Effect of a Heavy Metal on Ecto- and Vesicular-Arbuscular Mycorrhizal Fungi: The Physiology, Ultrastructure, and Ecology of Copper Stress and Tolerance

by

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(ABSTRACT)

This work consists of an introduction, six chapters dealing with various aspects of the response of mycorrhizal fungi to copper, and a brief conclusion. The first chapter examines the enzyme tyrosinase in several ectomycorrhizal fungi and shows that its activity is altered in these fungi in response to copper. Polyamines are also examined in this chapter, and it is shown that their levels are altered in some ectomycorrhizal fungi due to copper stress but not in others. The second chapter uses transmission electron microscopy to demonstrate that copper is bound to the hyphae of ectomycorrhizal fungi grown on solid media, but the location of the binding varies between fungal species. *In vitro* copper tolerances of a number of ectomycorrhizal species are compared in this chapter and differences in tolerance are evident between species and between different isolates of the same species. In the third chapter, four ectomycorrhizal fungi and one nonmycorrhizal fungus are evaluated for their ability to improve the growth of Japanese Red Pine under conditions of copper stress. Improvement of pine seedling growth is not correlated with *in vitro* copper tolerance of the fungus, but is related to the degree of compatibility between host and fungus. Despite differences in *in vitro* tolerance between three isolates of the same species, there are no differences in the effect of the isolates on the tree host under conditions of copper stress. Ectomycorrhizal fungi were also inoculated in pairs on pine seedlings and the competitive abilities of the fungi are compared under stressed and nonstressed conditions. The fourth chapter discusses the results of inoculation of pine with a nonhost fungus which stimulates dichotomous branching of the root system. The compound responsible for the branching is demonstrated to be indole-3-acetic acid (IAA), a plant growth hormone. The final two chapters deal with endomycorrhizal fungi. In the first of the two, inoculation of onion with an endomycorrhizal fungus demonstrates that the fungus probably

plays no direct role in the response of the plant to heavy metals, based on biomass production, nutrient uptake, and photosynthetic rate. The last chapter demonstrates that the vascular plants found on abandoned mines in Virginia and North Carolina are well colonized by endomycorrhizal fungi; thus, an absence of these fungi is not a reason for the limited natural recolonization of the mine spoils.

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Table of Contents

Introduction 1

Effect of copper on tyrosinase activity and polyamine content of some ectomycorrhizal fungi . 22

Growth of some ectomycorrhizal fungi on copper amended media and ultrastructural localization of copper 37

Effect of copper on growth of Pinus densiflora and five ectomycorrhizal symbionts 64

Boletinellus merulioides alters root morphology of Pinus densiflora without mycorrhizal formation 101

Effect of copper on the interaction between Glomus diaphanum and Allium cepa: Growth and photosynthetic relationships 118

A survey of vesicular-arbuscular mycorrhizal fungi on mine spoils in southwest Virginia and central North Carolina 134

Conclusions **144**

Vita **148**

Introduction

Mycorrhizae, the symbiotic association of the roots of some plants with some fungal species (Trappe, 1987), have been reported to aid in the establishment, survival, or both, of certain vascular plant species in substrates that contain toxic metals (Bradley, Burt and Read, 1981; Gildon and Tinker, 1981; Gildon and Tinker, 1983b; Brown and Wilkins, 1985a; Jones, Browning and Hutchinson, 1986). The studies reported in this work were designed to understand the behavior of mycorrhizal fungi when exposed to potentially fungitoxic levels of a heavy metal and, if metal tolerance exists, to examine possible mechanism(s) for tolerance. Copper was the metal examined in all of the experiments and was chosen because, although it is an essential micronutrient for normal eukaryotic cell growth and development (Bowen, 1966), it is toxic to most organisms at very low levels. Thus, intrinsic or nonecotypic mechanisms of dealing with potentially toxic levels of such a metal might be expected.

Mycorrhizae, the fungus associates, the host plants and their interactions have been extensively studied (e.g. Schenck, 1983; Harley and Smith, 1983; Safir, 1987; Harley, 1989). Ectomycorrhizal and endomycorrhizal or vesicular-arbuscular (VA) mycorrhizal fungi are taxonomically vastly different organisms; their only major similarity appears to be that their presence is probably required for the survival of their host plants in natural terrestrial ecosystems (Janos, 1987). For the most part, ectomycorrhizal fungi are Basidiomycetes (Miller, 1983) that form the

mycorrhizal association primarily with members of the Pinaceae and Fagales (Trappe, 1987). Fungal hyphae form a sheath, or mantle, of variable thickness around the host root and radiate out into the substrate. In addition, hyphae also grow between the cells of the outer cortex to produce what is morphologically described as the "Hartig net" (Wilcox, 1983). In pines, short roots often dichotomize when colonized by an ectomycorrhizal fungus (Hatch, 1937). There are many potential ectomycorrhizal fungi, and relatively few ectomycorrhizal host plant families (Miller, 1983). With a large number of fungal associates, it would seem logical to assume that there would be a variety of possible responses to a potential toxin. Many ectomycorrhizal fungi can be grown in pure culture; that is, on artificial media without the presence of the host plant (Molina and Palmer, 1983). This provides a researcher with a major advantage over the study of endomycorrhizal fungi, which cannot yet be grown successfully in the absence of a suitable host plant (Daniels and Skipper, 1983).

VA mycorrhizal fungi produce a large volume of hyphae in the soil away from the root (extraradical or extramatrical hyphae) and colonize the outer cortical cells of the host through inter and intracellular penetration (Brown and King, 1983). These Zygomycetes produce neither a mantle nor a "Hartig net". Close to 90% of the living angiosperm families have members that associate with endomycorrhizal fungi (Trappe, 1987); yet only species in eight closely related genera in a single family of fungi appear to be involved (Trappe and Schenck, 1983). This scenario suggests that the range of responses to a heavy metal toxin may be relatively narrow among endomycorrhizal fungi. Therefore, the response to a toxin of one fungus might have predictive value for responses in others. Despite the fact that both ecto- and endomycorrhizal fungi may benefit their hosts in a similar manner, such as increasing plant uptake of nutrients or water, they are very different organisms. As a result, they might be expected to have differing mechanisms of response to potential toxins.

This introduction presents information concerning the responses of a number of eukaryotes to heavy metals, especially copper, and discusses any tolerance mechanisms that have been elucidated in them. First, studies on *Saccharomyces* and other yeasts will be covered. This is followed by a review of the results of studies dealing with tolerance and/or intolerance of nonmycorrhizal

filamentous, ectomycorrhizal and vesicular-arbuscular mycorrhizal fungi. Lastly, a brief mention is made of lichens, algae, and green plants. The material in this introduction formed the basis for designing the experiments described in the subsequent chapters. Experimental procedures and results are described in the following six chapters. The first two deal with the biochemical and ultrastructural responses to copper in *in vitro* cultures of ectomycorrhizal fungi. They are followed by two chapters in which the response of Japanese Red Pine (*Pinus densiflora* D. Don) to a number of ectomycorrhizal fungi with and without copper stress are described. The final two chapters discuss studies on VA mycorrhizal fungi; the first details several experiments challenging mycorrhizal and nonmycorrhizal onions with potentially phytotoxic levels of copper and the second is an ecological survey of VA mycorrhizal fungi on abandoned metal and coal mines in southwestern Virginia and central North Carolina.

Copper, as well as many of the other di- and trivalent metal cations, is an essential micronutrient which occurs as part of the prosthetic groups of enzymes, as an activator of enzyme systems, and as a facultative activator in enzyme systems (Gupta, 1979). At higher than physiological concentrations, the fungistatic action of metal cations is related to their strength of covalent binding to surface ionogenic groups of the fungal cell membrane, i.e. to such groups as imidazole, carboxyl, phosphate, or sulphydryl (Somers, 1961). Some of these reactions may result in a loss of membrane semipermeability which allows easier influx of metal ions into the cell and outward movement of cell contents (Miller, 1969). In addition, this binding impairs the membrane synthesis, transport and degradation functions for which the membrane ligands are designated. Once inside the cell, copper is a potent inactivator of enzymes and may also damage the nucleus and chromosomes (Ross, 1975). In cells possessing mucopolysaccharide components of the cell wall, metal ions probably become initially bound to this carbohydrate as the first stage of entry into the cell (Brown and Smith, 1976). The presence of a mucopolysaccharide sheath has been noted in a number of fungi, especially wood rotting basidiomycetes (Palmer, Murmanis and Highley, 1983a&b). Following this initial binding, entry into the cytoplasm may occur where several biochemical entities, notably sulphydryl groups, come under attack (Jernelov and Martin, 1975). Protein synthesis can be significantly impaired. For example, cells of the yeast *Saccharomyces ellipsoideus* do not show

an increase in oxygen uptake during the change from logarithmic to stationary growth due to impairment of cytochrome *c* synthesis by copper (Minagawa, 1958). Copper may act as a fungistat or a fungicide, depending on the conditions of exposure, e.g., pH of the media or amount of ions present (Miller, 1969). The literature indicates that fungicidal levels of copper vary greatly within the Kingdom Fungi. However, as a group, fungi are considered to be highly tolerant of this metal (Ashida, 1965). For example, Somers (1961) found the fungicidal concentration of copper for the spores of species of *Botrytis* and *Alternaria* was 300 and 600 μg/ml (= ppm) respectively. Growth of the wood destroying *Fomitopsis annosa* was inhibited at copper concentrations of 100 μg/ml and greater (Vasilyauskas, 1964).

In green plants in contrast, copper is required in very small amounts: 5 to 20 μg/ml is adequate for normal growth and greater than 20 μg/ml is considered toxic (Adriano, 1986).

Yeasts

Most of the early studies of copper tolerance in yeasts were performed in Japan studying the ascomycetous genus *Saccharomyces*. Using *S. ellipsoides*, it was determined that copper resistant cells of the fungus accumulated far more copper than sensitive cells, whether living or dead (Naiki et al., 1954). This resistance could be correlated with increased production of amino acids by the resistant strains (Arakatsu and Ashida, 1956), as well as to the formation of a "brown colored substance" (Naiki, 1957a), which was thought to be hydrogen sulfide (Naiki, 1957b). Hydrogen sulfide was demonstrated ultrastructurally to be precipitated outside the cell wall by copper resistant cells (Ashida, Higashi and Kikuchi, 1962). However, sensitive cells were not examined for comparison. Naiki (1961) concluded that while hydrogen sulfide production may contribute to the detoxification of copper, resistant strains of yeast appear to have some other means of overcoming the toxic effect of the ion. One possibility is the formation of copper cuproporphyrin complexes to reduce the amount of free copper in the cell and thus injury (Minagawa, 1958). Using copper resistant strains of *S. cerevisiae*, Seno (1963) found that a dark brown coloration accompanied resistance more frequently than light brown coloration; hydrogen sulfide production did not seem to be a requisite for copper resistance; and copper resistance can segregate independently of it. In more recent studies,

a copper resistant strain of *S. cerevisiae* has been demonstrated to produce copper binding proteins (Naiki and Yamagata, 1976), which are metallothioneins (Kagi and Nordberg, 1979). Metallothioneins are low molecular weight, cysteine-rich, metal binding proteins which have been found in such phylogenetically diverse organisms as humans, horse, crab, and yeast. Production of metallothionein in yeast is apparently mediated by a gene amplification mechanism based on unequal sister chromatid exchange (Fogel and Welch, 1982). Kihn, Mestdagh and Rouxhet (1987) found that the cell walls accounted for only a small fraction of copper retention by whole cells of *S. cerevisiae*. In addition, copper was not bound only at the outer face of the plasma membrane, but was also distributed within the plasma membrane, in the cytoplasm, or both. Electron spin resonance studies also showed that in all three systems, copper was chelated by peptides or proteins. Finally, Ross and Walsh (1981) found that both a parent strain and a copper-resistant strain of *S. cerevisiae* had the ability to adapt to copper and the stable resistance of the copper-resistant strain had a different basis. The relevance to the present work is their suggestion that the ability to adapt to grow in the presence of elevated copper concentrations is a rather general property of yeast which can occur whether or not the particular strain is genetically copper-resistant. This theme is repeated throughout the literature on fungi and heavy metals. Both yeasts and filamentous fungi appear to have intrinsic mechanisms of coping with metal stress and virtually any isolate has the potential of becoming a resistant strain. However, there have been relatively few studies on the intrinsic resistance of the parent strains.

