Trace Metal Effects on Ectomycorrhizal Growth, Diversity, and Colonization of Host Seedlings

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(ABSTRACT)

Ectomycorrhizal fungi are essential to seedling establishment in disturbed sites. This dissertation examines the effects of trace metals and soil disturbance on ectomycorrhizal fungi in the laboratory and the field. The first experiment assessed Cu and Zn impact on growth of three ectomycorrhizal species in pure culture. *Suillus granulatus* and *Pisolithus tinctorius* were more tolerant to Cu than *Paxillus involutus*, however, none of the species grew at ≥ 250 ppm Cu. *Suillus granulatus* had the highest Zn tolerance, followed by *Paxillus involutus* and *Pisolithus tinctorius*. Sectoring observed in *Suillus granulatus* was deemed spontaneous and not involved in metal tolerance.

The second experiment examined the adsorption of copper and zinc to acidic Uchee fine loamy sand. Contrary to expectations, the soil adsorbed up to 667 ppm Cu and 238 ppm Zn. Adsorption occurred mainly in the non-crystalline fraction of the soil. This analysis is a new approach in mycorrhizal research, and the crucial need for such tactics is discussed.

The third experiment surveyed ectomycorrhizae on a mine reclamation project in Wise County, Virginia. *Pinus strobus* trees planted 1, 8, 13, and 25 years prior to the experiment were sampled. Colonization was lower than in well developed soils, but occurred on all seedlings. Increased colonization and a late stage mycobiont (*Tuber*) occurred on roots taken from the 25 year old subsite. A new observation was made of *Suillus americanus* on one year old seedlings. Lack of species overlap among sites suggests localized inoculum sources.

The last experiment explored *Pinus strobus* and *Pinus virginiana* seedlings naturally regenerating on acidic, bare-mineral soil exposed by a road cut in Floyd County, Virginia.

Ectomycorrhizal colonization ranged between 30 to 80 percent. Wide variation among individual samples suggests patchy inoculum distribution. *Scleroderma citrinum,* a common early-stage fungus, was dominant throughout. Other "early stage" genera included *Rhizopogon, Pisolithus,* and *Thelephora.* Mid to late stage genera including *Suillus* and *Lactarius* were identified. *Cenococcum*, often a dominant taxon, was a minor taxon here. The unusual presence of the ericoid mycobionts *Hymenoscyphus* and *Oidiodendron* is discussed. These results suggest that native inoculum can be an important resource for seedling recruitment.

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Chapter 1

Introduction and Literature Review

Statement of Research Problem

The symbiotic relationship between ectomycorrhizal fungi and the roots of certain types of forest trees is important to members of several plant families, including the Betulaceae, Fagaceae, and Pinaceae. Ectomycorrhizal fungi are a diverse group of organisms, with species belonging to several families found mainly in the phyla Basidiomycota and Ascomycota. In ectomycorrhizal symbioses, the fungus forms a thick sheath of hyphae, called the mantle, around the rootlets of the host plant. The fungal hyphae will also penetrate between the cortical cells of the roots, forming a nutrient-transfer network called the Hartig net. The fungus provides mineral nutrients, most importantly nitrogen and phosphorus, to the plant, and improves plant water uptake in low water environments. In turn, the plant provides the fungal symbiont with vital carbohydrates such as glucose and important vitamins such as biotin and thiamine. This relationship often determines whether or not a seedling will survive in its given environment. Plants inoculated with ectomycorrhizal fungi have greater biomass, more rootlet development, and higher P and N uptake than the uninoculated controls (Smith and Read, 1997).

The ectomycorrhizal symbiosis is often most dramatically beneficial in nutrient poor environments (Del Vecchio et al., 1993). Ectomycorrhizal symbioses allow plants to increase nutrient uptake and exploit nutrient pools that would be unavailable to a non-mycorrhizal plant, allowing the plant to survive in an area where it would not gain the necessary amount of nutrients for survival on its own (Smith and Read, 1997). The fungus can also assist with water uptake in times of drought (Smith and Read, 1997). Other benefits include increased carbon

transfer between plants (Simard et al., 1997) and pathogen resistance (Smith and Read, 1997). Also, ectomycorrhizal fungi are able to protect plants against toxic elements in the environment, including trace metals. This particular feature of the relationship is important for enhancing seedling establishment on metal-polluted sites, such as the mine spoils of the eastern United States (Schramm, 1966). The mechanism and extent of protection varies with the type of host-fungus system as well as the metal pollutants present in the soil.

Many ectomycorrhizal fungi are tolerant of higher metal concentrations than their host plant species. Additionally, previous studies have demonstrated that ectomycorrhizal colonization of seedlings can ameliorate metal phytotoxicity (Leyval et al., 1997). However, the mechanism of fungal protection from metal phytotoxicity is unknown. Several studies suggest that the fungi bind the metal elements in their hyphae and prevent them from entering the plant host (Gruhn, 1989; Howe, 1997; Tam, 1995). In other cases, ectomycorrhizal colonization has no effect on plant metal levels and may even increase the amount of foliar metals compared to the control (Colpaert and VanAssche 1992). Another possibility is that the mycorrhizal relationship improves the overall health of the plant, enabling it to withstand the stress of increased metal concentration in the soil. The first part of this dissertation will examine these possibilities through examination of three species of ectomycorrhizal fungi in axenic culture and in a controlled symbiosis with a host plant.

Fungi determined to be the most tolerant of trace metals in pure culture do not necessarily provide the best protection to a plant host when inoculated into contaminated soils. The relationship between the fungus and the plant (i.e., the ability of the fungus to infect and colonize a host plant) is likely more important in this case than the ability of the fungus to withstand a high threshold level of metal contamination. This dissertation will examine the

implications of fungal-host relationships: first, by establishing a baseline of tolerance to two trace metals, Cu and Zn, in three fungal species. Additionally, two field studies were performed to determine the diversity and colonization ability of ectomycorrhizal fungi naturally occurring in stressed sites.

Literature Review

Occurrence of trace metals in soils

Trace metals occur in soils either naturally or as a result of both air pollution and direct pollution of the ground by geologic disturbance or application of waste residues (Kelly et al., 1979; Tam, 1995). Naturally occurring trace metals are not normally present in toxic concentrations; however, phytotoxic levels are common in polluted areas. Soil pollution is widespread in North America, and results from several anthropogenic sources including mining and smelting activities, industrial emissions, automobile emissions, and emissions from municipal utilities (Jentschke et al., 1998). Some of the most severe pollution has resulted from mining activities, where high metal concentrations in the soil eliminate non-tolerant plant populations (Freedman and Hutchinson, 1981). A comparison of naturally occurring metal levels to levels found near mining and industrial areas illustrates the dramatic rise in pollution on these sites. The level of total Cu in a typical uncontaminated area is between 2-100 mg/kg, while normal total Zn soil levels range from 25 to 200 mg/kg (Bowen, 1966; Bowie and Thornton, 1985). Studies of mine spoils in Canada and of mining and industrial areas in Europe found soil Cu levels ranging from 90-2700 mg/kg and Zn levels from 200-27,600 mg/kg dry weight (Gregory and Bradshaw, 1965; Huttermann et al., 1999). As these metals accumulate in the soil, the effects of this pollution can severely inhibit plant and fungal growth (Dixon, 1988; Jentschke et al., 1998). Studies of the behavior of metals in forest rooting zones indicate that while Zn

only accumulated slightly in humus layers, it did so significantly in fine roots. Cu, on the other hand, was found to behave similarly to lead, which accumulates in both the humus soil layer and in fine roots (Lamersdorf, 1989, and sources within). As metals accumulate in soil, even low pollution rates can lead to phytotoxicity over time (Arudini et al., 1994). Long term ecosystem effects are likely unless some remediation method proves successful.

Inhibitory effects of Cu and Zn on higher plants

Although many species of higher plants have evolved mechanisms for tolerance of increased levels of heavy metals, significant pollution levels such as above reported can cause seedling mortality and therefore hinder revegetation efforts on contaminated sites (Ritchie and Thingvold, 1985; Thurman, 1981). Many factors can influence a plant's response to metal contamination, including concentration of metals in a given area, types of metals in the area, enzymatic adaptations, soil nutrient content, soil type, and ectomycorrhizal colonization (Dixon and Buschena,1988; Marschner et al., 1998; Thurman, 1981). Soil acidity is a major factor affecting plant response to metal pollution, as metal solubility and bioavailability are usually higher in acidic soils (Strock, 2002).

Cu and Zn are micronutrients, and therefore essential to plant cell processes. However, excessive concentrations are toxic to plants. Cu and Zn root damage damage occurs primarily as changes in morphology and reduction in root length, number, and biomass (Arudini et al., 1998; Barcelo and Poschenreider, 1990; Denny and Wilkins, 1987a; Ouzounidou, 1994;). Root growth reduction has been reported to be as high as 73% in the case of high Cu contamination (Ouzounidou, 1994). Additionally, increased Cu and Zn concentrations result in reduced root hair density and cortical cell damage (Barcelo and Poschenreider, 1990; Patterson and Olson, 1983). Roots of plants exposed to elevated levels of Cu and Zn usually appear brown due to

possible lignification of the root cells, and Zn also stiumulates suberization of the roots (Barcelo and Poschenreider, 1990). Root damage from Cu and Zn results in compromised water relations for plants as well as a reduction in nutrient uptake due to reduced membrane potential of the root cells (Barcelo and Poschenreider, 1990; Kennedy and Gonsalves, 1987). These effects are most severe in seedlings. Germination of *Betula* and *Pinus* seeds in the presence of high Cu levels showed a reduction in radicle elongation of up to 25%, which could prevent seedling establishment on contaminated sites (Patterson and Olson, 1983). In plants that do persist past the radicle stage, root damage and the resulting decline in nutrient and water uptake result in decreased overall plant health. Symptoms of plant decline include stunted growth, reduced biomass, leaf chlorosis and necrosis, and premature senescence (Arundini et al., 1998; Barcelo and Poschenreider, 1990; Ouzounidou, 1994). Soil Cu concentrations of 30 mg/kg have been shown to severely stunt growth of *Pinus densiflora* Sieb. ex. Zucc. seedlings, even if the seedlings were inoculated with protective mycorrhizal fungi (Gruhn, 1989). Copper concentrations in the range of 10-50 mg/kg reduced both root and shoot weight of *Pinus banksiana* Lamb. and *Picea glauca* (Moench) Voss (Dixon and Buschena, 1988). Additionally, susceptibility to attack by fungal pathogens is greater for plants in metal-contaminated soils (Patterson & Olson, 1983). The cause of this increased susceptibility has not been experimentally determined, but possible causes include higher tolerance of pathogenic fungal species to metals or easier infection via metal-induced damage to root cells. Although the extent of phytotoxicity will vary with the plant species involved and the surrounding environment, Cu and Zn are micronutrients that, when present in excess concentrations, can create significant damage to most plant species.

Fungal response to Cu and Zn—inhibition and tolerance

As many as 5000-6000 species of fungi are currently known to be ectomycorrhizal (Bentivenga, 1998). This variety means that one cannot assume that all ectomycorrhizal fungi will react in the same way to a given trace metal load. Most ectomycorrhizal fungi exhibit a higher tolerance for trace metals than their host plant species, and many are able to decrease trace metal toxicity in contaminated soil (Dixon, 1988; Jones and Hutchinson, 1988; Marschner et al., 1996). However, the threshold of fungal metal tolerance and the ability to ameliorate phytotoxicity vary among fungal species in both the lab and field. These responses are dependent on the species of fungi present, the metals present in the environment, other organisms present in the soil, and abiotic factors such as soil pH, organic matter, and mineral composition (Blaudez et al., 2000; Gruhn, 1989; Gussarson et al., 1995; Hartley et al., 1997; Marschner et al., 1996).

Variation in fungal response to trace metals is likely due to intrinsic physiological factors. For most species of fungi, sensitivity to one or more types of metals does not confer sensitivity to all trace metals (Jones and Muehlchen 1994; McCreight and Schroeder, 1982; Tam, 1995). In one study, *Laccaria laccata* (Scop. ex Fr.) Bk & Broome was inhibited by Cu and aluminum concentrations of just 10 parts per million (ppm), but was not sensitive to the same concentrations of Zn. Although most species are inhibited by relatively small increases in trace metal concentrations, one ectomycorrhizal fungus, *Thelephora terrestris* Pers. ex Fr., was tolerant of Cu up to 500 ppm, aluminum up to 100 ppm, and Zn up to 1000 ppm (Jones and Muehlchen, 1994). Hartley et al. (1997) demonstrated that the interaction of two metal cations—Zn, an essential micronutrient, and Cd, a nonessential element, could alter the fungal response compared to a similar dose of a single element. In this case, Zn appeared to ameliorate cadmium

toxicity. The researchers speculated that fungal cells may have a higher affinity for Zn as it is an essential element for fungal nutrition. In other cases, it has been suggested that fungi that have a higher growth rate can accumulate more metals within their hyphae while maintaining a nontoxic overall concentration (Colpaert and VanAssche, 1987). These studies collectively demonstrate that fungal response to trace metals is dependent not only on the taxa present in the system, but alos the type and number of metals present.

Although Cu is an essential element for fungal cell development, even relatively slight elevations in Cu concentration will have a toxic effect on most ectomycorrhizal fungi (Gruhn, 1989). Studies with axenic cultures of ectomycorrhizal species have shown that excessive amounts of Cu can cause a decrease in mycelial dry weight and in the diameter of mycelial growth on artificial media (Gruhn and Miller, 1989; Jones and Hutchinson, 1988; Jones and Muehlchen, 1994). Some species will exhibit a dark pigmentation in culture as a result of Cu stress (Gruhn and Miller, 1991). Other symptoms of Cu toxicity include swollen mycorrhizal tips (Jones and Muehlchen, 1994; Tam, 1995), distorted hyphal development, and secretion of an extracellular "slime" (Gruhn, 1989; Tam, 1995). This "slime" has been hypothesized to be a mechanism of tolerance for the fungus, but has not been characterized, and may be a symptom of general physiological distress.

Many fungi are quite tolerant to Cu, and often will survive and enhance plant growth in Cu-amended media (Gruhn, 1989; Jones and Hutchinson, 1986; Tam, 1995). Several possible mechanisms of tolerance have been investigated in past studies and may be useful in explaining the variation in response to Cu among species. For example, some species of ectomycorrhizal fungi have been shown to sequester Cu in different locations in the hyphal cell wall (Gruhn, 1989). Several other studies have shown that different species of ectomycorrhizal fungi have

different growth responses when grown in metal-amended media, whether artificial or soil-based (Hartley et al., 1997; Jones and Muehlchen, 1994; Leyval et al., 1997; McCreight and Schroeder, 1982; Tam, 1995). As with Cu, variation in response was seen with other metals, including Cd, Pb, and Zn.

Since a relationship has been shown by Gruhn (1989) between sequestration method and fungal response to Cu, it is therefore possible that differences in response to any given metal are based on ability to sequester it within the hyphal cell walls. One proposed way in which Cu binds in the cell wall was through the formation of polyphosphate granules, visualized by transmission electron microscopy (Gruhn, 1989). While Galli (1994) discusses a subsequent study that indicates that ectomycorrhizal polyphosphate granules appeared as an artifact of specimen preparation, other studies support the contention of Gruhn (1989) that phosperus does play a role in metal tolerance. One such study was done by Jones and Hutchinson (1988), who found that high mycelial phosphorus concentrations were correlated with the ability to take up high levels of metals. The nature of this correlation remains to be defined. Another study suggests that binding of metals occurs by production of small proteins called metallothioneins. These Cu-binding proteins were found in some strains of fungi isolated from contaminated sites, although there was no strong correlation between Cu tolerance and whether the fungi were isolated from contaminated or non-contaminated sites (Howe et al., 1997). In yeast, greater Cu tolerance occurred with the presence of multiple copies of Cu-binding metallothionein genes (Han et al., 1992). The presence of metallothioneins in ectomycorrhizal fungi suggest that Cu-binding proteins play a role in response to Cu exposure and may relate to Cu tolerance in the fungi; however, Howe et al. (1997) were not able to detect binding proteins in all tolerant strains of fungi.

Zn, also a micronutrient essential for cellular processes, appears to be one of the least fungitoxic metals because some fungi tolerate zinc at concentrations extending up to 1000 ppm (Jones and Muehlchen, 1994). Again, there exists a variation in Zn tolerance among ectomycorrhizal species. One study showed that *Paxillus involutus* (Batsch ex. Fr.) Fr. was sensitive to very low levels (25 ppm) of Zn in the media, whereas other species were not inhibited at concentrations up to 225 ppm Zn (Blaudez et al., 2000). Symptoms of Zn toxicity in fungi are primarily described as a reduction in mycelial growth (Blaudez et al., 2000; Colpaert et al., 2000; Denny and Ridge, 1995; Hartley et al., 1997) or as a reduction in ability to colonize a host plant (Hartley et al., 1999a; Hartley-Whitaker et al., 2000a; Hartley-Whitaker et al., 2000b). The reasons for higher fungal tolerance of Zn than other trace elements have not been definitively elucidated. Research indicates that, in ectomycorrhizal fungi, Zn is bound in the extrametrical mycelium (Colpaert and VanAssche, 1992; Denny and Wilkins, 1987c). X-ray microanalysis suggested that Zn is bound to electronegative sites on the cell wall and never enters the fungal cytoplasm, thus minimizing toxic effects to the fungus (Denny and Wilkins, 1987c). Furthermore, a polysaccharide "slime" is produced in fungi exposed to toxic levels of Zn. In one study, the amount of this "slime" produced by a fungus was directly correlated to its ability to take up Zn from its surroundings (Denny and Ridge, 1995). Staining reactions with *Pisolithus tinctorius* (Mich.:Pers) Coker and Couch indicated that Zn may also be bound by sulfhydryl compounds, suggesting that metallothionein-like proteins are involved in fungal response to Zn (Morselt et al., 1986).

Variation in both interspecific and intraspecific response to trace metals has led to interest in whether or not an adaptive mechanism of tolerance exists among ectomycorrhizal fungi. Several plant species from metal-enriched sites have shown an increased tolerance to the

metals present in the original site (Baker, 1987). Many of these plant species are ectomycorrhizal, leading to speculation that fungal adaptation also exists (Leyval et al., 1997). The possibility of some sort of microevolutionary adaptation in ectomycorrhizal fungi is still under question, as several studies have investigated and found no difference in tolerance between isolates from contaminated versus uncontaminated soil (Blaudez, et al., 2000; Brown and Wilkins, 1985; Denny and Wilkins, 1987b; Howe, et al., 1997; Jones and Hutchinson, 1986). However, there is some evidence to suggest that fungal adaptation does occur on contaminated sites. Bucking and Heyser (1994) found that growing *Suillus bovinus* (Fr.) Kuntze on a medium previous enriched with Zn improved its ability to reduce shoot concentrations of Zn in *Pinus sylvestris* L. compared to isolates not pre-exposed to Zn. *In vitro* tests with several isolates of *Suillus luteus* (L: Fr.) Roussel showed that isolates collected from a industrial site polluted with metals from a Zn smelter exhibited a higher tolerance for Zn and cadmium than isolates from nonpolluted control site. These isolates did not show any increased tolerance for metals not present in high quantities on the polluted site, such as Cu. Genetic analysis of the isolates showed a clear population distinction between the isolates from the polluted and nonpolluted sites (Colpaert et al., 2000). A subsequent study with isolates of *Suillus bovinus* from the same polluted site that were also tested *in vitro* showed that these isolates were more efficient at protecting pines from Zn toxicity than isolates from a nonpolluted site, suggesting further that at least some ectomycorrhizal species are able to adapt to high levels of metal contamination (Adriaensen et al., 2003).

Although studying metal effects on ectomycorrhizal fungi in pure culture can give important basic information regarding fungal metal tolerance, such studies admittedly can not predict fungal response in symbiosis. Axenic screening is important when one is examining a

large number of isolates for future use in bioremediation studies, but as Jones and Hutchinson (1988) point out, axenic tolerance alone does not necessarily correlate to good host protection for a given mycorrhizal symbiosis. To better understand the nature of mycorrhizal response to trace metals and the effect on a host plant, studies involving fungal-host relationships must be carried out, whether in the laboratory or the field. The following section discusses studies to date on the topic.

Protection of the host plant from Cu and Zn toxicity

It has been well established that ectomycorrhizal colonization has the potential to protect the more susceptible host plant from elevated levels of trace metals in soils (Adriaensen et al., 2003; Denny and Wilkins, 1987c; Dixon and Buschena, 1988; Dixon, 1988; Hartley-Whitaker et al, 2000a; Leyval et al., 1997; Marschner et al., 1997; Smith and Read, 1997). Amelioration of toxicity has been measured in several ways. An increase in biomass has been reported for mycorrhizal versus non-mycorrhizal seedlings in the presence of trace metals (Brown and Wilkins, 1985; Gruhn, 1989; Hartley et al., 1999a; Hartley-Whitaker et al., 2000a). Increase in biomass is usually only affected by ectomycorrhizal colonization and not the presence of trace metals; therefore, it is not considered a good measure of metal amelioration (Hartley et al, 1999a; Hartley-Whitaker et al., 2000a). In some cases, mycorrhizal colonization did not affect biomass at all (Colpaert and VanAssche, 1992). Since the site of contact for metal toxicity is in the plant roots, root elongation is considered the best measurement of phytotoxicity (Arudini et al., 1998; Ouzounidou, 1994). Mycorrhizal infection has been shown to enhance root length with respect to elevated soil metals (Hartley-Whitaker et al., 2000a). The most common measure of mycorrhizal amelioration of metal phytotoxicity is to measure the level of metals in plant tissues for ectomycorrhizal and non-mycorrhizal treatments. Several studies have shown a reduction in

foliar metal content in ectomycorrhizal seedlings compared to a non-ectomycorrhizal control (Bucking and Heyser, 1994; Colpaert and VanAssche, 1992; Denny and Wilkins, 1987c; Hartley-Whitaker et al., 2000a; Leyval, 1997; Smith and Read, 1997). However, not all ectomycorrhizal species reduce metal content, even when mycelial production and colonization levels were high (Hartley et al, 2000a;). In one study involving several mycobionts of *Pinus sylvestris*, colonization with *Thelephora terrestris* Pers. ex. Fr. significantly increased Zn content in needles (Colpaert and VanAssche, 1992). Another study showed that although a Zn-tolerant strain of *Suillus bovinus* reduced Zn stress in its host plant, needle concentrations of Zn were actually higher than the non-mycorrhizal control (Adriaensen et al., 2003).

A possible explanation for this discrepancy is that metal stress in plants may be ameliorated by mycorrhizal induced improvement in nutrient uptake. Nutrient stressed plants are less robust, and therefore may be less likely to withstand other environmental stresses such as metal pollution. Additionally, metals in the soil can limit availability of important nutrients such as phosphorus (Harrison et al., 1999). The ability of mycorrhizal fungi to facilitate uptake of soil nutrients is one of the most important facets of the fungal-host relationship. Nutrient uptake has been used as a tool to detect Cu toxicity in mycorrhizae (Van Tichelen et al., 1999), and nutrient uptake has been shown to be reduced in strains of mycorrhizae sensitive to metals (Adriaensen et al., 2003). Improved plant nutrition may prove to be an important factor in protecting host plants from metal toxicity, especially in seedlings where nutrients are crucial for establishment and development.

The mechanism(s) of protection have not yet been fully determined, and several suggestions have been made as to how the fungi reduce metal contents of their plant host. Understanding these mechanisms is crucial for determining what fungal species will "work" in a

given remediation situation. As previously discussed, the cell walls and extracellular matrices are considered the chief binding sites for trace metals (Denny and Ridge, 1995; Denny and Wilkins, 1987c; Gruhn, 1989; Tam, 1995;). This suggests that a genus producing a large amount of mycelium, such as *Suillus*, would provide the most surface area for adsorption and therefore the most host protection (Leyval et al., 1997). Other suggestions include the presence of a physical barrier provided by the fungal mantle, complexing of metals by fungal biomass, modifications of the rhizosphere, production of organic acids that may precipitate metals, and interactions between heavy metals and compatible anions (Ahonen-Jonnarth et al., 2000; Dixon, 1988; Dixon and Buschena, 1988; Leyval et al., 1997).

The level of protection afforded a host depends on several factors, including the species of mycobiont used, soil nutrient content, moisture, and pH (Gruhn, 1989; Jentschke et al., 1998; Marschner et al., 1996; McCreight and Schroeder, 1982; Smith and Read, 1997). The types and available concentrations of toxic metals in the soil will also affect the ability of the fungus to protect the plant, as will interactions between those metals (Dixon and Buschena, 1988; Hartley et al., 1999b; Tam, 1995;). While fungi are generally more tolerant of metals than the host plant, there exists a "threshold" capacity of fungal sequestration for each metal. The fungus, even when able to survive in metal pollution, can only reduce uptake by the plant host up to a certain extent. When these levels are exceeded, the fungus may no longer be able to provide a barrier to plant uptake (Bucking and Heyser, 1994; Dey et al., 1994; Dixon and Buschena, 1988; Marschner et al., 1998). In this case, higher binding capacity for metals would be the determining factor for choosing a mycobiont in a remediation situation. A fungicidal threshold also exists for each fungal species, at which concentration fungal mycelium will die and the ectomycorrhizal relationship will not be feasible (Leyval et al., 1997; Marschner et al., 1996;).

At these concentrations, no plant protection will be possible. Finally, the ability of a fungus to colonize a host plant is an important factor in mycorrhizal studies. A fungus that better colonizes a host plant will likely give more benefit in terms of nutrient and water uptake, and therefore improve plant health. If nutrient acquisition is a key factor in phytotoxicity amelioration, this may be one of the most important aspects to consider when choosing a mycobiont for remediation efforts.

The results of studies involving Cu amelioration by mycorrhizal inoculation have been inconclusive thus far. Ectomycorrhizal colonization has been found to reduce Cu concentrations in shoot tissues, although this reduction was species-dependent on the mycobiont (Leyval et al., 1997). Jones and Hutchinson (1986) found that colonization by ectomycorrhizae actually reduced plant growth at high Cu levels, while these same ectomycorrhizal fungi were tolerant of high levels of Cu *in vitro* (Jones and Hutchinson, 1988). In a study of five ectomycorrhizal species with *Pinus densiflora,* Gruhn (1989) found that the fungus which best stimulates growth under normal conditions tends to most ameliorate Cu stress. In this case the fungus, *Suillus pictus* (Peck) Smith and Thiers, had only moderately strong Cu tolerance. The same study showed that *Piloderma bicolor* (Peck) Julich , a Cu-sensitive fungus, also stimulated host growth in the presence of Cu. This strengthens the argument that nutrient uptake may be key to host protection in stressed environments, and also confirms the findings of Jones and Hutchinson (1988) that axenic screening of metal tolerant fungi will not necessarily correlate to success in protecting a host plant. In addition to examining the Cu tolerance of a host, the behavior of a fungus in a symbiotic situation must be considered.

Ectomycorrhizal fungi have been shown to improve seedling performance in Zn-amended environments (Adriaensen et al., 2003; Brown and Wilkins, 1985; Bucking and Heyser, 1994;

Colpaert and VanAssche, 1992; Denny and Wilkins, 1987c; Hartley-Whitaker et al., 2000a). However, the way in which these fungi ameliorate Zn toxicity is unclear. Some authors report that infection with ectomycorrhizal fungi increased root length and reduced Zn transport into shoots and foliar tissues (Bucking and Heyser, 1994; Colpaert and VanAssche, 1992, Denny and Wilkins, 1987a; Hartley-Whitaker et al., 2000a). However, other data indicate that the improvement in seedling success as measured by biomass and root production is only a function of mycorrhizal colonization in all soil types, not just those with high Zn (Dixon and Buschena, 1988; Hartley et al., 1998; Hartley et al, 1999a). As with Cu, axenic tests of fungal tolerance to Zn do not necessarily correlate to improved tolerance as symbionts (Colpaert and VanAssche, 1992). Additionally, some highly tolerant fungi increased Zn uptake into needles and leaves of their host plants (Adriaensen et al., 2003; Colpaert and VanAssche, 1992). Adriaensen et al. (2003) found that increased foliar content of Zn did not correlate to decreased plant performance; in fact, the opposite was found. This again suggests that decreased translocation of metals, while an important factor in host protection, is not the only mechanism for mycorrhizal amelioration of metal phytotoxicity. Rather, it appears that in the case of the *Pinus sylvestris / Suillus bovinus* study, mycorrhizal plants were able to withstand higher concentrations of Zn in their shoot and foliar tissues than non-mycorrhizal plants (Adriaensen et al., 2003).

