EFFECTS OF NATIVE ECTOMYCORRHIZAL FUNGI ON ASPEN SEEDLINGS IN GREENHOUSE STUDIES: INOCULATION METHODS, FERTILIZER REGIMES, AND PLANT UPTAKE OF SELECTED ELEMENTS IN SMELTER-IMPACTED SOILS

by

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ABSTRACT

In southwestern Montana, areas near Butte and Anaconda support little or no vegetation mainly because of impacts from copper mining. Aspen (*Populus tremuloides*) stands occur naturally in these areas and rely heavily on native mycorrhizal fungi to moderate below ground conditions. Aspen associates with over 60 species of mycorrhizal fungi, but only a subset occurs on acidic, low nutrient soils. For these reasons, and the fact that aspen is an important colonizing tree on disturbed landscapes, a study was undertaken to examine aspen and its native ectomycorrhizal fungi in pot studies for future use in reclamation projects.

The first goal was to develop a method for mycorrhization of aspen in the greenhouse by examining inoculation methods, fertilization regimes, and selection of fungi. Nine native ectomycorrhizal fungi from smelter impacted sites near Butte-Anaconda aspen stands were tested using seed from Silverbow county, MT. Results demonstrated that mycorrhization of aspen in the greenhouse is possible, and that banding is the most effective method, although also labor intensive. Some fertilization was necessary, and high levels of liquid soluble fertilizer dramatically reduce mycorrhization, while the time release fertilizer Osmocote allowed mycorrhization. In general, mycorrhization in peat:vermiculite reduced aspen growth at 3 months, which might reflect an initial carbon drain.

Secondly, effects of ectomycorrhizal fungi on aspen growth and metal uptake in pots from Smelter Hill and Mt. Haggin were examined. In contrast to our earlier findings, mycorrhization in field soils increased seedling growth, although controls were slightly compromised. Inoculation technique was important and only banded seedlings formed mycorrhizae. Copper uptake was depressed about 50% in both roots and shoots of banded aspen compared to seeding a soil inoculum. Iron, zinc, nickel and phosphorus uptake appeared affected by fungi, but was dependent on species and particular metal, and, in some cases, appeared enhanced. *Laccaria, Paxillus, Pisolithus* and *Scleroderma* are of interest for their impact on aspen growth and metal uptake characteristics. Results indicate that inoculation of aspen seedlings with native mycorrhizal fungi has potential for use on smelter-impacted field soils. Once established, aspen's clonal nature could be an advantage in re-vegetating large tracts of land.

CHAPTER 1

LITERATURE REVIEW

Introduction

Pollution related to human activity is ubiquitous in soil, water and the atmosphere as a result of agricultural, commercial and industrial activities. Contamination levels of soil, air and water resources are dependent on the type and scale of disturbance. Mining associated with heavy metal extraction has impacted significant areas of the western United States. Activities associated with mining alter the physical, chemical and biological properties of impacted soils as a result of ore removal and the associated smelting processes.

Aerial emissions resulting from the smelting of mineral ore typically affect large tracts of land due to the fallout from the smoke plume. The mined ore ultimately determines the chemical composition of the fallout (Wickland, 1990), while prevailing winds control the pattern of soil contamination across the landscape (Ernst, 1989; Jordon, 1975). Slope position, aspect and elevation also play a role in the distribution of smelter emissions (Bissel, 1982; Taskey, 1972).

Many mine-impacted lands, specifically smelter-impacted sites, remain sparsely vegetated, and as a result negatively affect water quality, wildlife habitat and many other important processes that define a healthy self-sustaining ecosystem. Public concern over disturbed lands culminated in the late 1960's through the 1970's with passage of new laws regulating mining and the reclamation of impacted lands. Federal and state laws

now govern reclamation of disturbed lands contingent on type of mining, mineral ore, types, level of contamination, and size of area impacted.

Vegetation around Anaconda, Montana has been negatively impacted by aerial emissions as a result of the smelting process (Walsh, 1976). Tons of sulfur dioxide, arsenic trioxide and other heavy metals were released into the atmosphere from the copper smelter stack (Taskey, 1972). Metal levels in soils in many areas around the vicinity of the Anaconda smelter stack are phytotoxic to plants (RRU report, 1993; Taskey, 1972). In September 1983, the Environmental Protection Agency (EPA) placed the area surrounding the smelter on its Superfund National Priorities List (NPL). Remediation of the smelter impacted areas around Anaconda is broadly governed by the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA, P.L. 96-510) or Superfund. The act gives authority to federal agencies to respond directly to releases or threatened releases of hazardous substances that may endanger public health or the environment

Establishment of a permanent vegetative cover on these disturbed areas can reduce erosion, and initialize restoration of important components of the impacted soil as required in most reclamation laws. Different remedial strategies exist for reclaiming contaminated areas. Phytoremediation utilizes plants to reduce or immobilize contaminates in place (Cunnigham & Berti, 1993). Some plants species can accumulate metals in their tissues (Kumar et al. 1995), inhibit the mobility of metals by reducing erosion, or convert pollutants into non-toxic compounds (Dushenkov et al. 1995).

In the past, much effort was given to the re-vegetation of disturbed areas with little or no concern for belowground processes**.** Now, however, the soil resource is

recognized as a critical component in reclaiming disturbed areas (Logan, 1992; Taskey, 1972) for the long-term sustainability of the reclamation efforts, especially in heavy-metal contaminated systems where organic matter plays a large role in the mobility of metals in the soil (Friedland, 1989).

Microbial communities are essential for terrestrial successional processes (Birch et al. 1991). Contamination of the soil resource and the corresponding negative effects on local vegetation, and lack of microorganisms disrupts successional processes (Wickland, 1989) as regulated by accumulation (Zak et al. 1990), and cycling of organic matter. Organic matter accumulation, decomposition, and nutrient cycling are impeded as a result of heavy metal contamination in the soil. Re-establishment of a self-sustaining vegetative cover, with nursery-grown mycorrhizal-inoculated plants, might aid in restoring some function that would enable successional processes to resume. Plant establishment and sustainability as a reclamation strategy seeks to facilitate the recovery of impacted systems by modifying soil conditions for later successional species (Connell & Slatyer, 1977). However, reclaimed disturbed sites will follow a new trajectory of succession (Allen, 1995).

Soils

Soils are a complex heterogeneous medium (Alloway, 1995) because many linkages exist between the chemical, physical, and biological components of the resource at different temporal and spatial scales (Wardle, 2002). Abiotic and biotic interactions in the soil determine soil properties, which dictate its ability to support plant growth (Elmarsdottir et al. 2003). Disturbance, related to human activity, alters these individual

processes, which in turn has important consequences when reclaiming an area. Mining and smelting generally disrupt the natural flow of energy through impacted systems. Immediate negative impacts as a result of smelter emissions include heavy metal accumulation and acidification of the soil (Wickland, 1990). Many important microorganisms are negatively affected by heavy metals (Hartman, 1976; Moynahan et al. 2002; Seaward & Richardson, 1989). Lack of organisms and/or organic matter destroys soil structure, water holding capacity, and cation exchange capacity (CEC) ratios, further compounding the effects of heavy metal contamination.

The availability and fate of elements in a soil system are affected by various aboveground and belowground components (Kabata-Pendias, 2001; Wickland, 1990). These factors, together, develop the components of soil that govern metal, nutrient, and water cycling through the system.

The ability of soil to adsorb ions is influenced by soil physical, biological and chemical characteristics (Alloway, 1995). Heavy metal deposition as a result of smelting disrupts both nutrient (Goodman et al. 1973) and water availability (Wickland, 1989) in impacted soils. Essential metabolic and physical pathways can be disrupted as a result of elevated levels of metals in the soil (Kabata-Pendias, 2001). Plant tolerance to heavy metal accumulation in soils, however, is affected by plant species, the physico-chemical properties of the metal and environmental factors (Gadd, 1993).

Chemical

Chemical properties of soil are affected by a number of factors. Initially, soil chemistry is the result of processes of erosion on parent material. The chemistry of a soil

is then further regulated by topography, soil age, vegetation and climate. Soil pH and cation exchange capacity are two important interrelated chemical properties that affect both above and belowground biota (Munshower, 1994).

Soil pH, or hydrogen ion concentration, is important because it influences other chemical properties in the soil, such as the availability of elements in the soil (Kabata-Pendias, 2002). Sulfur dioxide accumulation in the soil, as a result of smelter emissions, increases the relative amount of hydrogen ions in the soil system, thereby decreasing soil pH (Kabata-Pendias, 2001). In addition, heavy metals released from smelter stacks are more bio-available as a result of the lowered pH in the soil, exacerbating the contamination of the soil resource (Fitter & Hay, 1981; Munshower, 1994).

Cation exchange capacity (CEC) in soils is a general measure of the quantity of cations that can be adsorbed by the soil. Soils that contain higher levels of organic matter and clay particles exhibit higher levels of CEC, which may be used as a surrogate for soil fertility. Disruption of nutrient cycling processes as a result of loss of vegetation or lack of microorganisms negatively affects soil fertility. Cations (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, H^{+}) and other essential and non-essential elements are no longer recycled by the decomposition of organic matter, facilitated by microorganisms, and could be lost from the system as a result of leaching or erosion (Kabata-Pendias, 2001).

Physical

Soil texture and structure also play important roles in regulating above and belowground biota. Soil texture, the fraction of sand, silt, and clay present, is indirectly involved in soil CEC. Soils that have high concentrations of clay will have higher CEC

values and a large capacity for water absorption because of the higher number of possible exchange sites associated with soil colloids and the high fraction of mico-pores (Kabata-Pendias, 2001). On the other hand, sandy soils typically have low CEC values (Kabata-Pendias, 2001) and lower water holding capacities as a result of the inability of large pores to retain water in the soil profile and generally exhibit.

The structure of soil is affected by the relative size and shape of soil aggregates. Organic matter plays an important role in the development of soil structure. Soil particles are bound together by organic and inorganic polymers into larger soil aggregates (Kabata-Pendias, 2001). Soils that exhibit limited aggregation will impede water infiltration. On the other hand, soils that demonstrate granular structure are typically favorable for plant growth. Granular soils have a balanced number of micro-pore spaces and macro-pore spaces (Munshower, 1994), which increase gas exchange sites and provide habitat for root development while providing enough water and nutrients to the plant.

Biological

The contributions of plants, via quality of plant litter, root exudates, and quantity of litter, are vital for soil processes. First, different types of plants will contribute various amounts of organic matter to the soil biosphere. Organic matter helps build soil structure (Norland, 1993), increases the water holding capacity and CEC of a soil (Munshower, 1994), and binds certain elements in a way that renders them unavailable for plant uptake (Friedland, 1990; Jones, 1998). Thus, plant litter quality has direct effects on nutrient availability in the soil system, and as a result indirectly affects the soil

organisms that are present in a given area (Wardle, 2002).

Microbes are stimulated by increased concentrations of various chemicals produced by sloughing off of root cap cells, exudation of compounds from intact cells, and production of slime by the root cap (Fitter & Hay, 1981). However, increasing levels of heavy metals have been shown to decrease microbial populations (Birch et al. 1991; Leyval et al. 1997). Litter decomposition rates are reduced along a concentration gradient that correlates with distance to smelter, indicating reduced microbial activity as metal contamination increases, suggesting microbial populations are increasingly absent, in low numbers or unable to function as contamination levels increase (Freedman & Hutchinson, 1980; McEnroe & Helmisaari, 2001). A decrease in the amount of litter being decomposed will negatively affect the amount of nutrients available for plant uptake, slow the amount of organic matter produced, further reducing microbial populations and metal immobilization by organic matter in the soil.

Many different factors regulate soil organisms. These organisms can indirectly affect plant communities through decomposition of organic material and corresponding release of nutrients in the soil. Wilson et al. (1965) stated that the lack of microorganisms on mine tailings might be a deterrent to the development of vegetation.

Plants

Plant tolerance to heavy metals in the soil is affected by the amount and availability, of metal in soil solution (Alloway, 1995). Metal toxicity is a major constraint affecting root growth (Jentschke, et al. 2000; Jones & Hutchinson, 1988) and

plant metabolism (Friedland, 1989). Plant roots directly modify the chemical and physical properties of the rhizosphere and, thus, indirectly affect the bio-availability of metals in the soil (Alloway, 1995). Toxic levels of metal ions in soil solution can interfere with the plants ability to acquire water and nutrients, and the utilization of these resources (Fitter & Hay, 1981). Metal uptake by plants will be discussed further in Chapter 4. In addition, Kabata-Pendias (2001) provides a comprehensive review of the uptake of trace elements in plants.

Re-establishment of a self-sustaining plant community on these disturbed areas can reduce negative impacts to the environment by first reducing erosion. Minimizing erosion will conserve the surface soil resource, which generally contains most of the organic matter and nutrients in a soil profile. Additionally, establishment of vegetative cover will reduce the mobility of contaminants through the system via complexation, uptake, precipitation, and adsorption (Alloway, 1995). The addition of organic matter into the soil system might modify the contamination enough so that the overall toxicity of the soil resource is reduced, which can facilitate colonization by later successional species that may be less metal tolerant or resistant (Ernst, 1989).

Mycorrhizal Fungi

Many re-vegetation attempts have proved largely unsuccessful due to soil conditions. Severe land disturbance often reduces or eliminates microbial communities that are essential for nutrient cycling and plant survival (Read & Smith, 1997). While native mycorrhizal fungi are essential elements in natural ecosystems (Allen, 1991; Danielson, 1985) and are important for plant survival on harsh sites (Schramm, 1966;

Marx et al. 1994) those species may be reduced or eliminated as a result of heavy metal contamination and soil acidification. Some species of mycorrhizal fungi have been found to be more tolerant of these conditions (Cripps, 2001; Meharg & Cairney, 2000).

In disturbed areas where the biological, physical and chemical functions of the soil are disrupted, mycorrhizae play a vital role in the survival of vegetation by moderating below ground conditions. Powell (1980) discussed the connection between the addition of mycorrhizal fungi and a corresponding increase in vegetation. Wali (1999) argues that mycorrhizal fungi also accelerate succession by giving perennial mycorrhizal plants an advantage over annual weedy species, which typically colonize disturbed sites first.

Mycorrhizal fungi are partners in a mutualistic association with plant roots that occur with over 90 percent of all plant species (Malajczuk, et al. 1996). These mutualistic relationships between soil fungi and plant roots are interdependent, where the host plant may receive benefits from increased nutrient acquisition (Smith & Read, 1997), heavy metal resistance (Bradley, et al. 1981), drought tolerance (Mason, et al. 2000), and protection from pathogens and other organisms (Marx, 1969), and the mycorrhizal fungus receives photosynthetically-derived carbon compounds from the plant (Harley, 1989). Mycorrhizal relationships involve three main components, the soil, plants and mycorrhizal fungi.

In vitro studies using agar media have been conducted in an attempt to quantify mycorrhizal sensitivity to a range of toxic metals (Bradley et al. 1982; Colpaert & Van Assche, 1987; Hartley et al. 1997). Soil acidity affects mycorrhiza formation and hyphal development of ectomycorrhizal fungi to different degrees (Van der Heijden & Kuyper,

2001). Fungal tolerance to heavy metals *in vitro* simplifies the soil system when trying to relate both fungal and plant tolerance on actual sites where conditions are variable. In addition, species of mycorrhizal fungi can be functionally unique (Jones & Hutchinson, 1986; Willenvorg, et al. 1990). Different mycorrhizal species appear to have varying affects on a host under different conditions (Jentschke, et al. 2000). Anselmi et al. (1990) and Cripps (2001) demonstrated that growth of *Populus* is enhanced by some mycorrhizal species, while others had no affect, or even a deleterious one under some conditions. However, Hartley et al. (1997), discusses the limitations of inferring ultimate plant fitness in the field from laboratory studies.

Mycorrhizal fungi are also subject to successional processes, with "early stage" fungi typically present in young stands and nurseries, and late stage fungi in older stands that have accumulated more organic matter and carbon (Bushena et al. 1992). Cripps (2001) described a subset of ectomycorrhizal species of fungi that colonize young aspen stands or with young aspen ramets on the periphery of a mature stand as "early colonizers". These fungi are generally less host specific (Molina & Trappe, 1982) and are tolerant of harsh conditions found in early stages of plant succession where there is a lack of organic matter and other corresponding limiting conditions at the site.

Survival And Fitness

Numerous studies have confirmed the ability of certain mycorrhizal fungi to increase survival and fitness of plants as measured by growth and survival in varying environmental conditions (Lee & Koo, 1985; Read, 1991; Ruehle, 1980). For the long-term survival of a plant community in disturbed habitats, the establishment of primary

producers (plants), the resumption of nutrient cycling and decomposition are vital (Norland, 1993).

Increased Nutrient Uptake

Hyphae from the mycelial network of mycorrhizal fungi access micro-pores in soil particles that plant roots are unable to reach and are, thus, able to exploit more of the soil resource (Landeweert et al. 2001). Secondly, mycorrhizal fungi are able to mobilize and transport nutrients that might otherwise be unavailable to the plant (Hartly et al. 1997; Smith & Read, 1997). Phosphorus acquisition and transport by mycorrhizal fungi to plants is well documented (Brandes et al. 1998; Jentschke et al. 2001; Jones et al. 1998). More recent studies have evaluated the role of mycorrhizae in nitrogen and other nutrient acquisition and will be discussed later (Abuzinadah & Read, 1989; Plassard et al. 2000).

Drought Tolerance

In attempting to establish vegetative cover on a disturbed site, drought tolerance may be a critical factor in the survival of any vegetation (Danielson, 1985). Mason et al. (2000) discussed the effect of soil moisture on mycorrhizal development, and how mycorrhizal inoculation benefits seedling growth, via increased stomatal conductance in mycorrhizal plants. Parke et al. (1983) measured drought tolerance in plants as a function of CO_2 fixation and found that mycorrhizal seedlings were able to continue fixing CO_2 longer than non-mycorrhizal seedlings under drought conditions. Mycorrhizal plants recovered from drought stress more quickly than non-mycorrhizal plants (Parke et al. 1983). In relation to drought tolerance and increased plant nutritional status, increased

root length of plants inoculated with mycorrhizal fungi has also been considered a main factor in conveying drought tolerance to plants (Lehto, 1992). Mycorrhizal fungi alleviate moisture stress for the host plant by decreasing the resistance of water flow from the soil to plant roots, increasing the effective absorptive surface of roots, and are able to penetrate smaller pore spaces in the soil than plant roots (Parke et al. 1983).

Protection From Pathogens And Other Organisms

Marx (1969) demonstrated the benefits of mycorrhizal fungi in protecting plant roots from plant pathogens and other organisms that might negatively affect plant survival and growth. Both the physical barrier (Allen, 1991) of the mycorrhizal fungal hyphae and sheath (in ectomycorrhizal fungi), and the physiological changes in root chemistry help prevent infection by pathogenic fungi and other harmful organisms of the plant root (Branzanti et al. 1999). Mycorrhizal fungi may compete against pathogenic fungi for resources and space, thereby limiting infection by pathogenic fungi (Borowicz, 2001).

Heavy Metals

Severe land disturbance often reduces or eliminates native mycorrhizal fungi that are essential elements in natural ecosystems (Allen, 1991; Danielson, 1985). Wilson et al. (1965) stated that the lack of microorganisms on mine tailings might be a deterrent to the development of vegetation. Norland (1993) is even more specific when he discusses the importance of mycorrhizae in disturbed areas for plant survival. He states that disturbed sites require a microbial community that is tolerant of the limiting conditions in the soil specific to the site. While the role of some mycorrhizal fungi is generally acknowledged in ameliorating the toxic effects of some heavy metals in contaminated

soils, the specific mechanism of metal amelioration by the mycorrhizal fungi is less clear (Jentschke et al. 2000). Jentschke et al. (2000), suggest that any conveyance of heavy metal tolerance to plants by mycorrhizal fungi could possibly be a result of increased water and/or nutrient status, not amelioration of heavy metal toxicity.

Metals are directly and or indirectly involved in all aspects of fungal growth and metabolism (Gadd, 1993). Toxic symptoms may vary widely between different fungi and for different metal species and for different combinations of metals in the soil (Gadd, 1993; Van der Heijden & Kuyper, 2001).

Mechanisms

Different mechanisms for various fungi have been found to affect mycorrhizal plants ability to tolerate heavy metals (Jentschke et al. 2000; Denny & Wilkins, 1987b). A discussion on the mechanisms, such as metal amelioration or increased plant nutrition in mycorrhizal systems is included in Chapter 4.

Mycorrhizal Types

Smith and Read, (1997) provide a table outlining the various groups of mycorrhizal fungi and their characteristics. Arbuscular mycorrhizal fungi (AM) and ectomycorrhizal fungi are critical to the successful re-vegetation of disturbed areas because of their particular plant associations. AM fungi mycorrhizae are formed by members of the Glomales. AM fungi produce arbuscules within plant cells, and some produce storage vesicles. AM fungi are unable to grow in culture and as a result are thought to be completely reliant on the plant host i.e., obligate biotrophs (Brundrett et al.,

1996). These fungi are generally associated with wide diversity of plant species including grasses, forbs, some woody shrubs, and a few trees (Smith & Read, 1997).

Ectomycorrhizal (EMF) associations are mutualistic associations between higher fungi and certain groups of Gymnosperms and Angiosperms (Brundrett et al. 1996; Smith & Read, 1997). The majority of fungi that form ECM associations are Basidiomycetes and Ascomycetes, plus a few Zygomycetes (Molina et al. 1992). They associate primarily with woody plants in the Salicaceae, Betulaceae, Fagaceae, Pinaceae, Myrtaceae and others.

Aspen

Aspen (*Populus tremuloides*) is an early successional species in many habitats and as a result it can moderate harsh environmental conditions often associated with disturbance (Schramm, 1966). The wide distribution of aspen across North America suggests that the species is adapted to many different environmental conditions (Campbell, 1984, Lieffers et al. 2001). Aspen have naturally re-colonized small areas of smelter impacted sites such as those at Anaconda, Mt., Trail, B.C. Kellogg, ID (Cripps, 2001), Palmerton, PA. (Jordan, 1975), and Sudbury, Ontario (Freedman & Hutchinson, 1980). Aspen has a high potential to survive on these sites as evidenced by large stands naturally established in contaminated areas.

Aspen typically propagate asexually via underground adventitious roots, which produce sprouts of genetically identical ramets (Shier, 1976), but are also capable of reproducing sexually by seed. The clonal growth habit lends itself to reclamation when compared to the unreliability of seedling establishment on areas of disturbance. Once

established, aspen clones can extend themselves over a large expanse of land. Established aspen forests can provide a permanent vegetation cover over contaminated soil to minimize heavy metal mobilization, minimize transport of polluted ground water, minimize surface water and wind erosion, increase biodiversity, and provide wildlife habitat. The deciduous nature of the tree means the annual addition of organic matter to the soil at leaf-fall.

Aspen is associated with over 60 species of ectomycorrhizal fungi (Cripps & Miller, 1993; Godbout & Fortin, 1985). Cripps (1993, 2001) found only a small fraction of these occur on acidic, low nutrient soils typical of smelter impacted sites.

Ectomycorrhizal Fungi

Ectomycorrhizal fungi are a distinct group of mutalistic fungi that are characterized by a hyphal sheath that envelops the root, a Hartig net, fungal hyphae which penetrate between the root epidermal and cortex cells, and external hyphae (Smith & Read, 1997). Most plants that form ectomycorrhizal associations are woody perennials (Smith & Read, 1997). Ectomycorrhizal fungi are relatively selective of host plant species (Allen, et al. 1995) and, as a result, any benefits to a plant are ultimately determined by the ability of the fungal species to form mycorrhizae with the potential host plant (Denny & Wilkins, 1987). Since ectomycorrhizal fungi depend on their host plants, it is feasible that sites devoid of vegetation for some time also lack native fungi, hyphae, and possibly spores.

Plants with ectomycorrhizal fungi have been used to aid establishment of woody plants on harsh sites, and successful research includes establishment of pine on eastern coal spoils (Marx et al. 1994). Cordell et al. (2001) reported increased survival of

inoculated pine and oak seedlings on a harsh site in Ohio, where 85% of inoculated plants survived compared to only 50% survival of non-inoculated trees. In addition, total reclamation costs were reduced by over 90% from traditional reclamation methods (Cordell et al. 2001). Perry et al. (1987) suggest that addition of ectomycorrhizal fungi are critical in plant survival where native mycorrhizal communities may have been disturbed and as a result are absent from the system.

Ectomycorrhizal fungi can colonize a much greater volume of soil than roots alone and increase the absorptive surface of plant roots (Campell et al. 2002). Landeweert et al. (2001) provides a review of the evidence implicating ectomycorrhizal fungi in the mobilization and transport of various nutrients to the host plant. Heavy metal contamination of the soil resource further reduces the availability of plant essential nutrients due to complex biochemical reactions (Kabata-Pendias, 2001), increasing the importance of ectomycorrhizal fungi in plant survival. In fact, Ahonen-Jonnarth and Finlay (2001) propose that ectomycorrhizal fungi alleviate other plant limiting conditions in the soil that result from high concentrations of metals, improving plant health indirectly.

Soils of the northern temperate forested zones are typically deficient in many nutrients essential for plant health, and specifically nitrogen (Van der Heijden et al. 2001). In contaminated soils with limited vegetation and reduced microbial populations necessary for the decomposition of organic matter, nitrogen becomes even more limiting (Carpenter & Allen, 1988). Nitrogen can be acquired and transported by some ectomycorrhizal fungi to the host plant (Plassard et al. 2000), but large differences in their effectiveness have been reported (Abuzinadah & Read, 1989). *In vitro* and *in vivo*

studies reviewed by Smith and Read (1997) show that different species of ectomycorrhizal fungi have a wide range of ability to use nitrogen, generally dictated by the form of nitrogen. Smith and Read (1997) also discuss the importance ectomycorrhizal fungi in obtaining phosphorus from organic sources and weathering of parent material. Ectomycorrhizal fungi are also able to transport other important nutrients such as magnesium and potassium to the host plant (Jentschke et al., 2000).

Some species of ectomycorrhizae have been shown to increase aspen growth *in vitro* (Cripps, 2001). However, it is not known if increased aspen growth *in vitro* translates to increased growth, establishment or survival of aspen in the field. Molina and Palmer (1982) recognized the importance of pure culture synthesis (aseptic conditions) in mycorrhizal investigations, but admit limitations in predicting results in natural systems based on lab results.

Objectives

This research is a contribution towards investigating the use of native ectomycorrhizal fungi to enhance the establishment of aspen on smelter affected sites. The main goals are to first develop a method for mycorrhization of aspen on nursery grown plants using ectomycorrhizal fungi ecologically adapted to smelter-impacted areas of Butte and Anaconda under greenhouse conditions; and second, to evaluate individual native ectomycorrhizal fungi for their effects on aspen in various soil types (including those high in heavy metals from impacted smelter sites).

Individual objectives include: 1) development of an efficient method for inoculation and mycorrhization of aspen seedlings with native fungi; 2) examination of

mycorrhizal levels as affected by fertility levels in pot studies for producing viable mycorrhizal seedlings; 3) assessment of survival and growth of aspen inoculated with various species of native ectomycorrhizal fungi in contaminated field soils in the greenhouse; and 4) production of inoculated aspen seedlings for evaluation of plant survival and growth on smelter-impacted sites in the field.

The establishment of aspen inoculated with native, site-adapted mycorrhizal fungi on areas devoid of vegetation should speed the process of succession while providing ground cover, inputs of organic matter, and the corresponding establishment of a sustainable microbial community.

CHAPTER 2

PRELIMINARY EXPERIMENTS

Introduction

The symbiotic relationship between plants and mycorrhizal fungi is dependent on plant, soil and fungal interactions. Mycorrhizal fungi exhibit varying levels of host specificity (Molina & Trappe, 1982). Some of these symbiotic fungi are found with a limited number of host plants while others occur with many species of plants, even across different *genera* (Castellano & Molina, 1989; Molina, 1979). In addition, various species of ectomycorrhizal fungi have preferences for different soil types (Buschena et al. 1992; Cripps and Miller, 1993; Cripps, 2001), and for discrete habitats in various stages of plant succession (Allen, 1991). Consequently, site conditions can inhibit certain mycorrhizal associations between fungal species and their host plants. On disturbed or stressed sites, conditions can be even more limiting. Therefore, mycorrhizal studies need to take the specific fungi, plants, and soil conditions involved in this process into consideration. In applied studies with a goal of producing nursery grown inoculated seedlings for out-planting these factors need to be addressed simultaneously.

Native ectomycorrhizal fungi have high potential for enhancing survival of plants in the field, but since fungal species vary in their requirements and benefits to the host, screening of fungi is necessary for development of a feasible mycorrhizal system. A first step is to determine which fungal species are capable of forming mycorrhizae with the host plant being studied; the fungi need to be observed with the host in the field, and

identified, typically utilizing macroscopic features of the sporocarp, and tissue cultured. Not all macrofungi are mycorrhizal, and saprophytic and parasitic genera can be ignored. Microscopic examination of spores may be necessary for species level identification of some ectomycorrhizal species (Miller, 1982).

The source of plant material is also an important consideration in greenhouse studies of mycorrhizal fungi. Locally collected seeds or local vegetative plant material is adapted to environmental conditions for a given site, giving these plants an advantage over non-native strains that may not tolerate local conditions when out-planted (Brown et al. 1996; Elmarsdottier et al. 2003). In addition, plant age is an important factor in mycorrhization of the plant. More specifically, root development can be critical because studies have shown that mycorrhizal fungi typically infect lateral roots but are generally unable to infect primary and older roots (Smith & Read, 1997). Aspen (*Populus tremuloides*) typically propagate asexually via underground adventitious shoots, which produce sprouts of identical clones (Shier, 1976), but this species is also capable of reproducing sexually by seed. Vegetative propagation from lateral roots (Schier, 1978) and aspen seed were both considered for production of viable plant material in this study, as was the age of the root system.

Possible sources of fungal inoculum in mycorrhizal studies, include plant roots (Erland & Södeström, 1990), soil (Harris & Jurgensen, 1977), sclerotia (Trappe, 1969), spores (Fries, 1987), and fruiting bodies of ectomycorrhizal fungi (Molina, 1979).

Plant roots, collected from field sites have been used to inoculate plants in greenhouse studies with ectomycorrhizal fungi and for out plantings. Plant roots from the field are typically colonized by more than one type of fungus (Smith & Read, 1997), and

distinctions between fungal groups and species are difficult. Measured plant responses to inoculation are therefore impossible to attribute to only one species of ectomycorrhizal fungus. Plants may respond in various ways to infection by particular fungi, which may act individually and synergistically in conveying benefits or injury to the plant, masking potential species-specific interactions between fungus and plant.

Native soil has also been investigated as a potential source of fungal inoculum for mycorrhizal studies. The same set of problems faced in using roots is encountered when utilizing native soils as a source of inoculum in greenhouse and out planting studies. Many different species of fungi exist in the soil and as a result the sheer volume and diversity of organisms present in any given soil undermines interpretation of effects on plants due to a particular organism. However, soil transfer could be a low cost alternative for applied work.

Spores collected from the fruiting bodies of ectomycorrhizal fungi have also been successfully utilized as a source of inoculum (Marx, 1976). Since the fruiting body is the source of spores it is relatively easy to determine the species of fungus. Production of spore inoculum is often limited by the number of viable spores that are produced on the gills of most ectomycorrhizal fungi. However, the ectomycorrhizal fungus *Pisolithus tinctorius* produces massive quantities of spores, making it commercially valuable, and *P. tinctorius* spores have been used in a number of studies (Marx & Bell, 1975). Another drawback, when spore inoculum is utilized is that mycorrhization of plants may not be feasible in pot studies because of slow spore germination and slow development of the mycelium complicated by on-going competition with other soil organisms, and conditions unfavorable for spore germination.

One advantage of using mycelium gained from fruiting bodies lies in the ease of identifying the fungal species, compared to other sources of fungal inoculum. On the other hand, use of fruiting bodies excludes fungal species that do not readily produce these sexual reproductive structures, or that do not fruit regularly, but might be present in natural settings; these species could be critical for plant survival and fitness in that system, especially in impacted soils. Tissue from sporocarps is used to culture the fungus, however, not all species grow in captivity.

The cultured mycelium, applied in a liquid (Cline et al. 1982; Bushena et al., 1991), or in sterilized soil (Marx & Ruehle, 1991; Riffle & Maronek, 1982) is typically utilized in experiments with ectomycorrhizal fungi because of its ability to effectively infect host plants in pot studies compared to other types of inoculum. In addition, individual species of ectomycorrhizal fungi can be studied for their effects. Brundrett et al. (1996) includes a good review of the procedures and steps involved in producing vegetative inoculum.

The production of a pure mycelium of individual fungal species from sporocarps requires tissue culturing the fungus and growing it under sterile conditions. The survival and growth of the fungal mycelium on petri dishes enriched with nutrient solution is critical for providing a supply of living fungal tissue for development of inoculum. Modified Melin Norkans (MMN) nutrient media developed by Marx (1969) has been shown to be an effective nutrient medium for most ectomycorrhizal fungi. Earlier studies have examined the nutritional requirements of ectomycorrhizal fungi in more detail (Trappe, 1977), but development of specific requirements for growth and survival of individual species in the culture was beyond the scope of this study.

In addition to production of an actively growing culture of a fungus, successful inoculation requires that the fungus remain viable long enough for the plant roots to come into contact with the mycelium for mycorrhization to occur (Trappe, 1977). Ectomycorrhizal fungi that are unable to survive and grow in petri dishes (with MMN) or in soil substrate can be excluded from further evaluation using this method.

A liquid type inoculum, typically consists of mycelial plugs of the fungi initially placed in a flask of MMN nutrient solution (without agar) maintained under sterile conditions for subsequent growth (Cline, et al. 1982). Blending can be considered for distributing the liquid inoculum to the host plant, but it may reduce viability of the inoculum by disrupting hyphal growth. Conversely, other studies suggest blending, or mixing of inoculum can increase the number of fungal propugules, providing more points of contact for hyphal growth, thereby increasing the overall effectiveness of the inoculum in forming mycorrhizae for some species of fungi (Molina & Trappe, 1982). Based on experimental findings it appears that different species of ectomycorrhizal fungi have varying levels of sensitivity to blending or mixing of hyphae (Boyle et al. 1987). Liquid inoculum is also susceptible to contamination by other organisms.

Development of a soil type inoculum is another possibility for mycorrhization. A soil inoculum is a mixture of liquid nutrient media and soil, colonized by the mycelium of ectomycorrhizal fungi under sterile conditions. Ectomycorrhizal fungi colonize the sterile soil to a point where the inoculum can be applied in non-sterile conditions. The goal is for the desired fungus to survive long enough to form mycorrhizae with the host plant. The slow growth of ectomycorrhizal fungi in comparison to pathogenic or saprophytic fungi is often a limiting factor in the formation of mycorrhizae in greenhouse

studies. Soil inoculum, specifically when vermiculite is included in the mix, provides mycorrhizal fungi an important niche where it has been suggested that they are protected from environmental stress and microbial competition (Marx & Ruehle, 1991). Marx & Bryan (1975), describe a method for producing soil inoculum that continues to be the basis for producing viable inoculum today (Brundrett et al. 1996 & Brunner, 2001).

The correct combination of media properties, such as pH, temperature, water holding capacity, niche habitat, and CEC, are critical considerations for fungal survival and growth in soil inoculum. A study by Mason (1980) using a soil inoculum under sterile conditions, found that the different nutritional needs of various ectomycorrhizal fungi meant that certain nutrient media combinations or concentrations might be precluded from use for certain species.

In vitro studies involving mycorrhizal fungi typically use a nutrient solution of MMN under sterile conditions in tubes or flasks often with peat and vermiculite (Molina & Palmer, 1982), which encourages fungal growth. However, under non-sterile conditions, the use of MMN media can encourage the colonization of contaminant fungi, which out-compete mycorrhizal fungi in high nutrient environments. Brunner (2001) reported high levels of mycorrhization using a diluted mixture of MMN applied to seedlings of Norway Spruce (*Picea abies*), which reduces contamination potential.

The method of inoculation is also important for successful mycorrhization of plants in the greenhouse. "Banding" has been shown to be an effective method for inoculation of plants (Riffle & Maronek, 1982). Placing a layer, or "band" of soil inoculum just below developing root systems, is expected to bring the fungus in contact

with the roots as they grow through the band (Marx & Kennedy, 1982). The goal is to infect roots with the mycorrhizal fungus and ultimately mycorrhizae are formed. Root system development is an important consideration in mycorrhizal studies because roots that develop too quickly, especially in the greenhouse, might grow beyond the source of inoculum in the container, thus reducing mycorrhization. This is a particular problem with faster growing species such as aspen (*Populus*), birch (*Betula*), and oak (*Quercus)*.

A liquid inoculum can be applied to the soil surface of plants, or injected into the soil before or after plants are added (Marx et al. 1991). Gagnon et al. (1995) produced a liquid inoculum in fermenters that were gently agitated, and applied the inoculum by mixing it with soil just before seeding, with good results.

Similarly, mycelial plugs and basidiospores can be applied as inoculum in various ways. Plugs or spores can be added directly to the surface of the soil, or injected into it at various levels, all of which will affect survival of the fungus, its speed of growth, and ultimately mycorrhization levels. Marx et al. (1995) review different methods of producing a spore slurry inoculum. Dry spores can also be used, although utilizing a carrier such as hydro-mulch to apply the spore inoculum or pelletizing spores is suggested to increase its effectiveness (Marx & Bell, 1985)

Another factor that can affect mycorrhization in the greenhouse is the level of fertilization (Dixon et al. 1995; Gagnon et al. 1995; Ruehle, 1982). While it may be necessary to fertilize plants in pots in greenhouse, conditions to sustain growth, the high fertilization levels typically used in greenhouses can preclude mycorrhization. Fertilization levels that allow for mycorrhization need to be determined for each plant species and will be addressed for aspen in Chapter 3.

The methods for use of mycorrhizal fungi with trees for pot studies, for out-planting in field studies, and for practical reclamation purposes has primarily focused on conifer trees, such as pine, and use of a commercial inoculum such as *Pisolithus tinctorius* (Marx et al. 1991). A majority of the references and methods cited above are for coniferous trees and the fungi that associate with them. Little is known about the conditions necessary for mycorrhization of aspen in pots under greenhouse conditions. If aspen is to be useful as a reclamation or reforestation tree, then the first step is development of a practical method for mycorrhization of its root systems in the greenhouse so the system can be studied and applied.

Cripps and Miller (1993) showed that over 60 species of mycorrhizal fungi occur with aspen in southwest Montana, and a subset of these are on sites disturbed by mining (Cripps, 2001, 2003). An *in vitro* study of several selected fungi showed that most could form mycorrhizae under sterile conditions with aspen in large test tubes (Cripps & Miller, 1993; Cripps & Miller, 1995; Cripps, 2001, 2003). A majority of the fungi tested promoted the growth of aspen under these condition with biomass increases of 200 to 450% after 3 months, depending on the fungus used as inoculum. One fungus (*Inocybe lacera*) known to be important on disturbed sites killed all the aspen seedlings under high nutrient conditions.

Furthermore, results from studies of conifer trees inoculated with particular mycorrhizal fungi *in vitro* in non-sterile pots and in the field are not consistent (Molina & Palmer, 1982). Results from *in vitro* studies do not always translate to results for pot studies or out-plantings (Aggangan et al., 1998). If the ultimate goal is to maximize

survival of out-plantings, then the next step for aspen is to study the species in non-sterile pots in the greenhouse, with an ultimate goal of out planting aspen on reclamation sites.

Research Goals

Some of the same species, which showed high potential value for reclamation of mined land in a previous study (Cripps, 2001, 2003) were re-collected and re-isolated for the present study. Preliminary experiments were necessary to determine the appropriate source and age of plant material (cuttings, seeds), potential sources of fungal material (sporocarps, spores, mycorrhizae), viability of the inoculum source (plugs, spores, liquid, soil), method of inoculation (banding of soil inoculum, liquid applied to the soil surface, spore dissemination, or plug placement), and greenhouse conditions necessary to develop a system where mycorrhization of aspen could occur.

While these small trials are not statistical in nature, they are a necessary starting point for development of methodology and selection of fungi for larger trials with aspen (Chapters 3 & 4), and helped eliminate non-viable alternatives.

Materials And Methods

Site Description

The east ridge of the Butte, Montana site lies east of the continental divide at an elevation of 5900', approximately 45° 57' N latitude and 112° 29' W longitude. Acidic sandy soils, developed from parent material of the Idaho and Boulder batholiths eroded from uphill sites as colluvium, predominate on the site. Stands of aspen are scattered in the immediate vicinity on gentle slopes generally facing northwest. The area has been

impacted by fumes primarily from copper smelters operating from the 1880's to the 1960's located in the surrounding area (Taskey, 1972).

The climate is generally affected by continental air masses with precipitation averaging 32.5 centimeters per year and temperatures ranging from –9.4 to 16.7 ^{o}C with a yearly mean average of 4.2 ^{o}C. Most precipitation occurs during spring and summer months as rain.

Collection Of Fungi For Use As Potential Inoculum

Eight species of mycorrhizal fungi collected by Mahony and Cripps and identified by Cripps (1992), as forming ectomycorrhizae with aspen were collected at two smelter impacted sites within a pure stand of aspen trees on the east ridge of Butte, Mt in the fall of 2002. One species of fungus was collected near Gardner, Montana, within a thermal area with coniferous tree species. Observation of macroscopic features, such as overall color, gill attachment, cap shape, and other physical characteristics, contributed to identification of the fungi. When possible, multiple specimens were collected of various sizes and maturity to insure proper identification, and to provide enough material for tissue culturing. Fungi were placed in wax paper and transported to Montana State University, where they were immediately photographed, described, numbered, and tissue cultured (Figure 1).

Figure 1. Sporocarps of collected ectomycorrhizal fungi found with aspen. A. *Laccaria proxima*. B. *Inocbye lacera*. C. *Leccinum auranticum*. D. *Paxillus vernalis*. E. *Pisolithus tinctorius* (conifers). F. *Scleroderma citrinum*. G. *Tricholoma flavovirens*. H. *Tricholoma scalpturatum*. Not shown *Hebeloma insigne.* Photos by C. Cripps

In addition, spore prints were made of each fungus, for microscopic examination of spore color, size and shape. Ten spores of each species were measured length and width when necessary to complete the identification. Specimens were dried as vouchers and deposited in the MONT herbarium at Montana State University. Specimen descriptions are located in Appendix A. The ectomycorrhizal fungi collected, their corresponding voucher number, location, collection date, and host are listed in Table 1.

Table 1. Ectomycorrhizal fungal species collected fall 2002, primarily with aspen (*Populus tremuloides*).

Voucher #	Species	Location	Time of year	Host Species
NBCM11	*Pisolithus tinctorius*	Gardiner, MT.	Sept 21	conifers
ERCM12	*Paxillus vernalis*	East ridge of Butte, MT.	Sept 12	aspen
ERCM21	*Scleroderma citrinum*	East ridge of Butte, MT.	Sept 12	aspen
ERCM31	*Tricholoma scalpturatum*	East ridge of Butte, MT.	Sept 12	aspen
ERCM41	*Hebeloma insigne*	East ridge of Butte, MT.	Sept 12	aspen
ERCM51	*Inocybe lacera*	East ridge of Butte, MT.	Sept 12	aspen
ERCM52	*Tricholoma flavovirens*	East ridge of Butte, MT.	Sept 12	aspen
ERCM61	*Leccinum auranticum*	East ridge of Butte, MT.	Sept 12	aspen
ANCM142	*Laccaria proxima*	Anaconda, MT.	Sept 12	aspen

Source Of Plant Material

Aspen roots were collected from an aspen grove at a site in southwestern Montana, during the fall of 2002 for vegetative propagation. The following method described by Schier (1978) for propagating plant material was used to evaluate the possibility of efficiently using cuttings for mycorrhizal studies:

1. Clones with desirable phenotypic characteristics were selected.
2. Cuttings were taken from lateral roots, 1-2.5 cm diameter.
3. Soil was washed from roots.
4. Roots were cut into 10-20 cm lengths.
5. Root segments were treated with fungicide and ends sealed with paraffin.
6. Root segments were planted to a depth of 1-2 cm in vermiculite in trays, and vermiculite was moistened.
7. Root segments were maintained at 15-25 ^{o}C.
8. Segments were watered lightly every day.

9. After 6 weeks suckers 2 cm and larger were removed from root segments.
10. The basal ends of sucker cuttings were treated with indole butyric acid (IBA) in powder or 50% alcohol.
11. Sucker cuttings were inserted in moist vermiculite:perlite (1:1) so that one half to one third of stem was in the medium.
12. Sucker cuttings were placed on a misting bench (or high-humidity polyethylene chamber).
13. After 2-3 weeks rooted cuttings were removed from rooting medium.
14. Rooted cuttings were planted in containers with peat moss:vermiculite (1:1) and fertilized with a complete fertilizer.
15. Supplemental lighting was provided so growth was prolonged during short days.