Gadd and his associates in Scotland (Gadd, 1980; Gadd, 1981; Gadd, 1984; Gadd and Mowll, 1985) have studied the effects of a variety of heavy metals on the dimorphic fungus *Aureobasidium pullulans*. They found that the origin of copper tolerant colonies on solid media depended on intrinsic properties of the fungus and not physiological or genetic adaptation mechanisms (Gadd, 1984). Based on permeability experiments, they found that all cell types of *A. pullulans*, yeast-like cells, hyphae, and chlamydospores, could bind copper to the cell wall, with the more darkly melanin pigmented types having the greatest binding ability (Gadd and Mowll, 1985). Although copper induced dark pigmentation, which may indicate some involvement of tyrosine oxidase which is the key copper-requiring enzyme for melanin synthesis, pigmentation was also

produced in the presence of mercury, cadmium, and lead, which have no apparent catalytic function in melanin formation (Gadd, 1981). Thus, the transition from hyaline to melanized structures is a response to unfavorable conditions and resembles changes that occur in normal metal-free populations in response to nutrient limitation and alterations in growth rate (Gadd, 1980). It is likely that the pigmented cell wall can act as a barrier to metal ions, preventing further entry into the cell and subsequent toxic effects. Gadd (1981) has also demonstrated that *A. pullulans* possesses reduced cation uptake systems, which would be advantageous in a metal-polluted ecosystem.

Studies on the yeast phase of *Aspergillus nidulans* also suggest that melanin has binding capabilities. Melanized hyphal walls of *A. nidulans* appear to protect hyphae against microbial lysis in soils (Bull, 1970), probably due to a combination of enzyme inhibition and substrate complexing by melanin. Melanin seems to form an "intractable" complex with chitin. The melanin is apparently produced after the cessation of exponential growth and is laid down on the outside of the hyphal walls (Rowley and Pirt, 1972).

Heavy metal uptake in yeast cells of *A. pullulans, S. cervisiae, Sporobolomyces roseus* and protoplasts of *Penicillium ochro- chloron* appears to be involve of phases of wall binding and intracellular uptake (Gadd, Mowll, and White, 1985). The authors suggest that differences in wall binding capacity occur between different species and cell types which can be related to differences in wall structure and thickness. Once inside the cell, the metal ions may be compartmentalized and/or bound to metal-binding proteins.

The work presented in the first chapter, 'Effect of copper on tyrosinase and polyamine content in some ectomycorrhizal fungi', investigates the role of natural versus metal-induced hyphal pigmentation in copper tolerance in ectomycorrhizal fungi. The growth rates and properties of tyrosine oxidase under conditions of copper stress are compared in ectomycorrhizal fungi which are naturally melanin pigmented with those of fungi pigmented only in the presence of copper stress. In addition, polyamine content of the fungi was analyzed and differences were related to copper in the growth media.

Nonmycorrhizal filamentous fungi

Filamentous fungi appear to be able to tolerate levels of heavy metals far in excess of those tolerated by higher plants. Fungi in uncontaminated habitats tend to have greater internal levels of heavy metals than angiosperms (Hinneri, 1975; Stegnar et al., 1973). They have frequently been reported growing on soil contaminated with metals and often are characterized by extraordinarily high metal concentrations (Ernst, 1985). For example, significantly higher levels of copper per unit dry weight have been reported in members of the Agaricaceae when compared with vascular plants (Byrne, Ravik, and Kosta, 1976). However, metal uptake is probably species dependent (Seeger, 1976; Seeger and Gross, 1981). Some studies of fungi along heavy metal gradients show differences in microfungal species composition due to heavy metal contamination (Jordan and Lechevalier, 1975; Nordgren, Baath and Soderstrom, 1983, 1985) while other workers have not demonstrated any significant effects on soil fungi (Freedman and Hutchinson, 1980). Imperfect fungi, i.e. *Aspergillus* and *Penicillium*, often immobilize heavy metals such as cadmium (Kiff and Little, 1985) and copper (Stokes and Lindsay, 1979) and have been suggested for use in removal of metals from waste waters. Accumulation of cadmium by the aquatic *Pythium, Dictyuchus* and *Scytalidium* is due to the adsorption of the metal to the surface of the mycelium (Duddridge and Wainwright, 1980).

Copper tolerance in the imperfect genera *Aspergillus*, *Penicillium*, and *Verticillium* and in the wood rotting basidiomycetes *Poria placentia*, *P. monticola*, and *P. vaillanti* appears to be correlated with the production of copper oxalate (Murphy and Levy, 1983; Sutter, Gareth-Jones and Walchli, 1983). The second authors suggest the following mechanism for tolerance in *Poria sp.*: the hyphae are surrounded by an extracellular mucilaginous sheath containing dissolved oxalic acid, which extracts or reacts with cations such as calcium or copper. These cations are then precipitated and deposited around the hyphae forming a tube of microcrystals and mucilage. Copper oxalate is significantly less toxic to fungi than are free copper ions (Levi, 1969). However, since *Poria spp.* may have 3 to 4 percent copper per hyphal dry weight (Chou, Chandler, and Preston, 1973), an additional intracellular mechanism of copper immobilization in these fungi cannot be ruled out.

Copper tolerance of monokaryotic hyphae of the wood rotting *Polyporus palustris* is significantly less than that of dikaryotic hyphae (Osborne and Da Costa, 1973). The authors suggest two

possibilities. One, either the presence of more nuclei gives the fungus a greater potential output of RNA and enzymes and, hence, more copper is required to inactivate them; or two, that copper tolerance depends to some extent on a large number of genes, and more of the genes should be present in the dikaryon.

It appears that pH of the growth medium plays an important role in determining the toxicity of heavy metals to filamentous fungi. A low pH has been shown to reduce copper toxicity in a number of imperfect and wood rotting fungi (Singh, 1977; Young, 1961; Starkey, 1973). These authors suggest that there is less binding of copper to cell membranes at lower pH. The increased copper tolerance of brown-rotting compared with white-rotting species may not be entirely due to the low solubility of copper oxalate but to the lowering of the pH of the substratum by the brown-rotting fungi (Young, 1961). Culture solutions from white rotting fungi increased in pH during a 24 day growth period while that of a brown rotting fungus decreased in pH (Shigo, 1970).

Ectomycorrhizal Fungi

Colonization of plants by ectomycorrhizal fungi can alter the distribution of toxic metals in the host plant. For example, lower amounts of zinc and manganese have been found in the needles of *Pinus virginiana* seedlings colonized by *Pisolithus tinctorius* than in uncolonized seedlings (Miller and Rudolph, 1986). Zinc translocation was reduced to the shoots of *Betula* colonized by *Paxillus involutus* (Brown and Wilkins, 1985a), and seedlings of the same genus translocated less nickel to the shoots in the presence of *Scleroderma flavidum* (Jones and Hutchinson, 1986; Jones, Browning and Hutchinson, 1986). However, mycorrhizal birch seedlings were found to be more sensitive to copper than nonmycorrhizal seedlings (Jones and Hutchinson, 1986). The continued growth of certain ectomycorrhizal fungi following heavy metal exposure may select for those fungi which will be effective in increasing metal tolerance of a host plant (Jones and Hutchinson, 1988).

The binding of metal ions in the fungal cell wall or to other extracellular sites has been suggested as an underlying mechanism in reducing the transfer of metal to the shoot in both ectomycorrhizae (Jones and Hutchinson, 1986; Denny and Wilkins, 1987d) and in the related ericoid mycorrhizae (Bradley, Burt and Read, 1981). Potential extracellular binding sites occur in

fungal cell walls, the matrix between the hyphae, the material at the plant-fungus interface, and the plant cell walls (Edwards and Gessner, 1984). Zinc has been localized on the hyphal surface of mycorrhizal roots of *Betula*, and Denny and Wilkins suggest that the metal may be adsorbed to electronegative sites in the hyphal cell walls and extra-hyphal polysaccharide matrix of the extramatrical hyphae. They did not find an accumulation of the metal in the fungal mantle. However, concentrations of heavy metals, especially nickel and iron were found in the peripheral mycorrhizal mantle region of cross- sectioned roots (Wasserman et. al, 1987). That *Scleroderma flavidum* birch mycorrhizae do not require metabolic energy to reduce Ni translocation from roots to shoots (Jones, Dainty and Hutchinson, 1988) tends to support the hypothesis of adsorption to cell walls and extra-hyphal components rather than an active uptake and sequestration of the metal within the fungal cytoplasm. Finally, calcium oxalate has been shown to accumulate in the mantle of ectomycorrhizal roots of *Pinus* and *Eucalyptus* (Malajczuk and Cromack, 1982), which suggests that ectomycorrhizal fungi have the potential for detoxifying copper by forming copper oxalates in the manner that has been previously demonstrated for wood rotting fungi.

The experiments described in the second chapter used a stain that specifically identified copper using transmission electron microscopy to determine if the metal could be localized in the living or nonliving components of the fungal cells. The study was designed to see if there was a general ultrastructural response to copper in ectomycorrhizal fungi or if vacuoles, cell walls and the hyphal sheath were all capable of sequestering copper, depending on the species of fungus.

Throughout the literature on heavy metal tolerance in ectomycorrhizal fungi, the predominant theme is that of a lack of correlation between tolerance of specific fungal isolates and the metal toxicity of the soil of origin. When four species of ectomycorrhizal fungi reported as mycobionts of trees that grow on mine spoils, *Cenococcum graniforme, Pisolithus tinctorius, Suillus luteus*, and *Thelephora terrestris*, were grown on solid media amended with aluminum and manganese, the tolerance responses of the fungi were not consistent with field observations of the successional sequence of these fungi on acid coal spoils (Thompson and Medve, 1984). However, it is very clear that some ectomycorrhizal fungi will grow on metal contaminated sites while others will not. While ecotypic differences among isolates of the same fungus may not be demonstrable, ecological differ-

ences between fungal species are quite clear. It is the fungi that are typically regarded as early successional species, colonizing a wide host range of usually young trees, that are commonly found on metal contaminated soils (Trappe, 1962; Marx, 1977; Thompson and Medve, 1984). Although it is logical that the young trees occupying toxic sites would be colonized by early successional mycorrhizal fungi, I would suggest that these fungi may have additional metabolic mechanisms that correlate their ability to colonize seedlings with an ability to tolerate conditions under which other fungal species may not survive. In a study comparing the growth of nine ectomycorrhizal fungi on agar media amended with cadmium, lead, and nickel, the tolerance of *Suillus luteus* was found to be significantly greater than that of *S. brevipes* or *S. grevellei* (McCreight and Schroeder, 1980). *S. luteus* is often collected on metal mine tailings, unlike the other two species (Trappe, 1962). In axenic screening tests of three species of *Lactarius* and *Scleroderma flavidum*, isolates from a metal contaminated smelter site did not outperform those from an uncontaminated site on media amended with copper or nickel (Jones and Hutchinson, 1988). Although isolates of *Amanita muscaria* and *Paxillus involutus* collected from zinc contaminated soils were able to grow on agar media containing 3 mM zinc, a significantly higher level than that tolerated by higher plants (Brown and Wilkins, 1985b), no correlation was observed between tolerance of the isolates and the amount of zinc in the soil at the collection site. A related study compared isolates of *P. involutus* from toxic and non-toxic soils. Denny and Wilkins (1987c) found that the ability of different strains of the fungus to grow in pure culture on agar, to form ectomycorrhizas with *Betula*, and to produce a beneficial growth and zinc uptake response in the same plant, all in the presence of raised zinc concentrations, was not related to the zinc status of the site of fungus collection. They suggest the ameliorating influence of *P. involutus* on zinc toxicity to *Betula* was more positively linked to the degree of compatibility between fungal strain and higher plant.

One report claims to have histochemically demonstrated the presence of metallothionein-like proteins in ectomycorrhizal fungi (Morselt, Smits and Limonard, 1986). The authors stained hyphae of *Pisolithus tinctorius* and *Cenococcum geophilum*, species considered to be metal tolerant and intolerant respectively. Only hyphae of *P. tinctorius* were found to have protein bound disulphides and metal-thiolate clusters, the presence of which could be increased by the addition

of metals to the growth medium. They suggest that the potential for induction of metallothionein is an intrinsic property of some fungi known to be metal tolerant.

These studies appear to indicate that there are intrinsic mechanisms of heavy metal tolerance in ectomycorrhizal fungi and that growth on toxic sites by a particular species relates to one or more tolerance mechanisms it may possess, rather than to evolution of metal tolerant ecotypes. This hypothesis was tested by the work reported in the third chapter, 'Effect of copper on growth of *Pinus densiflora* and five ectomycorrhizal symbionts'. The responses of a single host species in the presence of copper stress and inoculated with species of ectomycorrhizal fungi considered to be primary successional species were compared with those that are thought to be late successional. Additionally, the responses of isolates from three different populations of *Suillus granulatus* were compared to determine if there was variability within the species in its ability to ameliorate host growth in the presence of copper.