A possible explanation for the discrepancies between fungal tolerance for Zn or Cu and the amount of protection it gives the host plant is that host plant colonization may be inhibited by the presence of metals. Dixon and Buschena (1998) found that colonization of *Pinus banksiana* and *Picea glauca* by the mycorrhizal fungus *Suillus luteus* was inhibited by high levels of lead, cadmium, Cu, and Zn. Additionally, Gruhn (1989) found that the fungal species offering the best protection to *Pinus densiflora* under Cu stress was the one that was the best colonizer of the

host plant, regardless of axenic tolerance. Further supporting the argument for colonization inhibition is the fact that, in one study, both cadmium and Zn decreased colonization of *Pinus sylvestris* by *Paxillus involutus*, but colonization of seedlings prior to metal exposure did provide increased seedling biomass (Hartley et al., 1999a). Metal exposure has also been shown to decrease the diversity of species colonizing host plants in contaminated soils as well as the number of colonized root tips (Hartley-Whitaker et al., 2000b). The decline in colonization could have serious implications for plant vulnerability to metal stress in the field. In order to gain a true measure of mycorrhizal amelioration, both colonization success and plant response must be measured.

Other metals

I. Cadmium

Cadmium is a rare element in soil. It is not found naturally in pure form, and often is not detectable or detectable at only low levels in natural soils. Even in contaminated areas, soil cadmium levels rarely reach concentrations of more than a few parts per million (Page et al., 1981). Cadmium toxicity is a serious problem in contaminated areas, however, because it is more readily taken up by plants than most other metals. This is especially true in acidic soils, where cadmium is present in its available, cationic form (Godbold, 1991). Cadmium is not an essential element for plant nutrition, and the result of plant uptake is generally an inhibition of photosynthesis and an imbalance in essential micronutrients. Cadmium also decreases transpiration in plants and reduces seedling root and shoot growth (Colpaert and VanAssche, 1993; Godbold, 1991; Kelly et al., 1979).

As may be expected, cadmium is quite toxic to fungi as well as plants. Cadmium arrests growth of some ectomycorrhizal fungi at extremely low levels (McCreight and Schroeder, 1982).

Fungal sensitivity to cadmium does vary greatly among species, with *Suillus variegates* Fr: O. Kuntze having an median effective concentration (EC_{50}) of just 0.04 mmol/m^3 cadmium and *Amanita muscaria* (L:Fr) Pers having an EC_{50} of up to 1334-1379 mmol/m^3 cadmium (Hartley et al., 1997; McCreight and Schroeder, 1982). Another factor in toxicity levels was the presence or absence of other cations. The EC_{50} for cadmium of *Suillus variegatus* was, as stated, just 0.04 mmol/m^3 in the absence of Zn ions, but when in the presence of 500 mmol/m^3 Zn, the EC_{50} rose to 5.5 mmol/m^3 (Hartley et al., 1997). The amelioration of cadmium toxicity by Zn was shown for other fungal species as well. There exist several possible explanations for this interaction. Cadmium appears to be associated with the hyphal cell wall or cell membrane (Galli et al., 1993; Hartley et al., 1997). Competition between the divalent cations for ion transport through the membrane is a possible explanation for cadmium amelioration by higher concentrations of Zn. Another possibility is a selection by the cell for Zn cations, which are essential for fungal cell function. Hartley et al. (1997) proposed that Zn induced some physiological response that would reduce sensitivity to cadmium, however the nature of this response has not yet been explored.

Ectomycorrhizal colonization appears to decrease cadmium toxicity in plants. When compared to non-mycorrhizal controls, cadmium content in leaves and needles of mycorrhizal plants were significantly lower, while root content was higher (Dixon, 1988; Galli et al., 1993). While this suggests that mycorrhizal fungi retain cadmium in their cells, at least one case showed that high concentrations of cadmium resulted in similar needle concentrations in the mycorrhizal and non-mycorrhizal plants (Dixon, 1988). This may be a result of fungitoxic effects on cadmium influencing development of mycorrhizal mycelium and colonization of the host plant by ectomycorrhizae. One study suggests that the main mycorrhizal influence in cadmium-amended soils is increased nutrient uptake and biomass, but not decreased metal uptake

(Ahonen-Jonarth and Finlay, 2001). Cadmium, like Zn, has been shown to decrease the colonization of a host plant by ectomycorrhizal fungi (Hartley-Whitaker et al., 2000b). At best, ectomycorrhizal inoculation appears to provide host protection at low and intermediate levels of cadmium (Colpaert and VanAssche, 1993; Dixon, 1988; Dixon and Buschena, 1988).

II. Lead

Like cadmium, lead is not an essential element for biological function, and is found in the soil mainly as a result of pollution in industrialized areas. Lead levels considered "normal" in soils range between 2-200 parts per million, with a mean of 10 parts per million (Bowen, 1966; Freedman and Hutchinson, 1981). In mining areas in North America and Europe, lead levels have ranged from 2-1750 ppm (Gregory and Bradshaw, 1965). Lamersdorf et al. (1991) modeled lead accumulation in contaminated sites in Northern Germany and found that significant lead accumulation occurs and creates a toxic environment for plants. Lead contamination from soil usually accumulates in the roots, and very little enters above-ground plant tissues (Koeppe, 1981). However, root accumulation of lead is detrimental to seedling establishment in new environments and therefore inhibits revegetation and phytoremediation of lead polluted sites. In one study, amendment of soil media with a range of lead concentrations from 2-20 ppm decreased root and shoot weight of *Pinus banksiana* and *Picea glauca* seedlings (Dixon and Buschena, 1988). Additionally, treatment of soil with a range of 30-480 mg/kg of lead resulted in both decreased height and total biomass of loblolly pine (*Pinus taeda*) seedlings (Chappelka et al., 1991). High soil levels of lead (>150 mg/kg) have been shown to cause a noticeable reduction in photosynthesis (Seiler and Paganelli, 1987). This lead-induced decrease in photosynthesis may be related to decreases in transpiration, which suggests that metal ions in the leaves somehow cause closure or blockage of the stomata (Bazzaz et al., 1974; Rolfe and

Bazzaz, 1975). *In vitro* studies have shown reduction in grana stacks, stroma, and starch granules in chloroplasts when seedlings are exposed to lead (Koeppe, 1981). The reduction in photosynthesis in turn correlates to reduced shoot and root height and weight (Seiler and Paganelli, 1987). Other suggestions have been that lead interferes with enzyme activity or that it kills cells indirectly by depriving them of available phosphate (Koeppe, 1981).

Accumulation of lead in the soil can inhibit ectomycorrhizal formation with its plant host (Chappelka et al., 1991; Dixon, 1988; Dixon and Buschena, 1988; McCreight and Schroeder, 1982). Sensitivity of fungi to lead varies with species: lead concentrations as low as 5 ppm inhibited ectomycorrhizal formation by *Suillus luteus*, although with other ectomycorrhizal species the concentration level needed to reach 200 ppm in order to have a negative effect on ectomycorrhizal formation (Dixon and Buschena, 1988; McCreight and Schroeder, 1982). The morphological symptoms of lead toxicity in fungi are the same as Cu; i.e., swollen and deformed mycorrhizal tips and secretion of an extracellular "slime" (Tam, 1995). An apparent tolerance of lead pollution by certain ectomycorrhizal species *in vitro* has been observed (Hartley et al., 1997; Tam, 1995; Vodnik et al., 1998). While tolerance varies among species, studies have shown tha the levels of lead that cause a toxic effect in a number of ectomycorrhizal fungi are a full order of magnitude higher than in many potential hosts (Godbold, 1991; Marschner, 1994). The extracellular slime may be a site of metal accumulation, preventing the poisonous element from entering the fungal cell (Tam, 1995).

Inoculation of seedlings with ectomycorrhizal fungi has been shown to improve growth in lead-amended soil (Dixon, 1988; Dixon and Buschena, 1988; Jentschke et al., 1998; Marschner et al., 1996). There seems to be at least some correlation between axenic tolerance of lead and protection offered to the host. A study by Hartley et al. (1996) showed that *Paxillus*

involutus was quite tolerant to lead contamination. Subsequent studies showed that mycorrhizal infection of Norway spruce by *P. involutus* was shown to decrease lead content in the roots when compared to non-mycorrhizal controls (Jentschke et al., 1998; Marschner et al., 1996). These findings support the suggestion that binding of the metals to the hyphal cell walls of the extrametrical mycelium and mantle, as shown in *P. involutus*, is indeed one mechanism of lead amelioration. However, the ability to tolerate lead does not necessarily correlate to reduced metal translocation. Tam (1995) showed that *Pisolithus tinctorius* was extremely tolerant to high lead concentrations; however, another study indicated that *P. tinctorius* was not the most effective reducer of lead content in plant short roots and cortex (Marschner et al., 1996). The mechanism of amelioration of lead toxicity, therefore, is not yet known.

Research Objectives

Trace metal pollution of soils is common in North America where industrial activities and mining have been active. Metal contamination such as Cu, Zn, Pb, and Cd are present in waste sites in phytotoxic concentrations, making revegetation difficult if not impossible. The ectomycorrhizal relationship shows potential in ameliorating metal toxicity; however, several issues need to be explored to fully understand this potential. This study is aimed at determining 1) how the metals Cu and Zn affect three common species of ectomycorrhizal fungi, 2) how soil disturbance and metal contamination affect ectomycorrhizal colonization of host plants and 3) whether soil disturbance affects ectomycorrhizal diversity in Southwest Virginia. This dissertation research has the following objectives:

1. To determine whether a threshold level exists for growth inhibition by Cu and Zn for three species of ectomycorrhizal fungi, *Suillus granulatus, Pisolithus tinctorius,* and

Paxillus involutus as measured by colony diameter growth rate, biomass, and morphological response. (Chapter 2)

2. To determine whether sectoring in *Suillus granulatus* results in differential response to Cu and Zn and whether sectors represent genetic differentiation of a parent strain or expression of phenotypic plasticity. (Chapter 2)

3. To evaluate whether Cu and Zn adsorb to, a low pH, low organic matter soil from the Coastal Plain of Virginia, and to determine where any adsorption occurs on the soil surface through adsorption isotherms and selective extraction. (Chapter 3)

4. To determine whether ectomycorrhizal diversity on *Pinus strobus* in different stages of growth on a coal mine revegetation site is low, as would be expected on a stressed site. (Chapter 4).

5. To investigate whether seedling colonization by naturally occurring ectomycorrhizal fungi is depressed by soil disturbances including acidity and lack of organic matter (Chapter 5).

6. To determine whether early-stage ectomycorrhizal fungi dominate the belowground mycorrhizal flora on a primary successional site characterized by no organic matter and low pH (Chapter 5).

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Chapter 2

Inter- and intraspecific variation in copper and zinc tolerance among ectomycorrhizal fungi

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Abstract

Copper and zinc are micronutrients essential for fungal and plant growth; however, these metals are toxic at elevated concentrations. Increased metal concentrations may significantly reduce ectomycorrhizal growth and colonization of tree seedlings. Axenic screening of ectomycorrhizal isolates is an effective way to examine basic fungal tolerance to a given metal or metals. Axenic tolerance was determined by colony growth on amended agar plates as well as biomass in liquid culture. *Suillus granulatus* and *Pisolithus tinctorius* were more tolerant to copper than *Paxillus involutus*, however none of the species showed growth past 100 ppm Cu. *Suillus granulatus* was most tolerant to zinc, followed by *Paxillus involutus* and finally *Pisolithus tinctorius*. Sectoring was observed on several plates containing colonies of *Suillus granulatus* strain VT 1990. Of the ten isolates tested (8 sectors, VT 1990 "parent strain", and VT 2176), one sector showed a higher tolerance for copper than VT 1990, VT 2176, and the other sectors. One isolate from nature, VT 2176, and three sectors (A, B, and D) did not grow well on agar amended with high levels of zinc. Inter-simple sequence repeat (ISSR) fingerprinting indicated that some genetic variation existed with in the sectors, but that the variation did not correspond to distinct clusters representing different levels of metal tolerance.

Keywords: *Suillus granulatus, Pisolithus tinctorius, Paxillus involutus*, copper, zinc, axenic culture, inter-simple sequence repeats (ISSR).

Introduction

Copper and zinc are essential micronutrients for fungal and plant growth; however, these metals are toxic at elevated concentrations that result from various industrial activities (Colpaert et al., 2000; Leyval et al., 1997; Marschner, 1995). High levels of copper reduce plant uptake of water and nutrients by inhibiting root elongation and branching. Symptoms of zinc toxicity are retardation of growth and chlorosis in plants (Colpaert and VanAssche, 1993; Dixon and Buschena, 1988). Metal cations may reduce the availability of nutrients such as nitrogen and phosphorous through complexing reactions, which can adversely affect plant growth and development (Derome and Lindroos, 1998). Additionally, increased metal concentrations may significantly reduce ectomycorrhizal growth and colonization of tree seedlings in contaminated environments (Hartley-Whitaker et al, 2000; McCreight and Schroeder, 1982).

Several studies have indicated that ectomycorrhizal colonization can improve seedling performance in the presence of metal contamination, especially for conifers which grow in acidic soils. These soils, when contaminated, generally have high levels of bioavailable metals (Denny and Wilkins, 1987; Dixon, 1988; Dixon and Buschena, 1988; Gruhn, 1989; Jones and Hutchinson, 1988; Marschner et al, 1996). Ectomycorrhizal fungi are a diverse group, spanning three phyla and several orders and families in the fungal kingdom. Significant variation in response to trace metals both in pure culture and in symbiosis exists among ectomycorrhizal taxa. Gruhn (1989) demonstrated interspecific and intraspecific variation in response to copper, where *Pisolithus tinctorius* (Fr.) Coker and Couch, *Gyrodon meruloides* (Schwein.) Schnell, and one isolate of *Suillus granulatus* (Fr.) Kuntze were most tolerant to copper, while *Suillus pictus* (Peck) Kuntze, *Piloderma bicolor* (Peck) Jülich, and two isolates of *Suillus granulatus* were less

tolerant. Interspecific variation of zinc tolerance has been reported between species (Colpaert and VanAssche, 1992) and within isolates of *Paxillus involutus* Fries (Denny and Wilkins, 1987). Egerton-Warburton and Griffin (1995) report intraspecific variation in *Pisolithus tinctorius* response to aluminum.

Additionally, tolerance of an ectomycorrhizal fungus to one metal does not confer tolerance to all metals. For example, one study found that *Suillus luteus* (Fr.) Gray*, Suillus bovinus* (Fr.) Kuntze*, Suillus variegatus* (Fr.) Kuntze*,* and *P. tinctorius* were more tolerant of copper, cadmium, and zinc than *P. involutus*, but that the reverse was true for nickel tolerance (Blaudez et al., 2000). Therefore, success in establishing seedling growth on metal contaminated sites will depend heavily on which ectomycorrhizal symbionts are involved.

Axenic screening of ectomycorrhizal isolates is an effective way to examine basic fungal tolerance to a given metal or metals. Tolerance of a fungus in pure culture does not necessarily correspond to ectomycorrhizal development or host protection in a symbiotic environment (Jones and Hutchinson, 1988). This is likely due to a number of factors, including metal interactions with other metals, the growth media, presence of other organisms in the soil that may influence metal availability, and physiological changes that occur in both fungus and host during mycorrhizal formation (Hilbert and Martin, 1988). Nevertheless, examination of fungal response to a metal or metals in pure culture allows insight into how individual species may cope with an excess metal load.

Metal concentrations that are phytotoxic are generally under the threshold levels for fungal growth (Blaudez et al, 2000); however, in some cases the reverse was true (Colpaert and VanAssche, 1992; Hartley-Whitaker et al., 2000). Axenic screening of fungal growth could help predict which fungi would persist under certain metal conditions. Additionally, *in vitro* tests

make it possible to conduct tests on several strains at once, which will provide information on whether inter- and intraspecific variation is adaptive to a given metal.

Morphological changes such as increased pigmentation (Gruhn and Miller, 1991) and sectoring can also indicate possible tolerance mechanisms employed by ectomycorrhizal fungi. Sectoring is common in filamentous fungi grown in the laboratory and may occur either in response to an external factor or spontaneously (Li et al. 1994). Our hypothesis was that sectoring observed in our experiments was the result of an adaptive genetic segregation in response to pressure from elevated levels of zinc.

The purpose of this study was 1) to investigate whether *Suillus granulatus, Paxillus involutus,* and *Pisolithus tinctorius* can withstand high levels of copper and zinc, and 2) to investigate whether sectoring observed in *Suillus granulatus* on high-metal plates was evidence of adaptation to the high metal environment. Axenic tolerance was determined by colony growth on amended agar plates as well as biomass in liquid culture. Molecular analysis was performed on sectors of *Suillus granulatus* strain VT 1990 to determine whether the morphologically distinct sectors differed genetically or whether the sectors were evidence of phenotypic plasticity within the fungus. Inter-simple sequence repeat (ISSR) fingerprinting was used to investigate possible genetic diversity in sectors.

Materials and Methods

Fungal isolates

Isolates of *Suillus granulatus* (VT 1990), *Pisolithus tinctorius* (VT 3303), and *Paxillus involutus* (VT 2093) were obtained from the Virginia Tech Culture Collection, where they had been maintained on Hagem's agar (5 g L^{-1} dextrose, 4 g L^{-1} malt extract, 1 g L^{-1} yeast extract,

500 mg L^{-1} NH_4Cl, 500 mg L^{-1} $MgSO_4$, 500 mg L^{-1} KH_2PO_4, 1 mg L^{-1} thiamine, 5 mg L^{-1} $FeCl_3$, 5 μg L^{-1} biotin) slants at 4° Celsius. The *S. granulatus* and *P. tinctorius* isolates were obtained from sporocarps collected under pine. No information is available regarding the location of the sporocarps collected to obtain the *Paxillus involutus* culture. Each culture was immediately subcultured onto plates containing Hagem's agar and incubated in the dark at 20° C, at which time new mycelial growth was evident. Transfers were then made onto agar plates containing Palmer's Defined Medium (PDM) (Palmer, 1971) and these subcultures were incubated at 20°C for two weeks. Additional transfers were made to flasks containing liquid PDM, which were allowed to grow in the dark at 20°C for four weeks.

Palmer's Defined Medium was chosen as the growth media for metal tolerance studies based on preliminary assays where the fungi were grown on agar plates with filter paper disks soaked in aqueous copper- and zinc solutions. Cultures grown on Hagem's agar showed no response to presence of the metal, while cultures grown on PDM showed zones of inhibition with diameters corresponding to the metal concentration used. Transfers were made initially to unamended PDM to reduce effects of the new media type in the dose-response experiments.

Colony diameter and biomass measurements

Two weeks after transfer to PDM, 8 mm cores of mycelium were cut out of the plates with a cork borer and transferred to plates containing 0, 25, 50, 75, and 100 ppm Cu or Zn. The copper and zinc were added to the media as sulfate salts after autoclaving, to prevent precipitation of the metal ions. Agar pHs ranged from 5.4 (control) to 4.0 (1000 ppm copper), well within the range for mycorrhizal growth. Six replicates of each treatment were performed. Subsequently, the experiment was repeated using 0, 100, 250, 500 and 1000 ppm Cu or Zn, as well as plates containing 25 ppm Cu and 25 ppm Zn or 50 ppm Cu and 50 ppm Zn. These

experiments were performed in triplicate. Colony diameter measurements were made in two directions at 3, 5, and 8 weeks. Fungal growth was compared by dividing the colony diameter in millimeters for each metal-amended plate by the average colony diameter achieved on unamended media. Qualitative changes in colony density, edge pattern, texture, and pigmentation were also noted.

Fungal biomass was measured by analyzing mycelial growth in PDM broth amended with Cu or Zn, also as sulfate salts after autoclaving. Fungal inoculum was ground for ten seconds in a sterile Waring blender to macerate the mycelial mat that had formed. Five ml aliquots of this ground mycelium were transferred to liquid media containing 0, 25, 50, 75, and 100 ppm Cu or Zn. Five replicates of each treatment were performed. After 5 weeks, the mycelium was vacuum filtered through a preweighed Whatman filter and placed in a 55°C oven. After 24 hours, the filters were taken out of the oven, allowed to cool for 2 hours, and weighed to obtain the fungal mass in milligrams.

Statistical analysis was performed in both experiments using one-way ANOVA on each combination of fungal species and series of metal doses, followed by Tukey's Honest Significant Difference Test (Tukey's HSD) at $p = 0.05$.

Sectoring in VT 1990

Sectoring was observed on cultures of VT 1990 grown on high levels of copper and zinc (Figure 3). In order to determine whether the observed sectoring was an adaptive mechanism for metal tolerance, several subcultures of each sector were made onto PDM agar plates. Eight sectors were isolated in total. These were allowed to grow for 4 weeks, at which time 8 mm discs were cut with a cork borer and transferred to plates containing 0, 100, 250, 500, and 1000 ppm Cu or Zn added as sulfate salts. The "parent" isolate, VT 1990, was included in the experiments

as the reference point for the strain. A separate isolate of *Suillus granulatus*, VT 2176, was added to the experiment in order to represent a different population of *S. granulatus*. Isolate VT 1990 was collected in Montgomery County, Virginia, and VT 2176 was collected approximately 70 miles away in Summers County, West Virginia. Each treatment was replicated three times. Colony diameter measurements were made at 3, 5, and 8 weeks in two directions. Colony diameters were standardized to the control as before by dividing the colony diameter by the average colony diameter for the control for each isolate. Statistical analysis was performed using Duncan's New MRT and Tukey's HSD tests.

Inter-simple sequence repeat (ISSR) fingerprinting was performed on the VT 1990 parent strain, the 8 sectors, and VT 2176 to determine if the sectors were distinct genets of *Suillus granulatus*. DNA was extracted from fresh mycelia using a modification of the CTAB method described by Gardes and Bruns (1993). Mycelium samples were ground in 500 µl CTAB with plastic grinders. The samples were then placed in a 65° water bath for 35-40 minutes. Samples were centrifuged 2 minutes at 13000 rpm to pellet the sample. The supernatant was decanted into a clean tube, and 1 volume (equal to the supernatant) of 24:1 chloroform:isoamyl alcohol was added. These were shaken and centrifuged 5 minutes at 12000 rpm. The aqueous fraction was pipetted into a clean 2 milliliter eppendorf tube. DNA was precipitated by adding 350 µl cold 100% isopropanol to the tube, mixing, and placing the tubes in a -20° C freezer overnight. The next day, the samples were centrifuged at 13000 rpm for 5 minutes to pellet the DNA. DNA pellets were washed with 300 µl cold 80% ethanol. The pellets were spun for 2 minutes at 13000 rpm, the supernatant was decanted, and the samples were allowed to air dry until no trace of ethanol was left in the tube. DNA was eluted in 50 µl 0.5X Tris-EDTA.

The PCR conditions used for ISSR amplification was the following: amplification in 1X reaction buffer (50 mM KCl, 10mM Tris-HCl pH 9, and 0.1% Triton X-100) (Promega), 3mM $MgCl_2$ (Promega), 0.2 mM of each dNTP (Promega), 1.5 µM primer, and 1 U Taq DNA polymerase (Promega). Ten to fifty nanograms of template DNA was added to each reaction, with the optimal amount determined for each isolate through trial and error. After an initial denaturation step at 95° C for 1 minute, the DNA was amplified by 40 cycles of denaturing at 94° C for 1 minute, annealing at 45° C for 1 minute, and extension at 72° C for three minutes. Twenty primers were selected for screening. Of these primers, four gave consistently polymorphic banding patterns: CA_8, M13 (GAGGGTGGXGGZTCT, Weising, 1995), $GGAT_4$, and GT_6YR. $GACA_4$ presented a polymorphic banding pattern the first time it was tried, but not in subsequent reactions. The remaining fifteen primers either did not amplify the template or produced a similar banding pattern for all samples.

Amplification products were separated in 1.5% agarose gels in 0.5 X Tris-Acetate-EDTA (TAE) buffer and stained with ethidium bromide. Amplification products were visualized under ultraviolet light using AlphaImager software and were scored as 1 if present and 0 if absent. The data were formatted into a NEXUS file and entered into PAUP* (version 4.01b, Swofford, 2002) for Unweighted Pair Group Method using Arithmetic Averages (UPGMA) cluster analysis. Bootstrap analysis was performed to assess the support for the resulting dendrogram. Analysis of molecular variance (AMOVA) was conducted to determine the significance of differences between VT 1990 and VT 2176, and to determine the significance of differences within the nine samples of VT 1990. Significance levels were computed with non-parametric permutations using non-Euclidean distances (Huff et al., 1993).

Results

Interspecific tolerance to copper and zinc

The responses of *Suillus granulatus, Paxillus involutus*, and *Pisolithus tinctorius* to copper and zinc as expressed by colony growth on agar plates are shown in Tables 2.1 and 2.2. *Suillus granulatus* and *Pisolithus tinctorius* were more tolerant to copper than *Paxillus involutus*, which did not grow at copper levels greater than 25 ppm (Figure 2.1). *Pisolithus tinctorius* had more growth at 25 ppm Cu, after which *P. tinctorius* and *S. granulatus* showed similar colony growth relative to controls. No growth was achieved by any fungus at concentrations greater than 100 ppm. *Suillus granulatus* was the most tolerant of zinc in the growth media, followed by *P. involutus* and then *P. tinctorius.* Of the three species, only *S. granulatus* grew at zinc concentrations above 100 ppm. (Figure 2.2). Fungal response to copper varied more when measured as biomass in liquid media than in colony diameter (Table 2.3). *Suillus granulatus* showed a sharp decline in biomass between 25 and 50 ppm, and then leveled off around 20% of the control biomass. *Paxillus involutus* declined steadily until the copper level reached 50 ppm, leveled off between 50 and 75 ppm, and then dropped to no growth at 100 ppm. *Pisolithus tinctorius* was most tolerant of copper up to 50 ppm, but the biomass sharply declined between 50 and 75 ppm copper (Figure 2.3). Biomass of *Suillus granulatus* in zinc-amended media generally continued to increase up to 100 ppm zinc, at which point the average biomass was 1.5 times that of the control. Based on biomass measurements, *Suillus granulatus* was most tolerant to zinc, followed by *Paxillus involutus* and finally *Pisolithus tinctorius* (Figure 2.4). Standard errors were greater for biomass measurements than colony diameter measurements in almost all cases, suggesting that colony diameter measurements were a more consistent measure of fungal response to metals for this system.

Sectoring of Suillus granulatus isolate VT 1990

At the five-week observation, several high-zinc plates inoculated with a single agar plug of *Suillus granulatus* exhibited two distinct morphological patterns (Figure 3). The *Suillus* plates showed a growth phenomenon where half the culture appeared affected by the metal and half the colony grew in a manner similar to growth under no metal amendment (Figure 2.5). Each sector was subcultured and grown on media containing 0, 100, 250, 500, and 1000 ppm copper or zinc. The sectors differed in morphology and colony diameter attained at all levels of metal amendment, most notably at the threshold levels of copper and zinc (Figures 2.6 and 2.7). Relative colony diameters for the sectors, VT 1990, and VT 2176 at 5 weeks of growth on metal amended agar are given in Tables 2.4 and 2.5. Sector H appeared to be most tolerant of copper, and was the only one to show any growth at 1000 ppm copper after 5 weeks (Figure 2.8). Only sectors E and H and the parent strain VT 1990 showed growth at 100 ppm copper after 5 weeks. Growth patterns on zinc were more varied. All sectors, as well as VT 2176, showed greater growth at 100 ppm zinc than VT 1990. The rate of growth decline as exhibited by colony diameter was different for each sector (Figure 2.9). At the highest zinc concentration, there appeared to be two groups: the first, consisting of sectors A, B, D, and VT 2176, did not grow at all on 1000 ppm zinc media. The second, consisting of VT 1990 and sectors C, E, and H had colony diameters that were between 20-25 percent of the control plates. Finally, two sectors did not fit into either of these groups: sector F appeared to fall between the two groups, and sector G showed a low average colony diameter (5.8 percent of the control), but the standard error was the same value as the average colony diameter at 1000 ppm Zn.

