detector set at 220 nm (WATERS, Model 2487). Organic acids identification and quantification were performed by comparison with standards. The organic acids standards included oxalic, citric, malic, malonic, succinic, lactic, formic and acetic acids.

As quantitative treatments were investigated in this study, data was analyzed via mixed-model analyses of variance (Littell *et al.* 2006) using restricted maximum likelihood (PROC Mixed, SAS 9.3 software, SAS Institute, Cary NC). Before statistical analysis, the homogeneity of residual variances was tested graphically using PROC Plot in SAS. The normality of experimental errors was tested using PROC Univariate in SAS, and LOG transformation applied where necessary to meet normality assumptions. Each of the response variables was fitted to the following statistical model: $Y_{ij} = \mu + \pi_i + \alpha_j + (\pi\alpha)_{ij} + \varepsilon_{ij}$ where Y_{ij} is the average value of the dependent variable for the i^{th} fungus and the j^{th} particle size, μ is the overall mean, π_i, α_j and $(\pi\alpha)_{ij}$ collectively represent fixed effects, and correspond, respectively, to fungus, mine tailings' particle size and fungus x mine tailings' effects, and the accepted random error. Pairwise comparisons of fungus and fungus x mine tailings' particle size treatment means were obtained using the pdiff option on the least square means statement of the mixed procedure in SAS, whereas polynomial contrasts were constructed for ranking of particle sizes.

To examine relationships of correlation between ergosterol content and organic acid release, Pearson product-moment correlations were performed using PROC CORR in SAS 9.3. In addition, an analysis of stability was conducted to compare ergosterol extraction amount between solid and liquid methods. To do so, treatment method was considered as a repeated measures factor (Montgomery 2012), and we modelled the covariance structure within-subjects for ergosterol amount using the autoregressive order one structure (Littell *et al.* 2006), which was fitted in the ANOVA model.

3.5 Results

3.5.1 *In vitro* ECM fungal growth on solid medium

Both ECM fungi and growth media affected total ergosterol content, dry biomass, and mean radial growth and there was an interaction between the two factors (*P*-values < 0.0001). Total ergosterol content of *Tricholoma scalpturatum* was significantly higher in normal MNM compared to poor MNM with tailings (Fig. 3.1). *Cadophora finlandia* showed its highest production of ergosterol in poor MNM without tailings followed by normal MNM and then the smallest concentration of ergosterol was found in poor MNM with tailings (Fig. 3.1). *Lactarius aurantiosordidus* had lower total ergosterol content in poor MNM without tailings compared to the other two media (Fig. 3.1). Total ergosterol content of *Cenococcum geophilum* was higher in poor MNM with tailings than in poor MNM without tailings (Fig. 3.1). *Hebeloma crustuliniforme* significantly produced more ergosterol in poor MNM with tailings compared to treatments with normal MNM and poor MNM without tailings (Fig. 3.1). *Paxillus involutus* showed the highest production of ergosterol in normal medium, which was almost three times greater than in poor medium with tailings, and the lowest ergosterol content in poor medium without tailings (Fig. 3.1). *T. scalpturatum, H. crustuliniforme,* and *P. involutus* were the three ECM fungal species with the highest ergosterol content (± 25 µg) in treatments with tailings and differences between the three were not significantly different (Fig. 3.1). Ergosterol content of *C. finlandia* and *L. aurantiosordidus* (± 7.5 µg) was significantly smaller compared to *T. scalpturatum, H. crustuliniforme*, and *P. involutus* and differences between the two were not significantly different (Fig. 3.1). *C. geophilum* displayed the lowest production of ergosterol of the six ECM fungal species in treatments with tailings.

Fungal dry biomass of *T. scalpturatum, C. finlandia*, *L. aurantiosordidus*, and *C. geophilum* was higher in normal MNM than in poor MNM without tailings (Fig. 3.2). No differences were found between mycelial dry biomass of treatments in normal MNM and poor MNM without tailings for the two ECM fungal species: *H. crustuliniforme*

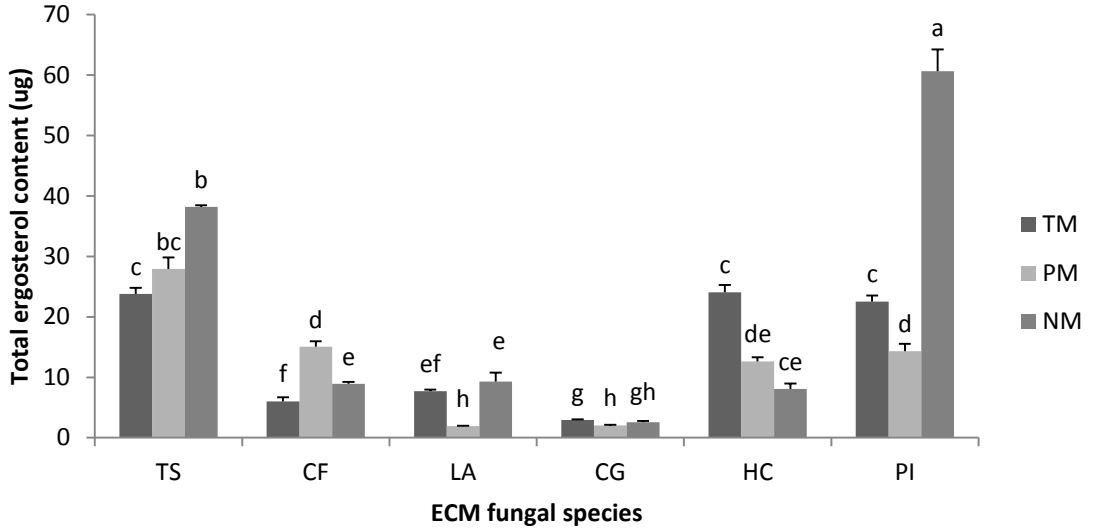

Figure 3.1: Total ergosterol content (ug) of ECM fungi (TS = *Tricholoma scalpturatum*, CF = *Cadophora finlandia*, LA = *Lactarius aurantiosordidus*, CG = *Cenococcum geophilum*, HC = *Hebeloma crustuliniforme*, and PI = *Paxillus involutus*) in three different solid culture media (TM = poor medium with tailings, PM = poor medium without tailings, and NM = normal medium) after eight weeks of growth (starting on the left side, means ± SE with same letters are not significantly different at α=0.05, Tukey test).

and *P. involutus* (Fig. 3.2). Interestingly, dry biomass of *C. finlandia* in poor MNM without tailings was not significantly different than that of *T. scalpturatum* and considerably higher than those of *L. aurantiosordidus, C. geophilum, H. crustuliniforme,* and *P. involutus* in normal richer medium (Fig. 3.2). Furthermore, dry biomass of *T. scalpturatum* in poor MNM was significantly similar to that of *L. aurantiosordidus, C. geophilum, H. crustuliniforme,* and *P. involutus* in normal rich medium (Fig. 3.2).

Mean radial growth of *T. scalpturatum* was more than 7 times higher in normal MNM and poor MNM with tailings compared to poor MNM without tailings (Fig. 3.3). Mean radial growth of *C. finlandia, L. aurantiosordidus, C. geophilum,* and *H. crustuliniforme* was at least two times greater in normal MNM compared to both poor media with and without tailings (Fig. 3.3). Mean radial growth of *P. involutus* was more than four times superior in normal MNM compared to poor MNM without tailings (Fig. 3.3). In addition, *P. involutus* manifested its lowest mean radial growth in poor MNM with tailings, which was more than four times smaller than in poor MNM without tailings (Fig. 3.3). In treatments with tailings, the two ECM fungal species, *T. scalpturatum* and *H. crustuliniforme*, had the highest radial growth (1-2 cm) and no significant difference was found between the two (Fig. 3.3).

Moreover, *C. finlandia* showed moderate radial growth (±0.75 cm) and it was not significantly different to *H. crustuliniforme* (Fig. 3.3). Lastly, the other three species, *L. aurantiosordidus, C. geophilum*, and *P. involutus*, had the lowest mean radial growth (close to 0 cm) (Fig. 3.3). See Appendix III for illustrations of ECM fungal growth on solid medium.

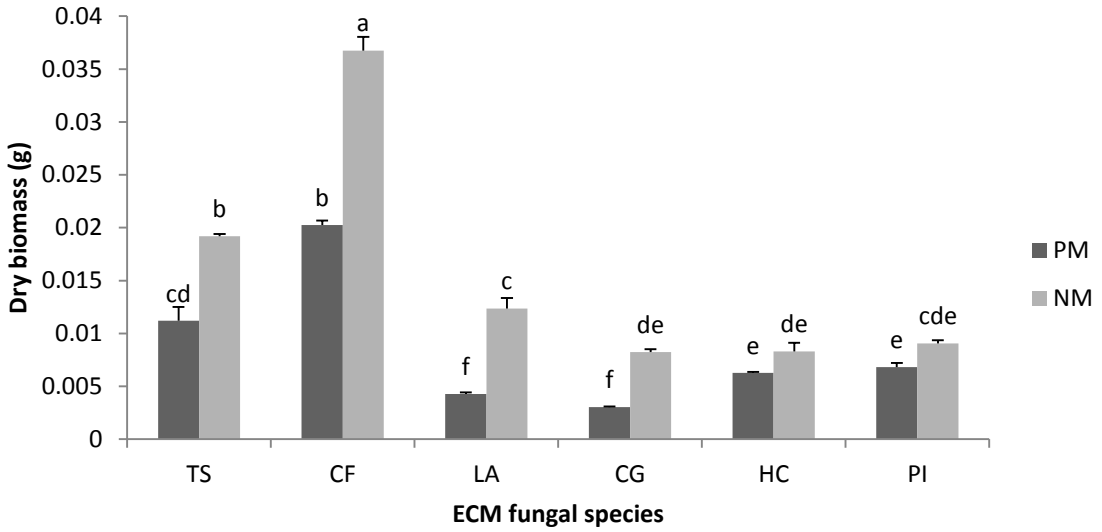

Figure 3.2: Dry biomass (g) of ECM fungi (TS = Tricholoma scalpturatum, CF = *Cadophora finlandia*, LA = *Lactarius aurantiosordidus*, CG = *Cenococcum geophilum*, HC = *Hebeloma crustuliniforme*, and PI = *Paxillus involutus*) in two different solid culture media (PM = poor MNM without tailings and NM = normal MNM) after eight weeks of growth (starting on the left side, means ± SE with same letters are not significantly different at α=0.05, Tukey test).

In this study, after analyzing correlation between ergosterol content, dry biomass, and mean radial growth, it was found that there was a strong positive relationship between ECM fungal ergosterol content and mean radial growth (r = 0.57, *P*-value < 0.0001). On the contrary, fungal dry biomass was correlated to neither ergosterol content (r = 0.16, *P*-value = 0.2783) nor mean radial growth (r = 0.27, *P*-value = 0.0678).

3.5.2 *In vitro* ECM fungal growth on liquid medium

Convergence criteria were met for each dependent variable, and Type III tests of fixed effects are shown in Table 3.1. Highly significant ($P < 0.0001$) fungal type x mine tailings particle size interaction was noted on ergosterol quantities that were extracted, indicating that fungal type has a differential effect on particle size. Simply put, the

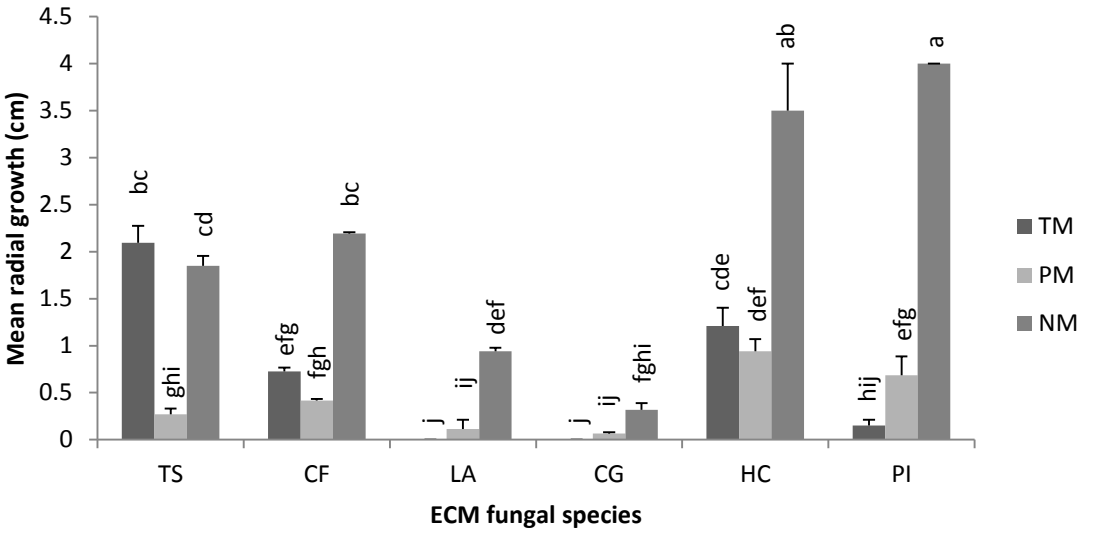

Figure 3.3: Mean radial growth (cm) of ECM fungi (TS = *Tricholoma scalpturatum*, CF = *Cadophora finlandia*, LA = *Lactarius aurantiosordidus*, CG = *Cenococcum geophilum*, HC = *Hebeloma crustuliniforme*, and PI = *Paxillus involutus*) in three different solid culture media (TM = poor medium with tailings, PM = poor medium without tailings, and NM = normal medium) after eight weeks of growth (starting on the left side, means ± SE with same letters are not significantly different at α=0.05, Tukey test).

ergosterol amount that is extracted from each particle size depends on the fungal type used for extraction. Fungi cultivated on complete MNM media without tailings showed significant higher quantities of ergosterol than those that grew on minimal MNM media supplemented with mine tailings (Fig. 3.4). When grown on mine tailings, the total amount of ergosterol extracted from *C. finlandia* ranged between 40 µg and 79 µg and depended on the size of mine particles (Fig. 3.4). Ergosterol content was higher in particle A than in particle B > C > D. Ergosterol content of *C. finlandia* was two-fold higher in complete MNM without tailings than in MNM with particle A. Moreover, *C. finlandia* had two-fold more ergosterol in particle A (79 µg) than in particle D (40 µg). *Tricholoma scalpturatum* had significantly more ergosterol when grown on particle C (58 µg) than on particle D (45 µg). However, no ergosterol was recorded when *T. scalpturatum* was cultivated on A and B particles (Fig. 3.4). In comparison with the other fungi, *H. crustuliniforme* showed smaller quantities of ergosterol when grown on mine tailings. Nevertheless, ergosterol content was high (130 ug) when *H. crustuliniforme* grew on complete MNM medium without tailings (Fig. 3.4). As shown in Table 3.1, both fungal type and particle size have significant effect on ergosterol content ($P < 0.0001$). The highest total ergosterol content was recorded for *C. finlandia* followed by *T. scalpturatum* then by *H. crustuliniforme* (Fig. 3.4). As expected, complete

MNM medium without tailings showed higher ergosterol content than media with tailings (Fig. 3.4).

Table 3.1: Analysis of variance (ANOVA) with *F* and *P* values that show the effects of the fixed factors fungal type, particle size and their interaction on total ergosterol (α = 0.05 level of significance).

Treatments	*df*	*F*	*P*
Fungal type	2	227.88	<0.0001
Particle size	4	138.85	<0.0001
Fungal type x particle size	8	52.76	<0.0001
Contrasts			
Mine tailings vs no tailings	1	172.68	<0.0001
A vs B	1	47.04	<0.0001
A vs C	1	372.16	<0.0001
A vs D	1	82.61	<0.0001
B vs C	1	154.59	<0.0001
B vs D	1	4.98	0.0311

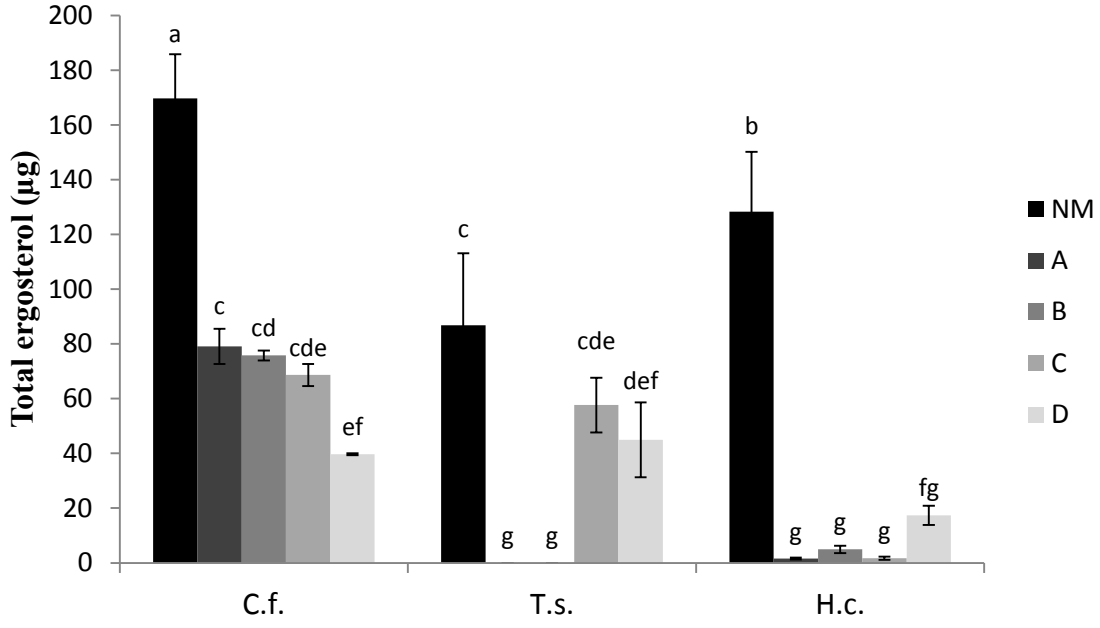

Figure 3.4: Total ergosterol content of the three ECM fungi *C. finlandia*, *T. scalpturatum* and *H. crustuliniforme* grown either in complete MNM media without tailings (NM) or in minimal MNM media with mine tailings at different particle sizes (A, B, C, or D) (Means ± SE with same letters are not significantly different at α = 0.05, LSD test).

When grown on minimal MNM medium amended with mine tailings, ECM fungi released all seven organic acids tested in this study. Except for lactic acid, the production of the other organic acids was found to be significantly affected by the fungal type and particle size interaction (Table 3.2). The presence of mine tailings highly ($P < 0.0001$) affected release of succinic and formic acids (Table 3.2). Higher quantities of succinic acid, malonic acid and formic acids were released by all three fungi in the presence of mine tailings than in complete MNM media without tailings. The production of succinic acid by *C. finlandia* was directly proportional to particle size (163 µg/ml released in A, 75 µg/ml in B, 64 µg/ml in C, and 51 µg/ml in D) (Fig. 3.5c). This is not the case for the other fungi, nevertheless *H. crustuliniforme* significantly produced succinic acid in the presence of mine tailings ($P < 0.0001$). As shown in Table 3.2, the presence of mine tailings did not affect citric acid productions. However, *C. finlandia* (for all particle sizes) and *H. crustuliniforme* (only in particle sizes A, C and D) released significant amounts of citric acid in the presence of mine tailings ($P < 0.0001$). Quantities of citric acid that were released differed significantly among particles sizes for the three fungi ($P < 0.0001$) but not for the controls without fungi. The amount of acetic acid released was also not affected by mine tailings ($P = 0.6842$). However, for *C. finlandia*, acetic acid secretion was significantly higher in A, B and C particle sizes (Fig. 3.5d). The presence of mine tailings significantly affected malonic acid production ($P < 0.0001$). Malonic acid was significantly secreted by *C. finlandia* in all particle sizes ($P < 0.0001$) and by *H. crustuliniforme* in C and D particle sizes (Fig. 3.5b). Unexpectedly, *T. scalpturatum* significantly produced less malonic acid when grown in the presence of mine tailings ($P < 0.0001$). Lactic acid release was not affected by the presence of mine tailings ($P = 0.3336$, Table 3.2). However, some lactic acids were significantly produced by *H. crustuliniforme* ($P < 0.0001$) (Fig. 3.5f). Oxalic acid was significantly produced in all three fungi ($P < 0.0001$) but in smaller amounts relative to the other organic acids (Fig. 3.5g). As expected, very small quantities of acetic, malonic and lactic acids were detected in controls without fungi. However, relatively high amounts of the other organic acids were produced in these controls (Fig. 3.5). This may be explained by the presence of bacteria in mine tailings (tailings were not sterilized).

Table 3.2: Analysis of variance (ANOVA) with *F* and *P* values that show the effects of the fixed factors fungal type, particle size and their interaction on the production of seven different organic acids (α = 0.05 level of significance).

Sources of variation	*df*	Citric acid (*d f*= 57)		Malonic acid (*d f*= 57)		Acetic acid (*d f*= 57)		Lactic acid (*d f*= 57)		Succinic acid (*d f*= 57)		Formic acid (*d f*= 57)		Oxalic acid (*d f*= 57)	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Fungal type	3	6.49	0.0007	65.71	<0.0001	45.33	<0.0001	34.95	<0.0001	13.75	<0.0001	20.23	<0.0001	9.94	<0.0001
Particle size	4	0.36	0.8342	10.39	<0.0001	2.40	0.0606	2.41	0.0597	49.48	<0.0001	45.62	<0.0001	41.34	<0.0001
Fungal type *vs.* particle size	12	3.7	0.0004	6.79	<0.0001	2.41	0.0134	1.52	0.1447	4.52	<0.0001	3.22	0.0014	3.21	0.0015
Mine tailings *vs.* No tailings	1	0.58	0.4496	18.28	<0.0001	0.17	0.6842	0.95	0.3336	23.82	<0.0001	20.23	<0.0001	1.53	0.2205

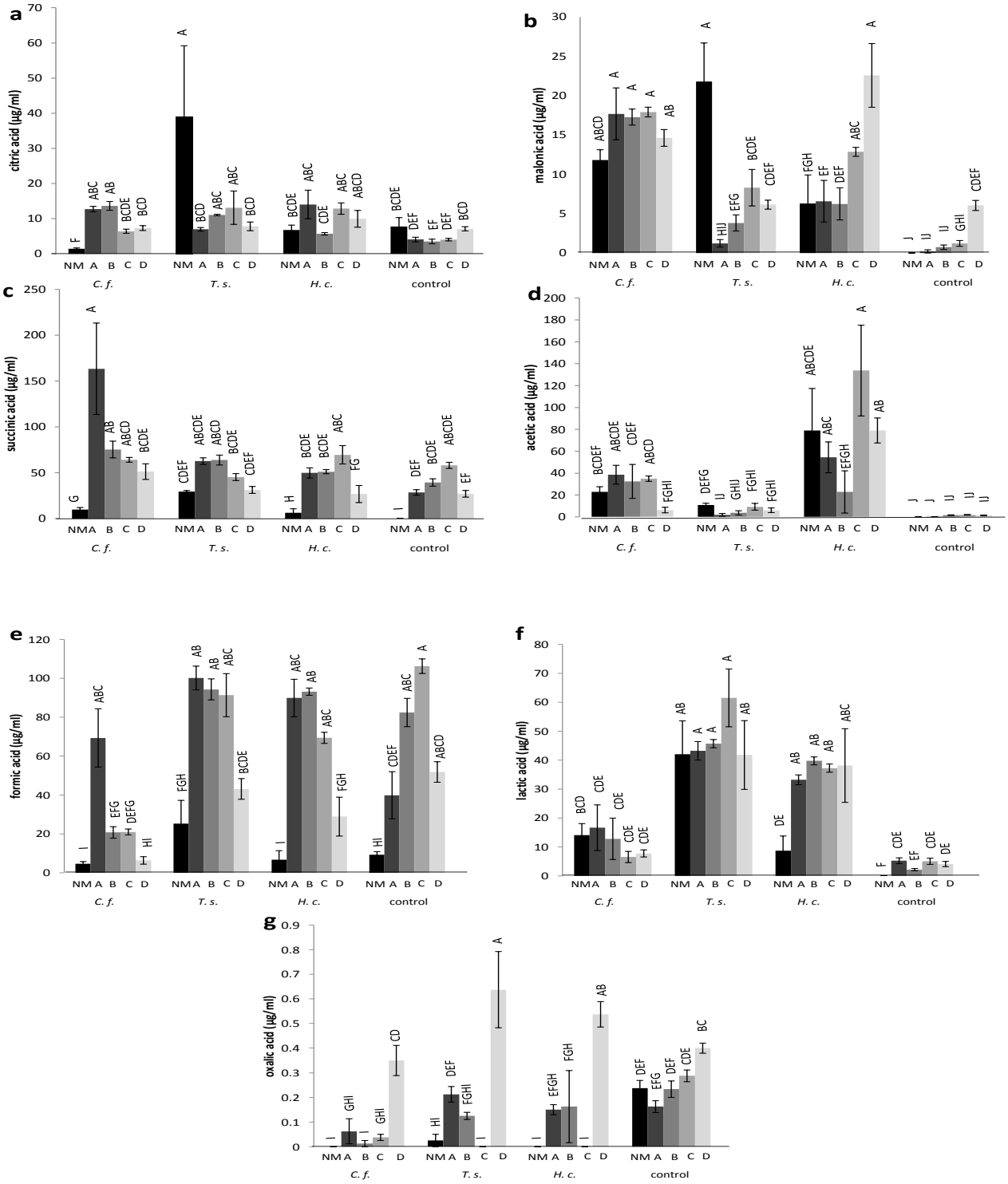

Figure 3.5: Amount of citric acid (a), malonic acid (b), succinic acid (c), acetic acid (d), formic acid (e), lactic acid (f) and oxalic acid (g) released by the three ECM fungi grown under either poor media (TM) with different size of tailings (A, B, C, or D) or no tailings (NM) media (Means ± SE with same letters are not significantly different at α=0.05, LSD test).

No correlation exists between ergosterol content and organic acid production (R^2 < 0.2, *P*-values varied from 0.01 to 0.74) for all organic acids except formic acid where R^2 reached 0.4 ($P < 0.0001$). Ergosterol content and formic acid were negatively correlated (r = - 0.64, $P < 0.0001$). Furthermore, stability analysis showed a significant difference (P = 0.0002) in ergosterol extraction between liquid and solid methods used for fungal growth.

3.6 Discussion

3.6.1 *In vitro* selection of ECM fungi on solid medium

On solid medium, total ergosterol production varied considerably among species. We expected that all ECM fungi growing on normal rich MNM medium would have produced a greater quantity of ergosterol than those growing on the poorer medium with or without tailings (Willenborg *et al.* 1990), but this was somewhat different in this experiment. *T. scalpturatum, P. involutus*, and *L. aurantiosordidus* yielded the highest amount of ergosterol on the normal medium. The highest amount of ergosterol was obtained on the poor medium with tailings for *H. crustuliniforme* and *C. geophilum* and on the poor medium without tailings for *C. finlandia*. These results were quite surprising if we consider ergosterol as a good proxy for estimating ECM fungal biomass. However, ergosterol production in fungal tissues depends on multiple factors such as species and growing medium (Zhao *et al.* 2005). For instance, Willenborg *et al.* (1990), Djajakirana *et al.* (1996), and Yamanaka *et al.* (2003) showed that the tolerance of ECM fungi to different environmental stress such as low or high pH, high heavy-metal concentrations and low soil fertility differed among species and among strains of the same species. Therefore, the growth media used in our experiments, more specifically high pH and nutrient availability, may have affected differently the production of ergosterol in our six ECM fungal species under study.

Yeast cells have previously been shown to decrease their ergosterol production under high pH and high concentrations of NaCl while maintaining a stable growth rate (Montanes *et al.* 2011). Inhibition of ergosterol production is thought to give, to certain fungi, an important growth advantage under high salinity and high pH conditions and several genes involved in down-regulating ergosterol synthesis are known to contribute to the tolerance of many fungi to toxic cations (Montanes *et al.* 2011). Djajakirana *et al.* (1996) demonstrated

that fungal ergosterol/dry biomass ratio was strongly negatively related to cation exchange capacity and soil pH. For their part, Barajas-Aceves *et al.* (2002) found that ergosterol content of 20 different fungi was not affected by high concentrations of heavy metals. As a result, some ECM fungal species may decrease considerably their ergosterol production, under stress, as a way of sustaining their growth rate while others do not. For that reason, we have to be careful when comparing fungal growth using ergosterol in different environmental conditions.

The accumulation of ergosterol is a mechanism often used by fungi for resisting and surviving drying events under atmospheric air (Dupont *et al.* 2012). In our study, *T. scalpturatum, H. crustuliniforme*, and *P. involutus* were the three ECM fungal species that revealed the highest ergosterol content on poor solid medium with tailings. We observed that these three fungi grew mainly at the surface of the tailings away from the bottom medium which contained most of the water. Therefore, these three species may have produced more ergosterol in order to protect themselves against drying.

Fungal provenance is another factor that could have affected ECM fungal behaviour to synthesize ergosterol. ECM fungal species isolated from different ecosystems and habitats often produced different quantities of ergosterol (Montgomery *et al.* 2000). Here, *C. finlandia* and *T. scalpturatum* were isolated from waste rocks and fine tailings of Sigma-Lamaque gold mine, respectively, while *L. aurantiosordidus* and *C. geophilum* were isolated from the surrounding natural forest, and then *H. crustuliniforme* and *P. involutus* were isolated from natural forest stands in Alberta, Canada. As such, the provenance and adaptability of each species could have affected their strategy of ergosterol production. Since interspecific differences are likely to exist in ergosterol production, we wanted to relate this method of ECM fungal biomass assessment to other techniques of measurement on solid medium to compare the effect of our treatment between different species. Radial growth has been reported as a reliable method of fungal biomass assessment which is well correlated with ergosterol content (Schnurer 1993). We found a strong correlation between fungal ergosterol content and mycelia radial growth. Interestingly, we found that, among the three ECM fungal species showing the greatest radial growth on poor medium with tailings, two species were isolated from the mining site. These results show clearly the greater adaptation and tolerance

of these fungi to low fertility conditions and high pH compared to most species native to natural forest. The other species having both a high ergosterol content and a higher radial growth in poor medium with tailings than without tailings is *H. crustuliniforme*. This species has already been shown to have a high tolerance to *in vitro* salinity (Bois *et al*. 2006a) and to increase the salt tolerance of Jack pine seedlings (Bois *et al.* 2006b).