The fourth chapter describes the alterations of the root system of pine that were observed when *Boletinellus merulioides* was used as a nonmycorrhizal control fungus in the previous experiments and discusses a possible biochemical basis for these changes.

Endomycorrhizal Fungi

The relatively few works on heavy metals and endomycorrhizal fungi indicate that these fungi appear to be nonselective in their ion uptake mechanisms and subsequent transfer of ions to the host plant. Consequently, endomycorrhizal fungi can increase the levels of heavy metals in the foliage of host plants (Killham and Firestone, 1983). High rates of zinc have been shown to decrease the percent mycorrhizal colonization in soybean (McIlveen, Spotts and Davis, 1975; McIlveen and Cole, 1978/1979). The degree of colonization of onions was reduced by additions of zinc, copper, nickel or cadmium (Gildon and Tinker, 1983). Although there have been reports of a heavy metal tolerant strain of *Glomus mosseae* (Gildon and Tinker, 1981; Gildon and Tinker, 1983), no further experiments have been conducted at the Rothamstead Experiment Station, because the strain was lost.

In the fifth chapter, experiments evaluate the response of mycorrhizal and nonmycorrhizal onions to copper stress using an isolate of *Glomus diaphanum* collected from a coal spoil pile high in heavy metals. The final chapter describes an ecological survey which establishes the presence and quantity of potential endomycorrhizal inoculum on abandoned metal and coal spoil piles in southwestern Virginia and central North Carolina.

Metal Tolerance Mechanisms of Other Organisms

Lichens

Lichens are symbiotic associations generally between ascomycetous filamentous fungi and green or blue-green algae and often accumulate heavy metals far in excess of their biological requirements (James, 1973). Metals incorporated in the lichen tissue are not removable by natural means, such as rain (Garty et al., 1977). Studies seem to indicate that the metals are most often associated with the mycobiont. In analysis of *Caloplaca aurantia*, metallic elements were concentrated in the extracellular space in the fungal medulla (Garty et al., 1979), but with the exception of zinc no metals were detected intracellularly or in the cell walls of the mycobiont. Lead was bound to insoluble anionic sites located in the hyphal cell walls of *Cladonia rangiformis* (Brown and Slingsby, 1972) and Goyal and Seaward (1982) interpreted metal binding sites in *Peltigera* as occurring near, on, or within the fungal hyphae. However, iron has been found in or on the phycobiont in one species of *Cladonia* (Lawrey, 1977).

Cyanobacteria

Certain microorganisms produce siderophores which are organic metabolites capable of complexing metals. Siderophores may not only enhance the bioavailibility of metals required for growth but can also reduce the ionic concentration of deleterious metals. The cyanobacteria *Plectonema boryanum* and *Anabaena cylindrica* both produce copper complexing extracellular material when grown under conditions of elevated copper. In addition, *P. boryanum* was able to actively respond to potentially toxic copper concentrations by complexing more of the metal and thus

reducing the free ion concentration (Jardim and Pearson, 1984). Cells of *P. boryanum* also appear to sequester heavy metals in polyphosphate bodies which provide a storage site for essential metals and act as a detoxification mechanism (Jensen et al., 1982)

Algae

Two species of *Scenedesmus* were found to be tolerant to copper due to the formation of copper-protein complexes in the cytoplasm (Silverberg, Stokes and Ferstenberg, 1976). In a relatively unusual mechanism of metal tolerance, a strain of *Chlorella vulgaris* isolated from a polluted environment, was found to metabolically exclude copper (Foster, 1977). A copper tolerant strain of *Ectocarpus siliculosus* has been shown to do the same (Hall, Fielding and Butler, 1979). In the later study extracellular material and the cell wall made little or no contribution to the exclusion which suggested that membrane and intracellular changes could account for the tolerance. Exclusion through metabolic processes is a relatively rare mechanism of metal tolerance among both prokaryotic and eukaryotic organisms, although it has been reported in a few algae.

Green Plants

Most of the plants in which metal tolerance has been studied are known to be endomycorrhizal but it is notable that no mention is made of the mycorrhizal component. Various mechanisms have been suggested to account for metal tolerance in plants (Antonovics, Bradshaw, and Turner, 1971; Wainwright and Woolhouse, 1975). Preferential binding of zinc to anionic sites in the cell walls of roots has been demonstrated to be characteristic of metal tolerant *Agrostis tenuis* (Turner and Marshall, 1972). Other studies have demonstrated the ability of metal tolerant plants to complex metal intracellularly by chelation with organic acid residues and sequestration in root cell vacuoles (Ernst, 1976). Metal tolerant clones of *Deschampsia, Anthoxanthum* and *Mimulus* have been shown to detoxify lead, zinc and copper by removal from the cytoplasm and sequestration in vacuoles; lead was found to be bound also to cell walls (Mullins, Hardwick and Thurman, 1985). Clones of *Festuca rubra* collected from serpentine soils show a stimulation of root surface phosphatases by manganese and nickel levels which were inhibitory to the phosphatases of

nonserpentine clones (Johnston and Proctor, 1984), but the ecological role of these metal tolerant phosphatases is unclear. Similar results were obtained when the acid phosphatases of zinc and copper tolerant clones of *A. odoratum* and *D. cespitosa*, respectively, were compared (Cox and Hutchinson, 1980). In the case of zinc tolerant *Betula* species, it appears that the plasma membrane may control the movement of zinc into the symplast (Denny and Wilkins, 1987a). As a final mechanism, cadmium, zinc, and copper-binding proteins similar to metallothioneins have been reported to occur in a number of vascular plant species (Robinson and Thurman, 1985) and may be involved in metal tolerance.

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Effect of copper on tyrosinase activity and polyamine content of some ectomycorrhizal fungi

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ABSTRACT
Several species of ectomycorrhizal fungi were grown on solid and liquid media in order to compare their tyrosine oxidases and polyamine contents in the presence of copper stress. Two of the fungi, *Pisolithus tinctorius* and *Boletinellus merulioides*, have naturally brown pigmented hyphae and showed evidence of extracellular tyrosinase production at both 0 and 60 μg/g copper. *Suillus pictus* and *S. granulatus* developed pigmented hyphae in the presence of copper. Only *S. pictus* produced extracellular tyrosinase at 60 μg/g copper. *Piloderma bicolor* and *Cenococcum graniforme* did not alter the color of their respective yellow and black hyphae in response to copper and did not produce extracellular tyrosinases. Intracellular tyrosinase of *P. tinctorius* and *S. granulatus* increased in activity but decreased in substrate affinity (Km) when these fungi were grown under conditions of copper stress. Addition of the polyamines putrescine and spermidine to the reaction mixture activated and repressed the enzyme, respectively. Polyamine content of *P. tinctorius* and *B. merulioides* was not altered by copper, while that of the two *Suillus* species was significantly decreased by the addition of copper to the growth medium. A decrease in fungal polyamine content may indicate a change in permeability of the cells both to copper and to the extracellular signals for polyamine biosynthesis.

Key words: Ectomycorrhizae, copper, polyamines, tyrosinase, melanin.

INTRODUCTION

Copper is an essential nutrient for normal growth and development of fungal cells (Bowen, 1966), but can be toxic at relatively low concentrations (Ross, 1975). Like the other heavy metals that are di- and trivalent cations, the solubility and availability of copper increases with decreasing pH of the growth medium. Since ectomycorrhizal fungi typically form symbiotic associations with members of the Pinaceae, a family which often occurs on acid soils (Meyer, 1973), mechanisms of tolerating copper and other heavy metal stress might be expected.

Any one or a combination of several known or as yet undescribed mechanisms may function in fungal tolerance to copper. Formation of a brown pigment, probably melanin, has been associated with tolerance in the yeast *Saccharomyces cervesiae* (Seno, 1963) and in the imperfect dimorphic fungus *Aureobasidium pullulans* (Gadd and Mowll, 1985). Increased hyphal pigmentation might be predicted in some fungi in the presence of excess copper since tyrosinase, the melanin biosynthetic enzyme, is a copper protein complex (Lerner and Fitzpatrick, 1950). However, tolerance in *A. pullulans* to other heavy metals such as lead and cadmium with no catalytic function in melanogenesis have also been correlated with an increase in pigmented hyphae (Gadd, 1981). Increasing hyphal pigmentation may be one way of limiting copper toxicity if higher levels of tyrosinase prevent the ion from toxifying other parts of the cell by sequestering copper in the enzyme. Melanin may also be capable of binding copper or otherwise blocking its entry into the cell.

Polyamines are aliphatic amines essential for normal growth and development of both prokaryotic and eukaryotic cells (McCann, Pegg and Sjoerdsma, 1987), although their specific function has not been established. They show a great affinity for pigmented tissues, possibly due to ionic interactions with melanin (Tjalve, Nilsson and Larson, 1981), but the physiological significance of this is unknown. Polyamines have been shown to enhance the activity of tyrosinase from mouse melanoma but had no effect on the enzyme from frog epidermis or the decomposer basidiomycete, *Agaricus bisporus* (Galindo, et al., 1987).

Tyrosinase (EC 1.14.18.1) is a mixed-function oxidase catalyzing three steps in the pathway of melanin formation; the hydroxylation of L-tyrosine to L-dopa, the oxidation of L-dopa to dopaquinone, and the oxidation of 5,6-dihydroxy-indol to indol-5,6-quinone. In order to investigate the role of tyrosinase and polyamines in the response of fungi to copper, this study compares the tyrosinases and polyamines produced by ectomycorrhizal fungi at low and high copper levels and examines the effect of polyamines on tyrosinase activity.

MATERIALS AND METHODS

The fungi used in this work were all maintained at 2°C on Hagem's agar (Hagem, 1910). Six are part of the Virginia Polytechnic and State University mycology culture collection. They included two strains of *Suillus granulatus* (L. ex. Fr.) O. Kuntze, *Suillus pictus* Peck (Smith & Thiers), *Pisolithus tinctorius* (Pers.) Coker & Couch, *Piloderma bicolor* (Pk.) Julich, and *Cenococcum graniforme* (Sow.) Ferde. & Winge. *Boletinellus merulioides* (Schw.)Murrill. was provided by Dr. H.V. Cotter. All of the fungi used are proven ectomycorrhizal associates of members of the genus *Pinus* (Trappe, 1962) except *B. merulioides* which is thought to be associated with *Fraxinus* (Cotter and Miller, 1985).

Fungal cultures grown in the defined liquid medium of Palmer (1971) (PDM) at 23°C in the dark were ground in a Waring blender, and 200 μl of the resulting suspension were added to agar plates of the same medium. After incubation for 3 weeks at 20C, 0.8 cm disks were removed with a cork borer and placed in the center of PDM plates amended with 0 and 60 μg/g (= ppm) copper. Copper was was added to the media as copper sulfate crystals after autoclaving. Plates were sealed with parafilm, incubated at 20°C in the dark, and inverted after 9 days of growth. Each fungus x copper combination was replicated four times and replicates were arranged randomly in the incubator.

In order to evaluate extracellular tyrosinase activity, a single drop of 1% p-cresol was applied to the leading edge of 30 day old fungal cultures (Marr, Grund and Harrison, 1986). Cultures were evaluated every 10 minutes for 1 hour and a darkening of the reagent was considered to be positive for the production of extracellular tyrosinase by the fungus.

Liquid cultures were used to compare the intracellular tyrosine oxidases of two of the fungi. Cultures grown in liquid PDM were ground in a Waring blender, and 100 μl of the suspension were added to 50 ml of liquid PDM amended with O, 15, or 30 μg/g copper as copper sulfate in 250 ml Erlenmeyer flasks. Flasks were incubated in the dark at 23°C. After four weeks, fungal mycelium was removed from the growth medium, rinsed twice in 0.1 M potassium phosphate buffer (pH 7.2) and ground to a fine powder with a mortar and pestle using three additions of liquid nitrogen. A small amount of buffer was added to the mortar, and the cultures were ground again after thawing slightly. The resulting slurry was centrifuged for 20 minutes at 12,000 g. Supernatants

(=enzyme preparations) were collected in 1.5 ml microfuge tubes and stored at -4C until use. Copper in the preparations was assayed using atomic absorption spectrophotometry. Total protein was measured using the method of Bradford (1976).

Intracellular tyrosinase activity was measured spectrophotometrically at 475 nm, using L-dopa in 50mM potassium phosphate buffer as substrate. Copper sulfate, EDTA, and the polyamines putrescine and spermidine were also dissolved in phosphate buffer when added to the reaction mixture. The molar extinction coefficient (ε_{475}) for the detected product, dopachrome, was taken as 3600/M.cm. Reactions were performed at room temperature in 1 ml quartz cuvettes using a Gilford 250 spectrophotometer. One enzyme unit was defined as the amount of tyrosinase transforming 1 μmol of L-dopa/min.