Aspen seed was also evaluated in early trials for viability and the ability to produce homogeneous seedlings. Aspen seed, collected in Utah, was purchased from Western Native Seed, germinated and grown in the greenhouse. In initial tests, germination rates of 70% for aspen seeds were typical on moistened paper towels. Later experiments utilized seed purchased from Bitteroot restoration and collected by them in Silverbow County, Montana. Aspen seedlings were sown on peat:vermiculite (1:1) with at least three seeds placed in each soil plug to insure seed germination. Aspen seed germination typically takes approximately 24 hours. G.E. multi-Vapor MVR1000/C/U lights (General Electric Company Nela Park Cleveland, OH 44112) were utilized to supplement natural light to simulate a 16-hour photoperiod. Primary root development is typically slow and as a result the aspen seedlings initially depend on a brush of long delicate hairs to absorb water and anchor the seedling to the seedbed (Perala & Alban, 1982). Plants were initially watered daily from below, until the aspen seedlings had developed to the point where they would not become dislodged from watering above. Watering needs were based on visual observation of the soil surface. When the soil surface was dry, plants were watered to field capacity. Day temperatures ranged from

18.3-26 ºC, night temperatures 10-23.9º C. Aspen were thinned after one week so only one seedling remained in each soil plug. A light dose of fertilizer 20-20-20 (NPK) was applied after the appearance of the second set of leaves. Procedures for aspen seedling germination and growth in the greenhouse generally followed those recommended by Tinus and McDonald (1979) for plants in containers.

Evaluating Sources Of Inoculum

After splitting open the sporocarp to minimize contamination, small pieces of fungal tissue were removed and cultured using the sterile technique described by Molina and Palmer (1982). The tissue was placed in petri dishes containing modified Melin Norkans (MMN) nutrient agar (Marx, 1969) consisting of: 3 g malt extract, 5 g d-glucose, 0.25 g $(NH_4)HPO_4$, 0.5 g KH_2PO_4, 0.15 g $MgSO_4(7H_2O)$, 0.05 g $CaCl_2$, 1.2 ml (1% solution) $FeCl_3$, .025 g NaCl, 100 ul (1% solution) Thiamin, 1000 ml distilled water, and 11 g agar. Petri dishes inoculated with fungi were grown at room temperature. The survival and growth of the ectomycorrhizal fungi in sterile culture was confirmed by observation of mycelial development. Hyphae of basidiomycetes typically have clamp connections, which aided in confirming the desired taxa was present. Further confirmation of the desired ectomycorrhizal fungus relied on morphological features of the mycelial colonies (Hutchinson, 1991). Plates that became contaminated with undesirable fungi and bacteria were removed from the collection. Plates were re-cultured under sterile conditions when necessary to insure pure cultures of the desired species of ectomycorrhizal fungus. Mycelium grown in petri dishes was used directly as agar plugs, or added to liquid media, or soil for further growth.

Spores of *Pisolithus tinctorius* were also tested as a direct source of inoculum. Spores were collected from sporocarps and scattered on the soil surface around the aspen seedlings. Plants were immediately watered to incorporate the spores into the soil matrix.

Evaluation Of Fungal Growth And Survival For Selection Of Vigorous Strains

In experiment 1, the growth of nine ectomycorrhizal fungal species on petri dishes was rated and categorized as follows: + for minimal mycelial growth, ++ for standard mycelial growth, and +++ for vigorous fungal mycelial growth. This was primarily to determine which fungi would grow in culture.

In experiment 2, fresh petri dishes were inoculated with a 5 mm colonized mycelial plug and evaluated for hyphal growth radially across the petri dish. Three measurements of mycelial growth were taken from the point of inoculation outward (at 120° intervals) from 6 different plates inoculated with the same ectomycorrhizal fungus, and results were averaged to provide a measurement of fungal hyphal growth in centimeters. This provided a more definitive comparison of vigorousness of growth.

Development And Evaluation Of Inoculum Types For Potential Use With Aspen

Soil and liquid inoculum were developed and used as the primary sources of fungi for inoculation of aspen in pots (Brundrett et al., 1996; Cline et al., 1982; Dixon et al., 1985; Riffle & Maronek, 1982). Additionally, readily available spores of one fungus (*Pisolithus tinctorius*), and colonized mycelial plugs of a few others were evaluated for mycorrhization of potted plants in the greenhouse. The methods for production of inocula are as follows:

Liquid Inoculum: Liquid media (MMN, minus the agar) was autoclaved in 200 ml flasks for 30 minutes at 20 psi. After cooling, mycelial plugs of fungi grown in petri dishes were added under sterile conditions to the liquid media.

1. Flasks were filled 1/3 full with MMN minus the agar.
2. Flasks were autoclaved for 30 minutes.
3. After liquid had cooled, actively growing mycelial plugs were added using sterile technique.
4. Flasks were covered with aluminum foil.

Experiment 3 visually graded hyphal growth in the sterilized flasks of liquid MMN to determine comparative rates. Liquid inoculum was prepared according to procedures outlined in Brundrett et al. (1996).

Soil Inoculum: Various levels of nutrient solution were applied to determine the most effective level for growth of mycorrhizal fungi in soil to be used as an inoculum. A minimum threshold of nutrients is necessary to promote hyphal growth in the soil. Soil with excess nutrients may be easily contaminated when applied to plants under non-sterile conditions. In a preliminary experiment 50, 100, 175, 200 and 300 ml of MMN were added to jars with a peat and vermiculite mix, utilizing standard sterile techniques. Hyphal growth was evaluated both visually and quantitatively. An optimum level (200 ml) was selected for use in experiment 4.

The goal of experiment 4 was to evaluate fungal growth in mason jars of peat and vermiculite, for development of a soil inoculum.. Jars filled with peat and vermiculite (1:9 ratio), were autoclaved for 60 minutes at 20 psi, after the addition of liquid MMN media minus the agar (Molina, 1979). Each jar contained approximately 500 ml of peat:vermiculite mix (in which large particles of peat and small particles of vermiculite

were removed) and approximately 200 ml of liquid MMN without agar. The following day, colonized mycelial plugs were added under sterile conditions to the peat/vermiculite jars. To allow for gas exchange while minimizing potential contaminants, holes were drilled (3/4") in jar lids and covered with filter paper (Whatman #4 70 mm).

In Experiment 5, the hyphal growth of ectomycorrhizal fungi was quantified in sterile test tubes of soil. Soil and fungi were prepared as above except test tubes were substituted for jars. Test tubes were utilized because the small diameter tubes allowed a more accurate visual inspection of comparative hyphal growth rates. Tubes were filled with approximately 25 ml of peat:vermiculite (1:9) and 10 ml MMN nutrient solution, covered with aluminum foil and autoclaved for 30 minutes. Mycelial plugs were added utilizing sterile technique after the soil cooled (Mason,1980). Mycelial growth was measured vertically on three sides of each tube, consisting of six replicates for each fungi, and results were averaged.

Mycelial Plugs And Spore Inoculum: Mycelial plugs colonized by fungi from sterile petri dishes were also used to inoculate aspen, and spores of *Pisolithus tinctorius* were used because they are produced in quantity within the sporocarp.

Evaluation Of Techniques For Inoculation Of Aspen With Mycorrhizal Fungi

Plugs, soil inoculum, liquid inoculum, and spores were used to evaluate the effectiveness of different methods of fungal application for aspen seedlings. After one month of growth, 18 aspen seedlings were replanted in 3” pots and colonized mycelial plugs of *Pisolithus tinctorius* and *Laccaria proxima*, or spores of *Pisolithus tinctorius* were added. Colonized mycelial plugs were placed above or below the soil surface to

determine if inoculum placement affected fungal survival and mycorrhization of aspen seedlings. Spores were placed directly on the soil surface. Plants were then watered to optimize spore germination and dissemination into the soil.

Liquid inoculum and soil inoculum were also assessed for their effectiveness in mycorrhization of aspen in non-sterile greenhouse soils. For liquid inoculum, plants were watered with a slurry comprised of fungal mycelium and liquid media, which was applied to the soil surface following procedures from Boyle et al. (1987):

1. Excess MMN was drained from flasks.
2. Distilled H_2O was added as a rinse to the flask and then poured off.
3. Cheesecloth was used to prevent mycelium from spilling out during rinsing.
4. Mycelium was rinsed 3 times.
5. Mycelium was then re-suspended at approximately 2 g wet weight with 100 ml of distilled H_2O to get a 2% mycelium slurry mix.
6. Mycelium was blended for 10 seconds.
7. Large pipettes were used to transfer mycelium to pots (8 ml per pot).

For soil inoculum, the fungal-peat-vermiculite mix was placed in a band at the root zone of 1 and 2 month old aspen seedlings that were repotted at time of inoculation. Different jars of soil inoculum with the same species of ectomycorrhizal fungi were mixed prior to banding. The general procedure followed that for soil inoculum as described in Marx and Bryan (1975).

1. The inoculated peat/vermiculite mix was wrapped in cheesecloth and gently washed with distilled H_2O, 3 minutes (Marx & Bryan, 1975).
2. Leaching may not be necessary if fungi utilized most of the carbohydrates (sugar) (Marx & Ruehle, 1991).
3. Three inch pots were filled ¾ with media (1 part peat:6 parts vermiculite).
4. Water is added to pots.
5. One inch of inoculated peat:vermiculite (1:9) mix was added in a band (Riffle & Maronek, 1982) to pots on top of lower layer.
6. The plant/cutting was placed directly over the peat/vermiculite inoculum.
7. Pots were then backfilled with soil.

Plants roots were visually examined for mycorrhizal development after three months following the methods described below.

Preliminary Assessment Of Mycorrhizal Formation

In experiment 6, the test tubes originally inoculated with mycelial plugs of ectomycorrhizal fungi to evaluate hyphal growth were planted with 30-day-old non-sterile aspen seedlings placed directly above the inoculated peat:vermiculite (1:9) mixture. Some tubes did not appear to contain viable hyphae, and mycorrhizae were not expected to form in these. Mycorrhization of aspen seedlings was visually assessed in the test tubes after a period of 120 days.

In experiment 7, a soil of equal parts Bozeman silt loam, washed concrete sand, and Canadian sphagnum peat moss was aerated and steam pasteurized at 80 degrees C for 45 minutes and used as substrate for experiments to evaluate mycorrhization of aspen seedlings. After two months in the soil plugs, aspen were transplanted into 3" pots above a band of fungal inoculum. Mycorrhization of the aspen roots was visually assessed.

Identification And Confirmation Of Mycorrhizal Types On Roots

In these trials, roots were assessed according to a checklist from Agerer (1987-1996) to determine the presence or absence of a particular mycorrhizal fungi. Identification of the mycorrhizal fungi was based on morphological features of the short roots attributed to individual species according to Agerer (1987-1996). Smith et al. (1997) and others recognize the formation of a Hartig net, a sheath, and extra radical mycelium (for some) to indicate the presence of ectomycorrhizae on roots.

Root tips were assessed for the percentage of mycorrhizal infection on the host plant root system.

Results

All the ectomycorrhizal fungi tested were capable of growth in culture (Figure 2), but exhibited differential growth rates according to conditions. Similarly, the type of inoculum, inoculation method, and soil conditions influenced fungal growth, which varied by species and conditions, all of which can ultimately influence mycorrhization.

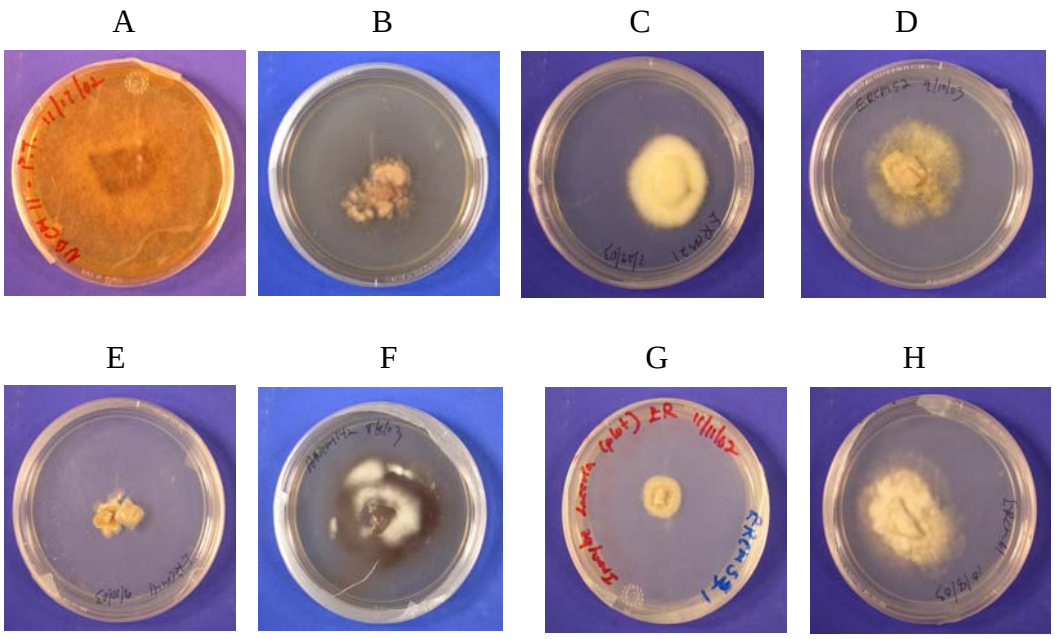

Figure 2. Ectomycorrhizal fungi on Petri-dishes of MMN. A. *Pisolithus tinctorius.* B. *Paxillus vernalis.* C. *Scleroderma citrinum.*. D. *Tricholoma flavovirens.* E. *Hebeloma insigne.* F. *Laccaria proxima.* G. *Inocybe lacera.* H. *Leccinum auranticum*. Not shown is *Tricholoma scalpturatum.* Photos do not necessarily reflect comparative values from Table 2.

Growth And Survival Of Ectomycorrhizal Fungi In Culture

In experiment 1, the hyphal growth of nine species of ectomycorrhizal fungi was evaluated in petri dishes of MMN media for their ability to grow in culture (Table 2).

Paxillus vernalis, *Pisolithus tinctorius*, *Laccaria proxima*, *Inocybe lacera*, and *Scleroderma citrinum* mycelium grew at a faster rate than the other species of ectomycorrhizal fungi.

Table 2. Hyphal growth of ectomycorrhizal fungi on petri dishes, in mason jars, and test tubes and mycorrhizal development in non-sterile test tubes and 3" pots.

Fungal species	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7
	Petri dishes	**Petri-dish (radial growth after 27 days)**	**Liquid media (MMN) in flasks**	**Mason jars of peat, vermiculite (1:9)**	**Test-Tube (cm) after 27 days**	**Mycorrhizal Formation in Test-tubes***	**Mycorrhizal Formation in greenhouse soil mix with soil and liquid inoculum after 60 days in 3" pots**
Pisolithus tinctorius	+++	4.50 cm	+	+++	5.58 cm	3 (59 days)	+
Paxillus vernalis	++	1.33 cm	+	++	0.33 cm	2 (118 days)	+
Scleroderma citrinum	++	2.12 cm	+	++	0 cm	0	-
Tricholoma flavovirens	+	0.57 cm	0	++	0.83 cm	1 (118 days)	-
Hebeloma insigne	+	0.48 cm	0	NA	0 cm	1 (118 days)	NA
Laccaria proxima	++	2.38 cm	++	++	9.10 cm	0	NA
Tricholoma scalpturatum	+	NA	0	++	NA	NA	NA
Inocybe lacera	++	0.57 cm	+	+	0.07 cm	0	NA
Leccinum aurantiacum	+	0.93 cm	NA	NA	NA	NA	NA

* 0=no colonization, 1=low, 2=moderate, 3=extensive, NA=not tested

In experiment 2, the radial growth rates of ectomycorrhizal hyphae in petri dishes were measured in centimeters over a period of 27 days (Table 2; Figure 3). Results were similar to those of experiment 1, with three of the species showing the most vigorous

growth, followed by *Paxillus vernalis*. *Tricholoma scalpturatum* was not tested.

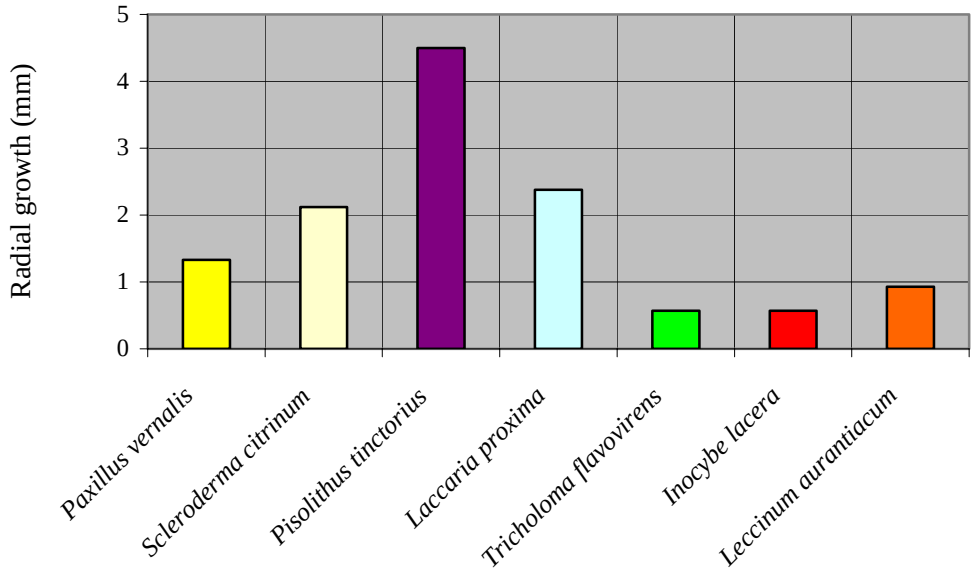

Figure 3. Growth of 7 species of ectomycorrhizal fungi on sterile petri dishes, enriched with MMN nutrient solution after 27 days.

In experiment 3, the hyphal growth of ectomycorrhizal fungi in sterile flasks with liquid MMN nutrient solution was visually quantified (Table 2). *Tricholoma scalpturatum*, *Tricholoma flavovirens*, and *Hebeloma insigne* did not grow in the liquid inoculum. *Leccinum aurantiacum* was not tested. *Pisolithus tinctorius*, *Paxillus vernalis*, *Scleroderma citrinum*, *Laccaria proxima* and *Inocybe lacera* were all able to grow in liquid inoculum. However, only *Laccaria proxima* colonized the liquid media sufficiently be utilized as inoculum for seedlings.

A small experiment (preliminary to experiment 4) investigated levels of MMN nutrient solution necessary for hyphal growth in the development in soil for use as inoculum (Table 3). Mixing (shaking) the soil in the jars colonized by fungal the hyphae,

appeared to disrupt hyphae and retard growth.

Table 3. Evaluation of nutrient levels for *Pisolithus tinctorius* growth under various nutrient regimes for soil inoculum. Date of initiation 10/21/02.

Jar 1 (350 ml MMN)	Not mixed	Hyphae present 11/17/02
Jar 2 (175 ml MMN, 175 ml deionized H_2O)	Mixed	No visible hyphae as of 11/17/02
Jar 3 (200 ml MMN, 150 ml deionized H_2O)	Not Mixed	Hyphae present 11/17/02
Jar 4 (100 ml MMN, 250 ml deionized H_2O)	Not Mixed	Hyphae present 11/17/02
Jar 5 (50 ml MMN, 300 ml deionized H_2O)	Mixed	No visible hyphae as of 11/17/02
Jar 6 (200 ml MMN, 150 ml deionized H_2O)	Not Mixed	Hyphae present, not on soil as of 11/17/02

Results showed that 100, 200 and 350 ml of MMN nutrient solution added to jars of peat:vermiculite (1:9) were sufficient for hyphal growth in sterile soil to be used to inoculate plants. Since higher nutrient levels can contribute to contamination and pathogenicity, and lower levels might be limiting over time, the 200 ml level was selected for further testing.

In experiment 4, the growth of 9-ectomycorrhizal fungi was evaluated based on hyphal colonization of peat:vermiculite and 200 ml MMN media in mason jars (Table 2). *Pisolithus tinctorius* consistently colonized the mason jars at a faster rate than the other fungi. *Laccaria proxima*, *Scleroderma citrinum*, *Paxillus vernalis*, *Tricholoma flavovirens* and *Tricholoma scalpturatum* all grew at a similar rate, but lagged behind *Pisolithus tinctorius*. *Inocybe lacera* grew slowly and *Hebeloma insigne* and *Leccinum aurantiacum* were not tested.

In experiment 5, long narrow test tubes were used to refine quantification of hyphal growth over a period of 27 days (Table 2, Figure 4). *Tricholoma scalpturatum* and *Leccinum aurantiacum* were not evaluated because of the lack of available mycelium at the initiation of the experiment. *Laccaria proxima* significantly out grew the other ectomycorrhizal fungi tested under these conditions. *Pisolithus tinctorius* colonized approximately half of the volume of the test tubes, while *Paxillus vernalis*, *Tricholoma flavovirens*, and *Inocybe lacera* initiated minimal hyphal growth. *Hebeloma insigne* and *Scleroderma citrinum* did not grow. It should be noted that soil in the test tubes became fairly dry over the course of the trials and could possibly have selected for ectomycorrhizal species more adapted to dry conditions.

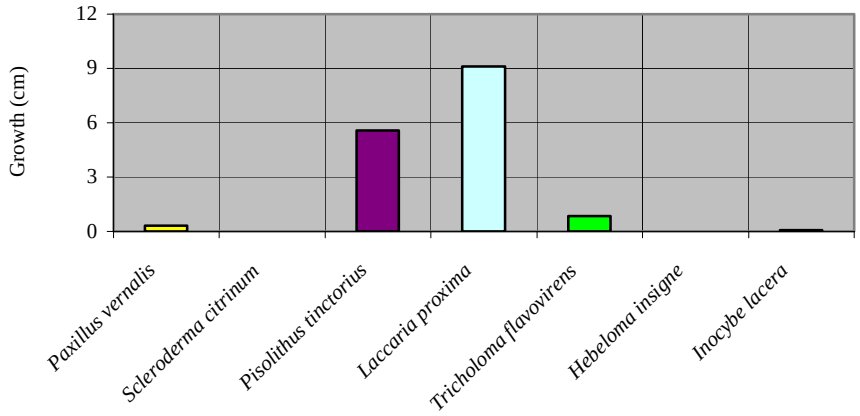

Figure 4. Growth of 7 ectomycorrhizal fungi in test tubes containing peat:vermiculite (1:9) enriched with MMN nutrient solution. Results are an average of cm of growth along the tubes.

Evaluation Of Aspen Seed And Rooted Cuttings As Source Of Plant Material

Vegetative clones produced from collected lateral roots were not as effective as seed in producing viable plants in a relatively short period of time. In addition, plants grown from seed are more easily manipulated in the greenhouse. While rooted cuttings

from lateral roots produce genetically identical clones, they proliferate at different rates possibly due to the varying levels of carbohydrate storage in the collected roots, and they can be infected with pathogenic fungi. Plants grown from seed could be culled to provide similar size plants in similar states of health. Rooted cuttings were variable in their growth responses, and as a result could not be grouped according to their developmental status such as height or number of leaves for inoculation.

Evaluation Of Methods For Inoculating Aspen With Mycorrhizal Fungi

In preliminary trials, various combinations of inoculum type and methods of application were evaluated for the potential to ultimately form mycorrhizae with aspen in pots. Soil, liquid, spores and mycelial plugs were tested as types of inoculum for aspen; of interest was the ability of fungi to survive and provide sufficient hyphae to form mycorrhizae. The methods of application employed were: a) use of mycelial plugs above and below the soil surface; b) spores; c) addition of liquid inoculum to the soil surface as a mycelial slurry; and d) banded soil inoculum.

Mycelial plugs taken directly from the petri dishes and placed on the soil surface quickly dried and were no longer visible. *Pisolithus tinctorius* and *Laccaria proxima* were the only ectomycorrhizal fungi tested due to availability of ready mycelial plugs. Plants inoculated by this method, regardless of whether mycelial plugs were placed above or below the soil surface, did not form mycorrhizae. In addition there was no indication that the fungi still persisted in the soil given the absence of visible hyphae for *Pisolithus tinctorius* and *Laccaria proxima*.

Spores placed on the surface failed to form mycorrhizae with aspen seedlings after 90 days. Only the spores of *Pisolithus tinctorius* were tested, since the other fungi do not produce copious quantities of spores. Sporocarps of *Pisolithus tinctorius* are essentially filled with spores amenable for use as a direct inoculum source. No visible hyphal growth was observed for *Pisolithus tinctorius* in the 3" pots.

For the liquid technique, inoculation with a mycelial slurry of *Laccaria proxima* failed to produce mycorrhizae on aspen in the greenhouse as assessed by visual inspection of aspen roots after 90 days. Further, the slow growth of the mycelium in the liquid media and high contamination levels made this method impractical for continued use in the present research. Other species were not tested.

Soil inoculum applied as a band directly below transplanted aspen seedlings proved to be an effective method for the mycorrhization of aspen in the greenhouse with *Pisolithus tinctorius* and *Paxillus vernalis*; *Scleroderma citrinum* and *Tricholoma flavovirens* did not form mycorrhizae in the time allotted. Other species were not tested due to lack of available mycelium. From these early experiments it was determined that soil inoculum would be utilized to inoculate plants for larger experiments in this study. Banding 1" soil inoculum was utilized as the primary inoculation technique, however other methods that might be more efficient and effective were evaluated later.

Conditions For Mycorrhization

In experiment 6 (Table 2), the ability of 7 ectomycorrhizal fungi growing in soil-filled test tubes to form mycorrhizae with transplanted aspen was evaluated. This

is similar to banding, except the soil is completely colonized by hyphae. Mycorrhizae were visually identified in all test tubes inoculated with *Pisolithus tinctorius* after 59 days (Table 2.). *Paxillus vernalis Hebeloma insigne* and *Tricholoma flavovirens* formed mycorrhizae with aspen seedlings in test tubes in 118 days (Table 2.). Abundant hyphae were present in *Laccaria proxima* test tubes but no mycorrhizae were observed after a period of 120 days. Mycorrhizae were not expected to form in test tubes if the mycelium did not proliferate, as was true for *Scleroderma citrinum* and *Inocybe lacera*.

In Experiment 7 (Table 2), the effectiveness of soil inoculum using the banded technique was evaluated for its effectiveness in mycorrhiza formation on aspen seedlings in greenhouse soils. *Pisolithus tinctorius* and *Paxillus vernalis* formed mycorrhizae, while *Tricholoma flavovirens* and *Scleroderma citrinum* did not form mycorrhizae at the end of 60 days (Table 2.). Other species were not evaluated due to lack of available inoculum. Subsequent tests (reported later) showed that a majority of the fungi were capable of forming mycorrhizae with aspen using a soil inoculum and banding technique with aspen.

Discussion

These preliminary experiments show that the nine native mycorrhizal fungi collected under aspen on contaminated sites can be cultured and have potential for use as inoculum. These results agree with findings of Cripps (2001, 2003), in which *in vitro* experiments showed that the same fungal species, with the exception of *Scleroderma citrinum* and *Tricholoma flavovirens* (which were not reported on), as able to survive on sterile petri dishes of MMN. The slow growth of *Hebeloma insigne* and *Leccinum*

aurantiacum prevented these isolates from subsequent evaluation in preliminary trials, although both eventually grew in the petri dishes. Further investigation showed that while most species grew in a sterile peat:vermiculite mixture, some outperformed others with *Pisolithus tinctorius* showing the most vigorous growth. In non-sterile conditions using tubes, *P. tinctorius* and *Laccaria proxima* exhibited the best mycelial growth, and *P. tinctorius, Paxillus vernalis, Hebeloma insigne*, and *Tricholoma flavovirens* formed mycorrhizae, but dry conditions appear to have been inhibitory to other species. *Pisolithus tinctorius* has been reported to form mycorrhizae with conifer seedlings one month after inoculation (Marx & Bryan, 1975) and rapidly forms mycorrhizae with aspen *in vitro* (Cripps & Miller, 1995). This could influence the selection of certain species for use as mycorrhizal inoculum on drought prone sites. The study could also reflect a bias for those fungi, which form mycorrhizae rapidly. It should be kept in mind that the local strain of *P. tinctorius* was originally isolated from conifer roots.

The use of native fungi is worthy of consideration when inoculating plants, despite the effort involved. Although over 60 species of mycorrhizal fungi have been identified with aspen, many are only found in discrete soil conditions (Cripps 2001). Native fungi are adapted to local climatic and environmental conditions. Native fungi collected beneath pure stands of aspen could provide nursery grown seedlings with a symbiont that is able to compete effectively with other naturally occurring fungi and soil biota when planted in the field. Within this subset of ectomycorrhizal fungi, successional processes will further select species (Allen, 1991). In fact succession moves in two parallel veins, succession on a large scale where plant and fungal species are gradually

replaced by later successional species, and succession at the local scale as succession moves outward from a stand as organic matter accumulates within the stand.

Succession at the local scale selects for fungal species that are adapted to particular tree age groups (Allen, 1991) and soil conditions. Some species of fungi appear to favor older areas within a stand where plant litter has accumulated, and other cycling processes have moderated soil conditions; whereas other ectomycorrhizal fungal species prefer the margins of tree stands where conditions may be less favorable to plant growth because of exposure, limited availability of nutrients, and the inability of the soil to hold water (Allen, 1991; Cripps, 2001). In planting inoculated seedlings on stressed or disturbed sites it is important that "early colonizing fungi" at the local scale are utilized in order to provide plants with a symbiont that is tolerant of poor soil development and is specifically found with young trees. All of the fungi collected and tested with aspen in this study fulfill these requirements, except perhaps *P. tinctorius*, which is common on stressed areas, but not with aspen in the west.

Successful inoculation of trees for out-planting requires that the ectomycorrhizal fungi not only form mycorrhizae with the host in the greenhouse, but that they also are able to persist after planting or at least until establishment. It has been suggested (Marx et al., 1982) that ectomycorrhizal benefits might not be realized until the mycorrhizal fungi infect 50% of the host plant root system, however this was for a discrete system with southern pines. It is not known whether a nursery grown plant with extensive mycorrhizae will be more successful than a nursery grown plant with only a few mycorrhizae when out-planted.

Commercial inoculum typically lacks fungi adapted to local environmental conditions, and, as a result, native fungi may quickly replace them (Marx & Ruehle, 1994), although native mycorrhizal fungi may be absent from drastically disturbed sites. In addition, the fungi are often exotic species, not found in the local area. It is hypothesized that native ectomycorrhizal fungi inoculum should be able to compete effectively with other organisms in field soils after out-planting, thus conveying possible future benefits to the host. Most of the commercially available mycorrhizal fungi are for conifer trees, and many are not known symbionts of aspen.

These preliminary trials have shown that mycorrhization of aspen is possible using several species of native fungi under non-sterile conditions in pots, even though aspen has a fast-growing root system; banding appears to be an effective method for mycorrhization of aspen. Further refinement is necessary to provide a reliable method for large-scale greenhouse inoculation of aspen. One consideration is determining the optimal level of fertilization necessary to allow mycorrhization to occur. This will be addressed in the next chapter.

CHAPTER 3

FERTILIZER AND INOCULATION EFFECTS ON ASPEN SEEDLING MYCORRIZATION AND GROWTH

Introduction

Inoculation of plants with ectomycorrhizal fungi has been shown to increase plant survival after out-planting on specific sites where mycorrhizal fungi may be absent (Kropp & Langlois, 1990). However, inoculation of plants requires a delicate balance of cultural methods in the greenhouse. Successful mycorrhizal formation in the greenhouse is strongly dependent on growing media, fertilization rates and inoculation techniques (Ruehle, 1980). Higher fertilization rates stimulate increased growth of plants in the greenhouse but may potentially inhibit mycorrhizal development, and plants may be less likely to survive on adverse sites (Ruehle & Marx, 1979). Highly fertilized and well-watered seedlings that are big and green may not be of value for reforestation because they may lack mycorrhizae and may have an unfavorable shoot:root ratio (Mikola, 1989).

There is overwhelming evidence that mycorrhizal fungi are important in stressed environments (Allen, 1991; Marx & Cordell, 1988; Molina & Trappe, 1982; Trappe, 1977). Seedlings often fail to survive or grow well after planting due to environmental stress when mycorrhizae are absent (Ruehle, 1980). Nutrient deficiency is common on metaliferous soils (Goodman et al. 1973), thus ectomycorrhizal fungi may be a critical component that enables plants to survive on these harsh sites.

Production of plants in the greenhouse acceptable for out-planting typically requires some level of fertilization and regular watering. Newton and Pigott (1991)

found that germinating birch seeds were unable to continue growth after development of the first pair of leaves without fertilization, illustrating the need of an external nutrient supply. Aspen seeds are likewise quite small (1-3 mm in length). Seedlings grown in containers in the greenhouse are typically given nutrient supplements in order to produce plant material of adequate size and vigor to survive when out-planted (Tinus, 1978).

Plants that receive no additional nutrient supplements in the greenhouse may not be able to survive due to decreased nutritional status and its corresponding negative effects on photosynthesis. In this case, a reduction of carbohydrates available to the fungal symbiont and a lack of host viability prevents mycorrhization (Smith & Read, 1997). When seedlings are unable to provide fungi with sufficient carbohydrates, the fungi may become a serious carbon drain (Maronek et al. 1981; Mason et al. 2000) or even act as a parasite to the seedlings (Cripps, 1993). Plassard et al. (2000) suggests that there can be a growth decrease in host plants that is related to carbon drain for fungal growth and N assimilation. Factors that control internal concentrations and composition of carbon compounds within plant roots will have a direct effect on mycorrhizal formation (Hacskaylo, 1973).

Studies have shown that fertilization of plants can prevent mycorrhization (Dixon et al. 1985; Ruehle & Marx 1979, Ruehle, 1980), although the results vary with different fertilization combinations and sources (Buschena et al. 1992; Molina & Chamard, 1983; Mason et at. 2000). Plants reared in the greenhouse are generally not stressed, potentially reducing the necessity of mycorrhizal interactions for the plant. Plants that are well fertilized and watered in the greenhouse may avoid forming mycorrhizae with fungi due

to the fact that any allocation of resources to the fungi does not yield any additional benefits (Son & Smith, 1988).

Given the nutrient needs of seedlings in artificial media, fertilization regimes in the greenhouse may need to be manipulated in order to produce viable seedlings with mycorrhizae for out-planting (Gagnon et al. 1995). Also, stressing plants at the right time, for the right length of time might increase mycorrhization of plants in the greenhouse.

Interactions between plant, soil and fungi are further complicated when the role of nutrient availability for mycorrhization of seedlings is considered not only for the greenhouse, but also for the field. Conditions that encourage mycorrhization are typically site specific; this is to be expected given the numerous species of native mycorrhizal fungi and their wide range of hosts and habitat types (Allen, 1991). A general review of the potential effects of ectomycorrhizal fungi on host plant growth and survival is necessary to introduce concepts that will be discussed later in detail. A brief discussion detailing some of the difficulties involved in mycorrhizal studies will help in understanding the importance of continued work in this area, and will be followed by a description of the research goals of this project.

Ectomycorrhizal Fungi In Native Habitats

Ectomycorrhizal fungi are important in the uptake of nutrients that are found in weak concentrations in soil solution or that are relatively immobile (Kropp & Langlois, 1990). Nutrients may be further conserved in the system against leaching by the

immobilization of elements in fungal tissue (Danielson et al. 1983; Fogel, 1980). The extraradical mycelia of mycorrhizal fungi has the ability to exploit nutrients beyond the root depletion zones, and, as a result, plants benefit mainly because of an increase of absorptive surfaces (Landeweert et al. 2001; Van Tichelen & Colpaert, 2000). Thus, ectomycorrhizal fungi are able to mobilize and transport nutrients that might otherwise be unavailable to the plant (Hartly et al. 1997; Smith & Read, 1997) particularly phosphorus and N (Abuzinadah & Read, 1988; Brandes et al. 1998; Fogel, 1980; Jentschke et al. 2001; Jones et al. 1998; Plassard et al. 2000). In addition, mycorrhizal seedlings are generally more resistant to drought than non-mycorrhizal seedlings as a result of the increased absorptive surface of roots (Kropp & Langlois, 1990).

Early work with mycorrhizal fungi concentrated on the ability of the fungi to alleviate phosphorus deficiencies in the soil (Harley & Smith, 1983) because experimental results showed that mycorrhizal plants absorbed more phosphorus from the system than non-mycorrhizal plants. In addition it is well known that phosphorus is often limiting in natural soils (Harley & Smith, 1983). While the total amount of phosphorus in soil can be high, it is generally immobile and unavailable for plant uptake because of its adsorption, precipitation or conversion to recalcitrant organic forms (Chalot et al. 2002). The ability of ectomycorrhizal fungi to enhance phosphorus uptake in host plants has been well documented (Smith & Read, 1997; Chalot et al. 2002), although considerable variation exists among different species of ectomycorrhizal fungi and host plants (Kropp & Langlois, 1990). In discussing the role of mycorrhizal fungi,

Bolan (1991) credits the increase in phosphorus absorption by plants to the decrease in distance phosphorus ions must travel and to the increased surface area available for absorption as a result of the mycorrhizal association. Jones et al. (1998) found ectomycorrhizal plants were more efficient than non-mycorrhizal plants at acquiring phosphorus per unit of carbon allocated below ground. Plant growth and seedling phosphorus content correlated with ectomycorrhizal fungi inoculation, and phosphorus inflow rates were higher in mycorrhizal plants (Jones et al. 1998). Ectomycorrhizal fungi have been shown to be efficient at phosphorus acquisition during early stages of infection, typically in spring when nutrients may be most limiting and carbon flow is most limiting, although uptake efficiencies will vary according to the development of mycorrhizas (Jones et al. 1991). In one study, root phosphorus values in mycorrhizal plants were higher than non-inoculated plants, indicating ectomycorrhizal roots can be a major sink for phosphate (Bougher et al. 1990; Finlay & Read, 1986).

Phosphorus deficiency has been shown to stimulate the mycelium (Wallander & Nylund, 1991), hyphal growth, foraging and translocation of phosphorus from local phosphorus-rich sites in the soil (Jentschke et al. 2001a; 2001b). The stimulation of fungal growth as a result of phosphorus deficiency increases the carbohydrate pool for plants (Wallander & Nylund, 1991), which may explain why Bougher et al. (1990) found growth responses of mycorrhizal plants to be greatest at low concentrations of phosphorus.

The ectomycorrhizal fungi, *Paxillus involutus* has been shown to play an active role in phosphorus acquisition with a corresponding increase in host growth (Jentschke et

al. 2001). Mycelial growth of this ectomycorrhizal fungus was increased by the addition of phosphorus and in turn actively transported phosphorus to neighboring plants deficient in phosphorus (Jentschke et al. 2001).

More recent studies have examined the role of ectomycorrhizal fungi in N acquisition (Abuzinadah & Read 1989; Chalot & Brun 1998). A large fraction of the total N in temperate forest soil systems is present in organic form (Read & Perez-Moreno, 2003; Wu & Koide, 2003), although the only forms that higher plants can utilize are ammonium and nitrate (Marschner, 1986).

Thus, bioavailability of N in temperate forests is a limiting factor in plant growth (Alexander, 1983). The trees dominating these ecosystems are predominantly ectomycorrhizal and nutrient uptake is thus dependent on the activity of the ectomycorrhizal fungal partners (Read, 1990). Ammonium can be the dominant form of available N in forest ecosystems (Alexander, 1983; Finlay et al. 1989), although nitrate (Plassard et al. 2000) and organics (Bending & Read, 1995; Wallenda & Kottke, 1998) are also important N sources. Some ectomycorrhizal fungi have been shown to be able to utilize ammonium, nitrate, and organic N (Abuzinadah & Read 1989; Chalot & Brun 1998). Ammonium assimilation into plant tissue requires less energy expenditure by plants than nitrate assimilation (Salsac et al. 1987). Also, ammonification can be carried out by many different soil organisms (Salsac et al. 1987) whereas, nitrification requires particular bacteria. The significance of mycorrhization for improved uptake of ammonium, nitrate and organic N has been shown in many studies (Finlay et al. 1988, 1989; Fogel, 1980; Javelle et al. 1999; Plassard et al. 2000; Wallander & Nylund, 1989) although considerable variation exists between N forms and species of ectomycorrhizal

fungi. N assimilation follows different pathways dependent on N form, possibly explaining some of the variable responses found in nutrient studies with ectomycorrhizal fungi (Wallenda & Kotte, 1998).

The ability of some ectomycorrhizal fungi to acquire and transport N ultimately reduces the steps for N assimilation into the plant (Abuzinadah & Read, 1986b). This, coupled with the ability of mycorrhizal plants to use a wider range of N sources than non-mycorrhizal plants could help explain why mycorrhizal plants have a selective advantage over non-mycorrhizal plants in N limited systems (Plassard et al. 2000).

Studies have suggested a role for some ectomycorrhizal fungi in the decomposition of organic substances (Bending & Read, 1995; Read & Perez-Moreno, 2003), which can potentially provide plants with an additional source of N, although there is debate concerning its relevance in nature (Wu & Koide, 2003). A more complete discussion on the role of ectomycorrhizal fungi in the decomposition of organic material will be discussed in Chapter 4.

Recent evidence suggests that some ectomycorrhizal fungi may also be involved in the direct weathering of primary minerals (Breemen et al. 2000; Wallander, 2000), although conclusive evidence remains elusive. The role of mycorrhizal plants in direct mineral weathering, and the potential for a corresponding increase in plant growth will be discussed in Chapter 4.

Extramatrical hyphae not only extend the root system of host plants, but they have also been shown to interconnect the root system of host plants (Finlay & Read, 1986 Simard et al. 1997). The ability of ectomycorrhizal fungi to absorb nutrients and water from the soil in addition to an ability to transfer carbon compounds between plants

(Finlay & Read, 1986) may increase seedling survival where photosynthesis is limited by shade (Kropp & Langlois, 1990). However, there is some controversy on this point.

The importance of nutrients other than N and P in natural systems in sustaining and promoting plant growth should not be underestimated when considering benefits conveyed to host plants by mycorrhizal fungi. Jentschke et al. (2001) concluded that ectomycorrhizal hyphae not only translocate phosphorus and N to the host plant, but also potassium and magnesium. Other studies have also shown that mycorrhizal root infection of forest trees increased the uptake of potassium (Rygiewicz & Bledsoe, 1984), while Bending and Read (1995a) found increases in calcium and magnesium uptake in mycorrhizal plants.

Although the benefits of ectomycorrhizal fungi on host plant growth in the field have been demonstrated in a number of studies (Hartly et al. 1997; Smith & Read, 1997), separating out the critical direct benefits of ectomycorrhizal fungi on host nutrition are difficult. Secondary indirect benefits as a result of increased nutrient acquisition of one or more nutrients by ectomycorrhizal fungi are difficult to quantify, but are certainly important for plant growth and survival.

Consequently, a well developed ectomycorrhizal system can improve the out-planting performance of nursery stock (Cordell et al. 1987; Dixon et al. 1981; Marx 1980; Trappe 1977), even though inoculated seedlings may be significantly smaller than non-inoculated plants when out-planted (Maronek et al. 1981). In some pot studies, inoculation by ectomycorrhizal fungi does not result in a corresponding increase in seedling growth; this is likely due to the carbon drain of photosynthate from the host plant by the fungi, which may be initial or sustained (Marx et al. 1982). Other studies

have shown a negative relationship between root collar diameter, stem dry weight and mycorrhizal infection (Mason et al. 2000). Seedlings heavily colonized by ectomycorrhizal fungi might be expected to experience more carbon drain than seedlings with only a few mycorrhizae (Marx et al. 1982) at least initially. Some studies have shown that seedlings with abundant mycorrhizae can be smaller, than those with fewer, however the ultimate objective is field survival (Marx et al. 1982), and the benefits of ectomycorrhizal fungi may not be realized until out-planting.

In contrast, there are numerous reports of increased growth of inoculated plants when compared to non-inoculated plants in the greenhouse (Dixon et al. 1981; Mason et al. 2000), especially under low nutrient concentrations (Aikio & Ruotsalainen, 2002). In fact, growth stimulation of host plants can occur before any indication of infection by the mycorrhizal fungi (Daughtridge et al. 1986).

Still other studies have shown no significant differences in seedling height, root collar diameter, stem and root dry weights and shoot:root ratios as a result of inoculation with ectomycorrhizal fungi (Molina, 1979). These findings indicate that while mycorrhizae might affect plant nutritional status, they do not necessarily directly affect biomass or the distribution of dry matter between roots and shoots of the host plant (Bougher et al. 1990). Other studies did not demonstrate the importance of mycorrhizal infection for either seedling growth or mineral uptake, but suggested that the symbiosis is important for seedling survival when environmental conditions may be limiting (Newton & Pigott, 1991a). Other factors such as changes in water relation and microbial interactions also need to be taken into account.

Nutrient availability for plants is affected by a number of physical, chemical and biological factors, which are interconnected through complex above and belowground feedbacks systems (Wardle, 2002). In addition, fungi vary considerably in their ability to utilize nutrients (Perez-Moreno & Read, 2000). Thus, any conclusions drawn from ectomycorrhizal studies regarding plant nutrition must take into account soil conditions, environmental conditions, fungal species and host plant species (Kropp & Langlois, 1990).