ISSR analysis of Suillus granulatus sectors

The ISSR fingerprints using four primers generated a total of 47 different bands, of which 36 were polymorphic. Analysis of the banding patterns using UPGMA showed that VT 2176 is distinct from all sectors of VT 1990 and the parent VT 1990 isolate. The initial UPGMA phylogram distinguishes two putative groups within VT 1990. The first group contains VT 1990 and sectors E and A. The second group consists of sectors B, C, D, F, G, and H (Figure 2.10). However, bootstrap analysis did not support the initial UPGMA phylogram; the two groups were collapsed into one large cluster containing all the VT 1990 isolates with 96% support (Figure 2.11). Sectors C and H do cluster together, which indicates that they may be more closely related to each other than the rest of the sectors. However, there is only low bootstrap support for this grouping.

Two-way AMOVA was conducted on variance estimates using non-parametric permutational procedures based on non-Euclidean distances (Huff et al., 1993). This analysis provides the Φst statistic, which gives the correlation of random haplotypes within a group relative to all the groups (Excoffier et al., 1992). High Φst values indicate sharp divisions between groups, while a Φst less than one indicates that genetic variation within a given group is significant (Sales et al., 2001). The variance among groups (Group 1 = VT 2176 Group 2 = VT 1990 sectors and parent strain) was estimated at 47.07%, while the variance within populations was estimated at 52.93%. The Φst statistic was 0.471, with the probability of obtaining a larger number estimated at $p < 0.01$. AMOVA results appear to indicate that the sectors do show a significant degree of variation within the group, although with the caution that UPGMA analysis does not show significant partitioning into related clades based on this variation.

Discussion

There was strong interspecific variation of ectomycorrhizal fungal tolerance to copper (Cu) and zinc (Zn). On solid media, *Suillus granulatus* and *Pisolithus tinctorius* were most tolerant to Cu, while *Paxillus involutus* was significantly inhibited by the presence of Cu. *Suillus granulatus* was most tolerant to Zn in agar media, *P. involutus* showed moderate tolerance, while *P. tinctorius* was inhibited greatly by Zn. Interspecific variation in colony growth rates have been documented by a number of other studies. Colpaert and VanAssche (1987) found that strains of several *Suillus* species from polluted soils were less inhibited than *Paxillus involutus* and *Pisolithus tinctorius* by Zn, but that *Pisolithus* and *Paxillus* were less inhibited than *Suillus* spp. by Cu. In another study, *Scleroderma flavidum* Ellis and Everh. was shown to be less sensitive to Cu and nickel (Ni) than *Lactarius rufus* Peck (Jones and Hutchinson, 1988). Furthermore, in biomass experiments, Blaudez et al (2000) found *Suillus luteus* and *Pisolithus tinctorius* to be more tolerant to Cu, cadmium (Cd), and Zn than *Paxillus involutus,* while the reverse was true for Ni.

The use of agar media in assessing tolerance has been criticized as problematic for a variety of reasons (Hartley et al., 1997). One potential issue is that diameter growth rates do not take into account mycelial density, which has been suggested to be important in mycorrhizal strategies to survive high metal loads (Colpaert and VanAssche, 1992; Denny and Wilkins, 1987). Nevertheless, utilizing agar plates is useful because it allows one to make several measurements over a given time period, and it provides a way to readily visualize morphological changes such as hyphal pigmentation. In order to address the problems associated with agar media studies, biomass experiments in liquid media were also performed. The results for biomass in liquid media were less consistent and had a higher standard error than the colony

diameter measurements on agar media, making them less useful than colony diameter measurements in this study. While the actual relative growth differed between colony diameter measurements and biomass measurements, the trends are similar between the two types of measurements.

Intraspecific variation in tolerance to trace metals has also been well documented. Colpaert and VanAssche (1987, 1992) suggested that fungi on polluted soils are selected for metal-tolerance, and demonstrated that isolates from polluted soils showed higher axenic tolerance to zinc than isolates of the same species from nonpolluted soils. In another study, *Pisolithus tinctorius* strains isolated from mine spoils were more tolerant of aluminum than those isolated from rehabilitated or forest soils (Egerton-Warburton and Griffin, 1995). However, several other studies found that the origin of the fungal isolates did not determine metal tolerance (Blaudez et al., 2000; Howe et al., 1997; Jones and Hutchinson, 1986). Colpaert and VanAssche (1987) suggest that this may be a result of uneven metal dispersal in the soil of origin; further studies including analysis of the soil from which the isolates were obtained would be useful in this regard. Studying intraspecific variation is of interest because it may help elucidate whether fungi are able to exhibit adaptive mechanisms to tolerate metal contamination.

The previous studies are based on variation between disparate strains found on polluted or nonpolluted sites, and are investigating adaptive events that presumably took place at some time in the past. We observed sectoring in one strain of *Suillus granulatus* isolated from a non-polluted site. Sectoring is generally considered the result of a genetic mutation or somatic recombination in fungal mycelium (Frankel and Ellingboe, 1977). Ten isolates were tested:8 sectors (A-H), VT 1990 “parent strain”, and one strain representing a separate population, VT 2176. One sector showed a higher tolerance for copper than VT 1990, VT 2176, and the other

sectors. The results for growth on zinc-amended agar are less clear, but differences in colony diameter do exist. One isolate from nature, VT 2176, and three sectors (A, B, and D) did not grow well on agar amended with high levels of zinc. Two of these sectors, A and B, were isolated from the same plate. VT 1990 grew well on high levels of zinc, along with three of the sectors (C, E, and H).

ISSR analysis has been used to elucidate intraspecific population structure among isolates of *Suillus* spp. (Bonfante et al., 1997; Zhou et al.,. 1999), *Pisolithus tinctorius* (Anderson et al., 1998), and *Cortinarius rotundisporus* (Clel. and Cheel.) Horak and Wood (Sawyer et al., 1999) isolated from the field. Colpaert et al. (2000) used ISSR fingerprinting to distinguish between populations of *Suillus luteus* isolated from trace metal polluted and non-polluted sites. ISSRs are PCR-based minisatellite markers based on short tandem repeats found in the genome (Weising et al., 1995). Most minisatellites are located in noncoding regions of the genome and thus exhibit a high level of variability (Burke, et al., 1991; Estoup et al., 1998). Minisatellite markers, like RAPD (Randomly Amplified Polymorphic DNA) markers, are dominant and are analyzed based on the presence or absence of bands (Weising et al., 1995). ISSR markers are advantageous for population level studies because no prior sequence information is necessary and the high level of variation allows one to discern differences at the sub-species level.

ISSR analysis shows that genetic variation does exist within the sectored isolates; however, the variation does not appear to reflect population differentiation. The phylogram resulting from UPGMA analysis shows division of the VT 1990 isolates into two major clades; however, these clades do not correlate with growth on metal-amended media. Furthermore, bootstrap analysis collapses the clades into one large clade containing all the VT 1990 isolates which are all separate from VT 2176. Interestingly, AMOVA suggests that the variation within

the VT 1990 group is slightly greater (52.93%) than the variation between the VT 1990 isolates and VT 2176 (47.07%). The Φst statistic of 0.471 indicates significant variation within the group as well. Although these results could be skewed by the small sample size, they are supported at $p < 0.01$. These results show that intraspecific variation in metal tolerance does exist, even at the "sub-population" level. They also show that some genetic segregation likely took place over the course of the experiments. One sector, Sector H, showed significantly higher growth on plates containing 100 ppm Cu than any of the other strains. However, the results do not suggest an adaptive mechanism for tolerance, as the original VT 1990 strain performed as well or better than the sectors on zinc plates. As the sectors were isolated from high-zinc plates, one would expect to see a higher tolerance for zinc in a sectored isolate than the original if adaptive tolerance were taking place. Spontaneous sectoring is common in fungi maintained in the laboratory, and likely explains the phenomenon exhibited by VT 1990 in this experiment.

Axenic screening for metal sensitive and tolerant ectomycorrhizal fungi is an important step in determining the reasons behind ectomycorrhizal success, or lack thereof, in the presence of high levels of trace metals. Axenic screening provides a simple and rapid process for determining fungal response to increasing metal doses, and identifying possible mechanisms of tolerance. Investigation of inter- and intraspecific variation in fungal metal response, and the genetic and physiological causes of such variation, is crucial in understanding the complex mechanisms of tolerance employed by ectomycorrhizal fungi. Although axenic screening is an important step in understanding mycorrhizal interactions with trace metals, it is important to note that tolerance to metals in pure culture does not necessarily predict the effects of metal contamination on ectomycorrhizal fungi in conjunction with a host plant (Colpaert and VanAssche, 1992; Hartley et al, 1997; Jones and Hutchinson, 1988). Combining axenic

screening with symbiosis experiments in the presence of trace metals is necessary to determine the effect of trace metals on ectomycorrhizal development and colonization, and is the only way to effectively understand how a given host-fungus partnership will respond to trace metal contamination. Such combined studies will also lead to better understanding of the relationship between fungal physiology and mycorrhizal ecology in the laboratory and in the field.

Table 2.1. Relative growth achieved over a 5 week period by three ectomycorrhizal fungi on solid growth media amended with copper, expressed as a percent of growth on unamended media[1,2]

Fungus	µg/g copper in agar media						
	25	50	75	100	250	500	1000
Suillus granulatus	61.5b[3]	48.2c	43.3d	37.8e	0f	0f	0f
Paxillus involutus	49.0b	0c	0c	0c	0c	0c	0c
Pisolithus tinctorius	93.5a	41.0b	44.0b	43.8b	0c	0c	0c

[1]Relative growth was measured as the colony diameter in mm and was determined by dividing the average colony diameter growth of a fungus grown on copper amended media by the average colony diameter of the same fungus grown on unamended media.

[2]Number of replicates equals 6 except for 250, 500, and 1000 ppm copper, which were performed in triplicate (n = 3)

[3]Numbers in the same row followed by the same letter are not significantly different at P = 0.05 according to ANOVA followed by Tukey's HSD test.

Table 2.2. Relative growth achieved over a 5 week period by three ectomycorrhizal fungi on solid growth media amended with zinc, expressed as a percent of growth on unamended media[1,2]

Fungus	µg/g zinc in agar media						
	25	50	75	100	250	500	1000
Suillus granulatus	92.8a[3]	90.8a	98.3a	80.4a	28.0b	25.0b	11.3b
Paxillus involutus	85.4b	78.0c	73.5c	65.3d	0e	0e	0e
Pisolithus tinctorius	61.5b	35.3c	20.7d	15.1d	0e	0e	0e

[1]Relative growth was measured as the colony diameter in mm and was determined by dividing the average colony diameter growth of a fungus grown on copper amended media by the average colony diameter of the same fungus grown on unamended media.

[2]Number of replicates equals 6 except for 250, 500, and 1000 ppm copper, which were performed in triplicate (n = 3)

[3]Numbers in the same row followed by the same letter are not significantly different at P = 0.05 according to ANOVA followed by Tukey's HSD test.

Table 2.3. Relative biomass of three species of ectomycorrhizal fungi grown in copper- and zinc- amended media (n=5)[1]

Fungus	mg/L copper in solution					mg/L zinc in solution			
	0	25	50	75	100	25	50	75	100
Suillus granulatus	100 ab[2]	77.8bc	15.2d	15.9d	23.5cd	100ab	62.7bcd	122ab	149a
Paxillus involutus	100a	65.3a	49.2ab	48.9ab	0b	65.7a	72.3a	83.8a	50.44ab
Pisolithus tinctorius	100ab	110a	84.4b	10.3d	12.7d	35.4c	20.2cd	17.7d	11.6d

[1]Relative growth was measured as biomass in mg and was determined by dividing the average biomass of a fungus grown in metal amended media by the average biomass of the same fungus grown on unamended media over a 5 week period.

[2]Numbers in the same row followed by the same letter are not significantly different at P = 0.05 according to ANOVA followed by Tukey's HSD test.

Table 2.4. Relative growth of sectors of *Suillus granulatus* (VT 1990) on agar media amended with high levels of copper (n=3)[1]

Sector	µg/g copper in agar media			
	100	250	500	1000
VT 1990 (parent)	41.1b[2]	0c	0c	0c
A	0b	0b	0b	0b
B	7.7b	10.2b	0b	0b
C	0b	0b	0b	0b
D	0b	0b	0b	0b
E	23.9b	0c	0c	0c
F	0b	0b	0b	0b
G	0b	0b	0b	0b
H	43.6b	28.8bc	18.9cd	5.3d
VT 2176	0b	0b	0b	0b

[1]Relative growth was measured as the colony diameter in mm and was determined by dividing the average colony diameter growth of a fungus grown on copper amended media by the average colony diameter of the same fungus grown on unamended media.

[2]Numbers in the same row followed by the same letter are not significantly different at P = 0.05 according toTukey's HSD test.

Table 2.5: Relative growth of sectors of *Suillus granulatus* (VT 1990) on agar media amended with high levels of zinc (n=3)[1]

Sector	µg/g zinc in agar media			
	100	250	500	1000
VT 1990 (parent)	63.1b	56.0bc	50.0c	22.6d
A	85.5ab	45.7bc	14.4c	0c
B	71.8b	39.7c	23.0c	0d
C	86.8a	63.5b	45.5c	24.5d
D	112a	83.3b	42.9c	0d
E	90.6a	65.8b	15.1c	21.6c
F	122 a	101a	50.8b	12.2c
G	75.5b	62.7c	42.7d	5.8e
H	118a	62.1b	57.2b	21.6c
VT 2176	124a	52.0b	0c	0c

[1]Relative growth was measured as the colony diameter in mm and was determined by dividing the average colony diameter growth of a fungus grown on copper amended media by the average colony diameter of the same fungus grown on unamended media.

[2]Numbers in the same row followed by the same letter are not significantly different at P = 0.05 according to ANOVA followed by Tukey's HSD test.

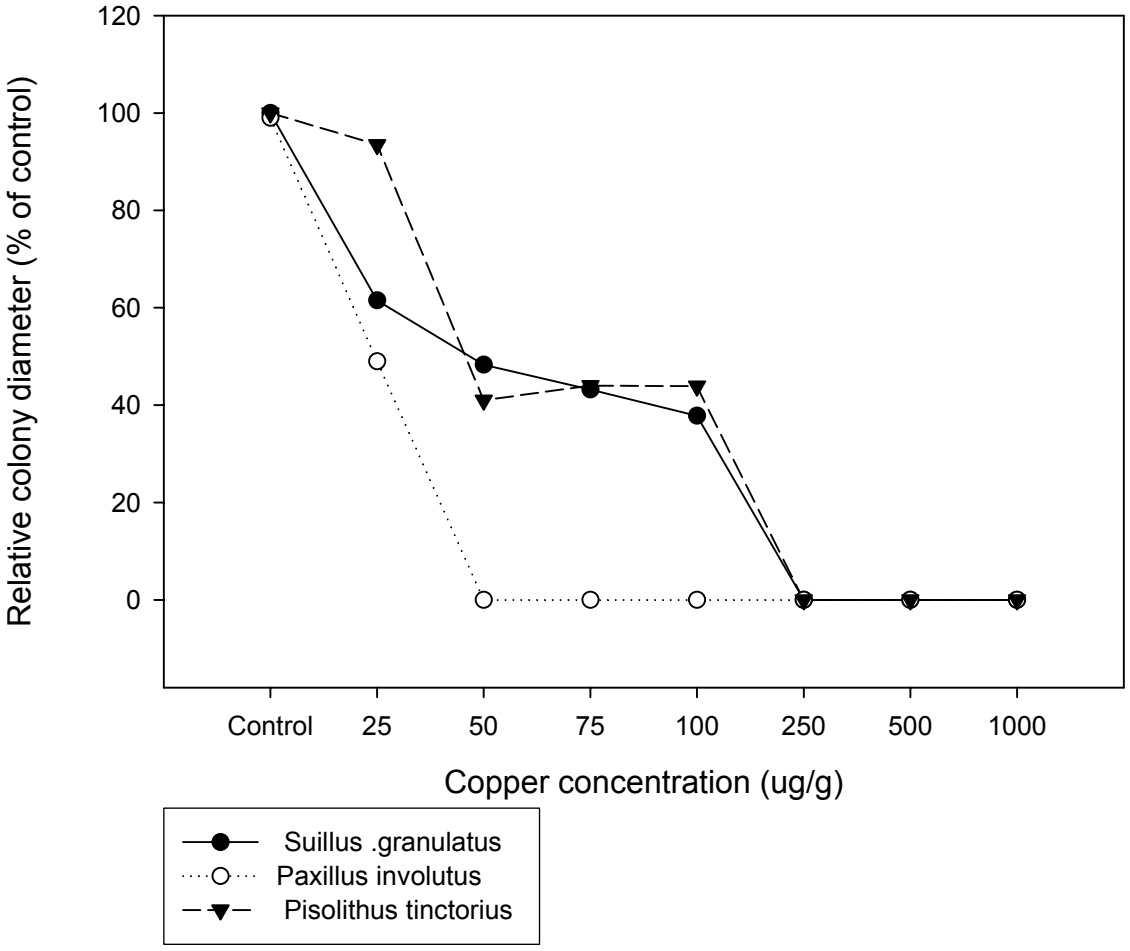

Figure 2.1. Relative colony diameter, expressed as a percent of the control, of three ectomycorrhizal fungi on copper amended media

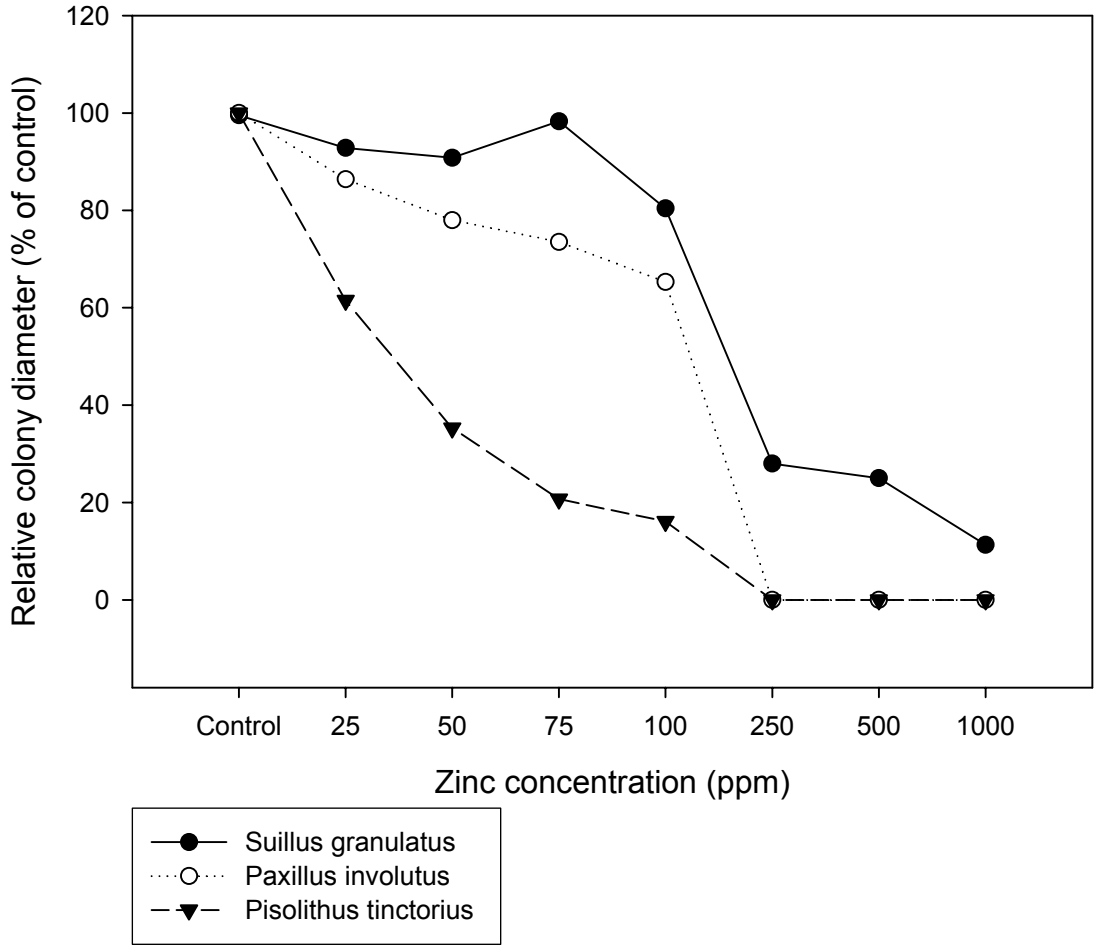

Figure 2.2. Relative colony diameter, expressed as a percent of the control, of three ectomycorrhizal fungi on zinc-amended medi

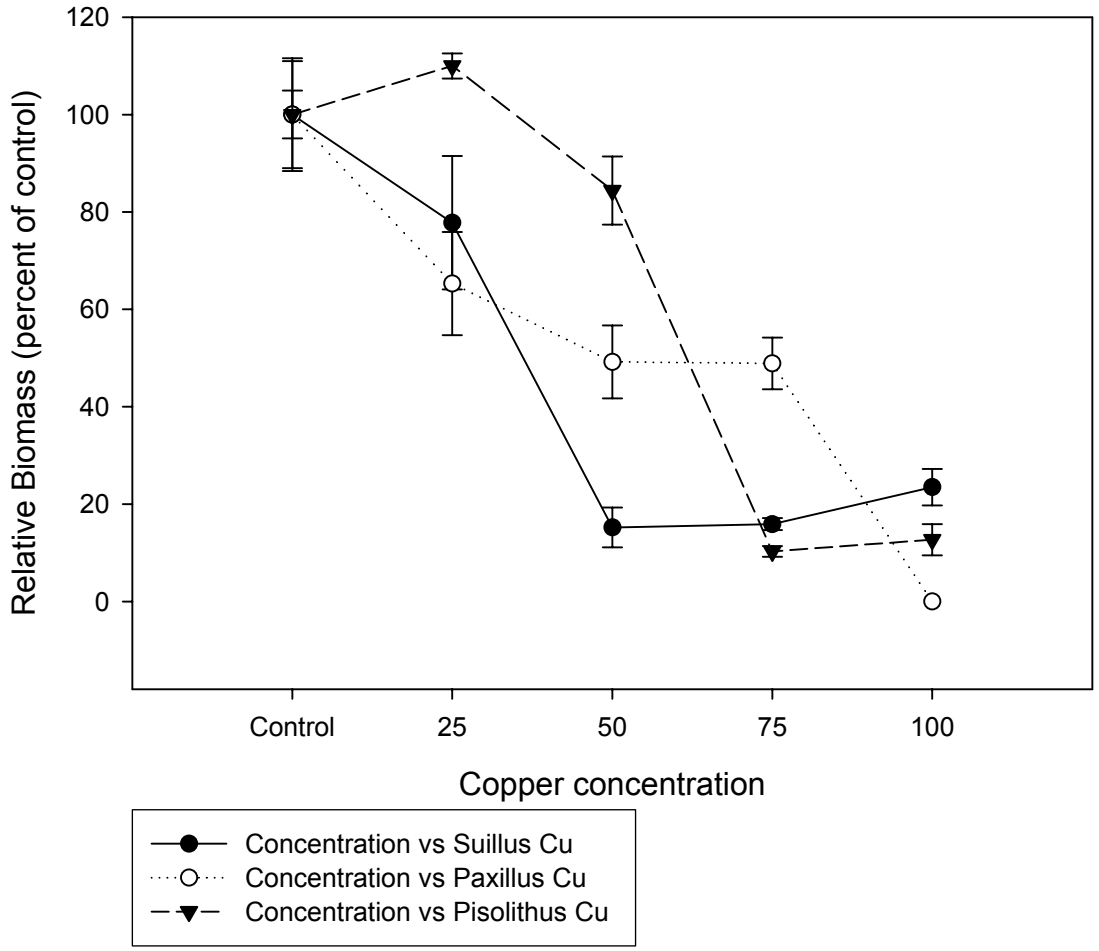

Figure 2.3. Relative biomass, expressed as a percent of the control, of three species of ectomycorrhizal fungi in copper-amended liquid media.

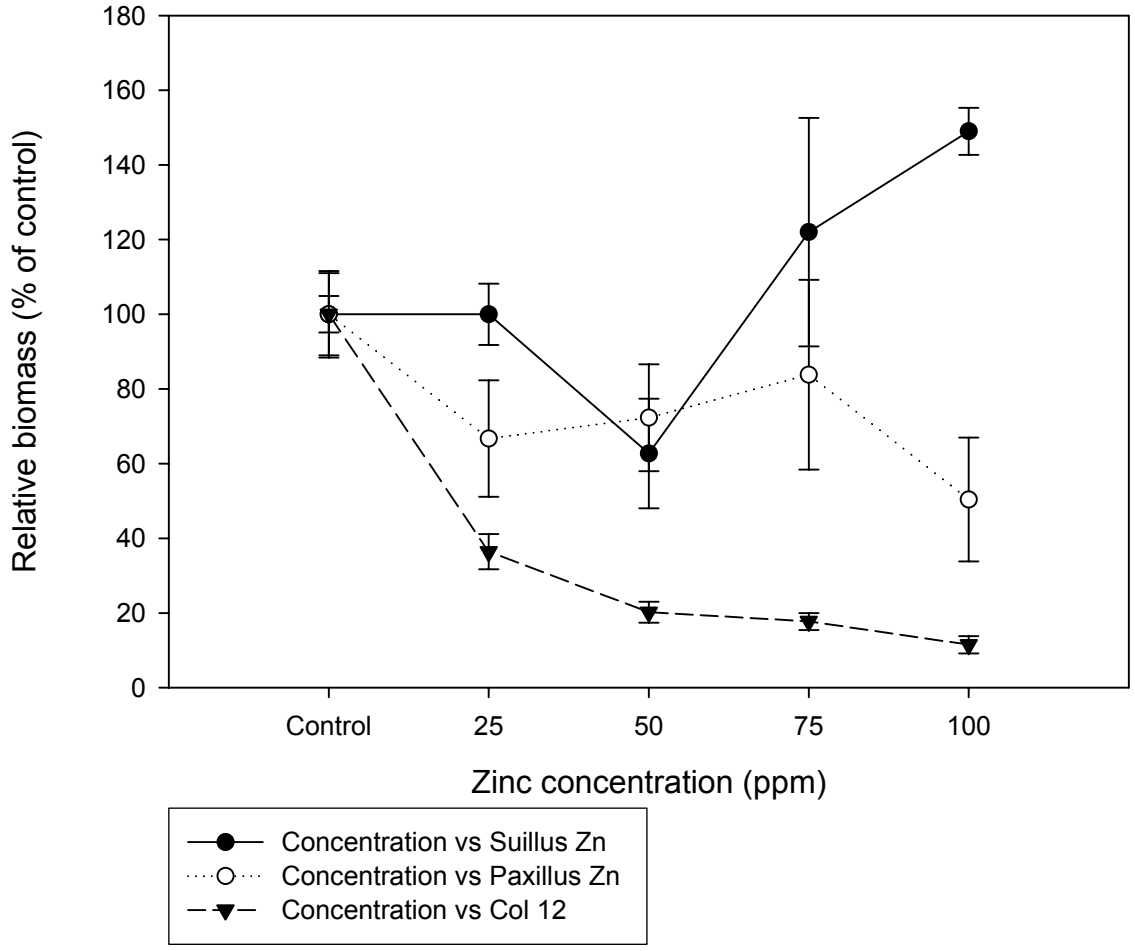

Figure 2.4. Relative biomass, expressed as a percent of the control, of three species of ectomycorrhizal fungi in zinc-amended liquid media.