Polyamine content of four fungi was determined by adding 50% trichloroacetic acid (TCA) to the enzyme preparations to a final concentration of 5% (w:w). To determine percent recovery of polyamines, 1-8 diamine hexane was added to each tube to a final concentration of 0.2 ng/μl. After two hours at room temperature, the preparations were mixed, centrifuged at 10,000 x g for 10 minutes, and the supernatants analyzed for putrescine, cadaverine, spermidine and spermine using HPLC at the Virginia-Maryland Regional College of Veterinary Medicine. Pellets were solubilized in 500 μl 1.0 N NaOH, heated for 15 minutes at 75°C, and centrifuged for 10 minutes at 10,000 x g. The resulting supernatants were assayed for total protein as above.

Statistical separations were performed using Duncan's New Multiple Range Test (Duncan, 1975) at P = .05.

RESULTS

Extracellular tyrosinase

The two fungi used which have naturally brown pigmented hyphae (*Pisolithus tinctorius* and *Boletinellus merulioides*) show evidence of extracellular tyrosinase production when grown on media containing 0 and 60 μg/g copper (Table 1). The most intense reaction was observed in *P. tinctorius*, and this reaction was enhanced when the fungus was grown on copper amended media. The reaction was faint in *B. merulioides* at both copper levels. *Suillus pictus* exhibited extracellular

tyrosinase activity only when grown on media containing copper, and the enzyme was apparently not present in amounts measurable by this method in *S. granulatus*, *Piloderma bicolor*, and *Cenococcum graniforme*.

Intracellular Tyrosinase

The addition of copper significantly increased both the Km and the specific activity of tyrosinase from *P. tinctorius*. However the specific activity of the enzyme in 15 μg/g copper cultures was always significantly higher than that in the 0 μg/g cultures, even when the copper levels were normalized in the reaction mixture by the addition of copper sulfate (Table 2). The addition of EDTA also increased both values in the enzyme from *P. tinctorius*. The enzyme parameters changed slightly with storage, but the same trends were present. Enzyme preparations from *S. granulatus* contained substantially less intracellular copper than did those from *P. tinctorius*. In the reverse of that in *P. tinctorius*, addition of copper or growth in a copper containing medium significantly lowered the Km of tyrosinase from *S. granulatus*. However, the specific activity increased in a similar manner to that seen in *P. tinctorius*. When copper levels are normalized, the enzyme from the *S. granulatus* cultures grown at high copper is also significantly more active than that from low copper cultures.

Addition of 10mM putrescine to the reaction mixture increased both the Km and the specific activity of tyrosinase from *Pisolithus tinctorius* by 160 and 100% respectively (Table 3). The addition of the same levels of spermidine had an opposite effect on both parameters decreasing Km by 83% and specific activity by 33%.

Polyamine Content

The polyamine content (primarily putrescine) of the two brown pigmented fungi utilized in this portion of the experiment (*P. tinctorius* and *B. merulioides*) did not decrease when grown in a medium containing copper (Table 4). In contrast, the levels of polyamines in the two species of *Suillus* decreased markedly in response to copper stress. Levels of putrescine in fungi grown in 30 μg/g copper were 73% lower than in the controls for *S. granulatus* and 68% lower for *S. pictus*. The four polyamines were present in the control cultures of all of the fungi examined, with the exception of spermidine in *S. granulatus* and *B. merulioides*. The two species of *Suillus* and

Boletinellus had higher levels of putrescine than any of the other three polyamines. In contrast, *P. tinctorius* had higher levels of cadaverine in relation to other polyamines.

Table 1: Effect of Copper on Extracellular Tyrosinase in Six Species of Ectomycorrhizal Fungi Grown on Solid Media

Fungus	Copper in Media (μg/g)	Reaction Time (min.)	Color of Spot Test	Intensity	Hyphal Color
Pisolithus tinctorius	0	5	rust	+ +	brown
	60	5	rust	+ + +	brown
Boletinellus merulioides	0	60	gold	+	gold
	60	60	gold	+	gold-brown
Suillus pictus	0	60	none	-	white-pale buff
	60	30	red	+ +	dark gold-brown
Suillus granulatus #6	0	60	none	-	white
	60	60	none	-	dark brown
Suillus granulatus #2	0	60	none	-	white
	60	60	none	-	dark brown
Cenococcum graniforme	0	60	none	-	black
	60	60	none	-	black
Piloderma bicolor	0	60	none	-	bright yellow
	60	60	none	-	bright yellow

Table 2:
Effect of Copper and EDTA on Intracellular Tyrosinase from Two Ectomycorrhizal Fungi Grown at 0 and 15 μg/g Copper

Fungus	Cu in Media (μg/g)	Cu in Enzyme Prep (μg/g)	Cu Added to Reaction Mixture (μg/g)	Total Cu in Reaction Mixture (μg/g)	EDTA Added to Reaction Mixture (mM)	Km (mM)	Specific Activity (μmol/min/ mg protein)
Assays performed within 2 weeks of harvest:							
Pisolithus	0	0.15	0.0	0.006	0	0.60b[1]	0.21c
tinctorius	0	0.15	0.0	0.006	10	2.00ab	0.65ab
	0	0.15	5.0	5.006	0	1.67ab	0.73ab
	15	4.65	0.0	0.200	0	2.85a	1.03a
	15	4.65	0.0	0.200	10	2.10ab	0.53bc
	15	4.65	5.0	5.200	0	1.50ab	0.63ab
Suillus	0	0.00	0.0	0.000	0	6.60a	1.60b
granulatus	0	0.00	5.0	5.000	0	1.8b	1.50b
	15	0.15	0.0	0.150	0	1.32b	4.20ab
	15	0.15	5.0	5.150	0	1.68b	8.26a
Assays performed 3 months after harvest:							
Pisolithus	0	0.15	0.0	0.006	0	1.85a	0.38b
tinctorius	0	0.15	0.2	0.206	0	3.58a	0.66b
	15	4.65	0.0	0.200	0	3.58a	1.08a

[1]Numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Table 3: Effect of the Addition of the Polyamines Putrescine and Spermidine on the Intracellular Tyrosinase of *Pisolithus tinctorius*

Polyamine (10mM)	Km (mM)	Specific Activity (μmol/min/mg protein)
none	5.44b[1]	0.64b
Putrescine	14.29a	1.27a
Spermidine	.88b	0.43b

[1]Numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Table 4: Effect of Copper on Polyamine Content of Four Ectomycorrhizal Fungi Grown in Liquid Culture

Fungus	Copper in Media (μg/g)	Polyamines (ng/μg protein) Putrescine	Cadaverine	Spermidine	Spermine
Pisolithus	0	3.67	58.89	4.66	3.32
tinctorius	15	5.17	24.98	1.84	1.96
	30	4.25	74.85	22.44	8.27
Suillus	0	13.93	nd[1]	nd	nd
granulatus	15	11.99	0.87	nd	1.29
	30	3.83	0.86	nd	nd
Suillus	0	42.92	23.02	3.32	74.26
pictus	15	10.44	nd	nd	0.75
	30	13.58	nd	nd	1.74
Boletinellus	0	2.27	nd	nd	2.29
merulioides	15	2.23	nd	nd	0.21

[1]nd = not detectable

DISCUSSION

Increase in hyphal pigmentation in as a result of copper stress does not appear to be a universal response of ectomycorrhizal fungi, although the two *Suillus* species which are normally unpigmented or lightly pigmented in culture did show a darkening of hyphae when grown on copper amended media. However, of the two *Suillus* species, only *S. pictus* noticeably increased production of extracellular tyrosinase. This supports the suggestion of Gadd (1981), that increase in hyphal pigmentation induced by copper stress may not be merely a response to increased copper as a cofactor for the enzyme reaction but may be a tolerance response to heavy metals in general.

The intracellular tyrosinases of both *Pisolithus tinctorius* and *S. granulatus* grown at high levels of copper always had a higher specific activity than the same enzyme obtained from cultures grown at low copper levels. Increased tyrosinase activity may be a mechanism of copper tolerance in these organisms. If the specific activity of tyrosinase increases and it's affinity for tyrosine decreases (i.e. Km), as it did in the case of *P. tinctorius*, this could be explained by an increase in the actual amount of enzyme in the cells. Since copper is a metalloenzyme, an increase in the amount of copper in the growth medium would accomodate *de novo* synthesis of tyrosinase. The low affinity of the enzyme for the substrate in cultures of *S. granulatus* grown at 0 μg/g copper suggests that the enzyme is present in the hyaline hyphae, but is less effective at low substrate concentrations. Tyrosinases prepared from *Streptomyces glaucescens* and *Neurospora crassa* are reported to contain Cu(I) and to form stable monomers (Lerch and Ettlinger, 1973; Fling, Horowitz and Heinemann, 1963). In the latter work, the copper atom in tyrosinase from *N. crassa* was not removed by dialysis against cyanide, and the enzyme from this fungus and other sources becomes inactivated during the oxidation of dopa and other substances. Inactivation of the enzyme during formation of the product coupled with formation of stable complexes with copper by tyrosinase may be one means of reducing free intracellar copper, and the increased hyphal pigmentation may be a side product of this intracellular ionic reduction.

The increase in enzyme activity with EDTA is not surprising, since our enzyme preparations were not purified. Substances that form weakly dissociable complexes with copper can result in a decrease in the rate of the dopa-tyrosinase reaction (Lerner and Fitzpatrick, 1950) and the EDTA

is probably removing such substances. Since 10mM EDTA was presumably enough to chelate all of the free copper ions in the reaction mixture, these data also support view that the copper of the metalloenzyme is irreversibly bound.

The decrease in polyamine content of the two *Suillus* species in response to copper in the growth medium suggests that the corresponding increase in hyphal pigmentation is preventing entry of the signal(s) which induce polyamine synthesis. Ionic strength is one inducer of polyamine biosynthesis (Rubenstein et al., 1972; Flores and Galston, 1982). Melanin pigmented cells of *Aspergillus nidulans* have been shown to be more resistant to enzymatic lysis than hyaline cells due either to substrate complexing or enzyme inhibition (Kuo and Alexander, 1967; Bull, 1970). If the increased pigmentation of the *Suillus* cells can prevent entry of copper to the interior of the cell by complexing the ion, a similar reaction may be taking place with the inductive agents of polyamine biosynthesis. The lack of a decrease in polyamine content in the two brown pigmented fungi *P. tinctorius* and *B. merulioides* suggests that their natural hyphal pigmentation differs from that produced due to metal stress in the two *Suillus* species.

The three fungi in the family Boletaceae, *B. merulioides*, *S. pictus*, and *S. granulatus*, had higher levels of putrescine relative to all of the other polyamines. *P. tinctorius*, which is placed in a different subclass from the other three and is not thought to have any ancestral connections to them (Miller, 1983), has higher levels of the less cadaverine when compared with the other polyamines. If relative polyamine levels are conserved at the family or generic level, their analysis might prove valuable in phylogenetic studies.

The physiological significance of the stimulation of tyrosinase activity by putrescine and inhibition by spermidine is unclear. Since putrescine is the first polyamine formed from ornithine and leads subsequently to production of all other polyamines (Tabor and Tabor, 1985), a build-up of putrescine may signal conditions of stress to the fungus. Production of hyphal pigmentation due to stress has been documented in the fungi (Pirt and Rowley, 1969; Rowley and Pirt, 1972); and the stress conditions may be stimulating to tyrosinase production. Conversely, the inhibition of activity by spermidine, which is produced from putrescine, may be a signal to the cell of absence

of stress, i.e. polyamine biosynthesis is proceeding normally and there is no need for additional tyrosinase that will produce hyphal pigmentation.

Our results suggest the following scenario for response of some ectomycorrhizal fungi to copper stress. Increased copper in the growth medium stimulates the activity of extra and intracellular tyrosinases. Intracellular tyrosinase may by its increase in activity play a role in chelation and detoxification of copper. The increased enzyme activity leads to an increase in melanin pigmentation of the hyphae in some fungi, which in turn limits the entry of copper and other ions into the cell. This reduction of ion flow also reduces the flow of inducers of polyamine biosynthesis which leads to a drop in polyamine content. Those fungi with naturally darkly pigmented hyphae also increase activity of tyrosine oxidase in response to copper stress but do not show a decrease in cellular polyamine levels.