On the other hand, *L. aurantiosordidus, C. geophilum*, and *P. involutus* that were isolated from the boreal forest all showed no radial growth on poor medium with tailings or less than on poor medium without tailings. Under these circumstances, the three ECM fungal species were repulsed by and definitely not adapted to the mine tailings. *P. involutus* is an acidophilic fungal species that tends to thrive better in acidic soils and high pH tends to inhibit considerably its growth and root colonization (Kernaghan *et al.* 2002; Willenborg *et al.* 1990). Dixon *et al.* (1993) suggested that only a handful of ECM fungi are able to grow in extreme environments such as alkaline soils. Our results clearly show the importance of selecting ECM fungi that are able to grow within the mine tailings before inoculating seedlings for revegetation purposes.

In addition to ergosterol content and radial growth, we measured fungal dry biomass on solid medium. Unfortunately, we could not measure dry biomass on poor medium with tailings because of the difficulty to separate fungi and tailings biomass so we could not measure the effect of this treatment. When comparing poor and rich medium, we found that the biomass of *T. scalpturatum, C. finlandia, L. aurantiosordidus*, and *C. geophilum* was higher on rich (pH = 5.5) than on poor (pH = 8) medium. Mycelial dry weight is usually at its highest for most ECM fungal species at pH between 3.5 and 5.5 (Willenborg *et al.* 1990). Interestingly, *C. finlandia* and *T. scalpturatum*, both isolated from white spruce roots on the mining site had two to four-fold more dry biomass than the four other ECM fungal species. These results demonstrate very well the high growth potential of these two fungi under low fertility conditions.

In our study, ergosterol content was strongly correlated with radial growth but not with dry biomass. Ergosterol is reliable measure of fungal surface area in temperate soils (Ruzicka *et al.* 2000). Higher fungal radial growth means greater surface area colonized by the fungi. Therefore, it makes sense that ergosterol content would be positively correlated to

fungal radial growth. Mycelium density was not measured in our media. However, Rosling *et al.* (2003) demonstrated that mycelia density can be affected by both fungal species and mineral type. Some species (*Piloderma fallax, Piloderma byssinum, Suillus bovinus*) formed a denser mycelium network than others (*H. crustuliniforme, P. involutus, C. geophilum*) in different mineral treatments (Rosling *et al.* 2003). As a result, species that form a denser mycelium network may produce less ergosterol (less membrane) and colonize a smaller surface area (lower radial growth) than others while still making as much or even more biomass. A good example in our experiment was *Cadophora finlandia*. This fungus produced relatively small quantities of ergosterol in its tissues and displayed moderate radial growth while yielding the highest dry biomass of the six ECM fungal species. Other studies demonstrated that ergosterol is not significantly correlated with fungal biomass in forest soils (Djajakirana *et al.* 1996; Zhao *et al.* 2005) which is contrary to the results of Montgomery *et al.* (2000). It is however important to notice that the soils tested by Montgomery *et al.* (2000) were either agricultural soils or undisturbed lands and did not concern mine tailings.

3.6.2 *In vitro* selection of ECM fungi on liquid medium

On liquid medium, the ergosterol content of C. *finlandia, T. scalpturatum*, and *H. crustuliniforme* in the normal rich medium without tailings was higher than in the poor medium with tailings. This result was expected because ergosterol has been shown to be a good estimate of fungi growth in the soil (Montgomery et al. 2000). Our results confirm that ergosterol is also a reliable method of assessment of fungal growth in a liquid medium.

Each species responded differently to particle size of tailings in the liquid medium. For instance, *C. finlandia* was capable of growing in all four tailing particle sizes with a greater growth on bigger particles while *T. scalpturatum* was only able to grow on smaller tailing particles. This result illustrates clearly the adaptation of these ECM species since *C. finlandia* was isolated from roots of white spruce naturally regenerating on waste rocks which contain mostly large particles while *T. scalpturatum* was isolated from root of white spruce naturally regenerating on fine tailings, which is exclusively composed of small particles. Therefore, each species has a potential to be used on mining sites composed of different tailings size particles. *H. crustuliniforme* showed poor growth in presence of tailings of all particle sizes. These results support our hypothesis that indigenous ECM fungi isolated from

mining sites (*C. finlandia* and *T. scalpturatum*) are more adapted and tolerant to mine tailings than allochthonous species (*H. crustuliniforme*). Blaudez *et al.* (2000) and Kernaghan *et al.* (2002) uncovered strong interspecific and intraspecific variation in tolerance to metal and tailings among ECM fungal species. The biotite-quartz mine tailings that were used in our experiments contained high concentrations of Fe and Ca. Therefore, the better adaptation and tolerance to mine tailings of indigenous *C. finlandia* and *T. scalpturatum* compared to *H. crustuliniforme* could be due to their tolerance to high concentrations of Fe and Ca.

Our results show that *C. finlandia*, *T. scalpturatum* and *H. crustuliniforme* produced different organic acids (LMMOAs) in the liquid medium and that the production differed according to the presence and the particle size of tailings in the medium. ECM fungi have already been found to exude LMMOAs in order to increase mineral weathering (Hoffland *et al*. 2004; Courty *et al.* 2010). A wide range of LMMOAs such as citrate, succinate, malate, malonate and formate have been shown to be produced by the ECM fungal species *Paxillus involutus*, *Suillus sp*., and *Pisolithus tinctorius* and to act as mineral complexing agents (Wallander and Wickman, 1999; Tahara *et al.* 2005). Citrate has been reported to be an important weathering agent due to its strong capacity to make complexes with free cations in the soil solution (Gadd 1999; Hoffland *et al.* 2004). We found that *Cadophora finlandia* and *H. crustuliniforme* produced citrate in the presence of mine tailings which was mainly composed of biotite and quartz (rich in Fe, Ca and Al). Previous studies reported that biotite weathering by ECM fungi was accompanied by the release of citrate, oxalate and malate and they concluded that one of the major roles of these organic acids was to dissolve iron (Watteau and Berthelin 1994; Wallander and Wickman, 1999).

It seems that succinic acid is an important organic acid in mine tailings solubilization since higher amounts were produced in presence of tailings than in NM for all ECM species. As iron, calcium and aluminium were present in high concentrations in the tailings, succinic acid production might be enhanced by ECM fungi in presence of these elements. Previous works supported this statement. Machuca *et al.* (2007) reported that high quantities of succinic acid were exuded by ECM fungi in presence of iron. Moreover, high concentration of succinate was found around fungal hyphae to dissolve calcium-rich feldspars (Jongmans

et al. 1997). In addition, succinate was highly produced by *Pisolithus tinctorius* under *in vitro* aluminium exposure (Cumming *et al.* 2001).

Malonic acid was also significantly produced by *C. finlandia* and *H. crustuliniforme* in presence of mine tailings. Malonate is considered as a strong weathering agent in aluminium contaminated soils (Hue *et al.* 1986). This is in agreement with our study since aluminum is a major component of the mine tailings used in our experiments. Malonic acid has also been reported to be produced in large quantity by *Paxillus involutus* in response to phosphorous deficiency which is a characteristic of the poor liquid medium we used in our experiment (van Schöll *et al.* 2006).

Oxalate has been considered as the most abundant organic anion secreted by ECM fungi, however its production seems to be species-specific (Courty *et al*. 2010). When oxalate is produced by ECM fungi under nutrient deficiency, it forms complexes with calcium (Lapeyrie et al. 1984; Tuason and Arocena 2009). Since calcium was a predominant component of mine tailings used in our work, the low oxalic acid production by the three fungi might be due to the formation of calcium oxalate crystals which are not detectable by HPLC. In agar-media, calcium oxalate crystals are easily detected (Lapeyrie *et al*. 1987; do Rio *et al.* 2008) but not in liquid media. In addition, low oxalic acid production could be due to the presence of ammonium that we used as nitrogen source in our poor MNM medium. It is known that ammonium ions inhibit the activity of glyoxylate dehydrogenase which is involved in oxalic acid synthesis from glyoxylate (Kritzman *et al.* 1977).

We found that a large quantity of lactic acid was produced by *H. crustuliniforme* in the presence of mine tailings. Lactic acid is rarely produced by fungi in a mineral weathering context. However, lactic acid is often produced by bacteria in nutrient deficiency conditions and is considered as an important agent for mineral phosphate solubilisation (Rodriguez and Fraga 1999). Formic acid was also found in large quantity with the three species of ECM fungi in presence of mine tailings. However, since formic acid was also found in the liquid medium without fungi, we have to consider this result with care because this high amount of formic acid could be due to other contaminants.

Despite low ergosterol content in presence of mine tailings, *H. crustuliniforme* unexpectedly produced significant amounts of organic acids. In fact, no correlation was found between ECM fungal ergosterol content and organic acid production except for formic acid. We could thus conclude that organic acids are not a good indicator of fungal growth. Exudation of organic acids could however be a tool for the assessment of the mechanism of weathering of each ECM species and to assess their weathering potential.

3.6.3 Methods for ECM fungal selection

Many studies report that the assessement of ergosterol content is the most accurate recommended method for estimating ECM fungal growth and biomass in soils (Martin *et al.* 1990; Ruzicka *et al.* 2000). This method has been shown to be accurate to compare ECM fungal growth in similar environmental conditions and habitats (Montgomery et al. 2000), but caution should be exercised. Our results show that many factors may affect differently the production of ergosterol among ECM fungal species. Ergosterol content was not representative of fungal growth in our solid medium experiment likely because of the high pH of the medium that could have affected differently the ergosterol synthesis on our six ECM fungal species. On the other hand, measurements of fungal radial growth in tailings were very useful and permitted to identify species that did not tolerate tailings. The method of radial growth measurement is cheaper and requires less equipment as compared to ergosterol analysis. Our results show that it can reliably be used to compare fungal growth and that it could be recommended as a first step for the selection of high potential ECM fungi for use in land reclamation and for screening and discarding strains that show no growth on tailings.

In this study, fungal ergosterol results were significantly different between the liquid and solid methods used for fungal growth. On solid medium, the pH of 8 was very high compared to the liquid medium pH of 5.4. As discussed above, this variation in pH may explain the differences in results obtained by the two methods. Future studies should investigate to a greater extent this effect of pH on ergosterol production by different ECM fungal species. All ECM species in the liquid medium responded similarly in regards to ergosterol synthesis (produced more ergosterol in normal medium than in poor medium with tailings) suggesting that liquid medium could be more appropriate for selecting high potential

ECM fungal species for revegetation based on ergosterol content. Barajas-Aceves *et al.* (2002) corroborated that the fungal ergosterol content in liquid medium with heavy metals was linearly positively correlated to fungal biomass However, Zhao *et al.* (2005) recommended to rely on more than one technique for monitoring fungal growth in soils to avoid misleading information. We herein report two efficient *in vitro* selection methods of ECM fungi: (1) measurement of mycelial radial growth on solid medium and (2) quantification of ergosterol content in liquid medium.

3.7 Conclusion

Among the two methods used for selection of ecologically adapted ECM fungi to mine tailings, the liquid-medium seems more suitable since it allowed the quantification of ergosterol content as well as the quantification of LMMOAs which play a crucial role in mineral weathering. Six ECM fungi were tested for their ability to grow on tailings and three of them, *C. finlandia*, *T. scalpturatum* and *H. crustuliniforme*, were considered to be tolerant to mine tailings. However, indigenous *C. finlandia* and *T. scalpturatum* species exhibited greater ergosterol content than *H. crustuliniforme* in liquid medium. Organic acids were exuded by ECM fungi and could likely enhance mineral weathering. *C. finlandia* exuded significant quantities of citric, succinic and oxalic acids. *Tricholoma scalpturatum* mainly produced succinic and oxalic acids and *H. crustuliniforme* exuded malonic, succinic, lactic and oxalic acids. According to ergosterol and organic acids contents, *C. finlandia* was clearly the most tolerant species to biotite-quartz rich mine tailing. This fungus is commonly found in heavy-metal polluted sites, and has been suggested to play a functional role in heavy metal resistance (Vralstad *et al.* 2002). These three species of ECM fungi will further be tested for their ability to improve the survival, health, growth, and nutrition of white spruce seedlings on waste rocks and fine tailings of Sigma-Lamaque gold mine.

3.8 Acknowledgements

The authors would like to thank the Natural Sciences and Engineering Research Council of Canada (NSERC) for their financial support. The authors would also like to express their gratitude to Jean-Guy Catford for his precious advices and Alain-René Atangana for his contribution to specific statistical analyses. The authors offer their

appreciation to Mathieu Boudreau, André Gagné, Marie-Ève Beaulieu, Josée Bourassa, and Laurent Fontaine who contributed in some way to the completion of this study. Finally, we are also indebted to Dr Line Lapointe (Université Laval) and Suzanne Simard (University of British Columbia) for careful review of an earlier draft which has helped improve it.

3.9 References

Adeleke R, Cloete TE, Bertrand A, Khasa D (2010) Mobilisation of potassium and phosphorus from iron ore by ectomycorrhizal fungi. World Journal of Microbiology and Biotechnology 26: 1901-1913.

Balogh-Brunstad Z, Keller CK, Dickinson JT, Stevens F, Li CY, Bormann BT (2008) Biotite weathering and nutrient uptake by ectomycorrhizal fungus, *Suillus tomentosus*, in liquid-culture experiments. Geochimica et Cosmochimica Acta 72: 2601-2618.

Barajas-Aceves M, Hassan M, Tinoco R, Vasquez-Duhalt R (2002) Effects of pollutants on the ergosterol content as indicator of fungal biomass. Journal of Microbiological Methods 50: 227-236.

Berner C, Johansson T, Wallander H (2012) Long-term effect of apatite on ectomycorrhizal growth and community structure. Mycorrhiza 22: 615-621.

Blaudez D, Jacob C, Turnau K, Colpaert JV, Ahonen-Jonnarth U, Finlay R, Botton B, Chalot M (2000) Differential responses of ectomycorrhizal fungi to heavy metals in vitro. Mycological Research 104(11): 1366-1371.

Bois, G., A. Bertrand, Y. Piché, M. Fung, and D. P. Khasa. (2006a). Growth, compatible solute and salt accumulation of five mycorrhizal fungal species grown over a range of NaCl concentrations. Mycorrhiza 16: 99-109.

Bois, G., F.J. Bigras, A. Bertrand, Y. Piché, M.Y.P. Fung, and D. Khasa. (2006b). Ectomycorrhizal fungi affect the physiological responses of *Picea glauca* and *Pinus banksiana* seedlings exposed to an NaCl gradient. Tree Physiology 26: 1185-1196.

Boyle JR, Voigt GK, Sawhney BL (1974) Chemical weathering of biotite by organic acids. Soil Science 117(1): 42-45.

Cairney JWG (1999) Intraspecific physiological variation: implications for understanding functional diversity in ectomycorrhizal fungi. Mycorrhiza 9: 125-135.

Colpaert JV, Vandenkoornhuyse P, Adriaensen K, Vangronsveld J (2000) Genetic variation and heavy metal tolerance in the ectomycorrhizal basidiomycete *Suillus luteus.* New Phytologist 147: 367-379.

Courty P-E, Buée M, Gamby Diedhiou A, Frey-Klett P, Le Tacon F, Rineau F, Turpault M-P, Uroz S, Garbaye J (2010) The role of ectomycorrhizal communities in forest ecosystem processes: New perspectives and emerging concepts. Soil Biology & Biochemistry 42: 679-698.

Cumming JR, Swiger TD, Kurnik BS, Panaccione DG (2001) Organic acid exudation by *Laccaria bicolor* and *Pisolithus tinctorius* exposed to aluminium *in vitro*. Canadian Journal of Forest Research 31: 703-710.

Dahlberg A (2001) Community ecology of ectomycorhizal fungi: an advancing interdisciplinary field. New Phytologist 150*:* 555-562.

Dixon RK, Rao MV, Garg VK (1993) Salt stress affects in vitro growth and in situ symbioses of ECM fungi. Mycorrhiza 3: 63-68.

Djajakirana G, Joergensen RG, Meyer B (1996) Ergosterol and microbial biomass relationship in soil. Biology and Fertility of Soils 22: 299-304.

do Rio MCS, de Oliveira DV, de Tomazella DPT, da Silva JAF, Pereira GAG (2008) Production of calcium oxalate crystals by the basidiomycete *Moniliophthora perniciosa*, the causal agent of Witches' Broom disease of cacao. Current Microbiology 56: 363-370.

Drever JI, Stillings LL (1997) The role of organic acids in mineral weathering. Colloids Surfaces A: Physicochemical Engineering Aspects 120: 161-181.

Dupont S, Lemetais G, Ferreira T, Cayot P, Gervais P, Bency L (2012) Ergosterol biosynthesis: A fungal pathway for life on land? Evolution 66(9): 2961-2968.

Gadd GM (1999) Fungal production of citric and oxalic acid: importance in metal speciation, physiology and biogeochemical processes. Advances in Microbial Physiology 41: 47-92.

Gong P, Guan X, Witter E (2001) A rapid method to extract ergosterol from soil by physical disruption. Applied Soil Ecology 17: 285-289.

Hoffland E, Kuyper TW, Wallander H, Plassard C, Gorbushina AA, Haselwandter K, Holmstrom S, Landeweert R, Lundstrom US, Rosling A, Sen R, Smits MM, Van Hees PAW, Van Breemen N (2004) The role of fungi in weathering. Frontier in Ecology and the Environment 2(5): 258-264.

Hue NV, Craddock GR, Adams F (1986) Effect of organic acids on aluminium toxicity in subsoils. Soil Science Society of America Journal 50: 28-34.

Jongmans AG, van Breemen N, Lundstrom U, van Hees PAW, Finlay RD, Srinivasan M, Unestam T, Giesler R, Melkerud P-A, Olsson M (1997) Rock-eating fungi. Nature 389: 682-683.

Kernaghan G, Hambling B, Fung M, Khasa DP (2002) In vitro selection of boreal ectomycorrhizal fungi for use in reclamation of saline-alkaline habitats. Restoration Ecology 10: 43-51.

Khan AG (2006) Mycorhizoremediation – an enhanced form of phytoremediation. Journal of Zhejiang University SIENCE B 7(7): 503-514.

Khasa DP, Sigler L, Chakravarty P, Dancik BP, Erickson L, Mc Curdy D (2001) Effect of fertilization on growth and ectomycorrhizal development of container-grown and bare-root nursery conifer seedlings. New Forests 22(3): 179-197.

Kritzman G, Chet I, Henis Y (1977) The role of oxalic acid in the pathogenic behaviour of *Sclerotium folfsii Sacc*. Experimental Mycology 1: 280-285.

Landeweert, R, Hoffland E, Finlay RD, Kuyper TW, Van Bremen N (2001) Linking plants to rocks: ectomycorrhizal fungi mobilize nutrients from minerals. Trends in Ecology and Evolution 16(5): 248-254.

Lapeyrie F, Perrin M, Pepin R, Bruchet G (1984) Formation de Weddellite (CaC_2O_4 $2H_2O$) extracellulaire en culture *in vitro* par *Paxillus involutus*; signification de cette production pour la symbiose ectomycorhizienne. Canadian Journal of Botany 62: 1116-1121.

Lapeyrie F. Chilvers GA, Bhem CA (1987) Oxalic acid synthesis by the mycorrhizal fungus *Paxillus involutus* (Batsch. Ex Fr.) Fr. New Phytologist 106: 139-146.

Lapeyrie F, Ranger J, Vairelles D (1991) Phosphate-solubilizing activity of ectomycorrhizal fungi in vitro. Canadian Journal of Botany 69: 342-346.

Littell RC, Milliken GA, Stroup WW, Wolfinger RD, Schabenberger O (2006) Analysis of repeated measures data. In: SAS® for Mixed Models, 2nd edition, Cary, NC: SAS Institute Inc. pp: 159-204.

Littke WR, Bledsoe CS, Edmonds RL (1983) Nitrogen uptake and growth in vitro by *Hebeloma crustuliniforme* and other Pacific Northwest mycorrhizal fungi. Canadian Journal of Botany 62: 647-652.

Machuca A, Pereira G, Aguiar A, Milagres AMF (2007) Metal-chelating compounds produced by ectomycorrhizal fungi collected from pine plantation. Letter in Applied Microbiology 44: 7-12.

Martin F, Delaruelle C, Hilbert J-L (1990) An improved ergosterol assay to estimate fungal biomass in ectomycorrhizas. Mycological Research 94(8): 1059-1064.

Montanes FM, Pascual-Ahuir A, Proft M (2011) Repression of ergosterol biosynthesis is essential for stress resistance and is mediated by the HOG1 MAP kinase and the MOT3 and ROX1 transcription factors. Molecular Microbiology 79(4): 1008-1023.

Montgomery HJ, Monreal CM, Young JC, Seifert KA (2000) Determination of soil fungal biomass from soil ergosterol analyses. Soil Biology & Biochemistry 32: 1207-1217.

Montgomery DC (2012) Design and analysis of experiments. 8th edition. John Wiley, New York.

Nadeau MB, Khasa DP (2015) Edaphic selection pressures as drivers of contrasting white spruce ectomycorrhizal fungal diversity and community structure in the Canadian boreal forest of Abitibi-Temiscamingue region. Unpublished (Chapter 1).

Olsson PA (1999) Signature fatty acids provide tools for determination of the distribution and interactions of mycorrhizal fungi in soil. FEMS Microbiology Ecology 29: 303-310.

Onwuchekwa NE, Zwiazek JJ, Quoreshi A, Khasa DP (2014) Growth of mycorrhizal jack pine (*Pinus banksiana*) and white spruce (*Picea glauca*) seedlings planted in oil sands reclaimed areas. Mycorrhiza: DOI 10.1007/s00572-014-0555-x.

Rodriguez H, Fraga R (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnology Advances 17: 319-339.

Rosling A, Lindahl BD, Taylor AFS, Finlay RD (2003) Mycelial growth and substrate acidification of ectomycorrhizal fungi in response to different minerals. FEMS Microbiology Ecology 47: 31-37.

Ruzicka S, Edgerton D, Norman M, Hill T (2000) The utility of ergosterol as a bioindicator of fungi in temperate soils. Soil Biology & Biochemistry 32: 989-1005.

Schnurer J (1993) Comparison of methods for estimating the biomass of three food-borne fungi with different growth patterns. Applied Environmental Microbiology 59(2): 552-555.

Smith, S.E. & D.J. Read. (2008). Mycorrhizal symbiosis, 3rd ed. Academic Press, London, UK.

Smits MM, Bonneville S, Benning LG, Banwart SA, Leake JR (2012) Plant-driven weathering of apatite – the role of an ectomycorrhizal fungus. Geobiology 10(5): 445-456.

Tahara K, Norisada M, Tange T, Yagi H, Kojima K (2005) Ectomycorrhizal association enhances Al tolerance by inducing citrate production in *Pinus densiflora*. Soil Science and Plant Nutrition 51(3): 397-403.

Taner MF, P Trudel P, Perrault G (1986) Géochimie de la biotite associée à certains gisements d'or de Val d'Or, Malartic et Chibougamau, Québec. Can Mineral 24: 761-774.

Taylor LL, Leake JR, Quirk J, Hardy K, Banwart SA, Beerling DJ (2009) Biological weathering and the long-term carbon cycle: integrating mycorrhizal evolution and function into the current paradigm. Geobiology 7: 171-191.

Tuason MMS, Arocena JM (2009) Calcium oxalate biomineralization by *Piloderma fallax* in response to various levels of calcium and phosphorus. Applied Environmental Microbiology 75: 7079-7085.

Vralstad T, Myhre E, Schumacher T (2002) Molecular diversity and phylogenetic affinities of symbiotic root-associated ascomycetes of the Helotiales in burnt and metal polluted habitats. New Phytologist 155: 131-148.

van Schöll L, Hoffland E, van Breeman N (2006) Organic anion exudation by ectomycorrhizal fungi and *Pinus sylvestris* in response to nutrient deficiencies. New Phytologist 170: 153-163.

Wallander H, Hagerberg D (2004) Do ectomycorrhizal fungi have significant role in weathering of minerals in forest soils? Symbiosis 37: 249-252.

Wallander H, Wickman T (1999) Biotite and microline as potassium sources in ectomycorrhizal and non-mycorrhizal *Pinus sylvestris* seedlings. Mycorrhiza 9: 25-32.

Watteau F, Berthelin J (1994) Microbial dissolution of iron and aluminium from soil minerals: efficiency and specificity of hydroxamate siderophores compared to aliphatic acids. European Journal of Soil Biology 30: 1-9.

Willenborg A, Schmitz D, Lelley J (1990) Effects of environmental stress factors on ectomycorrhizal fungi in vitro. Canadian Journal of Botany 68: 1741-1746.

Wurzburger N, Bidartondo MI, Bledsoe CS (2001) Characterization of *Pinus* ectomycorrhizas from mixed conifer and pygmy forests using morphotyping and molecular methods. Canadian Journal of Botany 79: 1211-1216.

Yamanaka T (2003) The effect of pH on the growth of saprotrophic and ectomycorrhizal ammonia fungi in vitro. Mycologia 95(4): 954-589.

Zhao XR, Lin Q, Brookes PC (2005) Does soil ergosterol concentration provide a reliable estimate of soil fungal biomass? Soil Biology & Biochemistry 37: 311-317.

4. Chapter 3: Ectomycorrhizal fungi and bacterial PGPR improve white spruce seedling health, growth, and nutrition on Precambrian rocky gold mine tailings

Martin Beaudoin Nadeau[1] and Damase P. Khasa[1]

1. Centre for Forest Research and Institute of Integrative and Systems Biology, Université Laval, Quebec city, QC, Canada, G1V 0A6.

Keywords: Ectomycorrhizae, Growth, Health, Metamorphic rock, *Picea glauca*, PGPR, Tree nutrition

Article prepared for submission in the scientific journal ‘’Tree physiology’’

4.1 Résumé

L'épinette blanche (*Picea glauca*) est une espèce d'arbre de la forêt boréale à grande valeur commerciale qui est connue comme étant apte à coloniser les sols rocheux après déglaciation. Durant la dernière décennie, il y a eu un intérêt grandissant à utiliser cette espèce d'arbre pour végétaliser et restaurer les sites miniers abandonnés. Dans cette étude, nous avons examiné, à travers un essai en serre, le rôle et l'importance des champignons ectomycorhiziens (ECM) et des rhizobacteries bénéfiques aux plantes (PGPR) à favoriser la santé, la croissance et la nutrition de semis d'épinette blanche poussant directement sur les résidus miniers grossiers et fins de la mine d'or Sigma-Lamaque dans la région canadienne de l'Abitibi. Les semis ont été inoculés avec différents traitements de champignons ECM et PGPR dans un dispositif expérimental factoriel. Après 32 semaines de croissance, des mesures de santé, de croissance et de nutrition des plants ont été réalisées. L'inoculation des semis avec *Cadophora finlandia, Tricholoma scalpturatum*, and *Azotobacter chroococcum* a amélioré considérablement la santé des semis. *C. finlandia* a accompli cette fonction en augmentant l'absorption foliaire de N à des niveaux optimaux, en augmentant l'absorption d'eau des racines et en diminuant la toxicité causée par le Fe dans les tissus foliaires. De leur côté, *T. scalpturatum* et *A. chroococcum* l'ont fait en augmentant, individuellement, l'absorption d'eau des racines et en diminuant, en association, la toxicité foliaire du Fe. *Pseudomonas putida* a été le seul PGPR à améliorer la croissance aérienne des semis. Il l'a fait en réduisant considérablement l'absorption foliaire du Ca qui était nuisible aux semis sous des concentrations très élevées dans le sol. Les effets bénéfiques des champignons ECM et PGPR sur la santé, la croissance et la nutrition des semis sont souvent spécifiques au site, à l'hôte, à l'espèce et/ou à la souche. Par conséquent, la sélection des micro-organismes symbiotiques performants et adaptés est essentielle au succès des activités de révégétalisation des sites miniers. Les champignons ECM et PGPR isolées à partir des racines de semis d'épinette blanche en santé sur le site minier étaient plus efficaces à améliorer la santé, la croissance et la nutrition des semis que les espèces allochtones.

4.2 Abstract

White spruce (*Picea glauca*) is a commercially valuable boreal tree species that has been known for its ability to colonize deglaciated rock tailings. Over the last decade, there

has been an increasing interest in utilizing this tree species for the revegetation and successful restoration of abandoned mine spoils. Here, we studied, in a glasshouse experiment, the role and importance of ectomycorrhizal (ECM) fungi and plant growth promoting rhizobacteria (PGPR) in promoting the health, growth, and nutrition of white spruce seedlings growing directly on waste rocks and fine tailings of Sigma-Lamaque gold mine located in the Canadian Abitibi region. Seedlings were inoculated with different treatments of ECM fungi and PGPR in a RCB design with crossed factors. After 32 weeks of growth, measurements of seedlings health, growth, and nutrition were performed. The inoculation of seedlings with *Cadophora finlandia*, *Tricholoma scalpturatum* or *Azotobacter chroococcum* improved considerably seedling health. *C. finlandia* did it by increasing foliar N uptake to optimal levels and root water uptake and by decreasing foliar Fe toxicity. For their part, *T. scalpturatum* and *A. chroococcum* did it by augmenting, individually, root water uptake and by declining, in association, foliar Fe toxicity. *Pseudomonas putida* was the only PGPR that ameliorated seedling growth. It did it by reducing considerably foliar Ca uptake, which is detrimental to seedlings due to high concentrations in soil. Beneficial effects of ECM fungi and PGPR on seedling health, growth, and nutrition may be site-specific, host-specific, species-specific and/or strain-specific. Therefore, the selection of efficient and adapted symbiotic microorganisms is critical for the success of revegetating mining areas. Overall, ECM fungi and PGPR isolated from roots of healthy white spruce seedlings on the mining site were more effective in enhancing seedling health, growth, and nutrition than allochthonous species.