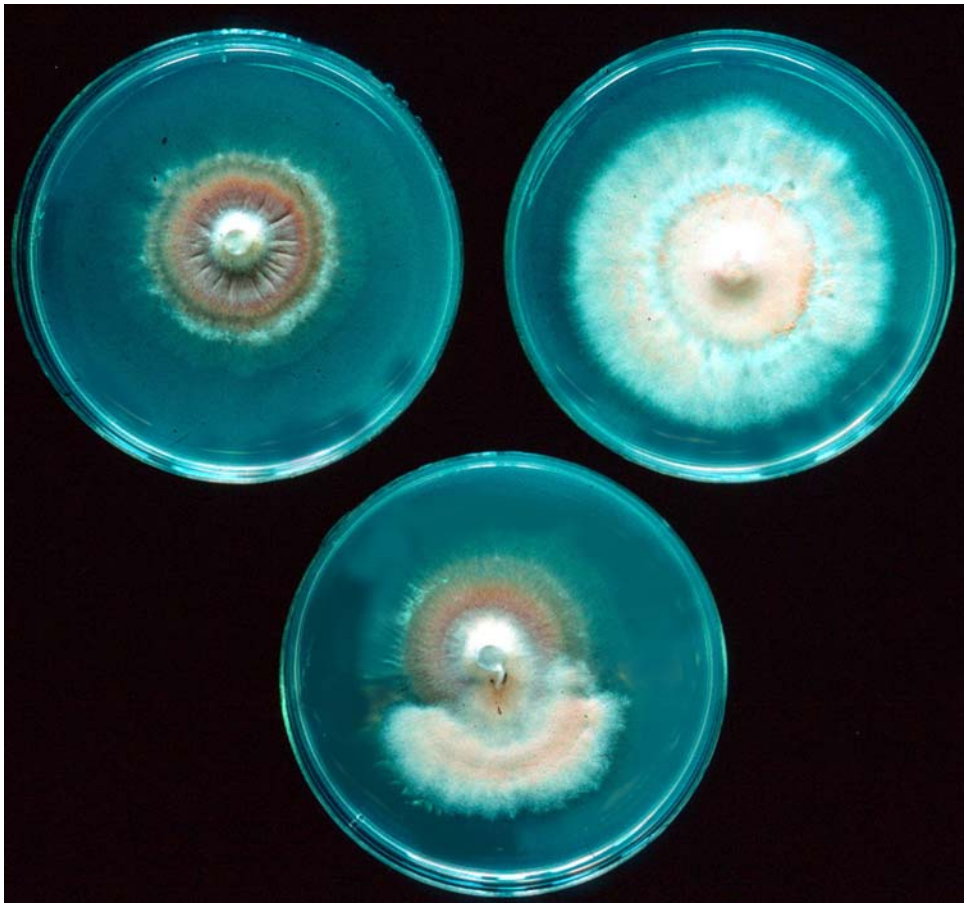

Figure 2.5**.** Sectoring response in *Suillus granulatus* VT 1990 on PDM agar amended with 250 ppm zinc. (5 weeks growth period). Top left: *Suillus granulatus* VT 1990 on PDM amended with 250 ppm Zn, typical colony morphology. Top right: *Suillus granulatus* VT 1990 on unamended PDM, typical growth and morphology. Bottom center: "Sectors" of *Suillus granulatus* VT 1990 on PDM amended with 250 ppm Zn.

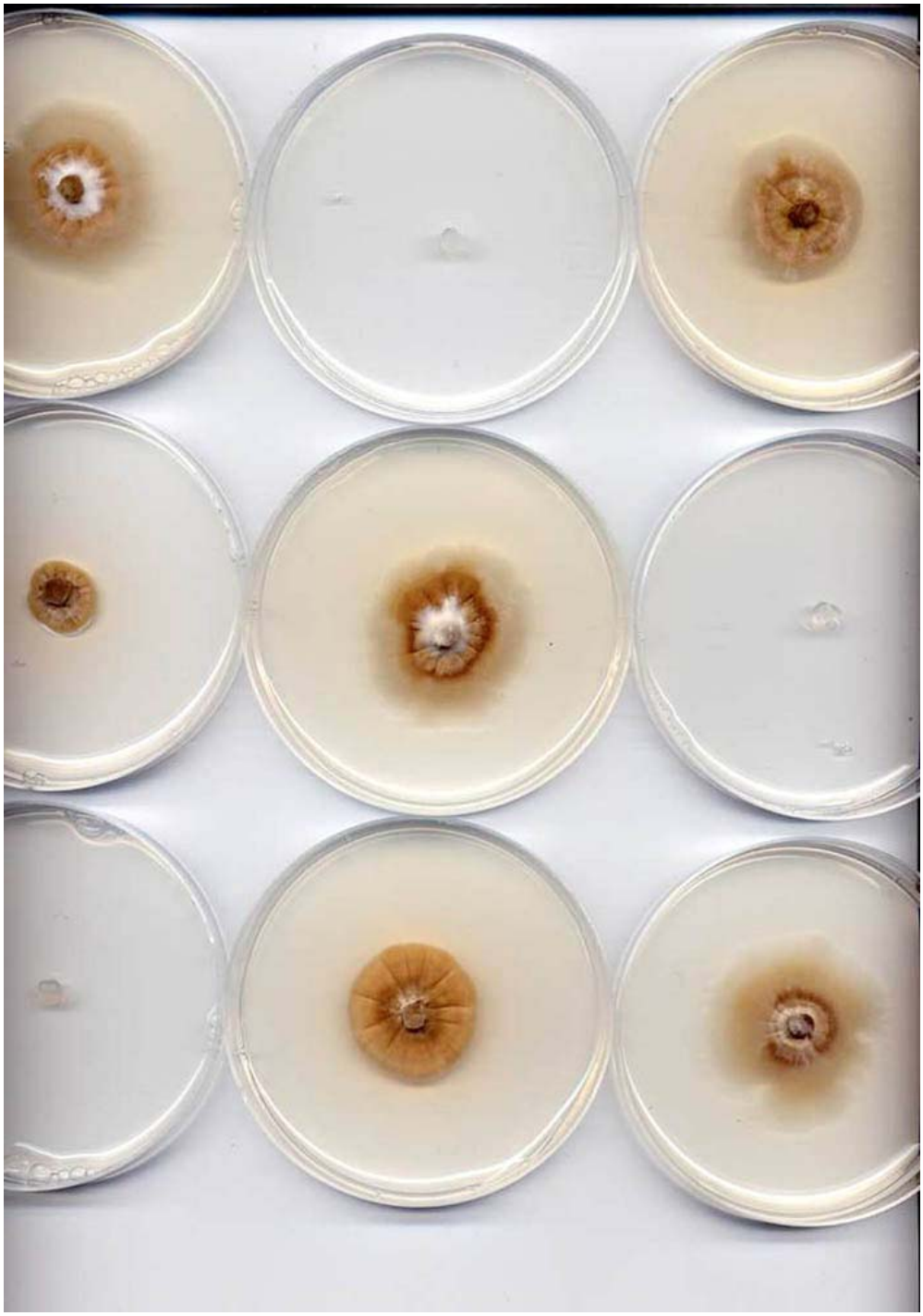

Figure 2.6. Cultures of 8 week old *Suillus granulatus* cultures grown on PDM agar amended with 100 ppm copper. Top row, left to right: VT 1990, Sector A, Sector B. Middle row, left to right: Sector F, Sector E, Sector D. Bottom row, left to right: Sector G, Sector C, Sector H.

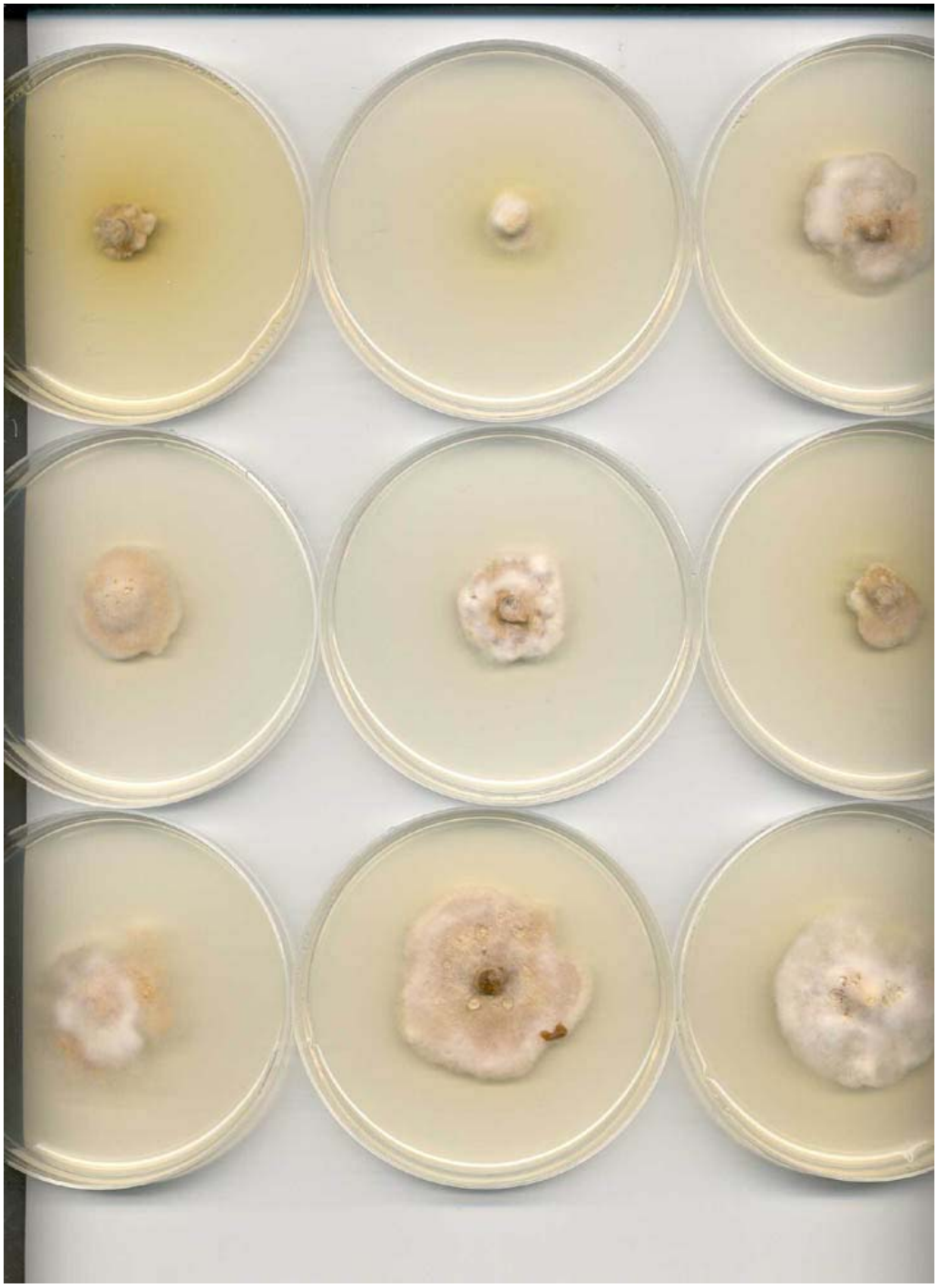

Figure 2.7. Cultures of 8 week old *Suillus granulatus* cultures grown on PDM agar amended with 1000 ppm zinc**.** Top row, left to right: VT 1990, Sector A, Sector B. Middle row, left to right: Sector F, Sector E, Sector D. Bottom row, left to right: Sector G, Sector C, Sector H.

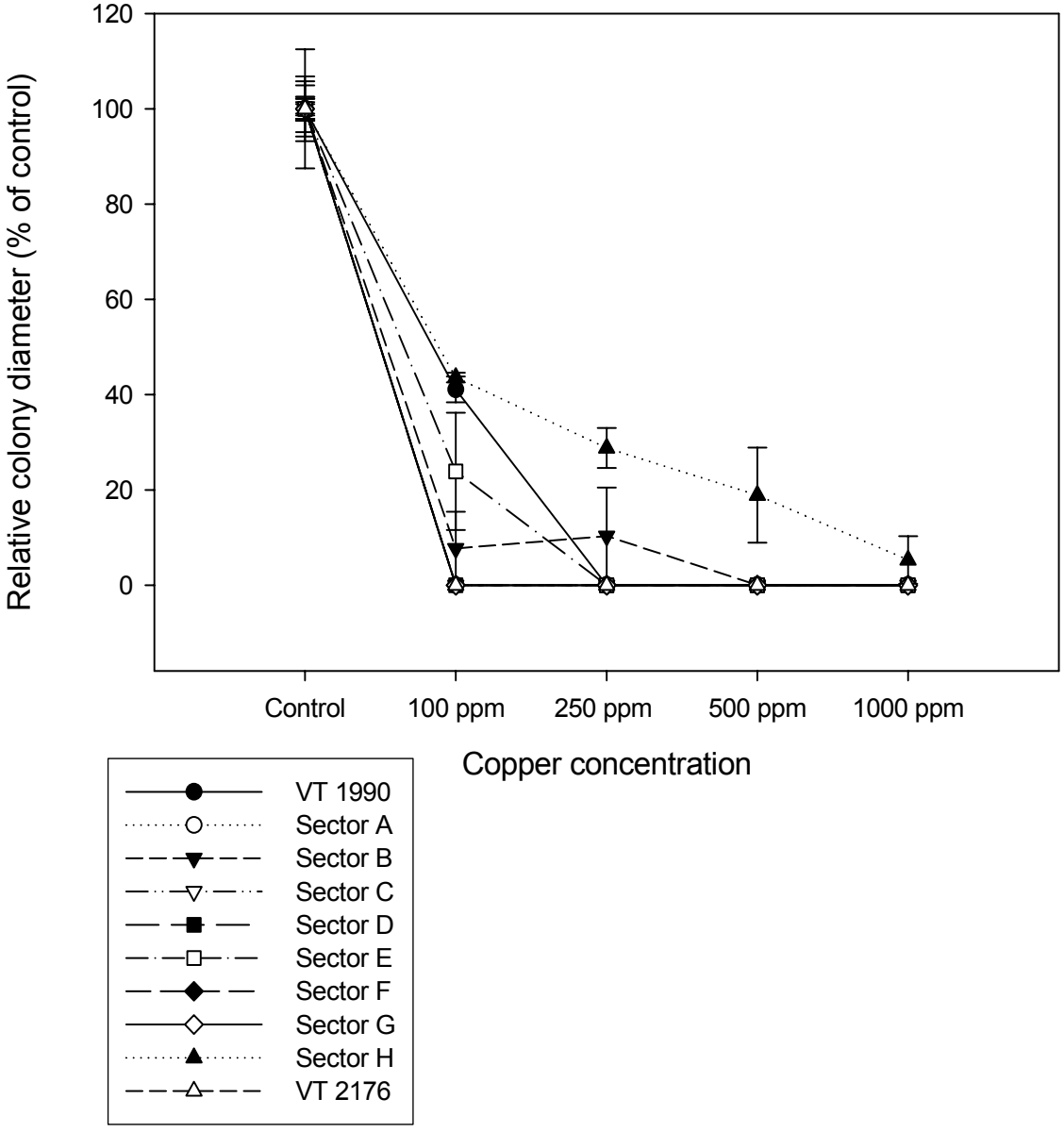

Figure 2.8. Relative colony diameters, expressed as a percent of the control, for *Suillus granulatus* isolates grown on copper-amended media.

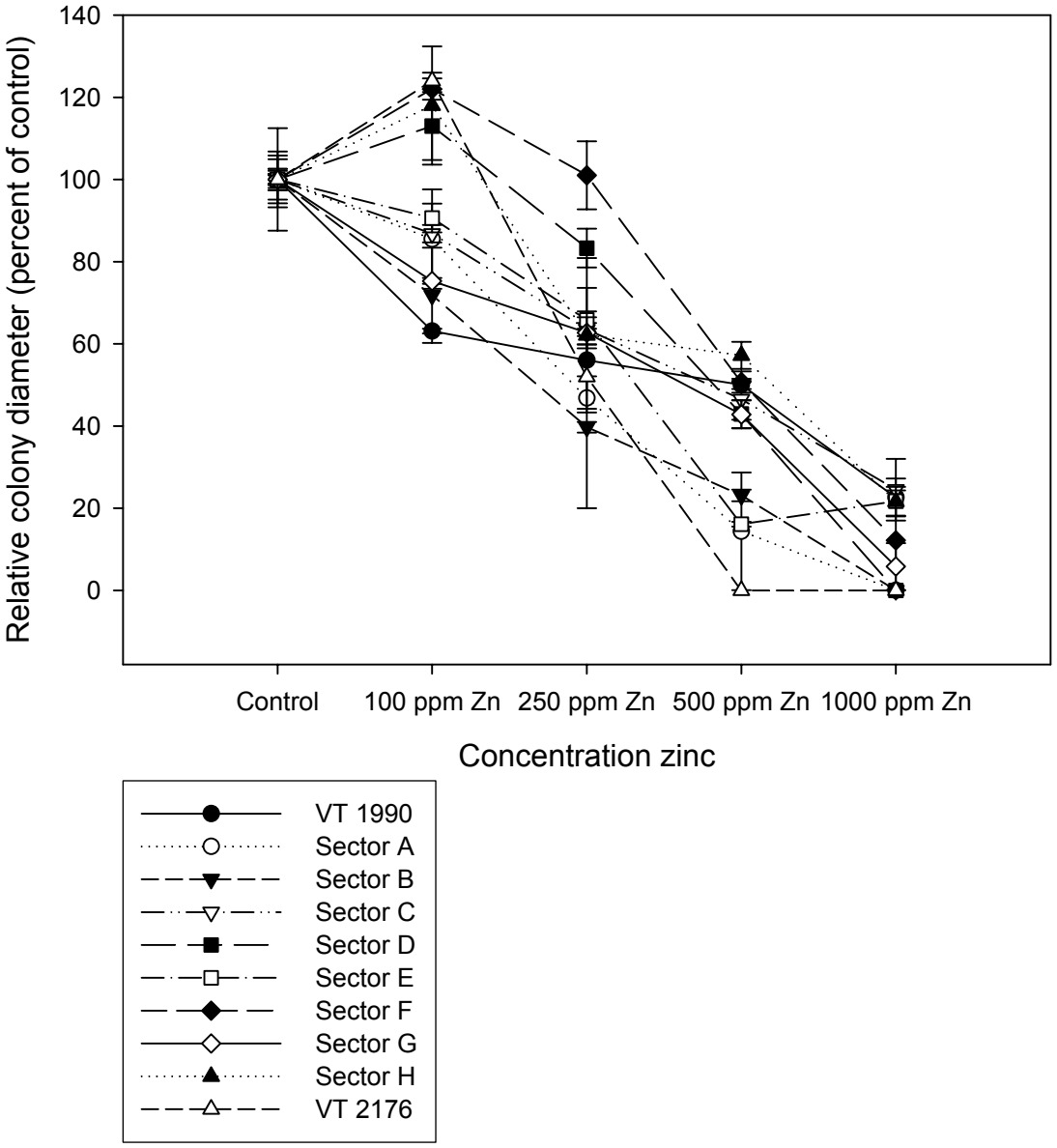

Figure 2.9. Relative colony diameters, expressed as a percent of the control, for *Suillus granulatus* isolates grown on zinc-amended media.

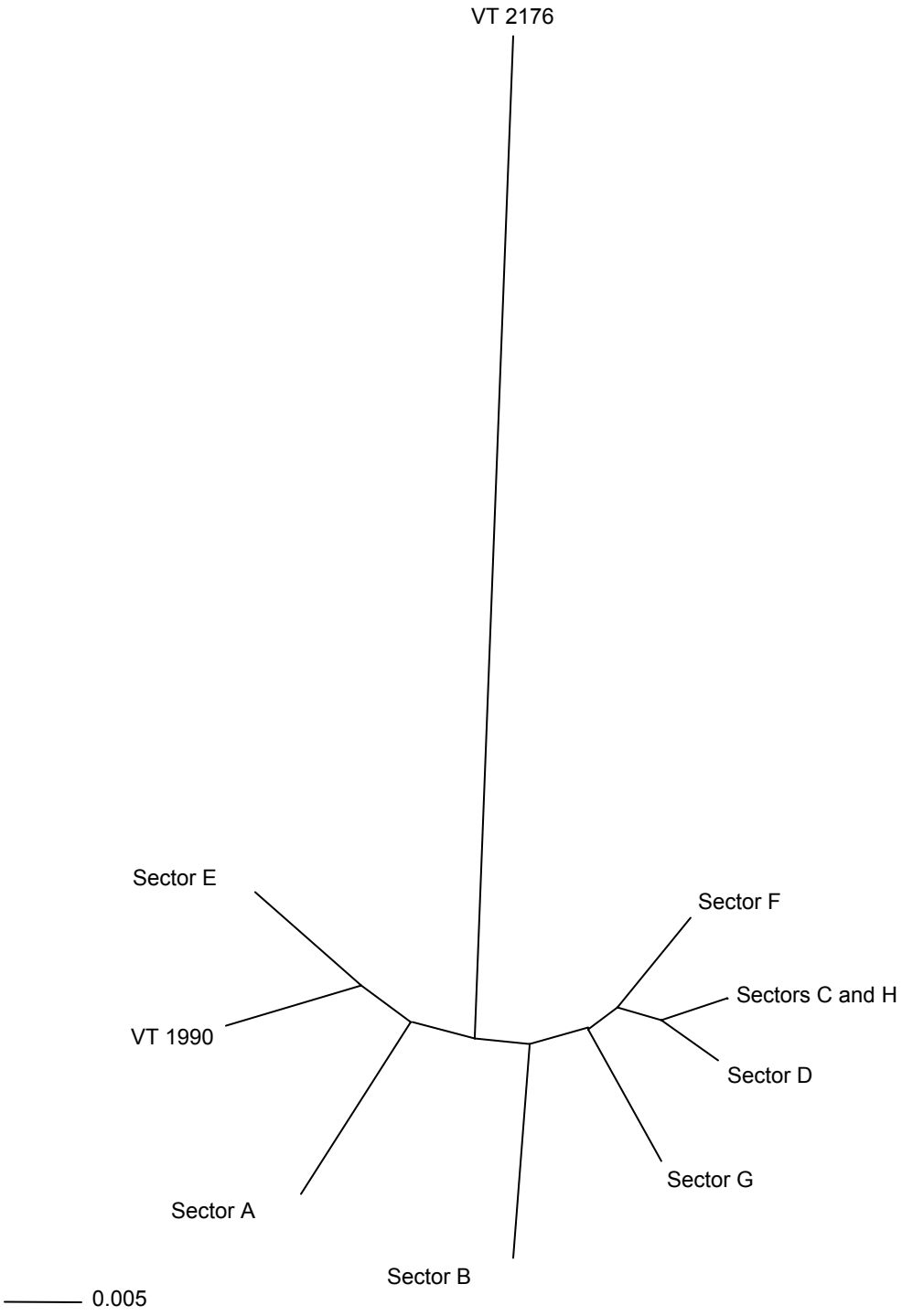

Figure 2.10. Unrooted UPGMA phylogram showing relationships of *Suillus granulatus* isolates based on ISSR fingerprinting and Nei-Li distance analysis.

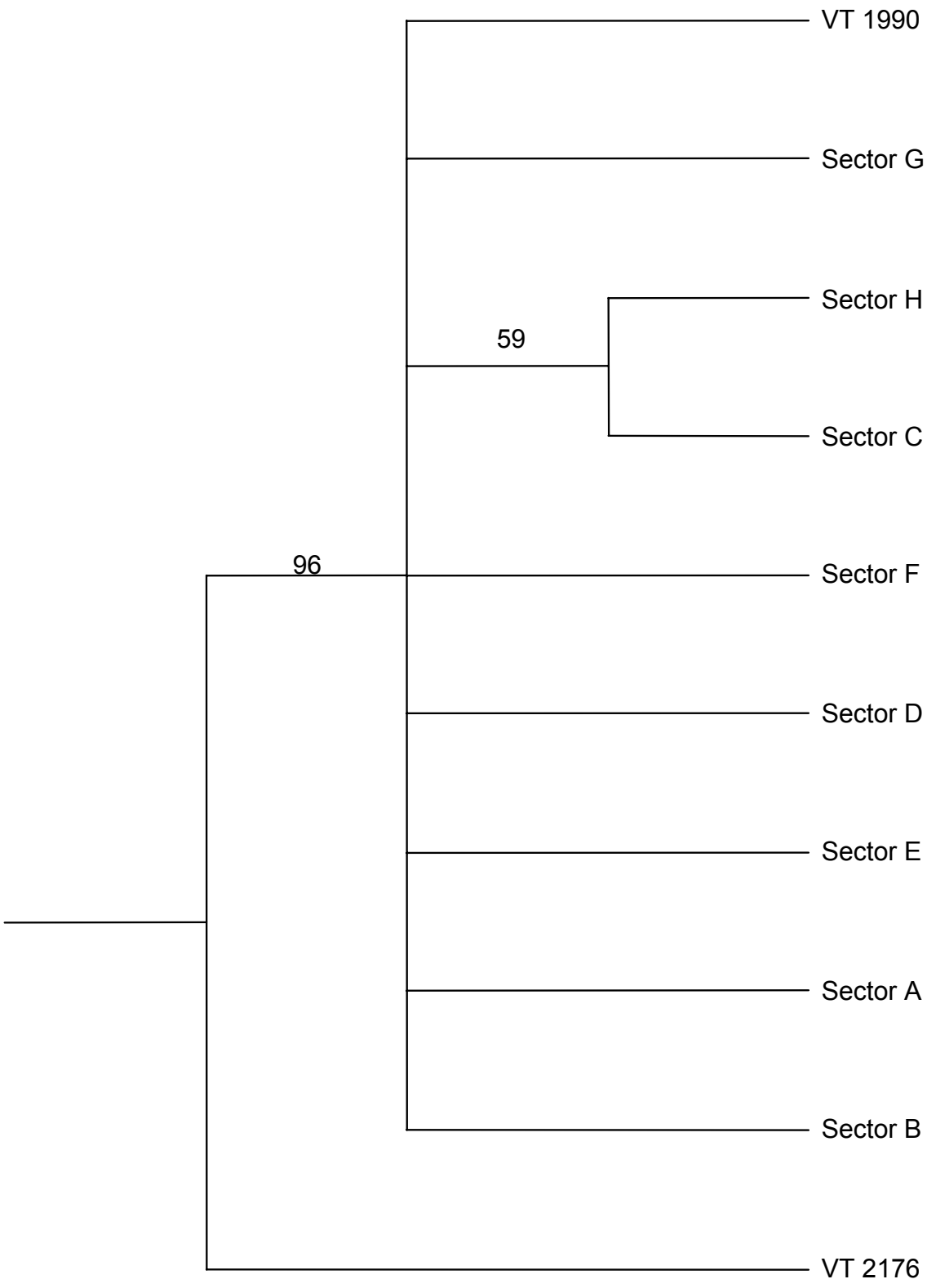

Figure 2.11. Bootstrap cladogram showing relationship between VT 1990 isolates and VT 2176 based on ISSR fingerprinting. Analysis was performed using UPGMA/Nei-Li distance analysis with 100 replicates.

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Chapter 3

Evaluation of Cu and Zn adsorption on an acid coastal plain soil from Virginia: Implications for mycorrhizal and revegetation research.

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Abstract

Adsorption of Cu and Zn to an acidic, low organic matter content loamy fine sand soil from the Coastal Plain of Virginia was investigated. Adsorption isotherms were performed with each metal in two systems: in the first, pH was not controlled and was allowed to change with increasing concentrations of metals. For the second set of isotherms, pH was held constant at 4.5 the unamended soil pH of 4.5. Selective extraction experiments were performed to determine which fraction(s) of the soil sorbed Cu and Zn.

Cu and Zn exhibited considerable sorption to the soil surface in both the pH-variable and pH-controlled isotherm experiments. For both Cu and Zn, adsorption increased exponentially as the concentration of the added metal increased. Cu showed the most adsorption to the soil surface regardless of pH control. For both Cu and Zn the non-crystalline material was responsible for most of the metal adsorption. Organic matter present in the soil adsorbed small quantities of both Cu and Zn (<5%).

Mycorrhizal growth and colonization are affected by soil conditions, including metal concentrations, organic matter content and soil pH; however, the study demonstrated that even soils with low pH and organic matter content can sorb significant amounts of metals. Metal sorption by soils can significantly impact mycorrhizal research. Metals that are assumed to be in solution and available to the fungus and host plant actually could be held to the soil surface and not bioavailable. Assumptions about metal availability could lead to the erroneous conclusions about mycorrhizal ability to ameliorate metal phytotoxicity. We discuss the necessity of careful soil analysis in avoiding these errors in plant and mycorrhizal research.

Keywords: Adsorption isotherms, Langmuir equation, Cu, Zn, selective extraction

Introduction

Trace metal contamination in soils is caused largely by anthropogenic activities such as mining, burning and smelting of coal, metal plating and finishing operations, and use of leaded gasoline. Trace metal pollutants are unique in that they are not broken down into harmless components over time, but rather accumulate in the soil. Accumulation of trace metals can create phytotoxicity of soils near the contamination source. Plant uptake of these metals is of serious concern. Potential dangers include the introduction of these elements into the food chain through uptake by food and forage crops, the ecological devastation of established forests through metal depositions from the air, reduction of crop yields due to phytotoxicity, and unsuccessful revegetation efforts due to lack of seedling establishment on contaminated soils.