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Growth of some ectomycorrhizal fungi on copper amended media and ultrastructural localization of copper

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ABSTRACT
Growth of several ectomycorrhizal fungi was compared on solid media amended with 0, 15, 30, 60, and 100 μg/g (ppm) copper as copper sulfate. *Pisolithus tinctorius*, *Boletinellus merulioides* and one isolate of *Suillus granulatus* were most tolerant to copper. *Suillus pictus* and two additional isolates of *S. granulatus* were moderately tolerant, while *Piloderma bicolor* was very intolerant. Ultrastructural localization of copper ions showed them to be deposited outside the cell wall and within the cytoplasm in *P. tintorius*, outside the cell wall in *S. granulatus*, on the cell wall in *S. pictus* and no deposition of copper ions was observed in *P. bicolor*. These results suggest that differential binding of copper ions by ectomycorrhizal fungi may be responsible for the differences in growth rates observed on copper amended media. In addition, it is probably incorrect to postulate a single mechanism for metal tolerance and subsequent improvement of host plant growth in such a diverse group of organisms as the ectomycorrhizal fungi.

Key words: Ectomycorrhizae, copper, ultrastructure, tolerance, polysaccharides, cell walls

INTRODUCTION

Copper is an essential micronutrient for normal fungal growth and development (Garraway and Evans, 1984) but can be highly toxic at supraoptimal concentrations (Ross, 1975). However, some fungi have the ability to grow at levels of copper which severely restrict the growth of other organisms (Ashida, 1965). As a group, fungi exhibit a number of mechanisms of copper tolerance. These include the production and deposition of copper oxalate in the extracellular hyphal sheath of some wood-rotting fungi (Sutter et al., 1983), the formation of low molecular weight cytoplasmic metal-binding proteins (Fogel and Welch, 1982) and peptide aggregates in the yeast *Saccharomyces cereviseae* (Grill, 1985). The deposition of hydrogen sulfide in the cell wall of *S. cereviseae* has also

been correlated with copper tolerance in some strains (Ashida, 1965). Cell walls have been recognized as sites for complexing metallic elements in other organisms as well. Heavy metals have been localized in close association with the walls of the mycobiont in a number of lichens (Brown and Slingsby, 1972; Garty, Galun and Kessel, 1979; Goyal and Seaward, 1982) and are probably bound to the cell walls of some aquatic fungi (Duddridge and Wainwright, 1980). Higher plants also may bind metals at anionic sites on the root cell wall (Turner and Marshall, 1972) or may complex them intracellularly in an innocuous form (Horgan and Rauser, 1981).

Ectomycorrhizal fungi, which form symbiotic associations primarily with the roots of coniferous and fagaceous trees, can enhance plant uptake of limiting nutrients in forest ecosystems (Bowen, 1973; Harley and Smith, 1983; Harley, 1989). These fungal associations may also improve the survival of their hosts in soils containing high levels of heavy metals. Coniferous trees often occur on acid soils (Meyer, 1973) in which solubility of di- and trivalent cations is increased (Adriano, 1986). It is therefore probable that the ectomycorrhizal partners of these trees might have intrinsic mechanisms of dealing with potentially phytotoxic amounts of metals.

Some naturally occurring ectomycorrhizal roots of oak show a significant deposition of metals within the mycorrhizal mantle (Wasserman et al., 1987). In addition, colonization by ectomycorrhizal fungi has been shown to increase tolerance of host plants to nickel (Jones and Hutchinson, 1986; Jones, Dainty and Hutchinson, 1988) and zinc (Brown and Wilkins, 1985a). However, at high copper levels, mycorrhizal fungi significantly reduced host growth (Jones and Hutchinson, 1986). Lower concentrations of metals in leaves and stems and higher amounts in roots have been reported in ectomycorrhizal birch seedlings (Brown and Wilkins, 1985; Jones & Hutchinson, 1986). These results indicate some sort of metal binding by the mycobiont. Using X-ray microanalysis, zinc has been localized in the hyphal walls and intrahyphal spaces of birch ectomycorrhizae (Denny and Wilkins, 1987), suggesting that the metal is bound by the cell walls and in the extracellular polysaccharide sheath of the fungus.

Axenic culture studies show differences in metal tolerance between species and genera of ectomycorrhizal fungi (McCreight and Schroeder, 1979; Thompson and Medve, 1984). However, the relative abilities of these fungi to tolerate metal stresses in pure culture have not been correlated

with the improvement of host growth in metal contaminated soils (Jones & Hutchinson, 1988) or with field observations of fruiting bodies (Thompson & Medve, 1984; Brown & Wilkins, 1985b). This is very likely due to the complex physiological changes which occur in both the host plant and the fungus when the mycorrhizal association is formed (Hilbert and Martin, 1988). Despite the lack of correlation between growth of the fungus and growth of the host-fungus combination under conditions of metal stress, it is likely that the intrinsic mechanisms which permit an ectomycorrhizal fungus to grow in the presence of heavy metals would be the same *in vitro* and in association with a host plant.

In the present work, growth of four ectomycorrhizal fungi, including three isolates of the same species, and one putatively ectomycorrhizal fungus was compared in axenic culture at five copper levels. Transmission electron microscopy (TEM) was used to localize the metal in the four ectomycorrhizal species.

MATERIALS AND METHODS

Fungi

Tissue isolates of the mycorrhizal fungi *Pisolithus tinctorius* (Pers.) Coker & Couch, *Piloderma bicolor* (Pk.) Julich, *Suillus pictus* Peck (Smith & Thiers), and three isolates of *Suillus granulatus* (L. ex. Fr.) O. Kuntze (OKM22366, OKM22233, and OKM22298) were maintained at 4°C on Hagem's agar (Hagem, 1910) as part of the Virginia Polytechnic Institute and State University mycology culture collection. The *S. granulatus* isolates were obtained from different populations of the fungus but all were collected under *Pinus densiflora* D. Don in Korea. *Boletinellus meruliodes* (Schw.) Murrill, which is associated with *Fraxinus*, but has not been proven to be ectomycorrhizal (Cotter and Miller, 1985), was obtained from Dr. H.V. Cotter.

Growth Studies

Transfers were made to agar plates of a defined medium (PDM) (Palmer, 1971) and incubated in the dark at 20°C. After 3 weeks, 8 mm discs were removed with a cork borer and transferred to the center of Palmer's plates amended with 0, 15, 30, 60 and 100 μg/g copper, added as $CuSO_4$ crystals after autoclaving. The pH's within plates, measured with a flat surface electrode after pouring, were 5.4, 5.2, 5.1, 4.8, and 4.5, respectively. Each treatment was replicated a minimum of 4 times. Plates were incubated in the dark at 20°C. Measurements of colony diameter in two directions were made at 9, 20, 31, 43, and 53 days. Colony growth rates were evaluated by dividing the average mm/day increase in diamter of a fungus on copper amended media by the average mm/day achieved by the fungus on unamended media. Surface areas were compared by dividing the area of the cultures grown on copper amended media by the area of control cultures. Statistical separations for each fungus were performed on both the percentage of control growth rate achieved and the percentage of control biomass using Duncan's New Multiple Range Test (Duncan, 1975) at $P = .05$.

Ultrastructure

Hyphae grown at 0 and 60 μg/g copper were processed for transmission electron microscopy 41 days after transfer to copper amended media. Blocks of fungal tissue plus agar (2mm x 2mm) were cut from the leading edge of the colonies and fixed for 2 hours in formaldehyde-glutaraldehyde

(Karnovsky, 1965 or McDowell and Trump, 1976). Karnovsky's solution was amended with 100 μl of 1% Triton-X per ml of fixative. Tissue was rinsed 3 times for 10 minutes in 0.1 M potassium phosphate buffer, followed by once in distilled water and was then stained to localize copper according to Scheuer, Thorpe and Marriott (1967). The stain was used at either 1/10 strength or full strength. Silver nitrate was omitted from the controls. A previous experiment, in which sections were rinsed in potassium cyanide following immersion in ammonium sulfide to specifically remove copper ions, demonstrated that copper was the only metal responsible for the staining reaction. After staining, tissues were post-fixed in osmium tetroxide in either 0.1 M cacodylate buffer or 0.1 M phospate buffer, dehydrated and then embedded in Spurr's resin (Spurr, 1969) or Ultra-Low Viscosity Resin (Oliveira, 1983). Gold and silver sections were collected on copper grids and stained in Reynolds lead citrate (Reynolds, 1963) for 15 minutes unless noted.

RESULTS

Measurements of either growth rate or relative biomass indicate that *Boletinellus merulioides*, *Pisolithus tinctorius* and isolate #3 of *Suillus granulatus* were the most copper tolerant fungi under the experimental conditions (Table 1-3). Relatively moderately tolerant in comparison were *Suillus pictus* and isolates #6 and #2 of *S. granulatus* Least tolerant of the fungi examined here was *Piloderma bicolor*, which was essentially unable to grow at 100 μg/g copper.

Ultrastructural examination of the fungi revealed three different locations of copper ions, depending on the fungus. Copper was found either in the cytoplasm and on the outside of the cell wall, only on the outside of the cell wall, or bound to the cell wall. When the fungi were grown on media containing 0μg/g added copper, metallic ions were not present in the TEM sections (Figure 1).

Copper ions were bound to the exterior of the cell wall of *P. tinctorius* exposed to the 1/10 strength stain (Figures 2 and 3). When full strength stain was used (Figures 4 and 5), copper was found both outside the cell wall and inside the fungal cell. The ion was not located in vacuoles or in organelles, with the exception of the mitochondria, but was found in the cytoplasm and appeared to surround the polyphosphate bodies (Figure 4). The developing clamp connection (Figure 5) demonstrates that the distributional pattern of copper in the cytoplasm and outside the hyphal wall was present in very young hyphae.

Suillus granulatus isolate #3 stained either with 1/10 or full strength stain (Figures 6, 7, and 8) had no copper in the interior of the cells or on the cell wall. Copper ions were bound to the exterior of the cell wall similar to *P. tinctorius*. In addition, this fungus produced a sheath which was found between the individual hyphal cells (Figures 6 and 8) and appeared to be responsible for the copper binding.

Copper in *Suillus pictus* could only be found within the exterior and interior hyphal cell walls (Figures 9 through 14). It was not present in the cytoplasm, vacuoles or any organelles. Generally, there was a greater density of the ion on the interior portion of the two-layered wall (Figure 10). The developing clamp connection (Figures 11 and 12) demonstrates that binding of copper to the hyphal walls took place very early in development, and ions were as dense on the septal walls as

on the exterior walls. With age and plasmolysis of the hyphae, copper remained bound to the hyphal walls (Figure 13 and 14).

In *Piloderma bicolor* copper could not be found in sections of hyphae grown at 60 μg/g copper (Figure 15).

Table 1: Growth rate achieved over a 43 day period by four ectomycorrhizal fungi and one putatively ectomycorrhizal fungus on solid media amended with copper, expressed as a percent of growth rate on unamended media (n = 4)[1]

Fungus	μg/g copper in agar media			
	15	30	60	100
Boletinellus merulioides	82ab[2]	99a	86ab	91a
Piloderma bicolor	92ab	59b	35b	07e
Pisolithus tinctorius	53b	74ab	99a	74ab
Suillus granulatus #2 (OKM22366)	78ab	73ab	83ab	45cd
Suillus granulatus #3 (OKM22233)	101a	86ab	83ab	73ab
Suillus granulatus #6 (OKM22298)	70ab	61b	36b	40d
Suillus pictus	86ab	78ab	60ab	61bc

[1]Percentage of growth rate was measured in mm/day and was determined by dividing the average mm/day growth of a fungus grown on copper amended media by the average mm/day growth of the same fungus grown on unamended media.

[2]Numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Table 2: Surface area of four ectomycorrhizal fungi and one putatively ectomycorrhizal fungus on solid media amended with 15 and 30 μg/g copper, expressed as a percent of surface area on 0 μg/g copper[1] (n = 4).

Fungus	Age of Culture (Days)					
	20	31	43	20	31	43
	15μg/g Cu			30μg/g Cu		
Boletinellus merulioides	88a[2]	83bc	93ab	78ab	80a	93ab
Piloderma bicolor	94a	115a	100a	94a	104a	77ab
Pisolithus tinctorius	79ab	83bc	49c	88ab	88a	74ab
Suillus granulatus #2 (OKM22366)	40c	41e	56c	37d	36b	58b
Suillus granulatus #3 (OKM22233)	65bc	86b	91ab	64bc	84a	85a
Suillus granulatus #6 (OKM22298)	66abc	53de	57c	51cd	49b	55b
Suillus pictus	56bc	65cd	64c	32d	39b	57b

[1]Percentage of control surface area was calculated by dividing the area of the culture on copper amended media by the area on media containing no copper.