4.3 Introduction

Plants have been widely known for their ability to recolonize land affected by major disturbances over time (Burns 1990, Hobbie et al. 1998, Jumpponen et al. 2002, Nienstaedt and Zasada 1990). After glaciation retreat, the only soil left are bedrocks and rock tailings (Hobbie *et al.* 1998). Yet, many plant species still colonize these low fertility ecosystems in which organic matter is inexistent (Hobbie et al. 1998). Anthropogenic activities such as mining of the Precambrian gold ores also create new ecosystems where most nutrients and minerals are trapped in the rock tailings under insoluble forms unavailable to plants. Plants need to take up nutrient elements (P, K, Mg, Ca, N, Mn, Zn, Mo, Cu, Fe, B) from soil into

its root system in order to survive and grow (Allen et al. 2003a, Allen et al. 2003b, Bois et al. 2006, Dahlberg 2001, Dixon and Buschena 1988, Gagné et al. 2006, Kernaghan et al. 2003, Khan 2006, Quoreshi and Khasa 2008, Roy et al. 2007). To thrive in harsh post-glacial or human-made new ecosystems conditions, plants have co-evolved with their microsymbionts, capable of scavenging nutrients from rocks or fixing nitrogen from the atmosphere.

Jumpponen et al. (2002) have studied the occurrence of ectomycorrhizal (ECM) fungi on the forefront of a retreating glacier. They found that trees were only able to grow on the rock tailings in association with ECM fungi (Jumpponen et al. 2002). All tree species (*Salix commutata, S. phylicifolia, Abies lasiocarpa, Larix lyalli, Pinus contorta*, and *Tsuga mertensiana*) that were growing on the tailings are commonly known for being symbiotically associated with ECM fungi on their fine roots (Dahlberg 2001, Smith and Read 2008). Species of alder and spruce are also known to form a symbiotic relationship with ECM fungi (Dahlberg 2001, Dixon and Buschena 1988, Nienstaedt and Zasada 1990, Quoreshi et al. 2007, Roy et al. 2007). Many studies have demonstrated that ECM fungi can utilize important soil elements (P, N, Mg, Ca, K, Zn, Cu, Ni, S, Mn, B, and Fe) that are unavailable to plants in their insoluble forms (Berner et al. 2012, Balogh-Brunstad et al. 2008, Hoffland et al. 2004, Landeweert et al. 2001, Smits et al. 2012, Wallander and Hagerberg 2004). ECM fungi have the ability to alter minerals by releasing organic acids such as oxalic, malic, and citric acids (Berner et al. 2012, Balogh-Brunstad et al. 2008, Hoffland et al. 2004, Landeweert et al. 2001, Smits et al. 2012, Wallander and Hagerberg 2004). This function allows ECM fungi to acquire essential elements stored in rock particles, transform them into soluble forms, and transfer them to plant roots in exchange for plant photosynthetic carbon sources (Allen et al. 2003a, Allen et al. 2003b, Bois et al. 2006, Dahlberg 2001, Dixon and Buschena 1988, Khan 2006, Quoreshi and Khasa 2008, Roy et al. 2007). As a result, ECM fungi may play a very important role in plant nutrition enabling colonization of deglaciated rock tailings.

Mine tailings usually have conditions very similar to deglaciated rock tailings (low fertility without organic matter) (Balogh-Brunstad et al. 2008; Taner et al. 1986). Bois et al. (2006) and Onwuchekwa et al. (2014) demonstrated that the inoculation of white spruce (*Picea glauca*) and jack pine (*Pinus banksiana*) with ECM fungi improves considerably their

growth and survival on oil sand tailings. Hence, ECM fungi may also be very important for the nutrition of trees growing on abandoned mine tailings. In the boreal forest, ECM fungi have high species richness and genetic diversity (Buée et al. 2009, Cairney 1999, Colpaert et al. 2000, Gagné et al. 2006, Kerhaghan et al. 2003, Villeneuve et al. 1989). Mycelial growth, plant colonization, enzyme production, plant growth stimulation, pH and temperature optimum for mycelium growth, nutrient uptake, and ECM fungal organic acid production may differ greatly among species and among strains of the same species (Cairney 1999, Colpaert et al. 2000, Lamhamedi and Fortin 1991). Therefore, some species and strains may be better adapted than others to extreme site conditions of mine tailings. Mine tailings of Sigma-Lamaque gold mine in Val d'Or, Abitibi, Quebec is made of Precambrian metamorphic rocks mainly composed of biotite. This biotite contains high concentration of Fe, Ca, Al, and Mg but also other important nutritional elements (P, K, Zn, Mn, Cu, Mo, and Na) for plant growth in low concentrations (Taner et al. 1986). ECM fungi have the ability to increase weathering of biotite minerals by producing organic acids and reducing pH in soil (Balogh-Brunstad et al. 2008, Smits et al. 2012, Wallander and Hagerberg 2004, Azaiez et al. 2015, Chapter 2). In this process, important elements such as P, K, Ca, and Mg are extracted from the biotite and absorbed by ECM fungi under soil nutrient limitation and then transferred to plants in soluble forms (Balogh-Brunstad et al. 2008). Therefore, ECM fungi have a tremendous potential to be used with their host for the revegetation of mine tailings composed of biotite.

In Sigma-Lamaque mine, tailings do not contain any nitrogen sources for plant growth (Taner et al. 1986). White spruce has been known as a plastic species because of its ability to recolonize areas at the end of glaciation (Nienstaedt and Zasada 1990, Sutton 1973). Some healthy white spruce seedlings were found naturally regenerating the mine tailings and revealed a distinct ECM fungal community different to adjacent nursery, forest edge, and natural forest ecosystems (Nadeau and Khasa 2015, Chapter 1). These coniferous trees certainly do not secure nitrogen from organic matter because it is inexistent on site. Nitrogen-fixing free living bacteria in soil such as *Azotobacter* sp. and *Azospirillum* sp. have the ability to fix atmospheric nitrogen and transform it into ammonium available to plants (Tripathy and Ayyappan 2005). In India, biofertilizers were developed using *Azotobacter chroococcum* and *Azotobacter vinelandii* in order to improve plant nitrogen nutrition (Damir et al. 2011).

Certain strains of other diazotrophic plant growth promoting rhizobacteria (PGPR) of the genus *Acetobacter, Bulkholderia, Enterobacter, Citrobacter,* and *Pseudomonas* are also capable of N_2 fixation (Hayat et al. 2010). These bacteria have been found in soil of ecosystems all around the world from the Arctic to the Antarctic including the boreal forest (Marshall 2000). These kinds of bacteria may play an important role in providing nitrogen to conifer trees growing in nitrogen-poor rock tailings.

Several bacterial strains of different genera (*Aminobacter, Labrys, Sphingomonas, Burkholderia, Collimonas, Dyella, Frateuria, Pseudomonas, Mycobacterium, Paenebacillus*, and *Staphylococcus*) have shown abilities to solubilize minerals from biotite within tree rhizosphere (Uroz et al. 2009). Calvaruso et al. (2006) demonstrated that *Bulkholderia glathei* inoculated on Scots pine roots (*Pinus sylvestris*) significantly increased Mg and K weathering of biotite by more than 40%. Two of the three *B. glathei* strains had a positive effect on tree growth and root size suggesting that some strains within species may be more effective in favouring plant growth and nutrition than others (Calvaruso et al. 2006). Furthermore, the inoculation of roots with PGPR (*Agrobacterium* spp., *Pseudomonas putida, Bacillus polymyxa, Arthrobacter citreus,* and *Pseudomonas fluorescens*) has previously increased the growth of many coniferous tree species in temperate forests (Chanway 1997). As a result, PGPR may be greatly beneficial for tree growth and nutrition in abandoned mine tailings composed of biotite.

The role and importance of ECM fungi and PGPR in tree nutrition on Precambrian metamorphic rocks of the Canadian Shield has never been studied before. In this study, we aimed to investigate the potential of ECM fungi and PGPR to ameliorate white spruce seedling survival, health, growth, and nutrition on mine tailings under glasshouse conditions. We hypothesized that the inoculation of white spruce seedlings with ECM fungi and PGPR improves seedling survival, health, growth, and nutrition. We predicted that native strains isolated directly from tailings give better seedling survival, health, growth, and nutrition than exotic strains with provenance of natural forest stands.

4.4 Materials and methods

4.4.1 White spruce seed germination and seedling growth

Seeds were germinated in recipients with tiny cavities (2 cm diameter by 3 cm deep) filled with a peat-vermicule-perlite substrate (80:15:5). The trial was conducted in a glasshouse at Université Laval. The glasshouse was disinfected with a bleach solution before the beginning of the experiment. Fertilizers were applied on seedlings two weeks after germination (NPK in 10 ml of water: 20-8-20) in order to favour seedling establishment and nutrition prior inoculation. Three weeks after germination, seedlings were transferred in 1.75L pots filled with waste rocks or fine tailings collected directly from Sigma-Lamaque gold mine. Seedlings were watered at field capacity every day. One week per month (every four weeks), seedlings were watered at field capacity only every two days in order to mimic field water stress condition that often occurs during drought periods of the summer in the Abitibi region. Glasshouse conditions for optimal growth of white spruce seedlings were set at an alternating temperature of 25°C/20°C (day/night). Seedlings received artificial light 16 hours per day in order to avoid the production of fructifications by ECM fungi, which could potentially contaminate surrounding experimental units. The experiment lasted for 32 weeks.

4.4.2 Bacterial and ECM fungal inoculation of white spruce seedlings

4.4.2.1 Bacteria

Three bacterial strains were selected for this experiment. Two strains of different PGPR species (*Pseudomonas putida* MBN0213 and *Rhizobium radiobacter* MBN0213) were isolated from the rhizosphere of healthy white spruce host naturally regenerating on the mining site following the methods of soil paste and direct sowing of single soil grains described by Aquilanti *et al.* (2004). One strain of free living N-fixing soil bacterium *Azotobacter chroococcum* ATCC 9043 was ordered from CEDARLANE Laboratories Ltd. (American Type Culture Collection, Burlington, Ontario, Canada). *P. putida* was cultivated in a liquid TSB medium (Tryptic soybean broth – Difco) as recommended by Glick et al. (1997). *R. radiobacter* was produced in a liquid yeast extract mannitol medium as described by Humphry *et al.* (2007). *A. chroococcum* was cultivated in a liquid Waksman medium (Nitrogen-free mannitol medium) following the method developed by AgriTech in India. Culture growth was executed under aseptic shaking conditions at 30°C during seven days for

maximum cell production before inoculation. Afterwards, the quantity of bacteria in each culture was counted under a microscope with a Petroff-Hausser Bacteria Counting Chamber (Hausser Scientific, Horsham, PA, USA). Solutions were centrifugated for 20 min (4000 rpm at 4°C) and the supernatant was discarded. Bacteria were diluted with sterile water until the inoculant reached a concentration of $1*10^8$ CFU/ml. 10 ml of the inoculant was applied onto roots of four-week-old white spruce seedlings two times within 14 days in order to increase rhizospheric colonization success.

4.4.2.2 Ectomycorrhizal fungi

Three ECM fungal strains were chosen for this experiment. *H. crustuliniforme* UAMH5247 was isolated from white spruce roots in a natural forest stand of the boreal forest in Canada. Both *T. scalpturatum* MBN0213 and *C. finlandia* MBN0213 were isolated from healthy naturally regenerating white spruce trees on fine tailings and waste rocks of Sigma-Lamaque gold mine, respectively. In another study, these three strains displayed, under axenic conditions, compelling *in vitro* growth and tolerance to mine tailings composed of biotite (Azaiez *et al.* 2015, Chapter 2). Inoculum was produced by cultivating fungal mycelia at 23°C in a liquid MNM medium under aseptic shaking conditions. After two months, the ECM fungal mycelia was collected and rinsed with sterile water to discard excess nutrients. Blended mycelia was mixed with sterile water (ratio 1:10) in order to obtain a final concentration higher than $5*10^5$ viable propagules/ml. Three-week-old white spruce seedlings were inoculated with 5 ml of the inoculant and a second time after four weeks in order to increase root inoculation success. The inoculum was released into the root zone using an analog adjustable-dispenser.

4.4.3 Experimental design and treatments

The experimental design was a generalized randomized complete block (RCB) with three crossed fixed factors (tailing type*ECM fungi*Bacteria). Tailing type was composed of two levels: waste rock (WR) and fine tailing (FT). ECM fungal factor had a total of four levels (none, *H. crustuliniforme* (HC), *T. scalpturatum* (TS), and *C. finlandia* (CF)). Bacteria also had four levels (none, *P. putida* (PP), *R. radiobacter* (RR), *A. chroococcum* (AC)). There were 32 treatments with three replicates in each of the four blocks for a total of 384 experimental units. Each replicate was randomly placed to experimental units within blocks.

Every experimental unit consisted of a 1.75 L pot filled with tailings containing one white spruce seedling. Experimental units within blocks were separated by a thin piece of plastic in order to avoid bacterial contamination among them. Each block was surrounded by two guard rows in order to maintain the most homogeneous environmental conditions possible in all experimental units. Detailed layout and illustrations of the experimental design are given in Appendix IV and V.

4.4.4 Measurements of seedling survival, health, growth, and nutrition

Seedling survival was assessed every four weeks through visual observations. Seedlings were considered dead when light red needles had no green colour left. At the end of the experiment, seedlings were brought into a growth chamber one hour before measuring chlorophyll fluorescence. Photochemical efficiency (FV/FM) was measured in a dark environment using a portable fluorometer PAM-2000 (Heinz Walz, Effeltrich, Germany) with the data acquisition software DA-2000. Fresh foliage tissues were placed under the fluorescence booster for recording FV/FM data. Seedlings were considered as perfectly healthy when FV/FM $\geq$ 0.8. Smaller values meant seedlings experienced ecophysiological stresses. Afterwards, roots of white spruce seedlings were washed gently with tap water in a 2 mm mesh sieve for removing all soil particles. Seedlings were stored in hermetic plastic bags at -20°C before beginning further analyses.

Needles were snatched from stem, weighted on an analytical balance with high precision of ±0.00005, individually positioned on a transparent plastic plate, and scanned. WinSEEDLE PRO LA2400 scanner system and software (Regent Instruments Inc., Québec, Canada) were utilized for determining specific surface foliar areas (SSFA) of green, yellow, brown, dark red and light red foliar tissues. Total SSFA was calculated by adding the SSFA of all colours together. Percentages of healthy green foliage and dark red foliage were calculated by comparing their SSFA with total SSFA. Seedling stem length was measured, in millimetres, from the base of roots to the tip of the terminal bud with a 15-cm ruler and then weighted in order to identify wet biomass. Roots were also weighted and percentage of colonization by ECM fungi was calculated by counting the number of mycorrhizal root tips under a microscope and comparing it with the total number of root tips. Subsequently, roots were transferred on a transparent plastic plate. WinRHIZO PRO LA2400 scanner system and

software (Regent Instruments Inc., Québec, Canada) were employed for measuring total root length, volume, and number of root tips.

For dry biomass analyses, white spruce seedling roots, shoots, and needles were dried at 65°C for seven days. Dry weight of roots, shoots, and needles were measured using an analytical balance with precision of ±0.00005g. Shoot water content was calculated by adding biomass of needles and stem together, subtracting their dry biomass from their wet biomass, dividing the result by their wet biomass and then multiplying by 100. Root water content was calculated by subtracting root dry biomass from wet biomass, dividing the result by wet biomass and then multiplying by 100. For nutrition analyses, replicates within treatments were pooled together in each block. Seedlings roots and needles were grounded separately in a Wiley Mill. Samples were digested in concentrated H_2SO_4 and 50% H_2O_2. Chemical analyses of N, P, K, Mg, Ca and Fe in roots and needles were performed on the digested tissues following techniques outlined in Kalra (1998) and Quoreshi and Khasa (2008). Other micronutrients were not measured because their concentrations in tailings were seen as neither a limiting nor a toxic factor.

4.4.5 Statistical analyses

4.4.5.1 Differences among treatments

All the statistical analyses were conducted with the SAS software (SAS Institute Inc. 2012). Survival data was quantified in percentage and compared using $\chi 2$ test with PROC FREQ. Data of seedling health, growth, nutrition, and percentage of fungal root colonization were subjected to three-way analyses of variance (tailing type*ECM fungi*Bacteria) using PROC GLM. Proper transformations were performed when needed in order to meet normality and homoscedasticity assumptions. No transformation was necessary for stem length, shoot water content, percentage of healthy green foliage, root N, P, K, Ca, and Mg contents, and foliar K, Ca, and Mg contents. Log transformations were performed with total root length, number of root tips, root, stem, and needle dry biomass, root Fe content, and foliar N, P, and Fe contents. Arcsine transformation was used with the photochemical efficiency variable. Finally, non parametric analyses (Wilcoxon rank sum test and post hoc test) was conducted using PROC NPAR1WAY with root water content, percentage of dark red foliage, and percentage of roots colonized by ECM fungi because it was not possible to

meet normality and/or homoscedasticity assumptions for these three variables even after transformations. Significance for all analyses was set at $\alpha = 0.05$ ($P \leq 0.05$). Means and standard errors of each treatment were calculated for all health, growth, nutrition, and colonization variables.

4.4.5.2 Correlation analyses

Correlations between percentage of roots colonized by ECM fungi and other health, growth, and nutrition variables were investigated using PROC CORR. Furthermore, correlation analyses between health variables (photochemical efficiency, percentage of healthy green foliage, and percentage of dark red foliage), growth variables (root, stem, and needle dry biomass), and nutrition variables (N, P, K, Mg, Ca, and Fe contents in roots and foliage) were performed in order to determine which soil elements and concentration affected positively or negatively seedling health and growth and if there was a relationship between white spruce seedling health and growth. For these analyses, individual data were used except for correlations between nutrition and other variables where block means per treatment had to be utilized. Significance for all Pearson correlation coefficients (r) was set at $\alpha = 0.05$ ($P \leq 0.05$).

4.5 Results

4.5.1 Seedling health

At the end of the experiment, after 32 weeks of growth, white spruce seedlings on waste rocks had a much better survival rate (97.92%) than seedlings on fine tailings (85.42%) (P-value < 0.0001) (Fig. 4.1). Difference between the two started to be significant at 20 weeks (P-value = 0.0158) (Fig. 4.1). Seedling mortality on both tailing types began after 8 weeks (Fig. 4.1). Then, the percentage of seedling survival stabilized after 16 weeks for the waste rock treatment while it appeared to commence stabilizing after 28 weeks for the fine tailing treatment (Fig. 4.1).

Photochemical efficiency, percentage of healthy green foliage, and percentage of dark red foliage were considered as health variables in this experiment. For those three variables, there was no interaction between factors (tailing type, ECM fungi, and bacteria). Photochemical efficiency was affected by both ECM fungi (P-value < 0.0001) and bacteria

(*P*-value = 0.0048), separately. The percentage of healthy green seedlings was also affected by both ECM fungi and bacteria, separately (*P*-values < 0.0001). In this study, white spruce seedlings inoculated with one of the two native ECM fungal species *Cadophora finlandia* and *Tricholoma scalpturatum* displayed significantly greater health than the controls without inocula (Fig. 4.2A,C). That greater health was represented by a significantly higher photochemical efficiency (0.70 for *C. finlandia* and 0.63 for *T. scalpturatum*) and percentage of healthy green foliage (72% and 48%, respectively) than the controls (0.52 and 28%) (Fig. 4.2A,C). *C. finlandia* was the most effective in improving the health of white spruce seedlings (Fig. 4.2A,C). On the other hand, exotic *Hebeloma crustuliniforme* did not significantly ameliorate white spruce seedling health compared to the controls without ECM fungi (FV/FM = 0.56 and healthy green foliage = 40%) (Fig. 4.2A,C).

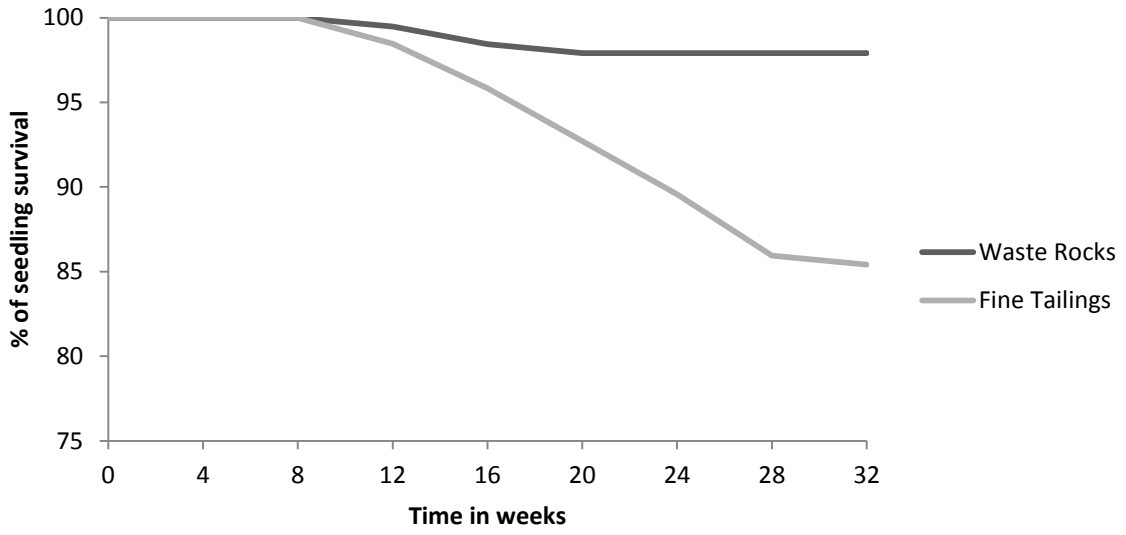

Figure 4.1: Percentage of seedling survival (%) over time (weeks) on waste rocks and fine tailings from the beginning to the end of the experiment during 32 weeks (*p*-value for Chi-square test (α =0.05): 0.5622, 0.1261, 0.0158, 0.0007, <0.0001, and <0.0001 after 12, 16, 20, 24, 28, and 32 weeks, respectively).

For the bacterial treatments, seedlings inoculated with *Azotobacter chroococcum*, a free living N-fixing PGPR, had a higher photochemical efficiency (0.63) than those inoculated with *Pseudomonas putida* (0.56) but not significantly different to neither the control (0.60) nor seedlings inoculated with *Rhizobium radiobacter* (0.61) (Fig. 4.2B). Furthermore, treatments with *A. Chroococcum* did not only increase the percentage of seedling healthy green foliage (58%) compared to treatments with *P. putida* (39%) but also compared to the control without PGPR (39%) (Fig. 4.2D). Overall, *C. finlandia* was more

successful in enhancing white spruce seedlings health than *A. chroococcum* (Fig. 4.2A,B,C,D).

The percentage of dark red foliage was affected by the three factors (ECM fungi, bacteria, and tailing type), separately (*P*-values < 0.0001). Higher percentages meant reduced seedling health. It was hypothesized that dark red foliage was caused by element toxicity. In this study, the inoculation of white spruce seedlings with *C. finlandia* permitted to significantly decrease by more than half the percentage of dark red foliage (8%) compared to the control (19%) and to the treatments inoculated with *H. crustuliniforme* (19%) or *T. scalpturatum* (17%) (Fig. 4.2E). For the bacterial treatments, the use of *R. radiobacter* (11%), isolated from a healthy white spruce naturally regenerating on the mining site, and *A. chroococcum* (10%) significantly reduced by close to 50% the percentage of dark red foliage on seedlings compared to the control (19%) and *P. putida* (23%) (Fig. 4.2F). Finally, it was found that white spruce seedlings on waste rocks (14%) had a lower percentage of dark red foliage than those on fine tailings (18%) (Fig. 4.2G).

Water is vital for plant growth and its availability has a huge effect on seedling health. In this study, it was discovered that shoot water content was only affected by tailing type (*P*-value < 0.0001) while root water content was influenced by the three factors (tailing type, ECM fungi, and bacteria), separately (*P*-values = 0.0278, < 0.0001, and = 0.003, respectively). There was no interaction among factors for these two variables. White spruce shoot water content was almost 3 % higher in fine tailings compared to waste rocks (Fig. 4.3A). On the opposite, root water content in fine tailings was 1.5 % lower than in waste rocks (Fig. 4.3B). For the ECM fungal treatments, the inoculation of seedlings with *T. scalpturatum* or *C. finlandia* increased root water content by 6% and 4%, respectively, compared to the control (74%) (Fig. 4.3C). Seedlings inoculated with *T. scalpturatum* had 4% higher root water content than those with *H. crustuliniforme* (76%) (Fig. 4.3C). Then, root water content of seedlings inoculated with *H. crustuliniforme* was not significantly different to those with *C. finlandia* and the control (Fig. 4.3C). For the bacterial treatments,

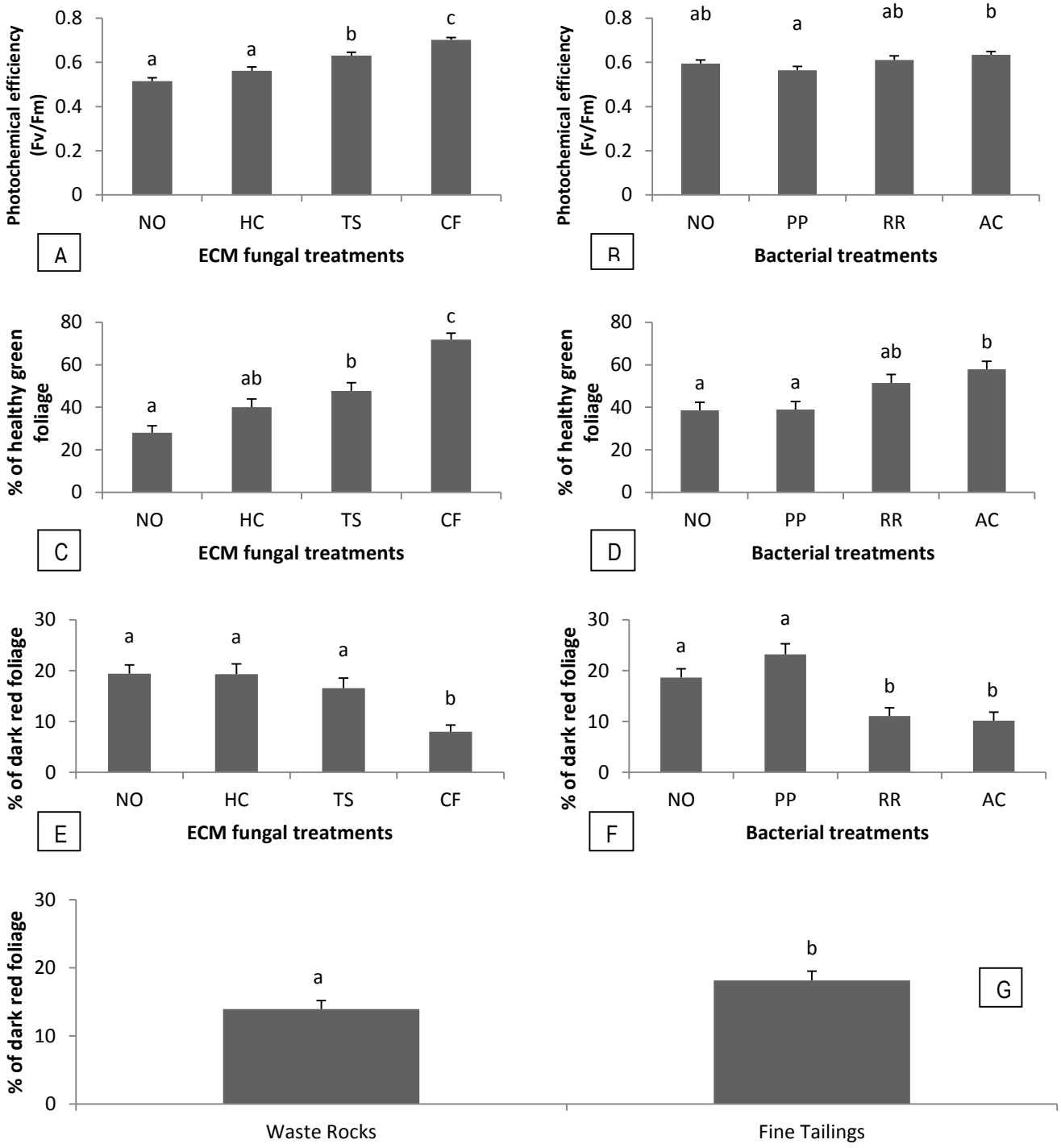

Figure 4.2: White spruce photochemical efficiency (Fv/Fm) [A,B], percentage of healthy green foliage (%) [C,D], and percentage of dark red foliage (%) [E,F,G] on the four different ECM fungal treatments (NO = no ECM fungi, HC = *Hebeloma crustuliniforme*, TS = *Tricholoma scalpturatum*, and CF = *Cadophora finlandia*), on the four different bacterial treatments (NO = no bacteria, PP = *Pseudomonas putida*, RR = *Rhizobium radiobacter*, and AC = *Azotobacter chroococcum*) and/or on the two different tailing type treatments (waste rocks and fine tailings) after 32 weeks of growth (Means ± SE with same letters are not significantly different at α =0.05, Tukey test).