Elevated levels of trace metals in the soil rhizosphere primarily result in damaged root tissues. This creates an inability in the plant to take up key nutrients such as N, P, and essential micronutrients. Certainly, increased trace metal content in root tissues has been positively linked with decreased macronutrient content (Brunner and Frey, 2000). Additionally, some metals can precipitate with P, which could significantly decrease P availability (Gadd, 1993).

Cu is rarely found at levels toxic to humans in soil; yet it is extremely toxic to plants. High Cu levels will reduce root elongation and branching, therefore inhibiting nutrient uptake (Arudini et al., 1995). Nutrient limitation severely inhibits plant growth, especially in seedlings, which are more sensitive to elevated metal levels than mature plants. Additionally, seedling roots grow and develop in surface soils, which can have the highest amount of metal contamination (Patterson and Olson, 1983). These phytotoxic effects severely limit the potential for revegetation of metal contaminated sites.

A number of authors have suggested that ectomycorrhizal colonization of seedlings can significantly reduce phytotoxic effects of trace metals (Colpaert and VanAssche, 1992; Dixon, 1988; Jentschke and Godbold, 2000; Jones and Hutchinson, 1986). Ectomycorrhizal fungi are ubiquitous in forest ecosystems, and form associations with a number of plant species, notably tree species in the Pinaceae, Fagaceae, and Betulaceae in the Northern Hemisphere, and Myrtaceae in the Southern Hemisphere. Ectomycorrhizal associations play an important role in nutrient acquisition, especially that of N and P (Smith and Read, 1997). Increased plant health in metal-stressed environments may be the result of mycorrhizae-assisted nutrient uptake.

Mycorrhizal roots have a much greater surface area than non-mycorrhizal roots, and this has the dual effect of creating more sites for nutrient absorption as well as allowing for nutrient foraging across a greater distance (Allen, 1991). Several studies have indicated that ectomycorrhizal colonization reduced metal phytotoxicity by binding the metals either intra- or intercellularly, preventing damage to the host plant (Bucking and Heyser, 1994; Colpaert and VanAssche, 1992; Gruhn, 1989). Mycorrhizal growth and colonization is also affected by soil conditions, including metal concentrations and soil pH. Although ectomycorrhizal species have generally been demonstrated to be more tolerant to elevated metal levels than seedlings, this is not always the case. In some cases, the mycobiont in an ectomycorrhizal symbiosis may be more sensitive to elevated metal levels than the host plant (Hartley-Whitaker et al., 2000a, Hartley-Whitaker et al., 2000b). Variations in mycorrhizal response to soil conditions result in inconsistent success rates for revegetation experiments. Therefore, ectomycorrhizal function needs to be studied in relation to soil conditions.

Few investigations have been done with plants grown in soils containing elevated metal levels rather than in artificial media (Jentschke and Godbold, 2000). Furthermore, several of

these investigations contain relatively little chemical analysis of the study soil beyond pH, total metal concentration, and organic matter level (Hartley et al., 1999; Hartley-Whitaker et al., 2000a, Hartley-Whitaker et al., 2000b). Few studies reported extractable metal concentrations (Dixon, 1988). Trace metal cations have a high affinity for organic and mineral surfaces found in soils. It is generally accepted that reactions at the particle-water interface control trace metal potential bioavailability in soil systems (Backes et al., 1995). For example, Fe- and Al-oxides strongly chemisorb trace metals such as Pb and Cu, reducing their bioavailability to growing plants (McBride, 1994). Metals that are held strongly on the soil surface will not be available to the plants and fungi in the systems and therefore will not be considered toxic, at least in the short term. Hence, ectomycorrhizal metal tolerance observed in some studies using natural soil may be a result of sorption reactions reducing the metals bioavailability.

Accordingly, the objectives of this study are to determine the quantity and form of Cu and Zn sorbed to a low organic matter acid coastal plain soil from Virginia. This soil was chosen because, based on its chemical and physical properties, it was expected to sorb only small amounts of Cu and Zn, making it ideally suited for evaluating ectomycorrhizal metal tolerance. Adsorption isotherms were performed in order to determine the amount of Cu and Zn adsorbed to the soil. Selective extraction experiments were performed in order to determine whether the sorbed metals were held in an exchangeable phase, meaning that they would remain bioavailable, or whether they were sorbed in a non-available form. This study provides important information on the bioavailability of adsorbed metal to the plant or fungus, allowing a more accurate assessment concerning possible interactions between the mycorrhizal system and the metal in the soil, as well as better experimental design for later mycorrhizal studies.

Materials and Methods

Soil collection and characterization

The study soil was collected from the upland cut bank of a wetland mitigation site in Charles City County, Virginia. The soil belongs to the loamy, kaolinitic, thermic Arenic Kanhapludults of the Uchee series, and is described as having strong to very strong acidity, low to moderate water capacity, and low organic matter content and natural fertility (Hodges et al., 1990; W. Daniels, pers. comm., Robert Hodges, pers. comm.). Seedling survival is most often limited by low water and nutrient availability The soil was passed through a 2 mm sieve to remove occasional plant debris and gravel. It was then sterilized by autoclaving and dried in a 60 degree drying oven. Prior to adsorption and extraction experiments, basic chemical and physical properties were determined using standard techniques (Tables 1 and 2). Soil pH was determined in a 1:1 soil/solution ratio using a Fisher Accument model 50 pH meter and a combination electrode (Fisher Scientific Corporation, Atlanta, Georgia). Cation exchange capacity was determined using the ammonium acetate (pH 7) method (Soil Survey Laboratory Staff, 1992). Particle size was determined by the hydrometer method (Day, 1965). The Walkley-Black Method was used to quantitatively determine organic matter content of the soil (Walkley and Black, 1934). Free iron oxides were quantitatively determined by citrate-ditionite buffered with sodium carbonate (CDB) (Mehra and Jackson, 1960). The percent of noncrystalline material present in the clay fraction was determined by measuring the weight loss resulting from treatment with ammonium oxalate in the dark (AOD) (Fey and LeRoux, 1977; Hodges and Zelazny, 1980). Soils were pre-treated, fractionated, and saturated for mineralogical analysis using standard procedures (Kunze and Dixon, 1986; Whittig and Allardice, 1986). The mineralogy of the <2 μm clay fraction was determined using X-ray diffraction. Oriented mounts

of the clay fraction were prepared by depositing approximately 0.50 g on a ceramic tile. Each sample was analyzed using a Scintag XDS-2000 diffractometer. The mounts were scanned at 2° 2θ min^{-1} using Cu Kα radiation and a graphite monochromater.

Adsorption Isotherms

Isotherm experiments were performed for the adsorption of Cu and Zn to the study soil in two systems: in the first, pH was not controlled and was allowed to change with increasing concentrations of metals. The second set of isotherms was carried out in a buffer system designed to hold the pH at 4.5, which approximated the pH of the unamended soil. Throughout the experiments, Cu and Zn were added to the soil as sulfate salts dissolved in a synthetic mycorrhizal growth medium (Palmer's Defined Medium; Palmer, 1971). We chose sulfate salts due to the expected low toxicity of the sulfate anions to ectomycorrhizal fungi. PDM is a synthetic medium that has been shown to support fungal growth and limit complexation reactions with trace metals, leaving them mostly available to the fungi and host plant (C.M. Gruhn, pers. comm.). Cu and Zn concentrations were designed to be 50, 100, 250, 500, and 1000 parts per million (ppm) for both experiments. For the pH-variable isotherms, 40 grams of soil were placed into acid washed, 100 mL screw cap test tubes. Metal-amended media was added in the volume of 40 mL, to achieve a 1:1 solid/solution ratio. Isotherm experiments were done separately for Cu and Zn for a total of ten treatments. Each treatment was performed in duplicate. Once the soil and media had been added, the tube mouths were covered in Parafilm and the caps were secured tightly. Each sample was shaken vigorously by hand and then placed on a reciprocating shaker for a 24 hour equilibration period. After 24 hours, the samples were removed from the shaker. Solution pH was measured for all twenty samples (Table 3.3). Ten milliliters of the mixture were then pipetted from the tubes and passed through a 0.20 μm filter

by vacuum filtration. Six to eight drops of concentrated nitric acid were added to the filtrate samples to prevent metal precipitation. The filtrate was analyzed for Cu or Zn by inductively coupled plasma atomic emission spectrometry (ICP-AES).

In order to ensure a constant pH of 4.5, the second set of isotherms was done using a potentiometric titrator (718 STAT, Metrohm Switzerland). Each isotherm for a given metal was carried out in a single reaction vessel containing 200 grams of soil and 400 mL media solution for a 1:2 solid/solution ratio. The vessel was placed on a stir plate with a stir bar to ensure equal distribution of the soil/media mixture. Metals were added incrementally to the reaction vessel, with two hours allotted between amendments as equilibration time. Preliminary results indicated that sorption was essentially complete after 2 hours. The pH of the reaction vessel was kept constant through the addition of 0.10 M NaOH. After each two hour interval, two six-milliliter samples were taken and filtered through a 0.20 μm filter and analyzed for Cu and Zn using ICP-AES.

Isotherm data was plotted based on the Langmuir equation,

$$q = kCb / (1 + kC). \qquad \text{Eq. 1}$$

In this equation, q represents the quantity adsorbed to the surface in parts per million (ppm), C represents the equilibrium solution concentration (ppm), k is a constant related to bonding strength, and b is the adsorption maximum. Based on this, data are plotted as q, the adsorbed quantity, versus C, the equilibrium concentration. The linear form of the Langmuir equation,

$$C/q = 1/kb + C/q \qquad \text{Eq. 2}$$

was used to calculate the adsorption maximum (b) and binding constant (k) for both Cu and Zn. Regression analysis of C/q versus C was performed in Sigma Plot version 8.0 (Sigma Plot

Software Systems) in order to plot the data in linear form and calculate adsorption maximum and binding constant values.

Selective Extractions

Selective extraction experiments enhance understanding of metal adsorption to soils by indicating to which soil fraction (s) the metals are binding, thereby giving information on the potential bioavailability of sorbed metals. Selective extraction for Zn and Cu was performed for exchangeable, carbonate, crystalline Mn- and Fe-oxides, noncrystalline Mn- and Fe-oxides, and organic matter phases. Ten grams of soil were weighed out and placed in pre-weighed, acid-washed 50 mL centrifuge tubes. The tubes were weighed again after the addition of the study soil to get the exact mass of the added soil. Twenty mL PDM amended with 100 ppm of either Cu or Zn were added to the tubes and the system was allowed to equilibrate for 48 hours. After 48 hours, the tubes were centrifuged for 5 minutes at 3000g, at which point the supernatant was decanted and the tubes were weighed a third time.

The following described extraction methods were modified from Amacher, 1999. For the exchangeable phase, 20 mL of 1M NH_4Cl was added to the tubes, vortexed, and placed on a rotational shaker at 120 cycles per minute for two hours. Manganese oxide extraction was done with 25 mL of pH 2, 0.1 M $NH_2OH{\cdot}HCl$. The mixture was vortexed and shaken for 30 minutes. Crystalline Fe oxides extraction was done by adding 10 mL of 0.3 M ammonium oxalate + 0.3 M oxalic acid and 10 mL 0.3 M ascorbic acid. This was vortexed, placed in a boiling water bath for 30 minutes, and allowed to cool for 15 minutes. These steps were repeated one more time. Noncrystalline oxide extraction was performed by incubating the soil mixture on a shaker for two hours in the dark with 25 mL 0.3 M ammonium oxalate + 0.3 M oxalic acid. Finally, metals

associated with organic matter in the soils were extracted by adding 20 mL of 6% $NaClO_4$ at 85° C, which was vortexed and shaken for 24 hours. After shaking or incubation, all tubes were centrifuged at 1500g for 10 minutes. The supernatant was decanted, filtered, acidified, and diluted in a 1:20 ratio. The diluted filtrates were analyzed by ICP-AES spectroscopy.

Results

Adsorption Isotherms

Chemical, physical, and mineralogical properties of the Uchee loamy fine sand are listed in Tables 3.1 and 3.2. Cu and Zn exhibited considerable sorption to the soil surface in both the pH-variable and pH-controlled isotherm experiments. When no buffer system was in place, the system pH decreased significantly as metal concentration increased, as demonstrated in Table 3.3. For both Cu and Zn, adsorption increased at a declining rate as the concentration of the added metal increased (Figures 3.1-3.4). Regression analysis shows that the data fit well to the linear Langmuir equation (Table 3.4). Cu showed the most adsorption to the soil surface regardless of pH control. For both Cu and Zn, adsorption at the higher concentrations was increased when the pH was held constant at 4.5. This was expected since trace metal cation adsorption increases with an increase in pH. The amount of total added Cu adsorbed ranged between 34 and 87 percent with no pH adjustment. An inverse relationship exists between total amount of metal added and percent adsorbed, so the highest percent adsorption occurred at the lowest total metal added levels. When the pH was held at 4.5, Cu adsorption ranged between 48 and 76 percent. The adsorption maximums, calculated from the linear Langmuir equation, reflect this trend. Maximum Cu adsorption in this system was calculated to be 400 ppm with no pH control, and 667 ppm when the pH was constant at 4.5. The percent of total Zn adsorbed

ranged between 12 and 49 percent with no pH control, and between 27 and 64 percent with the pH buffered at 4.5. Maximum Zn adsorption was 145 ppm with no pH control, and 238 ppm at pH 4.5.

Selective extractions

For both Cu and Zn, the non-crystalline material was responsible for most of the metal adsorption (Table 3.5). Organic matter present in the soil adsorbed small quantities of both Cu and Zn (<5%). Non-crystalline materials in soils are generally composed of amorphous oxides of Al, Fe, and Si. These surfaces have a high specific surface area and reactive functional groups that strongly sorb trace metal cations. Based on the chemical and mineralogical properties of the soil one would expect very little adsorption on the exchangeable component due to the low CEC and low pH. Similarly one would expect very little adsorption on free Fe and Mn oxides due to their low concentration.

Discussion

The Uchee loamy fine sand used in these experiments was chosen for a mycorrhizal study based on its low cation exchange capacity, base saturation, pH, and organic matter content. Based on these characteristics, we assumed that Cu and Zn sorption on the soil surface would be low, resulting in most of the metals being present in bioavailable form. Contrary to expectations, a significant portion of both Cu and Zn were sorbed by the soil. Binding occurred in the non-crystalline fraction of the soil, meaning that the metals would likely be retained by the soil and not easily released into the solution phase. Experiments such as these set the stage for rigorous experimentation involving mycorrhizal and plant response to trace metals. Unless experimenters

know the fate of metals added to the experimental system, they can not draw conclusions regarding biotic response to a given metal dose.

Cu and Zn sorption is influenced by a number of soil properties, including pH, organic matter, particle composition, amorphous oxide content, and cation exchange capacity (McBride, 1989). In temperate soils, acidic pH often directly correlates to increased Cu and Zn in soil solution (Bruemmer et al., 1986; Gerritse and VanDriel, 1984; Miller et al. 1983; Scokart et al., 1983; Watmough and Dickinson, 1995). Alva et al. (2000) found that citrus seedlings suffered Cu phytotoxicity in two sandy soils with low organic matter content and pH values of 5.7 and 5.5. Seedling biomass was unaffected by increasing Cu concentration when grown in a low organic matter sandy soil with a pH of 8.2 (Alva et al., 2000).

Based on its low pH, cation exchange capacity, and organic matter content, we expected the Uchee loamy fine sand chosen for our experiments to adsorb only small amounts of Cu and Zn, allowing us to evaluate ectomycorrhizal metal tolerance in a natural soil. However, adsorption of Cu ranged between 34 to 87 percent of total Cu added, and the amount of Zn adsorbed was between 12 and 64 percent of the total added. Organic matter accounted for less than 5% of Cu and Zn sorption. The remaining 95% was sorbed to non-crystalline, amorphous metal oxides present in the soil. A number of studies have showed that divalent cation sorption to amorphous (hydrous) metal oxides follows the linear Langmuir model (Trivedi and Axe, 2000). Amorphous metal oxides have a sorption capacity for Cu second only to organic matter, and therefore are important in controlling Cu solubility in low organic matter soils (Baker, 1990). Furthermore, research has demonstrated that both Cu and Zn chemisorb and co-precipitate as metal oxides, reducing their potential bioavailability (McBride, 1992; Sparks et al., 1995). Column flow-through experiments showed that hydrous oxide surfaces were the primary site for

Zn sorption on two sandy loam soils with similar mineralogy but different organic-matter content, and for Cu on the soil with low organic matter. Cu and Zn release from the soil was low at acidic pH levels until the end of the experiment, when the pH dropped to near pH 3 (Gong and Donahoe, 1997).

Most studies involving ectomycorrhizal response to elevated metals have been performed in artifical growth media (Hartley et al., 1997). These studies provide important information on the effect metals may have on ectomycorrhizal-host symbioses, but to understand the potential for ectomycorrhizae-mediated amelioration of metal phytotoxicity, these relationships must be studied in natural soils. A series of studies involving cadmium and Zn sensitivity of ectomycorrhizae and *Pinus sylvestris* L. (Scots pine) seedlings was performed by Hartley-Whitaker et al (2000a, 2000b). One study involved a dose response of non-mycorrhizal and ectomycorrhizal seedlings in a 1:5 peat:vermiculite mixture. This study found that ectomycorrhizal fungi increased host biomass for all treatments, but that cadmium and Zn did not affect plant biomass in the roots or shoots. Root length was inhibited by cadmium and to a lesser extent by Zn (Hartley-Whitaker et al., 2000a). The lack of biomass response to potentially toxic metal amendments may be a result of metal sorption by the growth medium. Peat materials have the ability to capture large amounts of metals, limiting metal mobility at pH values above 3 (Ringqvist and Oborn, 2002). Zn adsorption by peat is more pH-dependent than that of Cu, but significant amounts of Zn may be sorbed even in acidic soils (Dumontet et al., 1990). Hartley-Whitaker et al. (2000b) performed a second experiment using natural soil inoculum in a natural sandy loam soil from a Scots pine forest. In this experiment, colonization of the host plants by ectomycorrhizal fungi were found to be more sensitive than seedling growth to increasing amounts of Cd or Zn. It was reported that that the soil used in the study had a pH of 3.3 ± 0.1

before planting, a pH of 3.8 ± 0.1 at harvest, and an organic matter content of 5.0 ± 0.9 %. The assumption made based on this analysis is that the metals would be "relatively available" to the seedlings (Hartley-Whitaker et al., 2000b). However, soils high in organic matter can strongly adsorb trace metal cations even at low pH values (Thomas, 1975).

The results of our study show that the assumptions made in the above studies prove false for certain soil types. In the pH variable experiment, our reaction pH dropped below 4.0 for both Cu and Zn. The organic matter content of our soil was 0.9%, yet we had significant sorption of both Cu and Zn in these conditions. Adsorption maxima were reached at 400 and 667 parts per million Cu (pH variable and pH 4.5 isotherms, respectively) and 145 and 238 ppm Zn. The results of the two studies by Hartley-Whitaker et al., (2000a, 2000b) do indicate that ectomycorrhizal colonization is sensitive to increased metal concentrations. These findings imply that soil metal contamination may affect the ectomycorrhizal symbiosis at the mycobiont level rather than the host level. This could have serious implications for seedling establishment on metal-contaminated sites, as ectomycorrhizal diversity and colonization could be affected. The study states that their results do not support previous work suggesting that ectomycorrhizal fungi ameliorate metal toxicity in the plants, because while ectomycorrhizal colonization was compromised by metals, seedling health was not. The fact that seedlings were not affected by the metals could indicate sorption by the soil to the extent that the metals were not present at phytotoxic levels. Studies with more detailed soil analysis can elucidate in more detail the abiotic and biotic belowground interactions involved in ectomycorrhizal response to metal contamination.

Currently, there is no consensus on the role of ectomycorrhizal fungi in ameliorating metal toxicity in the field. Lack of consensus is likely due to a number of factors, including the

diversity of ectomycorrhizal and host plant species, variability in the nature of metal contamination among sites, and variability in soil conditions among sites and experiments. Careful soil analysis may allow for more comparison between experiments and better explain why certain host-plant symbioses react differently to metals in various environments. Focusing on how ectomycorrhizal relationships are affected by the whole soil environment will allow better predictions of revegetation success in the field.

Table 3.1: Chemical and physical properties of the Uchee loamy fine sand

pH	CEC* ($cmol_c\ kg^{-1}$)	Non-crystalline material (%)	Free Fe-oxides (%)	BS* (%)	OM* (%)	Particle sizes (%)		
						Clay	Sand	Silt
4.6	1.29	9.3	0.028‡	2.21	0.91	5.1	69.0	25.9

*CEC= cation exchange capacity; BS= base saturation; OM= organic matter content.

‡ Free Fe-oxides reported as goethite.

Table 3.2: Clay Mineralogy of Uchee loamy fine sand

	Chlorite	**HIV***	**Vermiculite**	**Illite**	**Kaolinite**	**Quartz**	**Feldspar**	**Gibbsite**
%	2	41	3	17	37	6	4	2

* HIV = Hydroxy-interlayer Vermiculite

Table 3.3: Solution pH after 48 hours equilibration for pH variable isotherms

	Metal Concentration (ppm)	pH*
Cu	50	4.26
	100	4.16
	250	4.00
	500	3.92
	1000	3.87
Zn	50	3.78
	100	3.84
	250	3.86
	500	3.88
	1000	3.90

* pH of the unamended metal solution was approximately 4.8. Soil pH was measured at 4.6 (see Table 1). The pH of the stock solutions were measured as follows: Cu = 3.76 and Zn = 3.87

Table 3.4: Cu and Zn adsorption maxima determined from the linear Langmuir equation.

Experiment	Adsorption Maximum (ppm)	r^2 value	p value
Cu, variable pH	400	0.954	<0.0001
Cu, pH = 4.5	667	0.852	0.0254
Zn, variable pH	145	0.962	<0.0001
Zn, pH =4.5	238	0.932	0.0076

Table 3.5: Adsorption of Cu and Zn to amorphous materials (percent of total adsorption)

	Non-crystalline metal oxides	Organic matter
Cu	95%	5%
Zn	94%	6%

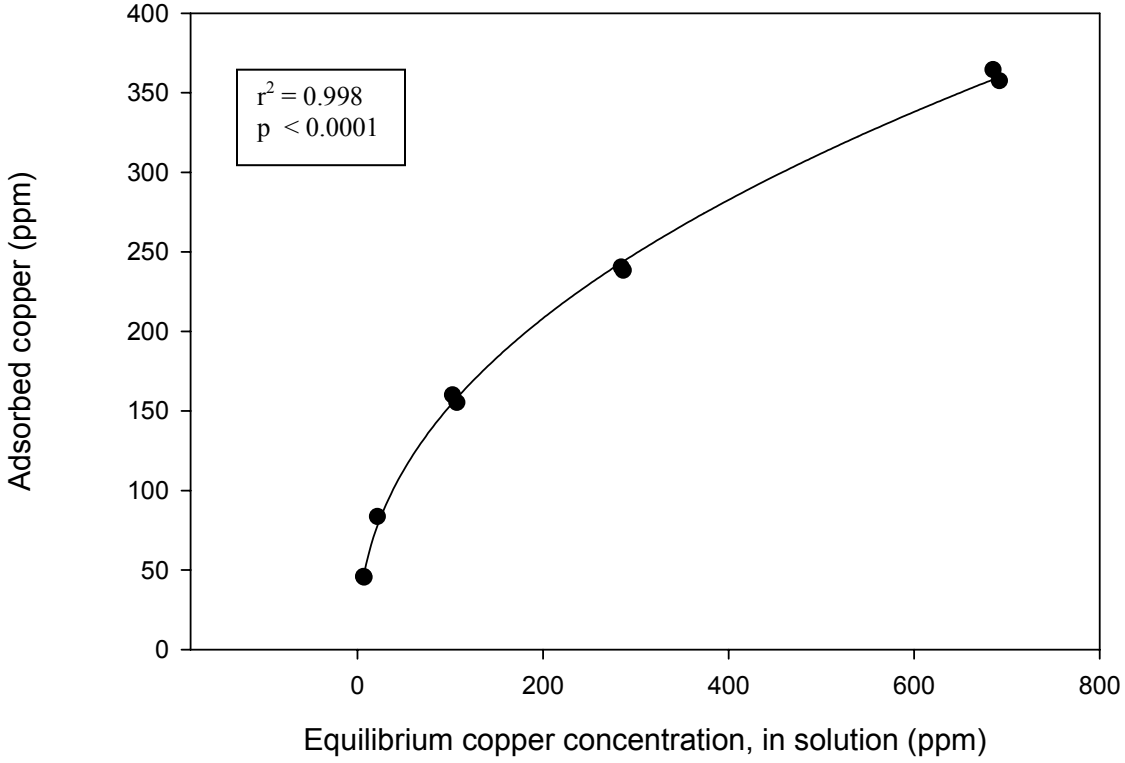

Figure 3.1. Langmuir isotherm depicting Cu adsorption to the soil surface under variable pH conditions.

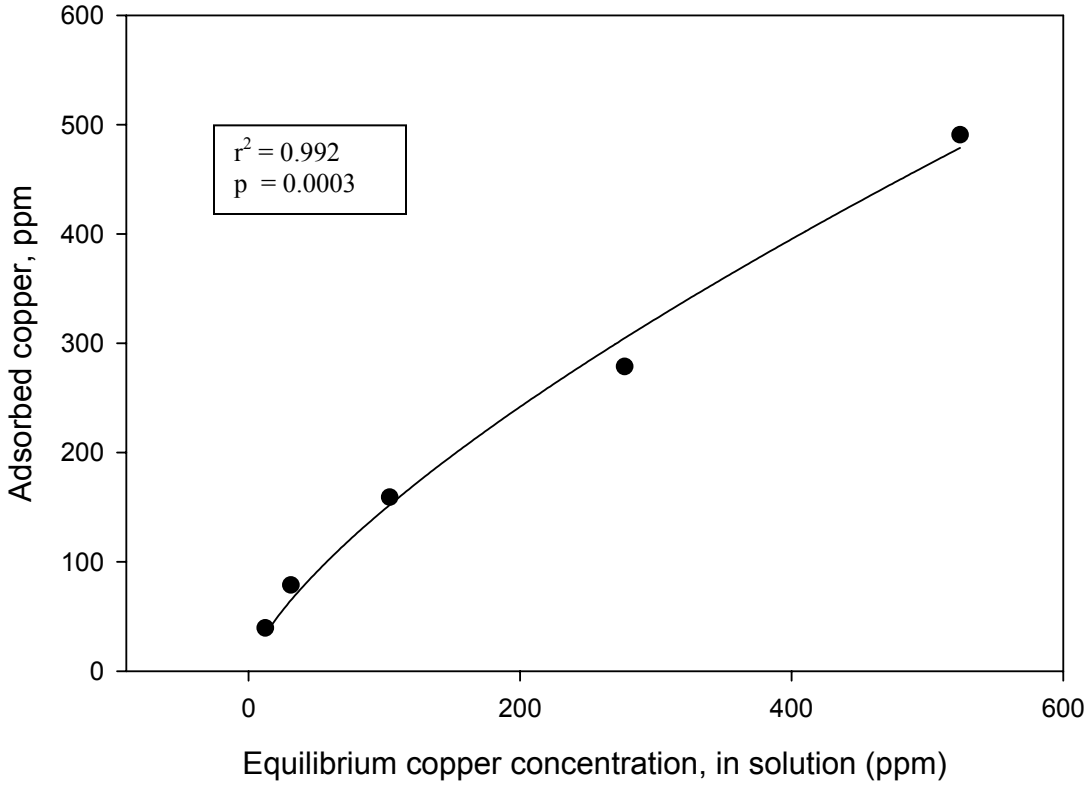

Figure 3.2 Langmuir isotherm depicting Cu adsorption to the soil surface when pH = 4.5.