[2]Numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

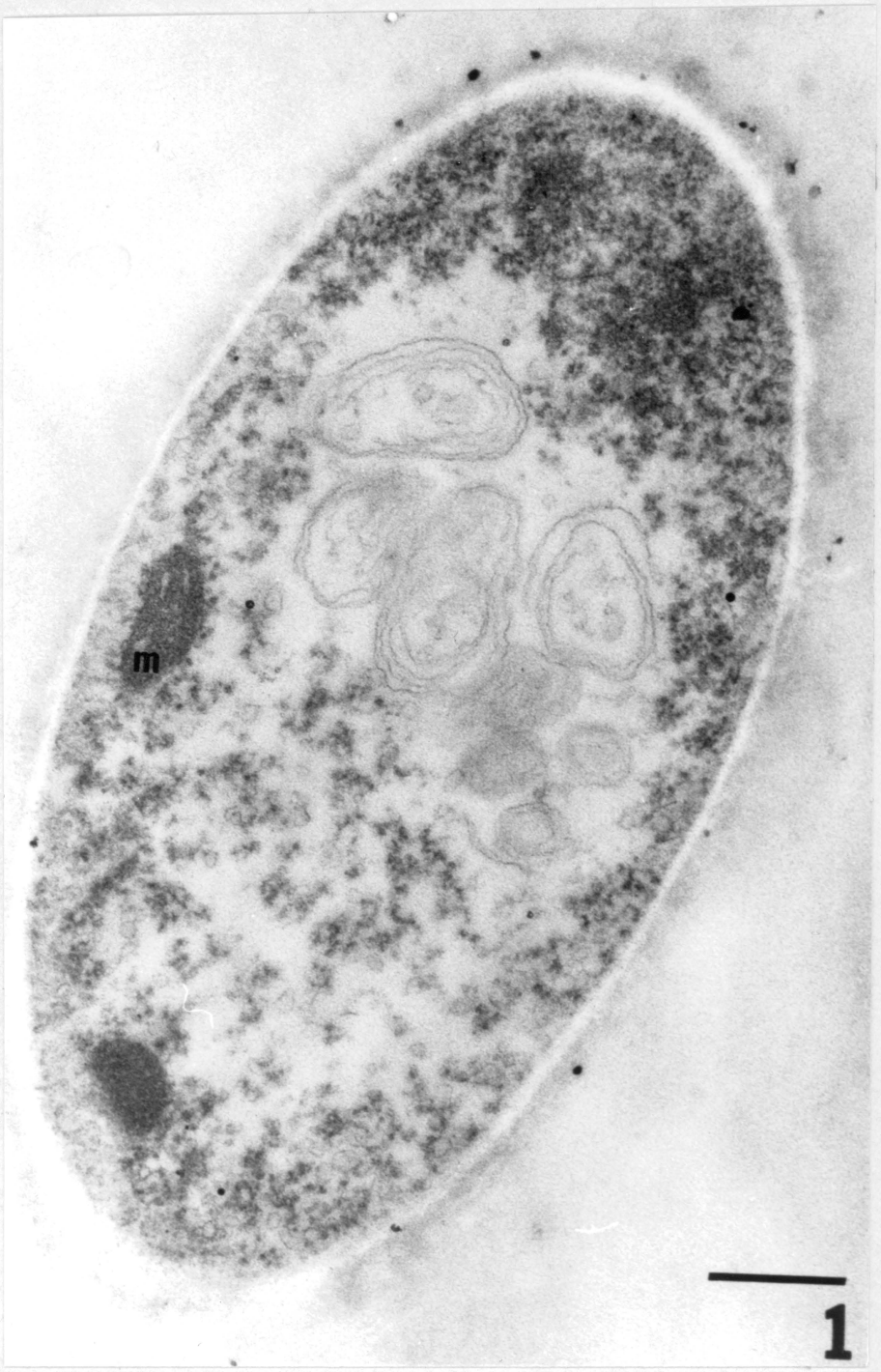
Table 3: Surface area achieved by four ectomycorrhizal fungi and one putatively ectomycorrhizal fungus on solid media amended with 60 and 100 μg/g copper, expressed as a percent of surface area on 0 μg/g copper[1] (n = 4).

Fungus	Age of Culture (Days)					
	20	31	43	20	31	43
	60 μg/g copper			100 μg/g copper		
Boletinellus merulioides	56b[2]	58a	68ab	51a	56a	71a
Piloderma bicolor	20c	30b	29c	0e	8d	0d
Pisolithus tinctorius	80a	69a	93a	46a	43b	56b
Suillus granulatus #2 (OKM22366)	18c	25b	50b	13cd	17cd	71a
Suillus granulatus #3 (OKM22233)	32c	60a	74ab	26b	44b	62ab
Suillus granulatus #6 (OKM22298)	31c	33b	30c	18bc	27c	39c
Suillus pictus	21c	26b	32c	17bc	25c	31c

[1]Percentage of control surface area was determined by dividing the area of the cultures grown on copper amended media by the area on media containing no copper.

[2]Numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Figure 1: *Suillus pictus* grown on media containing 0 μg/g copper. Full strength copper stain. m = mitochondrian. Bar = 0.5 μm.



Figures 2 and 3: *Pisolithus tinctorius* grown on media containing 60 μg/g copper. 1/10 strength copper stain, no lead stain. m = mitochondrian, arrows indicate copper deposition. Bar = 0.5μm

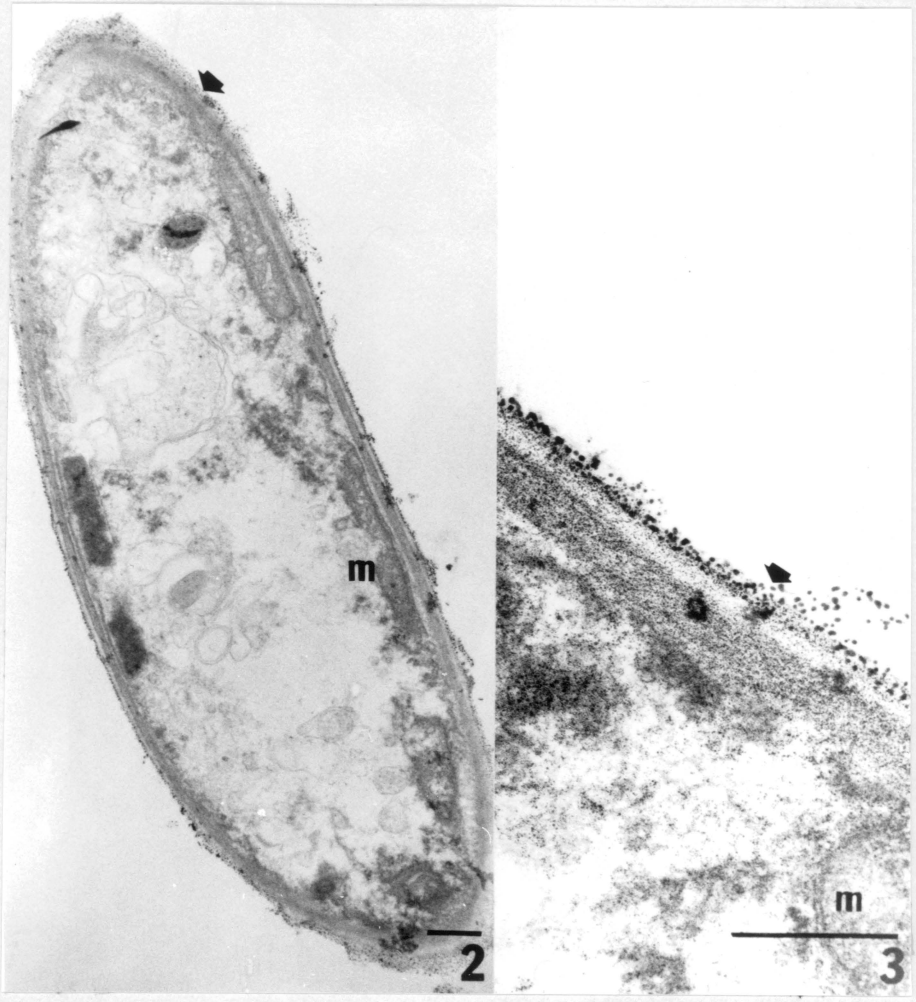


Figure 4: *Pisolithus tinctorius* grown on media containing 60 μg/g copper. Full strength copper stain. m = mitochondrian, pb = polyphosphate body, arrow indicates copper deposition. Bar = 0.5μm

Figure 5: Developing clamp connection of *Pisolithus tinctorius* grown on media containing 60 μg/g copper (enlargement of Figure 4). Full strength copper stain. pb = polyphosphate body, sw = septal wall, ve = vesicle. Bar = 0.5μm

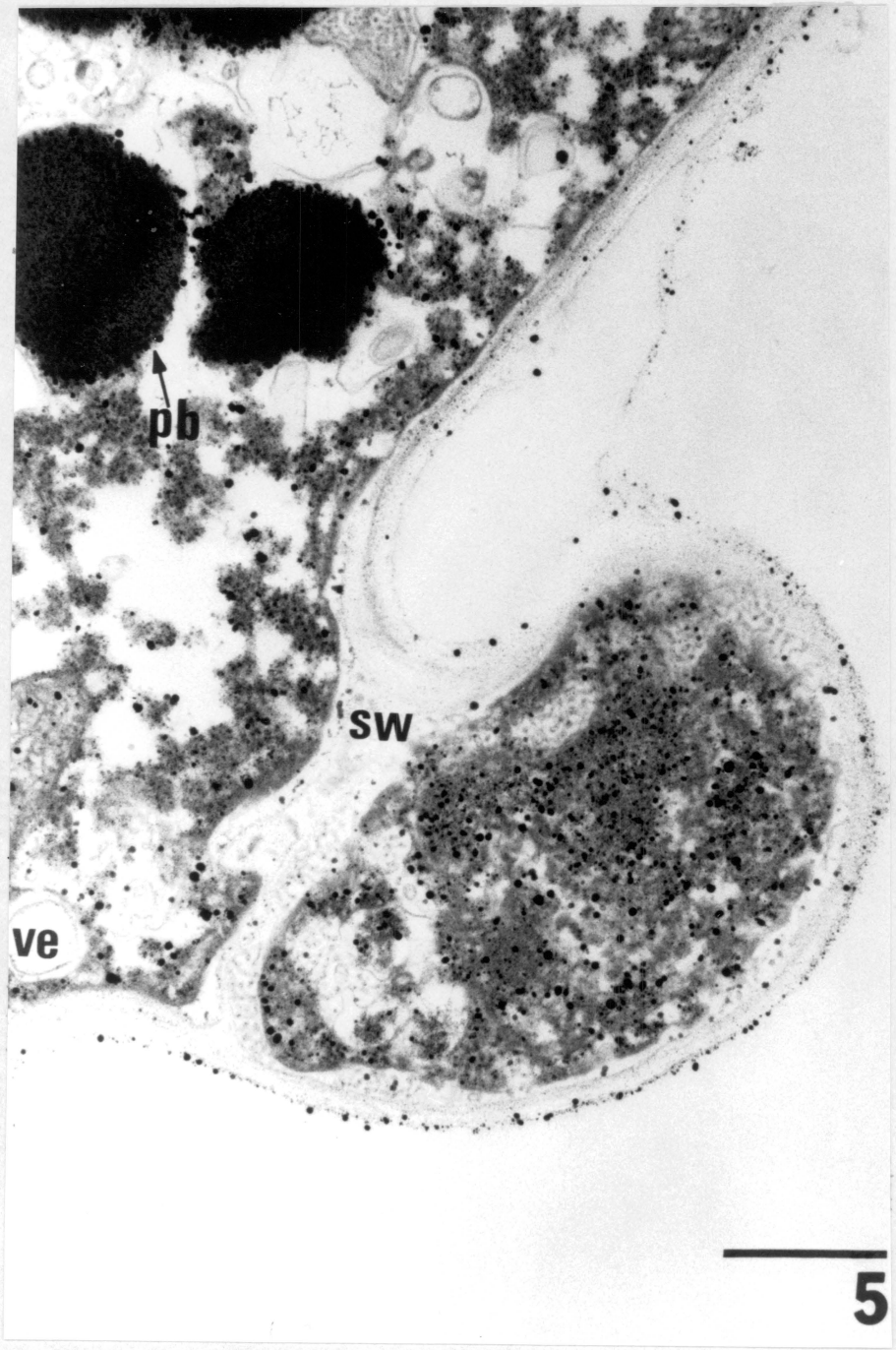
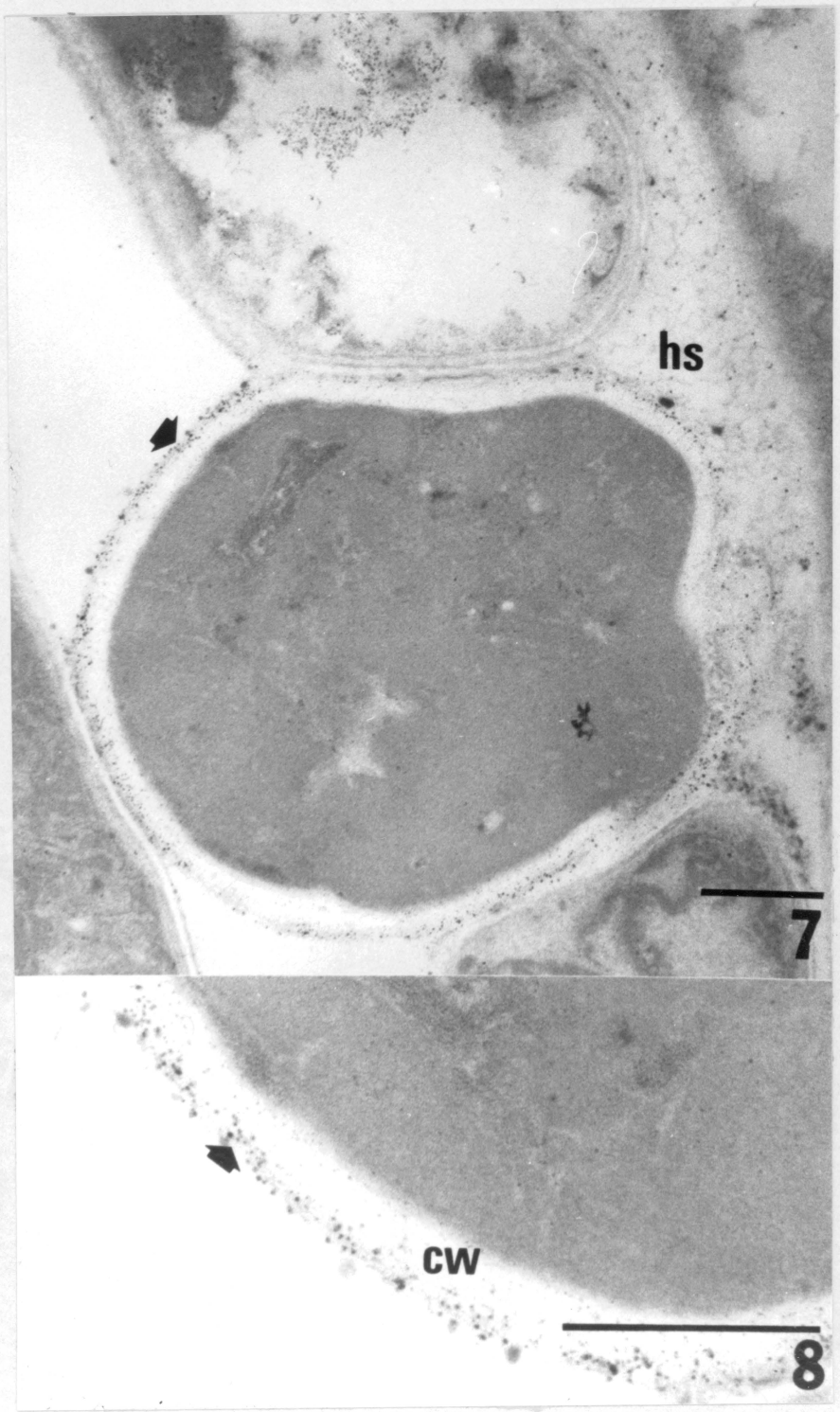