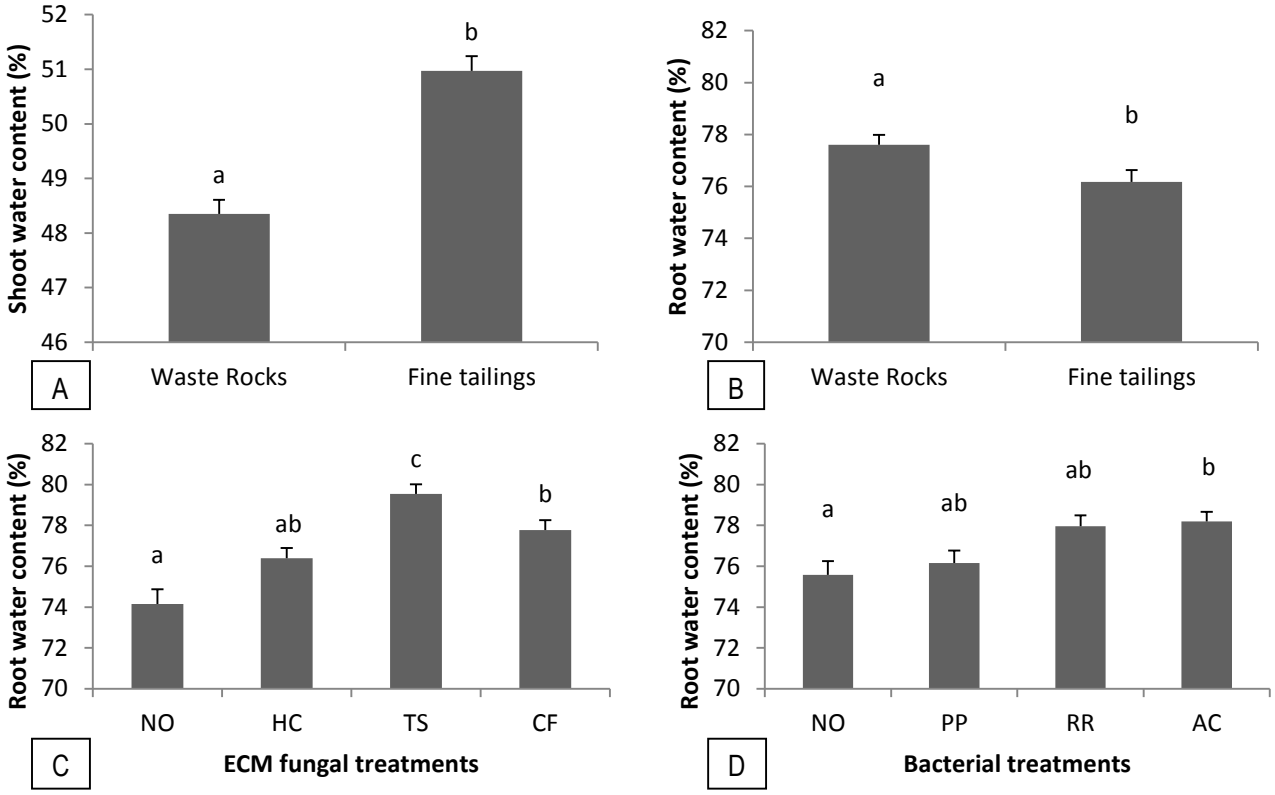

Figure 4.3: Percentage of water in white spruce shoot [A] and roots [B,C,D] (%) on the two different tailing type treatments (waste rocks and fine tailings), on the four different ECM fungal treatments (NO = no ECM fungi, HC = *Hebeloma crustuliniforme*, TS = *Tricholoma scalpturatum*, and CF = *Cadophora finlandia*), and/or on the four different bacterial treatments (NO = no bacteria, PP = *Pseudomonas putida*, RR = *Rhizobium radiobacter*, and AC = *Azotobacter chroococcum*) after 32 weeks of growth (Means ± SE with same letters are not significantly different at α =0.05, Tukey test).

A. chroococcum enhanced white spruce root water content by 2.5% compared to the control (75.5%) and there was no significant difference between root water contents of seedlings inoculated with *P. putida* or *R. rhizobium* and the other two bacterial treatments (Fig. 4.3D).

4.5.2 Seedling growth

Seedling growth included measurements of root, stem, and needle dry biomass, stem length, total root length, root volume, and root tip number. There was no interaction among factors (tailing type, ECM fungi, and bacteria) for all variables (*P*-value > 0.05) with the exception of the root tip number. Needle, stem, root biomass, stem length, total root length, and root volume were affected by tailing type (*P*-values < 0.0001, < 0.0001, = 0.0008, < 0.0001, = 0.0002, and < 0.0001, respectively) and were significantly higher for seedlings planted on waste rocks compared to those planted on fine tailings (Fig. 4.4A,B.C,D,E,F).

Furthermore, needle biomass, stem biomass and stem length were also affected by the bacterial factor (*P*-value < 0.0001, = 0.0048, and < 0.0001, respectively). Seedlings inoculated with *P. putida* had significantly greater needle biomass, stem biomass, and stem length than the control without PGPR (Fig. 4.5A,B,C).

There was an interaction between the three factors for the root tip number (*P*-value = 0.0128). On waste rocks, all treatments with the inoculation of at least one microorganism (ECM fungi, PGPR or both) increased significantly by 50% to 100% the number of root tips encountered on white spruce seedlings (Fig. 4.6). The highest increase was associated with treatments inoculated with the ECM fungus *T. scalpturatum* with or without bacteria (NO, PP, RR or AC) (Fig. 4.6). On fine tailings, white spruce seedlings inoculated with both *H. crustuliniforme* and *R. radiobacter*, both *H. crustuliniforme* and *A. chroococcum*, *T. scalpturatum* only, both *T. scalpturatum* and *P. putida*, both *T. scalpturatum* and *R. radiobacter*, both *T. scalpturatum* and *A. chroococcum*, and *C. finlandia* only had 40% to 80% greater number of root tips than the control without neither ECM fungi nor PGPR (Fig. 4.6). The greatest increase was obtained with seedlings inoculated with *C. finlandia* only or with both *T. scalpturatum* and *A. choococcum* (Fig.4.6).

4.5.3 Seedling nutrition

Tailing type had an impact on root and foliar N content, root and foliar Ca content, root and foliar Mg content, and foliar K and Fe contents (all *P*-values < 0.0001). Seedlings growing on fine tailings had greater root and foliar N content, root and foliar Ca content, and foliar Mg, K, and Fe contents than those on waste rocks (Fig.4.7A,B,C,D,F,G,H). On the other hand, seedling root Mg content was significantly higher on waste rocks compared to fine tailings (Fig.4.7E). If we look at element concentration ranges of perfectly healthy coniferous seedlings, we discovered that white spruce seedlings in this experiment had three to four times more root and foliar Ca and foliar Fe than a normal healthy coniferous seedling on both waste rocks and fine tailings (Fig.4.7C,D,H).

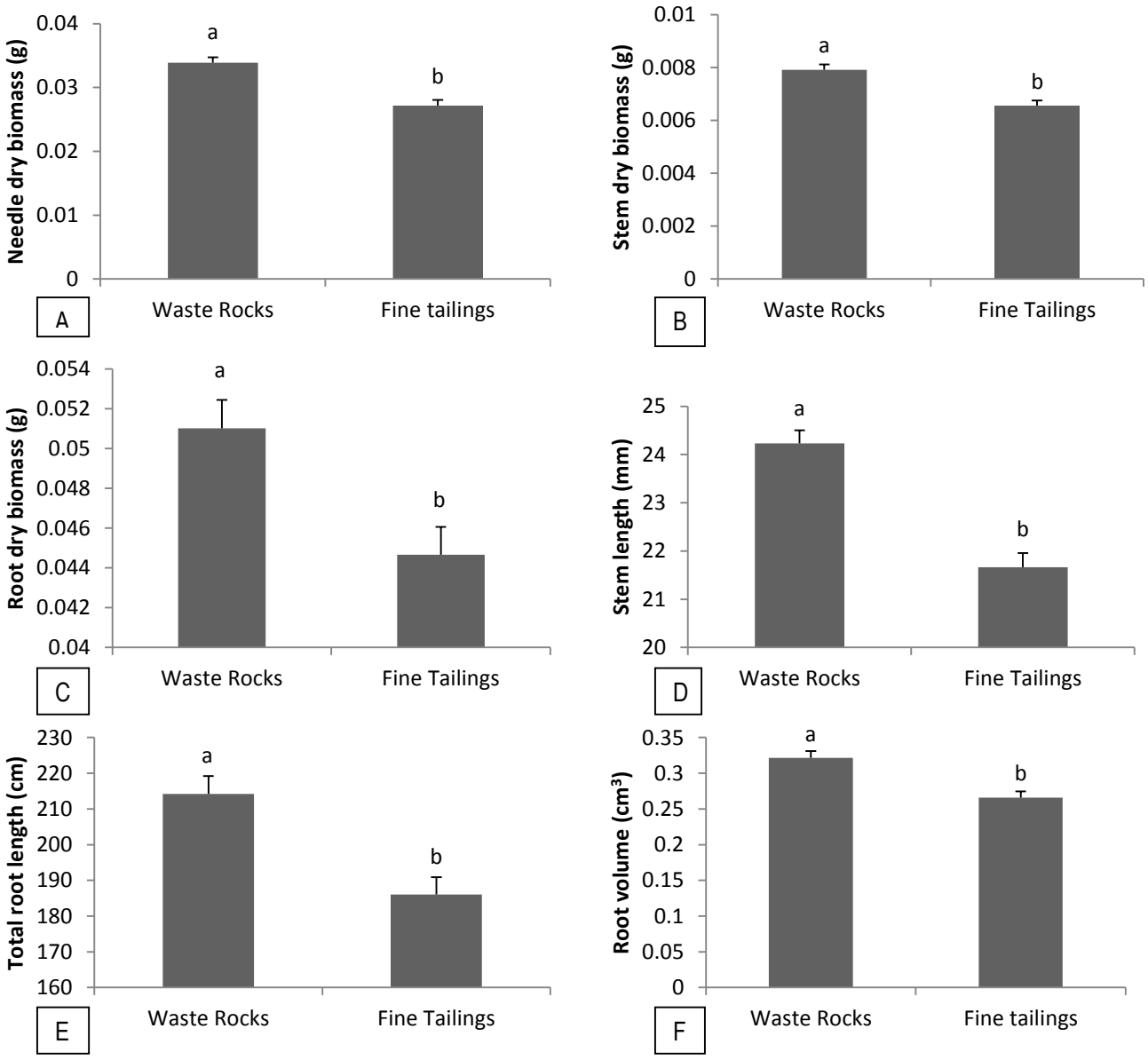

Figure 4.4: White spruce needle, stem, and root dry biomass (g) [A,B,C], stem length (mm) [D], total root length (cm) [E], and root volume (cm^3) [F] on the two different tailing type treatments (waste rocks and fine tailings) after 32 weeks of growth (Means ± SE with same letters are not significantly different at α =0.05, Tukey test).

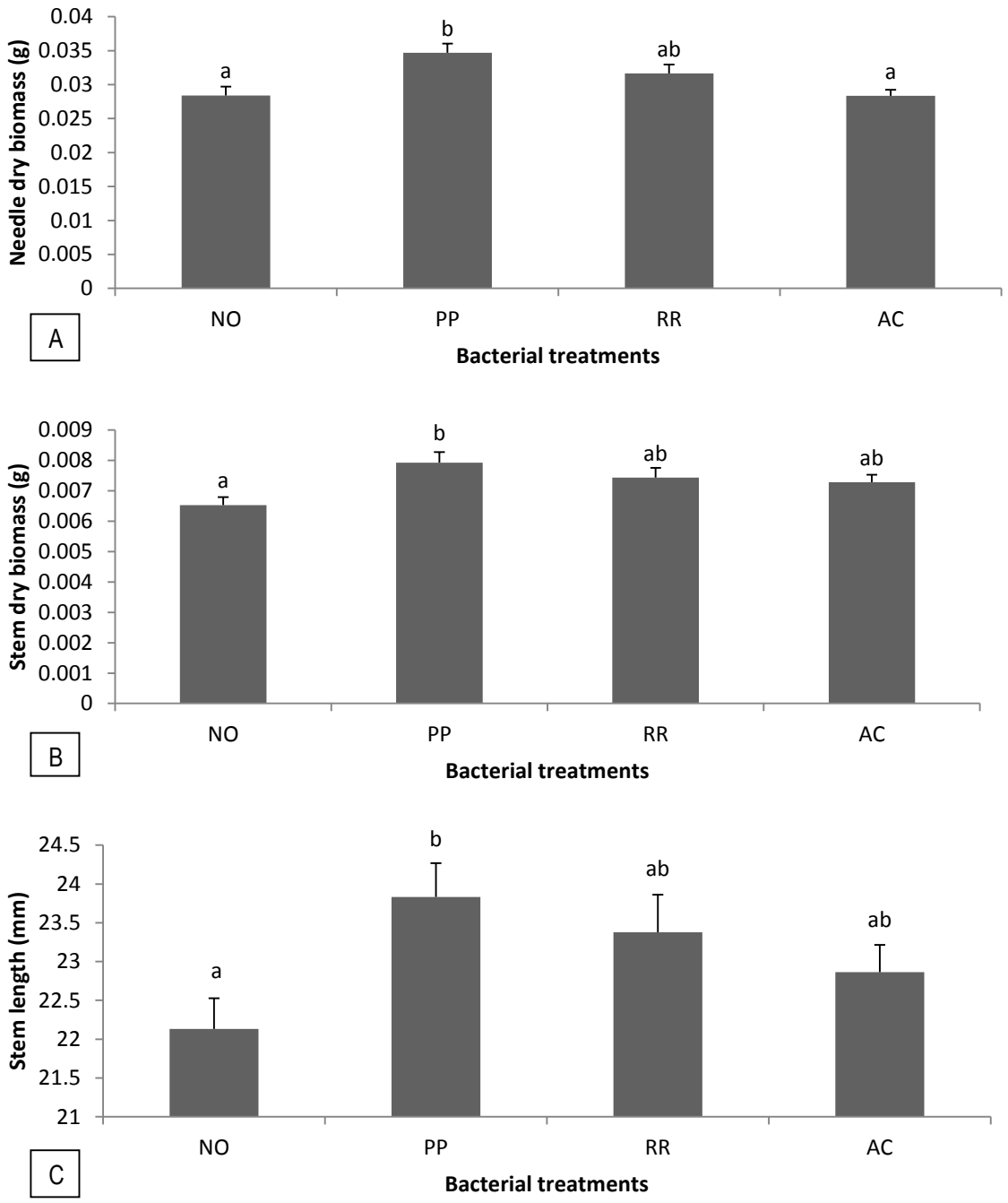

Figure 4.5: White spruce needle dry biomass (g) [A], stem dry biomass (g) [B], and stem length (mm) [C] on the four different bacterial treatments (NO = no bacteria, PP = *Pseudomonas putida*, RR = *Rhizobium radiobacter*, and AC = *Azotobacter chroococcum*) after 32 weeks of growth (Means ± SE with same letters are not significantly different at α =0.05, Tukey test).

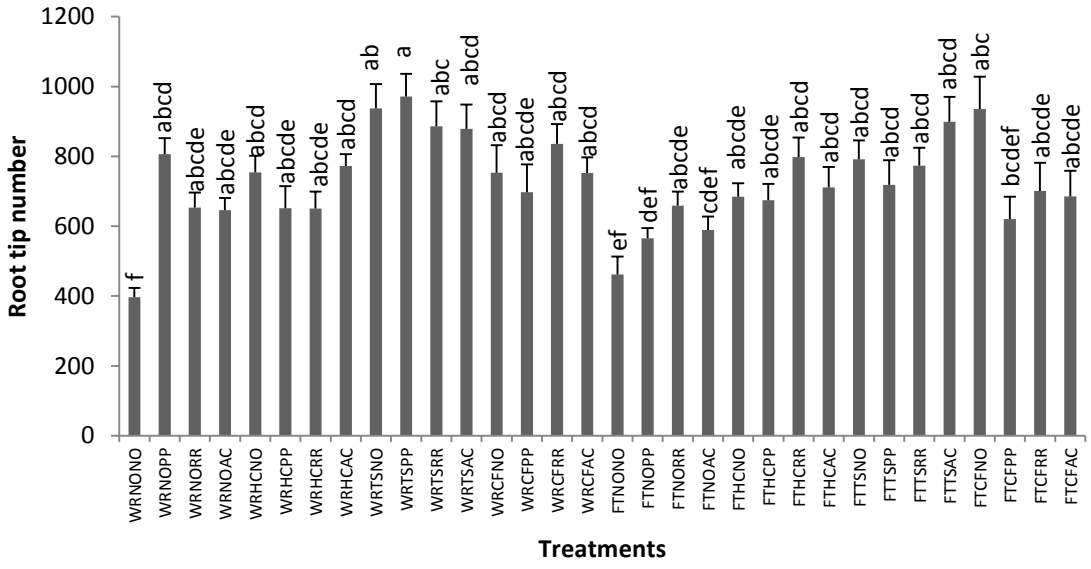

Figure 4.6: Number of white spruce root tips on the 32 treatments (Interaction between the three factors: first factor (tailing type) – WR = waste rocks and FT = fine tailings; second factor (ECM fungi) – NO = no ECM fungi, HC = *Hebeloma crustuliniforme*, TS = *Tricholoma scalpturatum*, and CF = *Cadophora finlandia*; third factor (bacteria) – NO = no bacteria, PP = *Pseudomonas putida*, RR = *Rhizobium radiobacter*, and AC = *Azotobacter chroococcum*) after 32 weeks of growth (Means ± SE with same letters are not significantly different at α=0.05, Tukey test).

Both tailing type and bacteria affected root Fe content of white spruce seedlings and there was an interaction between the two treatments (*P*-value = 0.0206). Seedlings inoculated with *A. chroococcum* on waste rocks had a root Fe content around two times smaller than seedlings inoculated with *P. putida* on waste rocks and those inoculated with *P. putida*, *R. radiobacter*, or *A. chroococcum* on fine tailings (Fig.4.8A). White spruce seedlings in this experiment contained eight to 15 times more root Fe than normal perfectly healthy coniferous seedlings (Fig.4.8A). Foliar Fe content was affected by both ECM fungi and bacteria and there was an interaction between the two treatments (*P*-value = 0.0472). Seedlings inoculated with both *T. scalpturatum* and *A. chroococcum* had a Fe concentration in needles more than twice smaller than seedlings without symbionts, inoculated with only *R. radiobacter* or *A. chroococcum*, and inoculated with both *H. crustuliniforme* and *R. radiobacter* (Fig.4.8B). Furthermore, seedlings inoculated with *C. finlandia* only had also a foliar Fe content more than twice smaller than seedlings without symbionts and inoculated with *A. chroococcum* only (Fig.4.8B). Here, white spruce seedlings, on average, had two to five times more Fe in needles than normal healthy coniferous seedlings (Fig.4.8B). Root K

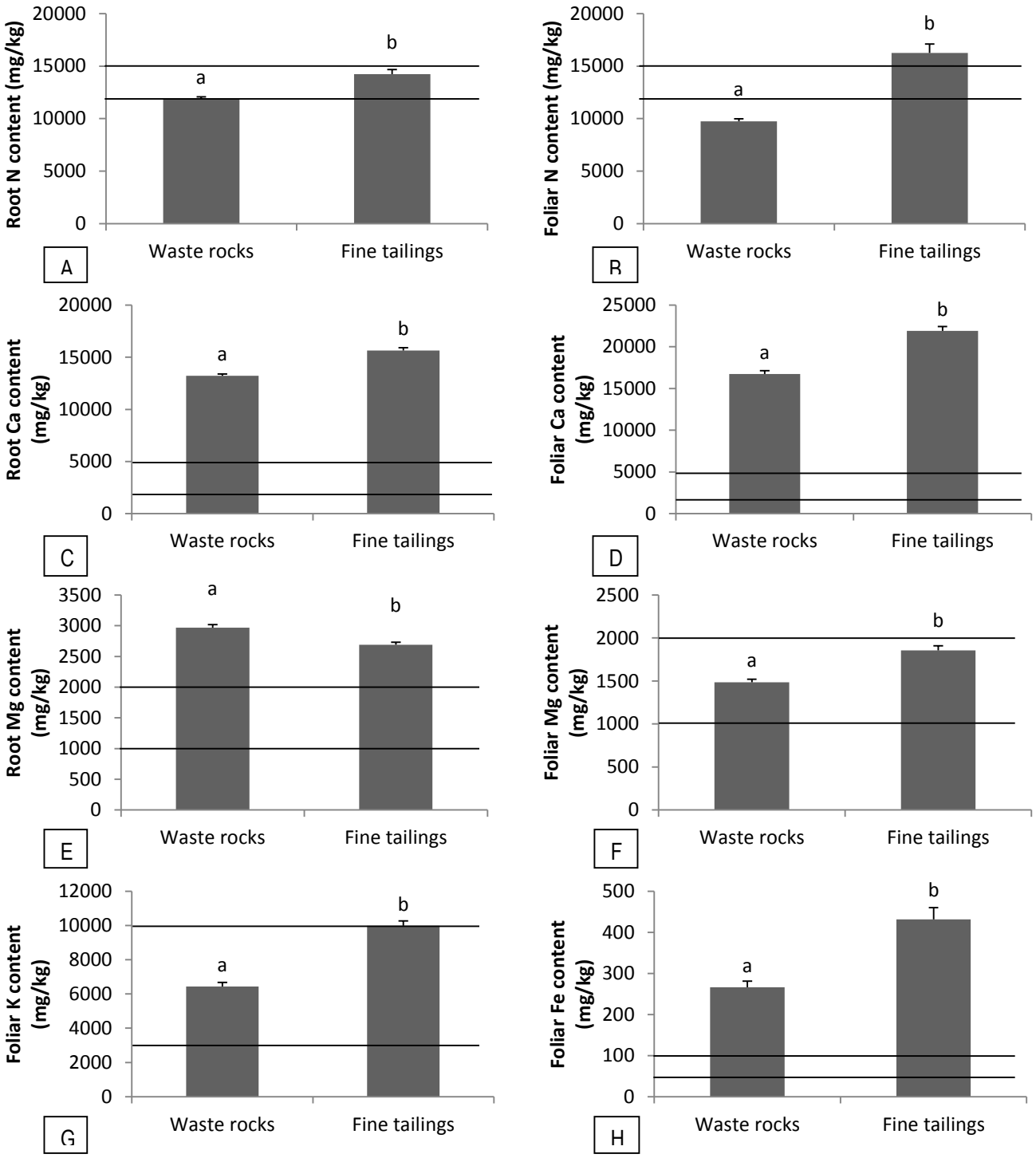

Figure 4.7: White spruce root and foliar N content (mg/kg) [A,B], root and foliar Ca content (mg/kg) [C,D], root and foliar Mg content (mg/kg) [E,F], and foliar K and Fe content (mg/kg) [G,H] on the two different tailing type treatments (waste rocks and fine tailings) after 32 weeks of growth (Means ± SE with same letters are not significantly different at α =0.05, Tukey test, black lines represent the concentration range of perfectly healthy coniferous seedlings (Van den Driessche 1991)).

content was also affected by both ECM fungi and bacteria and there was an interaction between the two treatments (*P*-value = 0.0358). White spruce seedlings inoculated with both *H. crustuliniforme* and *A. chroococcum* and inoculated with both *C. finlandia* and *A.chroococcum* had around 25% more K in roots than seedlings without symbionts and inoculated with both *T. scalpturatum* and *R. radiobacter* (Fig.4.8C).

Foliar N content was strongly influenced by ECM fungi (*P*-value < 0.0001). The inoculation of seedlings with *C. finlandia*, a ECM fungus isolated from roots of healthy white spruce trees naturally regenerating on the mining site, improved significantly (by around 25%) the foliar N content of white spruce compared with treatments without ECM fungi, with *H. crustuliniforme*, or with *T. scalpturatum* (Fig.4.8D). Here, foliar N content appeared to be within the concentration range of normal healthy coniferous seedlings except for seedlings without ECM fungi, which was slightly lower than the range minimum (Fig.4.8D). Bacteria were found to have an impact on white spruce foliar Ca and Mg contents (*P*-values < 0.0001 and = 0.0058, respectively). Seedlings inoculated with *P. putida* had significantly less Ca in needles than those without bacteria or inoculated with *R. radiobacter* or *A. chroococcum* and also significantly less Mg in needles than seedlings without bacteria (Fig.4.8E,F). Foliar Ca content of white spruce seedlings was three to four times greater than normal perfectly healthy coniferous seedlings (Fig.4.8E,).

White spruce seedling foliar and root P content (*P*-values = 0.1834 and 0.2544 respectively) was not affected by neither tailing type nor the fungal and bacterial microbial inoculants. Therefore, there were no significant differences among treatments. Twenty-three out of 32 treatments had a mean foliar P content within the concentration range of normal perfectly healthy coniferous seedlings (Fig.4.9). In roots, P content tended to be slightly lower than, slightly higher than or equal to the range minimum (Fig.4.9).

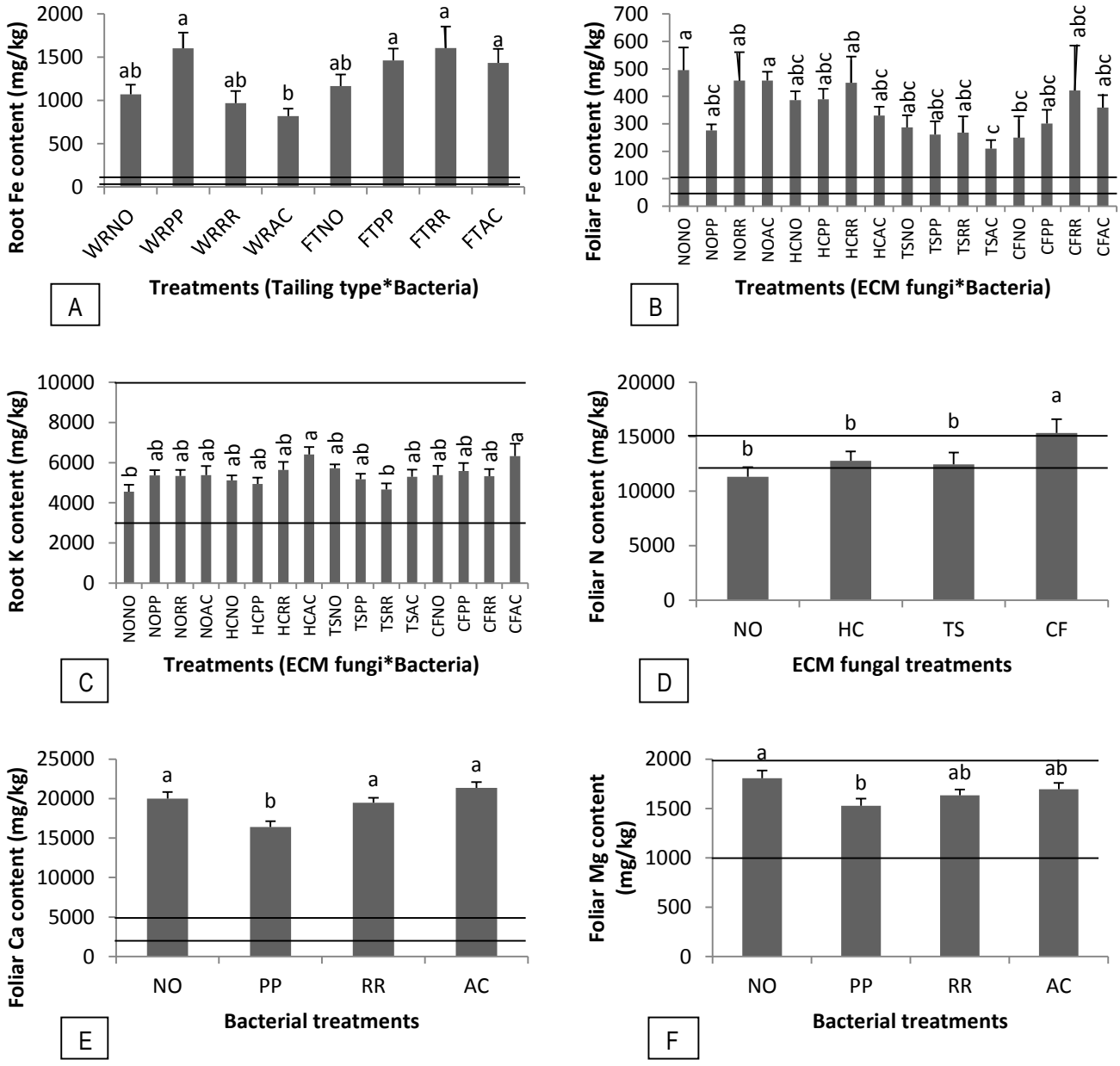

Figure 4.8: White spruce root and foliar Fe content (mg/kg) [A,B], root K content (mg/kg) [C], foliar N content (mg/kg) [D], foliar Ca content (mg/kg) [E], and foliar Mg content (mg/kg) [F] on different ECM fungal (NO = no ECM fungi, HC = *Hebeloma crustuliniforme*, TS = *Tricholoma scalpturatum*, and CF = *Cadophora finlandia*), bacterial (NO = no bacteria, PP = *Pseudomonas putida*, RR = *Rhizobium radiobacter*, and AC = *Azotobacter chroococcum*), and tailing type (WR = waste rocks and FT = fine tailings) treatments with or without interactions between factors (tailing type, ECM fungi, and bacteria) after 32 weeks of growth (Means ± SE with same letters are not significantly different at α = 0.05, Tukey test, black lines represent the concentration range of perfectly healthy coniferous seedlings (Van den Driessche 1991)).