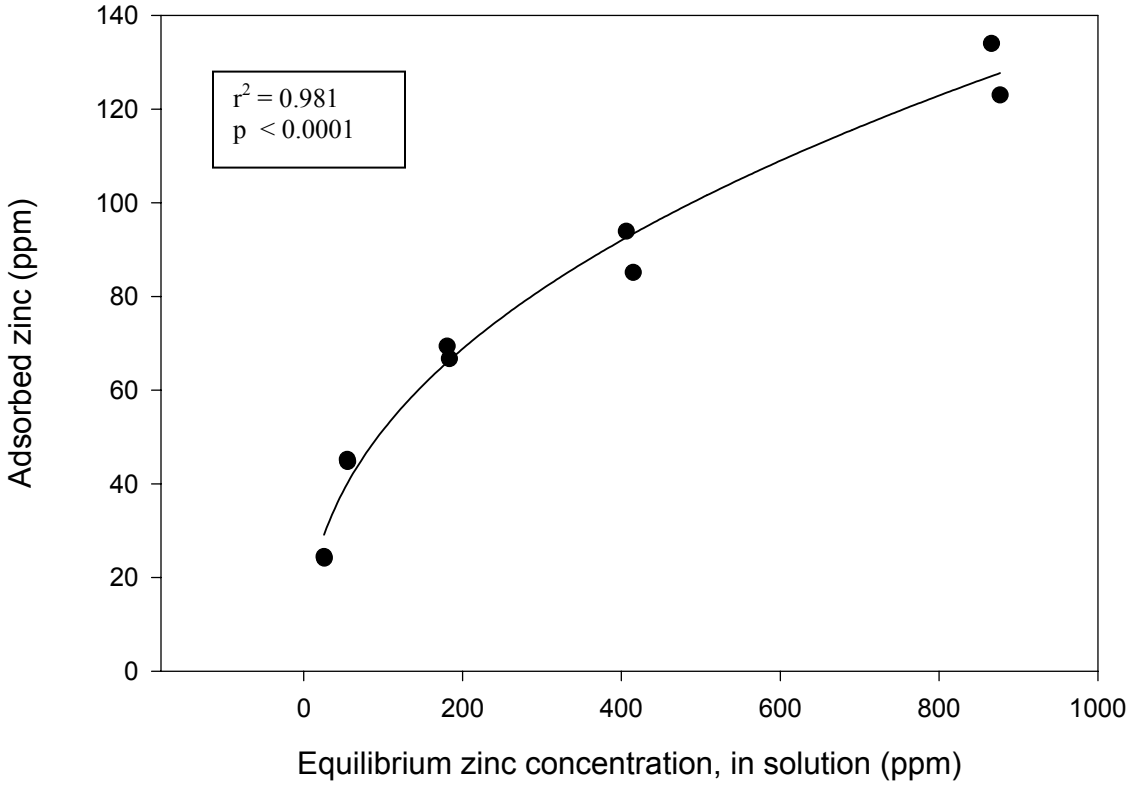

Figure 3.3. Zn adsorption to the soil surface under variable pH conditions

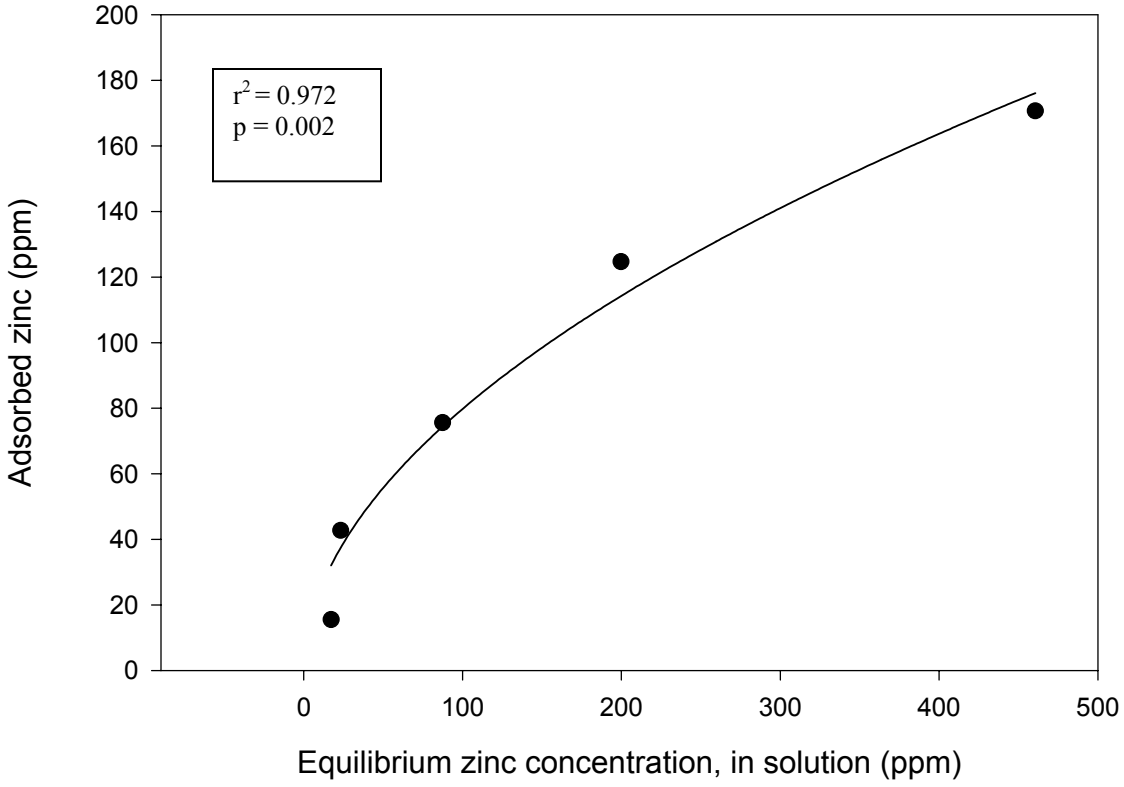

Figure 3.4. Zn adsorption to the soil surface at pH = 4.5

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Chapter 4

Diversity and role of ectomycorrhizal fungi on a revegetated mine site in Southwest Virginia

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Abstract

The ectomycorrhizal community of *Pinus strobus* L. stands in a replanted coal surface mine in Wise County, Virginia, was examined using DNA sequencing of the internal transcribed spacer (ITS) region of mycorrhizal root tips. Four sites of differing ages—1, 8, 13, and 25 years—were studied. Species richness was low on each site, with one or two ectomycorrhizal taxa dominating the root systems. There was no overlap of species among sites, suggesting a local mycorrhizal inoculum source. Database comparisons and phylogenetic analysis revealed that the overall species record included taxa in the Boletales, Stereales, and Pezizales. This study further confirms the utility of DNA-based techniques in identifying and describing below-ground mycorrhizal communities. The low diversity on the study sites supports the working hypothesis that mycorrhizal fungi are sparse on sites experiencing significant environmental stress. Our data also indicate that mycorrhizal fungi could persist and colonize seedlings on disturbed soils. Surveys of the mycorrhizal community on revegetation sites could influence revegetation choices and lead to greater revegetation success in areas disturbed by industrial activities.

Keywords: ectomycorrhizal diversity, *Pinus strobus*, revegetation, colonization, ITS

Introduction

Soil disturbances resulting from mining and smelting activities include loss of organic matter through erosion, increased acidity, and deposition of potentially toxic heavy metals (Leyval et al., 1997). Organic matter loss results in lowered water holding capacity and low nutrient pools. Trace metals also contribute to low nutrient status in soils by inhibiting mineralization of essential elements and inhibiting litter decomposition by soil biota (Derome & Lindroos, 1998; Laskowski et al., 1994). The combination of metal contamination, high acidity, and soil infertility can result in completely barren soils (Vangronsveld et al., 1995), and abandoned lands may remain without vegetation for decades (Bramble & Ashley, 1955).

Establishment of vegetation on barren lands has some benefits, and, in the case of coal mined land, is required by federal and state regulations (Strock, 2002). Established vegetation aids in stabilizing the soil surface and affects the hydrologic cycle by increasing evapotranspiration. Reforestation of mined lands in southwest Virginia has been considered a desirable option, not only because this option results in the closest "restoration" of the original ecosystem, but also because trees provide valuable natural and economic resources within the state (Preve et al., 1984).

The characteristics of disturbed soils described above make revegetation necessary. However, these properties create difficulties in establishing significant plant life on stressed sites. Soil acidity, while in several cases only phytotoxic at pH levels below 4.0, causes limited plant growth by increasing metal solubility and availability (Strock, 2002). As metals accumulate in the soil, even low metal pollution levels lead to phytotoxicity over time (Arudini et al., 1994). Elements commonly present at phytotoxic levels in acidic soils include aluminum, iron, manganese, copper, nickel, cobalt, zinc, and cadmium (Patterson & Olson, 1983). Trace metal

cations negatively affect root biomass, root length, root number, root morphology, and elongation rate, with root growth reductions as great as 25% (Arudini et al., 1994; Barcelo & Poschenrieder, 1990; Ouzounidou, 1994; Patterson and Olson, 1983). Suggested causes for reduction in root growth by increased metal concentrations include actual physical damage to the root cells, alterations of plant hormone balances, or inhibition of cell division (Barcelo & Poschenrieder, 1990). Reduction in root surface area inhibits uptake of water and essential nutrients including phosphorus and nitrogen. Additionally, susceptibility to attack by fungal pathogens is greater for plants in metal-contaminated soils (Patterson & Olson, 1983). The cause of this increased susceptibility has not been experimentally determined, but possible causes include higher tolerance of pathogenic fungal species to metals or easier infection via metal-induced damage to root cells. Metal toxicity therefore exacerbates the effects of drought and nutrient stress, conditions which commonly exist on revegetation sites. The combined toxic effects of, drought, low nutrient pools, elevated metal concentrations, and increased metal availability due to low pH often result in high seedling mortality, leading to failed revegetation efforts.

The severe conditions present on sites needing reclamation create an opportunity to explore the ecological roles of mycorrhizae in stressed situations. Ectomycorrhizal (ECM) fungi play essential roles in water and nutrient uptake for many tree species, and the establishment of the mycorrhizal relationship is often necessary for seedling survival (Smith & Read, 1997). Several studies have suggested that ectomycorrhizal fungi have the ability to ameliorate metal phytotoxicity (Hartley-Whitaker et al., 2000). While field studies with pre-inoculated seedlings suggest that ECM fungi have potential for improving revegetation success (Marx, 1977), a

majority of studies have been conducted in the laboratory and greenhouse, and have used a limited number of chosen ECM species for study.

The purpose of this study was to survey mycorrhizal diversity and colonization rates of trees planted as part of a mine revegetation project in Wise County, Virginia. Little is known concerning the natural populations of ECM fungi on mine revegetation sites. Investigation of mycorrhizae colonizing existing seedlings on mine revegetation sites will give information concerning which species are able to persist and colonize host seedlings. DNA sequencing methods rather than fruiting body surveys were used because no sporocarp production was observed either by the researchers on collecting trips or by revegetation staff throughout the season. Additionally, identification directly from the root tips gives a clearer picture of the below-ground ecology and the mycorrhizal viability on stressed sites. This information will not only give new insights on early-successional mycorrhizal ecology; it will also lead to new perspectives on effective revegetation strategies using ECM fungi.

Materials and Methods

Harvest of mycorrhizal roots.

Roots of *Pinus strobus* L. (Eastern white pine) were collected in spring and fall 2000. The collection sites were located on the property of the Virginia Iron, Coke, and Coal Company (VICC) located in Wise County, Virginia. The property had been mined for coal in the early and mid-twentieth century, and was undergoing revegetation efforts by the company (Figure 4.1). Soil characteristics of the revegetation sites include low pH and high iron and manganese content, and all sites had little to no ground cover at the time of planting (Knox, pers. comm.). In spring 2000, samples of *Pinus strobus* roots were collected at three different sub-sites, each one

at a different stage of recovery (Table 4.1). Samples were again taken in fall 2000 from the first two sub-sites; however, the third site was not resampled due to the poor health of the tree roots. A fourth sub-site was chosen where seedlings had been planted the previous fall (Table 4.1).

Pinus strobus roots were collected at random intervals from the trunk of the planted trees. At least 100 cm of roots were taken from each sub-site, placed in separate containers, and taken to the Virginia Tech Mycology Lab, Blacksburg, VA for processing. Root samples were rinsed carefully in sterile double-distilled water and prepared for molecular and morphological analysis.

Morphological observations

Each length of root was observed in its entirety for mycorrhizal colonization. Although percent colonization rates were not taken for this experiment, it was estimated that all collected roots had at least 50% colonization, and could be used for molecular species identification. Gross morphology—shape and color of the root tips--was observed using a dissecting microscope at 4X power, and used to classify the tips into corresponding "morphotypes" that were analyzed further using DNA sequencing.

Molecular analysis

DNA Extraction.—DNA extraction was carried out using a procedure modified from Gardes and Bruns (1993). The root tips were frozen and stored at -20° Celsius. Samples were thawed, individually ground, and placed in a 65°C water bath for 45 minutes. Equal volumes of 24:1 chloroform:isoamyl alcohol were added to the tubes, which were shaken until a milky emulsion formed. Samples were centrifuged at 12,000 RPM using a Biofuge fixed-angle centrifuge (American Scientific Products) for 15 minutes at room temperature. DNA was then precipitated from the supernatant using equal volumes of cold 100% isopropanol. At this point, samples were placed in the freezer for at least 48 hours in order to maximize DNA yield. After the

requisite precipitation period, DNA was pelleted, washed, and resuspended in 50 µl 0.1X Tris-EDTA buffer or in sterile double-distilled water.

Amplification of ITS region.—In order to identify fungal DNA to the species level, the internal transcribed spacer (ITS) region (rDNA) was chosen for amplification and sequencing. This region was chosen because 1) it is rapidly evolving, allowing for differentiation at the species level (Gardes & Bruns, 1993), and 2) a large database of mycorrhizal ITS sequences exists for comparison with our samples. Amplification was conducted using the following: 500 mM KCl, 100 mM Tris-HCl (pH = 8.3), 20 mM $MgCl_2$, 200 µM dNTPs, 125 µM primers (ITS1-F and ITS4-B or ITS4), 1 unit Taq polymerase (Promega), and 15-20 ng DNA template per 25 µl reaction. Both ITS1-F and ITS4-B primers and the thermocycler program were obtained from Gardes and Bruns (1993).

DNA sequencing and analysis.—Amplified ITS regions were quantified and sequenced using dye-terminated automated sequencing. PCR-based sequencing reactions were completed in the Mycology Lab, and the products were cleaned and run through an Applied Biosystem 377 sequencer at the Virginia Bioinformatics Institute Core Lab Facility, Blacksburg, VA. Sequencing reactions contained the following: 4µl BigDye Terminator ReadyReaction Mix version 2.0 or 3.0 (Applied Biosystems), 5µM primer (ITS1-F for the forward reaction, ITS4-B or ITS4 for the reverse reaction), and 15-20 ng DNA template. A standard sequencing program was used as recommended by the Core Lab Facility at Virginia Tech (VBI-CLF, 2002).

Sequences were edited and aligned using Lasergene (DNASTAR, Inc). Initial sequence analysis was performed by searching for close species matches to root tip ITS sequences on the GenBank BLAST database (National Center for Biotechnology Information). Closely matching sequences were downloaded and saved for phylogenetic analysis. In order to confirm species

identity, phylogenetic analysis of the sampled root tips was performed. For phylogenetic analysis, sequences were converted to the NEXUS format using MacClade (Maddison and Maddison, 1992). Phylogenetic and statistical analysis was performed using PAUP version 4.0b10 (Swofford, 2002).

Results

Morphological analysis

Although the morphological data were not sufficient for species identification, several "morphotypes" were identified which allowed us to organize the sampled tips into several groups (Table 4.2). Site one was characterized predominantly by grey to dark grey monopodial tips (A), while brown bifurcate tips were present in lesser quantity (B). Root tips at the second site were almost exclusively brown and tuberculate (C), although a few bifurcate root tips of the same brown color were present. This site also had the highest number of ECM root tips based on our visual estimates. Site three was characterized by grey monopodial root tips (D) as the dominant ECM morphotype, although some black pinnate tips (E) were also noted. This site had rather sparse mycorrhizal colonization, and the roots appeared spindly and in poor health. Finally, the fourth site, which was sampled in fall 2000 only, was characterized by brown bifurcate tips (F) as well as low numbers of tuberculate tips. No attempt was made to determine relationships among sub-sites based on the morphological data, as many unique species can look similar under the dissecting microscope.

Molecular analysis

Several sequences were obtained for each site. Database searches revealed that each sub-site was dominated by one or two species of mycorrhizae (Table 4.2). Subsequent sampling in an attempt to record less frequent types on each site did not reveal any new species matches based

on sequence data (Abler, unpublished data). Database matching indicated that representative taxa included the ascomycete orders Tuberales and Pezizales and Basidiomycete orders Thelephorales and Boletales. Phylogenetic analysis of ITS sequences obtained from the Wise county site with reference samples from the GenBank database provided statistical support for sequence identification (Figure 5.2). Sample type A from site 1 was supported with 62 percent bootstrap in a clade sister to *Amphinema*, its closest BLAST match. A sequence in the database identified only as an ectomycorrhizal isolate was sister to type A with 99 percent bootstrap support. Sample B from site 1, which was putatively identified as *Wilcoxina rehmii* Yang and Korf based on a 90% BLAST match, was sister to a group containing *Wilcoxina rehmii* and another mycorrhizal otidean fungus. Site 2 samples were grouped into the same morphotype (type C), but were placed into two distinct groups based on BLAST matches and phylogenetic analysis. Type C-1 was placed into a clade containing several members of the genus *Tuber* with 100% bootstrap support. Type C-2 could not be identified to genus, but appears to be a member of the Sarcosomataceae. Fungal type D, which was initally matched to the same *Amphinema* sequence as type A, was not identified to a specific taxon but appears distinct from type A based on phylogenetic analysis. Type E could not be placed into any clade based on this analysis. Fungal root tip samples from site 4 (Type F) were positively identified as isolates of *Suillus americanus* (Pk.) Snell with 100% bootstrap support.

Discussion

The results indicate that a wide taxonomic range of fungal taxa occur on and colonize disturbed sites; however, only 6 taxa were delimited. These findings support the hypothesis that species richness on disturbed and recovering soils is low relative to vegetation-rich sites. There

exist several possible reasons for this lack of fungal richness. Soil conditions, such as low pH and elevated metal concentrations, may have inhibited development of fungal mycelium and inhibited colonization ability. Greenhouse studies of ectomycorrhizal colonization of Scots pine (*Pinus sylvestris* L.) in metal-contaminated soils showed reduced mycorrhizal colonization, and that, in some cases, colonization by the fungal partner was more inhibited than seedling growth (Hartley et al., 1999; Hartley-Whitaker et al., 2000). Field studies have indicated that soil conditions including compaction (Amaranthus et al., 1996), pollution exposure (Kieliszewska-Rokicka et al., 1997), and acidity (Dighton & Skeffington, 1987) can negatively impact mycorrhizal diversity and function.

Mycorrhizal diversity on a revegetation site may also be reduced by low amounts of viable inoculum. Kranabetter and Wylie (1998), in a study of naturally regenerating western hemlock (*Tsuga heterophylla* (Raf.) Sarg) seedlings, found that mycorrhizal richness was reduced by 40% in open gaps compared to mycorrhizal richness under forest canopy. Bradbury (1998) found that mycorrhizal colonization of lodgepole pines planted from seed in a clear-cut was greater than 90% after two years of growth; however, all but two mycorrhizal taxa were also found in nearby undisturbed 90-year old and 6-19 year old regenerating stands. This suggests that viable inoculum was present and either persisted in the soil or migrated into the clear-cuts from the other plots. In the current study, no native ectomycorrhizal hosts were present on the site at the time of seedling planting, and the top layers of the soil horizon had been removed during mining, resulting in a likely reduction of the inoculum pool. There were no nearby stands of mycorrhizal hosts near the revegetation site (Figure 4.1). Sources of mycorrhizal inoculum most likely consisted of pre-existing mycorrhizae on the roots of the seedlings, originating from the nursery, or a limited inoculum pool existing in the soil at the reclamation site.

Although the number of mycorrhizal taxa discovered in this study was low, the sampled mycorrhizal root tips ranged across several families, including Basidiomycota: Boletales (Boletaceae: *Suillus*), Stereales (Atheliaceae: *Amphinema*), and Ascomycota: Pezizales (Tuberaceae: *Tuber*; Otideaceae: *Wilcoxina*). Two BLAST matches, *Rhizoctonia* and *Strumella*, are not known to be ectomycorrhizal. *Rhizoctonia violacea* Tul. (teleomorph = *Helicobasidium brebisonii* (Desm.)Donk) is a root parasite and matched samples from only on roots from the eight year old site (Site 3), on which we observed damaged roots to the extent that the seedlings were not resampled during the second collecting trip. Given the low BLAST match (93%) of our samples to *Rhizoctonia,* we can not state that the samples classified as "type E" belong to this genus. Furthermore, phylogenetic analysis was unable to assign this type to any clade (Figure 5.2). Further analysis needs to be performed in order to identify this fungus and its ecology. Similarly, some fungal samples from site 2 matched most closely to *Strumella griseola* von Höhnel (Table 4.2). Species of *Strumella*, which is a mitosporic member of the Sarcosomataceae, are known mainly as canker-forming fungi. Members of the Pinaceae are not considered hosts for *Strumella* canker, which makes the likelihood of finding this fungus in our samples extremely low. However, the BLAST match for *Strumella* with our root samples was low (90%), and can not be considered a strong match for identification purposes. Further phylogenetic analysis indicated that the samples from site 2 were sister to the clade containing *Strumella griseola* and *Sarcosoma latahense* Paden and Tylutki (Figure 4.2). Therefore, it is most prudent to say only that we have a probable match with the family Sarcosomataceae, in which mycorrhizal species have been reported.

The mycorrhizal composition of disturbed soils may be an important factor in revegetation success. Our results show that ectomycorrhizal fungi are able to persist on

seedlings in stressed sites and provide viable inoculum for host plants. Early research on the utility of mycorrhizae in reforestation of mined sites established that prior inoculation of pines with ectomycorrhizal fungi improved seedling growth and establishment (Marx, 1977; Marx et al., 1977, Marx et al., 1982). Seedling inoculation with *Pisolithus tinctorius* (Mich: Pers) Coker and Couch was recommended for mine site revegetation based on successful field trials and the ease with which large amounts of *Pisolithus tinctorius* inoculum can be produced commercially (Ruehle & Marx, 1979). The rationale behind preinoculation of outplanted seedlings was that *P. tinctorius*-colonized seedlings outperformed those left to be colonized by naturally occurring fungi (Marx, 1977; Marx et al., 1977). Although these results were promising, the success of pre-inoculated seedlings versus naturally colonized seedlings varied by both site and host species (Marx et al., 1977).

In contrast to Marx's methodology, seedlings on the Wise County site were not deliberately inoculated with mycorrhizal fungi prior to planting, yet all root samples contained mycorrhizae. Because seedlings were planted prior to the undertaking of this study, the exact source of mycorrhizal inoculum cannot be determined. Studies involving regeneration of lodgepole pine (*Pinus contorta* Douglas ex London) following logging suggested that mycorrhizal inoculum originated from surviving inoculum that remained viable in the soil during and after clear-cut, or from propagules that migrated into the plots from nearby stands (Bradbury, 1998; Bradbury et al., 1998; Visser, 1995). The Wise County site is located in the midst of large blast mining operations, and no close stand of mature ectomycorrhizal trees exists near the site. Therefore, mycorrhizae on the planted trees have several potential sources of inoculum. The seedlings were either inadvertently colonized in the nursery by mycorrhizal propagules migrating from surrounding plants, inoculum was brought in by animal vectors, or mycorrhizal inoculum

persisted through disturbance and was present in the soil at the time of seedling establishment (Allen, 1991, Bradbury, 1998). Long range spore dispersal is also a possibility but not the most likely source. Analysis of the root tip rDNA revealed that there was no overlap of ectomycorrhizal fungal species between sites. This suggests that the sites are highly isolated and further suggests a local inoculum source for each site. The presence of the same mycorrhizal taxa on samples taken in spring and in fall indicates that seasonal factors did not influence the mycorrhizal composition of host roots, and that we likely identified most if not all of the taxa present on the site.

The presence of viable mycorrhizal inoculum is crucial to the survival of seedlings on stressed sites. In our study, mycorrhizal fungi actively colonized host seedlings. Even if the source of inoculum was in the nursery where the seedlings were propagated, the mycorrhizal fungi were able to persist long enough for the seedlings to establish on a stressed site, and all trees were actively colonized up to 25 years after planting. These seedlings in turn can act as a source of mycorrhizal inoculum for further plantings, especially in site 2 where the 25 year old stand had closed and a forest soil had developed. In contrast, the mycorrhizal community on site 3 was poorly developed, and *Pinus strobus* seedling survival was consistently low throughout several planting attempts (Knox, pers. comm.). The results draw attention to the need to understand the biological community of a site prior to replanting.

The presence of certain types of mycorrhizae could well determine the success of planted seedlings. An example from our study is that on Site 4, only *Suillus americanus* was present. In a case such as this, the *Pinus strobus* seedlings were colonized successfully; however, *Suillus americanus* is highly specific and would not colonize other conifer or hardwood species. Other ectomycorrhizal fungi that do not colonize *Pinus strobus* may have been present in the soil on

site 4; however, this is only speculated without analysis of the soil mycorrhizal community. Although an extreme example, it does highlight the real world impact of a lack of understanding of mycorrhizal composition on extremely stressed sites.

The types of mycorrhizal inoculum existing on a revegetation site also affect revegetation practices utilizing precolonized seedlings. Obtaining seedlings colonized with a chosen fungus can be laborious; the process requires fumigating soil and protecting seedlings from airborne contaminants (Marx and Bryan, 1975). The presence of compatible mycorrhizal inoculum on the site may eliminate the need to obtain precolonization in some cases. However, not all colonizing mycobionts are appropriate for revegetation work. Colpaert and VanAssche (1992) found that *Thelephora terrestris* Fries, a common soil and nursery fungus, actually increased foliar levels of zinc in *Pinus sylvestris* L. In this case, inoculating seedlings with an appropriate mycorrhizal partner before planting may be required.

Succession by fungi present on the outplanting site plays a role in long-term seedling success in revegetation situations. Therefore, it is important to consider the diversity and taxonomic identification of potential mycobionts on the revegetation site even when planting pre-colonized seedlings. Danielson (1988) in his study regarding competition in the field between inoculated fungi (*Laccaria proxima* (Boud) Pat. and *Thelephora terrestris*) and native fungi in a jack pine plantation. This study indicated that the inoculated ectomycorrhizae were replaced by native fungi within one year. These results were similar to Bledsoe et al. (1982), where native fungi dominated root systems of Douglas fir (*Pseudotsuga menziesii* (Mirb) Franco) inoculated with *Laccaria laccata* (Scop:Fr) Cooke and *Hebeloma crustuliniforme* (Bull.:Fr.) Quel. after just five months. Villeneuve et al. (1991) found that ectomycorrhizae that formed in the nursery under natural conditions (*T. terrestris*, *Suillus sp.*) did not survive after

outplanting in a young Douglas fir plantation, but that inoculated *Laccaria bicolor* (Maire) Orton did survive and colonized new roots. Therefore, the type of mycorrhizae present, rather than simply the presence or absence of ectomycorrhizal fungi, affects the ability of inoculation regimes to effectively improve revegetation success. In our study, using Site 4 as an example *Pinus strobus* would adapt well to the presence of *Suillus americanus*, however, other plant species would not.

This study demonstrates that current molecular methods are useful in determining the ectomycorrhizal status of seedlings on disturbed sites. Several studies have indicated that direct molecular identification of mycorrhizal root tips is superior to sporocarp identification or root tip morphotyping (Horton & Bruns, 2001; Peter et al., 2001; Wurzburger et al., 2001). Our study supports this assertion, particularly since no sporocarps were found during any of the Wise County collecting surveys. Furthermore, several genera that produce inconspicuous fruiting bodies that may easily be overlooked (e.g. *Amphinema*) were identified from the root tip data. Additionally, the limited root tip morphotyping was not sufficient for species identification. More detailed morphotyping procedures would have been excessively time consuming and, as discussed by Sakikibara et al. (2002), may have been effective only with concurrent use of molecular identification methods.