Difficulties Of Mycorrhizal Studies

Difficulties in interpretating results from various studies of ectomycorrhizal fungi and nutrients can be attributed to 1) the effects of different fungi on the same plant 2) the effects of the same fungus on different plants 3) the effects of the same fungus on the same plant under different environmental conditions (Abuzinadah & Read, 1989; Molina, 1979; Van der Heijden & Kuyper, 2001). In addition, Van der Heijden and Kuyper (2001) found that substrate pH , substrate N to phosphorus ratios, and their interactions also have a significant effect on mycorrhizal colonization. Koide and Wu (2003) found that whole ectomycorrhizal fungi communities can be affected by substrate type. Interactions between ectomycorrhizal fungi and the host plant are complex and appear to be influenced by biochemical, physiological and environmental processes that interact in continual feedback loops (Maronek et al. 1981). The conditions under which the fungus optimizes its fitness and the conditions under which the plant optimizes its fitness apparently do not always coincide (Van der Heijden & Kuyper, 2001). Therefore in order to produce healthy ectomycorrhizal seedlings in the greenhouse it is necessary to

examine more closely the interactions of ectomycorrhizal fungal species, the host plant species, and the role of nutritional supplements.

Research Goals

The primary reason for inoculating plants in the greenhouse with ectomycorrhizal fungi is to enhance plant survival when out-planted. Of particular interest is the ability of native ectomycorrhizal fungi to infect aspen seedlings in the greenhouse in a timely manner, and how utilizing different fertilizer regimes and types effects mycorrhization and host plant growth. Fertilization can inhibit mycorrhization, but our research shows it is necessary for healthy aspen seedling development in containers in the greenhouse. Preliminary experiments (Chapter 2) show that particular fertilization regimes are conducive to mycorrhizal development for selected species of native fungi with aspen seedlings and have potential commercial value. The following experiments were designed to further quantify aspen seedling mycorrhization and growth, with and without ectomycorrhizal fungi, under different fertilizer regimes and fertilizer types, utilizing two different methods of inoculation.

The next phase (Chapter 4) will involve examining the growth parameters and survival of aspen inoculated with selected fungi in contaminated soils from near the Anaconda smelter site.

Materials And Methods

Aspen Seedlings

Aspen seed purchased from Bitterroot Restoration Inc., collected in Silverbow County, Montana, was sown on peat:vermiculite (1:1) in cubed trays in the greenhouse. Three seeds were placed in each cube to insure germination of at least one seed. Immediately afterwards, seeds were placed on the media surface, and trays were moved to a misting chamber. Trays were kept in the misting chamber for a period of two weeks to ensure complete germination and primary root development. Trays were then moved to the greenhouse where they received 16-hour photoperiods for an additional 2 weeks and watered every other day. Aspen seedlings were culled according to size to increase plant uniformity across all treatments before the initiation of the each experiment. Greenhouse specifications are given in Chapter 2.

Native Ectomycorrhizal Fungi

Three species of native mycorrhizal fungi, *Laccaria proxima*, *Tricholoma flavovirens* and *Paxillus vernalis*, initially collected from a smelter impacted site by Mahony and Cripps and identified by Cripps (1992) as forming ectomycorrhizae with aspen were utilized for these experiments. One species, *Pisolithus tinctorius* was collected near Gardner, Montana, within a thermal area with coniferous tree species, was also utilized because of it's potential as a good ectomycorrhizal fungi for inoculating plants to be used in reclaiming disturbed sites (Marx & Bryan, 1975). These ectomycorrhizal fungi were also selected based on earlier experiments (see Chapter 2)

that showed that these species could be cultured, have potential for use as inoculum, and were available in sufficient quantities to be utilized as soil inoculum.

Inoculation Procedures

After one month, aspen seedlings were transplanted and inoculated following 1) the 1" banding procedures (see chapter 2), or 2) the 1:6 mixing procedure. The 1:6 mixing procedure is simply mixing 1 part soil inoculum with six parts potting substrate (peat:vermiculite in a 1:1 ratio). If the inoculum could be simply added to the soil by mixing, and the typical seedling method used, it would be a highly efficient method of mycorrhization, if successful. For this reason, it was compared to the "banding" method. For the non-inoculated controls either a 1" band of non-colonized peat:vermiculite soil inoculum was placed directly below the one month old aspen seedlings, or 1 part non-colonized peat:vermiculite soil inoculum was mixed with six parts potting substrate.

Rootrainer ® trays were utilized because their design allows visual inspection of root systems without disruption of plant growth for Experiment 1 and 2, and in Experiment 3, 4" super cell containers ® were utilized.

Fertilizer Treatments

Two different kinds of fertilizer were utilized to examine the effects of fertilizer type on mycorrhization and growth of aspen seedlings in the greenhouse. The two types used were 1) a time release fertilizer with approximately equal amounts of ammonium and nitrate, and 2) a soluble liquid fertilizer with lower levels of ammonium but with a much higher level of N than the time release fertilizer. Osmocote ® 19:6:12, with source of N as ammoniacal (10%) and nitrate N (9%), plus P_2O_5 (6%), K_2O (12%) as sources of

P and K, was utilized as the time release fertilizer. The soluble liquid fertilizer utilized was the commercial brand Miracid ® 30:10:10 with the source of N as ammoniacal (3%) and urea N (27%), plus P_2O_5 (10%), K_2O (10%) as sources of P and K. Both fertilizers are produced by the Scotts-Sierra Horticultural Products Company (Marysville, Ohio, U.S.A.).

Fertilizer Application Rates

The time release fertilizer Osmocote ®, was mixed into potting substrate (peat:vermiculite) prior to inoculation at manufacturer's recommended rate for potted plants, 4-6 g/L of substrate. For the soluble fertilizer; one tablespoon of Miracid ® was mixed with 2 gallons of water and applied to the substrate, once a week after plants were inoculated.

Watering Regime

Aspen seedlings were generally watered every other day, although plants that received no fertilizer amendments as a result of their reduced growth did not require as much water as plants that received the Osmocote ® and Miracid ® fertilizer treatments.

Experimental Design

Experiment 1. Experiment 1 was designed to examine the ability of four different species of ectomycorrhizal fungi, *Paxillus vernalis*, *Pisolithus tinctorius*, *Laccaria proxima* and *Tricholoma flavovirens* to form mycorrhizae with aspen in the greenhouse and to quantify the plant growth response utilizing only the 1" banding technique of inoculation with no fertilization. One month after aspen seedlings were sown, the

seedlings were randomly arranged into 5 treatments. Twenty one seedlings were utilized as non-inoculated controls, 21 seedlings were inoculated with *Paxillus vernalis* and 21 seedlings were inoculated with *Pisolithus tinctorius*. Nineteen aspen seedlings were utilized for the *Laccaria proxima* treatment and 6 aspen seedlings were inoculated with *Tricholoma flavovirens* (due to lack of inoculum). After inoculation, seedlings were returned to the greenhouse utilizing a completely randomized design. Plants were moved weekly to further randomize the design and to reduce the variability of growing conditions present in the greenhouse.

Experiment 2. Experiment 2 was designed to examine the ability of *Pisolithus tinctorius* to form mycorrhizae with aspen in the greenhouse and to quantify the plant growth response to inoculation utilizing two different inoculation techniques, 1" banding with and without Osmocote ® fertilizer or 1:6 mixing and one fertilizer regime (Osmocote ®). One month after aspen seedlings were sown, the seedlings were randomly arranged into 6 treatments. Seven aspen seedlings were used for both the non-inoculated control treatment and for the *Pisolithus tinctorius* treatment utilizing the 1" banding method of inoculation with Osmocote ® fertilizer. Twenty one aspen seedlings were used for both the non-inoculated control treatment and for the *Pisolithus tinctorius* treatment utilizing the 1" banding method of inoculation without fertilizer. Fourteen aspen seedlings were used for both the non-inoculated control treatment and for the *Pisolithus tinctorius* inoculated treatment utilizing the 1:6 method of inoculation. After inoculation seedlings were returned to the greenhouse utilizing a completely randomized

design. Plants were moved weekly to further randomize the design and to reduce the variability of growing conditions present in the greenhouse.

Experiment 3. Experiment three was designed to examine the effects of different fertilization levels and types on the ability of two ectomycorrhizal fungi, *Pisolithus tinctorius* and *Laccaria proxima* to form mycorrhizae with aspen in the greenhouse and to quantify the plant growth response utilizing only the 1" banding method of inoculation. One month after aspen seedlings were sown, the seedlings were randomly arranged into 9 treatments. For the no fertilizer treatments 16 seedlings were utilized as non-inoculated controls, 16 seedlings were inoculated with *Pisolithus tinctorius* and 16 seedlings were inoculated with *Laccaria proxima*. For the Osmocote ® time release fertilizer treatments, 15 seedlings were utilized as non-inoculated controls, 16 seedlings were inoculated with *Pisolithus tinctorius* and 15 seedlings were inoculated with *Laccaria proxima*. For the Miracid ® liquid soluble fertilizer treatments 13 seedlings were utilized as non-inoculated controls, 14 seedlings were inoculated with *Pisolithus tinctorius* and 14 seedlings were inoculated with *Laccaria proxima*. After inoculation seedlings were returned to the greenhouse utilizing a completely randomized design. Plants were moved weekly to further randomize the design and to reduce the variability of growing conditions present in the greenhouse.

Seedling Harvesting

Aspen seedlings were harvested between 120-138 days after germination for each experiment. Roots were washed and visual estimates were made of the percentage of roots with mycorrhizae for each seedling and the total percentage of seedlings with

mycorrhizae and confirmed when necessary with microscopic inspection or by re-culturing hyphal strands of mycorrhizal fungi on sterile petri dishes with MMN media. Mycorrhizae were identified following procedures in chapter 2. Height (cm), root collar diameter (mm), above (g), below (g) and total biomass (g) were also measured.

Data Analysis

Experiment 1. In Experiment 1 the percentage of aspen seedlings that formed mycorrhizae was recorded for the 4 treatments. The growth response of non-inoculated and inoculated aspen seedlings inoculated by banding 1" with no fertilizer was analyzed using a 1-way analysis of variance (SPSS General Linear Model Univariate ANOVA, SPSS, Inc. Version 11.5) for height, root collar diameter, aboveground, belowground and total biomass. Levene's test of equality of error variance was used to determine whether the data required transformation. Height and shoot biomass were transformed in order to pass Levene's test of equality of error variance. Post-hoc Tukey with α=0.05 was conducted for each variable and is included in the Appendix.

Experiment 2. In Experiment 2 the percentage of mycorrhizal seedlings and the growth response of non-inoculated and aspen inoculated utilizing two inoculation techniques, banded 1" or mixing one part inoculum with six parts potting substrate, with and without fertilizer were compared. The two fungal treatments were; a non-inoculated control group and *Pisolithus tinctorius*. Height, root collar diameter, shoot, root ant total biomass were analyzed using a 2-way analysis of variance (SPSS General Linear Model Univariate ANOVA SPSS, Inc. Version 11.5). The 2-way analysis of variance included

three main effects – fertilizer regime, inoculation method and fungal treatment – and fertilization regime by fungal treatment and method of inoculation by fungal treatment interaction effects. All main effects and interactions with P-values less than 0.05 are reported. Levene's test of equality of error variance was used to determine whether the data required transformation. Height, shoot, and total biomass required transformation in order to pass homogeneity of variance tests for ANOVA. Root collar diameter and belowground biomass did not pass Levene's test of equality of variances even after transformations. Post-hoc Tukey with α=0.05 was conducted for each variable and is included in the Appendix.

Experiment 3. In Experiment 3 the percentage of mycorrhizal seedlings and the growth response of non-inoculated and aspen inoculated by banding 1" with three different fertilizer regimes - no fertilizer, Osmocote ® fertilizer, and Miracid ® fertilizer - were compared. The three fungal treatments were a non-inoculated control group, *Laccaria proxima* and *Pisolithus tinctorius*. Height, root collar diameter, shoot, root and total biomass were analyzed using a 2-way analysis of variance (SPSS General Linear Model Univariate ANOVA SPSS, Inc. Version 11.5). The 2-way analysis of variance included 2 main effects – fertilizer regime and fungal treatment – and fertilization regime by fungal treatment interaction effects. All main effects and interactions with P-values less than 0.05 are reported. Levene's test of equality of error variance was used to determine whether the data required transformation. Height, shoot, root, and total biomass required transformation in order to pass homogeneity of variance tests for

ANOVA. Post-hoc Tukey with $\alpha=0.05$ was conducted for each variable and is included in the Appendix.

Results

Experiment 1

The first experiment analyzed the growth response of aspen seedlings inoculated with four different native ectomycorrhizal fungi, and compared the percentage of seedlings that formed mycorrhizae. All species were banded 1" and no fertilizer was used in the experiment. The number of replicates for each treatment, mean values and standard deviations are included in table 4, and illustrated in figure 5.

Table 4. Number of replicates, means and standard deviations of growth parameters and percent mycorrhization of non-inoculated and aspen seedlings inoculated with 4 native ectomycorrhizal fungi by banding 1" with no fertilizer. Different letters denote significant differences at P<0.05 within a row. Untransformed data.

Fungal treatment	Controls n=21	*P. vernalis* n=21	*P. tinctorius* n=21	*L. proxima* n=19	*T. flavovirens* n=6
Height (cm)	3.41±1.50 ABC	2.43±0.81 C	2.66±0.81 BC	3.82±1.18 A	3.38±0.46 AB
Root collar diameter (mm)	1.57±0.19 A	1.31±0.12 BC	1.31±0.23 BC	1.50±0.21 AB	1.25±0.16 C
Aboveground biomass (g)	0.05±0.02 AB	0.03±0.01 BC	0.03±0.01 C	0.06±0.02 A	0.04±0.01 BC
Belowground biomass (g)	0.13±0.03 A	0.08±0.03 B	0.07±0.03 B	0.13±0.05 A	0.10±0.01 AB
Total biomass (g)	0.19±0.05 A	0.11±0.03 B	0.10±0.04 B	0.19±0.06 A	0.14±0.05 B
% Aspen with mycorrhizae	0	100	71	20	17

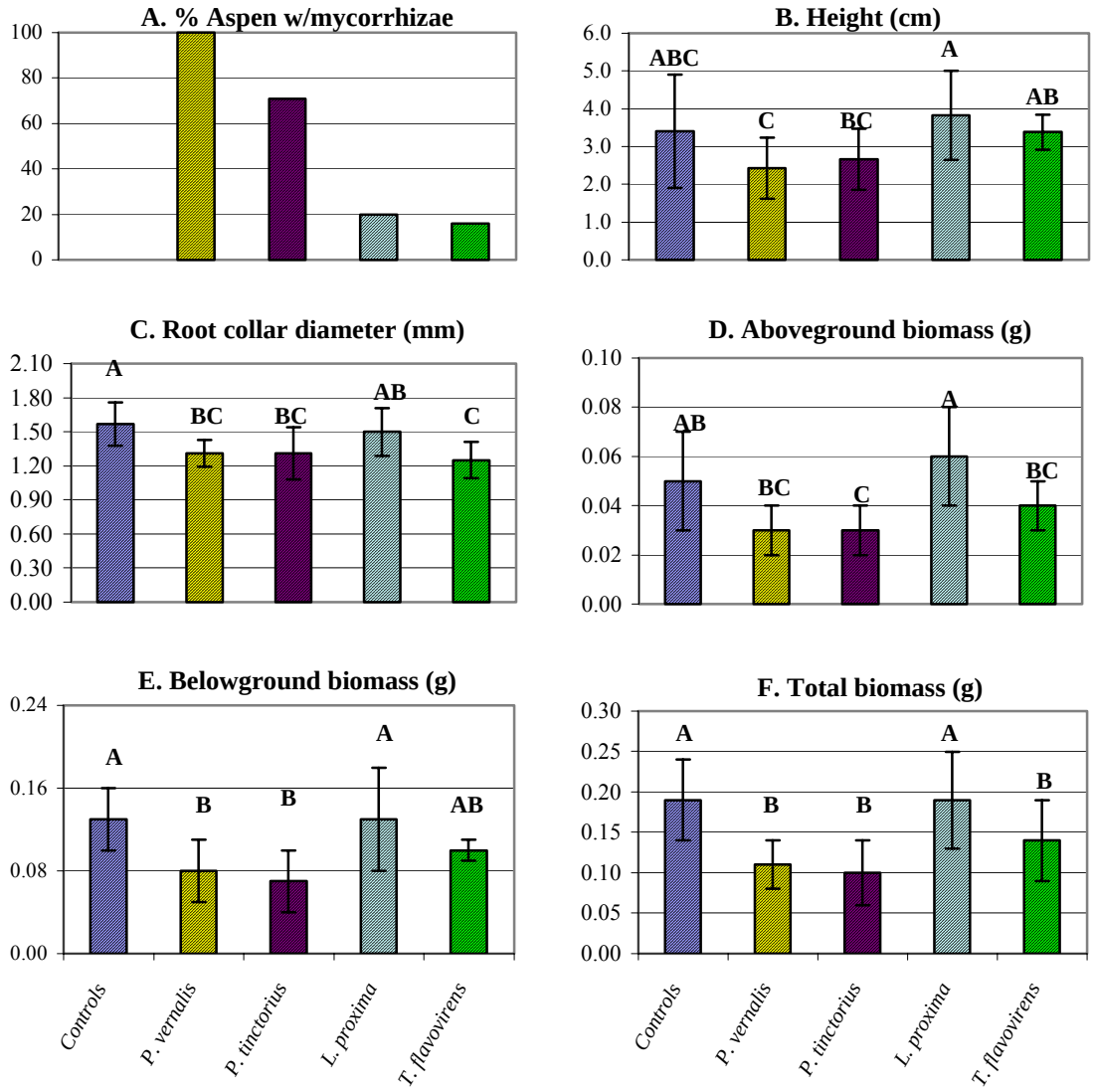

Figure 5. Percent mycorrhization and growth response of non-inoculated and aspen seedlings inoculated with 4 native ectomycorrhizal fungi in the greenhouse by banding 1" with no fertilizer, after 138 days. Bars represent standard deviations of the mean and different letters denote significant differences at $P<0.05$.

Controls: Banded 1" (n=21)

Paxillus vernalis: Banded 1" (n=21)

Pisolithus tinctorius: Banded 1" (n=21)

Laccaria proxima: Banded 1" (n=19)

Tricholoma flavovirens: Banded 1" (n=6)

Percentage Of Aspen Seedlings With Mycorrhizae

Mycorrhizae did not form on any of the controls and all aspen seedlings inoculated with *Paxillus vernalis* formed mycorrhizae on their roots (Table 4 & 5; Figure 5A). Seventy-one percent of aspen seedlings inoculated with *Pisolithus tinctorius* formed mycorrhizae, 20 percent of the aspen seedlings inoculated with *Laccaria proxima* formed mycorrhizae, and 17 percent of the aspen seedlings inoculated with *Tricholoma flavovirens* formed mycorrhizae. Numbers of mycorrhizae per tree ranged from a few to 20% coverage of the root system, although the average percent of mycorrhizal infection was less. Seedlings inoculated with *Paxillus vernalis* had higher infection rates than the other fungal treatments (Table 5).

Table 5. Mean % mycorrhization for roots of inoculated and non-inoculated aspen seedlings, number of trees with mycorrhizae, % mycorrhizal infection on trees with mycorrhizae and % of trees with mycorrhizae

Fungi	N	Mean % root mycorrhization	% Mean mycorrhizal infection of aspen w/mycorrhizae	# Aspen w/mycorrhizae	% Aspen w/mycorrhizae
Control	21	0	0	0	0
Paxillus vernalis	21	11.43	11.43	21	100
Pisolithus tinctorius	21	1.5	1.9	17	71
Laccaria proxima	19	0.4	2	4	20
Tricholoma flavovirens	6	0.2	1	1	17

Height

For all aspen seedlings, height was affected by fungal treatment (Table 6; Figure 5B). Aspen seedlings inoculated with *Paxillus vernalis* were significantly shorter than seedlings inoculated with *Laccaria proxima*, *Tricholoma flavovirens* and the controls (Table 4; Figure 5B).

Table 6. Univariate Analysis of Variance, Height (cm); transformed (square root)

Source	Type III Sum of Squares	df	F	Sig.
fungi	2.014	4	5.934	.000
Error	7.045	83		

Root Collar Diameter

There was a significant fungal effect on root collar diameter (Table 7). The root collar diameter of non-inoculated (controls) aspen seedlings were significantly larger than all fungal treatments except the *Laccaria proxima* inoculated plants (Table 4; Figure 5C). Aspen seedlings inoculated with *Paxillus vernalis* and *Pisolithus tinctorius* had smaller root collar diameters, followed by aspen seedlings inoculated with *Tricholoma flavovirens* with the smallest diameter (Table 4; Figure 5C).

Table 7. Univariate Analysis of Variance, Root Collar Diameter (mm).

Source	Type III Sum of Squares	df	F	Sig.
fungi	1.371	4	10.287	.000
Error	2.766	83		

Aboveground Biomass

Aboveground biomass (g) of aspen seedlings was affected by fungal treatment (Table 8). The seedlings inoculated with *Pisolithus tinctorius* were significantly smaller than the controls (Table 4: Figure 5D). The aboveground biomass of aspen seedlings inoculated with *Laccaria proxima* was significantly larger than that for *Paxillus vernalis*, *Pisolithus tinctorius*, and *Tricholoma flavovirens*, but was not significantly different from the controls (Table 4; Figure 5D).

Table 8. Univariate Analysis of Variance, Aboveground biomass (g); transformed (inverse square root)

Source	Type III Sum of Squares	df	F	Sig.
fungi	65.325	4	13.911	.000
Error	97.441	83		

Belowground Biomass (g)

There was a significant fungal effect on belowground biomass (Table 9). The root biomass of non-inoculated control seedlings and those inoculated with *Laccaria proxima* were significantly greater than plants inoculated with *Paxillus vernalis* and *Pisolithus tinctorius* (Table 4; Figure 5F).

Table 9. Univariate Analysis of Variance, Belowground biomass (g)

Source	Type III Sum of Squares	df	F	Sig.
fungi	.070	4	12.730	.000
Error	.114	83		

Total Biomass

There was a significant fungal effect on the total biomass of aspen seedlings (Table 10). The total biomass of non-inoculated control seedlings and those inoculated with *Laccaria proxima* was significantly greater than for plants inoculated with *Paxillus vernalis*, *Pisolithus tinctorius* and *Tricholoma flavovirens* (Table 4; Figure 5F). Results follow a pattern similar to biomass measurements for root and shoot systems.

Table 10. Univariate Analysis of Variance, Total biomass (g)

Source	Type III Sum of Squares	df	F	Sig.
fungi	.151	4	16.317	.000
Error	.192	83		

Experiment 2

The second experiment compared the level of mycorrhization of aspen seedlings inoculated with a local strain of *Pisolithus tinctorius* and non-inoculated controls to 2 different inoculation methods; banding 1" with and without fertilizer and 1 inoculum:6 parts media with fertilizer all fertilized with Osmocote ®. Host plant growth response to the various treatments after 138 days in the greenhouse was also analyzed. Percent mycorrhization, means and standard deviations of the growth response for the various treatments are shown in Table 11 with number of replicates in figure 6.

Table 11. Percent mycorrhization, means and standard deviations of growth parameters of inoculated and non-inoculated control aspen seedlings after 138 days in the greenhouse utilizing two inoculation techniques; banded 1" with Osmocote ® fertilizer and without fertilizer or 1:6 inoculum:media with Osmocote ® fertilizer. Data not transformed. Method 1=banded 1", method 2=1 inoculum:6 media; Fert 0=no fertilizer, Fert 1=Osmocote ® fertilizer.

	Inoculation	Fertilizer	Controls	*Pisolithus tinctorius*
% Aspen with mycorrhizae				
	Method 1	Fert 0	0	71
		Fert 1	0	86
	Method 2	Fert 1	0	29
Height (cm)				
	Method 1	Fert 0	3.41±1.50	2.66±0.81
		Fert 1	16.40±7.43	11.58±4.35
	Method 2	Fert 1	21.14±4.21	17.12±5.48
Root collar diameter (mm)				
	Method 1	Fert 0	1.59±0.15	1.31±0.23
		Fert 1	2.96±0.65	2.82±0.61
	Method 2	Fert 1	3.24±0.42	3.00±0.24
Aboveground biomass (g)				
	Method 1	Fert 0	0.05±0.02	0.03±0.01
		Fert 1	0.52±0.36	0.36±0.19
	Method 2	Fert 1	0.81±0.39	0.65±0.24
Belowground biomass (g)				
	Method 1	Fert 0	0.14±0.04	0.07±0.03
		Fert 1	0.75±0.40	0.59±0.30
	Method 2	Fert 1	1.10±0.43	0.88±024
Total biomass (g)				
	Method 1	Fert 0	0.19±0.05	0.10±0.04
		Fert 1	1.27±0.72	1.03±0.54
	Method 2	Fert 1	1.84±0.83	1.44±0.47

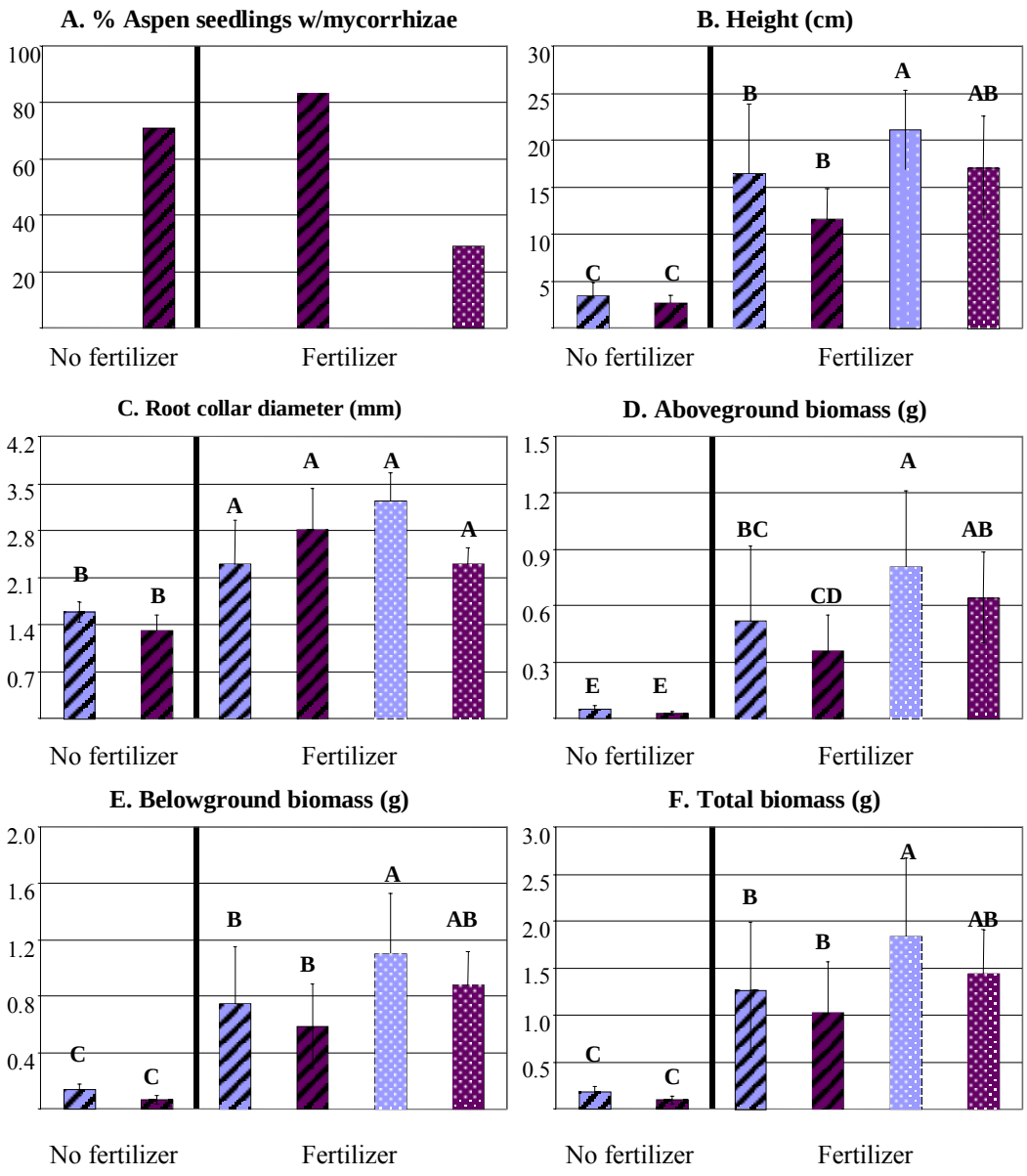

Figure 6. Percent mycorrhization and growth response of inoculated and non-inoculated aspen seedlings in the greenhouse utilizing two inoculation techniques; banded 1", with Osmocote® fertilizer and without fertilizer or 1:6 inoculum: media with Osmocote® fertilizer, after 138 days. Bars represent standard deviations of the mean and different letters denote significant differences at $P<0.05$.

Controls (non-inoculated): banded 1"; no fertilizer n=21, Osmocote® fertilizer n=7

Pisolithus tinctorius: banded 1"; no fertilizer n=21, Osmocote® fertilizer n=7

Controls (non-inoculated): 1 inoculum:6 media Osmocote® fertilizer n=14

Pisolithus tinctorius: 1 inoculum:6 media Osmocote® fertilizer n=14

Percentage Of Aspen Seedlings With Mycorrhizae

No mycorrhizae were observed in any of the controls regardless of treatment (Table 11 & 12, figure 6A). Seventy one percent of aspen seedlings inoculated with a 1" band of *Pisolithus tinctorius* without fertilization formed mycorrhizae. Eighty six percent of aspen seedlings inoculated with a 1" band of *Pisolithus tinctorius* with Osmocote ® fertilizer formed mycorrhizae. Less than 30% of aspen seedlings inoculated by the 1 inoculum:6 media method with *Pisolithus tinctorius* with Osmocote ® fertilizer formed mycorrhizae. Numbers of mycorrhizae per tree was less than 2% infection of the root system for all treatments (Table 12).

Table 12. Mean % mycorrhization of inoculated aspen seedlings, number of trees with mycorrhizae, % mycorrhizal infection on trees with mycorrhizae and % of trees with mycorrhizae

Fungi	N	Mean % root mycorrhization	% Mycorrhizal infection of aspen w/mycorrhizae	# Aspen w/mycorrhizae	% Aspen w/mycorrhizae
No fertilizer, banded 1"					
Control	21	0	0	0	0
Pisolithus tinctorius	21	1.5	1.9	17	71
Osmocote, banded 1"					
control	7	0	0	0	0
Pisolithus tinctorius	7	1.6	1.8	6	86
Osmocote, 1 inoculum:6 media					
control	14	0	0	0	0
Pisolithus tinctorius	14	0.29	1	4	29

Height

The height (cm) of aspen seedlings was affected by fungal treatment, method of inoculation and fertilization (Table 13). Non-inoculated controls were significantly taller

than aspen seedlings inoculated with *Pisolithus tinctorius* when comparing inoculated and non-inoculated aspen across all treatments (Table 11; Figure 6B). Aspen seedlings inoculated 1 inoculum:6 media were significantly taller than banded 1" aspen seedlings (Table 11; Figure 6B). Fertilized aspen seedlings were significantly taller than seedlings with no fertilizer (Table 11; Figure 6B). There was no interaction between fungal treatment and fertilization level or fungal treatment and method of inoculation (Table 13).

Table 13. Analysis of variance, Height (cm); transformed (.18).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
fert	1.493	1	1.493	187.357	.000
meth	.107	1	.107	13.372	.000
fungi	.058	1	.058	7.221	.009
fert * fungi	.005	1	.005	.627	.431
meth * fungi	.001	1	.001	.093	.761
Error	.621	78	.008		

Root Collar Diameter

Root collar diameter was affected by fertilization level, fungal treatment and method of inoculation (Table 14). The non-inoculated aspen seedlings (controls) had significantly larger root collar diameters than *Pisolithus tinctorius* inoculated aspen seedlings regardless of method of inoculation or fertilizer level (Table 11; Figure 6C). Aspen seedlings inoculated 1 inoculum:6 media had significantly larger root collar diameters than banded 1" aspen seedlings (Table 11; Figure 6C). Fertilized aspen seedlings had significantly larger root collar diameters than seedlings with no fertilizer (Table 11; Figure 6C). There was, however, no significant interaction between fertilization and fungal treatment or method of inoculation and fungal treatment (Table 14).

Table 14 Analysis of variance, Root Collar Diameter (mm).

Source	Type III Sum of Squares	df	F	Sig.
fert	21.701	1	180.935	.000
meth	.488	1	4.066	.047
fungi	1.107	1	9.228	.003
fert * fungi	.049	1	.406	.526
meth * fungi	.022	1	.184	.669
Error	9.355	78		

Aboveground Biomass

Aboveground biomass was affected by level of fertilization, method of inoculation and fungal treatment (Table 15). Aspen seedlings inoculated 1 inoculum:6 media had significantly larger shoots than banded 1" aspen seedlings (Table 11; Figure 6D). Fertilized aspen seedlings also had significantly larger shoot biomass than seedlings with no fertilizer (Table 11; Figure 6D). The non-inoculated aspen seedlings (controls) had significantly larger shoot biomass than seedlings inoculated with *Pisolithus tinctorius* (Table 11; Figure 6D). There was no significant interaction between fungal treatment and fertilization level or fungal treatment and method of inoculation (Table 15).

Table 15 Analysis of variance, Aboveground Biomass (g); transformed (-.08).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
fert	.513	1	.513	255.520	.000
meth	.027	1	.027	13.592	.000
fungi	.031	1	.031	15.694	.000
fert * fungi	.008	1	.008	3.874	.053
meth * fungi	2.08E-005	1	2.08E-005	.010	.919
Error	.156	78	.002		

Belowground Biomass

Belowground biomass of aspen seedlings was affected by fungal treatment, method of inoculation and fertilization level (Table 16). Aspen seedlings with Osmocote ® fertilizer had significantly more root biomass than plants with no fertilizer regardless of inoculation technique or fungal treatment (Table 11; Figure 6E). Aspen seedlings inoculated 1 inoculum:6 media had significantly more root biomass than seedlings banded 1" (Table 11; Figure 6E). Non-inoculated aspen seedlings had significantly more root biomass than inoculated aspen seedlings regardless of fertilization level or method of inoculation (Table 11; Figure 6E). There was no significant interaction between fungal treatment and fertilization level or fungal treatment and method of inoculation (Table 15).

Table 16. Analysis of variance, Belowground Biomass (g).

Source	Type III Sum of Squares	df	F	Sig.
fert	3.335	1	55.262	.000
meth	.971	1	16.086	.000
fungi	.353	1	5.844	.018
fert * fungi	.020	1	.338	.563
meth * fungi	.011	1	.186	.668
Error	4.707	78		

Total Biomass

The total biomass of aspen seedlings was affected by fungal treatment, method of inoculation and fertilization level (Table 17). Aspen seedlings with Osmocote ® fertilizer were significantly larger than plants with no fertilizer regardless of inoculation technique or fungal treatment (Table 11; Figure 6F). Aspen seedlings inoculated 1 inoculum:6 media had significantly more total biomass, than seedlings banded 1" (Table 11; Figure 6F). Non-inoculated aspen seedlings were larger than *Pisolithus tinctorius*

inoculated aspen seedlings regardless of fertilization or method of inoculation (Table 11; Figure 6F). There was significant interaction between fertilization regime and fungal treatment, although there was no significant interaction between fungal treatment and method of inoculation (Table 17).

Table 17. Analysis of variance, Total Biomass (g); transformed (-.15).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
fert	1.259	1	1.259	189.793	.000
meth	.048	1	.048	7.194	.009
fungi	.128	1	.128	19.366	.000
fert * fungi	.053	1	.053	7.918	.006
meth * fungi	.002	1	.002	.250	.619
Error	.517	78	.007		

Experiment 3

The third experiment compared the level of mycorrhization of aspen seedlings banded 1" with two species of ectomycorrhizal fungi, *Laccaria proxima* and *Pisolithus tinctorius*, and a control group (non-inoculated) and analyzed host plant growth response after 4 months in the greenhouse, with three different fertilizer regimes: no fertilizer, Osmocote ® fertilizer, and strength Miracid ® fertilizer. Percent mycorrhization, mean values and standard deviations for each treatment are included in table 18, with the number of replicates in figure 7 and 8.

Table 18. % Mycorrhization, means and standard deviations of growth parameters of inoculated and non-inoculated aspen seedlings in the greenhouse banded 1" with 3 fertilizer treatments, after 4 months. Data not transformed. Fert 0=no fertilizer, fert 1=Osmocote® fertilizer, fert 2=Miracid ® fertilizer. Different letters denote significant differences at P<0.05.

	Fertilizer	Controls	*L. proxima*	*P. tinctorius*
% Aspen with mycorrhizae				
	Fert 0	0	44	100
	Fert 1	0	40	81
	Fert 2	0	21	57
Height (cm)				
	Fert 0	10.18±1.95B	4.49±1.28C	5.06±1.81C
	Fert 1	27.95±6.77A	26.33±5.29A	25.85±4.17A
	Fert 2	30.6±5.4A	30.37±5.31A	29.76±5.75A
Root collar diameter (mm)				
	Fert 0	2.01±0.28B	1.50±0.16C	1.60±1.04C
	Fert 1	3.07±0.44A	3.40±0.34A	3.18±0.38A
	Fert 2	3.21±0.30A	3.39±0.38A	3.23±0.44A
Aboveground biomass (g)				
	Fert 0	0.26±0.07B	0.10±0.02C	0.11±0.04C
	Fert 1	0.81±0.30A	1.01±0.27A	0.81±0.31A
	Fert 2	1.02±0.12A	1.13±0.38A	0.99±0.32A
Belowground biomass (g)				
	Fert 0	0.26±0.06A	0.32±0.03B	0.33±0.06B
	Fert 1	0.40±0.19A	0.40±0.20A	0.40±0.14A
	Fert 2	0.33±0.10A	0.41±0.34A	0.32±0.13A
Total biomass (g)				
	Fert 0	0.52±0.13B	0.25±0.05C	0.27±0.07C
	Fert 1	1.21±0.45A	1.40±0.44A	1.17±0.45A
	Fert 2	1.38±0.32A	1.47±0.53A	1.30±0.44A

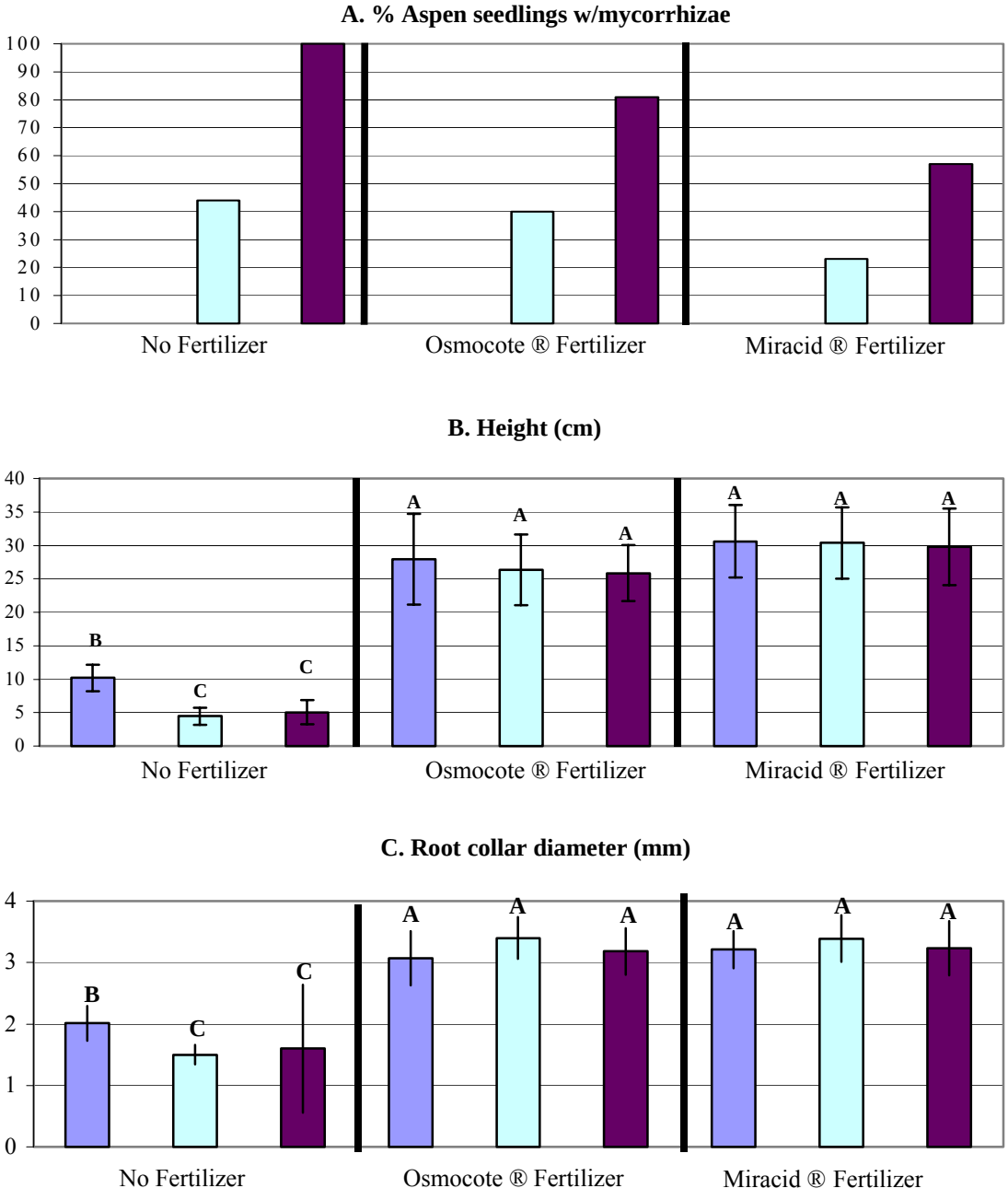

Figure 7. Percent mycorrhization and growth response of inoculated and non-inoculated aspen seedlings in the greenhouse under three nutrient regimes: no fertilizer, Osmocote ® fertilizer and Miracid ® fertilizer, after 4 months. Means with the same letter are not significantly different at $P<0.05$, bars represent standard deviations.

Controls; no fertilizer n=16, Osmocote ® fertilizer n=15, Miracid ® fertilizer n=13.

Laccaria proxima; no fertilizer n=16, Osmocote ® fertilizer n=15, Miracid ® fertilizer n=14.

Pisolithus tinctorius; no fertilizer n=16, Osmocote ® fertilizer n=16, Miracid ® fertilizer n=14.

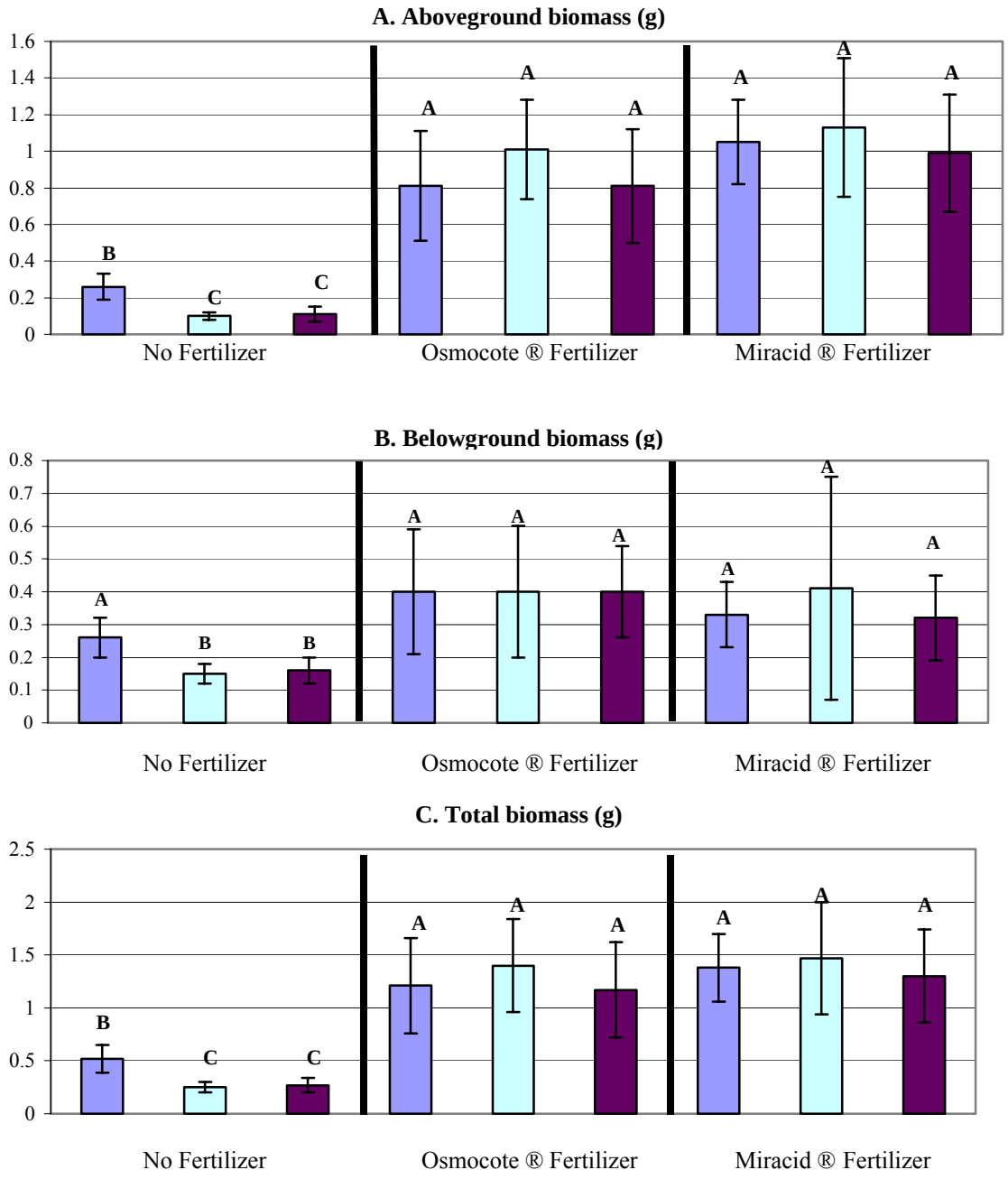

Figure 8. Growth response of inoculated and non-inoculated aspen seedlings in the greenhouse under three nutrient regimes: no fertilizer, Osmocote ® fertilizer and Miracid ® fertilizer, after 4 months. Means with the same letter are not significantly different at $P<0.05$, bars represent standard deviations.