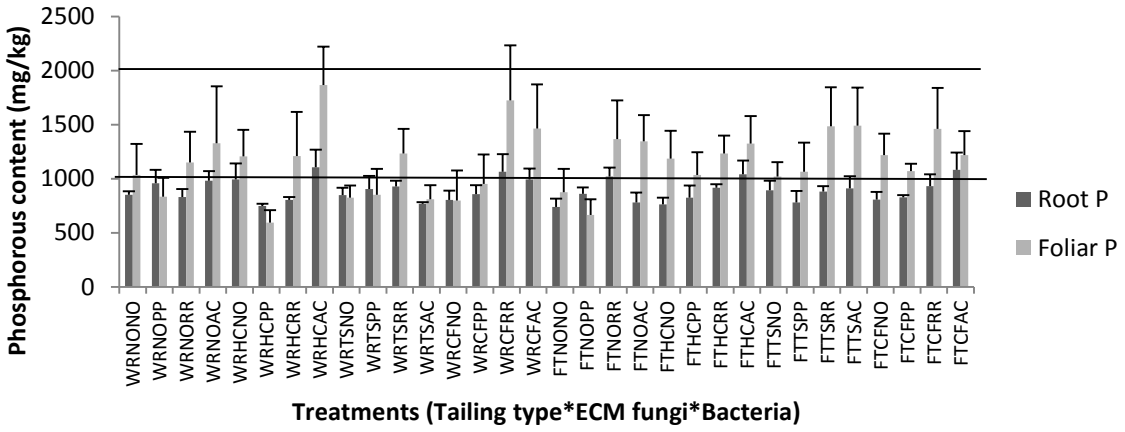

Figure 4.9: Root and foliar phosphorous content (mg/kg) on the 32 treatments (Nothing was significant among the three factors: first factor (tailing type) – WR = waste rocks and FT = fine tailings; second factor (ECM fungi) – NO = no ECM fungi, HC = *Hebeloma crustuliniforme*, TS = *Tricholoma scalpturatum*, and CF = *Cadophora finlandia*; third factor (bacteria) – NO = no bacteria, PP = *Pseudomonas putida*, RR = *Rhizobium radiobacter*, and AC = *Azotobacter chroococcum*) after 32 weeks of growth (Means ± SE, α =0.05, no letters because no factors affected significantly P content, black lines represent the concentration range of normal perfectly healthy coniferous seedlings (Van den Driessche 1991)).

4.5.4 Seedling mycorrhization rate

There was no interaction between factors (*P*- value > 0.05) for seedling mycorrhization rate. The percentage of root tips colonized by ECM fungi was affected by tailing type and ECM fungi separately (*P*-values < 0.0001). Seedlings on waste rocks had a significantly higher percentage of root tips colonized by ECM fungi (45%) than those on fine tailings (37%) (Fig. 4.10A). Seedlings inoculated with *C. finlandia* displayed the greatest percentage of root tips colonized by ECM fungi with a value of 63% and had a significantly higher mycorrhization rate than the control (3%) and seedlings inoculated with *H. crustuliniforme* (50%) or *T. scalpturatum* (49%) (Fig. 4.10B). Moreover, seedlings inoculated with *H. crustuliniforme* and *T. scalpturatum* exhibited a greater percentage of root tips colonized by ECM fungi than the control (Fig. 4.10B).

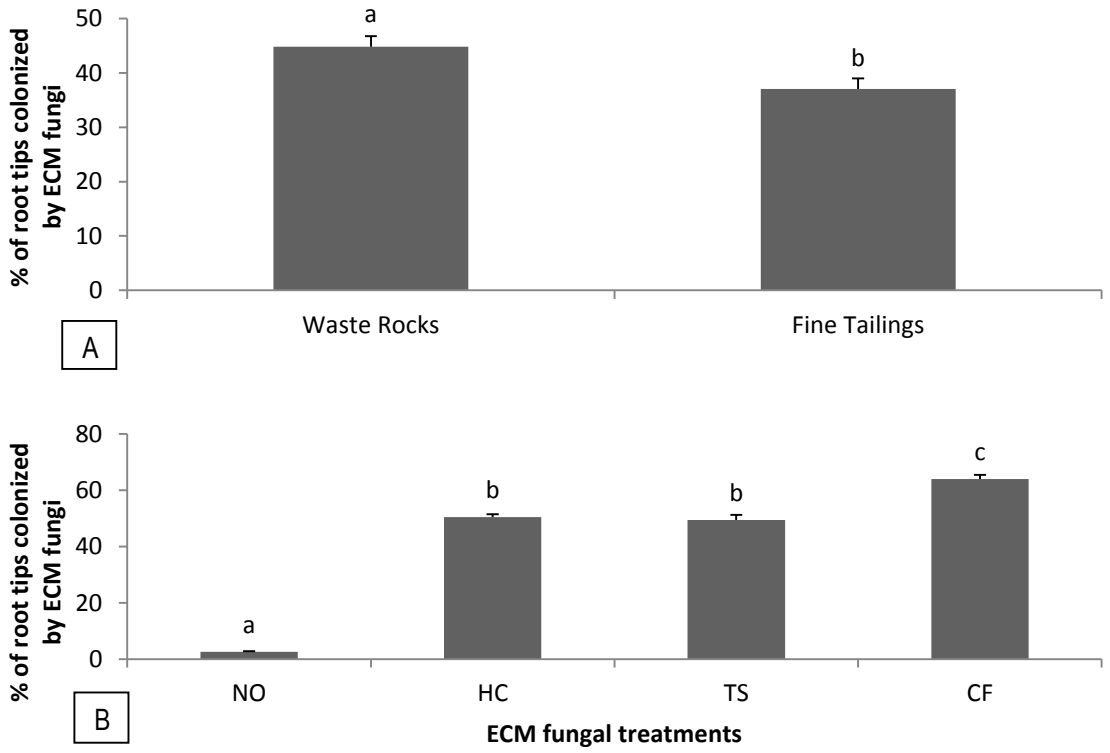

Figure 4.10: Percentage of white spruce root tips colonized by ECM fungi (%) on the two different tailing type treatments (waste rocks and fine tailings) [A] and on the four different ECM fungal treatments (NO = no ECM fungi, HC = *Hebeloma crustuliniforme*, TS = *Tricholoma scalpturatum*, and CF = *Cadophora finlandia*) [B] after 32 weeks of growth (Means ± SE with same letters are not significantly different at α = 0.05, Tukey test).

4.5.5 Correlation among variables

4.5.5.1 Percentage of root tips colonized by ECM fungi

For the three ECM fungal species (*H. crustuliniforme*, *T. scalpturatum*, and *C. finlandia*) inoculated on white spruce seedling roots, there was a positive correlation between the percentage of root tips colonized by the ECM fungi and other health and growth variables including photochemical efficiency, percentage of healthy green foliage, root volume, total root length, root dry biomass, and specific surface foliar area (Table 4.1). These results meant that the health, root growth, and specific surface foliar area of white spruce seedlings were considerably enhanced as the mycorrhization rate increased. The percentage of root tips colonized by *H. crustuliniforme* was not significantly correlated to needle dry biomass, stem dry biomass, shoot water content, root water content, and root tip number (Table 4.1). On the contrary, the percentage of root tips colonized by *T. scalpturatum* or *C. finlandia* was

positively correlated to needle dry biomass, stem dry biomass, root water content, and root tip number (Table 4.1). Accordingly, aerial growth, root water content, and root tip number were positively correlated with the mycorrhization rate of white spruce seedlings colonized by *T. scalpturatum* or *C. finlandia*. Finally, the percentage of root tips colonized by *C. finlandia* showed the highest positive relationship with all health and growth variables with the exception of root water content which was more positively correlated to the percentage of root tips colonized by *T. scalpturatum* (Table 4.1).

For seedling nutrition, the percentage of root tips colonized by *H. crustuliniforme* was negatively correlated with the concentration of N and Ca in roots and positively correlated with the concentration of P in roots (Table 4.2). Furthermore, the percentage of root tips colonized by *T. scalpturatum* was negatively correlated with root Ca content and positively correlated with root P content (Table 4.2). Subsequently, the percentage of root tips colonized by *C. finlandia* was negatively correlated with root Ca and Fe contents and also foliar K content (Table 4.2). These results meant that (1) the uptake of N and Ca in seedling roots increased and the uptake of P in roots decreased with the increased mycorrhization rate of *H. crustuliniforme*, (2) the uptake of P in seedling roots increased and the uptake of Ca in roots decreased with the increased mycorrhization rate of *T. scalpturatum*, and (3) the uptake of Ca and Fe in roots and K in needles is reduced with increasing mycorrhization rate of *C. finlandia*.

4.5.5.2 Health and growth of individual seedlings

The percentage of dark red foliage was positively correlated to root N (7050 to 80600 mg/kg), Ca (10432 to 34028 mg/kg), and Fe (98 to 14110 mg/kg) content and foliar K (3486 to 10686 mg/kg) and Fe (343 to 7358 mg/kg) content (Table 4.3). On the contrary, the percentage of dark red foliage was negatively correlated to root K (2715 to 35542 mg/kg) content (Table 4.3). All four following variables (the percentage of healthy green foliage, root, stem, and needle dry biomass) were negatively correlated to root N, Ca, and Fe content and the foliar concentration of K (Table 4.3). Furthermore, the percentage of healthy green foliage was positively correlated to root K and foliar P (518 to 3197 mg/kg) contents

Table 4.1: *P-value* and Pearson correlation coefficients (r) for the relationships between the percentage of white spruce root tips colonized by *Hebeloma crustuliniforme*, *Tricholoma scalpturatum*, or *Cadophora finlandia* and different health and growth variables (α = 0.05 level of significance).

% of root mycorrhizal colonization	Statistical values	FVFM	% of healthy green foliage	Root volume (cm3)	Total root length (cm)	Specific surface foliar area	Root dry biomass	Stem dry biomass	Needle dry biomass	% of shoot water content	% of root water content	Root tip number
Hebeloma crustuliniforme	*p-value*	**0.0014**	**< 0.0001**	**< 0.0001**	**0.0008**	**< 0.0001**	**0.0003**	0.0606	0.2653	0.8642	0.0571	0.1714
	r	**0.33**	**0.47**	**0.41**	**0.35**	**0.41**	**0.37**	0.2	0.12	-0.02	0.2	0.15
Tricholoma scalpturatum	*p-value*	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0.0078**	**0.0451**	0.6484	**< 0.0001**	**0.015**
	r	**0.54**	**0.71**	**0.47**	**0.41**	**0.49**	**0.43**	**0.28**	**0.21**	-0.05	**0.4**	**0.26**
Cadophora finlandia	*p-value*	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0.0004**	**0.0014**	0.6892	**0.0043**	**0.0013**
	r	**0.67**	**0.75**	**0.59**	**0.49**	**0.66**	**0.56**	**0.38**	**0.34**	-0.04	**0.31**	**0.34**

Table 4.2: *P-value* and Pearson correlation coefficients (r) for the relationships between the percentage of white spruce root tips colonized by *Hebeloma crustuliniforme*, *Tricholoma scalpturatum*, or *Cadophora finlandia* and different root and needle nutrition variables (α = 0.05 level of significance).

% of root mycorrhizal colonization	Statistical values	Roots						Needles					
		N	P	K	Ca	Mg	Fe	N	P	K	Ca	Mg	Fe
Hebeloma crustuliniforme	*p-value*	**0.0042**	**0.0283**	0.1567	**0.0047**	0.0594	0.1957	0.2744	0.0661	0.3384	0.2369	0.1357	0.1097
	r	**-0.49**	**0.39**	0.26	**-0.49**	0.34	-0.23	-0.2	0.33	-0.17	-0.22	-0.27	-0.29
Tricholoma scalpturatum	*p-value*	0.2448	**0.0175**	0.7757	**0.0047**	0.2048	0.207	0.8268	0.8905	0.4071	0.1705	0.5419	0.0966
	r	-0.21	**0.42**	0.05	**-0.49**	0.23	-0.23	0.04	-0.02	-0.15	0.25	-0.11	-0.3
Cadophora finlandia	*p-value*	0.0942	0.1156	0.2426	**<0.0001**	0.4354	**0.0109**	0.0764	0.7959	**0.0137**	0.5308	0.5217	0.6092
	r	-0.3	0.28	-0.21	**-0.66**	0.14	**-0.44**	-0.32	0.05	**-0.43**	-0.11	-0.12	-0.09

Table 4.3: *P*-value and Pearson correlation coefficients (r) for the relationships between the percentage of dark red foliage, the percentage of healthy green foliage, the root, stem, needle dry biomass and needle N, P, K, Ca, Mg, and Fe contents (at α = 0.05 level of significance) of white spruce seedlings including element concentration ranges (mg/kg) in the glasshouse trial versus element concentration ranges of perfectly healthy coniferous seedlings (Van den Driessche, 1991).

Growth and health Variables	Statistical measurements	Roots						Needles					
		N	P	K	Ca	Mg	Fe	N	P	K	Ca	Mg	Fe
% of dark red foliage	*p-value*	**0.0011**	0.0937	**0.0347**	**<0.0001**	0.4841	**0.0036**	0.8893	0.1435	**0.0035**	0.0903	0.1691	**0.0197**
	R	**0.28**	-0.15	**-0.19**	**0.47**	-0.06	**0.26**	0.01	-0.13	**0.26**	-0.15	0.12	**0.21**
% of healthy foliage	*p-value*	**0.0252**	0.0627	**0.0071**	**<0.0001**	0.2487	**0.0326**	0.2232	**0.0095**	**0.0086**	**0.0198**	0.1416	0.1709
	R	**-0.2**	0.16	**0.24**	**-0.42**	0.1	**-0.19**	0.11	**0.23**	**-0.23**	**-0.21**	-0.13	-0.12
Root dry biomass	*p-value*	**<0.0001**	**0.0319**	0.2319	**<0.0001**	0.2275	**0.0025**	**0.0228**	0.627	**0.0033**	0.2712	0.1672	**0.0289**
	R	**-0.53**	**0.19**	0.11	**-0.63**	0.11	**-0.26**	**-0.2**	0.04	**-0.26**	-0.1	-0.12	**-0.19**
Stem dry biomass	*p-value*	**<0.0001**	0.2781	0.4812	**<0.0001**	0.5223	**0.0139**	**0.0103**	0.6332	**0.0066**	**0.0006**	**0.039**	0.1218
	R	**-0.44**	0.1	-0.06	**-0.58**	0.06	**-0.22**	**-0.23**	-0.04	**-0.24**	**-0.3**	**-0.18**	-0.14
Needle dry biomass	*p-value*	**<0.0001**	0.7306	0.0629	**<0.0001**	0.9228	**0.0051**	**<0.0001**	0.0713	**0.0049**	**<0.0001**	**<0.0001**	**0.0387**
	R	**-0.49**	0.03	-0.16	**-0.54**	0.01	**-0.25**	**-0.38**	-0.16	**-0.25**	**-0.59**	**-0.35**	**-0.18**
Concentration range (mg/kg) : MIN – MAX		7050 80600	366 6929	2715 35542	10432 34028	1059 4716	98 14110	9191 44372	518 3197	3486 10686	9956 27117	2063 5222	343 7358
Concentration range of healthy coniferous seedlings (mg/kg)		12000 15000	1000 2000	3000 10000	2000 5000	1000 2000	50 100	12000 15000	1000 2000	3000 10000	2000 5000	1000 2000	50 100

Table 4.4: *P*-value and Pearson correlation coefficients (r) for the relationships between several health and growth variables (α = 0.05 level of significance).

Growth and health Variables	Statistical values	Water content		Dry biomass		
		Roots	Shoot	Roots	Stem	Needles
% of dark red needles	*p-value*	**<0.0001**	**0.002**	**<0.0001**	**<0.0001**	**0.0002**
	r	**-0.41**	**0.16**	**-0.5**	**-0.26**	**-0.19**
% of healthy needles	*p-value*	**<0.0001**	0.4856	**<0.0001**	**<0.0001**	**0.0443**
	r	**0.42**	-0.04	**0.51**	**0.26**	**0.11**
Root dry biomass	*p-value*	**<0.0001**	0.3711			
	r	**0.35**	-0.05			
Stem dry biomass	*p-value*	**0.0001**	**0.0282**			
	r	**0.2**	**-0.12**			
Needle dry biomass	*p-value*	**0.0014**	**<0.0001**			
	r	**0.17**	**-0.28**			

and negatively correlated to the foliar concentration of Ca (Table 4.3). Root dry biomass was positively related to root P (366 to 6929 mg/kg) content and negatively related to foliar N (9191 to 44372 mg/kg) and Fe content (Table 4.3). Stem dry biomass was negatively related to foliar N, Ca (9956 to 27117 mg/kg), and Mg (2063 to 5222 mg/kg) content (Table 4.3). Then, needle dry biomass was negatively related to foliar N, Ca, Mg, and Fe contents (Table 4.3).

Root water content was positively correlated with the percentage of healthy green needles and root, stem, and needle dry biomass (Table 4.4). On the other hand, root water content was negatively correlated to the percentage of dark red needles (Table 4.4). For those reasons, there was a positive relationship between root water content and white spruce seedling health. For shoot water content, it was negatively correlated to needle and stem dry biomass and positively correlated to the percentage of dark red needles (Table 4.4). Therefore, there was a negative relationship between shoot water content and white spruce seedling aerial growth and a positive relationship between shoot water content and the presence of dark red foliage. By looking at correlations between several health and growth variables, it appears that the percentage of dark red needles was negatively correlated to root, stem, and needle dry biomass (Table 4.4). On the opposite, the percentage of healthy needles was positively correlated to root, stem, and needle dry biomass (Table 4.4). As a result, there was a positive relationship between white spruce seedling health and growth in this glasshouse trial.

4.6 Discussion

4.6.1 Selection of ectomycorrhizal fungi, essential for seedling health

White spruce seedling health was greatly improved by both ECM fungi isolated from the mining site - *Cadophora finlandia* and *Tricholoma scalpturatum* - but not by the fungus originating from the natural forest - *Hebeloma crustuliniforme*. The highest increase was given by *C. finlandia*. Furthermore, *C. finlandia* permitted to greatly reduce the percentage of unhealthy dark red foliage. Therefore, ECM fungi isolated directly from roots of white spruce seedlings naturally regenerating on the mining site were much more effective in enhancing seedling health on waste rocks and fine tailings. Both *C. finlandia* and *T. scalpturatum* are commonly found, in high abundance and frequency, in heavy metal polluted soils (Krpata et al. 2008, Gorfer et al. 2009, Colpaert et al. 2011). These two ECM fungal species may be

better adapted to extreme site conditions of the mine tailings; thereby, enhancing seedling health. Contrarily to our results, white spruce seedlings inoculated with the same strain of *H. crustuliniforme* on peat moss and sand treated with a NaCl treatment showed greater health than non-mycorrhizal seedlings (Mushin et al. 2002). ECM fungal adaptation to site conditions and ability to improve seedling health may be site-specific. In another study, we demonstrated that *C. finlandia* produced the highest mycelial biomass (ergosterol content) on poor liquid medium with tailings followed by *T. scalpturatum* and then *H. crustuliniforme* with the smallest biomass (Azaiez et al. 2015, Chapter 2). As a result, the ability of an ECM fungus to grow (in biomass) in mine tailings may be positively associated with its ability to symbiotically improve seedling health. Here, it is important to remember that not all ECM fungi have the ability to boost seedling health on mine tailings, so ECM fungal selection is essential when planning revegetation activities. High heavy metal concentrations in soil lead to the evolution of ECM fungi adapted and tolerant to metal conditions and this evolution - adaptation is indispensable for both tree and ECM fungal survival and health on toxic soils (Colpaert et al. 2011).

The inoculation of white spruce seedling roots with *T. scalpturatum*, *C. finlandia* or *A. chroococcum* enhanced considerably root water uptake. *T. scalpturatum* was the most efficient microsymbiont in increasing root water uptake. Water retention in the mine tailings is generally very low. Thus, enhancing root water uptake may have contributed substantially to the improved white spruce seedling health on the tailings. Onwuchekwa et al. (2014) demonstrated that all their ECM fungal treatments (*H.crustuliniforme*, *Suillus tomentosus*, and/or *Laccaria bicolor*) inoculated on white spruce and jack pine (*Pinus banksiana*) increased seedling water uptake on oil sand tailings. Furthermore, Yi et al. (2008) showed higher root water uptake capacity on trembling aspen seedlings (*Populus tremuloides*) inoculated with *H. crustuliniforme* or *L.bicolor* compared to seedlings without ECM fungi. Here, *H. crustuliniforme* on white spruce seedlings did not improve root water uptake. *H. crustuliniforme* is known as a drought intolerant species (Coleman and Bledsoe 1989). Drought conditions are common in the tailings due to low water retention capacity. This may explain why *H. crustuliniforme* did not significantly improve seedling health and root water uptake. Again, root water uptake ability may be positively associated with the ability of ECM fungi to grow in tailings under special environmental conditions.

4.6.2 Selection of PGPR – fundamental for seedling growth

The inoculation of white spruce with *P. putida*, a PGPR strain isolated from roots of healthy white spruce seedlings naturally regenerating on the mining site, was the only treatment that increased considerably seedling aerial growth. Bacteria isolated from contaminated sites are usually tolerant to higher concentrations of heavy metals than those isolated from unpolluted sites (Rajkumar et al. 2009). *P. putida* is an endophytic PGPR which colonizes the roots of many tree species (Weyens et al. 2009a). Beall and Tipping (1989) and O'Neill et al. (1992) also discovered that *P. putida* inoculated on jack pine and hybrid spruce (*Picea glauca* x *engelmannii*), respectively, enhanced seedling aerial growth compared to control without PGPR. Meyer and Linderman (1986) also obtained similar results. Furthermore, Glick et al. (1997) and Lifshitz et al. (1987) demonstrated that canola inoculation with *P. putida* promoted shoot and root growth. In the *Pseudomonas* genus, growth-promoting mechanisms involve the production of hormones (auxins, cytokinins, and gibberellins), mineral solubilization (especially phosphorous), the prevention of pathogen attack, and sometimes, for certain strains, nitrogen fixation (Weyens et al. 2009a, Lifshitz et al. 1987, Weyens et al. 2009b).

In our study, *A. chroococcum* and *R. radiobacter* did not influence white spruce seedling growth. On the contrary, several other studies have demonstrated the beneficial effects of the inoculation of *A. chroococcum* and *R. radiobacter* on shoot growth of many non-woody plant species (Aquilanti et al. 2004, Radwan 1998, Fares 1997, Brown and Burlingham 1968, Humphry et al. 2007, Baset Mia and Shamsuddin 2010). Furthermore, it was shown previously that seedlings growth of *Quercus serrata* and *Fagus sylvatica* was enhanced by the inoculation with *A. chroococcum* and *R. radiobacter*, respectively (Akhromeiko and Shestakova 1958, Pandey et al. 1986, Leyval and Berthelin 1993). However, these studies were not conducted on mine tailings, which have completely different environmental conditions than agricultural fields and forest stands. Growth promoting mechanisms in these two species are the same than *P. putida* except that all strains of *A. chroococcum* fix atmospheric nitrogen (Humphry et al. 2007, Baset Mia and Shamsuddin 2010). These two PGPR species did not promote seedling growth like *P. putida* did. As a result, they may not be adapted to tailing conditions as much as *P. putida*; thus, inhibiting their growth promoting capacity. For that reason, bacterial selection must be done on mine

tailings in order to identify strains that have the ability to promote seedling growth on mining sites. Furthermore, ECM fungi did not enhanced seedling aerial growth in this experiment. Early root colonization by ECM fungi may have a carbon cost that negates seedling growth during the first growing season but enhances it the following years (Rygiewicz and Andersen 1994).

In our glasshouse trial, all treatments with at least one symbiont yielded a higher number of fine root tips on white spruce seedlings on waste rocks and fine tailings than the control without symbiont. PGPR such as species of the genus *Pseudomonas* and *Azotobacter* produce phytohormones (auxins and cytokinins) which alter plant growth and development (Rajkumar et al. 2009). High level of auxin released by soil bacteria in seedling rhizosphere stimulates the formation of lateral and adventitious roots while inhibiting primary root elongation (Rajkumar et al. 2009). ECM fungi also produce hormones, which influence considerably root morphology (Gogala 1991). On that account, hormone production by symbionts probably plays a very important role in seedling root growth behaviour. The production of higher number of root tips by seedlings inoculated with PGPR and/or ECM fungi may be highly beneficial for white spruce allowing the uptake of extra water and limiting elements such as phosphorous in the tailings.

4.6.3 Soil microorganisms play a huge role in seedling nutrition

Particle size and mineral type tend to affect mineral weathering in soil (Adeleke et al. 2012). Mineral mobilisation is normally higher in finer particle size tailings (Modak et al. 2001). In our glasshouse experiment, seedling root Ca, foliar Ca, and foliar Fe content on fine tailings was three to four times higher than concentrations normally found in perfectly healthy coniferous trees (Van den Driessole 1991). Seedlings root Ca, foliar Ca, and foliar Fe contents were much lower on waste rocks compared to fine tailings. High concentrations of Ca and Fe in plant tissues are probably toxic to the white spruce seedlings. For that reason, white spruce seedlings may function better on waste rocks than fine tailings because less Ca and Fe are absorbed in their foliar tissues.

In our study, the inoculation of white spruce roots with *C. finlandia* improved, to optimal level, foliar N uptake by seedlings compared to control and seedlings inoculated with *T. scalpturatum* or *H. crustuliniforme*. *C. finlandia* is a dark septate endophytic ascomycete

fungal species that forms both ectomycorrhizal and ericoid mycorrhizal associations (Mitchell and Gibson 2006, Peterson et al. 2008, Vralstad 2004). Other studies also demonstrated the positive effect of *C. finlandia* on N uptake by Norway spruce (*Picea abies*) and different ericaceae species (Mrnka et al. 2009, Mitchell and Gibson 2006). On the other hand, Alberton et al. (2010) found that the inoculation of scots pine seedlings (*Pinus sylvestris*) with *C. finlandia* did not enhance plant N uptake. The ability of *C. finlandia* to improve seedling N uptake may be site-specific, strain-specific and/or host specific. It is thought that mycorrhizal fungi furnish as much as 60 to 85% of nitrogen to arctic plants such as *Dryas octopetala* (Bjorbaekmo et al. 2010). In these arctic ecosystems, which are similar to harsh mining site conditions, dark septate endophytic fungi, such as *Phialocephala fortinii* and *C. finlandia*, are among the most frequent fungi encountered (Bjorbaekmo et al. 2010). Blaudez et al. (2000) discovered that N uptake by *P. involutus* was inhibited by high concentrations of heavy metals including Cu, Cd, Ni, Pb, and Zn. *H. crustuliniforme* and *T. scalpturatum* are basidiomycota species like the fungus *P. involutus* and the high concentration of Fe and Ca in soil may have led to the reduction of N uptake by *H. crustuliniforme* and *T. scalpturatum*; thereby, inhibiting the improved foliar N uptake of seedlings inoculated with these two ECM fungal symbionts.

None of our bacterial treatments permitted to increase seedling N uptake in our glasshouse experiment. Brown et al. (1962) indicated that nitrogen fixation by *Azotobacter* species is greatly affected by phosphate availability in soil. Not all strains of *R. radiobacter* are capable of fixing atmospheric N_2 in soil (Humphy et al. 2007, Kanvinde and Sastry, 1990, Xing et al. 2006). Furthermore, the nitrogenase fixation system requires molybdenum for functioning (Kanvinde and Sastry 1990). Low phosphate and Mo availability in the tailings and/or the use of non-N-fixing strains may explain why our bacterial treatments were not able to enhanced N uptake in white spruce seedlings. Neither ECM fungi nor bacteria affected seedling P uptake in this experiment. In general, foliar P concentration of our seedlings was within the concentration range of normal perfectly healthy coniferous seedlings (Van den Driessole 1991). Smith and Hinckley (1995) advocated that foliar P content do not usually differ between mycorrhizal and non-mycorrhizal seedlings, which is in agreement with our findings.

Root K uptake in our white spruce seedlings was amplified by dual inoculation of roots with both *H. crustuliniforme* and *A. chroococcum* or with both *C. finlandia* and *A. chroococcum*; thus, improving seedling root K nutrition. Adeleke et al. (2012) found that the inoculation of tree seedlings with ECM fungi enhanced iron ore K mobilisation in soil but did not increase foliar K uptake. These results agree with our findings where specific micro-symbionts only improved seedling root K uptake. Wu et al. (2005) found that dual inoculation of *Zea mays* with both *Rhizophagus irregularis* and *A. chroococcum* improved plant K content. On that account, *A. chroococcum* associated with mycorrhizal fungi may play an important role in plant potassium nutrition.

In our study, the inoculation of white spruce with *C. finlandia* only or with both *T. scalpturatum* and *A. chroococcum* permitted to decrease foliar Fe uptake in seedlings compared to the control without symbionts. Furthermore, foliar Ca content was much lower in seedlings inoculated with *P. putida*. Both nutritional elements reached concentrations much higher than normal healthy coniferous trees (Van den Driessche 1991). Micro-symbionts mentioned above seem to have played an important role in reducing Fe and Ca toxicity to white spruce seedlings by decreasing and regulating their uptake in aerial tissues. Heavy metals at high concentrations in soil are normally toxic to most plants damaging their metabolism and reducing their growth (Rajkumar et al. 2009). Many metal resistant PGPR possess attributes that allow them to alter heavy metal availability and toxicity to plants (Rajkumar et al. 2009). These attributes include the production of iron chelators and siderophores (Rajkumar et al. 2009). Metal-resistant PGPR have developed mechanisms for heavy metal tolerance involving metal exclusion, active removal, biosorption, precipitation, and bioaccumulation in both external and intracellular spaces (Rajkumar et al. 2009). These mechanisms tend to alter metal solubility and bioavailability to plants; thereby, changing their toxic effects to plants (Rajkumar et al. 2009, Meyer and Linderman 1986, Burd et al. 1998). *P. putida* may have used one of those mechanisms for decreasing white spruce foliar Ca content. During the process of plant N uptake, calcium ions are generally taken up by plants at high pH but released in soil at low pH (Rygiewicz et al. 1984). Mine tailings hold a very high pH of around 8.5 and contain high concentrations of calcium. In our study, *P. putida* was quite effective in regulating white spruce foliar Ca uptake compared to other treatments. De Maria et al. (2011) discovered that the inoculation of *C. finlandia* on *Salix* roots did not

influence the phytoextraction of Cd and Zn in a polluted soil while the dual inoculation of both *C. finlandia* and one of the two PGPR, *Streptomyces* sp. or *Agromyces* sp., increased it. Our results and previous studies (Leyval and Berthelin 1991, De Maria et al. 2011, among others) showed an interaction between several ECM fungal and PGPR strains associated with the uptake of certain nutritional elements in white spruce seedlings.