Summary

Our results confirm our expectations that the number of mycorrhizal species on the disturbed sites would be low. Molecular techniques allowed us to identify mycorrhizal root tips beyond "morphotype" levels, and in some cases allowed for species identification of mycorrhizae. The types of mycorrhizal fungi present ranged across the spectrum of taxonomic

possibilities, including species that do not form fruiting bodies and those that form hypogeous structures. Sporocarp production was also not noted for genera that produce epigeous fruiting bodies, such as *Suillus.* Lack of sporocarp production is most likely explained by the open nature of the site and the lack of moisture during the collecting seasons. Diversity surveys of mycorrhizal fungi on disturbed sites are crucial to understanding fungal ecology in extreme stresses and to developing more successful revegetation practices. To further develop our understanding of mycorrhizae that persist and colonize from soil inoculum on disturbed sites, studies are under way utilizing seedlings with uncolonized root systems. The combined results of these studies will give greater understanding of the activities and viability of native inoculum on stressed sites common to Southwest Virginia.

Table 4.1. Time since planting *Pinus strobus* seedlings on coal mine revegetation sites in Wise County, Virginia.

Site number	Age since planting (years)
1	13
2	25
3	8
4	1

Table 4.2: Morphotypes and BLAST matches of mycorrhizal samples taken from revegetation sites in Wise County, VA.[1]

Site number	Sample ("Type")	Morphotype descriptions	DNA sequence matches (BLAST database)	GenBank accession Number
1	A	Grey/dark grey, monopodial	*Amphinema* (94%)	AJ534707
	B	Brown, bifurcate	*Wilcoxina* (90%)	AF266708
2	C	Brown, tuberculate/bifurcate	*Tuber borchii* (95%)	AF106890
			**Strumella griseola* (90%)	AF485078
3	D	Grey, monopodial	*Amphinema* (93%)	AJ534707
	E	Black, pinnate	**Rhizoctonia violacea* (93%)	AB056725
4	F	Brown, bifurcate/tuberculate	*Suillus americanus* (99%)	L54103

[1] Percentages represent the base pair overlap of our sample with database sequences. GenBank accession numbers refer to the closest match in the BLAST database. Sequence data and GenBank accession numbers for the root tip data will be made available by the authors upon request.

* = taxa not considered ectomycorrhizal

Figure 4.1. View of mine revegetation project on Coastal Coal Company project, Wise County, VA (Site 1). The trees in this photograph are approximately 13 years old. Note the active mining evidenced by the blast in the distance.

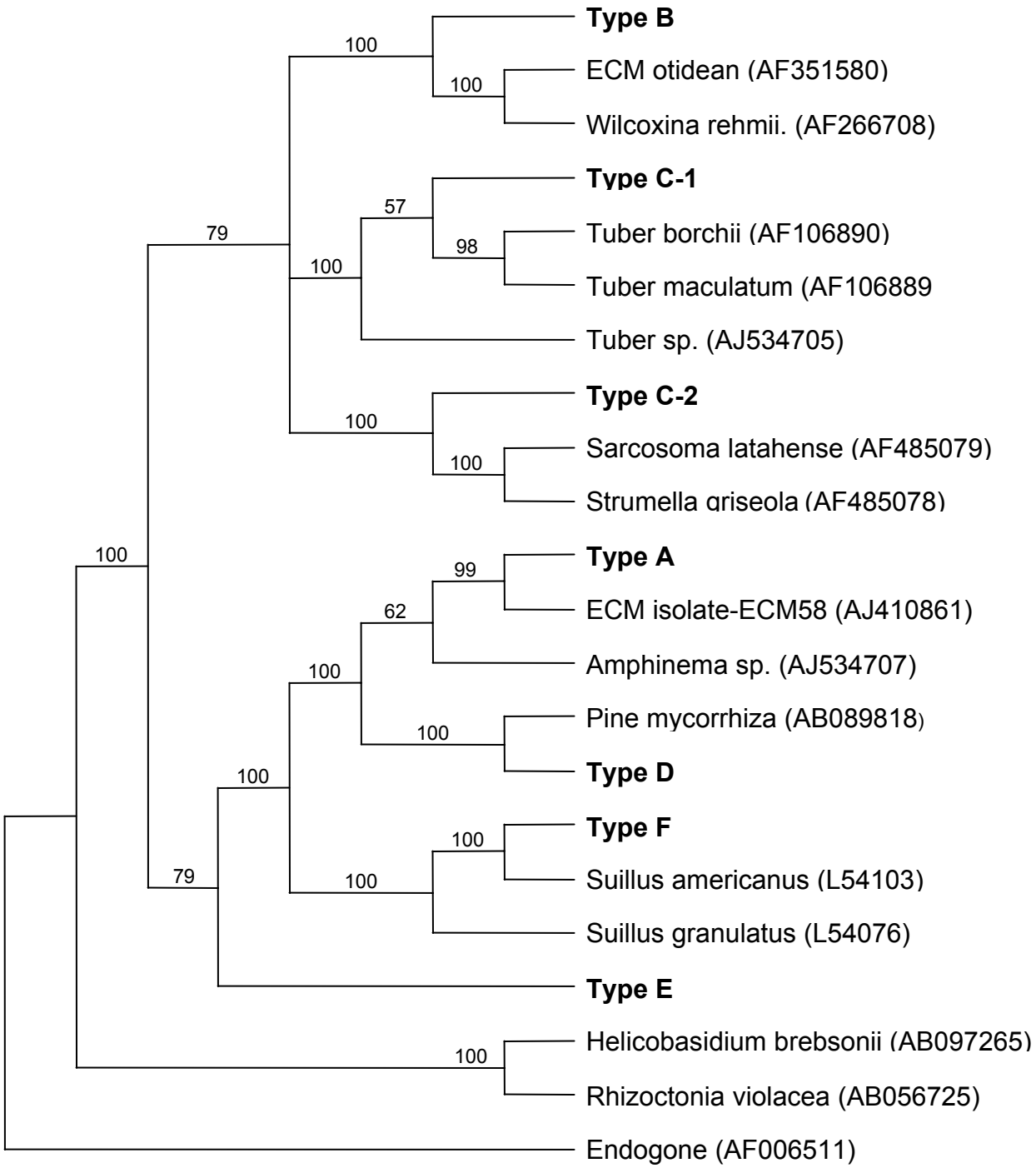

Figure 4.2. Strict consensus tree of ITS sequence data for root tip samples and reference sequences obtained from GenBank. Accession numbers for reference samples are listed after the taxon name. Root tip samples are labeled in accordance to their morphotype classification described in Table 2. Numbers on branches indicate bootstrap values (1000 replications). All sample numbers represent multiple identical sequences

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Chapter 5

Ectomycorrhizal diversity and colonization of naturally regenerated pine seedlings on a disturbed site in Floyd County, Virginia.

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Abstract

The belowground ectomycorrhizal community colonizing *Pinus strobus* and *Pinus virginiana* seedlings on a road construction fill site in Floyd County, Virginia was studied using morphotyping and DNA sequencing techniques. Sampling was performed on the fill site and the road embankment from which the fill was taken. The fill soil was characterized as having high acidity and no organic matter. Seedlings under one year old were sampled and the entire root systems examined for percent ectomycorrhizal coloniztion, number of morphotypes present, and number and identity of ectomycorrhizal taxa based on molecular analysis. Ectomycorrhizal colonization ranged from around 30 to 80 percent, with wide variation among samples. This suggests that ectomycorrhizal inoculum existed in patches on the disturbed soil. Twelve different ectomycorrhizal taxa were identified, with *Scleroderma* c.f. *citrinum* representing the dominant ectomycorrhizal mycobiont for both the road embankment and fill sites. Most of the identified ectomycorrhizal species were considered "early stage" or "multi-stage" fungi. Two ericoid mycobionts, *Hymenoscyphus* sp. and *Oidiodendron* sp. were putatively identified colonizing ectomycorrhizal hosts. This supports other studies that suggest that, contrary to traditional assumptions, a fungal species may be capable of forming both ectomycorrhizal and ericoid mycorrhizas.

Keywords: ectomycorrhizal colonization, diversity, soil disturbance, revegetation, *Pinus strobus, Pinus virginiana,* ITS.

Introduction

Ectomycorrhizal succession, defined as the directional change of mycorrhizal species in a given community over time, is common on disturbed sites where primary succession is occurring (Massicotte et al., 1999; Richter and Bruhn, 1993; Visser, 1995). Ectomycorrhizal community structure is dependent on the structure of the host plant community (Bradbury et al., 1998; Kranabetter and Friesen, 2002), and also largely dependent on soil properties (Baar, 1996). Ectomycorrhizal fungi are classified as "early stage" and "late stage" fungi based on when they appear in the plant community succession. Fungi that persist past primary succession, such as *Cenococcum geophilum* Fr., are considered "multi-stage". (Dighton and Mason, 1985; Keizer and Arnolds, 1994; Kranabetter, 1999). Reported pioneer species include *Thelephora terrestris* Pers. ex. Fr., *Laccaria laccata* (Scop:Fr) Cooke, *Suillus brevipes* (Peck) Kuntze, and *Scleroderma aurantium* L. ex. Pers. (syn. *Scleroderma citrinum* Pers.) (Keizer and Arnolds, 1994; Rao et al., 1997; Visser, 1995). Reported late-stage fungi include several *Suillus* spp. (including *S. tomentosus* (Kauffman)Singer, Snell, and Dick and *S. umbonatus* Dick and Snell), *Lactarius* spp., *Cortinarius* spp., and *Scleroderma citrinum* (Bradbury et al., 1998; Colpaert et al., 1996; Villeneuve et al., 1991; Visser, 1995). The reports of *Scleroderma citrinum* as both early- and late-stage fungi indicate that this is a multi-stage ectomycorrhizal fungus. Fungal species richness usually declines after a manmade or natural disturbance, with pioneer or "early stage" fungi becoming more abundant immediately after disturbance (Bradbury, 1998; Kranabetter and Wylie, 1998; Visser, 1995).

Researchers have been able to synthesize mycorrhizae on young tree seedlings with late-stage fungi in laboratory conditions; however, this does not reflect what occurs in the field (Deacon et al., 1983; Mason et al., 1983). Kranabetter and Friesen (2002) transplanted *Tsuga*

heterophylla (Raf)Sarg. seedlings from mature forests into canopy gaps, and found that two years post-transplant the seedling root tips were dominated by pioneer fungi. They hypothesized that late-stage fungi were not able to maintain colonization activity after transplantation into gaps either because of a lack of abundant root tips or a sufficient carbon source. In another competition study, inoculated *Laccaria bicolor* (Maire) Orton remained on Douglas-fir roots after outplanting in the field, however, *Thelephora terrestris,* which was present on control seedlings at the time of outplanting, was not observed in the field after 17 months (Villeneuve et al, 1991).

Early research on the utility of mycorrhizae in reforestation of mined sites established that prior inoculation of pines with ectomycorrhizal fungi improved seedling growth and establishment (Marx, 1977; Marx et al., 1977, Marx et al., 1982). Seedling inoculation with *Pisolithus tinctorius* (Mich: Pers) Coker and Couch was recommended for mine site revegetation based on successful field trials and the ease with which large amounts of *Pisolithus tinctorius* inoculum can be produced commercially (Ruehle & Marx, 1979). Additionally, *Pisolithus tinctorius* has a broad host range which would make it ideal for widespread application. The rationale behind pre-inoculation of outplanted seedlings was that *P. tinctorius*-colonized seedlings outperformed those left to be colonized by naturally occurring fungi (Marx, 1977; Marx et al., 1977). Although these results were promising, the success of pre-inoculated seedlings compared to naturally colonized seedlings varied by both site and host species (Marx et al., 1977). Not all fungi are equally adept at protecting their hosts from trace metal and other stresses; therefore, competition from a native fungus may impede seedling success on stressed sites.

The object of this study was to investigate the ectomycorrhizal diversity and colonization rates on an early successional site in Floyd County, Virginia. The chosen site consists of road-cut fill from a highway improvement project, and no improvements have been made to the sampled areas. The fill consists of a rocky soil with low pH and no organic matter, characteristics which make seedling survival difficult. We sampled naturally regenerating *Pinus strobus* L. and *Pinus virginiana* Miller seedlings in order to determine 1) which ectomycorrhizal fungi were present and actively colonizing very young (≤ 1 year old) seedlings, and 2) the amount of colonization taking place. Identification of mycorrhizal species was done through direct DNA sequencing of the root tips, as this has been shown to be a more accurate identification method than fruiting body analysis or root tip morphotyping (Horton & Bruns, 2001; Peter et al., 2001; Wurzburger et al., 2001). This study will allow us to investigate the hypothesis that disturbed sites support a narrow range of fungal taxa, and whether the taxa actively colonizing seedlings support the early- and late-stage hypothesis of ectomycorrhizal succession.

Materials and Methods

Site description and sampling methods

Seedling samples were taken from both sides of an acid road cut along Virginia State Route 8 in Floyd County, Virginia (Figure 5.1) at the intersection of Route 8 and Oakview Lane. The site parent material is part of the Ashe Formation of the Lynchburg group, and is predominantly a fine grained phyllite with substantial sulfate coatings (Orndorff, 2001). Soil samples were taken from the embankment along Route 8, and fill samples were taken from a vacant lot belonging to Mr. Gregory Miller (Figure 5.2). Soil sampling and analysis showed that the roadcut fill was highly acidic (pH = 3.2-3.3), and had no organic matter (0.0%). Sampling

occurred twice in 2001 and twice in 2003. In 2001, samples were taken from the road embankment, located on the west side of Route 8, and four places on the Miller property, located on the east side of Route 8, directly south of Oakview Lane. The first sampling site was near the driveway entrance to the property, and the second site was along the edge of the fill site. The site along the edge contained a narrow grove of well established eastern white pine (*Pinus strobus* L.) trees that had been planted by the owner, and henceforth was referred to as the "established" site. These two sites were sampled in order to have a basis of comparison to the roadcut fill on the Miller property. The fill sites sampled in 2001 were along a flat area at the top of the fill, and along a north facing slope.

In 2001, ten seedling root samples were taken from each sites and examined for percent colonization. DNA sequence data was taken from the 2001 samples. In 2003, sampling was done in June and October to assess any seasonal changes in the ectomycorrhizal community. Sampling occurred at two sites: 1) along the road bank on the west side of Route 8, 2) along a north slope gradient consisting of the fill on the Miller property. The "flat" fill site was being used by the property owner and was not available for sampling in 2003. Random transect lines were placed along the embankment and fill sites, and all seedlings less than one year old were sampled. For each seedling, the entire root system was harvested. Root samples were rinsed carefully under sterile double-distilled water, analyzed macroscopically, and prepared for molecular analysis.

Colonization by ectomycorrhizal fungi

Each seedling root was observed along its entire length for mycorrhizal colonization. Ectomycorrhizal short roots are obvious when observed using a dissecting microscope under 4X power, and colonization was expressed as a percent of all short roots present. Each mycorrhizal

root tip was classified by shape and color as a certain morphotype. Samples of each morphotype on each seedling were analyzed further using DNA sequencing.

Molecular identification of ectomycorrhizal species

DNA extraction was carried out using a procedure modified from Gardes and Bruns (1993). The root tips were frozen and stored at -20° Celsius. Samples were thawed, individually ground, and placed in a 65°C water bath for 45 minutes. Equal volumes of 24:1 chloroform:isoamyl alcohol were added to the tubes, which were shaken until a milky emulsion formed. Samples were centrifuged at 12,000 RPM using a Biofuge fixed-angle centrifuge (American Scientific Products) for 15 minutes at room temperature. DNA was then precipitated from the supernatant using equal volumes of cold 100% isopropanol. At this point, samples were placed in the freezer for at least 48 hours in order to maximize DNA yield. After the requisite precipitation period, DNA was pelleted, washed, and resuspended in 50 µl 0.1X Tris-EDTA buffer or in sterile double-distilled water.

In order to identify fungal DNA to the species level, the internal transcribed spacer (ITS) region (rDNA) was chosen for amplification and sequencing with fungal specific primers. This region was chosen because 1) it is rapidly evolving, allowing for differentiation at the species level (Gardes & Bruns, 1993), and 2) a large database of mycorrhizal ITS sequences exists for comparison with our samples. Amplification was conducted using the following: 500 mM KCl, 100 mM Tris-HCl (pH = 8.3), 20 mM $MgCl_2$, 200 µM dNTPs, 125 µM primers (ITS1-F and either ITS4-B or ITS4), 1 unit Taq polymerase (Promega), and 15-20 ng DNA template per 25 µl reaction. Both ITS1-F and ITS4-B primers and the thermocycler program were obtained from Gardes and Bruns (1993).

Amplified ITS regions were quantified and sequenced using dye-terminated automated sequencing. PCR-based sequencing reactions were completed in the Mycology Lab, using a standard sequencing program as recommended by the Core Lab Facility at Virginia Tech (VBI-CLF, 2002. Sequencing products were cleaned and run through an Applied Biosystem 377 sequencer at the Virginia Bioinformatics Institute Core Lab Facility, Blacksburg, VA. Some sequencing products were processed at the DNA Sequencing Facility at the University of Maine, Orono, ME. Sequencing reactions contained the following: 4µl BigDye Terminator ReadyReaction Mix version 2.0 or 3.0 (Applied Biosystems), 5µM primer (ITS1-F for the forward reaction, ITS4-B or ITS4 for the reverse reaction), and 15-20 ng DNA template.

Sequences were edited and aligned using Lasergene (DNASTAR, Inc). Initial sequence analysis was performed by searching for close species matches to root tip ITS sequences on the GenBank BLAST database (National Center for Biotechnology Information). Closely matching sequences were downloaded and saved for phylogenetic analysis. In order to confirm species identity, phylogenetic analysis of the sampled root tips was performed. For phylogenetic analysis, sequences were converted to the NEXUS format using MacClade (Maddison and Maddison, 1992). Phylogenetic and statistical analysis was performed using PAUP version 4.0b10 (Swofford, 2002).

Results

2001 sampling data

Ectomycorrizal colonization data for samples taken in 2001 is presented in Table 5.1. Average percent colonization for all sites ranged between 33.4%-65.5%. For each site, ectomycorrhizal colonization of each sample varied considerably. The largest range existed on

the "flat" site for both Virginia pine and white pine seedlings, where the ectomycorrhizal status of the sampled seedlings ranged from no colonization to between 80 and 90 percent colonization.

Results from DNA sequencing of seedling root tips are presented in Table 5.2. The preliminary sequence data suggested that ectomycorrhizal diversity on the site was low, as only one or two ectomycorrhizal sequences were obtained on seedlings for each site. The driveway site did not yield any good sequence data; analysis of the DNA extraction revealed that the DNA in these root tips was likely of poor quality. Six unique sequences were obtained. BLAST matching revealed putative identifications for each sequence. Two sequences matched species in the Sclerodermatales (*Scleroderma bovista* Fr.; *Pisolithus tinctorius*), three sequences matched species in the Boletales (*Suillus granulatus* (Fr.)Kuntze*, Suillus spraguei* (Berk, and Curt.)Kuntze (syn. *Suillus pictus* (Peck) Kuntze), *Boletus piperatus* Fr.), and one sequence matched a species in the Thelephorales (*Thelephora terrestris*). There was no overlap of species between any of the sites. Although detailed morphotyping was not done for each seedling, two distinct morphotypes were noted: a brown, multiply-bifurcate morphotype, which corresponded to samples identified as *Pisolithus tinctorius*, *Suillus granulatus*, and *Suillus spraguei* (Figure 5.3), and a white morphotype with extraradical hyphae, which corresponded to samples identified as *Scleroderma bovista* and *Boletus piperatus* (Figure 5.4).

2003 sampling data

The average percent colonization of root tips by ectomycorrhizal fungi in 2003 was 40-70% for both sites (5.3). Standard errors, given after the averages, and the ranges between minimum and maximum colonization of samples for each group, indicate again that variability in the amount of colonization was high on each site. No species or site effects were evident for percent colonization.

A total of 14 morphotypes were identified on the two sites (Tables 5.4 and 5.5). Of these, 5 were found on both the road and slope sites, 4 were unique to the road site, and 5 were unique to the slope site. Four morphotypes were found on both Virginia and Eastern white pine, while 9 were unique to Virginia pine and one was found only on Eastern white pine. Database analysis reveals that in several cases, morphotyping was not accurate as an indication of unique species status. *Scleroderma* was the closest match for up to six different morphotypes occurring on both Virginia and Eastern white pine. *Lactarius chrysorrheus* Fr. matched at least two different morphotypes, as did *Thelephora terrestris*, *Rhizopogon succosus* A.H. Smith, and *Hymenoscyphus ericae* (Read) Korf and Kernan. Several morphotypes matched more than one species, further indicating that morphotyping did not delineate separate species for this study.

DNA sequence analysis revealed 12 different mycorrhizal symbionts colonizing the root tips (Tables 5.4 and 5.5; Figure 5.5). Several samples could be positively identified to species based on BLAST matches and phylogenetic analysis. Identifications with over 95% bootstrap support were: *Pisolithus tinctorius* (sample S-9), *Tomentella sublilacina* (sample R-19), *Thelephora americana* (sample S-17), *Suillus spraguei* (sample S-18), *Rhizopogon vulgaris* (Vittad) Lange (sample S-19), *Rhizopogon succosus* (samples S-20, S-1, and S-2), and *Cenococcum geophilum* (samples R-17 and S-21).

Other samples could be placed within a genus with confidence. Samples S-10, S-11, and S-16 matched at a high percentage with *Thelephora terrestris* when compared to the BLAST database. Phylogenetic analysis reveals that these cluster in a sister group to *Tomentella sublilacina* (Ellis and Holw.) Wakef. that includes *Thelephora terrestris*, but only S-16 is in the same terminal clade as *T. terrestris*. This indicates that at the very least, these are in the genus

Thelephora. Samples R-1, R-2, S-13, and S-14 matched to *Lactarius chrysorrheus* in a BLAST search, and are in a clade containing *L. chrysorrheus* with 72 percent bootstrap support.

Samples R-10 and R-11 were matched with *Hymenoscyphus ericae*, and sample S-12 was matched with *Oidiodendron chlamydosporum* Morall in the BLAST database. Phylogenetic analysis shows that R-10 and R-11 are more closely related to each other than the *Hymenoscyphus* spp. from the database, but that they do cluster closely with *Hymenoscyphus*. Sample S-12 clusters with two *Oidiodendron* species with 99% bootstrap support, but the two species from GenBank are more closely related to each other than our sample. This may indicate that our samples are a different, but closely related, species from the species which currently exist on the database.

The dominant species on both sites was a species of *Scleroderma*, which comprised 19 of 40 total samples sequenced. BLAST matches for *Scleroderma* were low (90-94%); however, a single *Scleroderma* sporocarp (Sample S-8) was collected from the site and sequenced along with the root tips. The *Scleroderma* sporocarp sample is in the same terminal clade as 18 of the 19 sequenced root tips with 99% bootstrap support. The closest BLAST match for these specimens was *Scleroderma bovista*, which did not fall out in the same clade as the sequenced samples. While the sporocarp was quite degraded at the time of collecting, it was putatively identified as *Scleroderma citrinum*, which is a common species of *Scleroderma* found in the eastern United States. Currently, no ITS sequences of *Scleroderma citrinum* exist on the NCBI BLAST database, which explains the lower match to a related species. Sample R-18, identified by BLAST searching as *Scleroderma sp.*, did not cluster with either *Scleroderma bovista* or the *Scleroderma sp.*that it matched to in the BLAST database. However, it is more closely related to

the *Scleroderma* species than *Pisolithus*, which is a sister genus in the Sclerodermataceae, so it is likely some species of *Scleroderma*.

Discussion

Ectomycorrhizal colonization of Pinus virginiana and Pinus strobus rootlets

Ectomycorrhizal colonization was reduced overall in the roadcut material compared to what is expected in a mature forest soil. However, some samples exhibited high colonization levels, which was unexpected given the lack of a developed organic layer and the high acidity of the soil. Soil conditions including compaction (Amaranthus et al., 1996), pollution exposure (Kieliszewska-Rokicka et al., 1997), and acidity (Dighton & Skeffington, 1987) can negatively impact mycorrhizal diversity and function. Mycorrhizal diversity on a revegetation site may also be reduced by low amounts of viable inoculum. Kranabetter and Wylie (1998), found that mycorrhizal richness was reduced by 40% in open gaps compared to mycorrhizal richness under forest canopy. Ectomycorrhizal colonization of *Betula platyphylla* Sukatschev seedlings in Japan reached an average level of 4%, 13%, 53%, and 37% where 1, 2-3, 4-5, and 7-8 years had passed, respectively, since disturbance created by a construction and road-building project (Hashimoto and Hyakumachi, 2000). Our study found that mycorrhizal colonization was higher than would expected based on the literature. This may be explained by several factors. Established conifer vegetation above the roadcut site may be a source of mycorrhizal inoculum for regenerating seedlings. Additionally, although the soil in question was an acidic rocky waste soil that would not be expected to support mycorrhizal activity, soil patches that support mycorrhizal growth may exist. In many cases, ectomycorrhizal inoculum exists in patches that remain viable throughout severe disturbances, allowing colonization of a host plant (Allen, 1991).

Diversity of ectomycorrhizal fungi on the Floyd County site.

A broad range of ectomycorrhizal taxa are represented on the Floyd County site. Two phyla are represented, the Ascomycota and the Basidiomycota. *Cenococcum geophilum* is an imperfect fungus thought to be the asexual stage of some ascomycete; *Hymenoscyphus ericae* is an ascomycete in the family Leotiaceae (order Leotiales), and *Oidiodendron* is the imperfect stage of members of the family Myxotrichaceae, which is in the ascomycete order Onygenales. Several orders of basidiomycetes are also represented. Several samples are in the Sclerodermataceae, which are placed either in the Sclerodermatales or in the Boletales along with *Suillus* (Boletaceae) and *Rhizopogon* (Rhizopogonaceae; Hawksworth et al., 1995). *Lactarius* is in the family Russulaceae in the Russulalaes. *Tomentella sublilacina* and *Thelephora* spp. are in the Thelephoraceae, which is in the order *Thelephorales*. Interestingly, no members of the Agaricales are represented. Many mycorrhizal members of the Agaricales (*Amanita* spp., *Cortinarius* spp., *Hebeloma* spp., *Hygrophorus* spp., *Tricholoma* spp.) are late stage fungi (Cripps and Miller, 1993; Visser, 1995); however, *Laccaria* spp. and *Inocybe* spp. are considered early colonizers of disturbed sites (Cripps and Miller, 1993).

As all sampled seedlings, with the exception of the "established" site in the 2001 preliminary harvest, were regenerated from seed that had been naturally dispersed on the site, we can reasonably conclude that the ectomycorrhizae we found on the site represent native species rather than mycobionts introduced from nursery inoculum. Several of the taxa found on the young seedlings are considered early or multi-stage fungi, including *Scleroderma citrinum, Cenococcum geophilum, Pisolithus tinctorius, Thelephora terrestris,* and *Rhizopogon* spp. (McCreight and Schroeder, 1982; Schramm, 1966). *Suillus* and *Lactarius* contain some species which are considered early colonizers, such as *Suillus brevipes* and *Lactarius glyciosmus*

(Bradbury et al., 1998; Kranabetter, 1999). Based on our results, *Suillus spraguei, Suillus granulatus* (identified by BLAST matching of >500 base pairs with 99% similarity), and *Lactarius chrysorrheus* can also be considered early colonizers, although whether these are true "pioneer" species that are replaced by late stage fungi can not be determined.

Tomentella was identified by one study as a late-stage fungus, occurring under the humus layer in the forest floor in older (41+ years) stands (Visser, 1995). Visser hypothesized that *Tomentella*, a resupinate basidiomycete, depends on decomposing plant matter to fruit and therefore requires well decayed organic matter found in mature stands. Our results contradict this hypothesis, since *Tomentella sublilacina* colonized Eastern pine seedlings on the road embankment where there was little to no organic matter layer. The resupinate nature of this fungus may allow it to fruit under the mineral layer, protecting it from desiccation and UV radiation. Furthermore, conditions necessary for fruiting do not appear to be necessary for ectomycorrhizal colonization of a host plant. Several studies have demonstrated that ectomycorrhizal colonization does not correspond to aboveground sporocarp production (Grogan et al., 2000; Horton & Bruns, 2001; Peter et al., 2001; Wurzburger et al., 2001). The study by Baar et al. (1999) supports our findings in a study of post-wildfire, naturally regenerated *Pinus muricata* D.Don seedlings. They showed that one of the dominant field mycobionts was *Tomentella sublilacina*. In their study, *T. sublilacina* was observed on roots of pre-wildfire seedlings, suggesting that viable propagules were able to persist in the mineral soil through the disturbance. Since our study was based on opportunistic circumstances, we were not able to sample pre-disturbance soils on the site, but it is likely that *T. sublilacina* persisted in the same way on the Floyd County site.