Controls; no fertilizer n=16, Osmocote ® fertilizer n=15, Miracid ® fertilizer n=13.

Laccaria proxima; no fertilizer n=16, Osmocote ® fertilizer n=15, Miracid ® fertilizer n=14.

Pisolithus tinctorius; no fertilizer n=16, Osmocote ® fertilizer n=16, Miracid ® fertilizer n=14.

Percent Aspen Seedlings With Mycorrhizae

No mycorrhizae were observed on any of the controls (Table 18; Figure 7A). Forty-four percent of aspen seedlings inoculated with *Laccaria proxima* and without fertilizer formed mycorrhizae, while mycorrhizae formed on 40% of the inoculated aspen seedlings with Osmocote ® fertilizer and mycorrhizae formed on 23% of the inoculated aspen seedlings with Miracid ® fertilizer formed (Table 18; Figure 7A). All aspen seedlings inoculated with *Pisolithus tinctorius* and without fertilizer formed mycorrhizae, while mycorrhizae formed on 81 % of the inoculated aspen seedlings with Osmocote ® fertilizer formed and mycorrhizae formed on 57 % of the inoculated aspen seedlings with Miracid ® fertilizer (Table 18; Figure 7A). Numbers of mycorrhizae per tree ranged from 2% to 11% infection of the root system for all inoculated aspen seedlings treatments without fertilizer compared to 4% for Osmocote treated seedlings, and 1% to 2% for aspen seedlings fertilized with Miracid (Table 19).

Table 19. Mean % mycorrhization or roots of inoculated and non-inoculated aspen seedlings, number of trees with mycorrhizae, % mycorrhizal infection on trees with mycorrhizae and % of trees with mycorrhizae

Fungi	N	Mean % root mycorrhization	% Mycorrhizal infection of aspen w/mycorrhizae	# Aspen w/mycorrhizae	% Aspen w/mycorrhizae
No fertilizer					
Control	16	0	0	0	0
Pisolithus tinctorius	16	11.4	11.4	16	100
Laccaria proxima	16	2.1	4.9	7	44
Osmocote fertilizer					
control	15	0	0	0	0
Pisolithus tinctorius	16	4.3	5.3	16	81
Laccaria proxima	15	4.7	11.7	6	40
Miracid fertilizer					
control	13	0	0	0	0
Pisolithus tinctorius	14	2	4	8	57
Laccaria proxima	14	0.8	3.4	3	21

Height

Height (cm) of all aspen seedlings was affected by fertilization regime and fungal treatment (Table 20; Figure 7B). There were significant differences in fertilizer treatments with aspen seedlings fertilized with Miracid and Osmocote significantly taller than those in the non-fertilized treatments (Table 18; Figure 7B). The controls (non-inoculated) were significantly taller than aspen seedlings inoculated with either *Laccaria proxima* or *Pisolithus tinctorius* when compared across all treatments (Table 18; Figure 7B). In addition, there was significant interaction between fungal treatment and fertilizer regime. The controls (non-inoculated) were significantly taller that the seedlings inoculated with *Laccaria proxima* and *Pisolithus tinctorius* only for the non-fertilized treatment (Figure 7B).

Table 20. Univariate Analysis of Variance, Height (cm); transformed (square root)

Source	Type III Sum of Squares	df	F	Sig.
fungi * fert	5.832	4	6.583	.000
fungi	4.971	2	11.220	.000
fert	245.542	2	554.263	.000
Error	27.909	126		

Root Collar Diameter

Root collar diameter (mm) of all aspen seedlings was affected by fertilization regime (Table 21; Figure 7C). Seedlings fertilized with Miracid and Osmocote had significantly larger root collar diameters than the non-fertilized treatments (Table 18; Figure 7C). In addition, there was significant interaction between fungal treatment and fertilizer regime. The controls (non-inoculated) had significantly larger root collar

diameters than the seedlings inoculated with *Laccaria proxima* and *Pisolithus tinctorius* in only the non-fertilized treatment (Figure 7C).

Table 21. Univariate Analysis of Variance, Root collar diameter (mm)

Source	Type III Sum of Squares	df	F	Sig.
fungi * fert	3.190	4	6.826	.000
fungi	.248	2	1.063	.348
fert	73.778	2	315.685	.000
Error	14.724	126		
Total	1074.619	135		

Aboveground Biomass

Aboveground biomass (g) of aspens seedlings was affected by fertilization regime and fungal treatment (Table 22; Figure 8A). There were significant differences in fertilizer treatments with aspen seedlings fertilized with Miracid and Osmocote significantly larger than those in the non-fertilized treatments (Table 18; Figure 8A). In addition, there was significant interaction between fungal treatment and fertilizer regime. The controls (non-inoculated) were significantly larger than seedlings inoculated with *Laccaria proxima* and *Pisolithus tinctorius* only for the non-fertilized treatment (Figure 8A).

Table 22. Univariate Analysis of Variance, Aboveground biomass (g); transformed (-.032)

Source	Type III Sum of Squares	df	F	Sig.
fungi * fert	.006	4	14.191	.000
fungi	.003	2	11.065	.000
fert	.103	2	449.622	.000
Error	.014	126		

Belowground Biomass

Belowground biomass (g) of aspens seedlings was affected by fertilization regime and fungal treatments (Table 23; Figure 8B). There were significant differences in fertilizer treatments with aspen seedlings fertilized with Miracid and Osmocote significantly larger than those in the non-fertilized treatments (Table 18; Figure 8B). In addition, there was significant interaction between fungal treatment and fertilizer regime. The controls (non-inoculated) were significantly larger than seedlings inoculated with *Laccaria proxima* and *Pisolithus tinctorius* only for the non-fertilized treatment (Figure 8B).

Table 23. Univariate Analysis of Variance, Belowground biomass (g); transformed (inverse)

Source	Type III Sum of Squares	df	F	Sig.
fungi * fert	63.181	4	5.022	.001
fungi	35.606	2	5.661	.004
fert	217.659	2	34.605	.000
Error	396.264	126		

Total Biomass

Total biomass (g) of aspen seedlings was affected by fertilization regime and fungal treatments (Table 24; Figure 8C). There were significant differences in fertilizer treatments with aspen seedlings fertilized with Miracid and Osmocote significantly larger than those in the non-fertilized treatments (Table 18; Figure 8C). In addition, there was significant interaction between fungal treatment and fertilizer regime. The controls (non-inoculated) were significantly larger than seedlings inoculated with *Laccaria proxima* and *Pisolithus tinctorius* only for the non-fertilized treatment (Figure 8C).

Table 24. Univariate Analysis of Variance, Total biomass (g); transformed (inverse square root)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
fungi	1.148	2	.574	12.747	.000
fert	24.443	2	12.222	271.478	.000
fungi * fert	2.478	4	.620	13.764	.000
Error	5.672	126	.045		

Discussion

Detailing the cultural conditions for successful mycorrhization of aspen seedlings in the greenhouse is necessary when attempting to use inoculation for improvement of plant performance when out-planting on harsh sites. These experiments have shown that the ability of different species of ectomycorrhizal fungi to form mycorrhizae with aspen seedlings in the greenhouse is affected by the type and levels of fertilizer, and the inoculation technique. Success also depends on the fungus selected, not only for it's growth in culture, but also for its ecological role.

In natural systems mycorrhizal interactions are important components in successional process (Allen, 1991). Mason et al. (1982) described patterns of fruitbody production as a function of succession. Cripps (2001, 2004) found 'early colonizers' more likely to be associated with younger aspen along the edges of aspen stands and 'late colonizing' fungi within the interior where more organic matter had accumulated.

In one study, different species of ectomycorrhizal fungi all gave plants access to N but large differences in their effectiveness were observed, with an 'early stage' fungus providing significantly more N than either a 'late stage' fungus or a facultative associate (Abuzinadah & Read, 1989). Further, Lilleskov & Bruns (2003) demonstrated

differences between the ability of an 'early stage' fungi and 'late stage' fungi to initially colonize roots and to persist. Changes in the N status of soils such as increases in organic content over time can foster changes in the mycorrhizal flora. 'Early stage' ectomycorrhizal fungi are more successful in mineral soils with lower levels of organic matter (Abuzinadah & Read, 1986a) and are gradually replaced by 'late stage' fungi as nutrients and other abiotic conditions change. Succession of the fungal component can also be dependent on the quality and quantity of the carbon source as a result of decomposition (Norland, 1993) and the carbon to N ratio in particular (Read, 1991). Some early stage fungi such as *Hebeloma* spp. prefer high N environments, and are common with aspen in fertilized lawns. Selection of appropriate fungi for reclamation can be crucial and needs to be matched to both greenhouse and out-planting conditions.

Experiment 1

In Experiment 1, the growth response of aspen seedlings inoculated with four different species of native ectomycorrhizal fungi was investigated. All four species of native ectomycorrhizal fungi are considered 'early colonizers' and were able to form mycorrhizae under these conditions. Banding 1" of inoculum beneath transplanted 1 month old aspen seedlings was an effective method of inoculation. Mycorrhization was possible without fertilizer, although aspen seedling growth was stunted after 3 months as a result of low fertility, and ranged from 2.4 cm to 3.8 cm tall. Mycorrhization levels ranged from a high of 100 % for *Paxillus vernalis*, 71 % for *Pisolithus tinctorius*, 20 % for *Laccaria proxima*, to a low of 16 % for *Tricholoma flavovirens*. Although, within the fungal treatments, mycorrhization levels for individual tree root systems (0.3-1.6 %) was

considerably less. Marx et al. (1981) argues that for any realized benefit to the inoculated host plant after out-planting, 50% of the root systems need to be colonized by ectomycorrhizal fungi, at least with pine trees in the South.

The growth response of aspen seedlings varied with the fungal species used for all measured growth parameters, regardless of mycorrhizal formation. For most parameters, inoculated seedlings were the same size or significantly smaller than non-inoculated controls, in no case were they significantly larger. These results stand in contrast to earlier *in vitro* experiments by Cripps (1993), where inoculated aspen seedlings were 200-400 % larger than the controls under sterile conditions. The conflicting results may be a function of the nutrient status of the media. In Cripps (1993) liquid MMN was applied to the media to enhance mycorrhization under sterile conditions. The addition of liquid MMN may reduce the carbohydrates expended by the host plant that are necessary to promote mycorrhization thus, potentially reducing the amount of carbon drain on the aspen seedlings. In addition, adding liquid MMN may increase the nutritional status of the host plant indirectly by providing ample carbohydrates to the fungal symbiont so there is no observable carbon drain, or by directly providing N and phosphorus to the plant. In my experiments, nutrients were limiting which increased the carbon demand of the fungi on the aspen seedlings under non-sterile conditions.

For most measured growth parameters, *Paxillus vernalis* and *Pisolithus tinctorius* were significantly smaller than the controls, and *Laccaria proxima* and *Tricholoma flavovirens* were comparable to the controls. In addition, *Paxillus vernalis* and *Pisolithus tinctorius* formed more mycorrhizae than the other fungi, suggesting an increased carbon drain as a result of increased mycorrhization. *Paxillus vernalis* and *Pisolithus tinctorius*

also formed more extensive mycorrhizae than other ectomycorrhizal fungi tested with aspen seedlings in earlier experiments (see Chapter 2; Table 2; Experiments 6 and 7). Although the carbon drain of inoculated seedlings may reduce seedling growth initially in the greenhouse (Marx et al. 1982), inoculated plants have been shown, in some cases, to have higher survival rates when out-planted than non-inoculated seedlings (Kropp & Langlois, 1990; Maronek et al. 1981). Inoculated plants may also eventually be larger than controls in the greenhouse, after an initial lag response, depending on conditions. Another consideration here might be variability in fungal growth rates under particular conditions with some growing faster than others, in addition to their individual physiologies.

In summary, it appears that percent mycorrhization is in general, inversely proportional to aspen seedling growth under low fertility conditions at 138 days.

Experiment 2

In experiment two the growth response and percent mycorrhization of aspen with and without the ectomycorrhizal fungus, *Pisolithus tinctorius*, for two inoculation methods, and with or without fertilization with Osmocote was investigated. Our hypothesis for this experiment was that the inoculation method utilized and level of nutrients in the media would affect mycorrhization of aspen by *Pisolithus tinctorius*. *Pisolithus tinctorius* mycorrhizae did form on aspen seedlings in the greenhouse, with and without fertilizer and for two inoculation techniques, but amounts varied, as did the growth response of aspen.

All fertilized trees were significantly larger for all measured growth parameters, regardless of fungal treatment or type of inoculation. Without fertilization, aspen seedlings were less than 5 cm tall, compared with fertilized aspen seedlings at 11-21 cm tall.

Mycorrhizae were able to form in the 3 treatments where seedlings were inoculated, however more formed when they were banded and fertilized (86 %) or banded without fertilization (71 %), compared to only 29 % for the mixed inoculation technique. Thus, banding appears to be a more effective method of inoculating aspen seedlings in the greenhouse, even with fertilization using Osmocote (which appears not to affect results much, at least for *Pisolithus tinctorius* which is a fast growing fungus).

However, in general, trees were somewhat shorter and smaller when banded. This could be due to the initial carbon drain during mycorrhization and the fact that the banding method was a more effective method of inoculating aspen seedlings in containers.

Experiment 3

Experiment three investigated the effects of fertilization on mycorrhization and growth response of aspen seedlings, this time comparing two species of native ectomycorrhizal fungi and three different fertilizer treatments (No fertilizer, Osmocote ® fertilizer and Miracid ® fertilizer). Banding 1" of inoculum was used as the inoculum technique because of its successful use in experiment 2. Both fungi are considered 'early colonizers' and both are typically found on acidic, dry soils.

In general, it is known that commercial fertilizers produce the largest seedlings, but can cause a marked reduction in ectomycorrhizal development (Ruehle, 1980). Seedling growth, inorganic chemical content of needles, and soluble-carbohydrate content of shoots and roots can be significantly affected by soil fertility (Marx et al. 1977). Other studies have shown that the formation of ectomycorrhizas is affected by available N and P in the soil (Mason et. al. 2000). Fertility influences root growth and sugar content and consequently ectomycorrhizal colonization (Beckjord et al. 1985). The sugar content of short roots before inoculation was significantly correlated with mycorrhizal development. Results indicate that high levels of N and P in the soil decrease the sugar content of short roots (Vogt et al. 1993) and decrease their susceptibility to ectomycorrhizal development (Dixon et al. 1981a; Marx et al. 1977) because there is a shift in allocation of carbon from fungal growth to the process of N assimilation in plant tissue (Wallenda & Kottke, 1998; Wallander & Nylund, 1991). Additionally, high N availability increases the shoot/root ratio, thereby reducing the amount of photo-assimilates available to below-ground parts (Wallenda & Kottke, 1998). High N concentrations in the shoots of plants have been shown to be negatively correlated with mycorrhizal formation (Wallander & Nylund, 1989).

High N and P levels have been shown to inhibit mycorrhizal development (Alexander & Fairley, 1983; Dixon et al. 1981a; Gagnon et al. 1995; Lilleskov & Bruns, 2003; Mason et al. 2000; Marx et al. 1977; Wiklund et al. 1995) with the negative effects of N fertilization evident years later (Baum et al. 2000). A decrease in ectomycorrhizal types and sporocarp diversity due to fertilization has been shown in numerous studies (Baum & Makeshin, 2000; Cordell & Marx, 1994; Newton & Pigott, 1991). However,

mycelial growth and mycorrhization can also be inhibited without fertilizer amendments (Beckjord et al. 1983; Bougher et al. 1990; Wallander & Nylund, 1991; Wallenda & Kottke, 1998) because of the reduced amount of available carbohydrates produced by the plant. In other studies, fertilization had no effect on mycorrhization (Buschena et al. 1992; Molina & Chamard, 1983). Nilsen et al. (1998) suggests N concentrations in plant tissues might not have accumulated in high enough amounts to affect mycorrhizal infection in some studies. N to phosphorus ratios have also been implicated in affecting mycorrhizal formation (Brun et al. 1995), with mycorrhizae formed more readily in balanced fertility regimes with low N and P (Mason et al. 2000; Ruehle, 1980).

Baum and Makeshin (2000) found N fertilization affected mycorrhizal colonization the most, and that there was an optimal level of N concentration for formation of mycorrhizas (Wallenda & Kottke, 1998). The optimal amount of N depends on fungal species, plant species, and environmental/experimental conditions (Wallenda & Kottke, 1998), because different fungal species and ecotypes vary in their tolerance to fertilizer sources and rates (Trappe, 1977; Marx, 1980). The negative effects of N are only realized at high concentrations, but the use of species that might be adapted to high N concentrations make generalizations difficult (Wallenda & Kottke, 1998). Therefore, there is no consensus as to the best fertilization regime for mycorrhization (Kropp & Langlois, 1990). It is generally accepted that high concentrations of P and N in the substrate discourage ectomycorrhizal formation (Kropp & Langlois, 1990); at high nutrient levels, the relative benefits of mycorrhiza may decline, since the carbon allocation to fungi cannot yield additional nutritional advantages for the host-plant when it becomes saturated with nutrients (Aikio & Ruotsalainen, 2002).

Nitrate and ammonium are the two main sources of inorganic N that are readily available for plant uptake (Marschner, 1986). Numerous studies have examined the role of the type of N applied in fertilizers and how that can affect mycorrhizal development (Ruehle, 1980; Jentschke et al. 1989; Wallander & Nylund, 1991; Beckjord et al. 1983; Brunner I. (In ed.); Plassard et al. 2000; Termorshuizen & Ket, 1989; Finlay et al. 1989). Responses to fertilizer type have been varied, which might be expected given the diversity of ectomycorrhizal fungi and the varied habitats they are adapted to.

Ammonium requires less energy than nitrate for assimilation into plant tissue (Salsac et al. 1987). Wallander and Nylund (1989) conclude that ammonium has more of an inhibitory effect on the fungus than nitrate since it is more easily taken up by roots. This supports the view that an increase in uptake of N by the plant causes a decrease in free soluble carbohydrates in plant tissue, which in turn negatively affects the mycorrhizal fungus. Thus, the preferential uptake of ammonium by plants will decrease available carbohydrates reducing mycorrhizal formation more drastically than nitrate (Termorshuizen & Ket, 1989). Other studies have also shown ammonium to inhibit mycorrhizal formation in pine trees (Plassard et al. 2000).

However, some research has shown that ectomycorrhizal development is inhibited more with nitrate fertilization, and that ammonium was a better source of N for mycorrhizal development (Beckjord et al. 1983, 1995; Finlay et al. 1989; Termorshuizen & Ket, 1989). In this case, ammonium fertilization promoted more shoot growth leading to an increase in root sugars that are potentially available to the ectomycorrhizal fungi (Beckjord et al. 1985, 1995) for mycorrhizal development (Newton & Pigott, 1991; Marx et al. 1977).

Mycorrhizae did form for all treatments where trees were inoculated with *P. tinctorius* forming 100 % (no fert), 81 % (Osmocote) and 37 % (Miracid). It appears that fertilization did depress mycorrhization, only somewhat for Osmocote, but significantly with liquid fertilizer, Miracid. *L. proxima* followed the same pattern but at lower levels 44 % (no fert), 40 % (Osmocote) and 23 % (Miracid). Thus, Osmocote again appears to be a fertilizer type that doesn't severely affect mycorrhization, likely because of its time release quality, balanced N:P ratios and its lower N levels relative to the Miracid fertilizer.

Again there was a fertilizer effect with fertilized trees significantly taller, with two to three time the biomass. Root collar diameter and below and aboveground biomass were also significantly higher for fertilizer treatments.

There were no real differences in tree parameters among fertilized trees; all were similar in size, regardless of mycorrhization levels. For aspen seedlings without fertilizer there did appear to be a significant carbon drain and fungal effect for plants inoculated with native ectomycorrhizal fungi which agrees with the findings of Mason et al, (2000) and others (Maronek et al. 1981). However, there does not appear to be a significant carbon drain or fungal effect for inoculated plants with Osmocote or Miracid fertilizer. These results suggest that plants that receive no nutrient amendments will likely be more effected by the carbon drain involve in sustaining mycorrhizal fungi than plants that receive nutrient supplements. Most reported experiments have been done with conifers, which have a different growth dynamic than aspen. Aspen appears to require fertilization in the greenhouse to avoid serious nutrient deficiencies. Also, aspen root systems are

often more prolific in greenhouse conditions than conifers, and have been known to "by-pass" the fungal inoculum (Cripps, Pers. Comm.).

Summary

In summary, these experiments demonstrate that mycorrhization of aspen seedlings in the greenhouse is possible, although the results vary with inoculation methods, fertilizer regimes and fungal species. Inoculation by banding and fertilization with Osmocote was the most successful method. Generally, inoculated plants were smaller across all treatments when compared to the non-inoculated controls, however, inoculated plants have been shown to improve the field performance on adverse sites (Trappe, 1977; Molina & Trappe, 1982; Marx & Cordell, 1988). While mycorrhizal levels on individual trees were low in the greenhouse, one would expect that any native mycorrhizae that did form would benefit the host plant in the field. While Marx et al. (1981) argue that for any benefits to be realized to the host after out-planting, the root system needs at least 50% ectomycorrhizal colonization, his studies utilized a non-native fungi that might not be as effective as native ectomycorrhizal fungi in persisting on the root system of the inoculated plant when out-planted. Chapter 4 will discuss the mycorrhization and growth response of inoculated seedlings in contaminated field soil and the potential role of ectomycorrhizal fungi in the uptake or inhibition of nutrient and heavy metal uptake. Aspen seedlings were grown in the greenhouse, inoculated with ectomycorrhizal fungi then transplanted into 2 different contaminated field soils from a smelter impacted area.

CHAPTER 4

EFFECT OF VARIOUS ECTOMYCORRHIZAL FUNGI ON THE GROWTH AND UPTAKE OF ELEMENTS FOR ASPEN SEEDLINGS IN TWO SMELTER-IMPACTED SOILS IN GREENHOUSE POT STUDIES

Introduction

Mycorrhizal relationships involve three main components – the host plant, the mycorrhizal fungus, and the soil—and their interactions with one another. Some ectomycorrhizal fungi are thought to ameliorate metal toxicity in plants, allowing them to survive in areas they would otherwise be unable to inhabit. However, in order to understand the potential benefits of mycorrhizal relationships in this regard, it is helpful to first discuss the basic processes that govern each of these three components. Each, in turn, is broadly influenced by the physical, chemical, and biotic interactions of local environmental conditions and gradients. Plus, there are numerous linkages between soil, plants, and fungi, and these linkages may be direct or indirect (Wardle, 2002). Plant growth and survival, uptake of nutrients, water, and elements, rate of photosynthesis and transpiration, and soil chemistry have all been considered as measures for determining the ability of ectomycorrhizal fungi to ameliorate metal toxicity in plants, however, these complex interactions make interpretation uncertain (Jentschke & Godbold, 2000). It can be difficult to discern whether plant response to a mycorrhizal fungus is directly involved in heavy metal tolerance or if it is a secondary (indirect) plant response to mycorrhizal colonization, such as increased nutrient and water acquisition, that is responsible for the plant's tolerance and/or resistance to the heavy metal (Jentschke & Godbold, 2000).

Regardless, studies have shown that ectomycorrhizal fungi can be of significant benefit to plants in soils high in heavy metals (Colpaert & Van Assche, 1987; Jones & Hutchinson, 1986). A brief review of plant, fungal, and soil processes as they relate to heavy metal contamination will introduce concepts that relate to the experiments in this chapter.

Plants

Plant tolerance or resistance to heavy metals in the soil is affected by the concentration and speciation of metal in soil solution, movement of metal from soil to root surface, movement of metal from root surface into root cortex, and translocation from the root to the shoots of a plant (Alloway, 1995). Mechanisms of plant uptake of metal ions, depend on the selectivity of the plant for the ion, plant use of the ion, rate of ion uptake, root age, root densities and distribution (Fitter & Hay, 1981).

Ectomycorrhizal Fungi

Metals are directly or indirectly involved in all aspects of fungal growth and metabolism, and, in fact, many metals are essential to mycorrhizal health (Gadd, 1993). Individual metals may harm mycorrhizal fungi, but generally it is the coordinating effect of multiple metals that are most limiting to mycorrhizae survival and growth (Gadd, 1993). Toxic symptoms may vary widely between different fungi and for different metal species and for different combinations of metals in the soil (Gadd, 1993; Van der Heijden & Kuyper 2001). Gadd (1993) quantifies metal resistance as the ability of an organism to survive metal toxicity via a mechanism produced in direct response to the metal, in contrast to metal tolerance, which is the ability of an organism to survive metal toxicity by modification of toxicity in the environment or by means of intrinsic properties. Plant

growth, uptake of nutrients, water and elements, rate of photosynthesis and transpiration, and analysis of soil chemistry have been identified by Jentschke and Godbold (2000) as parameters which control the ability of ectomycorrhizal fungi to ameliorate metal toxicity in plants.

Studies demonstrating the ability of a mycorrhizal fungi to grow in the presence of an individual metal *in vitro*, while a good indicator of the fungi's tolerance to that metal, does not necessarily mean the mycorrhizal fungus will be able to convey any benefits to the plant in the field (Aggangan et al. 1998) where complex interactions between various components of the soil may affect the response of ectomycorrhizal fungi to toxic metals.

The ability of an ectomycorrhizal fungal species to form mycorrhizae with a species of plant is critical when trying to discuss any potential effects for alleviating metal toxicity in plants as a result of ectomycorrhizal fungi associations . Denny and Wilkins (1987a) argue convincingly that the compatibility of fungal strain and plant may be more important to the success of the relationship in areas with elevated metals, than fungal adaptation to the metal alone (Denny & Wilkins 1987a).

Soil

Soils are a complex heterogeneous medium (Alloway, 1995). The availability and fate of metals in a soil system are affected by various aboveground and belowground components, both abiotic and biotic. Ion concentrations in soil are dependent on parent material, climate, topography, vegetation, and soil age (Fitter & Hay, 1981). All of these

factors together help determine a soil's chemical, physical and biological characteristics. Climate and vegetation affect the development of soil, which in turn affects metal bioavailability and transport in the soil (Campbell et al. 1999).

To understand the potential benefits of ectomycorrhizal fungi on plants in the presence of heavy metals, it is first necessary to identify the type and quantity of metals present. The soil is a dynamic system, and short-term fluctuations in moisture, pH, and redox conditions occur with changes in the environment (Alloway, 1995). Toxicity of metals will vary depending on the organism, the physico-chemical properties of the metal and environmental factors (Gadd, 1993).

The ability of soil to adsorb ions is influenced by the pH, redox conditions, and relative concentration of ions present in the aqueous soil solution (Alloway, 1995). Metals are more labile under low pH conditions (Fitter & Hay, 1981). In addition, soil acidity affects mycorrhiza formation and hyphal development of ectomycorrhizal fungi to different degrees. Van der Heijden and Kuyper (2001) concluded that soil pH, substrate N and P, and their interactions had a significant effect on mycorrhizal colonization.

Bioavailability is affected by pH, organic matter, clay content, presence of other mineral elements, redox conditions, and presence of microflauna, microflora, and macrofauna (Alloway, 1995). Potential mechanisms of adsorption of metals to the soil are: cation exchange capacity of soil colloids, exchange of metal cations with surface ligands, formation of insoluble precipitates, and complexation with organic matter and clays (Alloway, 1995). In addition to the chemical composition of soils, the soil biota can also affect metal availability.

Decomposition And Mineral Weathering. Decomposition of organic matter is an essential process in all ecosystems and has a direct role in nutrient availability and plant growth (Attiwill & Adams, 1993). Decomposition, whether by saprophytic fungi, certain ectomycorrhizal fungi, or microbes, affects the type and availability of organics in the soil, and N compounds in particular which in turn affects mycorrhizal activity (Abuzinadah & Read, 1986b; Bending & Read, 1995). Organic matter levels also affect the moisture-holding capacity of the soil and the microbial activity, which is discussed below.

There is recent evidence that oxalic acid production by ectomycorrhizal fungi is positively correlated with the release of P from apatite (Wallander, 2000a), and organic acids produced by these fungi are implicated in release of other elements. Ectomycorrhizal fungi were also shown to improve potassium uptake from biotite (Wallander & Wickman, 1999). Both processes could affect heavy metal uptake by plant or fungus by changing the nutrient status of the soil.

Microbial Interactions. Interactions between ectomycorrhizal fungi and other soil microbes are complex (Breemen et al. 2000) and poorly understood, although, it is known that microbial communities and mycorrhizae affect each other (Meharg & Cairney, 2000; Read & Perez-Moreno, 2003). The level of microbial activity is dependent on quantity and quality of organic matter present in the system (Danielson et al. 1983). Microbial populations are important components in decomposition which affect the release of mineral nutrients essential for plant growth (Danielson et al. 1983).

Forstener (1995) suggests that heavy metal pollution of soils may inhibit microbial activity. If the decomposition rates are further slowed by heavy metal contamination of soils, it would reason that the decomposers, microbes, and fungi are less numerous or less able to decompose and eventually mineralize organic matter. With less actual mineralization, plants will eventually be affected by a reduction in the available inorganic nutrients necessary to survive. Plants will then contribute less to the organic matter pool, limiting the additional biomass necessary for decomposers, since the number of bacteria, actinomycetes and fungi in the soil are related to the humus content (Stevenson, 1972) of the soil, which is a function of the organic matter of the soil.

Competition of ectomycorrhizal fungi with microbial saprotrophs for access to organic polymers was indirectly shown by Gadgil and Gadgil (1975). Results from Bending and Read (1995a) and Abuzinadah and Read (1986) support the hypothesis that selective exploitation and translocation of N to the host plant by ectomycorrhizal fungi can Pb to N starvation among the saprotroph population, thus reducing decomposition. A study by Koide and Wu (2003) provides an alternative explanation for the 'Gadgil effect'. They found litter decomposition rates were reduced as ectomycorrhizal density increased, which agreed with the findings of Gadgil and Gadgil (1971, 1975). However, Koide and Wu (2003) showed that moisture content was significantly reduced as ectomycorrhizal density increased, thereby identifying moisture levels as the main cause of reduced decomposition. Moisture content plays a major role in forest litter decomposition, affecting the size, composition and activities of saprotrophic communities (Robinson, 2002), which supports the findings of Koide and Wu (2003).

Microorganisms drive various processes in the soil, such as carbon cycling, N mineralization, nitrification, and denitrification (Wardle, 2002). Microbial degradation of organic matter is dependent on the quality and quantity of resources entering the soil and supplies many of the nutrient ions in the soil (Wardle, 2002).

Microbes are stimulated by increased concentrations of various chemicals produced by sloughing off of root cap cells, root release of CO_2, exudation of compounds from intact cells, and production of slime by the root cap (Alloway, 1995; Fitter & Hay, 1981). Microorganisms have been shown to decrease with increasing levels of metals (Freedman & Hutchinson, 1979), although, two species of bacteria indirectly release metal ions from native minerals, *Thiobacillus* and *Ferrobacillus* (Fitter & Hay, 1981). If high levels of metals in the soil inhibit the microbial population, soil processes will also be disrupted. Freedman and Hutchinson (1979) found forest litter decomposition processes negatively affected by metal accumulation in soils. A decrease in the amount of litter being decomposed will negatively affect the amount of organic matter produced, further reducing microbial populations and metal immobilization by organic matter in the soil.

Nutrient Status. Ectomycorrhizal fungi have been shown to increase a plants nutritional status as a result of nutrient acquisition and transfer to the plant (Smith & Read, 1997; Jones et al. 1998; Jentschke et al. 2001). Plants inoculated with ectomycorrhizal fungi therefore are more likely to survive out-planting in disturbed areas where nutrients may be limited, including those with heavy metals.

Research Goals

While some studies have shown that inoculation with ectomycorrhizal fungi can benefit out-plantings on stressed sites (Marx & Cordell, 1988; Molina & Trappe, 1982), other studies do not show clear positive results (Castellano & Trappe, 1991; Ruehle et al. 1981). This suggests that successful ectomycorrhizal systems are dependent on the specifics of site (soil, climate), host plant, fungus, and their interactions. Greenhouse studies can be valuable for gaining insight into how plants might respond to out-planting in particular field soils when inoculated with various species of ectomycorrhizal fungi, and how particular fungi might affect host plant growth and uptake of various elements from these soils. In addition, systems are influenced by greenhouse conditions, and further examination of inoculation techniques is of high interest for nursery application.

The goals of this research were to first examine the effects of inoculation with various species of native ectomycorrhizal fungi originating from smelter-impacted sites near Butte and Anaconda (southwest MT) on the growth of aspen seedlings (with local seed from Silverbow Creek, MT) in smelter-impacted soils from Smelter Hill and the Mt. Haggin area with pot studies in the greenhouse. Seven different species of native fungi were used in this experiment, and most are known mycorrhizal associates of aspen on smelter-impacted sites (Cripps, 2001, 2003). A second goal was to examine how these individual species affect plant uptake of various elements in the soil, including P and heavy metals such as copper. Since data needed to be pooled for this part, precluding a statistical analysis, results will reflect a direct comparison of numbers among inoculated and un-inoculated controls to detect any trends in enhanced or depressed uptake of

elements in above and belowground biomass. In addition, two very different methods of inoculation were examined in this study, one valuable for its success in mycorrhization (as shown in Chapters 2 & 3), and one of interest for its potential efficiency and usefulness in nursery application.

Materials And Methods

Site Descriptions

For the first round of experiments, field soils were collected from two upland sites at Smelter Hill and Mt. Haggin in southwest Montana; both are within range of the now defunct copper smelter stack just south of Anaconda. Both field soil collection sites were impacted by fumes emitted from the copper smelter. Smelter Hill field soils were collected within a 1 mile radius of the Anaconda smelter stack. Mt. Haggin soils were collected upwind from the smelter stack approximately 5 miles to the southeast. In the second round of experiments, the field soils were collected from two upland sites with different elevations in the Mt. Haggin wildlife refuge as a prelude to field studies.

Smelter Hill (Experiment 1). The Smelter Hill injured area is just south of Anaconda, adjacent to the old smelter (Figure 9). The Smelter Hill site is located in Deerlodge County, T4N, R11W, Sec. 9, 10, 15 and 16. Three soil samples taken from three locations were combined into one composite sample for the Smelter Hill site. Location 1 was located at N 46º 03’ 41.0” by W 112º 47’ 17.5” on a 5-10% slope facing 297º west with approximately 80% ground cover, mostly grass. Location 2 was at N 46º 05’ 37.1” by W 112º 57’ 14.7” next to a remnant aspen stand and grass meadow on a 5-

10% slope facing 260º west. Location 3 was at N 46º 06’ 05.7” by W 112º 56’ 00.2” on a small bench facing 180º south with no vegetation present in the immediate vicinity. The Smelter Hill damage area encompasses 4,649 acres (Dept of Justice Natural Resource Damage Program).

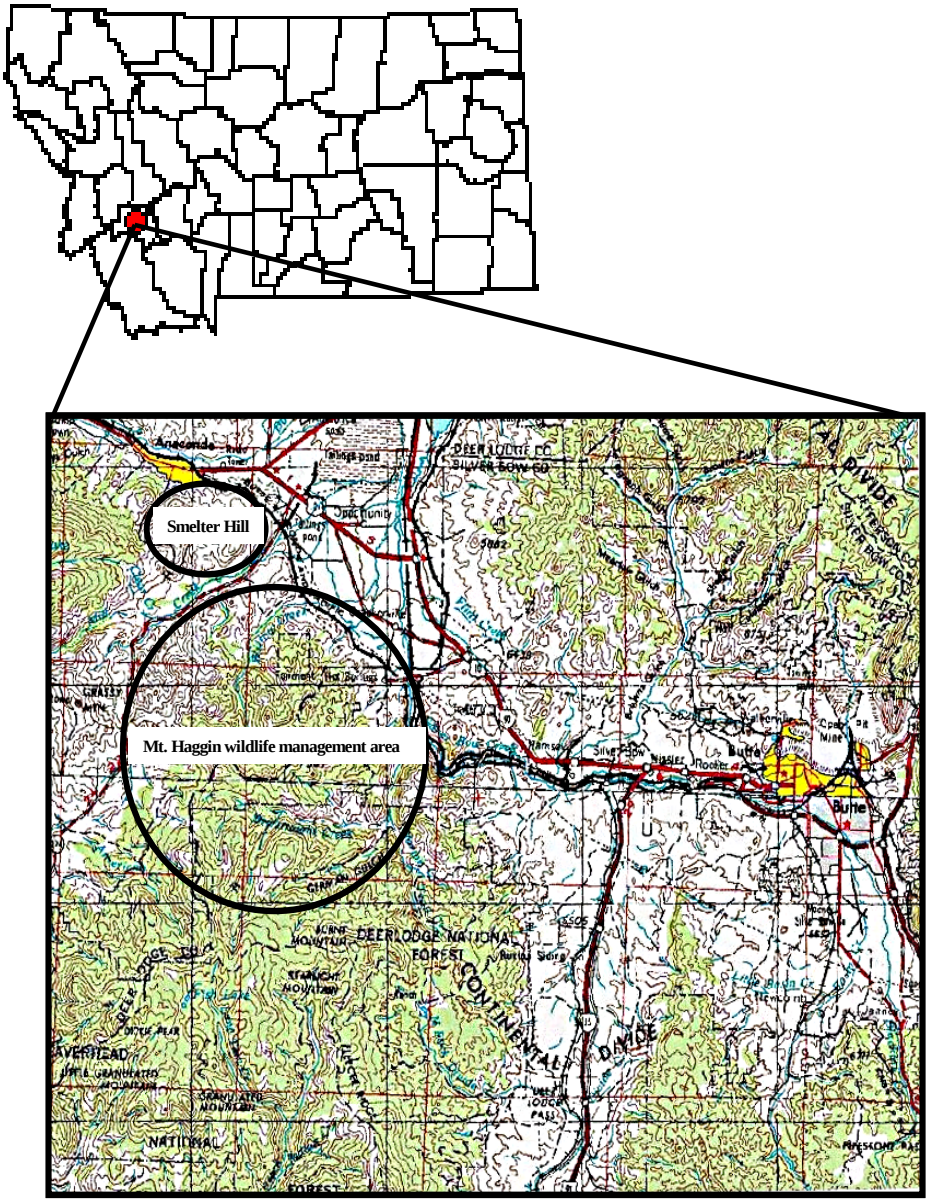

Figure 9. Smelter Hill and Mt. Haggin field site locations at Anaconda, Mt. Map from NRIS, Montana State Library

Climate for areas west of the continental divide are a modified North Pacific Coast type, although the Smelter Hill area is generally affected by continental air masses and less affected by maritime air masses. According to the Environmental Protection Agency and Montana Department of Environmental Quality, the climate is semi-arid, with long cold winters, and cool summers (EPA & MDEQ, 1998). Average annual precipitation is less than 35 cm (14 inches). Data records from the western regional climate center for Anaconda, Montana indicate the average maximum temperatures as 14 ºC, with minimal temperatures averaging –1 ºC. The site is mountainous with elevations above 2,133 meters (7,000 feet) above sea level.

Soils in the Smelter Hill injured area are phytotoxic as a result of smelter emissions (Montana, 1995). Soils developed on steeply sloping alluvial fans, colluvium, and bedrock of sedimentary and volcanic rock types (EPA and MDEQ, 1998). Soils are generally thin and may contain large percentages of rock fragments. The bedrock is a mix between intrusive Idaho Boulder batholiths that are typically characterized by sandy soils, and extrusive igneous rock that have been heavily influenced by ash materials. Intermountain and Piedmont soil associations dominate the western half of Montana. Aridisol and Mollisol soil orders are characteristic of the soils near the Smelter Hill injured area, and are found on nearly level to steep soils of fans, benches, and terraces. These soil orders are further characterized as aridic and frigid. Aridic soils are typically dry for more than half of the time when they are warm enough for plant growth and never moist in any part for 90 consecutive days when they are warmer than 8 ºC. Frigid soils have mean soil temperatures below 8 ºC, but above 0 ºC. Aridisols are light colored soils of dry regions that are depleted of plant-available moisture for most of the summer.

Aridisols are generally un-leached and alkaline in reaction, however in the Anaconda area the aerial deposition of heavy metals, sulfur, and other elements from the smelting process has acidified most of the top 1-13 cm of soil. Aridisol soils typically consist of a shrub-grass or short-grass prairie with juniper and stunted Ponderosa pine in places. Mollisols are dark colored, base-rich soils, which lack layers of clay accumulation. Typically they are characterized by prismatic and a blocky subsoil structure.

Mount Haggin (Experiment 1). The Mount Haggin injured area lies within the Mount Haggin Wildlife Management Area, just east of Mill Creek highway. The Mount Haggin injured area is in Deerlodge County, T3N, R11W, sec. 25, 26, 35, and 36. Three samples were collected from one location, N 46º 04' 55.5" by W 112º 54' 56.1" with a 5% slope facing 344º N. Surrounding vegetation consisted of a mixed grass-shrub habitat with approximately 70% ground cover. The Mt. Haggin Wildlife Refuge Damage Area encompasses 4,299 acres (Dept of Justice Natural Resource Damage Program).

According to the Environmental Protection Agency and Montana Department of Environmental Quality, the climate is semi-arid, with long cold winters, and cool summers. Average annual precipitation is less than 35 cm (14 inches). Data records from the western regional climate center for Anaconda, Montana indicate the average maximum temperatures as 14 ºC (57 ºF), with minimal temperatures averaging –1 ºC (29.7 ºF). The site is mountainous with an elevation of approximately 1,676 meters (5,500 feet) above sea level.

Dominant landforms in the area are foothills, mountain slopes, alpine ridges, plateaus, and cirques. The soils are derived from a variety of igneous, sedimentary and

metasedimentary rocks of volcanic origin with soils further developed from rhyolite and rhyolitic tuffs (Montagne et al. 1982). Inceptisol and Alfisol soil orders are typical of the area. These soil orders are further characterized as Udic-Cryic. Udic soils are usually moist soils, not dry for as long as 90 cumulative days and do not have a prolonged summer dry period, and are never wet except for short periods. Cryic soils have a mean annual soil temperature 0-8º C (Montagne et al. 1982). Inceptisol soils are light colored soils that lack horizons of clay accumulation, occur under cool and cold temperatures in areas of high to moderately low precipitation, and are formed in either grass or forest vegetation types that may be both alkaline or acidic in reaction. Alfisols are light colored, slightly to moderately acidic soils, with brownish subsoil horizons of clay accumulation. Some are leached but are moderate to high in bases. These soils are formed under coniferous or mixed forests with moderately low to high precipitation and cool to cold climates, primarily on mountains and foothills (Montagne et al. 1982).

Mt. Haggin (Experiment 2) Site 1 (MH 1). Both sites in Experiment 2 are within the boundaries of the Mount Haggin injured area in Deerlodge County, T3N, R11W, sec. 25, 26, 35, and 36. Site 1 location is N 43º 38’ 392” by W 111º 01’ 377” with a 5-10% slope facing 76º east. Elevation at Site 1 is 1560 meters (5117 feet). The smelter stack is 16º north of the line-of-site from the plot where the soil was collected. Remnant aspen is present in gullies to the east and west of Site 1, while old stumps, possibly *Douglas fir*, logged over 50 years prior remain in scattered areas at a density of 200 DTPA (dead trees per acre). Bare ground accounts for roughly 30% of the plot area. Other significant

components of the ground cover consists of *Mahonia repens* at 30%, *Carex garberi* at 15%, bunchgrass at 15%, and slash (dead tree limbs) at 5%.