4.6.4 Relationships between health, growth, nutrition of individual seedlings and ECM fungal root colonization

In this study, the percentage of white spruce roots colonized by ECM fungi was the highest on treatments with *C. finlandia* followed by *T. scalpturatum* and *H. crustuliniforme* and then the lowest on the control without ECM fungi. These findings agree with the results obtained by Onwuchekwa et al. (2014), which demonstrated that the inoculation of white spruce seedlings with ECM fungi improved considerably ECM fungal root colonization on tailings. Furthermore, our white spruce seedlings had a higher colonization rate on waste rocks than on fine tailings. Mineral solubilisation may be faster on fine tailings due to smaller particles size (Modak et al. 2001); therefore probably causing Fe and Ca toxicity to ECM fungi which may have led to a decrease in fungal growth and root colonization (Leyval et al. 1997).

Seedling health, aerial and root growth, and root water uptake were positively correlated with root colonization rates of the two ECM fungal species isolated from the mining site, *C. finlandia* and *T.scalpturatum*. On the other hand, the increase in root colonization rate by *H. crustuliniforme* only brought the enhancement of seedling health, root growth, and specific surface foliar area. We know little about population genetics of the majority of ECM fungal species, which is directly related to the functioning of forest ecosystems (Douhan et al. 2011). *T. scalpturatum* is a generalist ECM fungal species with high intraspecific genetic diversity at the local scale and with high sporal wind-dispersal abilities (Carriconde et al. 2008, Gryta et al. 2006). Both *T. scalpturatum* and *C. finlandia* are widely known for their ability to colonize roots of different tree species on heavy metal contaminated sites (Krpata et al. 2008, Gorfer et al. 2009). Little is known about the intraspecific genetic diversity of *C. finlandia*. However, we know that *C. finlandia* has the capability to enhance the expression of metal-regulated genes, which encode several extracellular and plasma membrane proteins,

under conditions of high heavy metal concentrations (Gorfer et al. 2009). This increase in gene expression may explain why *C. finlandia* is so often found on polluted sites and adapted to extreme conditions of mine tailings. High intraspecific genetic diversity and high metal-regulated gene expression of *T. scalpturatum* and *C. finlandia* are probably the two factors that contributed the most to fungal adaptation to mine tailings and to better seedling health, growth and root water content brought by the two indigenous ECM fungal species on the mine tailings compared to *H. crustuliniforme*, which has low intraspecific genetic diversity (Aanen et al. 2000).

The effect of root colonization rate on seedling nutrition was different from one inoculated ECM fungal species to another. Root P uptake was enhanced and root N and Ca uptake was reduced as the root colonization rate of *H. crustuliniforme* increased. Furthermore, root P uptake was improved and root Ca uptake was declined as the root colonization rate of *T. scalpturatum* increased. Then, root Ca and Fe and foliar K uptake was decreased as the root colonization rate of *C. finlandia* increased. Therefore, the effect is species-specific. The ability of *H. crustuliniforme* and *T. scalpturatum* to provide greater P to the roots of white spruce seedlings may be important under mine tailing conditions where soil P is present, in low concentrations, in relatively insoluble inorganic forms (Smith and Hinckley 1995). Hyphal tissues are known to be normally much more effective than roots at P uptake (Smith and Hinckley 1995). The decline in root N uptake as *H. crustuliniforme* root colonization rate increased could be explain by the fact that high N levels in soil tend to decrease the formation of ectomycorrhiza on roots in many ECM fungal species (Smith and Hinckley 1995). However, under normal N soil conditions, mycorrhizal plants usually take up both ammonium and nitrate faster than non-mycorrhizal plants (Smith and Hinckley 1995). The decline in root Ca and Fe uptake may have been very beneficial to the white spruce seedlings because these two elements were found in very high concentrations in soil and may have become toxic to plants under high uptake conditions.

In this study, health of individual seedlings was negatively correlated with root N, Ca, and Fe content and foliar K, Ca, and Fe contents and positively correlated with root K and foliar P contents. Root Ca content displayed the highest negative relationship with seedling health. Furthermore, aerial growth of individual seedlings was negatively correlated with root

N, Ca, and Fe contents and foliar N, K, Ca, Mg, and Fe contents. Root and foliar Ca and N contents showed the greatest negative correlations with seedling growth on mine tailings. Thus, factors limiting seedling health and growth seem to be more related to element toxicity, especially Ca but also N and Fe, than nutrient deficiency. Tree genetics may have played an important role in this phenomenon of toxicity. Some seedlings had extremely high concentrations of certain elements in their plant tissues. Li et al. (1991) discovered that the efficiency of nitrogen uptake by Loblolly pine seedlings (*Pinus taeda*) was under high degree of genetic control. Furthermore, this toxicity problem may be caused by an imbalance in mineral composition of the tailings affecting negatively seedling development. One may think that N is the limiting factor in these mineral ecosystems because the tailings do not contain any, but it is not the case on this mining site. High pH of the tailings is a favorable environment for the growth and development of many different diazotrophic bacterial species (Brown et al. 1962). Some diazotrophic bacteria may have been already present in the tailings (mine tailings were not sterilized in order to mimic mine field conditions) at the beginning of the experiment and may have brought an important source of N in soil for tree nitrogen nutrition. Phosphorous was the only nutrient that concentration in foliar tissues was negatively correlated with neither seedling growth nor seedling health and was positively correlated with seedling health. Therefore, the addition of a P source on the tailings could be potentially beneficial for both plant growth and health by reducing mineral imbalance of the tailings.

4.6.5 Understanding how microorganisms improved seedling health and growth

The microsymbionts *C. finlandia, T. scalpturatum*, and *A. chroococcum* were remarkably effective in improving white spruce seedling health on waste rocks and fine tailings. *C. finlandia* did it by enhancing seedling (1) foliar N uptake to optimal levels, (2) root water uptake, (3) root K uptake in association with *A. chroococcum* and also by reducing (4) foliar uptake of Fe which was toxic to seedling tissues in high concentrations. Meanwhile, *T. scalpturatum* improved seedling health by increasing seedling (1) root water uptake and by decreasing (2) foliar Fe uptake in association with *A. chroococcum*. These two ECM fungi also promoted seedling health through better root colonization rate which boosted root P uptake and diminished root Ca uptake for *T. scalpturatum* and minimized root Ca, root Fe and foliar K uptake for *C. finlandia*. For the rhizobacteria, *A. chroococcum* enhanced seedling health by improving seedling (1) root water uptake and (2) root K uptake in association with

H. crustuliniforme or *C. finlandia* and by reducing (3) foliar Fe uptake in association with *T. scalpturatum*. *P. putida* was the only symbiont that improved seedling growth. This PGPR isolated from the mining site did it by decreasing considerably seedling foliar uptake of Ca, which was detrimental to seedling plant tissues in high concentrations. We herein found that seedling health and growth was directly linked to the ability of their microsymbionts, ECM fungi and PGPR, to control and improve seedling nutrition and water access.

4.7 Conclusion

Several ECM fungi and PGPR played an essential role in improving white spruce seedling health, growth, and nutrition on biotite-quartz rich waste rocks and fine tailings of Sigma-Lamaque gold mine. The selection of highly efficient and adapted microsymbionts is crucial for the success of mine revegetation programs. ECM fungi and PGPR isolated from roots of healthy white spruce seedlings naturally regenerating on the mining site proved to be more effective in enhancing seedling health and growth, respectively, when planted on mine tailings, than allochthonous species. Health and growth limiting factors are more related to element toxicity, mineral imbalance, and low root water uptake capacity than nutrient deficiency. These limiting factors can be rectified by the use of particular microorganisms. Land reclamation managers should consider inoculating their tree seedlings with specific ECM fungi and PGPR for attaining better seedling health and growth on mine spoils and tailings.

4.8 Acknowledgements

The authors would like to thank the Natural Sciences and Engineering Research Council of Canada (NSERC) for their financial support of this work. The authors would also like to express their sincere gratitude to Gaétan Daigle and Marc Mazerolle for their statistical guidance, Alain Brousseau for conducting seedling nutrition analyses, and Mathieu Boudreau for his assistance. Furthermore, the authors are grateful to Steeve Pépin and Marie Coyea for their advice and assistance related to the equipment used for measuring seedling health and growth. They also thank François Larochelle, Marie-Andrée Paré, André Gagné, Jean-Guy Catford, Aida Azaiez, Marie-Ève Beaulieu, and Laurent Fontaine who contributed in some way to the completion of this study. Last but not least, the authors are thankful to Dr Line

Lapointe (Université Laval) and Dr Suzanne Simard (University of British Columbia) for their useful comments and constructive review of the first draft of this manuscript.

4.9 References

Aanen DK, Kuyper TW, Boekhout T, Hoekstra RF (2000) Phylogenetic relationships in the genus *Hebeloma* based on ITS1 and 2 sequences, with special emphasis on the *Hebeloma crustuliniforme* complex. Mycologia 92: 269-281.

Adeleke RA, Cloete TE, Bertrand A, Khasa DP (2012) Iron ore potential of ectomycorrhizal plants. Mycorrhiza 22: 535-544.

Akhromeiko AI, Shestakova VA (1958) The influence of rhizosphere microorganisms on the uptake and secretion of phosphorus and sulfur by roots of arboreal seedlings. United Nations International Conference 2: 193-199.

Alberton O, Kuyper TW, Summerbell RC (2010) Dark septate root endophytic fungi increase growth of Scots pine seedlings under elevated CO_2 through enhanced nitrogen use efficiency. Plant and Soil 328: 459-470.

Allen MF, Swenson W, Querejeta JI, Egerton-Warburton LM, Treseder KK (2003a) Ecology of mycorrhizae: A conceptual framework for complex interactions among plants and fungi. Annual Review of Phytopathology. 41: 271-303.

Allen EB, Allen MF, Egerton-Warburton L, Corkidi L, Gomez-Pompa A (2003b) Impacts of early- and late-seral mycorrhizae during restoration in seasonal tropical forest, Mexico. Ecological Applications 13: 1701-1717.

Aquilanti L, Favilli F, Clementi F (2004) Comparison of different strategies for isolation and preliminary identification of *Azotobacter* from soil samples. Soil Biology & Biochemistry 36: 1475-1483.

Azaiez A, Nadeau MB, Bertrand A, Khasa DP (2015) In vitro selection of ecologically adapted ectomycorrhizal fungi through production of fungal biomass and metabolites for use in reclamation of gold mine tailings. Unpublished (Chapter 2).

Balogh-Brunstad Z, Keller CK, Dickinson JT, Stevens F, Li CY, Bormann BT (2008) Biotite weathering and nutrient uptake by ectomycorrhizal fungus, *Suillus tomentosus*, in liquid-culture experiments. Geochimica et Cosmochimica Acta 72: 2601-2618.

Baset Mia MA, Shamsuddin ZH (2010) *Rhizobium* as a crop enhancer and biofertilizer for increased cereal production. African Journal of Biotechnology 9: 6001-6009.

Beall F, Tipping B (ed) (1989) Plant growth-promoting rhizobacteria in forestry. Forest Research Community, Toronto, Ontario, Canada.

Berner C, Johansson T, Wallander H (2012) Long-term effect of apatite on ectomycorrhizal growth and community structure. Mycorrhiza: DOI 10.1007/s00572-012-0438-y.

Bjorbaekmo MFM, Carlsen T, Brysting A, Vralstad T, Hoiland K, Ugland KI, Geml J, Schumacher T, Kauserud H (2010) High diversity of root associated fungi in both alpine and arctic *Dryas octopetala*. BMC Plant Biology 10: 1-12.

Blaudez D, Botton B, Chalot M (2000) Effects of heavy metals on nitrogen uptake by *Paxillus involutus* and mycorrhizal birch seedlings. FEMS Microbiology Ecology 33: 61-67.

Bois G, Bigras FJ, Bertrand A, Piché Y, Fung MYP, Khasa DP (2006) Ectomycorrhizal fungi affect the physiological responses of *Picea glauca* and *Pinus banksiana* seedlings exposed to an NaCl gradient. Tree Physiology 26: 1185-1196.

Brown ME, Burlingham SK, Jackson RM (1962) Studies on *Azotobacter* species in soil. Plant and Soil 17: 309-319.

Brown ME, Burlingham SK (1968) Production of plant growth substances by *Azotobacter chroococcum*. Journal of General Microbiology 53: 135-144.

Buée M, Reich M, Murat C, Morin E, Nilsson RH, Uroz S, Martin F (2009) 454 pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. New Phytologist 184: 449-456.

Burd GI, Dixon DG, Glick BR (1998) A plant growth-promoting bacterium that decreases nickel toxicity in seedlings. Applied Environmental Microbiology 64: 3663-3668.

Burns RM (1990) *Pinus banksiana* Lamb. Silvics of North America. Volume 1 Conifers. USDS. Retrieved October 27th 2012.

Cairney JWG (1999) Intraspecific physiological variation: implications for understanding functional diversity in ectomycorrhizal fungi. Mycorrhiza 9: 125-135.

Calvaruso C, Turpault M, Frey-Klett P (2006) Root-associated bacteria contribute to mineral weathering and to mineral nutrition in trees: a budgeting analysis. Applied Environmental Microbiology 72: 1258-1266.

Carriconde F, Gardes M, Jargeat P, Heilmann-Clausen J, Mouhamadou B, Gryta H (2008) Population evidence of cryptic species and geographical structure in the cosmopolitan ectomycorrhizal fungus, *Tricholoma scalpturatum*. Microbial Ecology: DOI 10.1007/s00248-008-9370-2.

Chanway CP (1997) Inoculation of tree roots with plant growth promoting soil bacteria: an emerging technology for reforestation. Forest Science 43: 99-112.

Coleman MD, Bledsoe CS (1989) Pure culture response of ectomycorrhizal fungi to imposed water stress. Canadian Journal of Botany 67: 29-39.

Colpaert JV, Vandenkoornhuyse P, Adriaensen K, Vangronsveld J (2000) Genetic variation and heavy metal tolerance in the ectomycorrhizal basidiomycete *Suillus luteus.* New Phytologist 147: 367-379.

Colpaert JV, Wevers JHL, Krznaric E, Adriaensen K (2011) How metal-tolerant ecotypes of ECM fungi protect plants from heavy metal pollution. Annals of Forest Science 68: 17-24.

Dahlberg A (2001) Community ecology of ectomycorhizal fungi: an advancing interdisciplinary field. New Phytologist 150: 555-562.

Damir O, Mladen P, Bozidar S, Srdan N (2011) Cultivation of the bacterium *Azotobacter chroococcum* for the preparation of biofertilizers. African Journal of Biotechnology 10: 3104-3111.

De Maria S, Rivelli AR, Kuffner M, Sessitsch A, Wenzel WW, Gorfer M, Strauss J, Puschenreiter M (2011) Interactions between accumulation of trace elements and macronutrients in *Salix caprea* after inoculation with rhizosphere microorganisms. Chemosphere 84: 1256-1261.

Dixon RK, Buschena CA (1988) Response of ectomycorrhizal *Pinus banksiana* and *Picea glauca* to heavy metals in soil. Plant and Soil 105: 265-271.

Douhan GW, Vincenot L, Gryta H, Selosse M-A (2011) Population genetics of ectomycorrhizal fungi: from current knowledge to emerging directions. Fungal Biology 115: 569-597.

Fares CN (1997) Growth and yield of wheat plant as affected by biofertilisation with associative, symbiontic N_2-fixers and endomycorrhizae in the presence of the different P-fertilizers. Annals of Agriculture Science 42: 51-60.

Gagné A, Jany J, Bousquet J, Khasa DP (2006) Ectomycorrhizal fungal communities of nursery-inoculated seedlings outplanted on clear-cut sites in northern Alberta. Canadian Journal of Botany 36: 1684-1694.

Glick BR, Liu C, Ghosh S, Dumbroff EB (1997) Early development of canola seedlings in the presence of the plant growth-promoting rhizobacterium *Pseudomonas putida* GR12-2. Soil Biology & Biochemistry 29: 1233-1239.

Gogala N (1991) Regulation of mycorrhizal infection by hormonal factors produced by hosts and fungi. Experientia 47: 331-340.

Gorfer M, Persak H, Berger H, Brynda S, Bandian D, Strauss J (2009) Identification of heavy metal regulated genes from the root associated ascomycete *Cadophora finlandica* using a genomic microarray. Mycological Research 113: 1377-1388.

Gryta H, Carriconde F, Charcosset, Jargeat P, Gardes M (2006) Population dynamics of the ectomycorrhizal fungal species *Tricholoma populinum* and *Tricholoma scalpturatum* associated with black poplar under differing environmental conditions. Environmental Microbiology 8: 773-786.

Hayat R, Ali S, Amara U, Khalid R, Ahmed I (2010) Soil beneficial bacteria and their role in plant growth promotion: a review. Annals of Microbiology 60: 579-598.

Hobbie EA, Macko SA, Shugart HH (1998) Patterns in N dynamics and N isotopes during primary succession in glacier bay, Alaska. Chemical Geology 152: 3-11.

Hoffland E, Kuyper TW, Wallander H, Plassard C, Gorbushina AA, Haselwandter K, Holmstrom S, Landeweert R, Lundstrom US, Rosling A, Sen R, Smits MM, Van Hees PAW, Van Breemen N (2004) The role of fungi in weathering. Frontiers in Ecology and the Environment 2: 258-264.

Humphry DR, Andrews M, Santos SR, James EK, Vinogradova LV, Perin L, Reis VM, Cummings SP (2007) Phylogenetic assignment and mechanism of action of a crop growth promoting *Rhizobium radiobacter* strain used as a biofertiliser on graminaceous crops in Russia. Antonie Van Leeuwenhoek 91: 105-113.

Jumpponen A, Trappe JM, Cazares E (2002) Occurrence of ectomycorrhizal fungi on the forefront of retreating Lyman Glacier (Washington, USA) in relation to time since deglaciation. Mycorrhiza 12: 43-49.

Kalra YP. 1998. Handbook of reference methods for plant analysis. Boca Raton (FL): CRC Press.

Kanvinde L, Sastry GRK (1990) Agrobacterium tumefaciens is a diazotrophic bacterium. Applied Environmental Microbiology 56: 2087: 2092.

Kernaghan G, Sigler L, Khasa DP (2003) Mycorrhizal and root endophytic fungi of containerized *Picea glauca* seedling assessed by rDNA sequence analysis. Mycrobial ecology 45: 128-136.

Khan AG (2006) Mycorhizoremediation – an enhanced form of phytoremediation. Journal of Zhejiang University SIENCE B 7: 503-514.

Krpata D, Peintner U, Lancer I, Fitz WJ, Schweiger P (2008) Ectomycorrhizal communities associated with *Populus tremula* growing on heavy metal contaminated site. Mycological Research 112: 1069-1079.

Lamhamedi MS, Fortin JA (1991). Genetic variations of ectomycorrhizal fungi: extrametrical phase of *Pisolithus sp.* Canadian Journal of Botany 69: 1927-1934.

Landeweert R, Hoffland E, Finlay RD, Kuyper TW, Van Bremen N (2001) Linking plants to rocks: ectomycorrhizal fungi mobilize nutrients from minerals. TRENDS in Ecology & Evolution 16: 248-254.

Leyval C, Berthelin J (1993) Rhizodeposition and net release of soluble organic compounds by pine and beech seedlings inoculated with rhizobacteria and ectomycorrhizal fungi. Biology and Fertility of Soils 15: 259-267.

Leyval C, Turnau K, Haselwandter K (1997) Effect of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological, and applied aspects. Mycorrhiza 7: 139-153.

Li B, McKeand SE, HL Allen (1991) Genetic variation in nitrogen use in efficiency of loblolly pine seedlings. Forest Science 37: 613-626.

Lifshitz R, Kloepper JW, Kozlowski M, Simonson C, Carlson J, Tipping EM, Zaleska I (1987) Growth promotion of canola seedling by a stain of *Pseudomonas putida* under gnotobiotic conditions. Canadian Journal of Microbiology 33: 390-395.

Marshall VG (2000) Impacts of forest harvesting on biological processes in northern forest soils. Forest Ecology and Management 133: 43-60.

Meyer JR, Linderman RG (1986) Response of subterranean clover to dual inoculation with vesicular-arbuscular mycorrhizal fungi and a plant growth-promoting bacterium, *Pseudomonas putida*. Soil Biology & Biochemistry 18: 185-190.

Mitchell DT, Gibson BR (2006) Ericoid mycorrhizal association: ability to adapt to a broad range of habitats. Mycologist 20: 2-9.

Modak JM, Vasan SS, Natarajan KA (2001) Calcium removal from bauxite using *Paenibacillus polymyxa*. In: Kawatra SK, Natarajan KA (eds) Mineral biotechnology: microbial aspects of mineral beneficiation, metal extraction, and environmental control. Society for Mining, Metallurgy, and Exploration, USA, pp 13-25.

Mrnka L, Tokarova H, Vosatka M, Matejka P (2009) Interaction of soil filamentous fungi affects needle composition and nutrition of Norway spruce seedlings. Trees 23: 887-897.

Mushin TM, Zwiazek JJ (2002) Colonization with *Hebeloma crustuliniforme* increases water conductance and limits shoot sodium uptake in white spruce (*Picea glauca*) seedlings. Plant and Soil 238: 217-225.

Nadeau MB, Khasa DP (2015) Edaphic selection pressures as drivers of contrasting white spruce ectomycorrhizal fungal diversity and community structure in the Canadian boreal forest of Abitibi-Temiscamingue region. Unpublished (Chapter 1).

Nienstaedt H, Zasada JC (1990) *Picea glauca* (Moench) Voss. Silvics of North America, Volume 1:Conifers. United States Forest Service. Retrieved October 27th 2012.

O'Neill GA, Radley RA, Chanway CP (1992) Variable effects of emergence promoting rhizobacteria on conifer seedling growth under nursery conditions. Biology and Fertility of Soils 13: 45-49.

Onwuchekwa NE, Zwiazek JJ, Quoreshi A, Khasa DP (2014) Growth of mycorrhizal jack pine (*Pinus banksiana*) and white spruce (*Picea glauca*) seedlings planted in oil sands reclaimed areas. Mycorrhiza: DOI 10.1007/s00572-014-0555-x.

Pandey RK, Bahl RK, Rao PRT (1986) Growth stimulating effects of nitrogen fixing bacteria (biofertiliser) on oak seedlings. Industrial Forestry 112: 75-79.

Peterson RL, Wagg C, Pautler M (2008) Associations between microfungal endophytes and roots: do structural features indicate function? Botany 86: 445-456.

Quoreshi AM, Khasa DP (2008) Effectiveness of mycorrhizal inoculation in the nursery on root colonization, growth, and nutrient uptake of aspen and balsam poplar. Biomass and Bioenergy 32: 381-391.

Quoreshi AM, Roy S, Greer CW, Beaudin J (2007) Inoculation of green alder (*Alnus crispa*) with *Frankia*-ectomycorrhizal fungal inoculant under commercial nursery production conditions. Native Plants Journal 8: 271-281.

Radwan FI (1998) Response of some maize cultivars to VA-mycorrhizal inoculation, biofertilization and soil nitrogen application. Alexandria Journal of Agricultural Research 43: 43-56.

Rajkumar M, Ae N, Freitas H (2009) Endophytic bacteria and their potential to enhance heavy metal phytoextraction. Chemosphere 77: 153-160.

Roy S, Khasa DP, Greer CW (2007) Combining alders, frankiae, and mycorrhizae for the revegetation and remediation of contaminated ecosystems. Canadian Journal of Botany 85: 237-251.

Rygiewicz PT, Bledsoe CS, Zasoski RJ (1984) Effects of ectomycorrhizae and solution pH on ammonium uptake by coniferous seedlings. Canadian Journal of Forest Research 14: 885-892.

Rygiewicz PT, Andersen CP (1994) Mycorrhizae alter quality and quantity of carbon allocated belowground. Nature 369: 58-60.

SAS Institute Inc. 2012. SAS 9.3 software, SAS Institute, North Carolina, USA.

Smith WK, Hinckley TM (1995) Resource physiology of conifers: acquisition, allocation and utilization. Academic Press Inc, San Diego, California, USA.

Smith SE, & Read DJ (2008) Mycorrhizal symbiosis, 3rd ed. Academic Press, London, UK.

Smits MM, Bonneville S, Benning LG, Banwart SA, Leake JR (2012) Plant-driven weathering of apatite – the role of an ectomycorrhizal fungus. Geobiology 10: 445-456.

Sutton RF (1973) Histoire naturelle de l'Épinette blanche (*Picea glauca* (Moench) Voss). Ministère de l'Environnement, Service Canadien des Forêts, Publication # 1250f, Ottawa.

Taner MF, Trudel P, Perrault G (1986) Géochimie de la biotite associée à certains gisements d'or de Val d'Or, Malartic et Chibougamau, Québec. Canadian Mineralogist 24: 761-774.

Tripathy PP, Ayyappan S (2005) Evaluation of *Azotobacter* and *Azospirillum* as biofertilizers in aquaculture. World Journal of Microbiology and Biotechnology 21: 1339-1343.

Uroz S, Calvaruso C, Turpault MP, Frey-Klett P (2009) Mineral weathering by bacteria: ecology, actors, and mechanisms. TRENDS in Microbiology 17: 378-387.

Van den Driessche R (1991) Mineral nutrition of conifer seedlings. CRC Press Inc., Bota Raton, Florida, USA.

Villeneuve N, Grandtner MM, Fortin JA (1989) Frequency and diversity of ectomycorrhizal and saprophytic macrofungi in the Laurentide mountains of Quebec. Canadian Journal of Botany 67: 2616-2629.

Vralstad T (2004) Are ericoid and ectomycorrhizal fungi part of a common guild? New phytologist 164: 7-10.

Wallander H, Hagerberg D (2004) Do ectomycorrhizal fungi have significant role in weathering of minerals in forest soils? Symbiosis 37: 249-252.

Weyens N, Van der Lelie D, Taghavi S, Vangronsveld J (2009a) Phytoremediation: plant-endophyte partnerships take the challenge. Current Opinion in Biotechnology 20: 248-254.

Weyens N, Van der Lelie D, Taghavi S, Newman L, Vangronsveld J (2009b) Exploiting plant-microbe partnerships to improve biomass production and remediation. TRENDS in Biotechnology 27: 591-598.

Wu SC, Cao ZH, Li ZG, Cheung KC, Wong MH (2005) Effects of biofertilizer containing N-fizer, P and K solubilizers and AM fungi on maize growth : a greenhouse trial. Geoderma 125: 155-166.

Xing Y, Yang L, Huang S, Li Y (2006) Identification of a new nitrogen fixing endo-bacterium strain isolated from sugarcane stalk. Sugar Tech 8: 49-53.

Yi H, Polanco MC, Mackinnon MD, Zwiazek JJ (2008) Responses of ectomycorrhizal *Populus tremuloides* and *Betula papyrifera* seedlings to salinity. Environmental and Experimental Botany 62: 357-363.

5. General conclusion and future prospects

This research project demonstrated very well the ability of specific ectomycorrhizal (ECM) fungi and plant growth promoting rhizobacteria (PGPR) to promote the health, growth, and/or nutrition of white spruce seedlings (*Picea glauca*) on waste rocks and fine tailings of Sigma-Lamaque gold mine in the Abitibi region of Canada. In the next few years, *in situ* field testing will be conducted on Sigma-Lamaque mine with our most efficient tree-microsymbiont associations in order to validate *in vitro* and *in vivo* results recorded from the community, laboratory, and glasshouse experiments. This project opens the avenue to new interesting research possibilities. In this general conclusion section, a summary of the results will be laid out, scientific and industrial significance of the results will be discussed and future studies in the field of phytoremediation and mine revegetation will be recommended.

5.1 Summary of the results

In the ECM fungal community study, 41 ECM fungal species from the phyla Basidiomycota and Ascomycota were found to be associated with white spruce seedling within the four sites sampled: Trecesson nursery, mining site, forest edge, and natural forest. Genetic divergence of the ECM fungal communities was greater on the mining site compared to the other three sites. These results were quite surprising. The adaptation of ECM fungi to mining site conditions may be more related to high genetic variability within species than phylogenetic group adaptation and evolution. High genetic variability within ECM fungal species allows individual strains, adapted to site conditions, to colonize the highly disturbed mine ecosystem (Jumpponen *et al.*, 2002). Species richness and diversity were at the highest in forest edge, followed by natural forest, then mining site, and finally at the lowest in Trecesson nursery. Nevertheless, species richness and diversity of the mining site was still relatively high. Furthermore, the ECM fungal community composition of the mining site differed significantly from the other three sites. Only one ECM fungal generalist species (*Amphinema byssoides*) was found to thrive in all four sites. Through the analyses of linear patterns, it was discovered that soil pH and root mycorrhization rate increased whereas C/N ratio and soil concentration of available K, Mg, P, Al, C, Fe, N, Ca, and Na decreased across the following ecological niche (gradient): nursery → natural forest → forest edge → mining site. Specific groups of ECM fungi were found to inhabit and thrive together in each of the

four ecosystems sampled. Species colonizing the mining site are adapted to high pH, low soil chemical fertility, and low organic matter content. Edaphic selection pressures are drivers of ECM fungal community structure and diverstity in the boreal forest.