Figure 6: *Suillus granulatus #3 (OKM22298)* grown on media containing 60 μg/g copper. Full strength copper stain. hs = hyphal sheath, arrow indicates copper deposition.

Figures 7 and 8: *Suillus granulatus #3 (OKM22298)* grown on media containing 60 μg/g copper. Full strength copper stain, no lead stain. cw = cell wall, hs = hyphal sheath, arrows indicate copper deposition. Bar = 0.5μm



Figures 9 and 10: *Suillus pictus* grown on media containing 60 μg/g copper. 1/10 strength copper stain. cw = cell wall, m = mitochondrian, n = nucleus, v = vacuole. Bar = 0.5μm

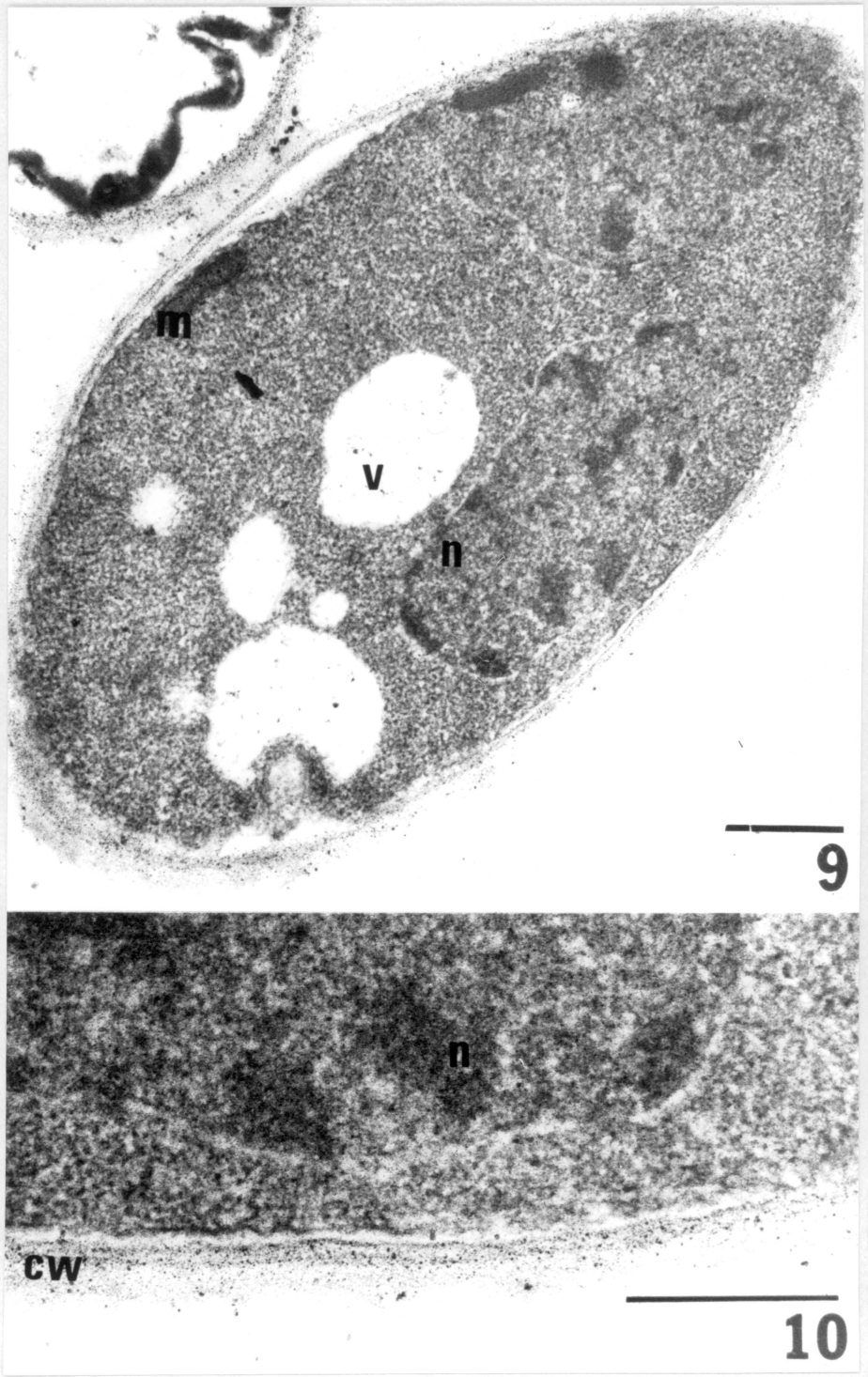


Figure 11: Developing clamp connection of *Suillus pictus* grown on media containing 60 μg/g copper. 1/10 strength copper stain. pb = polyphosphate body, ps = parenthesome. Bar = 0.5μm

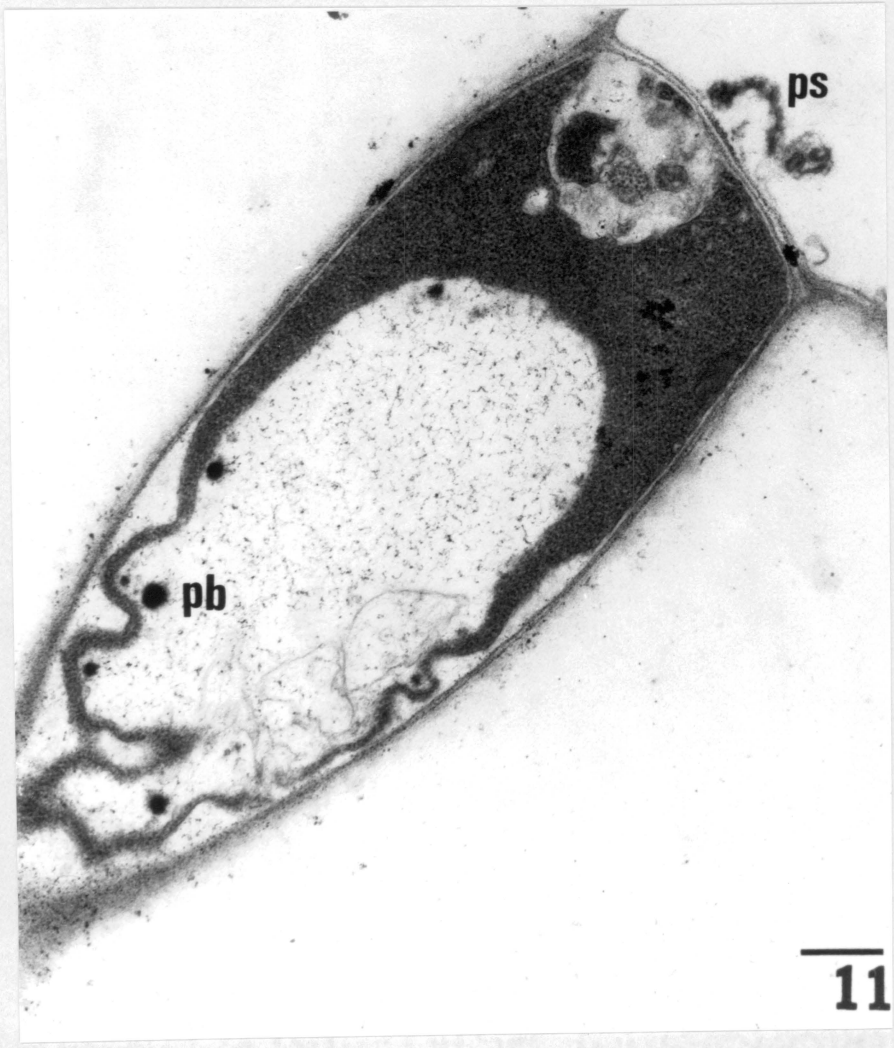


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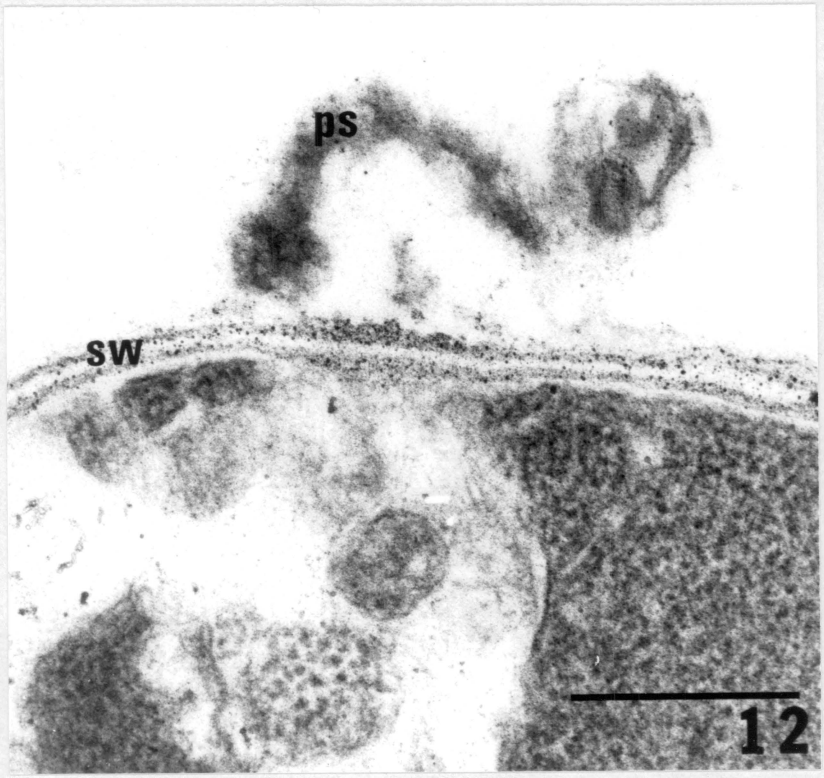
Figure 12: Septal wall of *Suillus pictus* grown on media containing 60μg/g copper (enlargement of Figure 11). 1/10 strength copper stain. ps = parenthesome, sw = septal wall. Bar = 0.5μm