Significance of "ericoid" mycorrhizal fungi

The presence of two ascomycete taxa closely related to *Hymenoscyphus ericae* and *Oidiodendron sp.* on the sampled seedlings was surprising. *Hymenoscyphus ericae* and *Oidiodendron chylamydosporum*, and *Oidiodendron scytaloides* W. Gams and Soderstrom have been characterized as mycobionts in ericoid mycorrhizal symbioses (Monreal et al., 1999). In our study, Samples R-10 and R-11, comprised two distinct morphotypes that clustered strongly together and were closely related to *Hymenoscyphus ericae*,. R-10 was light brown and highly branched, which may represent an intermediate stage (Vralstad et al, 2005.3), while R-11 was monopodial and dark grey, similar to the *Piceirhiza bicolorata* morphotype (Agerer, 1998). Several members of the Helotiales, including members of the *Hymenoscyphus ericae* aggregate, have been isolated from post-fire forest areas and Cu-mine spoils (Vralstad et al., 2005.4). Traditionally, ericoid mycorrhiza-formers and ectomycorrhizal mycobionts have been considered to comprise distinct taxonomic groups with no overlap between species (Smith and Read, 1997). Recently, molecular techniques allowed investigation into the taxonomic identity of previously unidentified mycorrhizas. Vralstad et al. (2000) used ITS-RFLPs to show that *Piceirhiza bicolorata*, a distinct morphotype of conifer and hardwood ectomycorrhizae, may belong to the *Hymenoscyphus ericae* species aggregate. Researchers were subsequently able to synthesize ectomycorrhizae *in vitro* using mycorrhizal isolates of ericoid origin (Vralstad et al., 2005.3). The impact of these *Hymenoscyphus*-like ectomycorrhizal partners may have been ignored or underestimated in the past because of 1) the difficulty in identifying mycorrhizal root tips to species before the advent of molecular techniques, and 2) the similarity in appearance of some *Hymenoscyphus ericae/Piceirhiza bicolorata* root tips to *Cenococcom geophilum* root tips (Vralstad et al, 2005.3

Ectomycorrhizal symbiosis with *Oidiodendron* species has not yet been described. While only two of our samples matched *Oidiodendron* spp., and only one was included in the phylogenetic analysis, support was high for the clade containing our samples and *Oidiodendron* spp. No ericaceous plants were observed on or near the sampling sites, therefore the possibility of contamination from an ericoid root system can be eliminated. Our findings support the possibility that ericoid and ectomycorrhizal plants may share mycobiont taxa. This formerly overlooked group may be of ecological importance in early-successional sites.

Significance of naturally occurring fungi to revegetation efforts

. Our results show that ectomycorrhizal fungi are able to persist on seedlings through disturbances and provide viable inoculum for host plants. Mycorrhizal fungi are essential to seedling establishment and survival, especially on low nutrient sites (Smith & Read, 1997). The presence of certain types of mycorrhizae could well determine the success of planted seedlings. Current revegetation practices involved planting young seedlings obtained from nursery stock (W. Daniels, pers. comm..) In this case, the ectomycorrhizal fungi the seedling encounters may determine its survival. For example, *Thelephora terrestris* is an aggressive colonizer in nurseries and on disturbed sites (B. Appleton, pers. comm.; Abler, unpublished data). *T. terrestris* has been shown in at least one study to increase plant uptake of zinc at toxic concentrations (Colpaert and VanAssche, 1992), and therefore would not be a good mycobiont for revegetation of zinc-contaminated sites. Additionally, native fungi may have a competitive advantage over inoculated ectomycorrhizae, and could therefore replace the inoculated mycobiont on planted seedlings. Several studies have shown that inoculated seedlings were overtaken with native mycorrhizae within one year of outplanting (Danielson, 1988; Bledsoe et al., 1982; Villeneuve et al., 1991). Therefore, the types of mycorrhizae present on a given site

can significantly affect the ability of inoculation regimes to effectively improve revegetation success.

Table 5.1: Ectomycorrhizal colonization of conifer seedlings on acidic road construction fill material in 2001.

Site	Tree	% colonization (Mean)	% colonization (Range)
Road	White Pine	57.3 ± 5.4	35.3-72.2
Road	Virginia Pine	65.5 ± 5.4	43.8-84.2
Driveway	Virginia Pine	33.4 ± 5.7	15.7-65.7
Established (Edge)	White Pine	53.8 ± 4.1	20.0-81.8
Established (Edge)	Norway Spruce	52.7 ± 4.6	23.5-65.5
Flat	White Pine	37.3 ± 4.4	0-80.0
Slope	White Pine	52.8 ± 5.8	15.4-84.0
Slope	Virginia Pine	44.1 ± 5.0	0-89.6

Table 5.2: Putative species identification of ectomycorrhizal fungi colonizing root tips on the Floyd County roadcut site based on ITS data.

Site	BLAST match(es)	% match
Road	*Suillus spraguei*	99
	Boletus piperatus	99
Established (Edge)[1]	*Thelephora terrestris*	99
Flat	*Suillus granulatus*	99
Slope	*Scleroderma bovista*	92

1 Trees from the "Established" site were approximately 10 years old (Miller, pers. comm), and transplanted from a nursery. Ectomycorrhizae on these samples may have originated in the nursery. All other sampled seedlings were less than one year old and naturally regenerated.

Table 5.3: Ectomycorrhizal colonization of *Pinus strobus* and *Pinus virginiana* seedlings naturally regenerating on roadcut materials in 2003.

Site	Tree	% colonization (Mean)	% colonization (Range)
June (n = 45)			
Road	Virginia Pine	54.1 ± 5.5	38.7-64.2
Road	White Pine	53.0 ± 11.8	33.6-74.5
Slope	Virginia Pine	73.9 ± 8.9	51.4-90.0
Slope	White Pine	47.4 ± 14.3	4.9-81.3
October (n = 47)			
Road	Virginia Pine	45.8 ± 10.6	28.3-65.0
Road	White Pine	60.4 ± 14.7	9.1-80.5
Slope	Virginia Pine	51.7 ± 8.4	20.4-69.6
Slope	White Pine	65.4 ± 9.0	49.0-80.0

Table 5.4. Morphotypes and BLAST matches for ectomycorrhizae of seedlings collected along Route 8 embankment, Floyd County, in June and October 2003.[1]

Sample name	Seedling type	Date collected	Morphotype	DNA Sequence Match (BLAST database)
R-1	Virginia pine	June 2003	Light brown, bifurcate	*Lactarius chrysorrheus* (97%)
R-2	Virginia pine	June 2003	Light brown, bifurcate	*Lactarius chrysorrheus* (96%)
R-3	Virginia pine	June 2003	Grey-brown, bifurcate	*Scleroderma bovista* (94%)
R-4	Virginia pine	June 2003	White, monopodial, long	*Scleroderma bovista* (93%)
R-5	Virginia pine	June 2003	White, monopodial, long	*Scleroderma bovista* (91%)
R-6	Virginia pine	June 2003	Grey-brown, bifurcate	*Scleroderma bovista* (91%)
R-7	Virginia pine	June 2003	White, bifurcate	*Scleroderma bovista* (91%)
R-8	Virginia pine	June 2003	White, bifurcate	*Scleroderma bovista* (91%)
R-9	Virginia pine	June 2003	Grey-brown, bifurcate	*Scleroderma bovista* (91%)
R-10	Virginia pine	June 2003	Light brown, highly branched	*Hymenoscyphus ericae* (97%)
R-11	Virginia pine	June 2003	Grey, monopodial	*Hymenoscyphus ericae* (97%)
R-12	Virginia pine	October 2003	White, bifurcate, with rhizomorphs	*Scleroderma bovista* (92%)
R-13	Virginia pine	October 2003	White, bifurcate, with rhizomorphs	*Scleroderma bovista* (91%)
R-14	Virginia pine	October 2003	White, bifurcate, with rhizomorphs	*Scleroderma bovista* (92%)
R-15	Virginia pine	October 2003	White, bifurcate, with rhizomorphs	*Scleroderma bovista* (93%)
R-16	White pine	October 2003	White, multiply bifurcate	*Scleroderma bovista.* (92%)
R-17	Virginia pine	October 2003	Black, hairy, monopodial	*Cenococcum geophilum* (93%)
R-18	Virginia pine	October 2003	White, short-bifurcate, smooth	*Scleroderma sp.* (94%)
R-19	White pine	October 2003	Light brown, bifurcate	*Tomentella sublilacina* (97%)

[1] Percentages represent the overlap between our samples and the database samples. Accession numbers for BLAST database matches given in Figure 3. Accession numbers for our samples are given in Appendix I.

Table 5.5. Morphotypes and BLAST matches for ectomycorrhizae of seedlings collected along north facing slope on Miller property, Floyd County, in June and October 2003.

Sample name	Seedling type	Date collected	Morphotype	DNA Sequence Match (BLAST database)
S-1	White pine	June 2003	Light brown, bifurcate	*Rhizopogon succosus* (99%)
S-2	White pine	June 2003	Light brown, multiply bifurcate	*Rhizopogon succosus* (99%)
S-3	White pine	June 2003	Grey-brown, bifurcate	*Scleroderma bovista* (91%)
S-4	Virginia pine	June 2003	White, bifurcate, smooth	*Scleroderma bovista* (91%)
S-5	Virginia pine	June 2003	White, bifurcate, with rhizomorphs	*Scleroderma bovista* (91%)
S-6	Virginia pine	June 2003	White, bifurcate, with rhizomorphs	*Scleroderma bovista* (91%)
S-7	Virginia pine	June 2003	White, bifurcate, with rhizomorphs	*Scleroderma bovista* (91%)
S-8	N/A	June 2003	*Fruiting body of *Scleroderma* found on slope site	*Scleroderma bovista* (90%)
S-9	Virginia pine	June 2003	Brown, hairy, bifurcate	*Pisolithus tinctorius* (99%)
S-10	Virginia pine	June 2003	Brown, bifurcate	*Thelephora terrestris* (97%)
S-11	Virginia pine	June 2003	Brown, bifurcate	*Thelephora terrestris* (97%)
S-12*	White pine	June 2003	Dark brown, monopodial	*Oidiodendron chlamydosporium* (94%)
S-13	Virginia pine	October 2003	Light brown, bifurcate	*Lactarius chrysorrheus* (94%)
S-14	Virginia pine	October 2003	Dark brown, bifurcate	*Lactarius chrysorrheus* (90%)
S-15	Virginia pine	October 2003	White, bifurcate	*Scleroderma bovista* (93%)
S-16	White pine	October 2003	Orange, bifurcate	*Thelephora terrestris* (99%)
S-17	White pine	October 2003	Brown, bifurcate	*Thelephora americana* (92%)
S-18	Virginia pine	October 2003	Bright orange, tuberculate	*Suillus spraguei* (99%)
S-19	White pine	October 2003	White, bifurcate	*Rhizopogon vulgaris* (97%)
S-20	Virginia pine	October 2003	Orange, bifurcate	*Rhizopogon succosus* (97%)
S-21	Virginia pine	October 2003	Black, hairy	*Cenococcum geophilum* (94%)

Figure 5.1. *Pinus strobus* seedling growing on bare mineral surface on road embankment, Floyd County, Virginia.

Figure 5.2. *Pinus strobus* seedling growing on bare mineral surface on roadcut fill site, Floyd County, Virginia.

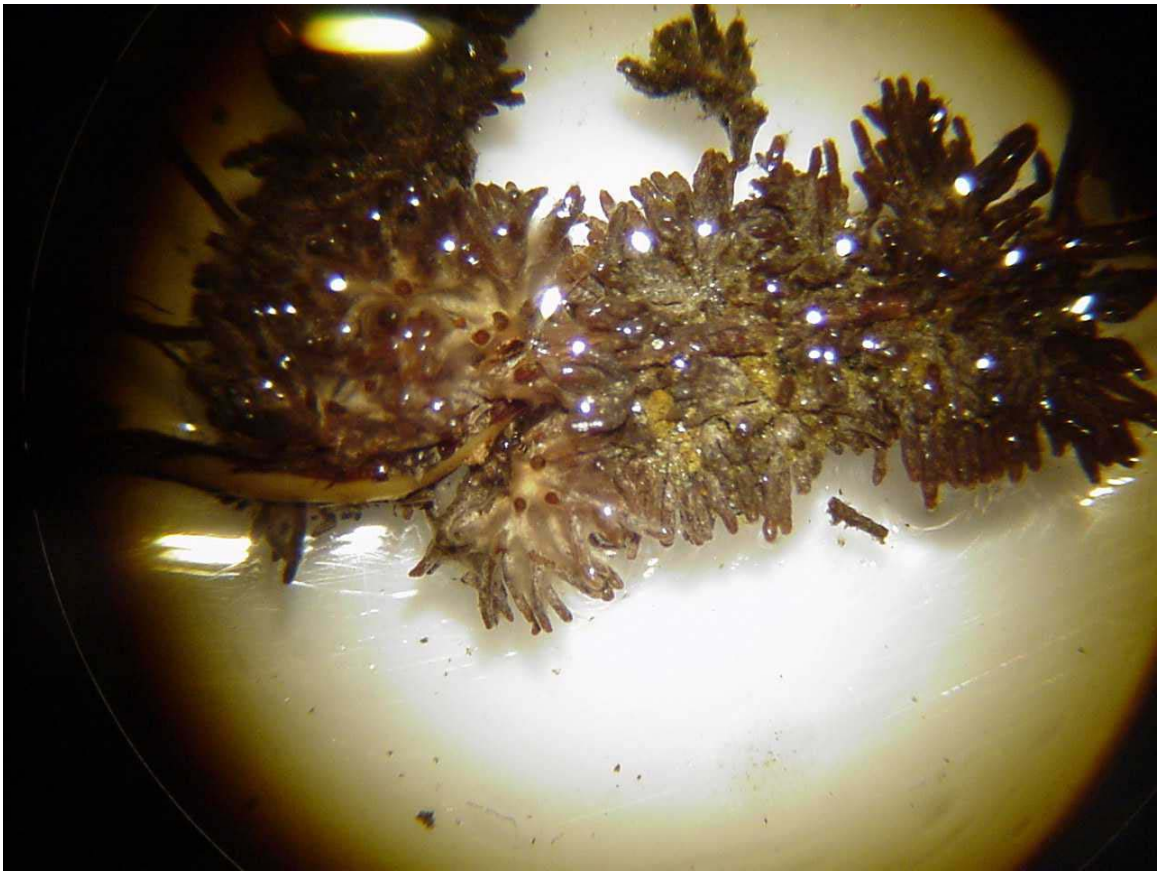

Figure 5.3: *Pisolithus/Suillus*-type mycorrhizae collected from Floyd county site in 2001.

Figure 5.4: *Scleroderma*-type mycorrhizae collected from Route 8 road embankment, Floyd County, Virginia.

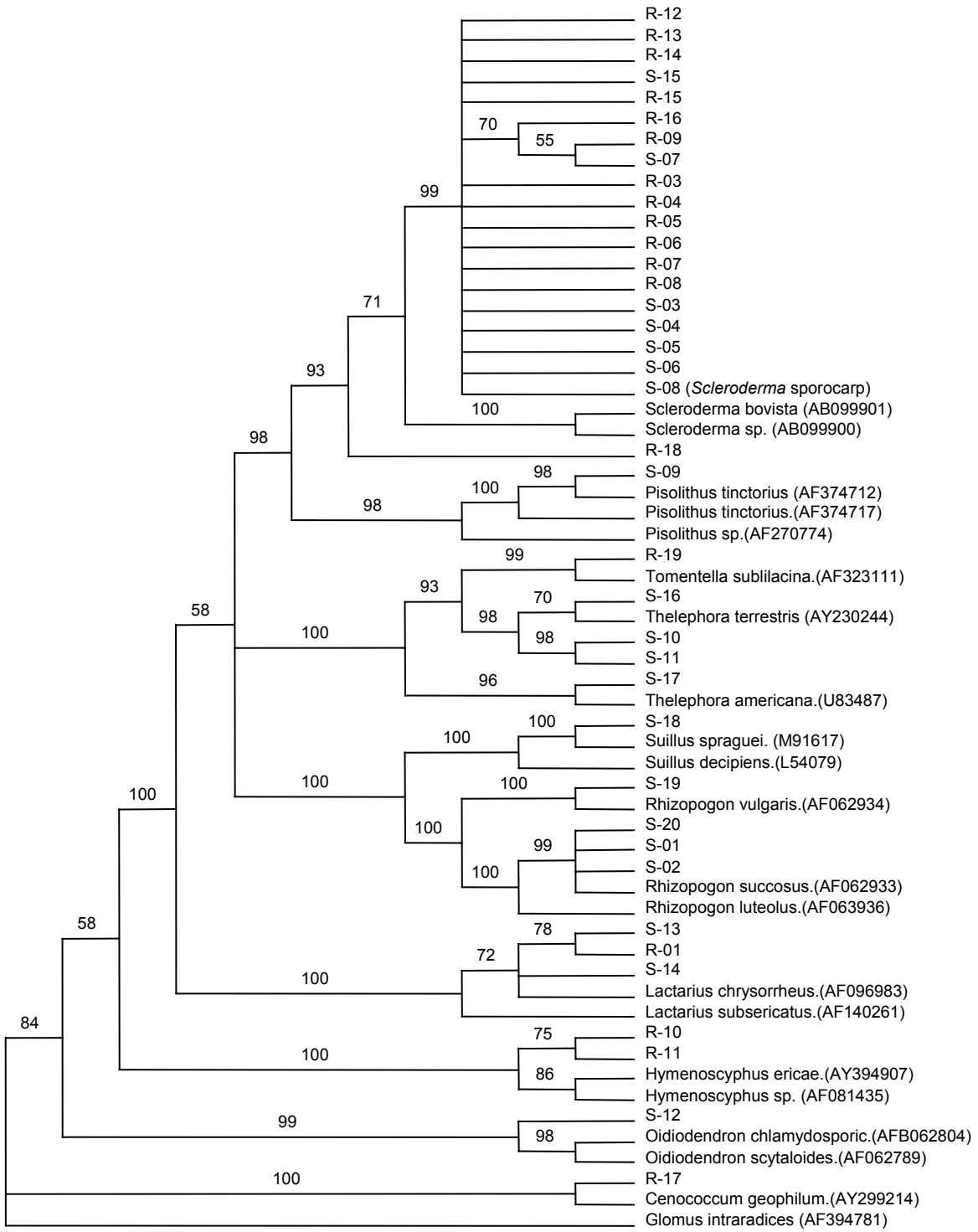

Figure 5.5. Strict consensus tree of ITS sequence data for Floyd County root tip samples and reference sequences obtained from GenBank. Accession numbers for reference samples are listed after the taxon name. Root tip samples are labeled in accordance to their morphotype classification described in Table 4. Numbers on branches indicate bootstrap values (1000 replications

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Chapter 6

Summary and Conclusions

The experimental results contained in this work support the hypothesis that ectomycorrhizal fungi play a crucial role in plant establishment on disturbed soils. Loss of plant cover following disturbance creates several problems, including increased soil erosion, decreased water retention and organic matter, degraded soil structure, loss of flora and fauna, and increased risk of invasive species establishment. Therefore, revegetation of a site is desirable as soon as possible post-disturbance. However, changes in the physical, chemical, and biological structure of the soil can make establishment of new vegetation difficult. Ectomycorrhizal fungi serve an important role in seedling establishment, especially in nutrient and water limited conditions. Therefore, the interactions between ectomycorrhizal fungi and the host plants in a disturbance situation can determine whether a revegetation project will be successful. The goal of this dissertation was to examine the response of ectomycorrhizal fungi to common disturbances, including trace metal contamination, acidity, and low organic matter in order to determine how these stresses affect the ectomycorrhizal community.

Ectomycorrhizal fungi are a taxonomically diverse group, and consequently, have varying responses to stresses such as trace metal contamination. Axenic screening of fungal growth in pure culture was done in order to determine the response of three species of ectomycorrhizal fungi, *Suillus granulatus, Paxillus involutus,* and *Pisolithus tinctorius* to increasing levels of copper and zinc (Chapter 2). The results supported the hypothesis that these taxonomically and ecologically diverse species respond differently to trace metals. *Suillus granulatus* and *Pisolithus tinctorius* were more tolerant to copper than *Paxillus involutus*,

however none of the species showed growth past 100 ppm Cu. *Suillus granulatus* was most tolerant to zinc, followed by *Paxillus involutus* and finally *Pisolithus tinctorius*. *Suillus granulatus* showed a sectoring response on high-zinc plates. Axenic screening of the sectors, followed by inter-simple sequence repeat (ISSR) fingerprinting, indicated that sectoring was a spontaneous event and not a tolerance response to trace metals. These results led to the rejection of the hypothesis that sectoring occurred in response to selective pressure from high concentrations of zinc (Chapter 2).

While axenic screening provides valuable information regarding the variation in fungal species responses to a given metal or set of metals, it can not predict mycorrhizal response in symbiosis. Ectomycorrhizal responses to trace metals in nature are complex and depend on both the host plant community and abiotic interactions of both the fungi and metals involved with the soil. The second experiment was an adsorption analysis of a natural soil that could be used in mycorrhizal experiments (Chapter 3). The soil was an acidic Uchee fine loamy sand (pH = 4.5) that had low organic matter content. These properties led to the hypothesis that it would adsorb only small quantities of the metal and therefore most of the metal would remain available to plants and fungi. Adsorption isotherms revealed that the soil isotherms adsorbed significant amounts of copper and zinc—up to 667 ppm copper and 238 ppm zinc at pH 4.5. Adsorption occurred mainly in the non-crystalline fraction of the soil (Chapter 3). This is a new approach to allow mycorrhizal researchers to make a given load of metals available to the fungus. The analyses performed in Chapter 3 have set the stage for careful mycorrhizal experimentation using the study soil as a natural growth medium. Soil analysis is crucial for mycorrhizal researchers studying metal toxicity in order to ensure that treatment responses are experimentally sound and not a result of metal sorption to the soil surface. Unless experimenters know the fate of metals

added to the experimental system, they can not draw conclusions regarding biotic response to a given metal dose.

The final experiments explore ectomycorrhizal diversity and activity on typical disturbed habitats. Ectomycorrhizal survival depends on its ability to adjust to its soil environment. Traditional hypotheses concerning ectomycorrhizal communities on disturbed sites contend that that ectomycorrhizal diversity must be low due to the poor soil conditions. A mine site reclamation project in Wise County, Virgina, was surveyed for mycorrhizal diversity and colonization by sampling *Pinus strobus* roots from four differently-aged subsites (Chapter 4). Colonization was less than would be expected in a well developed forest soil, however it occurred regularly on all sampled seedlings. While ectomycorrhizal diversity was limited to one or two taxa identified at each subsite, genera identified represented the phylum Ascomycota (*Tuber, Wilcoxina*) and the phylum Basidiomycota (*Amphinema, Suillus*). Higher colonization levels and the presence of late stage mycorrhizal taxa were noted on roots taken from the 25 year old subsite, suggesting that suitable mycorrhizal inoculum exists in the soil. The subsite containing 8 year old seedlings was poorly developed, contained the early-stage mycobiont *Amphinema*, and had low rates of seedling survival. However, a new observation was made of *Suillus americanus,* a so-called "late-stage" fungus, on one year old seedlings. There was no overlap of species among sites, suggesting a local inoculum source. These findings support the hypotheses that species richness on disturbed and recovering soils is low relative to vegetation-rich sites. Additionally, the results suggest that in areas of low diversity, "early-stage" and "late-stage" concepts of fungal succession do not apply. The use of experimental soils to assess the impact of heavy metals and other biotic factors on mycorrhizal function will lay the foundation for better approaches to the revegetation of impacted sites.

The final experiment studied colonization of first generation *Pinus strobus* and *Pinus virginiana* seedlings naturally regenerating on an acidic bare mineral soil in Floyd County, Virginia (Chapter 5). Root samples were taken from an exposed road cut and from a fill site; on both sites the soil was exposed parent material with zero organic matter content. Average ectomycorrhizal colonization ranged between 30 to 80 percent; however, wide variation among individual samples suggests that inoculum may exist in patches in disturbed soil. *Scleroderma citrinum,* a common early-stage fungus, was consistently dominant on both sites. Other "early stage" genera included *Rhizopogon, Pisolithus,* and *Thelephora. Suillus spraguei, Suillus granulatus,* and *Lactarius chrysorrheus* were also identified from the site. The genera *Suillus* and *Lactarius* are reported to contain both early stage and late stage species; however, reported species have not been previously reported in the literature to colonize very young seedlings. *Cenococcum*, often a dominant taxon in disturbed areas, was a minor taxon in this case.

In general, the experimental results described in Chapter 5 appear to support the "early-stage" and "late-stage" successional hypothesis, as early stage fungi made up the majority of taxa identified colonizing seedlings. However, combined results from Chapter 4 and Chapter 5 suggest that this hypothesis is not universally correct for all revegetation sites. Abiotic soil factors such as moisture and pH may be more important in determining early mycorrhizal communities; however, this is only speculation as the soil properties for the Wise County and Floyd County sites were not compared. Two unusual mycobionts, *Hymenoscyphus* and *Oidiodendron* also colonized host plants. These genera are considered to be ericoid-mycorrhizae formers. The identification of these in an ectomycorrhizal symbiosis suggests that the traditional segregation of ericoid and ectomycorrhizal taxa into two distinct groups needs to be revisited. Overall, our concepts of succession and fungal-host interactions have been altered by the results

of this study. The presence of several taxa actively colonizing seedlings on disturbed sites suggests that native inoculum sources can be an important resource for revegetation.

The overriding goal of this dissertation was to determine how disturbances, including but not limited to trace metal contamination, influence ectomycorrhizal growth, diversity, and host plant colonization. The results indicate that ectomycorrhizal interactions with host plants in stressed situations are complex. Not all species react in the same way to a given metal load, and not all metals influence growth of a single species in the same manner (Chapter 2). Abiotic factors such as soil chemical and physical properties can affect metal interactions as well as biological factors such as soil flora and fauna (Chapter 3). Two disturbed sites with similar plant host communities can have very different belowground ectomycorrhizal communities (Chapters 4 and 5). Ultimately, a comprehensive approach involving ectomycorrhizal mycobionts, host plants, and the soil environment must be employed in order to further elucidate the impact of disturbance on ectomycorrhizal function.

VITA

Rebecca A. Belling Abler was born on March 12, 1976, in Neenah, Wisconsin, to Michael and Judith Belling. She credits her parents with her early love of nature and biology through their strong encouragement for her to "go outside and play!" She attended Neenah High School where she was active in the debate and forensics clubs and graduated in the top ten of her class in 1994.

Rebecca attended the University of Wisconsin-Oshkosh and graduated *magna cum laude* with a B.S. in biology and a chemistry minor in 1998. While attending UW-Oshkosh, she worked in the laboratory of Dr. Jennifer Mihalick studying the chemical properties of heavy metal-binding polysaccharides produced by the cyanobacterium *Microcystis flos-aquae*. In 1997, she took Introductory Mycology from Dr. Steve Bentivenga, and her interest in mycology was irrevocably sparked. Based on Dr. Bentivenga's recommendation, she enrolled at Virginia Polytechnic Institute and State University in 1998 to pursue graduate studies with Dr. Orson K. Miller, Jr. Her doctoral dissertation is titled "Trace Metal Effects on Ectomycorrhizal Growth, Diversity, and Colonization of Host Seedlings". While completing her doctoral work, Rebecca has attended numerous professional meetings, including the Mid-Atlantic States Mycology Conference and the annual meetings of the Mycological Society of America. In 2000, she attended a NATO Advanced Study Institute in Visegrad, Hungary entitled "Environmentally-Acceptable Pollution and Remediation Endpoints: Scientific Issues and Policy Development," for which she received a National Science Foundation travel award. She has been active in teaching at the university, serving as a teaching assistant for General Biology, General Microbiology, and Introductory Mycology courses, and serving as lead instructor for the

Introductory Mycology course. In 2001, she received a university-wide award for graduate teaching excellence. She has also been involved as a consultant and researcher for projects in a number of fields including horticulture, mycological toxicology, and veterinary molecular diagnostics.

In 1995, while attending the University of Wisconsin-Oshkosh, Rebecca met fellow biology major Steven Abler. Rebecca and Steve were married August 18, 2001. Rebecca is looking forward to continued studies in mycology, and hopes to mark her career following her parents' wise advice to explore the world outside.