In the *in vitro* laboratory study, *Tricholoma scalpturatum*, *Hebeloma crustuliniforme*, and *Cadophora finlandia* exhibited the greatest radial growth on poor solid medium with tailings. Two of them (*T. scalpturatum* and *C. finlandia*) were isolated directly from roots of healthy white spruce seedlings naturally regenerating on the mining site. Three out of four ECM fungal species originating from natural forest (*Paxillus involutus, Lactarius aurantiosordidus*, and *Cenococcum geophilum*) showed no radial growth or not much on poor solid medium with tailings. Accordingly, they were not adapted to the mine tailings. On poor liquid medium with tailings, *C. finlandia* had the highest fungal growth in biomass (ergosterol content), followed by *T. scalpturatum*, and then *H. crustuliniforme* with the lowest. *C. finlandia* grew well in all-sized particles including large and small ones. *T. scalpturatum* grew well only in smaller particle sizes. These results are compatible with what we expected. *T. scalpturatum* was isolated from healthy white spruce seedlings naturally regenerating on the fine tailings, so it was adapted to small tailing particles. On the other hand, *C. finlandia* was isolated on waste rock piles and adapted to a wide range of mine tailing particle sizes. *H. crustuliniforme* hardly grew in all four particle sizes. The method of radial growth on solid medium was excellent for identifying species and strains that did not tolerate growing inside tailings and this method could be used as a cheap first screening step towards selecting ECM fungi. Furthermore, the method of ergosterol content on solid medium was inappropriate for measuring fungal growth in the tailings. Fungal species reacted differently to the presence of tailings towards ergosterol production perhaps due to high pH. More research needs to be done with this method in order to understand precisely why there are different ergosterol production trends among ECM fungal species. The method of ergosterol content in liquid medium is the most interesting method to be used for measuring fungal biomass in future studies. There was no correlation between ergosterol synthesis and organic acid production. Therefore, organic acids should not be used as an estimate of fungal growth. Finally, the three ECM fungi *C. finlandia*, *T. scalpturatum*, and *H. crustuliniforme* produced citric, malonic, and/or succinic acids in order to solubilize nutrients elements in the tailings for their growth and nutrition.

In the *in vivo* glasshouse experiment, white spruce seedling survival, root water content, needle, stem, and root dry biomass, stem length, total root length, and root volume was greater on waste rocks than fine tailings. Furthermore, percentage of unhealthy dark red foliage was smaller on waste rocks compared to fine tailings. Overall, seedling health and growth was better on waste rocks than fine tailings due to reduced root Ca, foliar Ca, foliar Fe, and root Mg toxicity. For microbial treatments, *C. finlandia* improved seedling health by increasing foliar N uptake to optimal levels, root water uptake, and root K uptake (in association with *Azotobacter chroococcum*) and by decreasing foliar Fe toxicity. *T. scalpturatum* improved seedling health by increasing root water uptake and by decreasing foliar Fe toxicity (in association with *A. chroococcum*). *A. chroococcum* enhanced seedling health by increasing root water uptake and root K (in association with *H. crustuliniforme* or *C. finlandia*) and by decreasing foliar Fe toxicity (in association with *T. scalpturatum*). *Pseudomonas putida* was the only microsymbiont that improved seedling growth and it did it by decreasing foliar uptake of Ca, which was detrimental to seedlings due to high concentrations in tissues. White spruce seedling health and growth was directly linked to the ability of ECM fungi and PGPR to control and improve seedling nutrition and water access. Mine-native ECM fungi and PGPR proved to be more efficient in promoting white spruce seedling health and growth, respectively, on mine spoils and tailings than exotic species.

5.2 Scientific and industrial significance of the research

In this research project, white spruce seedling health on mine tailings was increasing with increasing root water uptake, root K content, and foliar P content and with decreasing root N, Ca, and Fe and foliar K and Ca contents (Fig. 5.1). Furthermore, seedling aerial growth was increasing with increasing seedling health and root water uptake and with decreasing root N, Ca, and Fe and foliar N, K, Ca, Mg, and Fe contents (Fig. 5.2). Therefore, high concentrations of Ca, Fe, N, K, and Mg (especially Ca, Fe, and N) in seedling tissues were toxic and detrimental to seedling health and/or growth. Seedling health and growth was more correlated with element toxicity than nutrient deficiency.

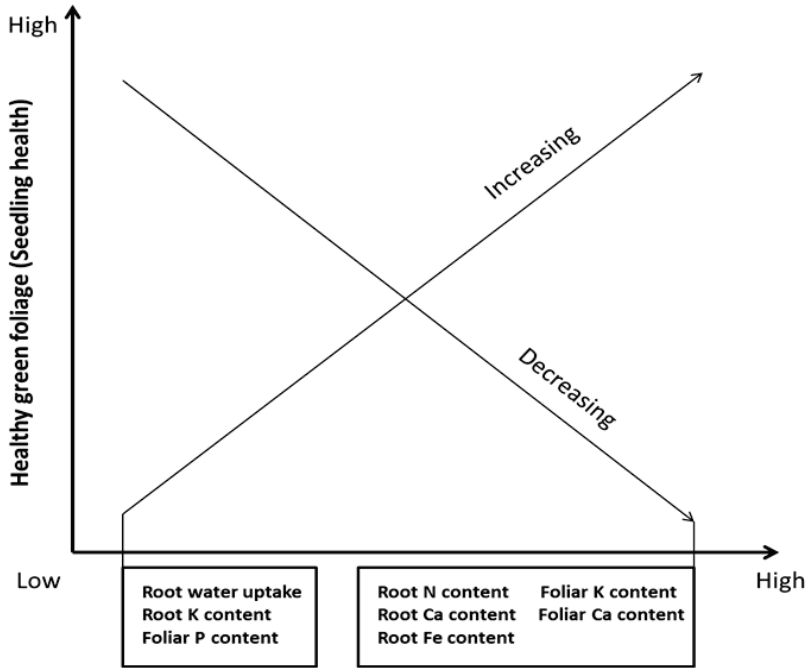

Figure 5.11: Effects of root water uptake, root N, K, Ca, and Fe contents, foliar P, K, and Ca contents on the health of white spruce seedlings planted on mine tailings.

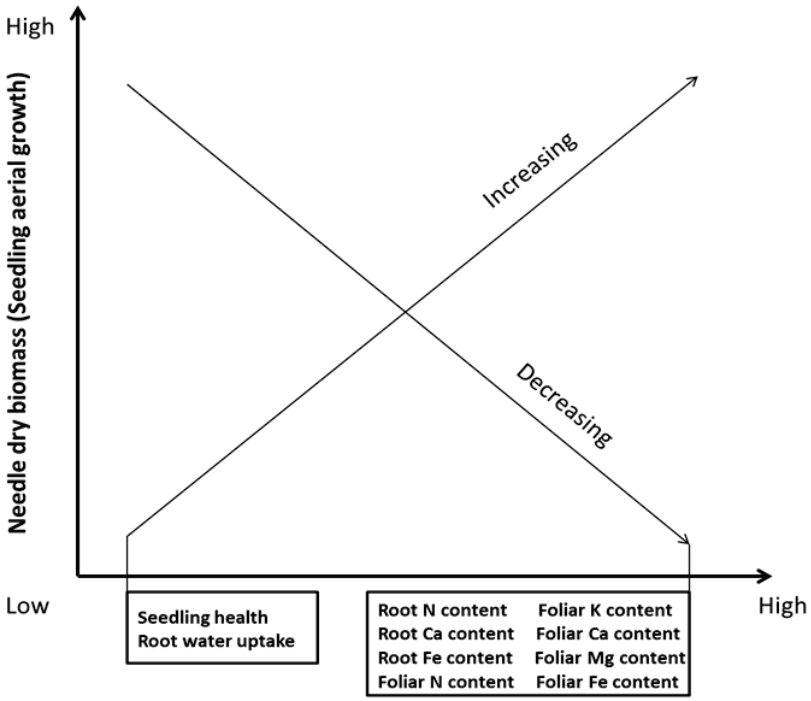

Figure 5.12: Effects of seedling health, root water uptake, root N, Ca, and Fe contents, and foliar N, K, Ca, Mg, and Fe contents on the aerial growth of white spruce seedlings planted on mine tailings.

In the first study, analyses of soil chemical fertility were performed and demonstrated that all exchangeable nutritional elements were present in soil of the mining site in very low concentrations compared to natural forest stands. Why there were so much toxicity and high concentrations of many nutritional elements in our white spruce seedlings? Soil chemical analyses do not consider mineral elements stored in the tailings but only the exchangeable ones directly available to plants. Analyses of rock mineral composition showed phosphorous as the limiting factors and the macronutrients with the lowest concentration in the rock tailings (data not shown). Same analyses showed high concentrations of other elements such as Ca and Fe in Sigma-Lamaque rock (data not shown). These analyses of tailing mineral composition were directly linked to white spruce seedling nutrition. Phosphorous was also the limiting factor and Ca and Fe were present in foliar tissues in concentrations three to four times higher than normal. These findings suggest that analyses of rock mineral composition are crucial for determining rock fertility, toxicity, and limiting factors which affect plant nutrition needs in mineral soils. Element toxicity to seedlings may be triggered by a mineral imbalance between concentrations of some nutritional elements in mine tailings. Adding a phosphorous source could potentially reduce this imbalance. However, this source should not contain much of the elements (especially Ca and Fe) already present in high concentrations in Sigma-Lamaque rock because it could potentially further favour imbalance.

Specific mine-adapted microsymbionts – ECM fungi and PGPR – are capable of improving considerably white spruce seedling health and growth by regulating seedling nutrition and by increasing root water uptake on mine tailings which has often low water retention capacity (Conesa *et al.*, 2010). Therefore, these specific microorganisms play a huge role in allowing white spruce to recolonize rocky soil of the mining site. Land reclamation managers should consider using them for mine revegetation purposes. These fungi and bacteria could potentially improve considerably the success of revegetation programs while possibly reducing the cost of restoration activities by minimizing the need to apply large quantities of organic amendments on site. Strong mycorrhization rate is also very important for enhancing the positive effect of ECM fungi to plants and inoculation should be done accurately. For example, in the glasshouse study, the percentage of roots colonized by *C. finlandia* was positively correlated to seedling health, growth, and root water content whereas it was negatively correlated with root Ca and Fe and foliar K toxicities (Fig. 5.3). Another

example is the increase in seedling root P uptake following greater root colonization by *H. crutuliniforme* and *T. scalpturatum*.

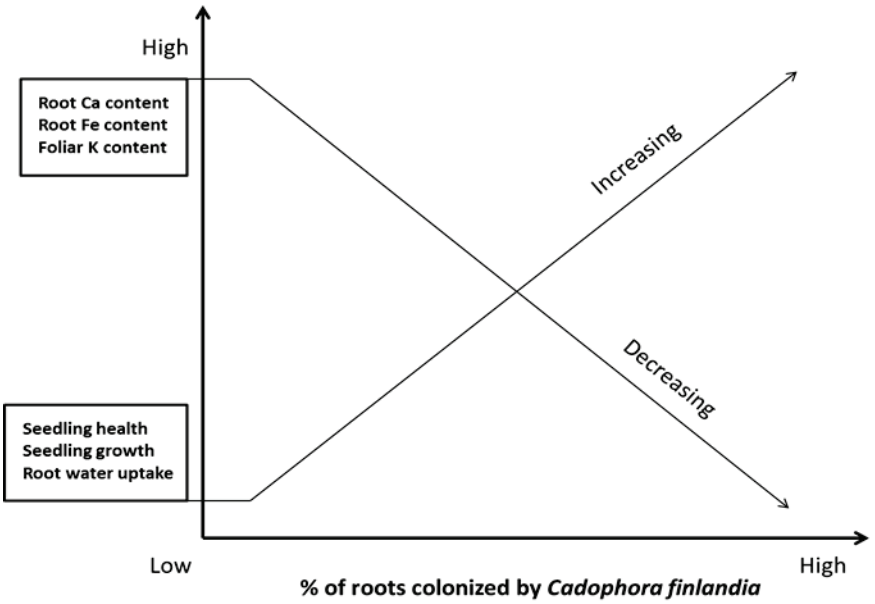

Figure 5.13: Effects of increasing percentage of roots colonized by *Cadophora finlandia* on white spruce seedling health, seedling growth, root water uptake, root Ca content, root Fe content, and foliar K content.

As we are currently implementing field testing on Sigma-Lamaque gold mine, it will be interesting to see if *in situ* field results will confirm the relevance of our previous *in vitro* and *in vivo* studies. If field results are positively conclusive, we will propose a new green technology for the revegetation of old mining sites using specific microsymbionts associated with their tree or shrub hosts. The technology involves (1) root sampling of perfectly healthy seedlings naturally regenerating on the mining site, (2) isolation of symbiotic microorganisms, ECM fungi and PGPR, from the collected roots, under aseptic laboratory conditions, (3) *in vitro* growth and selection of promising microorganisms on grinded mine tailings, under axenic conditions, (4) *in vivo* growth and selection of best tree-symbiont combinations on waste rocks and fine tailings, under glasshouse conditions, (5) field testing of the best tree-symbiont associations on tailings of the mining site, and finally (6) seedling and inoculum production for large-scale operational mine revegetation programs (Fig. 5.4). This is a very promising technology in the domain of land reclamation and ecological restoration and further research is warranted for reinforcing it, especially for yielding greater seedling growth. In order to reach this goal, problem of soil element imbalance has to be resolved.

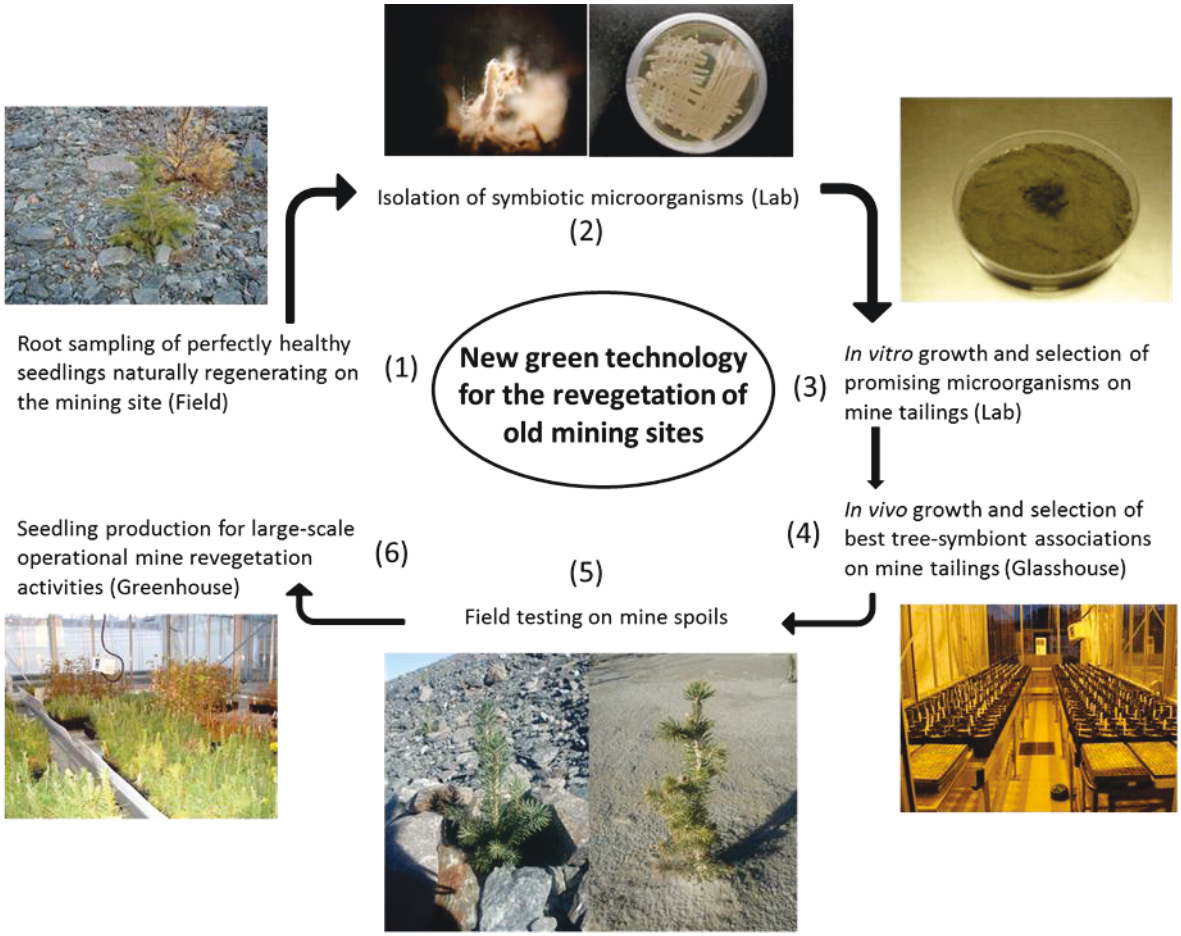

Figure 5.14: Diagram showing the proposed new green technology development for the revegetation of old mining sites.

5.3 Future studies

This research program focused on the revegetation of old abandoned mining site where scarce natural regeneration has already started to slowly recolonize the site. However, what should we do with human disturbed mining sites without natural regeneration? Here, we propose an interesting research program that could potentially lead to the development of a promising technology for the revegetation of newly-formed disturbed mining sites. This new program would focus on genetics and would involve (1) sampling of fructifications and spores of ECM fungi in natural forest stands, (2) *in vitro* germination and selection of haploid spores under environmental stressors (e.g., grinded mine tailings), (3) mating and reconstitution of dicaryons (diploids) from selected monocarons (haploid spores), under aseptic laboratory conditions, (4) *in vitro* growth and selection of promising and highly efficient diploid strains on grinded mine tailings, under axenic conditions, (5) *in vivo* growth and selection of best

tree-symbiont combinations on waste rocks and fine tailings under glasshouse conditions, and finally (6) field testing on mine spoils (Fig. 5.5). Furthermore, in the same program, different clones of the same tree species could be tested under glasshouse conditions for their ability to grow and thrive on mine tailings in order to see if tree genetics has an effect on seedling health, growth, and nutrition on mine tailings (Fig. 5.5).

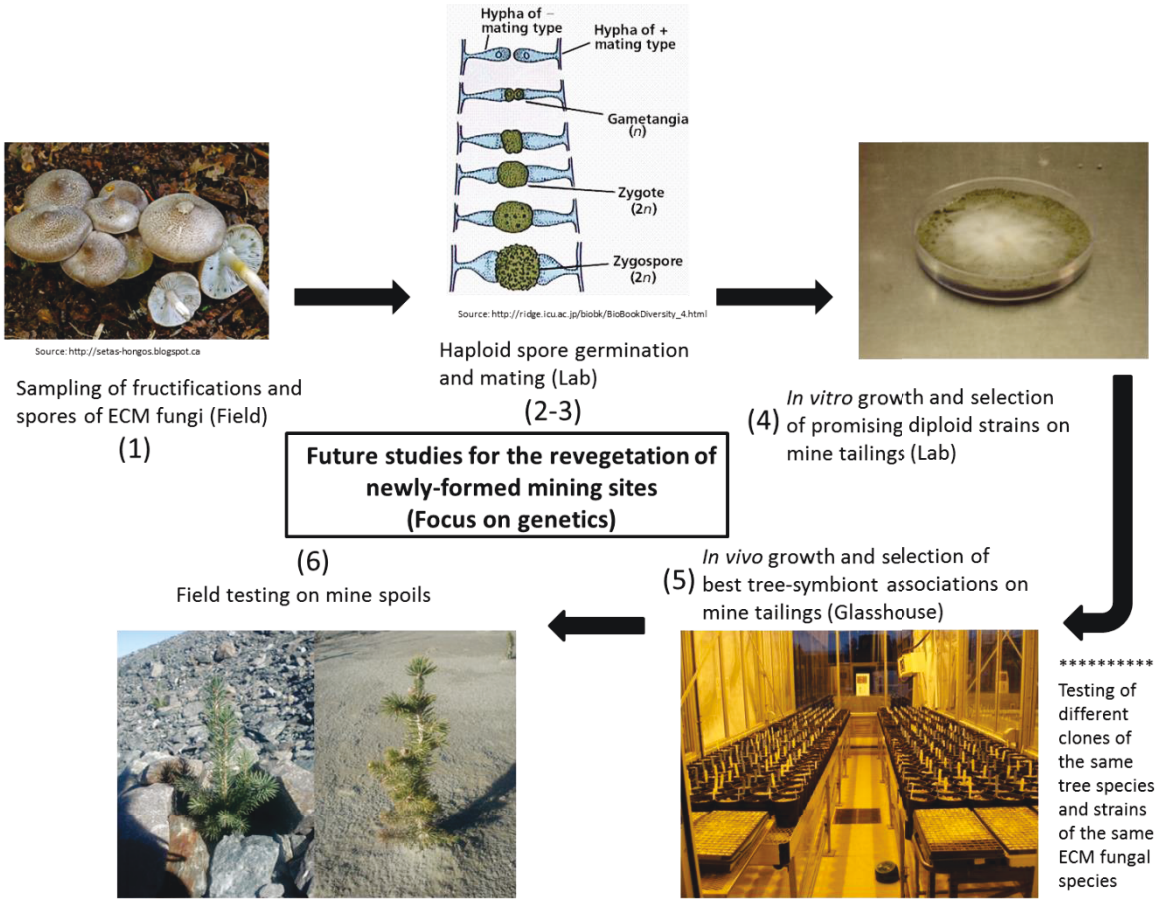

Figure 5.15: Diagram showing proposed future studies for the revegetation of newly-disturbed mining sites on which no tree has naturally regenerated the tailings yet.

As a young professional in the domain of ecological restoration, I suspect that tree genetics plays a very important role in their ability to colonize newly-formed rocky ecosystems following mining activities. Future studies should also focus on the identification of genes more expressed in tree seedlings highly efficient in phytoremediation. The program would consist of (1) planting few hundred seedlings on the mining site or sampling old orphan sites, (2) sampling foliar tissues of the seedlings after two or three growing seasons, (3) identifying genes more expressed in the healthiest and most productive seedlings in

laboratory, and finally (4) developing markers for the identification of promising seedlings in land reclamation. *Picea glauca* and *Populus* sp. are interesting tree species that could be studied in this program because their genome is relatively well known (Birol *et al.*, 2013; Tuskan *et al.*, 2006). As well, the genome of the model species *Laccaria bicolor* is also well known (Martin *et al.*, 2008). If researchers would successfully develop the markers, fungi and seedlings highly efficient in phytoremediation could be identified in the petri dish or at the nursery stages and used for producing biofortified seedlings to be outplanted on mining sites. Therefore, development of functional ecogenomic tools is important to accelerate the breeding programs of both tree and fungal partners.

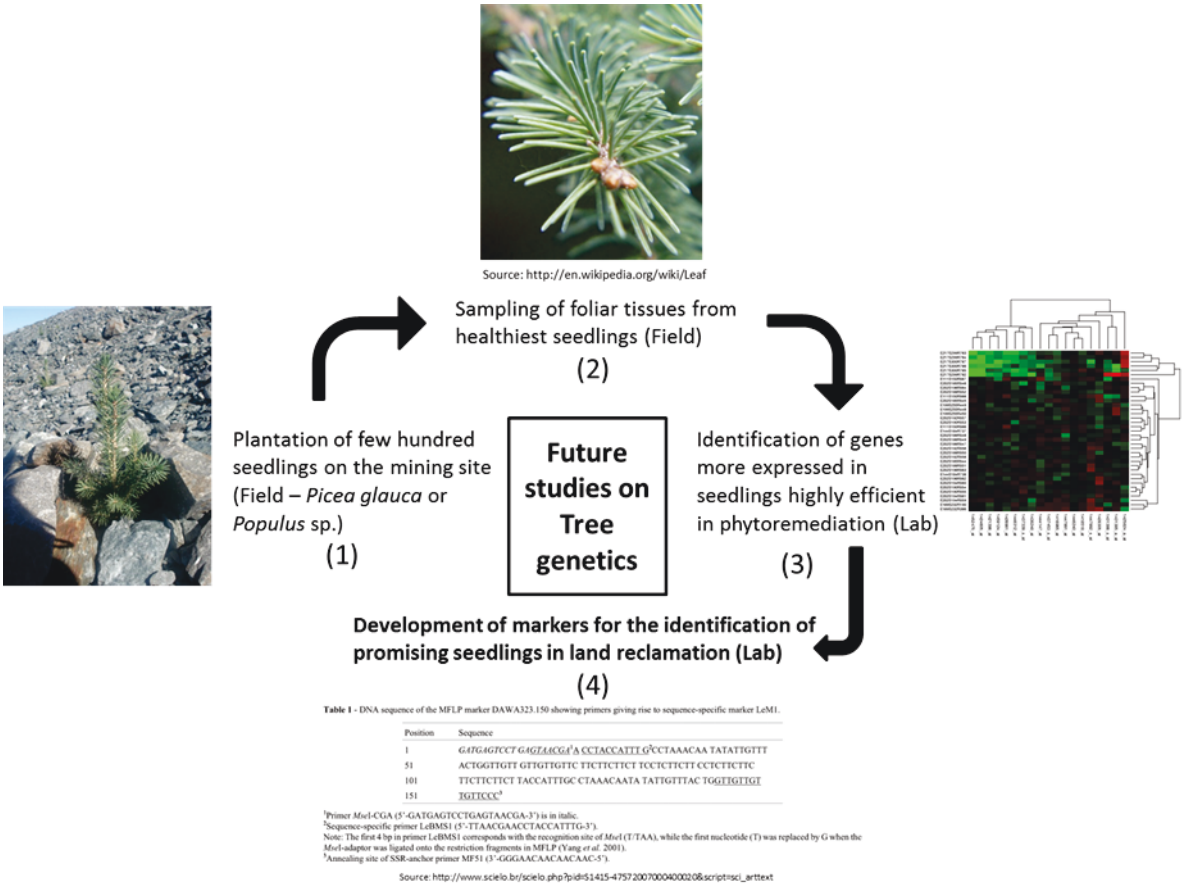

Figure 5.16: Diagram showing proposed future studies on tree genetics for the development of markers that could potentially be used for the selection of seedlings genetically adapted for phytoremediation.

5.4 References

Birol, I., A. Raymond, S.D. Jackman, S. Pleasance, R. Coope, G.A. Taylor, M.M. Yuen, C.I. Keeling, D. Brand, B.P. Vandervalk, H. Kirk, P. Pandoh, R.A. Moore, Y. Zhao, A.J. Mungall, B. Jaquish, A. Yanchuk, C. Ritland, B. Boyle, J. Bousquet, K. Ritland, J. Mackay, J. Bohlmann, and S.J. Jones. (2013). Assembling the 20 Gb white spruce (*Picea glauca*) genome from whole-genome shotgun sequencing data. *Bioinformatics 29*(12): 1492-1497.

Conesa, H.M., R. Schulin, and B. Nowack. (2010). Suitability of using diffusive gradients in thin films (DGT) to study metal bioavailability in mine tailings: possibilities and constraints. *Environmental Science and Pollution Research 17*(3): 657-664.

Jumpponen, A., J.M. Trappe, and E. Cazares. (2002). Occurrence of ectomycorrhizal fungi on the forefront of retreating Lyman Glacier (Washington, USA) in relation to time since deglaciation. *Mycorrhiza 12*: 43-49.

Martin, F., A. Aerts, D. Ahrén, A. Brun, E.G. Danchin, F. Duchaussoy, J. Gibon, A. Kohler, E. Lindquist, V. Pereda, A. Salamov, H.J. Shapiro, J. Wuyts, D. Blaudez, M. Buée, P. Brokstein, B. Canback, D. Cohen, P.E. Courty, P.M. Coutinho, C. Delaruelle, J.C. Detter, A. Deveau, S. Difazio, S. Duplessis, L. Fraissinet-Tachet, E. Lucic, P. Frey-Klett, C. Fourrey, I. Feussner, G. Gay, J. Grimwood, P.J. Hoegger, P. Jain, S. Kilaru, J. Labbé, Y.C. Lin, V. Legué, F. Le Tacon, R. Marmeisse, D. Melayah, B. Montanini, M. Muratet, U. Nehls, H. Niculita-Hirzel, M.P. Oudot-Le Secq, M. Peter, H. Quesneville, B. Rajashekar, M. Reich, N. Rouhier, J. Schmutz, T. Yin, M. Chalot, B. Henrissat, U. Kües, S. Lucas, Y. VandePeer, G.K. Podila, A. Polle, P.J. Pukkila, P.M. Richardson, P. Rouzé, I.R. Sanders, J.E. Stajich, A. Tunlid, G. Tuskan, and I.V. Grigoriev. (2008). The genome of *Laccaria bicolour* provides insights into mycorrhizal symbiosis. *Nature 452*(7183): 88-92.

Tuskan, G.A., S. Difazio, S. Jansson, J. Bohlmann, I. Grigoriev, U. Hellsten, N. Putman, S. Ralph, S. Rombauts, A. Salamov, J. Schein, L. Sterck, Aaerts, R.R. Bhalerao, R.P. Bhaler, D. Blaudez, W. Boerjan, A. Brun, A. Brunner, V. Busov, M. Campbell, J. Carlson, M. Chalot, J. Chapman, G.-L. Chen, D. Cooper, P.M. Coutinho, J. Couturier, S. Covert, Q. Cronk, R. Cunningham, J. Davis, S. Degroeve, A. Déjardin, C. dePamphilis, J. Detter, B. Dirks, I. Dubchak, S. Duplessis, J. Ehlting, B. Ellis, K. Gendler, D. Goodstein, M. Gribskov, J. Grimwood, A. Groover, L. Gunter, B. Hamberger, B. Heinze, Y. Helariutta, B. Henrissat, D. Holligan, R. Holt, W. Huang, N. Islam-Faridi, S. Jones, M. Jones-Rhoades, R. Jorgensen, C. Joshi, J. Kangasjarvi, J. Karlsson, C. Kelleher, R. Kirkpatrick, M. Kirst, A. Kohler, U. Kalluri, F. Larimer, J. Leebens-Mack, J.-C. Leplé, P. Locascio, Y. Lou, S. Lucas, F. Martin, B. Montanini, C. Napoli, D.R. Nelson, C. Nelson, K. Niemine, O. Nilsson, V. Pereda, G. Peter, R. Philippe, G. Pilate, A. Poliakov, J. Razumovskaya, P. Richardson, C. Rinaldi, K. Ritland, P. Rouzé, D. Ryaboy, J. Schmutz, J. Schrader, B. Segerman, H. Shin, A. Siddiqui, F. Sterky, A. Terry, C.-J. Tsai, E. Uberbacher, P. Unneberg, J. Vahala, K. Wall, S. Wessler, G. Yang, T. Yin, C. Douglas, M. Marra, G. Sandberg, Y. Van de Peer, and D. Rokhsar. (2006). The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science 313*(5793): 1596-1604.