Mt. Haggin (Experiment 2) Site 2 (MH 2). Site 2 location is N 42º 02' 149" by W 112º 57' 836" on mostly flat ground also facing 76º east. Some scattered *Pinus contorta*, 0.5 m (1-2') and 1.5 m (4-6') are in the general vicinity of the plot. Elevation at site 2 is 2132 meters (6996') above sea level. Bare ground accounts for roughly 50% of the plot area. Other significant components of the ground cover consist of bunchgrass at 30%, *Chamerion angustifolium* at 5%, and slash at 5%.

Soil Collection And Analysis

Field soil was collected from two sites in the Anaconda area for use in greenhouse studies: Smelter Hill and the Mt. Haggin Wildlife Refuge. Samples were collected to a depth of 15 cm (6") from three areas at each site and combined for a composite sample representing each site. Smelter Hill composite samples were taken from a larger overall area than the Mt. Haggin field soils because the lack of distinct habitat types in the immediate area around the smelter stack indicated a potential for large differences in soil characteristics. The metal shovel used to collect field soil was wiped clean between each site. Field soils were transported to Montana State University, where they were first sifted through a 2 mm sieve to remove larger rock fragments, placed in paper bags and then taken to the Soil, Plant & Water Analytical Lab in Leon Johnson Hall for analysis.

Smelter Hill And Mt. Haggin, Soil Analysis (Experiment 1). For Experiment 1, field soils from Smelter Hill and Mt. Haggin were analyzed for: pH (1:2), N both in

nitrate and ammonium (KCl extraction), P (P Olsen $NaHCO_3$ method), K (extractable 1M ammonium acetate), Electrical Conductivity (1:2), and percent organic matter (OM) (modified Walkley-Black Method). Extractable levels are reported for Al, As, Cd, Cu, Fe, Mn, Ni, Pb and Zn in mg/kg, utilizing the DTPA (diethyltriaminepentaacetic acid) extraction method. This method of extraction reflects the amount of each element that would potentially be available for plant uptake, however, this method utilizes some harsh extraction procedures and as a result will report elemental values that might not be available for plant uptake. (For a thorough discussion on different tools utilized to evaluate bioavailability in soils see Chapter 4 in *Bioavailability of Contaminants in Soils and Sediments*, NRC, 2003).

Mt. Haggin; Site 1 (MH 1), Site 2 (MH 2), (Experiment 2). For Experiment 2, field soils from Mt. Haggin site 1 (MH 1) and site 2 (MH 2), a saturated paste method of analysis was utilized (in contrast to Experiment 1). Consequently soil analysis values for the second experiment consist of only water soluble levels for pH, N, P, K, EC, Al, As, Cd, Cu, Fe, Mn, Ni, Pb, Zn, S, NH_4-N and NO_3-N in mg/l. Percent sand, silt and clay and soil texture are also reported. To obtain mg/kg, all values were multiplied by the percent water paste and are included in the results section. These values reflect the most available levels of elements in a soil system.

For the pot studies, soil samples from each of the sites were mixed in the greenhouse in a rotary cement mixer. A metal sieve (2mm) was utilized to remove larger soil and rock particles before the soil was placed in containers. For Experiment 1, field soil was aerated steam pasteurized at 80 ºC for 40 minutes. In Experiment 2, field soils

were again aerated steam pasteurized at 80 ºC but for 60 minutes.

Native Ectomycorrhizal Fungi

Seven species of native ectomycorrhizal fungi, *Paxillus vernalis*, *Scleroderma citrinum*, *Pisolithus tinctorius*, *Laccaria proxima*, *Tricholoma flavovirens*, *Tricholoma scalpturatum*, and *Inocybe lacera*, initially collected from a smelter impacted site by Mahony and Cripps and identified by Cripps (1992) as forming ectomycorrhizae with aspen, were utilized for these experiments. These ectomycorrhizal fungi were further selected based on earlier experiments (see Chapter 2 and 3) that showed that these species could be cultured, have potential for use as inoculum, and were available in sufficient quantities to be utilized as soil inoculum.

Aspen Seed

Aspen seed purchased from Bitterroot Restoration Inc., was collected by the company in Silverbow County, Montana. The seed was stored at 1 ºC in a refrigerator until planted.

Inoculation Procedures

Two different practical methods for mycorrhization of aspen in a greenhouse setting were selected for this study. The first method results from preliminary studies (Chapter 2 and 3) which showed that "banding" was a superior method for success of mycorrhization of aspen, however, it is labor intensive in that aspen seedlings must be raised for 1 month and then transplanted in soil in which a "band" of mycorrhizal inoculum has been carefully placed.

The alternative method involves sowing the seeds directly on soil inoculum. This method would be of high interest to nurseries in that most aspen are grown from seed by simply seeding pots and thinning the resulting seedlings. For this reason, it was compared to the "banding" method.

Banding 1". Germination of the aspen seedlings utilized for the banded 1" method of inoculation followed those discussed in Chapter 2, except that seedlings received a small amount of liquid fertilizer. All aspen seedlings received 1 dose (4-6 ml) of 20-20-20 Peters ® liquid fertilizer at 100 ppm after the second set of true leaves appeared. The remainder of the procedures followed those outlined in Chapter 3. All aspen seedlings were harvested 3 months after germination.

Inoculation At Time Of Sowing. For the 'inoculated at time of sowing' method, seed was sown directly on soil inoculum (with fungus mycelium). All aspen seedlings received 1 dose (4-6 ml) of 20-20-20 Peters ® liquid fertilizer at 100 ppm after the second set of true leaves appeared. All aspen seedlings were harvested 3 months after germination.

For the non-inoculated controls, either a 1" band of non-colonized peat:vermiculite soil inoculum was placed directly below the one-month-old aspen seedlings, or cubed trays were filled with non-colonized peat:vermiculite soil inoculum. A review of the interactions between roots and soil inoculum utilizing the banded 1" method of inoculation can be found in Chapter 2. Rootrainer ® trays were utilized for Experiment 1, and in Experiment 2, 4" super cell containers ® were used.

Experimental Design

Two different practical methods of mycorrhization of aspen (banding and sowing) were first compared for two field soils (Experiment 1: Smelter Hill and Mt. Haggin), and then for different elevational sites within the Mt. Haggin area (Experiment 2: site 1 & 2) using several different mycorrhizal fungi with each inoculation method. The level of mycorrhization, and growth response of aspen was compared for the treatments within each experiment. A soil analysis was conducted to characterize elemental levels within the soil for all sites. In addition, the levels of selected elements and heavy metals, as determined by tissue analysis of roots and shoots of aspen, were compared for treatments, and examined in regard to their levels in the soils for Experiment 1.

Experiment 1. Experiment 1 was designed to examine the ability of seven different species of ectomycorrhizal fungi, *Paxillus vernalis*, *Scleroderma citrinum*, *Pisolithus tinctorius*, *Laccaria proxima*, *Tricholoma flavovirens*, *Tricholoma scalpturatum* and *Inocybe lacera* to form mycorrhizae with aspen and to quantify the plant growth response in two different field soils from smelter impacted sites, in the greenhouse utilizing two dissimilar inoculation techniques, 1" banding or 'sowing seeds on inoculum'. Smelter Hill field soils were utilized in this experiment because of their close proximity to the smelter stack (<1 mile) and Mt. Haggin field soils were selected because of their distance from the smelter stack (>5 miles) and their direction from the smelter stack (345º south) which is generally upwind of the smelter stack.

Two inoculation methods were used for each of the two soils with the following fungal treatments. For the Mt. Haggin field soil, six treatments (5 fungal, 1 control) were

randomly arranged in the greenhouse first utilizing the 'sowing seeds on inoculum' method of inoculation. Aspen seed was sown directly on *Paxillus vernalis*, *Pisolithus tinctorius*, *Laccaria proxima*, *Tricholoma flavovirens* and *Tricholoma scalpturatum* inoculum. Twelve seeds were utilized for the non-inoculated controls, 12 seeds for both the *Paxillus vernalis* and *Pisolithus tinctorius* treatments, 11 seeds for the *Laccaria proxima* treatment, 8 seeds for the *Tricholoma flavovirens* treatment, and 9 seeds for the *Tricholoma scalpturatum* treatment, all dependent on availability of inoculum.

Another five fungal treatments were randomly arranged in rows in the greenhouse utilizing the 'sowing seeds on inoculum' method of inoculation for the Smelter Hill field soil. Aspen seed was directly sown on *Paxillus vernalis*, *Pisolithus tinctorius*, *Laccaria proxima* and *Tricholoma flavovirens* inoculum. Twelve aspen seeds were inoculated with *Laccaria proxima*, 12 seeds with *Tricholoma flavovirens*, 11 seeds with *Paxillus vernalis*, 7 seeds with *Pisolithus tinctorius,* and 11 seeds were utilized for the non-inoculated controls. It was not possible to use all fungi for all treatments because of a lack of sufficient inoculum.

For the 1" banding method of inoculation, aspen seedlings were grown for one month then randomly arranged into 15 treatments for the Mt. Haggin and Smelter Hill field soil. For the Mt. Haggin field soil 'banding method of inoculation', 12 seedlings were utilized as non-inoculated controls, 12 seedlings were inoculated with *Paxillus vernalis*, 11 seedlings each with *Tricholoma flavovirens* and *Inocybe lacera*, five with *Pisolithus tinctorius*, and six with *Scleroderma citrinum*. For the 1" banding method of inoculation for the Smelter Hill field soil, 12 seedlings were inoculated with *Paxillus vernalis*, 12 with *Laccaria proxima*, 12 with *Inocybe lacera*, six with *Scleroderma*

citrinum treatment and five with *Pisolithus tinctorius*, and 11 were utilized for the non-inoculated controls. Plants were moved weekly to further randomize the design and to reduce the variability of growing conditions present in the greenhouse. After 3 months plants were measured and weighed. Shoots and roots were washed, air dried for 2 days, ground, and then analyzed separately according to the method of inoculation, fungal treatment and site to determine how the different treatments influenced the uptake of elements from contaminated soils.

Experiment 2. Experiment 2 was designed to examine the ability of six different species of ectomycorrhizal fungi, *Paxillus vernalis*, *Scleroderma citrinum*, *Pisolithus tinctorius*, *Laccaria proxima*, *Tricholoma flavovirens* and *Inocybe lacera* to form mycorrhizae with aspen and to quantify the plant growth response in soil from two different field sites within one smelter-impacted site in greenhouse studies. Mt. Haggin field soils were utilized to compare the effects of fungi on aspen growth in field soil from two different elevations with similar aspects, and slopes, within the same area. In addition these locations were chosen for their potential as out-planting sites.

The 1" banding method of inoculation where aspen seedlings are grown for one month was used for all treatments. Five fungal treatments and a non-inoculated control group were utilized for each soil type. For site MH 1 field soil, 15 seedlings were utilized as non-inoculated controls, 11 seedlings were inoculated with *Scleroderma citrinum*, 10 seedlings were inoculated with *Tricholoma flavovirens*, 10 seedlings were inoculated with *Paxillus vernalis*, 9 seedlings were inoculated with *Inocybe lacera* and 8 seedlings were inoculated with *Pisolithus tinctorius*. For site MH 2 field soil, 16

seedlings were utilized as non-inoculated controls, 10 seedlings were inoculated with *Laccaria proxima*, 10 with *Pisolithus tinctorius*, 10 with *Scleroderma citrinum*, 10 with *Paxillus vernalis* and 10 with *Inocybe lacera*. Plants were moved weekly to further randomize the design and to reduce the variability of growing conditions present in the greenhouse. After 3 months, plants were analyzed.

Seedling Harvesting

Aspen seedlings were harvested 3 months after germination for each experiment. Roots were washed and visual estimates were made following procedures in Chapter 2 and 3. Mycorrhizae were identified following procedures in chapter 2. Height (cm), root collar diameter (mm), above (g), below (g) and total biomass (g) were measured. The shoot and root biomass of aspen seedlings were weighed after 1 week in a plant drying oven.

Plant Tissue Analysis

In Experiment 1, the plant tissue of the shoots and roots were analyzed separately utilizing the dry ash digestion method by the Soil, Plant & Water Analytical Lab, located in Leon Johnson Hall, Montana State University. Plants were oven dried for a week. Aspen seedlings were pooled together by fungal treatment in order to provide the lab with enough plant material for analysis, and to reduce costs. Pooling data prevented any statistical analysis of plant tissue for the different fungal treatments on element uptake, but general trends and conclusions were based on comparisons with non-inoculated control aspen seedlings. Potassium, magnesium, N, P, and sulfur are given in percentages, while copper, Fe, Ni, Pb and Zn are given in mg/kg because of their

relatively low levels, and are reported in the results section. Values above or below 10% of the controls are reported, to indicate general trends.

Data Analysis

Experiment 1. In experiment 1 the percentage of aspen seedlings that formed mycorrhizae in Mt. Haggin and Smelter Hill field soils was assessed and recorded for all treatments, including the non-inoculated controls. Additionally, the growth response of non-inoculated and inoculated aspen seedlings utilizing two different inoculation techniques, banded 1" or by 'sowing seeds directly on inoculum' was analyzed using a 3-way analysis of variance (SPSS General Linear Model Univariate ANOVA, SPSS, Inc. Version 11.5) for height, root collar diameter, aboveground, belowground and total biomass. Analysis only included aspen seedlings that were alive in each treatment at the end of the experiment. Aspen seedlings survival was not a significant issue because mortality was minimal and random across treatments. The 3-way ANOVA included three main effects – field soil, inoculation method, and fungal treatment – plus interaction effects for method of inoculation by fungal treatment, site by fungal treatment, and site by method of inoculation by fungal treatment. All main effects and interactions with P-values less than 0.05 are reported. Levene's test of equality of error variance was used to determine whether the data required transformation. Post-hoc Tukey with α=0.05 was conducted for each variable and is included in the Appendix. Percentage differences of elements within plant tissue greater than 10 % above or below the control group were reported for both the shoots and roots of each fungal treatment for each site.

Experiment 2. In Experiment 2 the percentage of aspen seedlings that formed mycorrhizae in two field soils from the Mt. Haggin area, was recorded for all treatments, including the non-inoculated controls. Additionally, the growth response of non-inoculated and inoculated aspen seedlings was analyzed using a 2-way ANOVA (SPSS General Linear Model Univariate ANOVA, SPSS, Inc. Version 11.5) for height, root collar diameter, aboveground, belowground and total biomass. Analysis only included aspen seedlings that were alive in each treatment at the end of the experiment. Aspen seedling survival was not a significant issue because mortality was minimal and random across treatments. The 2-way ANOVA included two main effects – field soil and fungal treatment – plus interaction effects of site by fungal treatment. All main effects and interactions with P-values less than 0.05 are reported. Levene's test of equality of error variance was used to determine whether the data required transformation. Post-hoc Tukey with $\alpha=0.05$ was conducted for each variable and is included in the Appendix.

Results

Experiment 1

The first experiment compared the level of mycorrhization of aspen seedlings inoculated with seven species of ectomycorrhizal fungi by two different inoculation methods in two different field soils collected from Smelter Hill and the Mt. Haggin wildlife refuge (Table 25). Host plant growth response to the various treatments was analyzed after 3 months in the greenhouse. Percent mycorrhization, means and standard deviations of the growth response for the various treatments are shown in Table 26. Standard deviations varied across all treatments in field soils, which is to be expected

given the large variations of aspen seedling growth under more uniform conditions in soil-less media (peat:vermiculite) utilized in Chapter 3 experiments. In addition, results of the plant tissue analysis were conducted on both the shoots and roots for each treatment from both field soils as shown in Table 27.

Table 25. Soil analysis of extractable elements from Mt. Haggin and Smelter Hill (Experiment 1; values in Mg/Kg unless otherwise noted)

Site	pH	EC (mmhos/cm)	OM %	NO3-N	NH4-N	K	Olsen Phos	SO_4-S	As
Mt. Haggin	6.3	0.08	2.44	4	8.9	3760	23.7	3.9	1
Smelter Hill	6.1	0.08	5.75	2.6	33	368	67.9	11.6	2

Site	Al	Cu	Fe	Mn	Pb	Zn	Cd	Ni
Mt. Haggin	2.1	96.6	42.1	41.7	51.7	91.6	4.67	0.54
Smelter Hill	3.5	395	107	30.6	29.1	75.3	5.33	0.93

Field soils collected from the Smelter Hill area had a lower pH value, higher levels of organic matter and electrical conductivity values similar to that of Mt. Haggin field soils (Table 25). For the major nutrients, N, P, K, Smelter Hill had more available P and less available potassium, and the available N in the two field soils differed by N form (Table 25). Smelter Hill had more available N as ammonium and less available N as nitrate than Mt. Haggin field soils. Other than Pb, Zn, and manganese, the Smelter Hill field soils had higher levels of metals, sulfur, and As.

Percentage Of Aspen Seedlings With Mycorrhizae

Mycorrhizae did not form on the controls in the Mt. Haggin and Smelter Hill field soil utilizing the 'inoculating at time of sowing' method of inoculation (Table 26, 27; Figure 10). However, mycorrhizae did form on the controls in the Mt. Haggin and Smelter Hill field soil utilizing the 1" banding method. Thirty-three percent of non-

inoculated aspen seedlings formed mycorrhizae in the Mt. Haggin field soil. Eighty one percent of non-inoculated aspen seedlings formed mycorrhizae in the Smelter Hill field soil. The mycorrhizae identified on the controls in both field soils were brown, smooth, and without clamps (a characteristic of Basidiomycetes), and is possibly an E-strain fungus, an ectendomycorrhizal fungi. Ectendomycorrhizas are used to describe mycorrhizal roots that exhibit characteristics of both ectomycorrhizal and endomycorrhizal fungi (Smith & Read, 1997). No mycorrhizae formed on any of the aspen inoculated with fungi ' at the time of sowing' for the Mt. Haggin field soils.

In the Mt. Haggin field soil, utilizing the banded 1" method of inoculation, 58 % of the aspen seedlings inoculated with *Paxillus vernalis* formed mycorrhizae, 50 % with *Scleroderma citrinum* formed mycorrhizae, 70 % with *Laccaria proxima* formed mycorrhizae, 45 % with *Tricholoma flavovirens* formed mycorrhizae, and 81 % with *Inocybe lacera* formed mycorrhizae. In the Smelter Hill field soil utilizing the 'inoculated at time of sowing' method only *Laccaria proxima* formed mycorrhizae at 50 %.

For the banded 1" method of inoculation in the Smelter Hill field soil, 100 % of seedlings inoculated with *Paxillus vernalis, Laccaria proxima* and *Scleroderma citrinum* formed mycorrhizae, 80 % inoculated with *Pisolithus tinctorius* formed mycorrhizae, 75 % inoculated with *Tricholoma flavovirens* formed mycorrhizae and 50 % of aspen seedlings inoculated with *Inocybe lacera* formed mycorrhizae. Mycorrhization appears to be affected by both soil type and method of inoculation, although conclusions are difficult because of inadvertent mycorrhization of some controls. Mycorrhization levels were higher utilizing the banded 1" method when compared to the 'inoculated at time of

sowing' method. In addition, the growth of aspen seedlings inoculated utilizing the banded 1" method were bigger than seedlings 'inoculated at the time of sowing'. Aspen seedlings grown in Smelter Hill field soils were much smaller than seedlings grown in Mt. Haggin field soil as a result of elevated metal levels in the Smelter Hill field soil.

Table 26. Percent mycorrhization, number of replicates, means and standard deviations of growth parameters of inoculated and non-inoculated aspen seedlings in two different field soils (Mt. Haggin and Smelter hill) after three months in the greenhouse utilizing two inoculation techniques: banded 1" or "inoculated at time of sowing'. Data not transformed. Method 1=Banded 1", method 2='inoculated at time of sowing'. NA=inoculum unavailable. Different letters denote significant differences at $p<0.05$.

MT. HAGGIN	Inoculation	Controls	*Paxillus vernalis*	*Scleroderma citrinum*	*Pisolithus tinctorius*	*Laccaria proxima*	*Tricholoma flavovirens*	*Tricholoma scalpturatum*	*Inocybe lacera*
% Aspen w/mycorrhizae	Method 1	33	58	50	0	70	45	NA	81
Number of replicates	n	12	12	6	5	10	11	0	11
% Aspen w/mycorrhizae	Method 2	0	0	NA	0	0	0	0	NA
Number of replicates	n	12	12	0	12	11	8	9	0
Height (cm)	Method 1	10.53±2.75 A	7.33±2.21 ABCDEF	9.28±1.88 AB	7.7±2.88 ABCDEF	10.67±2.57 A	8.19±3.37 ABCDE	NA	10.29±3.17 A
	Method 2	7.33±1.82 ABCDEF	7.77±2.21 ABCDE	NA	5.8±1.8 CDEFG	7.68±2.39 ABCDEF	8.96±1.86 ABCDE	8.47±2.13 ABC	NA
Root collar diameter (mm)	Method 1	2±0.33 AB	1.85±0.26 ABCDE	1.71±0.19 ABCDE	1.73±0.35 BCDEF	2.21±0.32 A	1.82±0.52 ABCD	NA	1.96±0.27 ABC
	Method 2	1.72±0.12 ABCDE	1.74±0.28 ABCD	NA	1.59±0.41 ABCDE	1.72±0.25 ABCDE	1.92±0.45 ABCD	2.04±0.31 AB	NA
Aboveground biomass (g)	Method 1	0.23±0.09 A	0.14±0.04 ABCDEF	0.14±0.02 ABCDE	0.14±0.07 ABCDEFG	0.24±0.06 A	0.15±0.08 ABCDEFG	NA	0.22±0.1 A
	Method 2	0.13±0.04 ABCDEFG	0.17±0.06 ABC	NA	0.11±0.05 BCDEFGH	0.15±0.07 ABCD	0.17±0.08 ABC	0.19±0.04 AB	NA
Belowground biomass (g)	Method 1	0.21±0.10 ABC	0.16±0.08 ABCDE	0.15±0.4 ABCD	0.13±0.07 ABCDEF	0.14±0.09 A	0.14±0.10 ABCDEF	NA	0.23±0.09 ABC
	Method 2	0.12±0.05 ABCDEF	0.17±0.08 ABCD	NA	0.16±0.08 ABCDEF	0.30±0.14 ABCDEF	0.19±0.16 ABCDE	0.21±0.10 ABC	NA
Total biomass (g)	Method 1	0.44±0.18 ABC	0.30±0.11 ABCDE	0.29±0.06 ABCD	0.27±0.13 ABCDEFG	0.54±0.19 A	0.29±0.17 ABCDEFG	NA	0.45±0.19 AB
	Method 2	0.26±0.10 ABCDEF	0.33±0.13 ABCD	NA	0.27±0.10 ABCEF	0.29±0.15 ABCDEF	0.36±0.23 ABCD	0.40±0.14 ABC	NA

Table 26. continued

SMELTER HILL	Inoculation	Controls	*Paxillus vernalis*	*Scleroderma citrinum*	*Pisolithus tinctorius*	*Laccaria proxima*	*Tricholoma flavovirens*	*Inocybe lacera*
% Aspen w/mycorrhizae	Method 1	81	100	100	80	100	75	50
Number of replicates	n	11	12	6	5	12	12	12
% Aspen w/mycorrhizae	Method 2	0	0	NA	0	50	0	NA
Number of replicates	n	11	11	0	7	12	12	0
Height (cm)	Method 1	4.56±1.53 FGHI	3.93±1.81 GHIJ	4.67±1.49 EFGHI	4.56±1.50 FGHI	4.99±1.33 DEFGH	5.27±1.64 CDEFGH	6.14±1.90 BDEFG
	Method 2	1.82±0.64 J	7.77±2.21 GHIJ	NA	3.97±1.02 GHIJ	2.70±0.75 HIJ	2.45±0.54 IJ	NA
Root collar diameter (mm)	Method 1	1.48±0.15 CDEFG	1.47±0.32 CDEFGH	1.58±0.13 BCDEF	1.30±0.11 EFGH	1.63±0.16 BCDEF	1.44±0.22 DEFGH	1.64±0.31 BCDEF
	Method 2	0.72±0.29 I	1.26±0.22 EFGH	NA	1.15±0.39 FGHI	0.97±0.23 HIJ	1.08±0.28 GHI	NA
Aboveground biomass (g)	Method 1	0.08±0.02 CDEFGH	0.07±0.05 FGHI	0.09±0.01 CDEFGH	0.07±0.04 EFGHI	0.10±0.03 BCDEFGH	0.08±0.02 DEFGHI	0.1±0.04 CDEFGH
	Method 2	0.02±0.01 J	0.05±0.02 HIJ	NA	0.06±0.02 GHIJ	0.02±0.02 IJ	0.04±0.02 IJ	NA
Belowground biomass (g)	Method 1	0.09±0.03 BCDEF	0.11±0.07 ABCDEF	0.08±0.3 BCDEFG	0.06±0.03 DEFG	0.13±0.05 ABCDEF	0.07±0.03 CDEFGH	0.12±0.08 ABCDEF
	Method 2	0.01±0.01 H	0.06±0.04 FG	NA	0.06±0.03 EFG	0.01±0.01 H	0.03±0.02 GH	NA
Total biomass (g)	Method 1	0.17±0.05 CDEFG	0.17±0.11 DEFG	0.17±0.04 CDEFG	0.13±0.07 EFGH	0.23±0.07 ABCDEFG	0.15±0.04 DEFGH	0.22±0.12 BCDEFG
	Method 2	0.03±0.02 I	0.11±0.05 GH	NA	0.12±0.05 GFH	0.03±0.02 H	0.07±0.04 HI	NA

Table 27. Mean % mycorrhization of inoculated and non-inoculated seedlings, number of trees with mycorrhizae, % mycorrhizal infection on trees with mycorrhizae and % of trees with mycorrhizae

Fungi	N	Mean % Mycorrhization of Aspen	% Mycorrhizal Infection of Aspen w/mycorrhizae	# Aspen w/mycorrhizae	% Aspen w/mycorrhizae
Mt. Haggin (inoculated at time of sowing)					
Controls	12	0	0	0	0
Paxillus vernalis	12	0	0	0	0
Pisolithus tinctorius	12	0	0	0	0
Laccaria proxima	11	0	0	0	0
Tricholoma flavovirens	8	0	0	0	0
Tricholoma scalpturatum	9	0	0	0	0
Mt. Haggin (banded 1")					
Controls	12	0.33	1	4	33
Paxillus vernalis	12	0.8	1.7	7	58
Scleroderma citrinum	6	0.5	1	3	50
Pisolithus tinctorius	5	0	0	0	0
Laccaria proxima	10	1.9	2.7	7	70
Tricholoma flavovirens	11	1.2	2.6	5	45
Inocybe lacera	11	2.3	2.5	10	81

Fungi	N	Mean % Mycorrhization of Aspen	% Mycorrhizal Infection of Aspen w/mycorrhizae	# Aspen w/mycorrhizae	% Aspen w/mycorrhizae
Smelter Hill (inoculated at time of sowing)					
Controls	11	0	0	0	0
Paxillus vernalis	11	0	0	0	0
Pisolithus tinctorius	7	0	0	0	0
Laccaria proxima	12	0.75	1.5	6	50
Tricholoma flavovirens	12	0	0	0	0
Smelter Hill (banded 1")					
Controls	11	0.09	1	9	81
Paxillus vernalis	12	6.5	6.5	12	100
Scleroderma citrinum	6	28.3	28.3	6	100
Pisolithus tinctorius	5	9	11.3	4	80
Laccaria proxima	12	9.17	9.17	12	100
Tricholoma flavovirens	12	6.5	8.7	9	75
Inocybe lacera	12	4.6	9.2	6	50

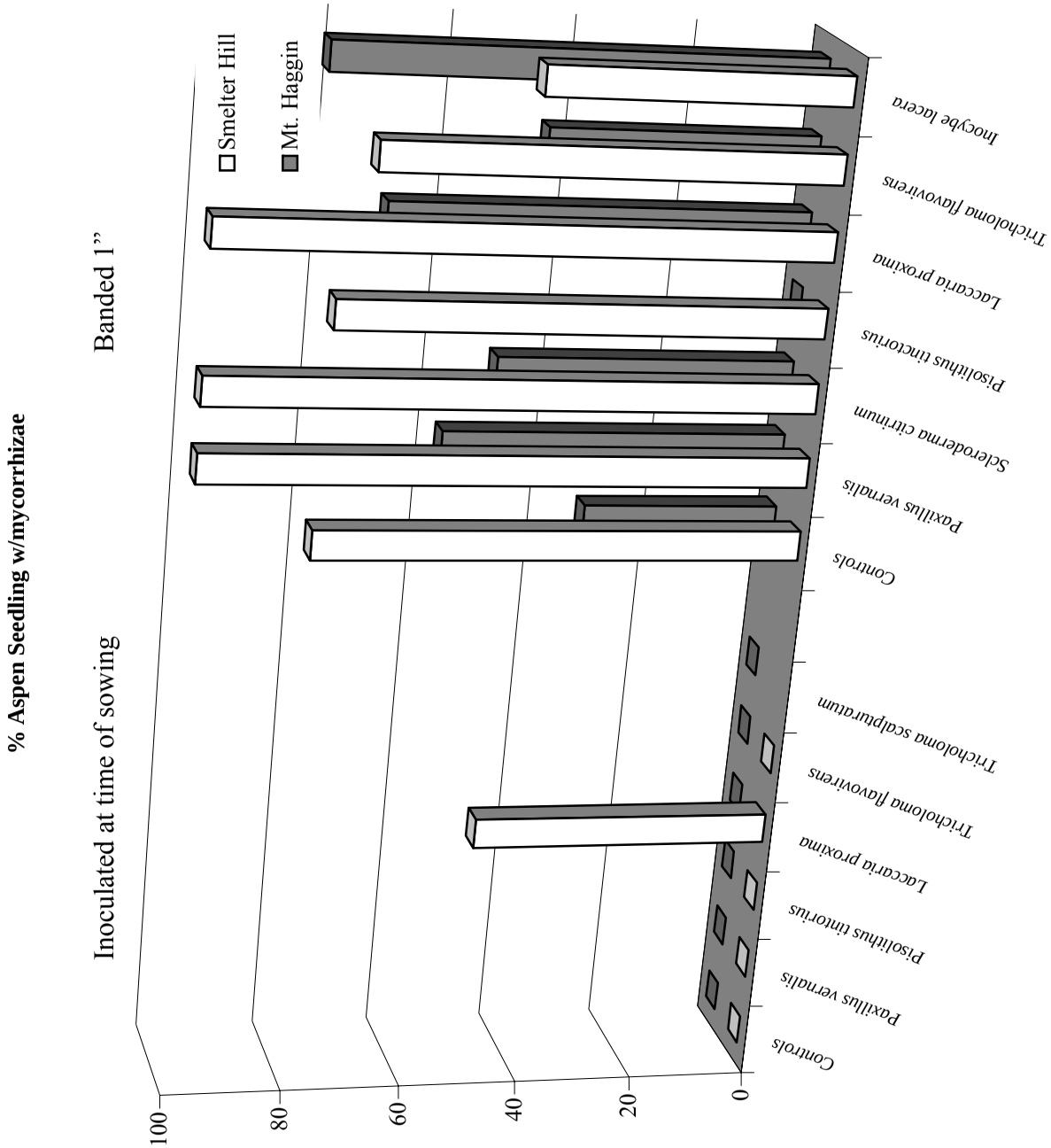

Figure 10. Percent mycorrhization of inoculated and non-inoculated aspen seedlings in two field soils in the greenhouse utilizing two inoculation techniques; banded 1” and inoculated at time of sowing, after three months.

Height

The height (cm) of aspen seedlings was affected by soil treatment, method of inoculation and fungal treatment (Table 28). Seedlings inoculated with ectomycorrhizal fungi were taller than the non-inoculated controls (Table 28; Figure 11). Aspen seedlings inoculated by the banding 1" method were taller than seedlings inoculated at the time of sowing (Table 28; Figure 11). Aspen seedlings in Mt. Haggin field soil were significantly taller than seedlings in the Smelter Hill field soil (Table 28; Figure 11). There were significant interactions between site, method of inoculation and fungal treatment, between site and fungal treatment and between method of inoculation and fungal treatment (Table 28).

Table 28. Analysis of variance, Height (cm); transformed (square root).

Source	Type III Sum of Squares	df	F	Sig.
site * meth * fungi	2.600	5	3.640	.003
site * fungi	2.957	6	3.450	.003
meth * fungi	2.770	4	4.847	.001
Site	42.206	1	295.436	.000
Meth	6.077	1	42.536	.000
Fungi	2.788	7	2.788	.008
Error	32.715	229		

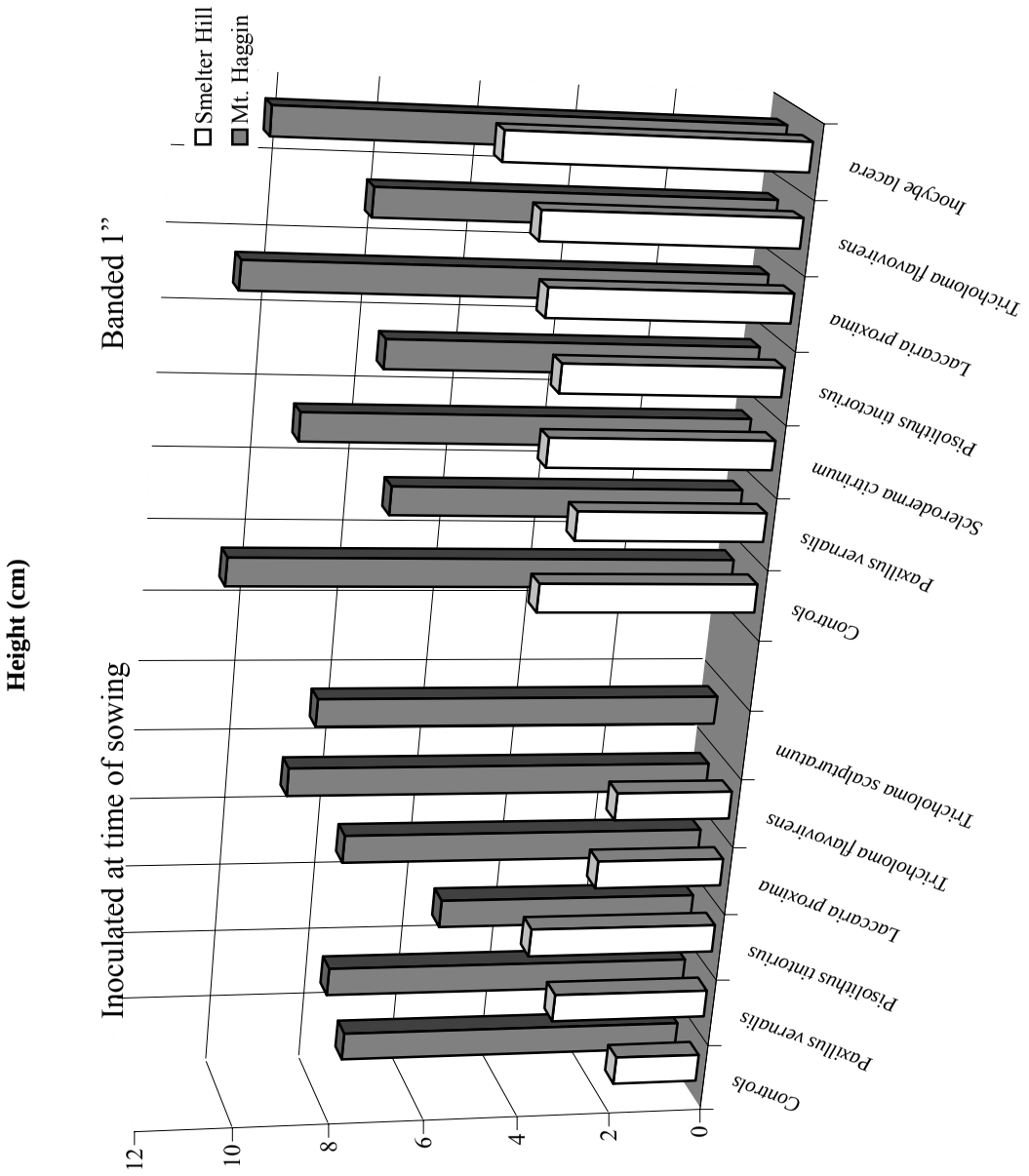

Figure 11. Height (cm) of inoculated and non-inoculated aspen seedlings in two field soils (Smelter Hill, Mt. Haggin) in the greenhouse utilizing two inoculation techniques: banded 1” and inoculated at time of sowing, after three months.

Root Collar Diameter

The root collar diameter (mm) of aspen seedlings was affected by soil treatment, method of inoculation and fungal treatment (Table 29). Seedlings inoculated with ectomycorrhizal fungi had larger stems than the non-inoculated controls (Table 26; Figure 12). Aspen seedlings inoculated by the banding 1" method also had larger stems than seedlings inoculated at the time of sowing (Table 26; Figure 12). Additionally, aspen seedlings in Mt. Haggin field soil had larger stems than seedlings in the Smelter Hill field soil (Table 26; Figure 12). There were significant interactions between site, method of inoculation and fungal treatment, between site and fungal treatment and between method of inoculation and fungal treatment (Table 29).

Table 29. Analysis of variance, Root collar diameter (mm).

Source	Type III Sum of Squares	df	F	Sig.
site * meth * fungi	1.727	5	4.073	.001
site * fungi	1.790	6	3.519	.002
meth * fungi	2.967	4	8.749	.000
site	13.101	1	154.497	.000
meth	3.132	1	36.942	.000
fungi	2.302	7	3.878	.001
Error	19.418	229		

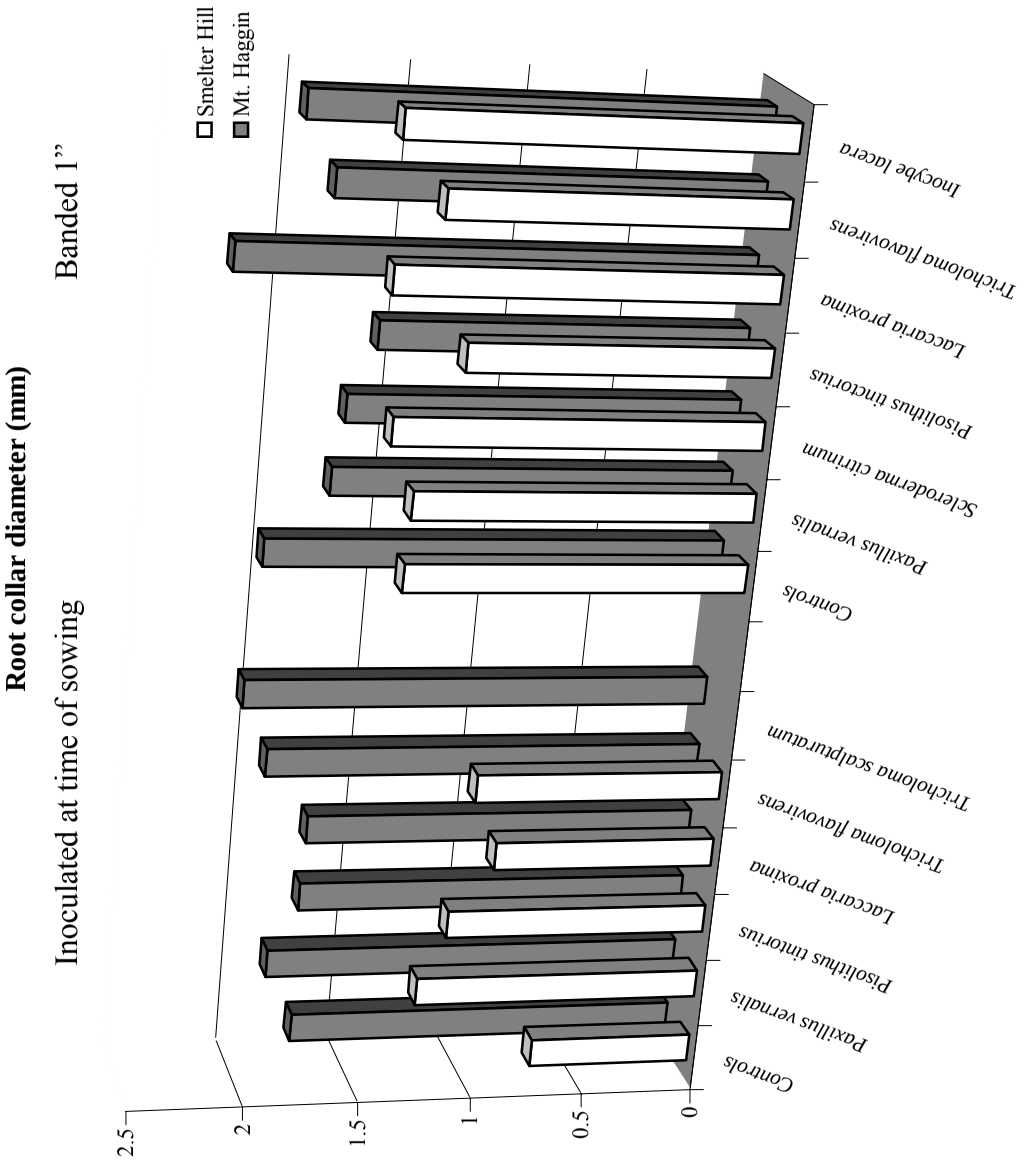

Figure 12. Root collar diameter (mm) of inoculated and non-inoculated aspen seedlings in two field soils (Smelter Hill, Mt. Haggin) in the greenhouse utilizing two inoculation techniques: banded 1” and inoculated at time of sowing, after three months.

Aboveground Biomass

The aboveground biomass (g) of aspen seedlings was affected by soil treatment and method of inoculation (Table 30). Aspen seedlings inoculated by the banding 1" method were larger than seedlings inoculated at the time of sowing (Table 26; Figure 13). Aspen seedlings in Mt. Haggin field soil were larger than seedlings in the Smelter Hill field soil (Table 26; Figure 13). There were significant interactions between site, method of inoculation and fungal treatment, between site and fungal treatment and between method of inoculation and fungal treatment (Table 30).

Table 30. Analysis of variance, Aboveground biomass (g); transformed (.313)

Source	Type III Sum of Squares	df	F	Sig.
site * meth * fungi	.119	5	5.147	.000
site * fungi	.106	6	3.813	.001
meth * fungi	.222	4	11.993	.000
site	1.123	1	243.041	.000
meth	.209	1	45.138	.000
fungi	.088	7	2.709	.010
Error	1.058	229		

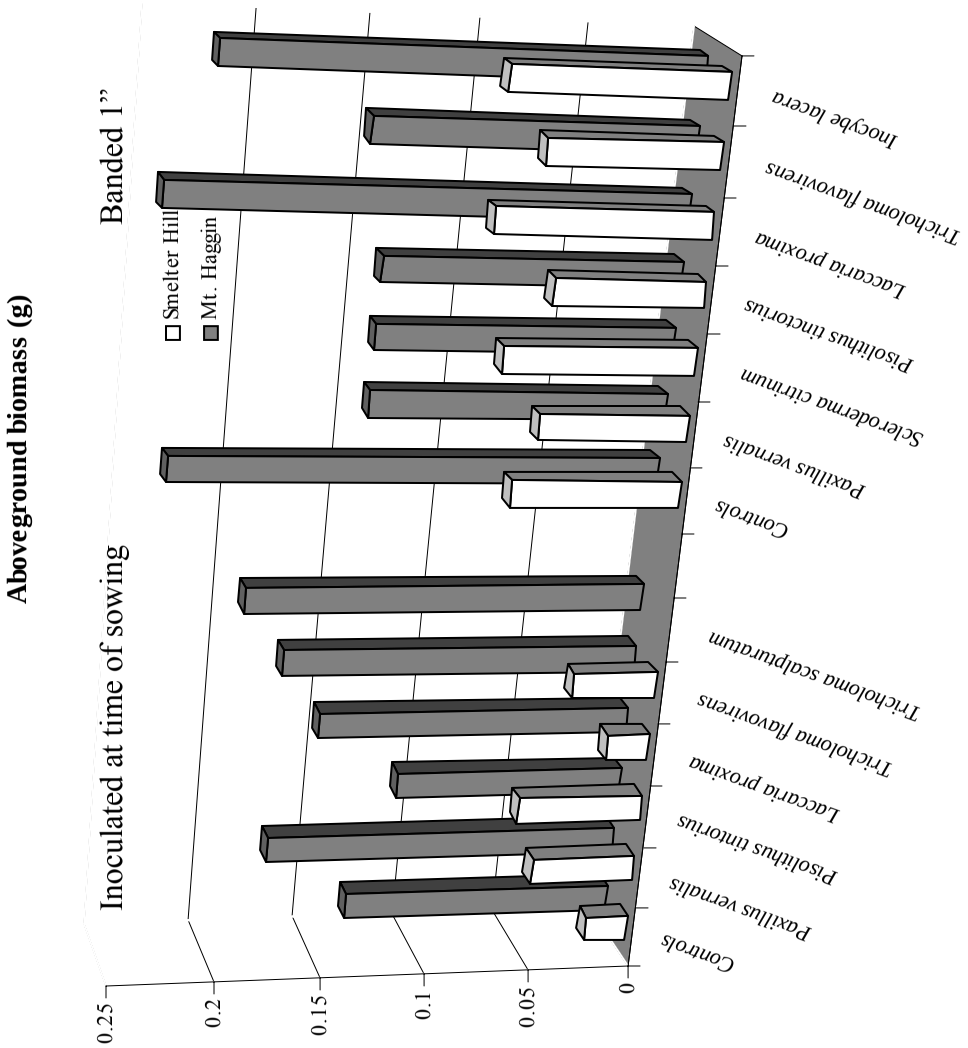

Figure 13. Aboveground biomass (g) of inoculated and non-inoculated aspen seedlings in two field soils (Smelter Hill, Mt. Haggin) in the greenhouse utilizing two inoculation techniques: banded 1” and inoculated at time of sowing, after three months.