APPENDIX

Appendix I: Localization of the ECM fungal community field study

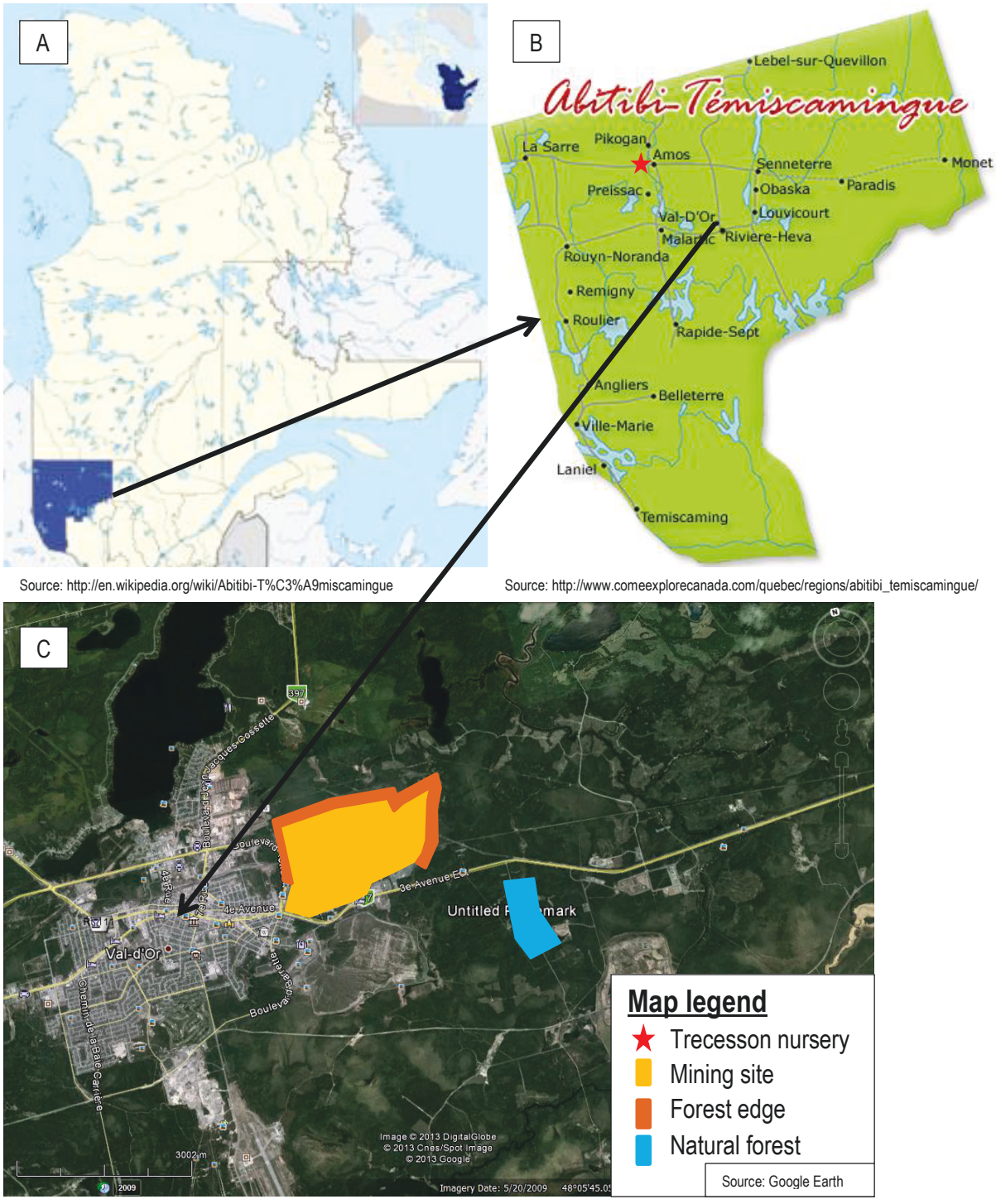

Figure A.1: Localization of the four sampling sites (Trecesson nursery, mining site, forest edge, and natural forest) of the ECM fungal community study: (A) map of the province of Quebec, (B) map of the Abitibi region, and (C) map of Val d'Or.

Appendix II: Pictures of the four sampling sites of the ECM fungal community field study

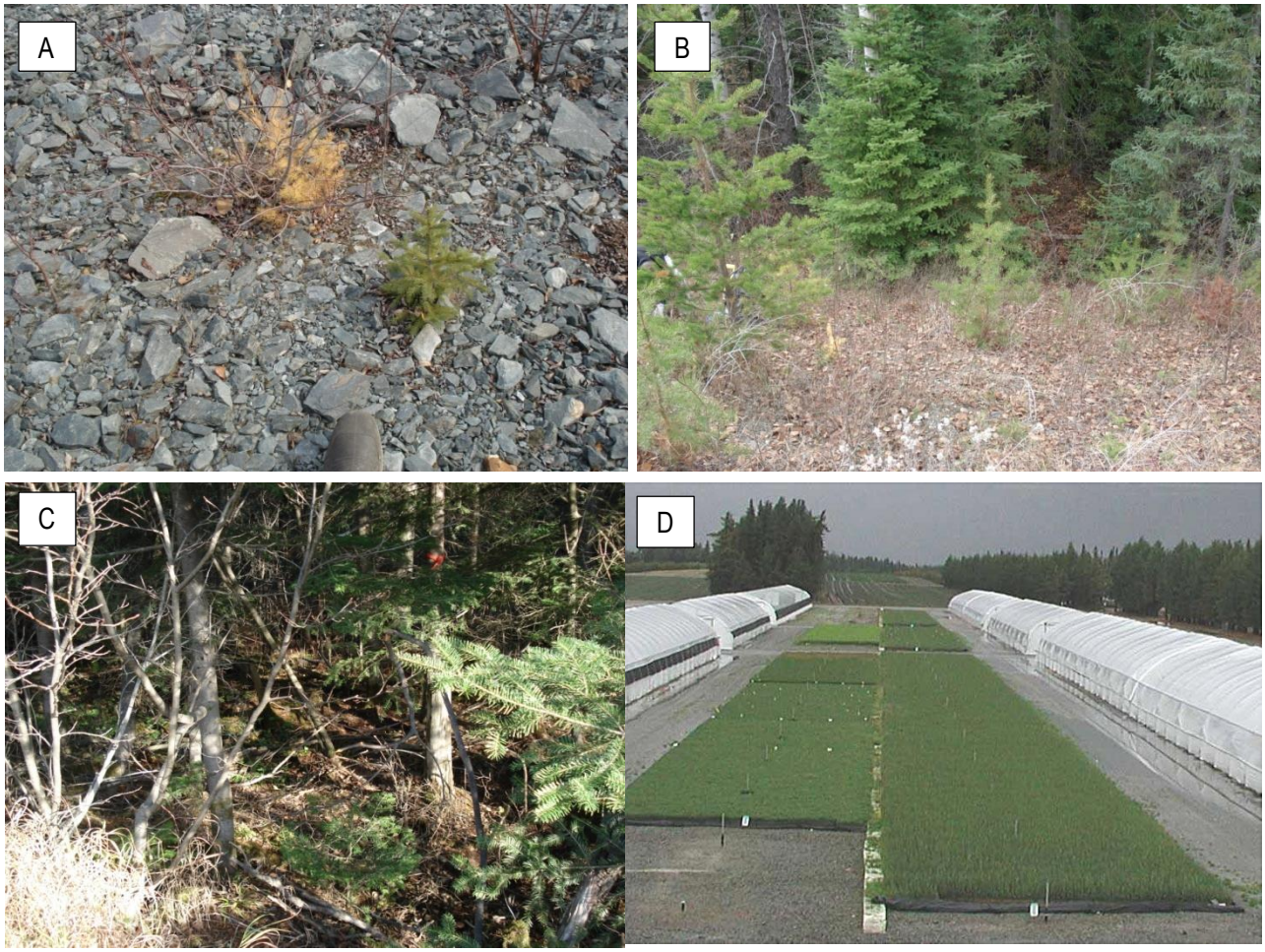

Figure A.2: Illustrations showing the four sampling sites of the ECM fungal community study: (A) mining site, (B) forest edge, (C) natural forest, and (D) Trecesson nursery.

Appendix III: Pictures of ECM fungal mycelium growth on solid medium in the *in vitro* culture experiment

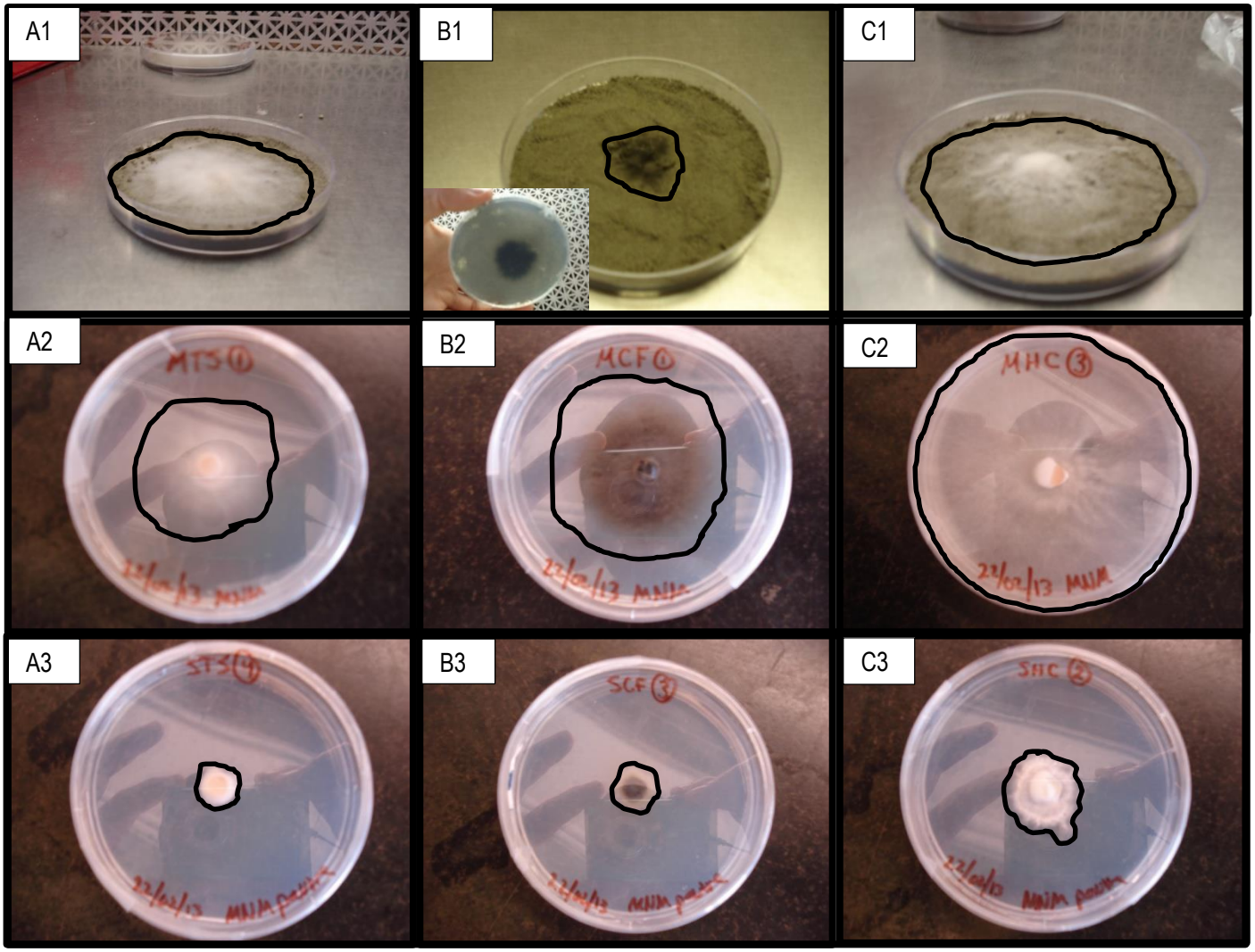

Figure A.3: Illustrations of *in vitro* ECM fungal growth on solid medium (A = *Tricholoma scalpturatum*, B= *Cadophora finlandia*, C= *Hebeloma crustuliniforme*, 1 = poor MNM medium with tailings, 2 = Normal MNM medium without tailings, and 3 = poor MNM medium without tailings). The black line shows the extension of fungal mycelial growth in the 8-cm petri-dish. *C. finlandia* was the only fungi that has grown directly inside the tailings. *T. scalpturatum* produced denser mycelia than *H. crustuliniforme*.

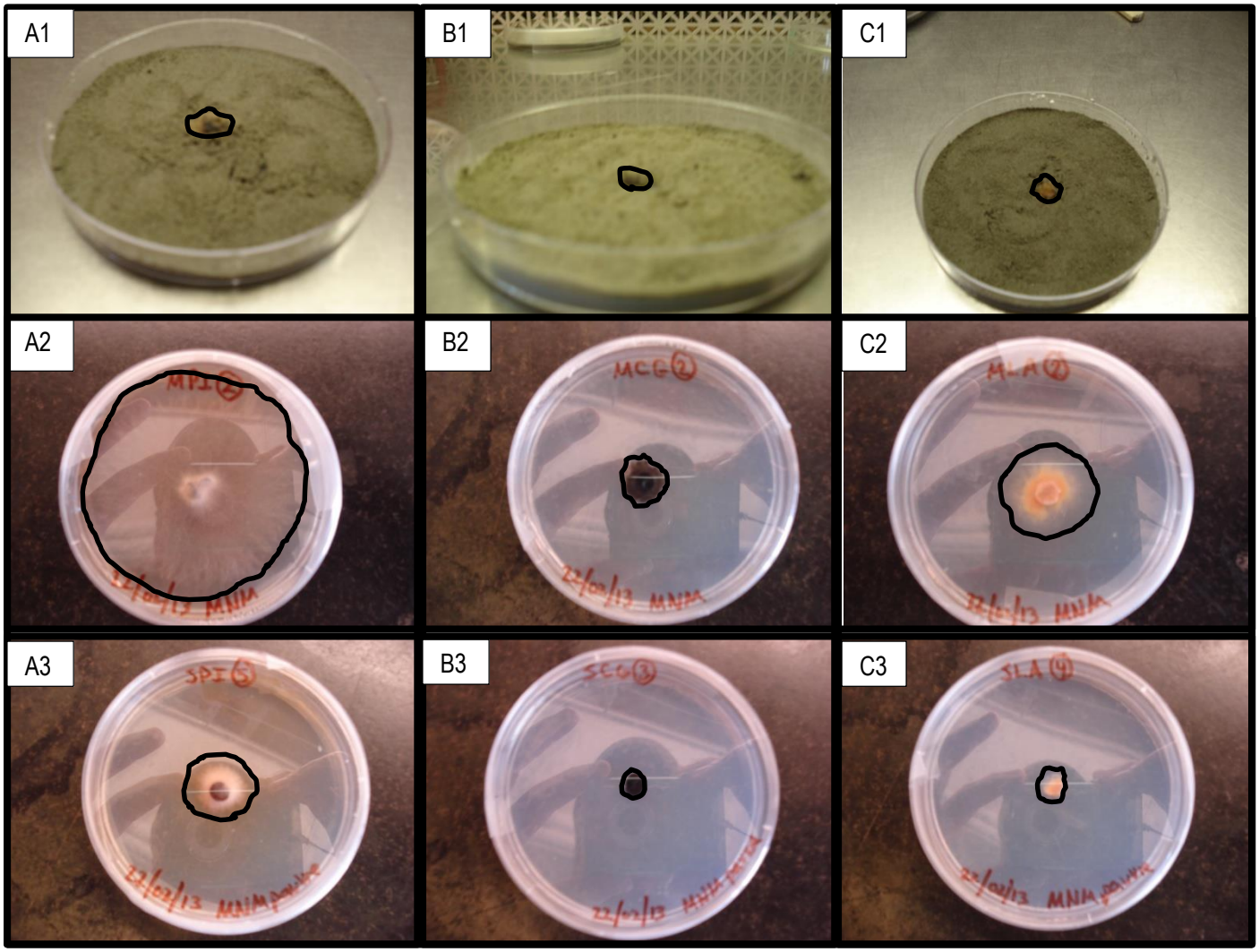

Figure A.4: Illustrations of *in vitro* ECM fungal growth on solid medium (A = *Paxillus involutus*, B= *Cenococcum geophilum*, C= *Lactarius aurantiosordidus*, 1 = poor MNM medium with tailings, 2 = Normal MNM medium without tailings, and 3 = poor MNM medium without tailings). The black line shows the extension of fungal mycelial growth in the 8-cm petri-dish. *P.involutus* seems to have not grown much in the tailings. No mycelia were seen growing from the inoculated plug of both *C. geophilum* and *L. aurantiosordidus* in the tailings.

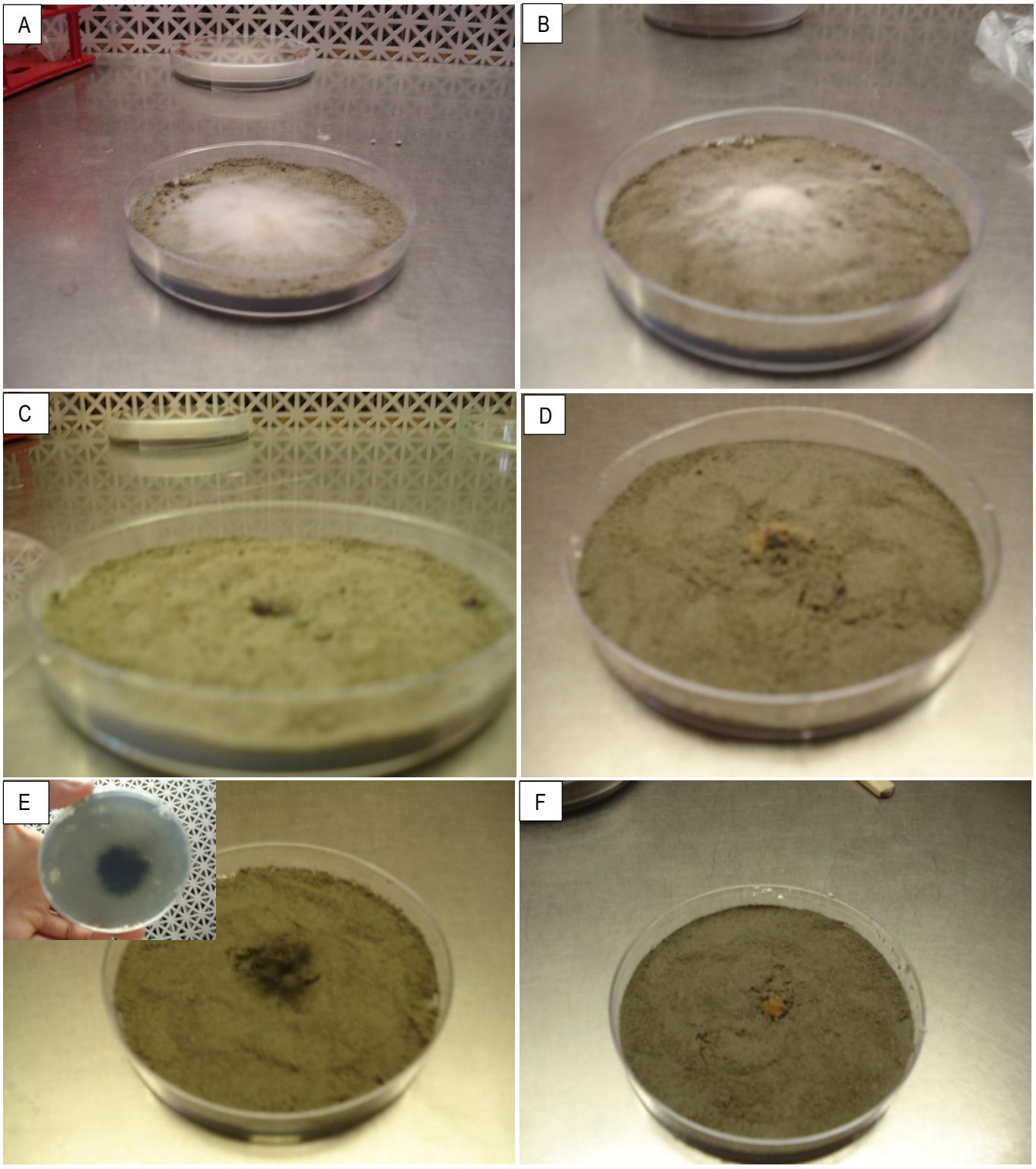

Figure A.5 : Illustrations of *in vitro* ECM fungal growth on poor solid MNM medium with tailings (A = *Tricholoma scalpturatum*, B = *Hebeloma crustuliniforme*, C = *Cenococcum geophilum*, D = *Paxillus involutus*, E = *Cadophora finlandia*, and F = *Lactarius aurantiosordidus*). *T. scalpturatum*, *H. crustuliniforme*, and *C. finlandia* were the three ECM fungal strains that had, visually, the most promising growth in gold mine tailings.

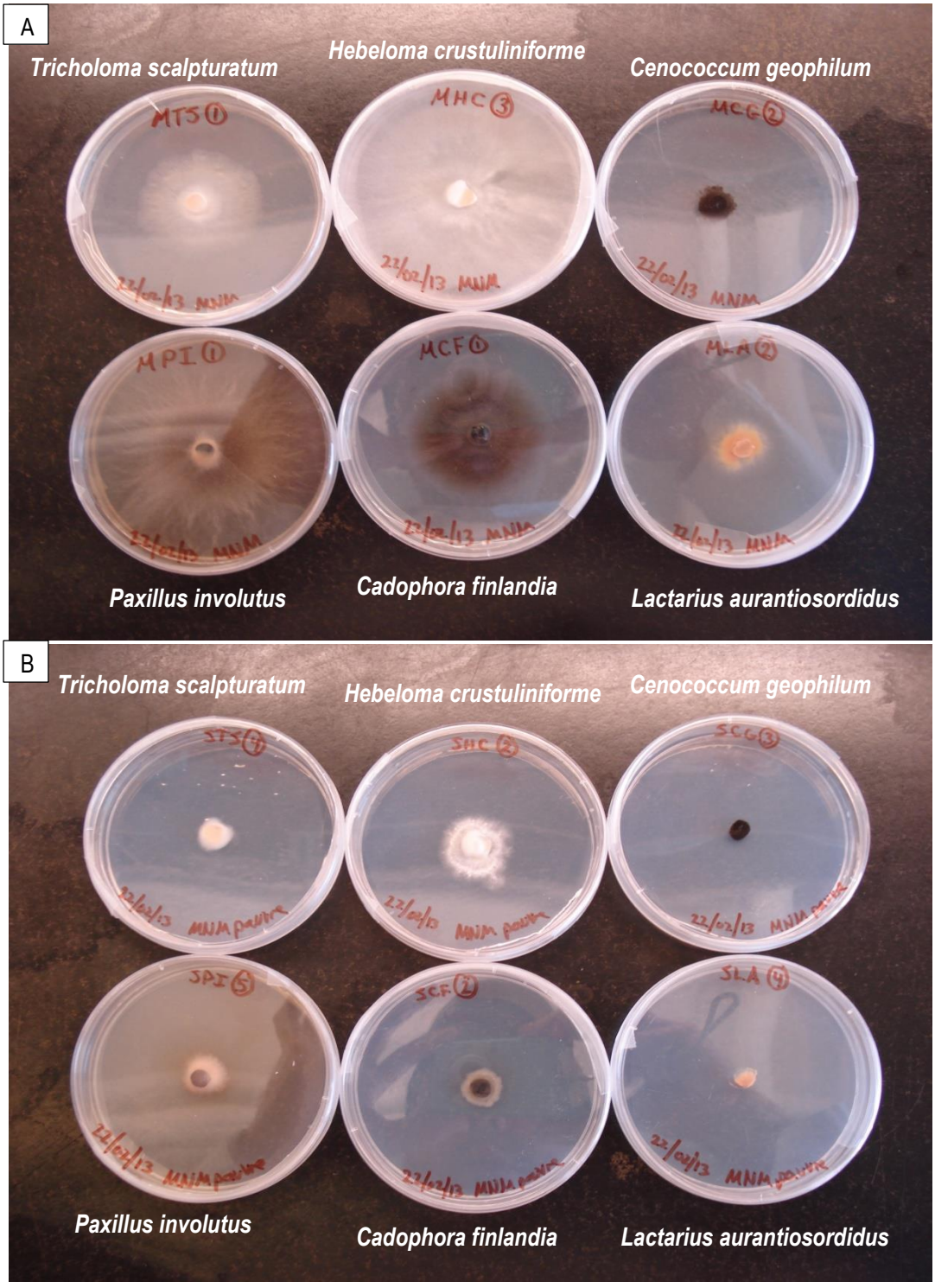

Figure A.6: Illustrations of in vitro ECM fungal growth of six different species (*Tricholoma scalpturatum, Hebeloma crustuliniforme, Cenococcum geophilum, Paxillus involutus, Cadophora finlandia*, and *Lactarius aurantiosordidus*) on solid medium without tailings (A = normal MNM medium and B = poor MNM medium). On both normal and poor media, *H. crustuliniforme* and *P. involutus* had, visually, the best mycelium growth followed by *T. scalpturatum* and *C. finlandia* with moderate growth, and finally *C. geophilum* and *L. aurantiosordidus* with the smallest growth.

Appendix IV: Diagram showing the glasshouse experimental design

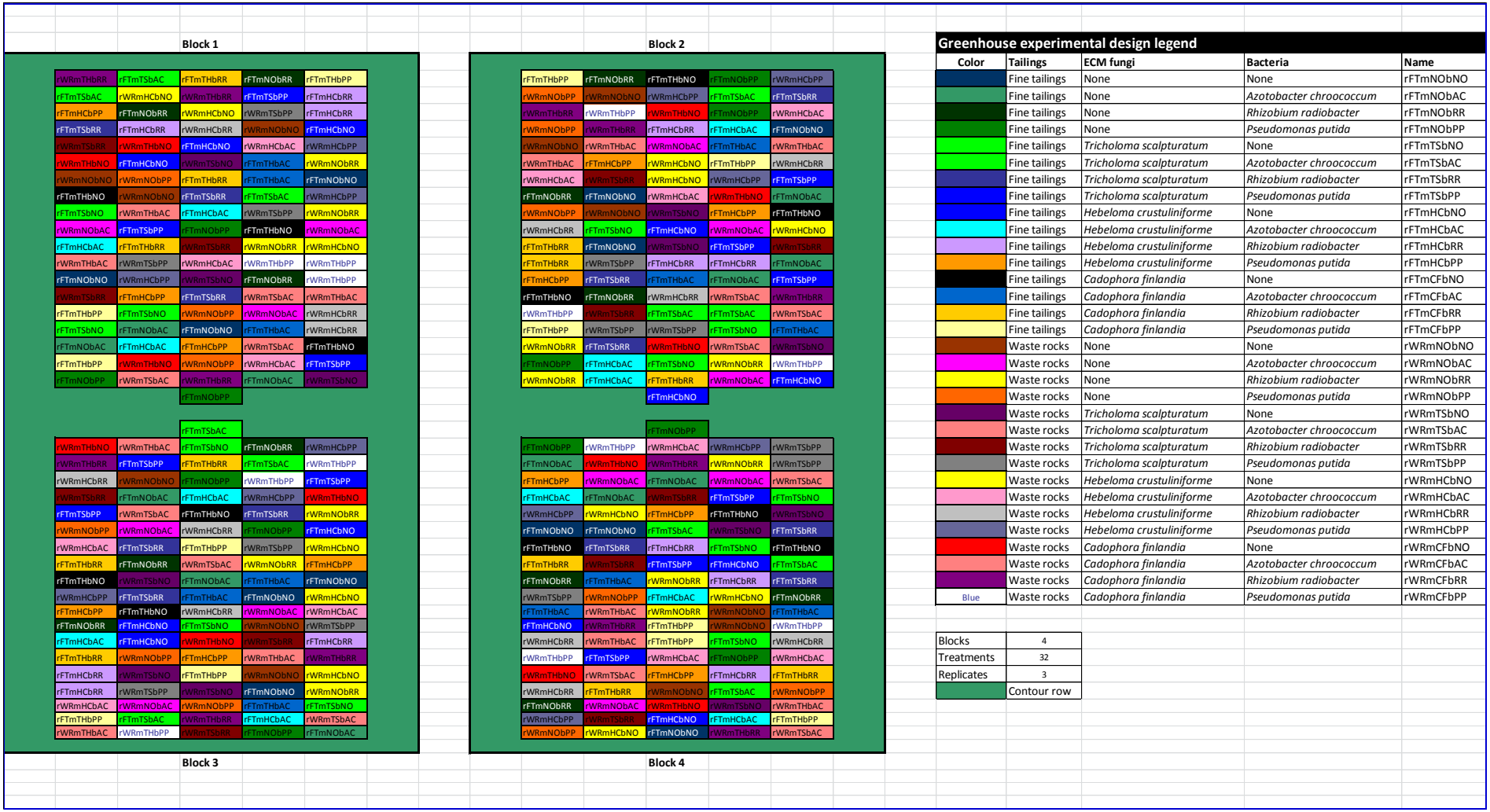

Figure A.7: Randomized complete block (RCB) design with three crossed fixed factors: tailing type (2 levels), ECM fungi (4 levels), and bacteria (4 levels) for a total of 32 treatments, 4 blocks, 3 replicates per treatment per block, and 384 experimental units.

Appendix V: Pictures of the glasshouse trial

Figure A.8: Illustrations of the glasshouse experiment - Root tips colonized by (A) *Hebeloma crustuliniforme,* (B) *Tricholoma scalpturatum*, and (C) *Cadophora finlandia*; (D) Glasshouse experimental design; (E) Experimental units; (F) Healthy white spruce seedlings in (G) fine tailings and (H) waste rocks; (I) Dead, (J) Unhealthy, and (K) Dark red white spruce seedlings.