Belowground Biomass

The belowground biomass (g) of aspen seedlings was affected by soil treatment, method of inoculation and fungal treatment (Table 31). Seedlings inoculated with ectomycorrhizal fungi were larger than the non-inoculated controls (Table 26; Figure 14). Aspen seedlings inoculated by the banding 1" method were larger than seedlings inoculated at the time of sowing (Table 26; Figure 14). Aspen seedlings in Mt. Haggin field soil were larger than seedlings in the Smelter Hill field soil (Table 26; Figure 14). There were significant interactions between site, method of inoculation and fungal treatment, between site and fungal treatment and between method of inoculation and fungal treatment (Table 31).

Table 31. Analysis of variance, Belowground biomass (g); transformed (Lg10).

Source	Type III Sum of Squares	df	F	Sig.
site * meth * fungi	3.354	5	9.124	.000
site * fungi	1.294	6	2.934	.009
meth * fungi	3.128	4	10.637	.000
site	10.292	1	139.987	.000
meth	3.661	1	49.793	.000
fungi	1.739	7	3.379	.002
Error	16.837	229		

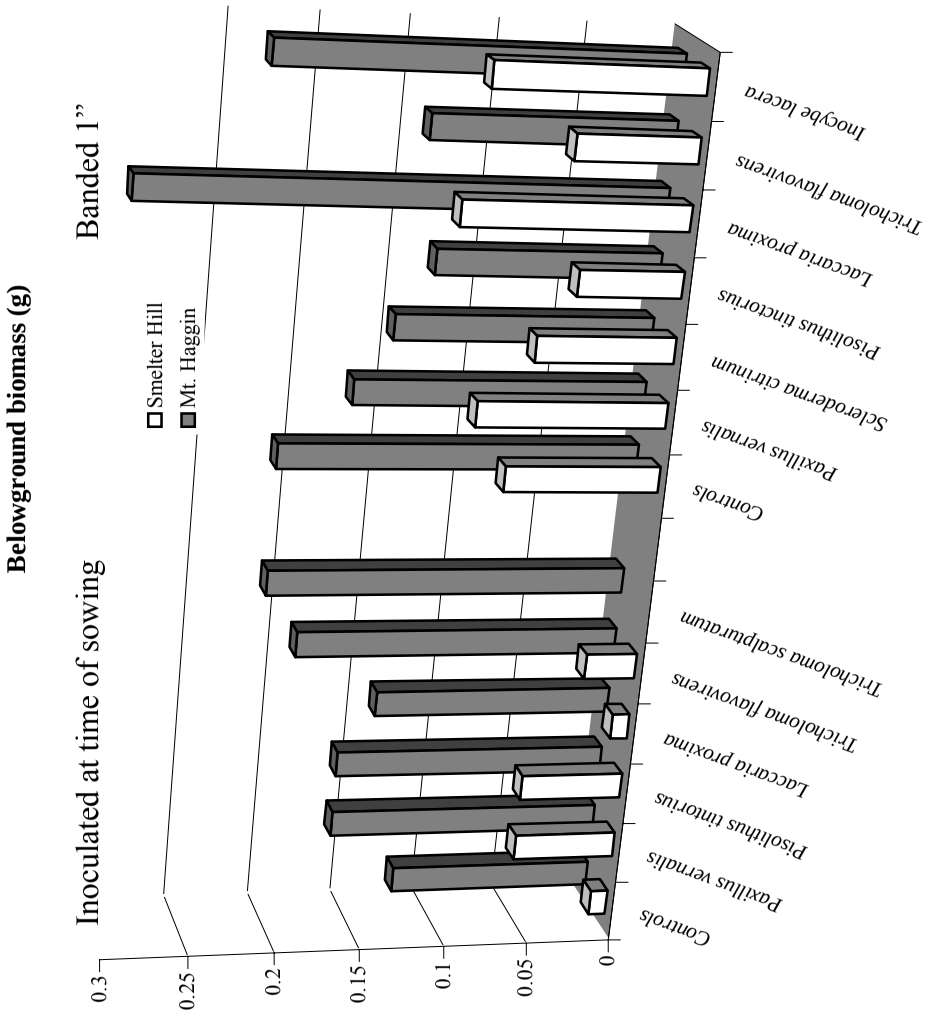

Figure 14. Belowground biomass (g) of inoculated and non-inoculated aspen seedlings in two field soils (Smelter Hill, Mt. Haggin) in the greenhouse utilizing two inoculation techniques: banded 1” and inoculated at time of sowing, after three months.

Total Biomass

The total biomass (g) of aspen seedlings was affected by soil treatment, method of inoculation and fungal treatment (Table 32). Seedlings inoculated with ectomycorrhizal fungi were larger than the non-inoculated controls (Table 26; Figure 15). Aspen seedlings inoculated by the banding 1" method were larger than seedlings inoculated at the time of sowing (Table 26; Figure 15). Aspen seedlings in Mt. Haggin field soil were larger than seedlings in the Smelter Hill field soil (Table 26; Figure 15). There were significant interactions between site, method of inoculation and fungal treatment, between site and fungal treatment and between method of inoculation and fungal treatment (Table 32).

Table 32. Analysis of variance, Total biomass (g); transformed (Lg10).

Source	Type III Sum of Squares	df	F	Sig.
site * meth * fungi	2.955	5	11.755	.000
site * fungi	1.318	6	4.368	.000
meth * fungi	2.871	4	14.279	.000
site	10.521	1	209.262	.000
meth	2.923	1	58.138	.000
fungi	1.161	7	3.298	.002
Error	11.513	229		

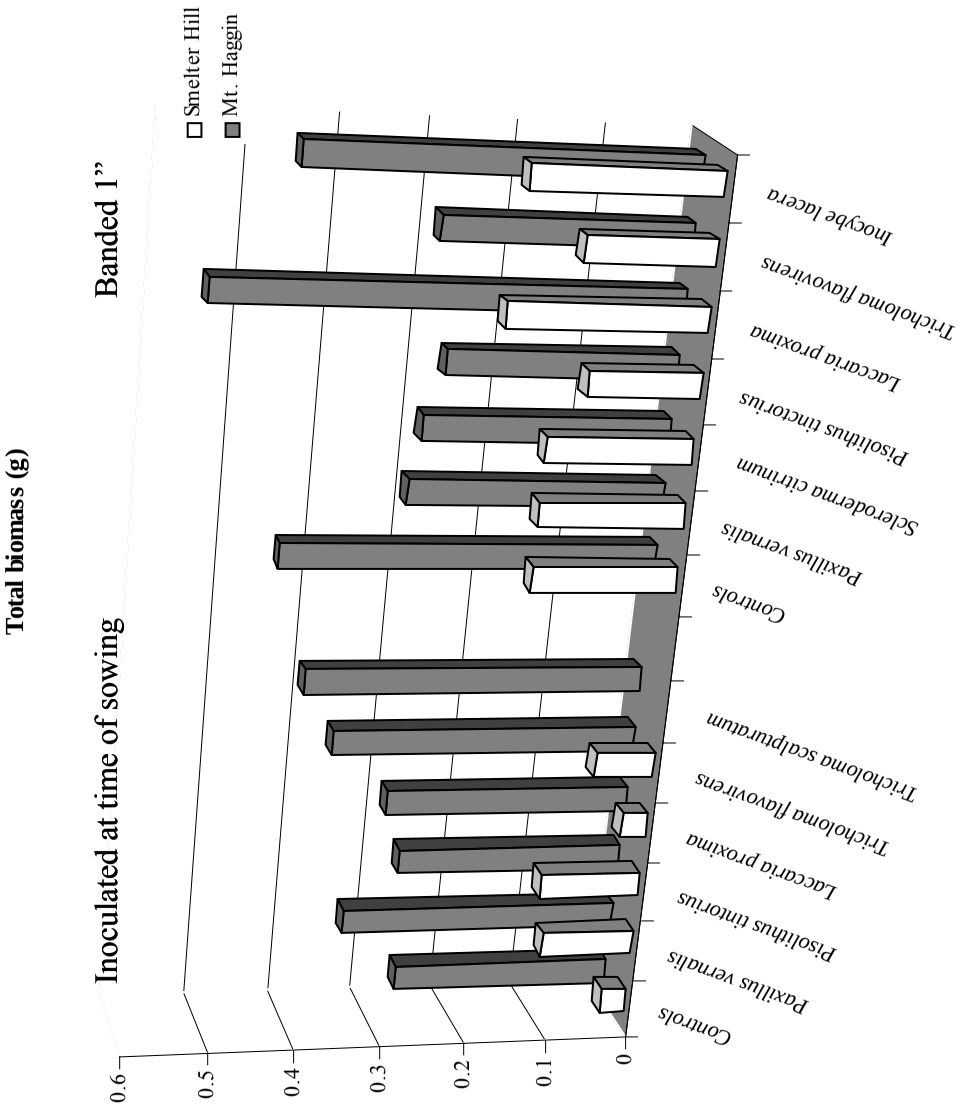

Figure 15. Total biomass (g) of inoculated and non-inoculated aspen seedlings in two field soils (Smelter Hill, Mt. Haggin) in the greenhouse utilizing two inoculation techniques: banded 1” and inoculated at time of sowing, after three months.

Plant Tissue Analysis

Mt. Haggin Field Soil; Banded 1"; Shoot Analysis. Results of the plant tissue analysis for Mt. Haggin and Smelter Hill field soils, for two different inoculation techniques are shown in Table 33. In the Mt. Haggin field soil shoots of aspen seedlings inoculated with *Paxillus vernalis* by the banded 1" method of inoculation had 23% more total sulfur, 24% more total N, 34% more magnesium, 30% more Fe, 27 % more Zn, 29% more P, 90% more Ni, 41% more copper and 66% more Pb than the shoots of the controls (Table 33). Shoots of *Scleroderma citrinum* inoculated aspen seedlings had 26% more magnesium, 48% more Fe, 28% more Zn, 33% more P, 80 % more Ni and 52% more copper than the shoots of the controls. Shoots of *Pisolithus tinctorius* inoculated aspen seedlings values were fairly consistent with the shoots of the controls except P levels were 23% higher. Shoots of *Laccaria proxima* inoculated aspen seedlings had 32 % higher Zn values, 38% more copper and 24% higher P values than the shoots of the controls. Shoots of *Tricholoma flavovirens* inoculated aspen seedlings had 19% more total N, 25% more Fe, 21% more Zn, 29% more copper and 21 % more P than the shoots of the controls. *Inocybe lacera* inoculated aspen seedlings had 15% more P than the shoots of the controls.

Table 33. Plant tissue analysis; Mt. Haggin and Smelter hill field soils utilizing two different inoculation techniques: banded 1" or inoculated at time of sowing.

Site; plant material Fungal treatment	Total S %	Total N %	Mg %	K %	Fe mg/kg	Zn mg/kg	P %	Ni mg/kg	Pb mg/kg	Cu mg/kg
Mt. Haggin banded 1"; aboveground biomass										
Controls	0.14	1.03	0.31	1.28	54	386	0.15	2	2	10
Paxillus vernalis	0.18	1.34	0.47	1.28	79	528	0.22	20	6	17
Scleroderma citrinum	0.14	1.08	0.43	1.27	103	538	0.23	10	3	21
Pisolithus tinctorius	---	---	0.35	1.29	56	328	0.20	3	3	10
Laccaria proxima	0.15	1.07	0.40	1.20	51	566	0.20	3	2	16
Tricholoma flavovirens	0.14	1.27	0.40	1.25	72	486	0.20	3	3	14
Inocybe lacera	0.13	1.00	0.34	1.32	57	407	0.18	3	3	11
Mean	0.15	1.13	0.39	1.27	67	463	0.20	6	3	14
Mt. Haggin banded 1"; belowground biomass										
Controls	0.14	0.72	0.41	1.12	1411	191	0.17	7	16	154
Paxillus vernalis	0.14	0.80	0.41	1.24	1399	231	0.22	7	21	198
Scleroderma citrinum	0.14	0.80	0.29	1.26	961	238	0.23	6	18	183
Pisolithus tinctorius	---	---	0.52	1.12	1710	167	0.18	7	15	132
Laccaria proxima	0.14	0.73	0.26	0.97	834	217	0.18	5	20	168
Tricholoma flavovirens	0.14	0.89	0.45	1.12	1540	213	0.19	8	20	187
Inocybe lacera	---	---	0.26	1.00	952	194	0.18	5	18	150
Mean	0.14	0.81	0.37	1.12	1258	207	0.19	6	18	167
Mt. Haggin inoculated at time of sowing; aboveground biomass										
Controls	0.18	1.51	0.36	1.44	133	778	0.20	8	5	19
Paxillus vernalis	0.20	1.40	0.40	1.22	107	838	0.22	5	4	20
Pisolithus tinctorius	0.19	1.42	0.44	1.17	94	844	0.22	6	3	19
Laccaria proxima	0.19	1.43	0.33	1.47	76	714	0.21	5	5	19
Tricholoma flavovirens	0.18	1.31	0.35	1.23	84	564	0.20	4	3	15
Tricholoma scalpturatum	0.18	1.41	0.39	1.34	90	748	0.22	4	3	19
Mean	0.18	1.41	0.38	1.31	97	748	0.21	5	4	19
Mt. Haggin inoculated at time of sowing; belowground biomass										
Controls	0.22	1.13	0.35	1.55	1435	345	0.23	6	37	319
Paxillus vernalis	0.22	1.13	0.20	1.27	926	337	0.22	7	35	311
Pisolithus tinctorius	0.17	0.94	0.22	1.21	901	315	0.21	5	32	264
Laccaria proxima	0.21	1.10	0.16	1.30	757	325	0.20	4	31	281
Tricholoma flavovirens	0.12	0.72	0.25	0.95	988	231	0.16	5	25	227
Tricholoma scalpturatum	0.16	0.89	0.23	1.11	1250	279	0.17	6	23	236
Mean	0.18	0.99	0.24	1.23	1043	305	0.20	6	31	273

Table 33. continued

Site; plant material Fungal treatment	Total S %	Total N %	Mg %	K %	Fe mg/kg	Zn mg/kg	P %	Ni mg/kg	Pb mg/kg	Cu mg/kg
Smelter Hill banded 1"; aboveground biomass										
Controls	0.19	1.67	0.61	1.03	114	252	0.21	9	2	16
Paxillus vernalis	0.24	1.27	0.69	0.96	475	234	0.20	60	2	16
Scleroderma citrinum	0.22	1.75	0.63	1.12	173	306	0.22	19	5	20
Pisolithus tinctorius	0.25	1.83	0.57	1.24	198	254	0.23	18	<2	16
Laccaria proxima	0.24	1.52	0.67	0.97	99	326	0.21	3	2	16
Tricholoma flavovirens	0.21	1.56	0.83	1.00	530	264	0.26	4	5	22
Inocybe lacera	0.21	1.59	0.63	1.10	112	253	0.26	8	<2	15
Mean	0.22	1.60	0.66	1.06	243	270	0.23	17	3	17
Smelter Hill banded 1"; belowground biomass										
Controls	0.21	1.20	0.56	1.17	1999	146	0.21	7	30	446
Paxillus vernalis	0.17	0.86	0.46	1.08	1621	125	0.18	7	31	344
Scleroderma citrinum	0.20	1.11	0.39	1.15	3313	157	0.22	9	31	402
Pisolithus tinctorius	0.17	1.10	0.97	1.40	3168	113	0.19	12	26	187
Laccaria proxima	0.20	1.05	0.48	1.02	1864	122	0.20	6	38	432
Tricholoma flavovirens	0.25	1.22	1.10	1.48	3569	165	0.25	13	41	578
Inocybe lacera	0.18	0.95	0.40	1.19	1436	141	0.22	5	30	415
Mean	0.20	1.07	0.62	1.21	2424	138	0.21	8	32	401
Smelter Hill inoculated at time of sowing; aboveground biomass										
Controls	0.23	1.65	0.65	0.92	315	185	0.16	5	9	35
Paxillus vernalis	0.30	1.71	0.73	0.84	169	346	0.19	3	7	33
Pisolithus tinctorius	0.29	1.49	0.68	0.85	119	240	0.16	3	3	23
Laccaria proxima	0.34	1.89	0.62	1.05	506	229	0.18	4	10	39
Tricholoma flavovirens	0.29	1.95	0.88	0.90	874	274	0.19	5	7	42
Mean	0.29	1.74	0.71	0.91	397	255	0.18	4	7	34
Smelter Hill inoculated at time of sowing; belowground biomass										
Controls	---	---	2.16	1.26	8029	140	0.12	36	69	679
Paxillus vernalis	0.26	1.39	0.34	0.82	1881	139	0.14	6	46	649
Pisolithus tinctorius	0.26	1.16	0.24	0.90	1002	111	0.15	2	35	458
Laccaria proxima	0.29	1.49	---	---	---	---	---	---	---	
Tricholoma flavovirens	0.31	1.64	0.27	1.00	1391	132	0.17	6	45	630
Mean	0.28	1.42	0.75	1.00	3076	131	0.15	13	49	604

Mt. Haggin Field Soil; Banded 1"; Root Analysis. Roots of *Paxillus vernalis* inoculated aspen seedlings had 10% more potassium, 17% more Zn, 22% more copper and 22% more P than the roots of the controls (Table 33). Shoots of *Scleroderma citrinum* inoculated aspen seedlings had 30% less magnesium, 11% more potassium, 32% less Fe, 20% more Zn, 16 % more copper and 26% more P than the roots of controls. *Pisolithus tinctorius* and *Tricholoma flavovirens* inoculated aspen seedlings root tissue values were consistent with the tissue analysis of roots from the controls, except for copper, which was 21% less than the controls for *Pisolithus tinctorius* and 18% more for *Tricholoma flavovirens*. Roots of *Laccaria proxima* inoculated aspen seedlings had 36% less magnesium and 41% less Fe than the roots of the controls. *Inocybe lacera* inoculated aspen seedlings had 42% less magnesium, and 38% less Fe than the roots of the controls.

Mt. Haggin Field Soil; Inoculated At Time Of Sowing; Shoot Analysis. In the Mt. Haggin field soil, shoots of aspen seedlings inoculated with *Paxillus vernalis* with the 'inoculated at time of sowing' method had 15% less potassium and 20% less Fe than the shoots of the controls (Table 33). Shoots of *Pisolithus tinctorius* inoculated aspen seedlings had 19% less potassium, 29% less Fe than the shoots of the controls. Shoots of *Laccaria proxima* inoculated aspen seedlings had 43% less Fe than the shoots of the controls. Shoots of *Tricholoma flavovirens* inoculated aspen seedlings had 15% less potassium, 37% less Fe, 28% less Zn and 21% less copper than the shoots of the controls. Shoots of *Tricholoma scalpturatum* inoculated aspen seedlings had 32% less Fe than the shoots of the controls.

Mt. Haggin Field Soil; Inoculated At Time Of sowing; Root Analysis. Roots of *Paxillus vernalis* inoculated aspen seedlings had 42% less magnesium and 18% less potassium than the roots of the controls (Table 33). Roots of *Pisolithus tinctorius* inoculated aspen seedlings had 36% less magnesium, 17% less copper and 22% less potassium than the roots of the controls. Roots of *Laccaria proxima* inoculated aspen seedlings had 53% less magnesium, 12% less copper and 16% less potassium than the roots of the controls. Roots of *Tricholoma flavovirens* inoculated aspen seedlings had 43% less total sulfur, 36% less total N, 26% less magnesium, 29% less copper and 39% less potassium than the roots of the controls. Roots of *Tricholoma scalpturatum* inoculated aspen seedlings had 28% less total sulfur, 21% less total N, 33% less magnesium, 26% less copper and 28% less potassium than the roots of the controls.

Smelter Hill Field Soil; Banded 1"; Shoot Analysis. Shoots of *Paxillus vernalis* inoculated aspen seedlings had 20% more total sulfur, 76% more Fe, and 85% more Ni than the shoots of the controls (Table 33). Shoots of *Scleroderma citrinum* inoculated aspen seedlings had 15% more total sulfur, 34% more Fe, 18% more Zn, 52% more Ni and 20% more copper than the shoots of the controls. Shoots of *Pisolithus tinctorius* inoculated aspen seedlings had 24% more total sulfur, 42% more Fe, and 50% more Ni than the shoots of the controls. Shoots of *Laccaria proxima* inoculated aspen seedlings had 21% more total sulfur and 22% more Zn than the shoots of the controls. Shoots of *Tricholoma flavovirens* inoculated aspen seedlings had 26% more magnesium, 78% more Fe and 27% more copper than the shoots of the controls. Shoots of *Inocybe lacera* inoculated aspen seedlings had values consistent with the shoots of the controls.

Smelter Hill Field Soil; Banded 1"; Root Analysis. Roots of *Paxillus vernalis* inoculated aspen seedlings had 28% less total N, 14% less Zn and 23% less copper than the roots of the controls (Table 33). Roots of *Scleroderma citrinum* inoculated aspen seedlings had 30% less magnesium, 10% less copper and 40% more Fe than the roots of the controls. Roots of *Pisolithus tinctorius* inoculated aspen seedlings had 42% more magnesium, 16% more potassium, 36% more Fe, 22% less Zn, and 58% less copper than the roots of the controls. Roots of *Laccaria proxima* inoculated aspen seedlings had 13% less potassium and 16 % less Zn than the roots of the controls. Roots of *Tricholoma flavovirens* inoculated aspen seedlings had 16% more total sulfur, 49% more magnesium, 21% more potassium, 44% more Fe, 27% more Pb and 23% more copper than the roots of the controls. Roots of *Inocybe lacera* inoculated aspen seedlings had 21% less total N and 29% less magnesium than the roots of the controls

Smelter Hill Inoculated At Time Of Sowing; Shoot Analysis. Roots of *Paxillus vernalis* inoculated aspen seedlings had 24% more total sulfur, 11% more magnesium, 46% less Fe, 47% more Zn and 17% more P than the shoots of the controls (Table 33). Shoots of *Pisolithus tinctorius* inoculated aspen seedlings had 62% less Fe, 23% more Zn and 34% less copper than the shoots of the controls. Shoots of *Laccaria proxima* inoculated aspen seedlings had 32% more total sulfur, 13% more total N, 38% more Fe and 19% more Zn than the shoots of the controls. Shoots of *Tricholoma flavovirens* inoculated aspen seedlings had 26% more magnesium, 64% more Fe, 32% more Zn and 16% more copper than the shoots of the controls.

Smelter Hill Inoculated At Time Of Sowing; Root Analysis. Roots of *Paxillus vernalis* inoculated aspen seedlings had 84% less magnesium, 35% less potassium, 77% less Fe, 13% more P, 83% less Ni, and 33% less Pb than the roots of the controls (Table 33). Roots of *Pisolithus tinctorius* inoculated aspen seedlings had 89% less magnesium, 29% less potassium, 88% less Fe, 21% less Zn, 18% more P, 94% less Ni and 49% less Pb than the roots of the controls. Roots of *Laccaria proxima* inoculated seedlings did not have enough root material to compare with controls.

Metal levels in the roots and shoots of aspen seedlings inoculated with two different methods were compared and is included in the Discussion. As a result of the sampling method (pooling) data was not analyzed statistically for element levels in plant tissue.

Experiment 2

The second experiment compared the level of mycorrhization of aspen seedlings inoculated with six different species of ectomycorrhizal fungi in field soil collected from two locations within the Mt. Haggin Wildlife Refuge (Table 34). Site 1 was approximately 1-2 miles closer to the smelter stack than Site 2, and 1,880 feet lower in elevation than Site 2. Host plant growth response to the various treatments after 3 months in the greenhouse was also analyzed. Percent mycorrhization, means and standard deviations of the growth response for the various treatments are shown in Table 35. The variability of aspen growth in field soils was again illustrated by the large standard deviations throughout Experiment 2.

Table 34. Soil Analysis of water soluble elements; Mt. Haggin; Site 1, Site 2; analytic data (mg/kg reported unless otherwise noted; original values in mg/L)

Site	sat.pst. pH	%H2O sat.pst.	Sand %	Silt %	Clay %	EC sat pst mmhos/cm	NO3-N	NH4-N	K	P	Texture
Site 1	5.2	102.4	68	24	8	0.37	13.97	0.94	28.25	1.86	sandy loam
Site 2	5.5	38.0	58	26	16	0.31	5.7	0.12	5.36	0.08	sandy loam

Site	S	Al	Cu	Fe	Mn	Pb	Zn	Cd	Ni	As
Site 1	20.4	1.39	2.07	0.91	2.58	0.10	3.04	0.09	<0.03	1.76
Site 2	5.7	0.14	0.06	<0.1	1.16	<0.1	0.54	0.01	<0.03	0.19

Site 1 soils collected from within the Mt. Haggin Wildlife Refuge had a lower pH and percent clay than Site 2 soils (Table 34). Site one soils had a higher electrical conductivity and higher levels of all water soluble elements tested. As levels in Site 1 soils were 9 times higher than in Site 2 soils.

Table 35. Number of replicates, means and standard deviations of growth parameters and percent mycorrhization of non-inoculated and aspen seedlings inoculated with 6 native ectomycorrhizal fungi by banding 1" in two field soils from Mt. Haggin. Different letters denote significant differences at P<0.05. Untransformed data.

	Site	Controls	*Paxillus vernalis*	*Scleroderma citrinum*	*Pisolithus tinctorius*	*Laccaria proxima*	*Tricholoma flavovirens*	*Inocybe lacera*
% Aspen w/mycorrhizae	Site 1	0	90	55	100	NA	70	22
Number of replicates		n=15	n=10	n=11	n=8	n=0	n=10	n=9
% Aspen w/mycorrhizae	Site 2	0	100	50	90	50	NA	40
Number of replicates		n=16	n=10	n=10	n=10	n=10	n=0	n=10
Height (cm)	Site 1	6.15±2.79 ABCD	8.30±1.95 A	5.26±2.27 BCDE	6.64±2.27 ABC		5.99±1.99 ABCD	6.88±2.03 AB
	Site 2	3.63±1.30 DE	3.65±0.87 DE	3.13±0.87 E	4.06±1.64 CDE	3.33±1.12 E		3.65±1.10 DE
Root collar diameter (mm)	Site 1	1.59±0.33 ABC	1.86±0.23 A	1.55±0.25 ABCD	1.53±0.21 ABCDE		1.68±0.40 AB	1.69±0.17 AB
	Site 2	1.32±0.23 CDE	1.35±0.21 BCDE	1.19±0.16 E	1.37±0.19 BCDE	1.22±0.18 DE		1.19±0.15 E
Aboveground biomass (g)	Site 1	0.14±0.09 AB	0.22±0.08 B	0.10±0.04 AB	0.13±0.05 AB		0.14±0.11 AB	0.14±0.05 AB
	Site 2	0.07±0.03 A	0.07±0.03 A	0.09±0.05 A	0.08±0.03 A	0.07±0.02 A		0.07±0.02 A
Belowground biomass (g)	Site 1	0.16±0.09 B	0.28±0.09 A	0.17±0.07 AB	0.13±0.05 BC		0.16±0.07 B	0.16±0.08 B
	Site 2	0.07±0.04 CD	0.06±0.03 D	0.06±0.03 D	0.07±0.03 CD	0.07±0.04 CD		0.07±0.03 CD
Total biomass (g)	Site 1	0.30±0.16 B	0.50±0.15 A	0.28±0.09 BC	0.26±0.10 BCD		0.30±0.17 B	0.30±0.12 B
	Site 2	0.14±0.06 DE	0.13±0.06 DE	0.15±0.07 CDE	0.14±0.05 CDE	0.12±0.05 E		0.13±0.05 DE

Percentage Of Aspen Seedlings With Mycorrhizae

Mycorrhizae did not form on any of the controls. All aspen seedlings inoculated with *Pisolithus tinctorius* in Site 1 soils formed mycorrhizae on their roots (Table 35, 36; Figure 16), and in Site 2 soils 90 percent of the seedlings formed *Pisolithus tinctorius* mycorrhizae. In addition, seedlings inoculated with *Paxillus vernalis* in Site 2 soils all formed mycorrhizae on their roots, and in Site 1 soils 90 percent of the seedlings formed *Paxillus vernalis* mycorrhizae. Aspen seedlings inoculated with *Scleroderma citrinum* in Site 1 soils formed mycorrhizae on 55 percent of the seedlings, and in Site 2 soils 50 percent of the seedlings formed *Scleroderma citrinum* mycorrhizae. Seedlings inoculated with *Tricholoma flavovirens* in Site 1 soils formed mycorrhizae on 70 percent of the seedlings, and inoculum was unavailable for inoculation of seedlings in Site 2 soils. Seedlings inoculated with *Inocybe lacera* in Site 1 soils formed mycorrhizae on 22 percent of the seedlings, and in Site 2 soils 40 percent of the seedlings formed *Inocybe lacera* mycorrhizae. Inoculum was also unavailable for inoculation of aspen seedlings in Site 1 soils with *Laccaria proxima*, however, in Site 2 soils 50 percent of the seedlings inoculated with *Laccaria proxima* formed mycorrhizae.

Table 36. Number of replicates, mean % mycorrhization of inoculated and non-inoculated seedlings, % mycorrhizal infection on trees with mycorrhizae, number of trees with mycorrhizae and % of trees with mycorrhizae

Fungi	**N**	**Mean % Mycorrhization of Aspen**	**% Mycorrhizal Infection of Aspen w/mycorrhizae**	**# Aspen w/mycorrhizae**	**% Aspen w/mycorrhizae**
Site one					
Controls	15	0	0	0	0
Paxillus vernalis	10	7.5	8.3	9	90
Scleroderma citrinum	11	4.3	7.8	6	55
Pisolithus tinctorius	8	9	9	8	100
Tricholoma flavovirens	10	2.7	4	7	70
Inocybe lacera	9	0.8	4	2	22
Site two					
Controls	16	0.33	1	0	0
Paxillus vernalis	10	5.3	5.3	10	100
Scleroderma citrinum	10	2.5	5	5	50
Pisolithus tinctorius	10	5.7	6.3	9	90
Laccaria proxima	10	1.7	3.4	5	50
Inocybe lacera	10	1.4	3.5	4	40

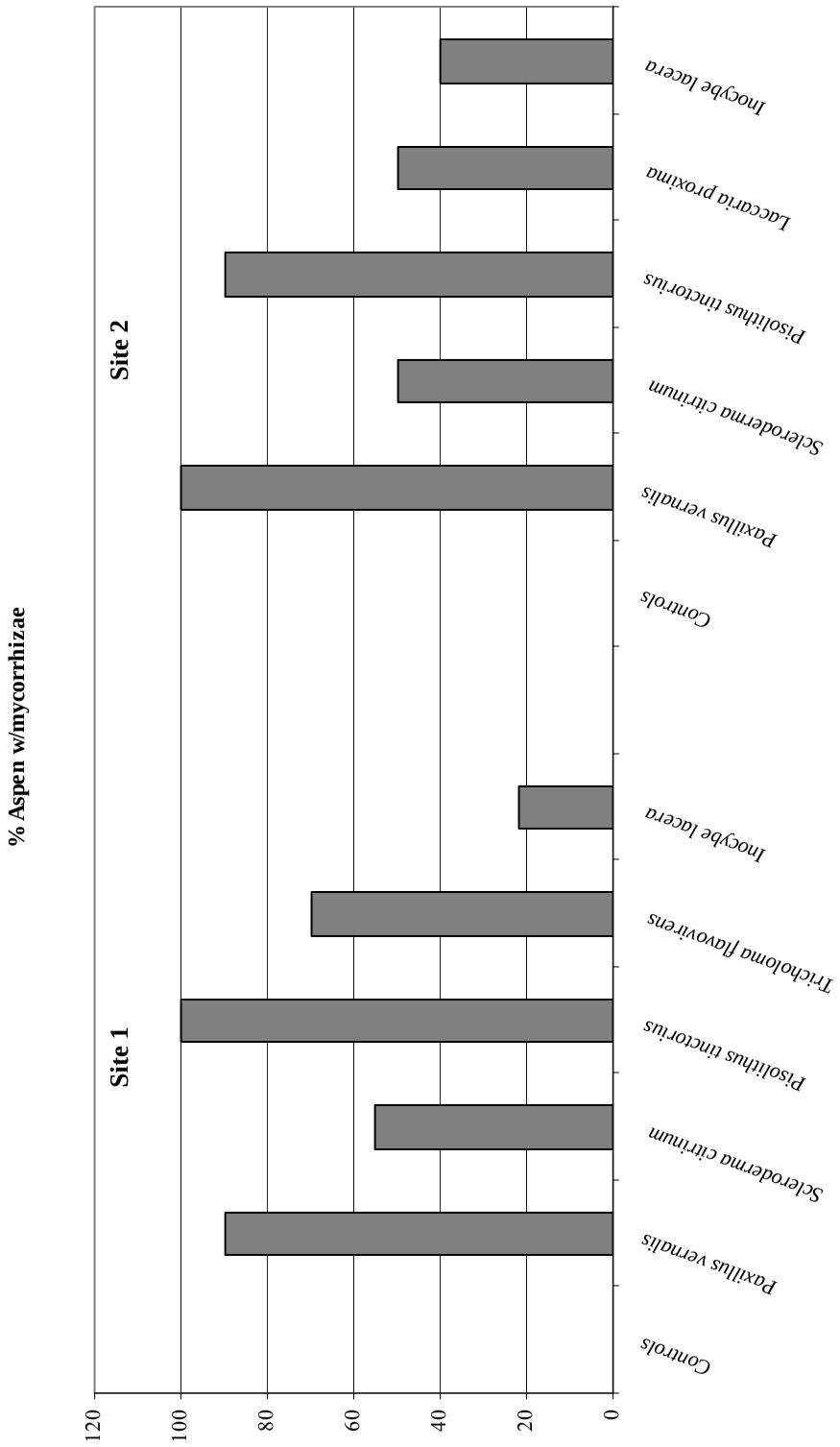

Figure 16. Percent mycorrhization of inoculated and non-inoculated aspen seedlings in two samplings of field sites at Mt. Haggin in the greenhouse, banded 1", after three months.

Height

For all aspen seedlings, height was affected by the soil from each sampling site (Table 35, 37; Figure 17). Height was not significantly affected by fungal treatment (Table 37). There was no significant interaction between site and fungal treatment (Table 37).

Table 37. Univariate Analysis of Variance, Height (cm); transformed (square root)

Source	Type III Sum of Squares	df	F	Sig.
site	11.133	1	74.479	.000
fungi	1.803	6	2.010	.070
site * fungi	.765	4	1.280	.282
Error	17.490	117		

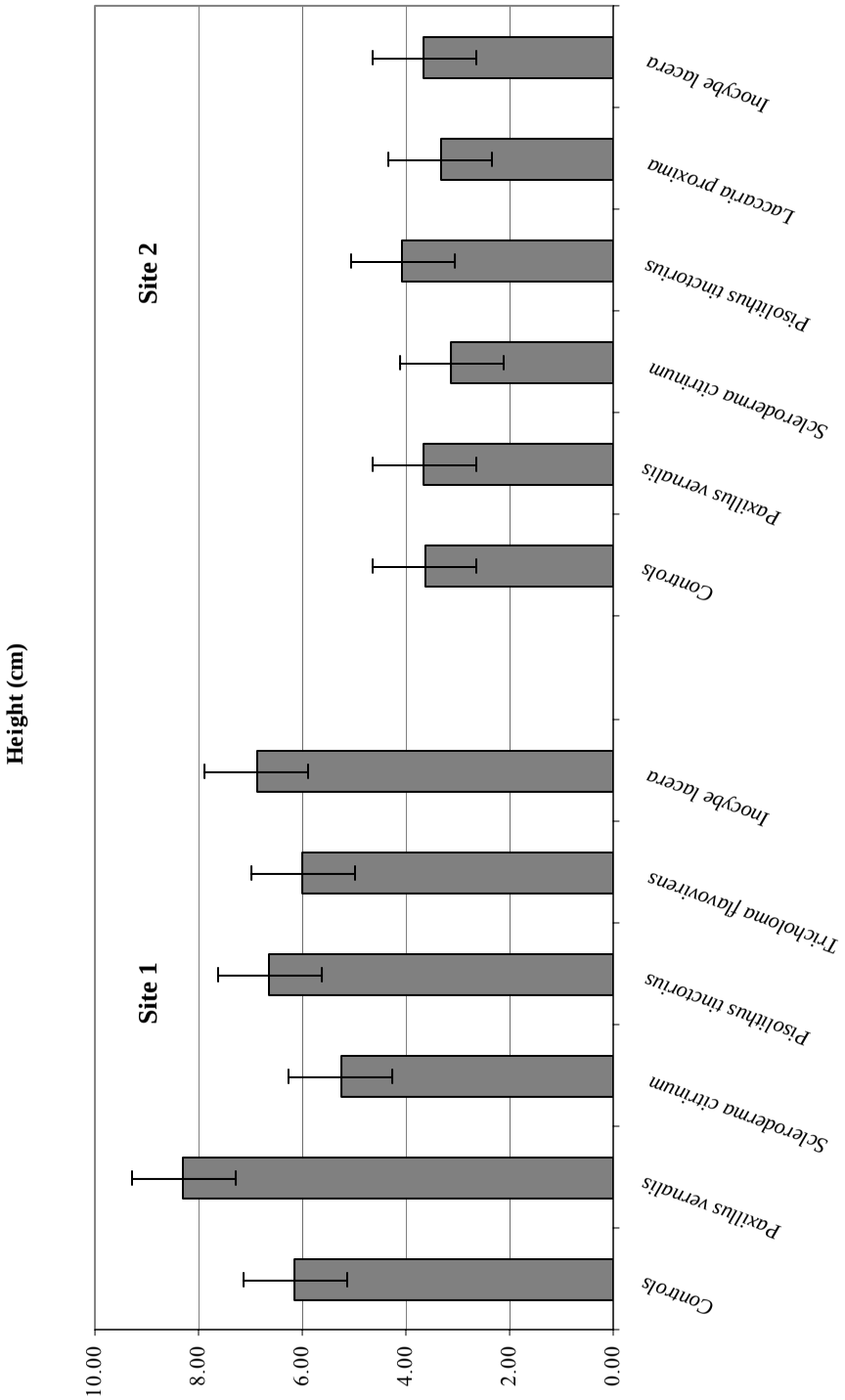

Figure 17. Height (cm) of inoculated and non-inoculated aspen seedlings in two samples of soils in the greenhouse, banded 1", after three months.

Root Collar Diameter

Root collar diameter (mm) of all aspen seedlings was affected by the soil from each site (Table 35, 38; Figure 18). Fungal treatment did not have a significant affect on root collar diameter (Table 38). There was no significant interaction between site and fungal treatment (Table 38).

Table 38. Univariate Analysis of Variance, Root collar diameter (mm)

Source	Type III Sum of Squares	df	F	Sig.
site	3.381	1	57.862	.000
fungi	.621	6	1.771	.111
site * fungi	.446	4	1.909	.113
Error	6.837	117		

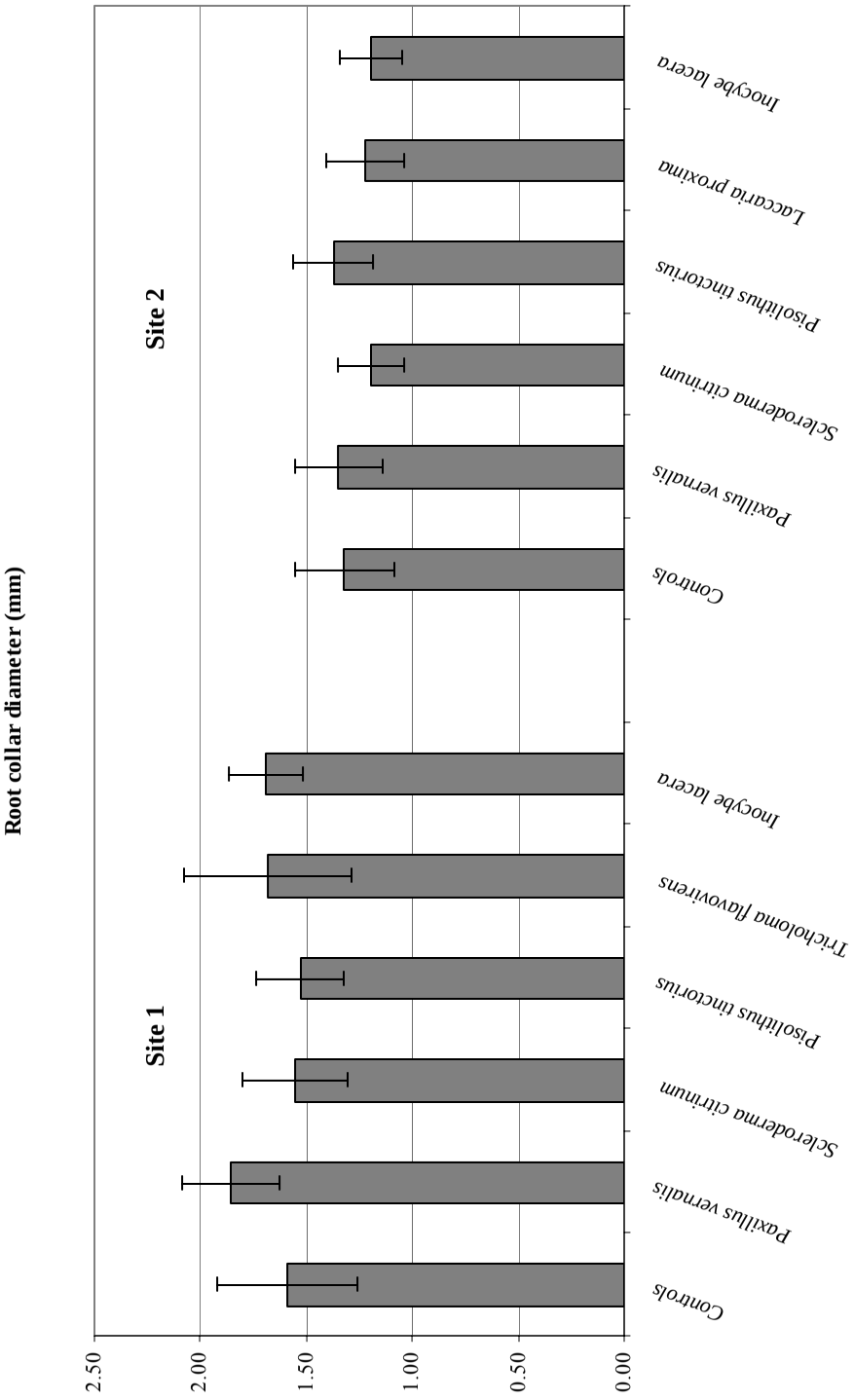

Figure 18. Root collar diameter (mm) of inoculated and non-inoculated aspen seedlings in two samplings of soils from Mt. Haggin in the greenhouse, banded 1", after three months.

Aboveground biomass

For all aspen seedlings, shoot biomass (g) was affected by the soil from each site (Table 35, 39; Figure 19). Shoot biomass was not significantly affected by fungal treatment (Table 39). There was no significant interaction between site and fungal treatment (Table 39).

Table 39. Univariate Analysis of variance, Aboveground biomass (g); transformed (inverse square root).

Source	Type III Sum of Squares	df	F	Sig.
site	32.129	1	35.403	.000
fungi	4.064	6	.746	.613
site * fungi	3.846	4	1.059	.380
Error	106.183	117		

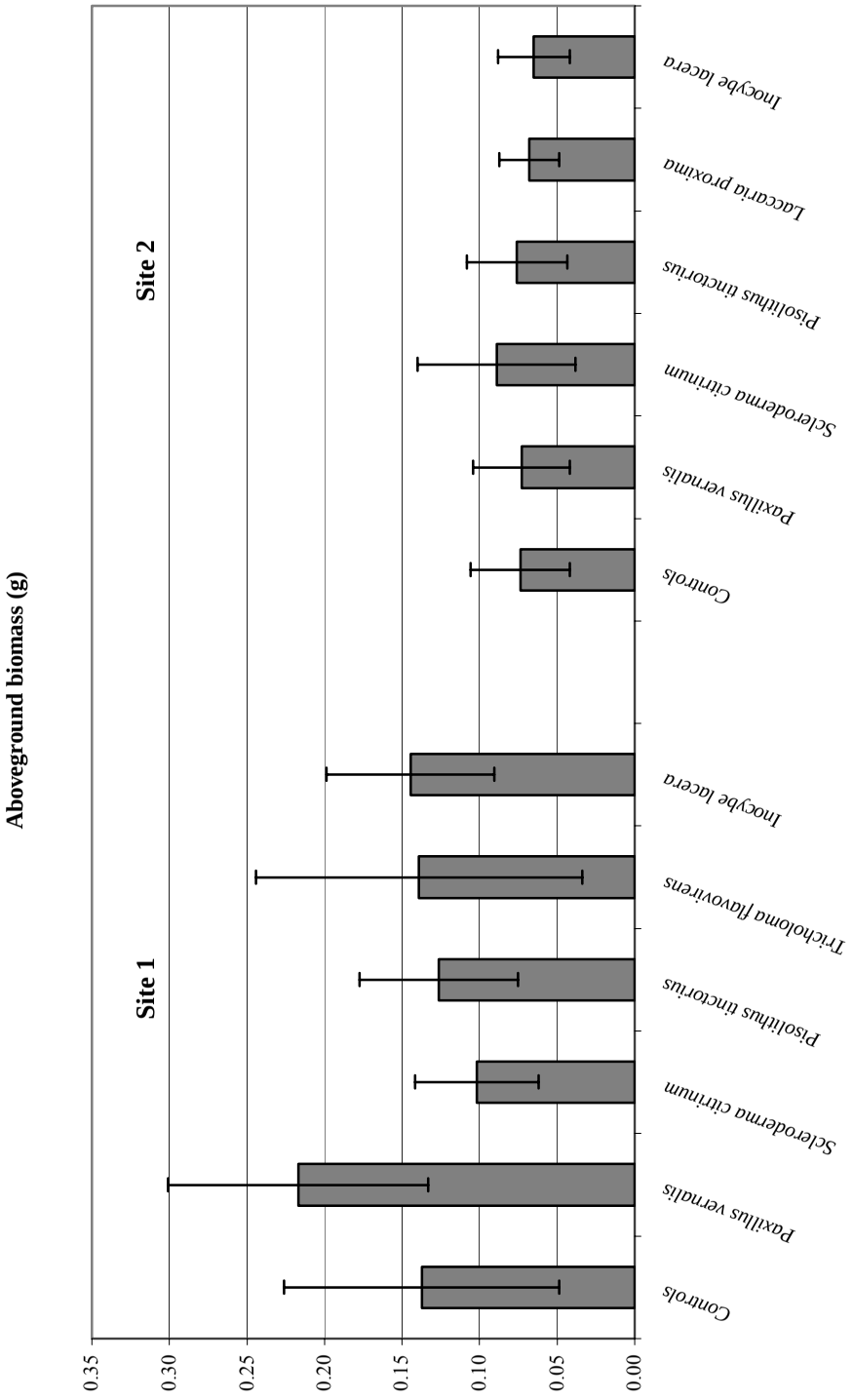

Figure 19. Aboveground (g) of inoculated and non-inoculated aspen seedlings in two samplings of field soils from Mt. Haggin in the greenhouse banded 1", after three months.

Belowground biomass

For all aspen seedlings, root biomass (g) was affected by the soil from each site (Table 35, 40). Root biomass was not significantly affected by fungal treatment (Table 40; Figure 20). There was significant interaction between site and fungal treatment (Table 40).

Table 40. Univariate Analysis of variance, Belowground biomass (g); transformed (square root).

Source	Type III Sum of Squares	df	F	Sig.
site	.720	1	116.877	.000
fungi	.073	6	1.972	.075
site * fungi	.099	4	4.000	.004
Error	.721	117		

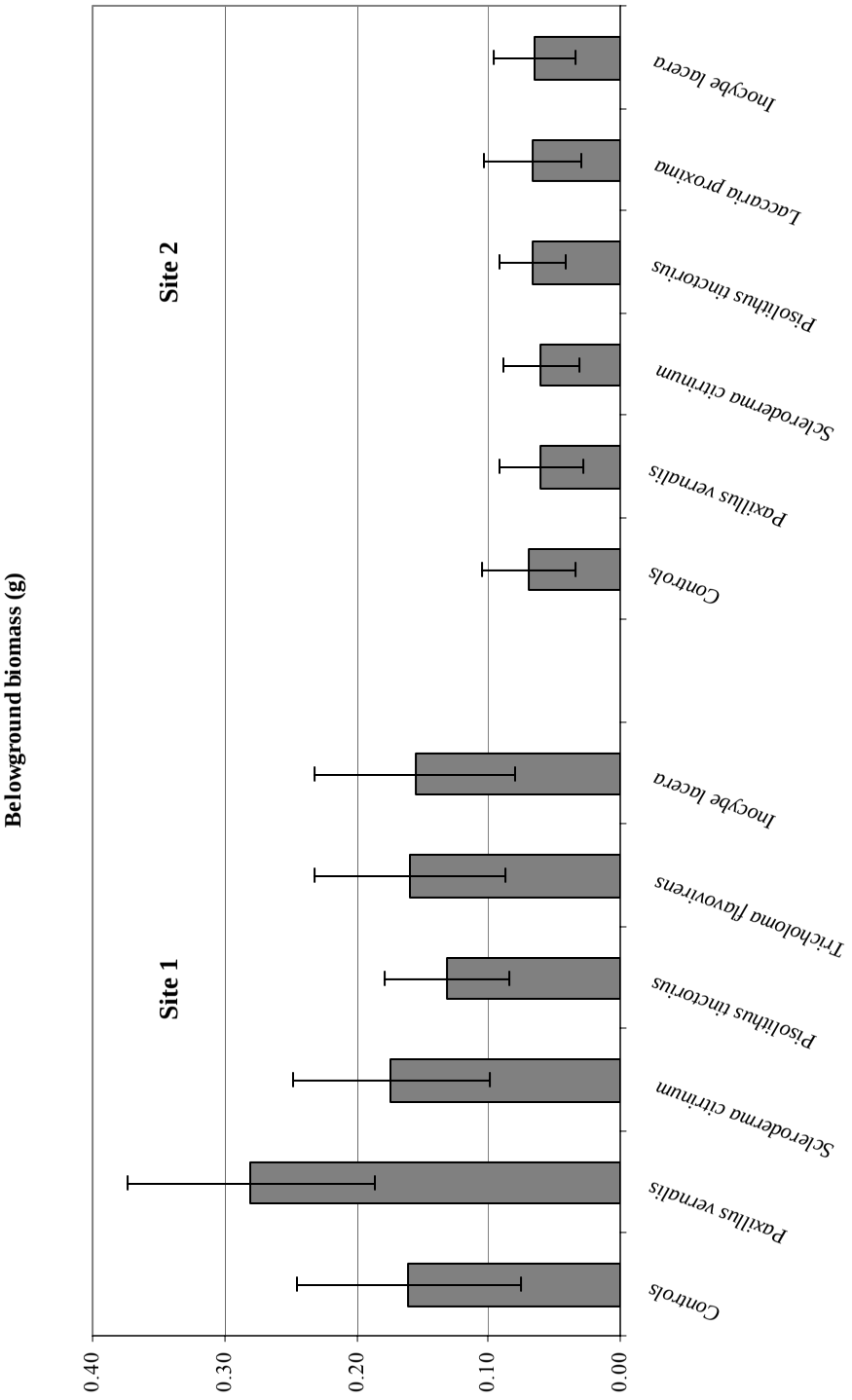

Figure 20. Belowground biomass (g) of inoculated and non-inoculated aspen seedlings in two samplings of field soils from Mt. Haggin in the greenhouse banded 1", after three months.

Total biomass

For all aspen seedlings, total biomass (g) was affected by the soil from each site (Table 35, 41; Figure 21). Total biomass was not significantly affected by fungal treatment (Table 41). There was significant interaction between site and fungal treatment (Table 41).

Table 41. Univariate Analysis of variance, Total biomass (g); transformed (square root)

Source	Type III Sum of Squares	df	F	Sig.
site	.943	1	91.326	.000
fungi	.118	6	1.904	.086
site * fungi	.147	4	3.556	.009
Error	1.208	117		

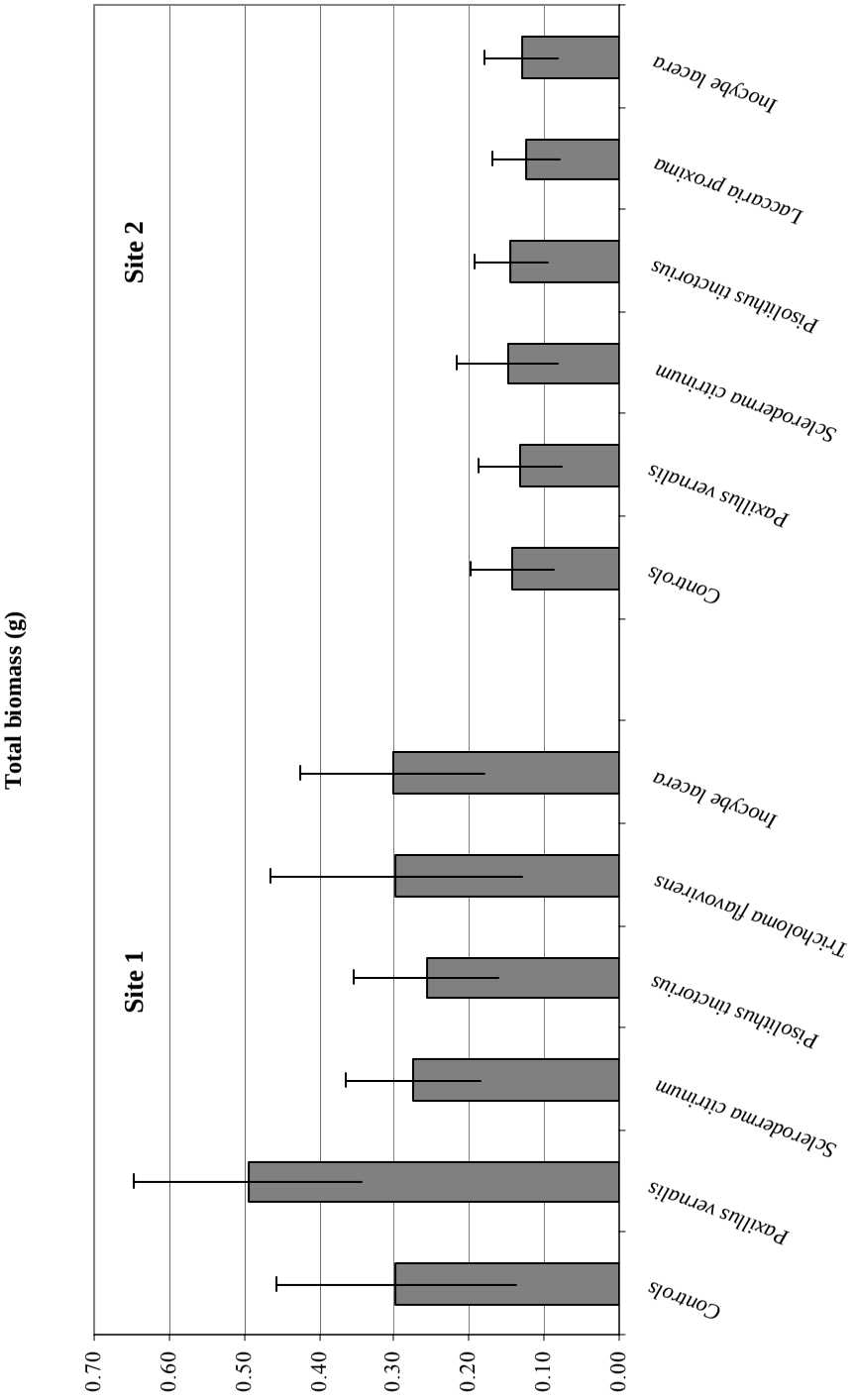

Figure 21. Total biomass (g) of inoculated and non-inoculated aspen seedlings in two samplings of field soils from Mt. Haggin in the greenhouse, banded 1", after three months.

Discussion

Detailing the effects of various ectomycorrhizal fungi on the growth and uptake of elements for aspen seedlings in smelter-impacted soils in the greenhouse is an important step in developing successful inoculum programs for out-planting on stressed sites. The benefits realized from inoculation with mycorrhizal fungi are often most important on "extreme" sites (Smith and Read, 1997). The findings of these studies contribute to our understanding of some of the effects of native ectomycorrhizal fungi on plants in stressed environments, specifically smelter impacted sites, which is limited to a few studies, particularly for aspen (Cripps, 1996, 2003).

Experiment 1

Soil Analysis (Experiment 1). Extractable levels of various elements utilizing the DTPA (diethyltriaminepentaacetic acid) - method were obtained from soil analysis of the Smelter Hill and Mt. Haggin field soils for Experiment 1. Many of the methods utilized to analyze the field soils were designed to detect nutrient plant availability in systems where elements are deficient. Plants growing in soils with enriched levels are not as aggressive in the uptake of excess levels of elements in the soil solution (Committee on Bioavailability of Contaminants in soils and Sediments, 2003), thus, the bio-available fraction of elements in the soil might be less than the values reported utilizing the DTPA method of analysis. Potassium in Mt. Haggin values as reported in Table 25 were extremely high, potentially suggesting some error in sampling. For this study however,

the amount of each element in each soil was reported in order to characterize the two different field soils relative to one another.

Field soils from the Smelter Hill site had a slightly lower pH value than the Mt. Haggin field soils, although the Smelter Hill field soil had a higher organic matter content than the Mt. Haggin field soils in Experiment 1. Organic matter in soil is an important sink for metals (Kabata-Pendias, 2001), although metal bio-availability was still higher in the Smelter Hill field soils.

Soil acidity affects mycorrhiza formation and hyphal development to a degree dependent on the plant species and the mycorrhizal fungus (Sharpe and Marx, 1986). The acidifying nature of NH_4 in soils and the high levels of $NH_4 - N$ in Smelter Hill field soils may contribute to the lower pH values in these soils. The higher levels of sulfur in the Smelter Hill field soils will also lower pH values. N and P levels are dependent on the fate of the various ions related to pH (Marschner, 1995), and according to Domsch et al. (1983), N and P mineralization is especially sensitive to heavy metal pollution. Soil acidification also increases metal availability in the soil (Fitter and Hay, 1981), such as Al (Jentschke et al. 2000).

The higher metal and As levels in the Smelter Hill field soil compared to the Mt. Haggin field soils is expected, given the close proximity to the Anaconda smelter stack. In addition, Mt. Haggin field soils were collected upwind from the Anaconda smelter stack, thus suggesting a reduced effect of aerial deposition of emissions.

Percentage Of Aspen Seedlings With Mycorrhizae (Experiment 1). Mycorrhizae formed on the controls in both field soils utilizing the banded 1" method of inoculation,

although mycorrhizae did not form on the controls utilizing the 'inoculated at time of sowing' method. I hypothesize that the 1" band of non-colonized soil inoculum in the controls stimulated existing mycorrhizal fungi present in the soil that had survived steam pasteurization by providing essential nutrients for fungal growth. While the mycorrhizal fungus was not identified to species, it has microscopic characteristics consistent with an e-strain fungi. Colonization of plants with some mycorrhizal fungi (*Thelephora terrestris* and E-strain) is common occurrence in nursery inoculation programs (Smith & Read, 1997). Therefore, comparisons between inoculated seedlings and non-inoculated 'controls' are comparisons between specific ectomycorrhizal fungi and a naturally occurring mycorrhizal fungi that either survived soil pasteurization, or was present in the peat fraction of growing media or germinated from airborne spores. The existence of native mycorrhizal fungi, even in the Smelter Hill field soils, which are heavily contaminated, is possible given that there was vegetation present at some soil collection sites, including soil collected from the edge of a declining remnant aspen stand. The presence of ectendomycorrhizal fungi on aspen seedlings is probable, given the pioneering aspect of this tree species with other 'early' successional plant species such as grasses and woody shrubs, which are typically associated with arbuscular fungi. Cripps (2001) reports that there have been some atypical reports of aspen associating with arbuscular fungi. In addition, grass germination from the soil seed bank was observed during the experiment indicating soil organisms including mycorrhizal fungi and some seeds did survive soil pasteurization that was conducted before the initiation of the experiment. Consequently, a longer duration of steam pasteurization was initiated for

Experiment 2 in order to limit the number of factors that could affect the results. Alternatively, interactions with native soil biota in pot studies give a more realistic simulation of conditions that exist *in vivo*.

Field sites. That mycorrhizae formed more readily in the Smelter Hill field soils than in Mt. Haggin field soils may be due to differences in organic matter, pH, and/or metal levels. Organic matter was higher in Smelter Hill field soils than Mt. Haggin field soils, even though the Smelter Hill soils had higher metal levels. Native ectomycorrhizal fungi, originally collected from a site in Butte, Mt., where soil pH was 5.3 (Cripps, 1995, 2003), may be more adapted to more acidic soils as the Smelter Hill field soils had a lower pH than Mt. Haggin field soils. Colpaert and Van Assche (1987) found a strong correlation between ectomycorrhizal fungi found on polluted sites and their ability to tolerate heavy metals *in vitro*, suggesting site-specific fungal adaptation to metals. Meanwhile, Denny and Wilkins (1987) found no indication of fungal adaptation to Zn at the inter- and intraspecific level *in vitro* and Howe, et al. (1997) found that copper tolerance was not due to whether the fungi were isolated from contaminated or uncontaminated sites. These contrasting findings may be explained by the amount of time a fungal species has been present on a polluted site, and the fungal species themselves. Mycorrhizal fungi, like plants, require certain environmental parameters for growth and survival. Therefore, prolonged exposure to elevated metal concentrations would select for a specific group of mycorrhizal fungi.

In addition, the Smelter Hill field soils had higher NH_4 concentrations than Mt. Haggin field soils, which might be the preferred form of N for the species of

ectomycorrhizal fungi in these experiments. Conversely, *Inocybe lacera* might favor nitrate over ammonium as a source of N thus explaining the higher mycorrhization levels of *Inocybe lacera* in the Mt. Haggin field soil, which had higher nitrate levels than the Smelter Hill field soil.

Method of Inoculation. 'Inoculation at time of sowing' was not an effective method of inoculating aspen in the greenhouse. Soil inoculum placed in cubed trays and utilized in a non-sterile environment for seed germination quickly became colonized by opportunistic saprophytic fungi, thus reducing the effectiveness of the soil inoculum. Although the soil inoculum was initially fully colonized by ectomycorrhizal fungi, opportunistic saprophytic fungi were able to out-compete the ectomycorrhizal fungi, except in the case of *Laccaria proxima*. *Laccaria proxima* colonizes soil inoculum at a fast rate (see Chapter 2), and as a result was able to out-compete the saprophytic fungi and form mycorrhizae with the roots of aspen. Alternatively, the fast growing *Laccaria proxima* may have more fully utilized the enriched nutrient media (MMN) in the soil inoculum before being utilized in a non-sterile environment, thereby reducing the available sugars for saprophytic fungi. Placing a band of sterile or pasteurized peat:vermiculite in a 1:1 ratio over the soil inoculum just before sowing seeds might provide enough of a physical barrier to prevent excessive colonization by opportunistic saprophytic fungi.

Soil inoculum banded 1" below one-month old aspen seedlings was an effective method of inoculation of field soils in the greenhouse. Utilizing the banded 1" method of inoculation reduces the opportunity for the soil inoculum to become colonized by other

microorganisms because of the physical barrier of placing the seedling above the inoculum. However, the month old seedling is not sterile and some contamination of the soil inoculum is possible. In addition, since pasteurization of the field soil in Experiment 1 did not kill all soil organisms, it might be that some contamination of the 1" band of soil inoculum did occur from below.

The native ectomycorrhizal fungi exhibited different rates of mycorrhization of aspen seedlings. In general however, most species utilized in Chapter 4 experiments formed mycorrhizae on at least 50 % of inoculated aspen seedlings, with *P. vernalis*, *S. citrinum* and *L. proxima* exhibiting the highest levels. Within fungal treatments there were also large variations in the percentage of roots colonized by ectomycorrhizal fungi, thus one could assume that inoculation effects would be varied. The growth rates of the native ectomycorrhizal fungi utilized in these experiments varied (Chapter 2), and root colonization might simply be a function of "faster" colonization by some fungi than others, consistent with Chapter 3 results.

Growth Responses (Experiment 1).

Inoculation Method. Aspen seedlings that were inoculated by banding inoculum were taller, with more biomass and greater root development than seedlings 'inoculated at time of sowing'. Seedlings 'inoculated at the time of sowing' grew for a month directly on soil inoculum that consisted of colonized peat:vermiculite (1:6). Aspen seedlings inoculated with the banded 1" method were sown on peat:vermiculite in 1:1 ratio for one month. The roots of seedlings inoculated by banding 1" grew though the

soil inoculum when they were one month old, whereas seedlings 'inoculated at the time of sowing' might have been stunted because they were inoculated with the fungus at an early stage. Also, opportunistic colonization by saprophytic fungi of the peat:vermiculite (1:6) soil inoculum for the 'inoculation at time of sowing' method could have suppressed plant growth as a result of direct competition with the mycorrhizal fungi or the seedlings for essential nutrients.

Table 42. Comparison of mycorrhization and growth trends of 7 different ectomycorrhizal fungi with non-inoculated 'controls' in two different smelter-impacted soils utilizing two different inoculation techniques, Mycor.=mycorrhization, RCD=root collar diameter, na=inoculum unavailable, 0=approximately equal, + = 10% > controls, ++ = 11-40% > controls, +++ = 41%>controls, - = < 10% controls, - - = <11-40% controls, --- = < 41% controls, and * = significantly different from the controls with P<0.05. Controls for Mt. Haggin, "banded" were 33% mycorrhizal and controls for Smelter Hill, "banded" were 81% mycorrhizal with an E-strain fungus.

Mt. Haggin

	Inoculation at time of sowing						Banded 1"					
Fungal Treatment	Mycor.	Height	RCD	Shoot	Root	Total	Mycor.	Height	RCD	Shoot	Root	Total
P. vernalis	-	+	+	+	+	+	++	-	-	-	-	-
S. citrinum	na	na	na	na	na	na	++	-	-	-	-	-
P. tinctorius	-	- *	0	- *	+	0	-	-	- *	-	-	-
L. proxima	-	+	0	+	+	+	++	0	+	0	++	+
T. flavovirens	-	++	+	+	+	+	++	-	-	-	-	-
T. scalpturatum	-	+	++	++	++	++	na	na	na	na	na	na
I. lacera	na	na	na	na	na	na	+++	-	-	-	+	0

Smelter Hill

	Inoculation at time of sowing						Banded 1"					
Fungal treatment	Mycor.	Height	RCD	Shoot	Root	Total	Mycor.	Height	RCD	Shoot	Root	Total
P. vernalis	-	+	++ *	++	++ *	+	++	-	0	-	+	0
S. citrinum	na	na	na	na	na	na	++	0	+	+	-	0
P. tinctorius	-	++	++	++	++ *	+	0	0	-	-	-	-
L. proxima	++	+	+	-	0	0	++	+	+	+	++	++
T. flavovirens	-	+	+	+	+	+	-	+	-	0	-	-
T. scalpturatum	na	na	na	na	na	na	na	na	na	na	na	na
I. lacera	na	na	na	na	na	na	-	++	+	+	+	++

Comparison Of Field Soils. That aspen seedlings were taller and larger when grown in Mt. Haggin field soil versus Smelter Hill field soil, regardless of method of inoculation or fungal treatment. These results may reflect the higher level of contamination in the Smelter Hill field soils, for sulfur, As, Cu and Fe. The lower pH value in the Smelter Hill field soils would increase the bio-availability of these metals, thus increasing their potential toxicity to the plant and inhibiting plant growth. Aspen seedlings growth in Smelter Hill field soils was less variable than plant growth in Mt. Haggin field soils, possibly because of the depressed growth response of aspen seedling in the more contaminated field soils, or due to soil heterogeneity..

For aspen seedlings 'inoculated at time of sowing', the growth response for all variables was positive when compared to the growth of non-inoculated control seedlings, except for the height and aboveground biomass of *Pisolithus tinctorius* inoculated aspen seedlings. The positive growth may be due to stimulation by the fungi even though they didn't form mycorrhizae, or the result of addition of organic matter in the form of fungal material assuming the ectomycorrhizal fungus did not survive and can change soil characteristics.

The growth response of *Paxillus vernalis*, *Scleroderma citrinum* and *Tricholoma flavovirens* inoculated aspen seedlings utilizing the banding 1" method of inoculation in Mt. Haggin field soils was negative for all variables. This consistent negative correlation in the growth response suggests a carbon drain as a result of mycorrhization as previously reported in Maroneck et al. 1981 and others (Marx 1982; Mason et al. 2000; Plassard et al. 2000). For *Inocybe lacera* inoculated aspen seedlings there was a negative growth

response for height, root collar diameter and shoot biomass, but an increase in root biomass so that the total biomass was equal to the non-inoculated controls. This ectomycorrhizal fungus shifted the carbon allocation from aboveground components to belowground components more effectively than the other fungal treatments except *Laccaria proxima*. *Laccaria proxima* inoculated aspen seedlings in Mt. Haggin field soils always had a positive or equal growth response for all variables measured. *Laccaria* can be a fast grower under some circumstances, and it is also possible that mycorrhizae formed more quickly, and thus plants were past the stage of carbon drain and were responding to benefits afforded by the fungus.

For aspen seedlings 'inoculated at time of sowing' in Smelter Hill field soils, the growth response for all variables was also generally positive when compared to the growth of non-inoculated control seedlings, with the exception of aboveground biomass for *Laccaria proxima* inoculated seedlings. This is similar to the response in Mt. Haggin soils for this type of inoculation. Only *Laccaria proxima* mycorrhizae were observed for this treatment, again indicating a potential carbon flow shift from aboveground growth to belowground growth with mycorrhization. The presence of the fungus without mycorrhization enhanced growth with this method, and there are other reports in the literature of this phenomenon.

With the banded 1" method, results were less consistent. All fungal treatments formed mycorrhizae with aspen seedlings, except for those with *Inocybe lacera,* which exhibited a positive growth response. *Scleroderma citrinum*, *Pisolithus tinctorius*, and *Tricholoma flavovirens* inoculated aspen seedlings had lower root biomass values than

the controls, while *Paxillus vernalis* and *Laccaria proxima* inoculated aspen seedlings increased belowground biomass, indicating a shift in resources to belowground components. *Pisolithus tinctorius* inoculated aspen seedlings were generally smaller than the non-inoculated controls, possibly indicating some carbon drain as a result of mycorrhization. *Laccaria proxima* inoculated aspen seedlings followed the same tendencies throughout both soil types, with a positive response for all variables with any kind of inoculation. *Laccaria* grows faster and may be beyond the lag carbon drain phase, at least for these conditions, where fungal benefits outweigh the carbon drain. The root collar diameter was larger in *Scleroderma citrinum* and *Laccaria proxima* inoculated seedlings than the controls but less in the *Pisolithus tinctorius* and *Tricholoma flavovirens* seedlings, making general conclusions about the effects of ectomycorrhizal fungal inoculation on root collar diameter for aspen seedlings in contaminated field soils difficult.

Inoculation With Ectomycorrhizal Fungi. Inoculated aspen seedlings were taller and larger when compared to non-inoculated seedlings across all treatments (taken together) except for aboveground biomass. However, the controls in some groups (banded 1") did form mycorrhizae. Individual growth responses to inoculation with different species of ectomycorrhizal fungi in metal-contaminated soils were varied (Table 26, Fig. 5), which agrees with the findings of Jones and Hutchinson (1986) and Willenvorg et al. (1990). Additionally, different ectomycorrhizal species have varying affects on a host plant under different conditions (Jentschke et al. 2000; Van der Heijeden & Kuyper, 2001). Compounding the problem of identifying realized benefits to a host

plant from the mycorrhizal association, is the fact that multiple species of mycorrhizal fungi may be present on the same plant at the same time, or different species of ectomycorrhizal fungi may be active at different times in natural systems (Allen et al. 1995). As a result, measurements of different plant parameters can add to the uncertainty of realized benefits to a host plant by ectomycorrhizal fungi in metal contaminated soil systems by under-representing or over-representing relevant data. Sustained survival on these harsh sites is the definitive analysis.

Plant Tissue Analysis (Experiment 1)

The variability in uptake of elements in the roots and shoots of inoculated and non-inoculated "controls" of aspen seedlings in these experiments is similar to results documented in earlier studies (Gadd, 1993; Trappe, 1977). The uptake of selected elements from smelter-impacted soils varies with inoculation with species of mycorrhizal fungi, however, the results can not be analyzed statistically. From these experiments, it is clear that ectomycorrhizal fungi can effect plant element uptake, and that both essential and non-essential element uptake is dependent on the species of fungi, the element, and its concentration in the soil. In addition, these results suggest that the effects of both metal amelioration and enhanced nutrient uptake may be coordinated on a species level scale for plant uptake of various elements in smelter impacted soils. Enhancement and exclusion of various elements appeared to coincide, suggesting multiple strategies in dealing with elevated levels of elements in a smelter impacted soil.

Table 43. Comparison of mycorrhization and growth trends of 7 different ectomycorrhizal fungi with non-inoculated 'controls' in two different smelter-impacted soils utilizing two different inoculation techniques, na=plant material unavailable, 0=approximately equal, + = 10% > controls, ++ = 11-40% > controls, +++ = 41%>controls, - = < 10% controls, - - = <11-40% controls, --- = < 41% controls. Controls for Mt. Haggin, "banded" were 33% mycorrhizal and controls for Smelter Hill, "banded" were 81% mycorrhizal.

Site; plant material Fungal treatment	Total S %	Total N %	Mg %	K %	Fe mg/kg	Zn mg/kg	P %	Ni mg/kg	Pb mg/kg	Cu mg/kg
Mt. Haggin banded 1"; aboveground biomass										
Paxillus vernalis	+	++	+	0	+	++	+	++	+	+
Scleroderma citrinum	0	+	+	-	++	++	+	+	+	+
Pisolithus tinctorius	na	na	+	0	+	-	+	+	+	0
Laccaria proxima	+	+	+	-	-	++	++	+	0	+
Tricholoma flavovirens	0	++	+	-	+	+	+	+	+	+
Inocybe lacera	-	-	+	+	+	+	+	+	+	+
Mt. Haggin banded 1"; belowground biomass										
Paxillus vernalis	0	+	0	+	-	+	+	0	+	+
Scleroderma citrinum	0	+	-	+	--	+	+	-	+	+
Pisolithus tinctorius	na	na	+	0	+	-	+	0	-	-
Laccaria proxima	0	+	-	-	--	+	+	-	+	+
Tricholoma flavovirens	0	+	+	0	+	+	+	+	+	+
Inocybe lacera	na	na	-	-	-	+	+	-	+	-
Mt. Haggin inoculated at time of sowing; aboveground biomass										
Paxillus vernalis	+	-	+	-	-	+	+	-	-	+
Pisolithus tinctorius	+	-	+	-	-	+	+	-	-	-
Laccaria proxima	+	-	-	+	-	-	+	-	0	-
Tricholoma flavovirens	0	-	-	-	-	-	0	-	-	-
Tricholoma scalpturatum	0	-	+	-	-	-	+	-	-	-
Mt. Haggin inoculated at time of sowing; belowground biomass										
Paxillus vernalis	0	0	-	-	-	-	-	+	-	-
Pisolithus tinctorius	-	-	-	-	-	-	-	-	-	-
Laccaria proxima	-	-	-	-	-	-	-	-	-	-
Tricholoma flavovirens	-	-	-	-	-	-	-	-	-	-
Tricholoma scalpturatum	-	-	-	-	-	-	-	0	-	-

Table 43. continued

Site; plant material Fungal treatment	Total S %	Total N %	Mg %	K %	Fe mg/kg	Zn mg/kg	P %	Ni mg/kg	Pb mg/kg	Cu mg/kg
Smelter Hill banded 1"; aboveground biomass										
Paxillus vernalis	+	-	+	-	++	-	-	++	0	0
Scleroderma citrinum	+	+	+	+	+	+	+	+	+	+
Pisolithus tinctorius	+	+	-	+	+	+	-	+	0	0
Laccaria proxima	+	-	+	-	-	+	0	-	0	0
Tricholoma flavovirens	+	-	+	-	+	+	+	-	+	+
Inocybe lacera	+	-	+	+	-	+	+	-	0	-
Smelter Hill banded 1"; belowground biomass										
Paxillus vernalis	-	-	-	-	-	-	-	0	+	-
Scleroderma citrinum	-	-	-	-	++	+	+	+	+	-
Pisolithus tinctorius	-	-	++	+	++	-	-	+	-	--
Laccaria proxima	-	-	-	-	-	-	-	-	+	-
Tricholoma flavovirens	+	+	++	+	++	+	+	+	+	+
Inocybe lacera	-	-	-	+	-	-	+	-	0	-
Smelter Hill inoculated at time of sowing; aboveground biomass										
Paxillus vernalis	+	+	+	-	--	++	+	-	-	-
Pisolithus tinctorius	+	-	+	-	--	+	0	-	--	--
Laccaria proxima	+	+	-	+	++	+	+	-	+	+
Tricholoma flavovirens	+	+	+	-	+++	+	+	0	-	+
Smelter Hill inoculated at time of sowing; belowground biomass										
Paxillus vernalis	na	na	---	-	---	-	+	--	-	-
Pisolithus tinctorius	na	na	---	-	---	-	+	--	--	--
Laccaria proxima	na	na	na	na	na	na	na	na	na	na
Tricholoma flavovirens	na	na	---	-	---	-	+	--	-	-

Mt. Haggin Field Soil; Shoot And Root Analysis. Similar trends were observed utilizing two different inoculation techniques (banded 1", inoculation at time of sowing) for total levels of analyzed elements in the roots and shoots of aspen seedlings in Mt. Haggin field soil (Tables 33, 43). N levels were always lower in the roots of aspen

seedlings than in the shoots. Fe, Pb, and copper levels were higher in root tissue than shoot tissue. These results agree with the findings of Burt and Read (1981), who reported significantly lower metal concentrations in shoots than roots, suggesting mycorrhizal fungi somehow reduce the metal translocation from the root to the shoots. Conversely, Zn values were generally double in the shoots when compared to the roots. However, controls showed the same trends for the most part, even those that did not become mycorrhizal.

Similar trends were also observed for both inoculation methods for total levels of analyzed elements in the roots and shoots of aspen seedlings in Smelter Hill field soil (Tables 33, 43).

Aspen seedlings grown in Smelter Hill field soil generally had higher levels of sulfur, N, manganese, Fe, P, Ni, Pb, and copper regardless of whether above or belowground plant tissue was analyzed. The higher metal levels in plant tissue in aspen seedlings grown in Smelter Hill field soil likely contributed to the lower rates of growth compared to seedlings grown in Mt. Haggin field soil, and is consistent with the higher levels of these elements present in the Smelter Hill soil analysis. Seedlings grown in Mt. Haggin field soil exhibited higher levels of potassium and Zn in both the roots and shoots when compared to Smelter Hill values for roots and shoots, which is consistent with their higher levels in Mt. Haggin field soil.

Inoculation At Time Of Sowing. Most elements in the shoots and roots were found at lower levels than the controls, except for *Laccaria proxima* inoculated aspen seedlings, which appeared to slightly increase plant uptake of S, K, P in the shoots in

both soil types (although these are not likely significant differences). In addition, inoculation with *Laccaria proxima* slightly increased plant shoot uptake of Fe, N, Zn, Pb, and Cu in Smelter Hill field soils compared to the "controls". The plant tissue analysis of aspen seedlings 'inoculated at time of sowing' confirms earlier results that showed that seedlings that were inoculated with this technique were smaller than the banded 1" inoculated seedlings, possibly as a result of reduced uptake of essential elements in both the shoot and roots, speculated to be because of lack of mycorrhization.

Banded 1". Plant uptake of N and P of seedlings inoculated with ectomycorrhizal fungi in Mt. Haggin field soil was slightly enhanced in both the roots and shoots. In general, inoculation of aspen seedlings with ectomycorrhizal fungi appeared to enhance metal uptake and translocation slightly to the shoots. Elemental levels in the roots of inoculated aspen seedlings exhibited more variability, although there were some slight decreases in Fe, Zn, Ni, Pb and copper in the roots of a few treatments by different species of ectomycorrhizal fungi.

In Smelter Hill field soils, which had higher levels of N and P, uptake of N, P, K was dependent on fungal treatment for both the roots and shoots, although in general N, P, K levels were lower than the "controls" when compared to the Mt. Haggin field soils. These results suggest that when element levels are excessive, inoculation with some species of ectomycorrhizal fungi will prevent uptake of certain elements and in soils with lower levels of elements some species of ectomycorrhizal fungi might enhance element uptake, although the results varied with fungal treatments. Magnesium levels in the shoots of inoculated plants in Smelter Hill field soils was higher or lower than the

"controls", while the magnesium levels in the roots of aspen seedlings was similar to the results in Mt. Haggin field soils where inoculation with *P. tinctorius*, and *T. flavovirens* appeared to enhance magnesium uptake. In addition, the higher levels of metals associated with Smelter Hill field soil could negatively affect essential nutrient uptake as a result of soil toxicity. Inoculation of aspen seedlings with ectomycorrhizal fungi in Smelter Hill soils, which exhibited higher levels of metals generally, appeared to increased plant uptake and translocation of some metals to the shoots when banded. When compared to the "controls" most species of ectomycorrhizal fungal decreased the copper and sulfur uptake to the roots. Pb, Ni, Zn and Fe levels were also reduced in the root tissue of inoculated aspen in some fungal treatments.

It appear from these results that inoculation with *Laccaria proxima*, which had high mycorrhization levels, promoted plant growth of inoculated aspen seedlings in smelter impacted soils, and reduced metal uptake in the roots and shoots more than any other fungal treatment including the "controls".

Inoculation Methods And Metal Uptake

Since controls were compromised for the banding treatment, and mycorrhization did not occur for the seed sowing method, it might be illustrative to compare uptake of particular metals between the two inoculation methods, and for each soil. Although not statistical, it could be illustrative since mycorrhization occurred primarily for banding, however, a fungal effect for sowing cannot be precluded.

Large differences were observed in the copper content of roots and shoots of aspen seedlings when the two different inoculation techniques, banding or sowing were

compared for each soil type for all fungi taken together (Figures 44, 45). However, as a result of pooling plant tissue for analysis these differences cannot be compared statistically. Although not statistically guaranteed, copper uptake does appear to be affected by inoculation technique, which might be correlated with the level of mycorrhizal formation. As a result of the inability of most ectomycorrhizal fungi to form mycorrhizae for the 'inoculating at time of sowing' method in both field soils, (except *Laccaria proxima* 50 % in Smelter Hill field soil), these could effectively be considered non-mycorrhizal because out of 117 aspen seedlings utilizing this method only 6 formed mycorrhizae on their roots, a 0.05 % infection rate. Conversely, with the banded 1" method of inoculation 94 aspen seedlings formed mycorrhizae out of 137 for an infection rate of 69%. Controls were averaged too, since some formed mycorrhizae utilizing the banded 1" method and could thus be considered mycorrhizal. In Mt. Haggin field soils utilizing the banded 1" method, shoot uptake of copper was 26% less than aspen seedlings 'inoculated at time of sowing'. Root uptake was 39% less using the banded 1" method. In Smelter Hill field soils there were notable differences as well. Shoot uptake of copper was 50% less, and root uptake was 34% less utilizing the banded 1" method when compared to the 'inoculated at time of sowing' method (Table 45).

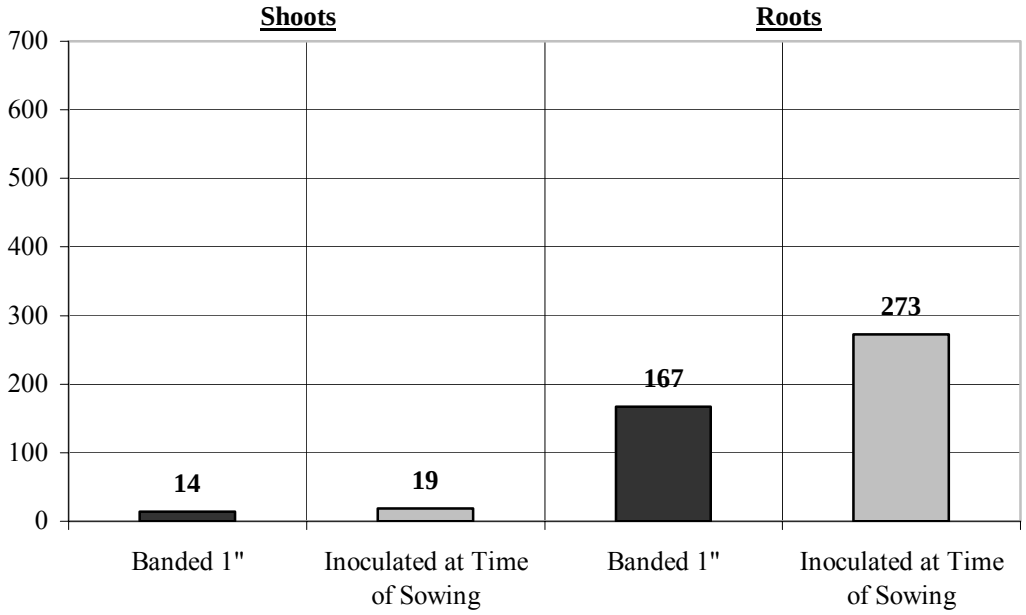

Figure 22. Copper in the shoots and roots of aspen comparing two inoculation methods, banded 1" and inoculated at time of sowing in Mt. Haggin field soils averaged for all fungal treatments. Values in mg/kg.

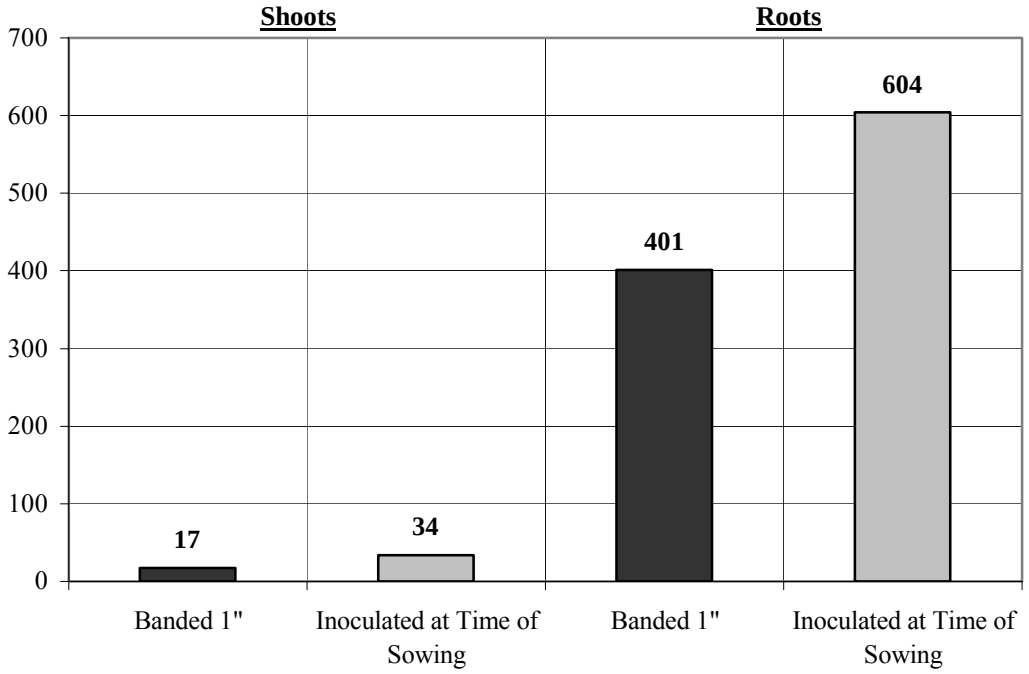

Figure 23. Copper in the shoots and roots of aspen comparing two inoculation methods, banded 1" and inoculated at time of sowing in Smelter Hill field soils averaged for all fungal treatments. Values in mg/kg.

Given the much higher mycorrhization rates of aspen seedlings utilizing the banded 1” method and the depressed accumulation of copper in the roots and shoots, coupled with published reports of the ability of some ectomycorrhizal fungi to inhibit heavy metal uptake (Bradley et al. 1981; Smith & Read, 1997), this suggests that successful inoculation of aspen seedlings with mycorrhizal fungi may decreases copper uptake in the plant tissue of aspen seedlings in smelter-impacted soils. To further elucidate this effect, a comparison was done for particular species of fungi for the two inoculation methods in the two soils (Table 45).

Table 44. Copper, Fe and Zn levels in the roots and shoots of inoculated aspen utilizing 2 different inoculation techniques (mg/kg) and the differences between the two values in percents

Copper (mg/kg)

	Smelter Hill				Mt. Haggin			
	Roots		Shoots		Roots		Shoots	
Ectomycorrhizal Fungi	sown	banded	sown	banded	sown	banded	sown	banded
Paxillus vernalis	649	344 (-47%)	33	16 (-52%)	311	198 (-47%)	20	17 (-15%)
Pisolithus tinctorius	458	187 (-60%)	23	16 (-30%)	264	132 (-50%)	19	10 (-48%)
Laccaria proxima	-	432	39	16 (-59%)	281	168 (-41%)	19	16 (-16%)
Tricholoma flavovirens	630	578 (-9%)	42	22 (-48%)	227	187 (-18%)	15	14 (-6%)

Fe (mg/kg)

	Smelter Hill				Mt. Haggin			
	Roots		Shoots		Roots		Shoots	
Ectomycorrhizal Fungi	sown	banded	sown	banded	sown	banded	sown	banded
Paxillus vernalis	1881	1624 (-14%)	169	475 (+280)	926	1399 (+150%)	107	79 (-27%)
Pisolithus tinctorius	1002	3168 (+316%)	119	198 (+166%)	901	1710 (+190%)	94	56 (-41%)
Laccaria proxima	-	1864	506	99 (-81%)	757	834 (+10%)	76	51 (-33%)
Tricholoma flavovirens	1391	3569 (+257%)	874	530 (-40%)	988	1540 (+56%)	84	72 (-14%)

Zn (mg/kg)

	Smelter Hill				Mt. Haggin			
	Roots		Shoots		Roots		Shoots	
Ectomycorrhizal Fungi	sown	banded	sown	banded	sown	banded	sown	banded
Paxillus vernalis	139	125 (-11%)	346	234 (-32%)	337	231 (-31%)	838	528 (-37%)
Pisolithus tinctorius	111	113 (+101%)	240	254 (+106%)	315	167 (-47%)	844	328 (-61%)
Laccaria proxima	-	122	229	326 (+142%)	325	217 (-33%)	714	560 (-22%)
Tricholoma flavovirens	132	165 (+125%)	274	264 (-104%)	231	213 (-8%)	564	486 (-14%)

Copper. A decrease of 30-52% in roots and 9-47% in shoots for Smelter Hill soil by banding with *P. vernalis*, *P. tinctorius* and *L. proxima* appeared to be the more effective in reducing copper uptake, when compared to the other inoculation method (Table 45). The same was true for Mt. Haggin soils at lower levels (lower in soil). Results of tissue analysis for banding in both soils are similar.

Iron. Fe uptake utilizing the banded method was higher in roots (316%) and shoots (166%) with *P. tinctorius*, and for shoots with *P. vernalis* (280%), and in roots for *T. flavovirens* (257%) compared to 'sown seeds' for Smelter Hill soil (Table 45).

Zinc. Zn uptake was lower (14-61%) in the shoots of banded aspen when compared to 'sown seeds' in Mt. Haggin soils (Table 45). Root uptake of Zn in Mt. Haggin soils with the banded method was also lower (8-47%), especially seedlings inoculated with P. tinctorius, which depressed Zn uptake in the roots 47% when compared to 'inoculated at time of sowing'. Banding 1" of inoculum appeared to increase the uptake of Zn in the roots and shoots of aspen in most fungal treatments when compared to 'inoculation at time of sowing' (with the exception of *P. vernalis* and in the shoots of *T. flavovirens*) in Smelter Hill soils.

Fungal Species And Metal Uptake

The genus *Laccaria* is common on disturbed sites with young trees, and has been reported on uranium tailings, As mine wastes, and various smelter-impacted areas including near Butte and Anaconda (Cripps 1996, 2003). Many species of *Laccaria* are considered "weedy" early colonizers capable of forming mycorrhizae on a variety of young tree species, which are later replaced by more competitive fungi as under-story conditions improve. *Laccaria* species grow well in culture and are considered to have high value for reclamation. Reports of *Laccaria laccata* on mine spoils could actually refer to *Laccaria proxima*, a similar species. In this study, aspen which formed mycorrhizae, reduced copper uptake in shoots 59% in Smelter Hill soil and 27% in Mt.

Haggin soil, and 41% in roots for Mt Haggin, compared to aspen inoculated by sowing of seed which for this species also formed mycorrhizae. It reduced Fe uptake in shoots 81% in Smelter Hill soil and 33% for Mt Haggin soil, but slightly increased uptake in Mt Haggin roots by 10%, compared to those inoculated by sowing seed. It appeared to reduce the uptake of Zn for both roots and shoots at Mt. Haggin, but not for Smelter Hill soil, compared to those inoculated by the seeding method (Table 45).

Paxillus involutus can have a broad host range, while *P. vernalis* is more specific to aspen and birch. The former has been recorded on As wastes, uranium tailings and is prolific with aspen at Butte and Anaconda (Cripps 2003). In this study, *P. vernalis* grew well in culture and formed mycorrhizae efficiently. It reduced copper uptake in roots (47%) and shoots (52%) in Smelter Hill soil and in roots (47%) and shoots (15%) at Mt. Haggin, compared to those inoculated by the seeding method (Table 45). Also, Fe uptake was reduced in roots (14%) in Smelter Hill soils and in roots (27%) for Mt. Haggin soil, but it enhanced Fe uptake was enhanced in shoots (280, 150%, respectively), compared to the inoculated seed method. Similarly, Ni uptake was enhanced 2000% in shoots for Smelter Hill soil, and 400% for shoots in Mt. Haggin soil in banded plants, compared to the seeding method. *Paxillus* did appear to reduce Pb uptake for Mt Haggin soils slightly in both roots and shoots (Table 44).

Pisolithus tinctorius is a well known fungus commonly used in reforestation of conifer trees (Mark 1980; Cordell et al. 2000), and has been used with success on coal spoils, but is known to form mycorrhizae with aspen (Cripps 2001). The local variety used in this study grew well in culture and was an efficient colonizer of aspen roots,

although it is not known to occur on regional smelter sites, and only in the surrounding region in natural soils high in heavy metals. This species reduced copper in banded plant roots (60%, 50%) and shoots (30, 48%) respectively for Smelter Hill and Mt. Haggin soils compared to using the seed method. For Fe, *Pisolithus tinctorius* enhanced uptake in roots (316%) and shoots (166%) in Smelter Hill soils, and in roots (190%) for Mt. Haggin soils with banding compared to use of the seed method. Fe uptake was depressed (41%) for shoots in Mt Haggin soil. *Pisolithus tinctorius* enhanced Ni uptake 600% in shoots for Smelter Hill soils, but not for those in Mt. Haggin soil with banding compared to seeding. For *Pisolithus tinctorius* banding reduced Zn levels by about half in Mt. Haggin soil for roots and shoots compared to seeding, but there were negligible differences for Smelter Hill soil.

Tricholoma species are not typically considered early colonizers, or recorded on waste areas. However, *T. scalpturatum* is common with aspen thoughout southwest Montana, grows well in culture and increases aspen growth in tubes (Cripps 2001) or pots as for this study. This *Tricholoma* species appears less involved with metal uptake, but it did appear to increase uptake of copper (48%, 8%) for roots in Smelter Hill and Mt Haggin soils respectively for banding versus seeding. Fe uptake was enhanced in roots (256%, 56%) and depressed in shoots (40%, 14%) with *T. scalpturatum*, in the two soils respectively with banding versus seeding.

Scleroderma species are well-known early colonizers, particularly with conifers, but is reported here with aspen. It has potential as a commercial inoculum since, unlike most other species, its sporocarps contain huge numbers of spores. Species are known to

occur on coil spoils, As waste, and *S. cepa* was recently used in reclamation of a copper mine in Utah (Cordell et al. 2000).

Inocybe lacera is one of the most prolific fungi on smelter-impacted sites in southwest Montana, and is also known to occur in eastern coal spoils, and on uranium tailings with willow (Cripps 2003). Like *Laccaria,* it is considered a "weedy" early colonizer with a broad host range. It often does not grow well in culture but was used successfully here for mycorrhization of aspen. While it was detrimental to aspen *in vitro* perhaps becoming pathogenic (Cripps 2001), for this study it formed mycorrhizae with aspen with no negative effects, again showing that results for *in vitro* studies do not necessarily translate to those for pot or field studies.

Ectomycorrhizal fungi can reduce metal concentrations in shoots although this differs by the ability of a particular species to reduce translocation, and it is selective by the metal involved (Wilkins 1991). Some fungi can reduce Zn, Ni and Cu except at the highest concentrations, which means the process is soil dependent. In other cases, heavy metals can be increased in roots, but typically not shoots as was found here for Fe and Ni with *Paxillus* and *Pisolithus tinctorius* in Smelter Hill soils. Measurement of metals in roots may also include metals adhered to the mycorrhizae which prevent uptake (Wilkins, 1991). Of particular interest here, is copper because of the high levels in Smelter Hill soils, and Cu is known to be toxic to plants. *Laccaria, Paxillus*, and *Pisolithus tinctorius* all appeared to ameliorate uptake of copper in both roots and shoots by about 30 to 60%. This suggests that these species could have a beneficial effect on out-plantings of aspen on the Smelter Hill site.

Some metals such as copper and Zn may be considered plant-essential trace metals and can behave like other essential elements relative to plant uptake (Friedland, 1990). For cadmium and Zn uptake and immobilization, studies suggest that strategies involved in dealing with potential toxic levels of metals are governed by the importance of the individual element to plant and fungal survival and their quantities in the soil (Frey et al., 2000). Interactions between elements, positive, negative and synergistic are also important in elemental bio-availability (Kabata-Pendias, 2001). Final distribution and accumulation of elements in plant tissue depends on the element, plant species and plant developmental stage (Kabata-Pendias, 2001).

Metal toxicity is a major constraint affecting root growth (Jentschke, et al. 2000; Jones & Hutchinson, 1988). Plant roots directly modify the chemical and physical properties of the rhizosphere, and thus indirectly affect the bioavailability of metals in the soil (Alloway, 1995). Metal uptake can be divided into two main phases, metabolism-independent binding to cells walls (passive), and energy-dependent intracellular uptake across a cell membrane (active) (Gadd, 1993).

Lower ion concentrations in roots allow plants to pull ions from the soil solution either actively, or passively into the root. It appears that most cations enter root cells passively, through diffusion, while anions generally enter the root cell via active transport involving metabolic activity (Fitter & Hay, 1981). Active uptake of metal ions by a plant can be inhibited by the toxic effects of metal ions (Alloway, 1995). Toxic levels of metal ions in soil solution can interfere with the plants ability to acquire water and nutrients,

and the utilization of these resources (Fitter & Hay, 1981). Copper, Pb and Ni are identified in Kabata-Pendias (2001) as some of the most toxic elements to plant growth.

Gadd (1993) outlines several biological mechanisms that are relevant to fungal survival in heavy metal contaminated soils, such as extra-cellular precipitation, complexation and crystallization, transformation of metal species by oxidation, reduction, methylation and dealkylation, absorption to cell walls, extracellular polysaccharides, decreased transport or impermeability, intracellular compartmentation and/or sequestration. Gadd (1993) and others (Hartley et al. 1997) note that more than one strategy might be employed by an organism. Fungal strategies for survival are intimately associated with mycorrhizal effects on plant growth in heavy metal contaminated soil.

According to Denny and Wilkins (1987b), metals are adsorbed to electronegative sites on the hyphal cell wall and along the polysaccharide slime as the growing hyphae colonize new areas of soil. In this way, binding sites for metals are constantly renewed at the first site of contact of fungal hyphae, thereby lowering the total metal content in the soil solution (Denny & Wilkins, 1987a). Galli et al. (1994) proposes specific mechanisms by which a fungus moderates transport into the host plant via intracellular fixation, such as metal accumulation in cell walls of the fungi. Denny and Wilkins (1987b) found Zn accumulated in hyphal cell walls and at the interface between the Hartig net and cortical cells of roots. In a more recent study, retention of Cd and Zn was demonstrated in fungal tissue (Frey et al. 2000). Cadmium extracellular complexation to cell walls in the Hartig net was demonstrated, while Zn was also able to bind to cell walls

in the Hartig net, it was also sequestrated in the cytoplasm of mantle hyphae (Frey et al. 2000).

Retainment of metal ions in the extramatrical mycelium appears to be the key factor in conveying metal tolerance to a host plant by ectomycorrhizal fungi in metal-contaminated soils (Vodnik et al. 1995). The potential metal binding site is a function of the concentration of metals in the soil solution. In soils with high levels of metals, the ectomycorrhizal fungi may be unable to keep up with the additional metal ions that become available through time. In soils with lower concentrations of metals, the ectomycorrhizal fungi may be able to bind most of the metal of concern outside of the plant. The high nutrient content in Denny and Wilkin's (1987b) experiment may favor the identification of metal binding sites further from the actual plant root because of the decreased need for translocation of minerals and water to the plant under artificial conditions, which might alter the allocation of metals in the mycorrhizal and ultimately plant tissues. The interface between mycorrhizal fungi and plant root is recognized by Denny and Wilkins (1987) as a possible important metal-binding site as a last defense, but they speculate that since this is not an active growing area, the interface, it would soon become saturated with metals.

Plants stimulate the production of extra-hyphal slime, which in itself may Pb to enhanced binding of metals to hyphae (Denny & Wilkins, 1987). In addition, Tam (1995) demonstrated by energy-dispersive X-ray spectrometry, the accumulation of metals in the extra-hyphal slime of the ectomycorrhizal fungi, *Pisolithus tinctorius*.

Brown and Wilkins (1985) hypothesized that decreased levels of Zn in leaves of mycorrhizal plants were correlated with improved plant growth. This increases a plant's ability to tolerate toxic levels of metals by excluding toxic metals from the shoot. However, Hentschel et al. (1993) argues that a decrease in metal concentration in leaves of mycorrhizal plants may be a result of increased growth, effectively diluting the metal through the production of additional plant material. A pot experiment by Ahonen-Jonnarth and Finlay (2001), found mycorrhizal plants grew significantly better than non-mycorrhizal seedlings in Ni and cadmium contaminated soils as a result of enhanced nutrient status, not metal amelioration. Plant concentrations of cadmium and Ni were not affected by the mycorrhizal associations (Ahonen-Jonnarth and Finlay 2001), as had been shown in other heavy metal contaminated systems (Jones & Hutchinson 1986). Jentschke and Godbold (2000) also argue that evidence linking fungal immobilization of metals is lacking and other mechanisms need to be explored, such as the release of metal chelating substances, and nutritional or hormonal effects mediated by mycorrhizal fungi. Goldbold et al. (1998) agrees that concentrations of metals in needles or leaves are an insufficient measurement of metal amelioration by ectomycorrhizal fungi. Percentage of mycorrhizal root tips on host plants was found by Aggangan et al. (1998) to be a good measure of heavy metal tolerance *in vitro* and *vivo.*

Experiment 2

Site 1 soils had a lower pH than site 2 soils, although both pH values were

fairly low (5.2. 5.5) and could negatively affect plant growth (Table 34). Site 1 soils had a higher sand component and lower clay component than Site 2 soils indicating that the latter have more water holding capacity. The higher clay content of Site 2 soils was reflected in the percent water necessary for the saturated paste analysis (Table 34). While the overall clay content in site two soils was not excessive, the effect on plant growth was dramatic. The poor drainage of Site 2 soils as a result of the higher clay content and the excessive hardening of the soil when dry negatively affected plant growth. Plant growth, specifically plant root growth and mycorrhizal development are negatively affected by poor soil aeration (Smith & Read, 1997), which is directly related to soil texture. The electrical conductivity of Site 1 soils was higher than Site 2 soils. In addition, Site 1 soils had higher values for all other variables analyzed, and is potentially capable of providing more essential elements to the plant, but also potentially a source of other elements in concentrations that might be toxic to plant growth and survival, such as copper.

Percentage Of Aspen Seedlings With Mycorrhizae (Experiment 2)

Mycorrhizae formation varied by soil and species. *Paxillus vernalis* and *Pisolithus tinctorius* inoculated seedlings, regardless of field soil site, exhibited higher levels of mycorrhization than the other fungal treatments. *Scleroderma citrinum*, *Laccaria proxima* and *Tricholoma flavovirens* exhibited moderate mycorrhization levels, while seedlings inoculated with *Inocybe lacera*, especially in Site 1 soils, exhibited lower mycorrhization levels than the other treatments. In general, soil type had a weak effect on mycorrhization levels. Mycorrhization levels in Experiment 2 were generally higher than mycorrhization levels in Experiment 1. Conetainers were used in experiment 2, and

they hold a larger soil volume than the Rootrainer containers used in Experiment 1 increasing nutrient supply. In addition, plants growth may have been inhibited by the smaller soil volumes in the Rootrainers when compared to the conetainers utilized for experiment 2.

Table 45. Comparison of mycorrhization and growth trends of 6 different ectomycorrhizal fungi with non-inoculated 'controls' in two different smelter-impacted soils, Mycor.=mycorrhization, RCD=root collar diameter, na=inoculum unavailable, 0=approximately equal, + = 10% > controls, ++ = 11-40% > controls, +++ = 41%>controls, - = < 10% controls, - - = <11-40% controls, --- = < 41% controls, and * = significantly different from the controls with P<0.05.

	Mt. Haggin											
	Site one						Site two					
Fungal Treatment	Mycor.	Height	RCD	Shoot	Root	Total	Mycor.	Height	RCD	Shoot	Root	Total
P. vernalis	++	+	+	+	+ *	+ *	+++	0	0	0	0	0
S. citrinum	++	-	0	-	+	-	+	-	-	+	0	+
P. tinctorius	+++	+	0	-	-	-	++	+	+	0	0	+
L. proxima	na	na	na	na	na	na	+	0	-	-	0	-
T. flavovirens	++	E	+	0	0		na	na	na	na	na	na
I. lacera	+	+	+	+	0	0	+	0	-	-	-	-

Field sites. There were no large differences in the mycorrhization of inoculated aspen seedlings in the two field soils collected from Mt. Haggin for Experiment 2 (MH 1, MH 2). Mycorrhization levels were fairly similar in both field soils with *Paxillus vernalis* and *Pisolithus tinctorius* forming mycorrhizae on more aspen seedlings than the other fungal treatments. Individual aspen seedlings in the *P. vernalis*, *P. tinctorius* and *S. citrinum* fungal treatments exhibited the highest levels of mycorrhizal infection in both field soils. Together, the similar mycorrhization levels and infection levels indicate that

soil characteristics important for the development of mycorrhizae were similar in both soils.

Growth response (Experiment 2).

For height, root collar diameter and aboveground biomass there were no significant differences in the growth response of inoculated aspen seedlings between fungal groups by site. There however, was a large difference in the growth of aspen seedlings by field site with larger seedlings from the less-impacted low elevation site. This might be explained by the higher clay content or increased levels of metals in the more impacted site. These results suggest that nutrients were limiting in Site 2 soils and that any elevated levels of elements in site 1 soils did not inhibit seedlings growth more than the limiting factor in Site 2 soils.

Inoculation With Ectomycorrhizal Fungi. Inoculation with *P. vernalis* generally increased plant growth on Site 1 (MH 1) soils more than the controls or any other fungal treatment, although due to the large standard deviation values, this was not significant when analyzed statistically. There were no significant differences with *P. vernalis* between the growth of inoculated and non-inoculated aspen seedlings for Site 2 (MH 2). The lack of any observable differences in Site 2 (MH 2) soils is likely the result of the depressed growth of all aspen seedlings in this soil, small number of replicates and the associated large standard deviations in this experiment.

Experiment 2 was originally conducted to characterize the mycorrhization and growth of inoculated and non-inoculated seedlings in collected field soils and to compare

these results with out-plantings at the same locations. Due to time constraints out-planting experiments were not conducted.

Summary

The results of this study have demonstrated that mycorrhization of aspen seedlings in the greenhouse is possible, and that banding with 1" of inoculum was the most effective method of inoculation, although it is labor intensive. In addition, some fertilization is necessary for aspen seedling development, although high levels of fertilization with a liquid soluble fertilizer high in ammonium dramatically reduced mycorrhization of seedlings. Time release fertilizer such as Osmocote was a viable alternative, resulting in good mycorrhization. In general, mycorrhization of seedlings in peat:vermiculite reduced aspen growth in the greenhouse at 3 months, especially under low fertilizer regimes. Mycorrhization in field soils, however, increased the growth of aspen seedlings inoculated with particular native ectomycorrhizal fungi. These results indicate that inoculation with some native ectomycorrhizal fungi in field soil, even in contaminated soils where nutrients essential to plant growth may be limited due to heavy metal contamination, are able to increase aspen seedling growth. Two strategies appear to be acting simultaneously in the enhanced growth of inoculated aspen seedlings, amelioration of some heavy metals and increased plant fitness as a result of increased acquisition of some essential elements. Fungi affect metal uptake but that is dependent on the fungal species and particular metal.

Numerous variables in field soils affect plant growth and mycorrhization directly

or indirectly as a result of the inter-connectedness of the system. Growth and element uptake of aspen seedlings is dependent on the species of ectomycorrhizal fungi and soil characteristics. In the most contaminated soils *Laccaria proxima* enhanced aspen seedling growth more than any other fungal treatment utilizing the banded 1" method of inoculation, and reduced copper uptake when banded. Similarly, *P. vernalis* and *P. tinctorius* were effective at mycorrhization and reduction of copper uptake when banded.

As a result of the inadvertent mycorrhization of non-inoculated aspen seedlings it appears that utilization of soil transfer could be a viable method of inoculating aspen in field soils collected from the Anaconda Mt. area, although we do not know the ecology of this fungus. Utilization of soil transfer from a similar ecological site where the existing mycorrhizal fungi have adapted to the local environmental conditions is important for the ultimate survival and persistence of the mycorrhizal fungi (Helm & Carling, 1993). A problem with utilizing the soil transfer method to inoculate plants is the unpredictability of the organisms (including pathogens) that may be present in the soil, whereas in inoculation programs with specific native ectomycorrhizal fungi, inoculation can be tailored to the species of plant and soil characteristics of the site. Inoculation using a soil inoculum with specific ectomycorrhizal fungi ecologically adapted to acidic sites with high levels of heavy metals, improved aspen seedling growth when compared to the non-inoculated "control" aspen seedlings, suggesting that while native mycorrhizal fungi may be present in these contaminated soils, other ectomycorrhizal species may be more beneficial to aspen seedling growth and perhaps establishment. Regardless of inoculation method, the use of "native" ectomycorrhizal fungi is critical because it is assumed that

these fungi are ecologically adapted to the site characteristics and will be able to persist on the roots of the host plant, which circumvents the problem with commercial inocula consisting of exotic strains or species of mycorrhizal fungi that are not adapted to the environmental conditions on site.

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APPENDIX A

TABLES

Chapter 3: Experiment one

Levene's Test of Equality of Error Variances(a)

Dependent Variable: Height Transformed (square root)

F	df1	df2	Sig.
1.645	4	83	.171

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+fungi

Tukey HSD (Height)

		Subset		
fungi	N	1	2	3
1	21	1.0102		
3	21	1.0113	1.0113	
5	21	1.0138	1.0138	1.0138
6	6		1.0146	1.0146
4	19			1.0157
Sig.		.146	.199	.699

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = 1.61E-005.
a Uses Harmonic Mean Sample Size = 13.806.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Levene's Test of Equality of Error Variances(a)

Dependent Variable: Root Collar Diameter

F	df1	df2	Sig.
1.860	4	83	.125

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+fungi

Tukey HSD (Root Collar Diameter)

fungi	N	Subset 1	2	3
6	6	1.2517		
1	21	1.3110	1.3110	
3	21	1.3129	1.3129	
4	19		1.4974	1.4974
5	21			1.5890
Sig.		.903	.065	.680

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .033.
a Uses Harmonic Mean Sample Size = 13.806.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Levene's Test of Equality of Error Variances(a)

Dependent Variable: Aboveground Biomass Transformed (inverse square root)

F	df1	df2	Sig.
.320	4	83	.864

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+fungi

Tukey HSD (Aboveground Biomass)

fungi	N	Subset 1	2	3
4	19	1.5083		
5	21	1.5497	1.5497	
6	6		1.6219	1.6219
1	21		1.6419	1.6419
3	21			1.7122
Sig.		.758	.071	.081

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .008.
a Uses Harmonic Mean Sample Size = 13.806.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Levene's Test of Equality of Error Variances(a)

Dependent Variable: Belowground Biomass

F	df1	df2	Sig.
1.739	4	83	.149

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+fungi

Tukey HSD (Belowground Biomass)

		Subset	
fungi	N	1	2
3	21	.0733	
1	21	.0795	
6	6	.1017	.1017
4	19		.1284
5	21		.1395
Sig.		.273	.066

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .001.
a Uses Harmonic Mean Sample Size = 13.806.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Levene's Test of Equality of Error Variances(a)

Dependent Variable: Total Biomass

F	df1	df2	Sig.
2.773	4	83	.032

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+fungi

Tukey HSD (Total Biomass)

fungi	N	Subset 1	Subset 2
3	21	.1000	
1	21	.1143	
6	6	.1400	
4	19		.1916
5	21		.1929
Sig.		.196	1.000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .002.
a Uses Harmonic Mean Sample Size = 13.806.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Experiment 2

Levene's Test of Equality of Error Variances(a)

Dependent Variable: Height Transformed (.18)

F	df1	df2	Sig.
1.364	5	78	.247

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+fert+meth+fungi+fert * fungi+meth * fungi

Tukey HSD(a,b) (Height)

fert_meth	N	Subset for alpha = .05 1	2	3	4
2	21	2.6619			
1	21	3.4143			
4	7		11.5571		
3	7			16.4	
6	14			17.1214	17.1214
5	14				21.1429
Sig.		0.997	1	0.997	0.126

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 11.455.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
Levene's Test of Equality of Error Variances(a)

Dependent Variable: RCD

F	df1	df2	Sig.
10.123	5	78	.000

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+meth+fert+fungi+meth * fungi+fert * fung

Tukey HSD (a,b) (RCD)

		Subset for alpha = .05	
fert_meth	N	1	2
2	21	1.3129	
1	21	1.589	
4	7		2.8186
3	7		2.9586
6	14		2.9986
5	14		3.2357
Sig.		0.405	0.055

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 11.455.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
Levene's Test of Equality of Error Variances(a)

Dependent Variable: Aboveground Biomass Transformed (-.08)

F	df1	df2	Sig.
1.522	5	78	.193

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+fert+meth+fungi+fert * fungi+meth * fungi

Tukey HSD(a,b) (Aboveground Biomass)

		Subset for alpha = .05			
fert_meth	N	1	2	3	4
2	21	0.0267			
1	21	0.0533			
4	7		0.3557		
3	7		0.52	0.52	
6	14			0.645	0.645
5	14				0.8086
Sig.		1	0.477	0.749	0.482

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 11.455.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Levene's Test of Equality of Error Variances(a)

Dependent Variable: Belowground Biomass

F	df1	df2	Sig.
13.418	5	78	.000

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+meth+fert+fungi+meth * fungi+fert * fungi

Tukey HSD(a,b) (Belowground Biomass)

		Subset for alpha = .05		
fert_meth	N	1	2	3
2	21	0.0733		
1	21	0.1395		
4	7		0.5929	
3	7		0.7471	
6	14		0.8807	0.8807
5	14			1.1043
Sig.		0.987	0.067	0.26

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 11.455.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Levene's Test of Equality of Error Variances(a)

Dependent Variable: Total Biomass Transformed (-.15)

F	df1	df2	Sig.
2.045	5	78	.081

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+fert+meth+fungi+fert * fungi+meth * fungi

Tukey HSD(a,b) (Total Biomass)

			Subset for alpha = .05		
	fert_meth	N	1	2	3
	2	21	0.1		
	1	21	0.1929		
	4	7		1.0271	
	3	7		1.2671	
	6	14		1.4357	1.4357
	5	14			1.8414
	Sig.		0.997	0.294	0.302

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 11.455.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Experiment 3

Levene's Test of Equality of Error Variances(a)

	F	df1	df2	Sig.
sqrtht	1.585	8	126	.136
RCD	2.131	8	126	.037
above_tran	1.685	8	126	.108
invbelow	.904	8	126	.516
invsqrtot	1.261	8	126	.270

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+fungi * fert+fungi+fert

Levene's Test of Equality of Error Variances(a)

Dependent Variable: Height Transformed (square root)

F	df1	df2	Sig.
1.585	8	126	.136

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+fungi * fert+fungi+fer

Tukey HSD (Height)

		Subset for alpha = .05		
fungi_fert	N	1	2	3
2	16	2.0975		
3	16	2.2146		
1	16		3.1746	
6	16			5.0663
5	15			5.1067
4	15			5.2495
9	14			5.4316
8	14			5.4896
7	13			5.5110
Sig.		.999	1.000	.205

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 14.923.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Levene's Test of Equality of Error Variances(a)

Dependent Variable: Root Collar Diameter

F	df1	df2	Sig.
2.131	8	126	.037

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+fungi * fert+fungi+fert

Tukey HSD (Root Collar Diameter)

fungi_fert	N	Subset 1	2	3
2	16	1.4963		
3	16	1.6013		
1	16		2.0106	
4	15			3.0707
6	16			3.1838
7	13			3.2123
9	14			3.2329
8	14			3.3914
5	15			3.3987
Sig.		.995	1.000	.189

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .117.
a Uses Harmonic Mean Sample Size = 14.923.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Levene's Test of Equality of Error Variances(a)

Dependent Variable: above_tran

F	df1	df2	Sig.
1.685	8	126	.108

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+fungi * fert+fungi+fert

Tukey HSD (Aboveground Biomass)

fungi_fert	N	Subset 1	2	3
8	14	.9987		
7	13	.9992		
5	15	1.0008		
9	14	1.0024		
4	15	1.0087		
6	16	1.0092		
1	16		1.0418	
3	16			1.0695
2	16			1.0708
Sig.		.159	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .000.
a Uses Harmonic Mean Sample Size = 14.923.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05

Levene's Test of Equality of Error Variances(a)

Dependent Variable: Belowground Biomass Transformed (inverse)

F	df1	df2	Sig.
.904	8	126	.516

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+fungi * fert+fungi+fert

Tukey HSD (Belowground Biomass)

		Subset	
fungi_fert	N	1	2
6	16	3.0179	
5	15	3.1605	
7	13	3.2852	
4	15	3.3050	
8	14	3.6351	
9	14	3.8344	
1	16	3.9585	
3	16		6.7812
2	16		7.2306
Sig.		.876	.999

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = 3.145.
a Uses Harmonic Mean Sample Size = 14.923.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Levene's Test of Equality of Error Variances(a)

Dependent Variable: Total Biomass Transformed (inverse square root)

F	df1	df2	Sig.
1.261	8	126	.270

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+fungi+fert+fungi *

Tukey HSD (Total Biomass)

fungi_fert	N	Subset 1	Subset 2	Subset 3
7	13	.8661		
5	15	.8722		
8	14	.8804		
9	14	.9190		
4	15	.9683		
6	16	.9958		
1	16		1.4174	
3	16			1.9767
2	16			2.0252
Sig.		.764	1.000	.999

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .045.
a Uses Harmonic Mean Sample Size = 14.923.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Chapter 4 exp 1

Levene's Test of Equality of Error Variances(a)

Dependent Variable: height_tran

F	df1	df2	Sig.
1.246	24	229	.204

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+site * meth * funig+site * funig+meth * funig+site+meth+funig

Tukey HSD (Height)

site_meth_fungi	N	Subset									
		1	2	3	4	5	6	7	8	9	10
21	11	1.3302									
25	12	1.5563	1.5563								
24	12	1.6315	1.6315	1.6315							
22	11	1.8087	1.8087	1.8087	1.8087						
15	12	1.9404	1.9404	1.9404	1.9404						
23	7	1.9764	1.9764	1.9764	1.9764						
14	11		2.1075	2.1075	2.1075	2.1075					
17	5		2.1125	2.1125	2.1125	2.1125					
16	6		2.1357	2.1357	2.1357	2.1357	2.1357				
18	12			2.2140	2.2140	2.2140	2.2140	2.2140			
19	12			2.2753	2.2753	2.2753	2.2753	2.2753	2.2753		
3	12				2.3097	2.3097	2.3097	2.3097	2.3097		
20	12				2.4540	2.4540	2.4540	2.4540	2.4540	2.4540	
1	12					2.6880	2.6880	2.6880	2.6880	2.6880	2.6880
8	12					2.6885	2.6885	2.6885	2.6885	2.6885	2.6885
10	5					2.7279	2.7279	2.7279	2.7279	2.7279	2.7279
4	11					2.7429	2.7429	2.7429	2.7429	2.7429	2.7429
2	12						2.7609	2.7609	2.7609	2.7609	2.7609
12	11							2.7911	2.7911	2.7911	2.7911
6	9								2.8891	2.8891	2.8891
5	8									2.9794	2.9794
9	6									3.0345	3.0345
13	11										3.1692
7	12										3.2137
11	10										3.2472
Sig.		.051	.154	.053	.052	.062	.074	.159	.090	.152	.205

Means for groups in homogeneous subsets are displayed.

Based on Type III Sum of Squares

The error term is Mean Square(Error) = .143.

a Uses Harmonic Mean Sample Size = 9.348.

b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c Alpha = .05.

Levene's Test of Equality of Error Variances(a)

Dependent Variable: Root Collar Diameter

F	df1	df2	Sig.
1.382	24	229	.116

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a Design: Intercept+site * meth * funig+site * funig+meth * funig+site+meth+funig

Tukey HSD (Root Collar Diameter)

site_meth_fungi	N	Subset 1	2	3	4	5	6	7	8	9
21	11	.7218								
24	12	.9742	.9742							
25	12	1.0783	1.0783	1.0783						
23	7	1.1486	1.1486	1.1486	1.1486					
22	11		1.2600	1.2600	1.2600	1.2600				
17	5		1.2960	1.2960	1.2960	1.2960				
19	12		1.4367	1.4367	1.4367	1.4367	1.4367			
15	12		1.4667	1.4667	1.4667	1.4667	1.4667	1.4667		
14	11			1.4809	1.4809	1.4809	1.4809	1.4809		
16	6				1.5800	1.5800	1.5800	1.5800	1.5800	
10	5				1.5860	1.5860	1.5860	1.5860	1.5860	
18	12				1.6342	1.6342	1.6342	1.6342	1.6342	
20	12				1.6392	1.6392	1.6392	1.6392	1.6392	
9	6					1.7083	1.7083	1.7083	1.7083	1.7083
1	12					1.7233	1.7233	1.7233	1.7233	1.7233
4	11					1.7245	1.7245	1.7245	1.7245	1.7245
3	12					1.7308	1.7308	1.7308	1.7308	1.7308
8	12					1.7350	1.7350	1.7350	1.7350	1.7350
12	11						1.8209	1.8209	1.8209	1.8209
2	12						1.8450	1.8450	1.8450	1.8450
5	8						1.9213	1.9213	1.9213	1.9213
13	11							1.9600	1.9600	1.9600
7	12								2.0042	2.0042
6	9								2.0367	2.0367
11	10									2.2050
Sig.		.221	.058	.326	.061	.086	.069	.057	.126	.053

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .085.
a Uses Harmonic Mean Sample Size = 9.348.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.Levene's Test of Equality of Error Variances(a)

Dependent Variable: above_tran

F	df1	df2	Sig.
1.578	24	229	.047

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+site * meth * funig+site * funig+meth * funig+site+meth+funig

Tukey HSD (aboveground biomass; transformed)

site_meth_fungi	N	Subset 1	2	3	4	5	6	7	8	9	10
21	11	.2779									
24	12	.2846									
25	12	.3399	.3399								
22	11	.3943	.3943	.3943							
23	7		.4103	.4103	.4103						
15	12		.4147	.4147	.4147	.4147					
17	5		.4187	.4187	.4187	.4187	.4187				
19	12		.4409	.4409	.4409	.4409	.4409	.4409			
14	11			.4579	.4579	.4579	.4579	.4579	.4579		
20	12			.4665	.4665	.4665	.4665	.4665	.4665		
16	6			.4696	.4696	.4696	.4696	.4696	.4696		
18	12			.4860	.4860	.4860	.4860	.4860	.4860	.4860	
3	12			.4862	.4862	.4862	.4862	.4862	.4862	.4862	
12	11				.5220	.5220	.5220	.5220	.5220	.5220	.5220
10	5				.5229	.5229	.5229	.5229	.5229	.5229	.5229
1	12				.5255	.5255	.5255	.5255	.5255	.5255	.5255
8	12					.5300	.5300	.5300	.5300	.5300	.5300
9	6						.5331	.5331	.5331	.5331	.5331
4	11							.5402	.5402	.5402	.5402
5	8								.5647	.5647	.5647
2	12								.5668	.5668	.5668
6	9									.5928	.5928
13	11										.6095
7	12										.6160
11	10										.6361
Sig.		.050	.198	.372	.057	.056	.061	.227	.102	.123	.063

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .005.
a Uses Harmonic Mean Sample Size = 9.348.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Levene's Test of Equality of Error Variances(a)

Dependent Variable: below_tran

F	df1	df2	Sig.
1.122	24	229	.321

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+site * meth * funig+site * funig+meth * funig+site+meth+funig

Tukey HSD (Belowground Biomass)

site_meth_fungi	N	Subset 1	2	3	4	5	6	7	8
24	12	-1.9101							
21	11	-1.8745							
25	12	-1.5552	-1.5552						
22	11		-1.3308	-1.3308					
23	7		-1.3073	-1.3073	-1.3073				
17	5		-1.2555	-1.2555	-1.2555	-1.2555			
19	12		-1.1744	-1.1744	-1.1744	-1.1744	-1.1744		
16	6		-1.1082	-1.1082	-1.1082	-1.1082	-1.1082	-1.1082	
14	11			-1.0790	-1.0790	-1.0790	-1.0790	-1.0790	
15	12			-1.0214	-1.0214	-1.0214	-1.0214	-1.0214	-1.0214
20	12			-.9959	-.9959	-.9959	-.9959	-.9959	-.9959
12	11			-.9931	-.9931	-.9931	-.9931	-.9931	-.9931
10	5			-.9835	-.9835	-.9835	-.9835	-.9835	-.9835
1	12			-.9463	-.9463	-.9463	-.9463	-.9463	-.9463
18	12			-.9358	-.9358	-.9358	-.9358	-.9358	-.9358
4	11			-.9114	-.9114	-.9114	-.9114	-.9114	-.9114
3	12			-.8812	-.8812	-.8812	-.8812	-.8812	-.8812
8	12				-.8574	-.8574	-.8574	-.8574	-.8574
5	8				-.8549	-.8549	-.8549	-.8549	-.8549
9	6					-.8299	-.8299	-.8299	-.8299
2	12					-.8250	-.8250	-.8250	-.8250
7	12						-.7900	-.7900	-.7900
6	9						-.7237	-.7237	-.7237
13	11							-.6748	-.6748
11	10								-.5634
Sig.		.439	.077	.072	.067	.112	.070	.105	.059

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .074.

a Uses Harmonic Mean Sample Size = 9.348.

b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c Alpha = .05

Levene's Test of Equality of Error Variances(a)

Dependent Variable: total_tran

F	df1	df2	Sig.
1.429	24	229	.095

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+site * meth * funig+site * funig+meth * funig+site+meth+funig

Tukey HSD (Total Biomass)

site_meth_fungi	N	Subset								
		1	2	3	4	5	6	7	8	9
21	11	-1.5512								
24	12	-1.5461								
25	12	-1.2294	-1.2294							
22	11		-1.0041	-1.0041						
23	7		-.9638	-.9638	-.9638					
17	5		-.9394	-.9394	-.9394	-.9394				
19	12		-.8521	-.8521	-.8521	-.8521	-.8521			
15	12			-.8113	-.8113	-.8113	-.8113			
14	11			-.7778	-.7778	-.7778	-.7778	-.7778		
16	6			-.7736	-.7736	-.7736	-.7736	-.7736		
20	12			-.7275	-.7275	-.7275	-.7275	-.7275	-.7275	
12	11			-.6665	-.6665	-.6665	-.6665	-.6665	-.6665	-.6665
18	12			-.6593	-.6593	-.6593	-.6593	-.6593	-.6593	-.6593
10	5			-.6519	-.6519	-.6519	-.6519	-.6519	-.6519	-.6519
1	12				-.5949	-.5949	-.5949	-.5949	-.5949	-.5949
3	12				-.5942	-.5942	-.5942	-.5942	-.5942	-.5942
4	11				-.5833	-.5833	-.5833	-.5833	-.5833	-.5833
8	12					-.5612	-.5612	-.5612	-.5612	-.5612
9	6						-.5470	-.5470	-.5470	-.5470
5	8						-.5182	-.5182	-.5182	-.5182
2	12						-.5021	-.5021	-.5021	-.5021
7	12							-.4269	-.4269	-.4269
6	9							-.4187	-.4187	-.4187
13	11								-.3844	-.3844
11	10									-.2896
Sig.		.256	.061	.124	.056	.060	.131	.103	.157	.062

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .050.
a Uses Harmonic Mean Sample Size = 9.348.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

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Levene's Test of Equality of Error Variances(a)

Dependent Variable: height_tran_a

F	df1	df2	Sig.
1.152	11	117	.328

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+fungi+site

Tukey HSD (Height)

		Subset				
fungi_site	N	1	2	3	4	5
10	10	1.7543				
8	10	1.8004				
7	16	1.8784	1.8784			
12	10	1.8931	1.8931			
11	10	1.8979	1.8979			
9	10	1.9792	1.9792	1.9792		
4	11	2.2464	2.2464	2.2464	2.2464	
2	10		2.4162	2.4162	2.4162	2.4162
1	15		2.4214	2.4214	2.4214	2.4214
3	8			2.5380	2.5380	2.5380
6	9				2.5966	2.5966
5	10					2.8633
Sig.		.155	.073	.056	.649	.273

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .149.
a Uses Harmonic Mean Sample Size = 10.379.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Levene's Test of Equality of Error Variances(a)

Dependent Variable: Root Collar Diameter

F	df1	df2	Sig.
1.664	11	117	.090

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+fungi+site

Tukey HSD (Root Collar Diameter)

fungi_site	N	Subset 1	2	3	4	5
10	10	1.1920				
12	10	1.1920				
8	10	1.2230	1.2230			
7	16	1.3206	1.3206	1.3206		
11	10	1.3470	1.3470	1.3470	1.3470	
9	10	1.3740	1.3740	1.3740	1.3740	
3	8	1.5300	1.5300	1.5300	1.5300	1.5300
4	11		1.5518	1.5518	1.5518	1.5518
1	15			1.5940	1.5940	1.5940
2	10				1.6790	1.6790
6	9				1.6889	1.6889
5	10					1.8590
Sig.		.076	.095	.305	.069	.095

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .058.
a Uses Harmonic Mean Sample Size = 10.379.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Levene's Test of Equality of Error Variances(a)

Dependent Variable: above_tran_a

F	df1	df2	Sig.
1.533	11	117	.129

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+fungi+site

Tukey HSD (Aboveground Biomass)

		Subset	
fungi_site	N	1	2
5	10	2.2811	
6	9	2.7538	2.7538
3	8	2.9575	2.9575
1	15	3.1088	3.1088
2	10	3.2626	3.2626
4	11	3.2960	3.2960
10	10		3.8889
9	10		3.9364
8	10		3.9608
11	10		3.9649
7	16		4.0024
12	10		4.1472
Sig.		.397	.050

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .908.
a Uses Harmonic Mean Sample Size = 10.379.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Levene's Test of Equality of Error Variances(a)

Dependent Variable: below_tran_a

F	df1	df2	Sig.
1.230	11	117	.275

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+fungi+site

Tukey HSD (Belowground Biomass)

fungi_site	N	Subset 1	2	3	4
11	10	.2381			
10	10	.2393			
12	10	.2462	.2462		
8	10	.2475	.2475		
9	10	.2523	.2523		
7	16	.2548	.2548		
3	8		.3576	.3576	
6	9			.3839	
1	15			.3873	
2	10			.3907	
4	11			.4092	.4092
5	10				.5222
Sig.		1.000	.066	.938	.058

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .006.
a Uses Harmonic Mean Sample Size = 10.379.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Levene's Test of Equality of Error Variances(a)

Dependent Variable: total_tran_a

F	df1	df2	Sig.
1.681	11	117	.086

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+fungi+site

Tukey HSD (Total Biomass)

fungi_site	N	Subset 1	2	3	4	5
8	10	.3465				
12	10	.3532	.3532			
11	10	.3576	.3576			
7	16	.3701	.3701			
9	10	.3738	.3738	.3738		
10	10	.3765	.3765	.3765		
3	8		.5001	.5001	.5001	
4	11			.5190	.5190	
2	10				.5269	
1	15				.5272	
6	9				.5395	
5	10					.6950
Sig.		1.000	.056	.063	.999	1.000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .010.

a Uses Harmonic Mean Sample Size = 10.379.

b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c Alpha = .05.

Plant Toxicity Table

Critical Concentration of some Trace Metals in Plant Tissues (from Kabata-Pendias, 2001)

Metal	In Sensitive Plant Species	For 10% Yield Loss
As		1-20
Cd		10-20
Cu	15-20	10-30
Ni	20-30	10-30
Zn	150-200	100-500