Figures 13 and 14: Plasmolyzed hyphae of *Suillus pictus* grown on media containing 60 μg/g copper. 1/10 strength copper stain. Bar = 0.5μm

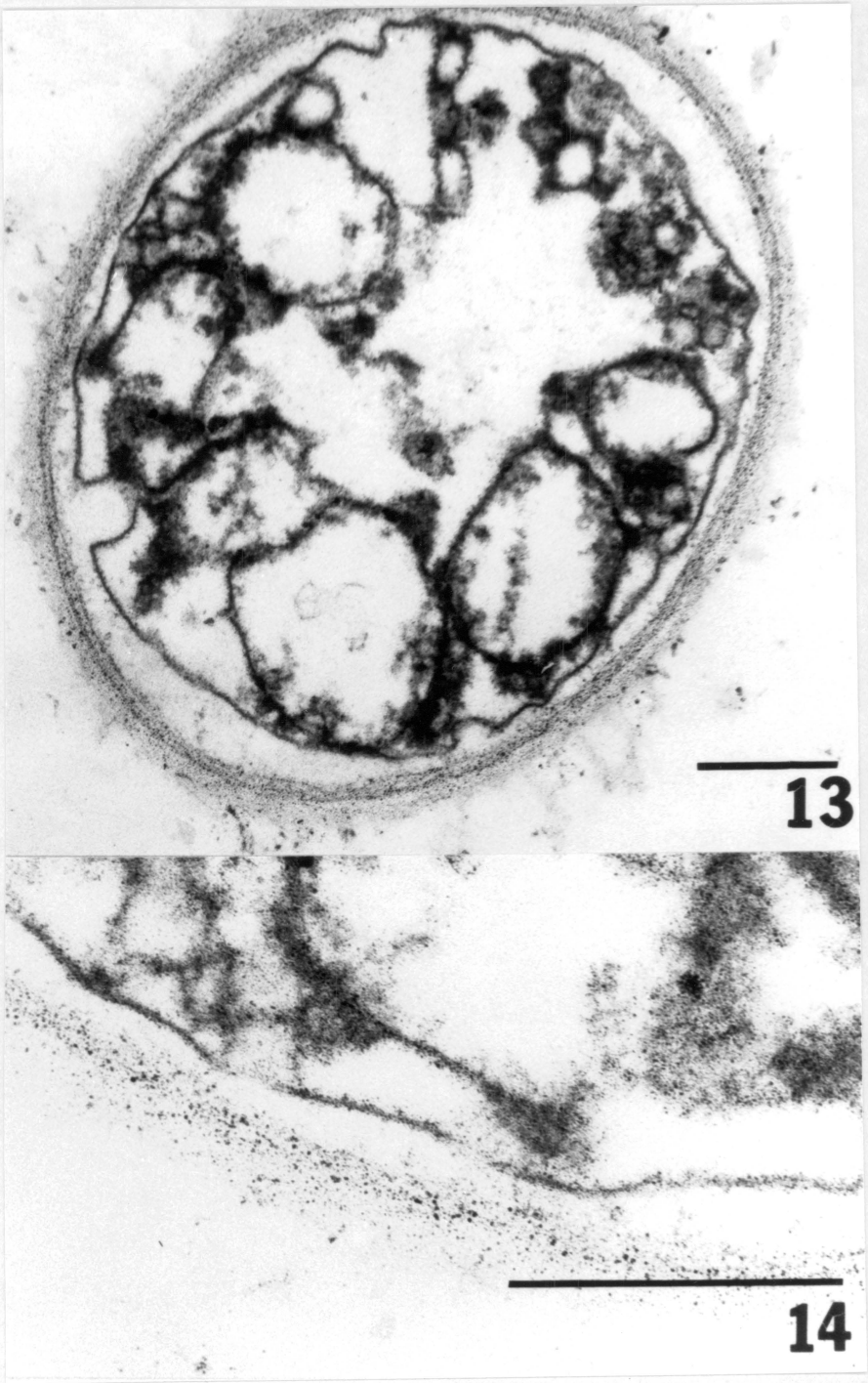


Figure 15: *Piloderma bicolor* grown on media containing 60 μg/g copper. 1/10 strength copper stain. 1 = lipid body. Bar = 0.5μm

DISCUSSION

Ectomycorrhizal fungi as a group do not have a single mechanism of responding to heavy metals as evidenced by the diverse reactions to copper observed in this study. *Pisolithus tinctorius* both binds copper to the exterior of the hyphal wall and presumably sequesters it in the cytoplasm. *Suillus granulatus* has a fairly extensive extrahyphal sheath and binds copper in this matrix, while in *Suillus pictus* copper is bound to the cell walls. *Piloderma bicolor*, the least copper tolerant of the fungi used in this study, does not appear to have a mechanism of binding or sequestering copper ions.

A number of fungi have been shown to have an extracellular capsular material at the surface of the hyphal wall which is composed of β1-3 and β1-6 glucans (Wessels et al., 1972); it is possible that the copper seen exterior to the cell wall in *P. tinctorius* could be bound to this surface material. It may also be that copper is held by components of an extracellular sheath produced by this fungus. Although this study did not indicate a significant extrahyphal matrix in *P. tinctorius*, the presence of a polysaccharide hyphal sheath has been demonstrated in ectomycorrhizal fungi (Foster, 1981; Piche, Peterson and Ackerly, 1983). Cultural and environmental conditions may alter the production of the sheath in wood rotting basidiomycetes (Palmer, Highley and Murmanis, 1985), and in ericaceous mycorrhizae the host exerts a controlling influence on the sheath (Bonfante-Fasolo, Gianinazzi-Pearson and Martinengo, 1984). The presence of copper in the cytoplasm and mitochondria, but not in the vacuoles or other organelles, indicates a mechanism of selective sequestration of the ion by the fungus. Copper could be moving in association with the polyphosphate bodies as they move through the hyphae (Ling Lee, Chilvers and Ashford, 1975). Sequestration and detoxification of heavy metals in polyphosphate bodies has been demonstrated in the blue-green alga *Plectonema boryanum* (Jensen et al., 1982). Morselt, Smits and Limonard (1986) have histochemically demonstrated the presence of metallothionein-like proteins in *P. tinctorius* and they may account for the large amount of copper visible in the cytoplasm in this study.

Suillus granulatus apparently binds copper to the components of the extracellular hyphal matrix, thus preventing entry of large amounts of the ion into the interior of the cell. The isolate

examined ultrastructurally was the most tolerant of the three isolates of *S. granulatus* considered in this study. It would be interesting to examine the other two isolates to see if metal tolerance in this species could be correlated with amount of hyphal sheath production.

Copper in *Suillus pictus* is found exclusively in the cell wall, possibly to a greater extent on the interior portion of the wall. Since this inner part of the wall in *Schizophyllum commune* has been demonstrated to be primarily composed of chitin microfibrils (Hunsley and Burnett, 1970), it may be that copper ions are bound to the n-acetyl glucosamine subunits which compose the chitin in *S. pictus*.

The physical binding of toxic metal ions by an ectomycorrhizal fungus could ensure the survivual of an individual as it grew through an area of toxic soil, as with continued growth of the mycelium the metal would remain bound to the nonliving hyphae. This could create pockets of metal free areas in a soil matrix for further extension of either fungal hyphae, host roots, or both. However, this could not be postulated as potential means of detoxifying soils, as the toxic metals would be released as soon as the fungal hyphae are degraded by other microorganisms.

The fungus in this study which exhibited the least amount of *in vitro* copper tolerance, *Piloderma bicolor*, also appears not to have a means of binding or sequestering copper ions under these experimental conditions. However, this does not necessarily mean that it will be intolerant to heavy metals in the presence of a suitable host plant; some potential tolerance mechanisms may only be stimulated by interaction with the host.

It should not be surprising that such an ecologically and taxonomically diverse group of organisms as ectomycorrhizal fungi should have a variety of intrinsic mechanisms of responding to a potentially toxic heavy metal. If all ectomycorrhizal fungi bound heavy metals exclusively to the cell wall as we have demonstrated in *S. pictus*, a high degree of correlation might be expected between axenic culture tolerances and field studies in combination with the host plant. However, when fungi possess metal tolerance mechanisms which are under genetic control, it is unlikely that pure culture studies will accurately reflect the situation when the fungus is in association with a suitable host. The recent observation of the synthesis of novel proteins during the mycorrhizal association (Hilbert and Martin, 1988) might suggest that the interaction between the host plant and

the fungal genome may enhance or repress the production of the hyphal sheath or of cytoplasmic metal binding proteins in the fungus. This would lead to a completely different picture of the relative metal tolerances of ectomycorrhizal fungi than that which is observed in pure culture.

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Effect of copper on growth of Pinus densiflora and five ectomycorrhizal symbionts

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ABSTRACT

Five ectomycorrhizal symbionts were synthesized in pure culture with *Pinus densiflora* at low and high media copper in the growth medium. *Suillus pictus* improved host growth most significantly at both 0 and 15 μg/g copper although the *in vitro* copper tolerance of this fungus is not high. In addition, there was no correlation between improvement of growth under metal stress in pure culture and whether the fungus is an early or late stage colonizer of the host. Copper decreased mycorrhizal colonization by both *S. pictus* and *S. granulatus* but not by *Pisolithus tinctorius* and *P. bicolor*. If a fungus induced the host to produce branched mycorrhizal short roots, there was more branching in the presence of the metal. All of the fungi tested, including the nonmycorrhizal *Boletinellus merulioides*, probably bind copper to the fungal hyphae as evidenced by a decrease in copper lost from mycorrhizal roots under acidic conditions when compared to nonmycorrhizal roots. Root and shoot phosphorus, calcium and magnesium levels were increased by mycorrhizal colonization, and the increase was most noticeable at higher copper levels. Copper decreased the percent vascular tissue in trees colonized by *S. granulatus*. Copper uptake to the shoot was increased by colonization with *P. tinctorius*. When two ectomycorrhizal fungi were inoculated on the same tree, it was probable that both symbionts were responsible for formation of the mycorrhizal association. Copper altered the ratio of short root branching patterns when two fungi were present on the same host, and may change the relative competitive abilities of the fungi at high stress levels. The different ways in which ectomycorrhizal fungi respond to heavy metal stress are discussed.

Key words: Ectomycorrhizae, copper, morphology, nutrients, tolerance

INTRODUCTION

Colonization of tree roots by a suitable ectomycorrhizal partner can improve the growth of the tree in metal contaminated soil. Mycorrhizal *Betula* have been shown not only to outperform nonmycorrhizal controls but to incorporate less zinc (Brown and Wilkins, 1985a) and nickel (Jones, Dainty and Hutchinson, 1988) into the aboveground parts of the plant when grown in high metal media substrate. Needles of mycorrhizal *Pinus virginiana* contain less zinc and manganese than nonmycorrhizal controls (Miller and Rudolph, 1986). In contrast, ectomycorrhizal fungi can

stimulate heavy metal uptake in *Pinus sylvestris* (Ernst, 1985) and decrease *Betula* growth in copper contaminated soil (Jones and Hutchinson, 1986). Heavy metals can prevent mycorrhizal formation in willow, poplar and sitka-spruce (Harris and Jurgensen, 1977; Burton, Morgan and Roig, 1984).

Isolates of mycorrhizal fungi collected from metal contaminated soils neither exhibit superior growth in high metal media nor significantly improve tree growth in toxic soils when compared with isolates not obtained from such sites (Brown and Wilkins, 1985b; Jones and Hutchinson, 1988a). This may be due to the morphological (Wilcox, 1983) and physiological (Hilbert and Martin, 1988) changes in fungi when they become associated with a suitable host plant.

With reference to a given host tree, ectomycorrhizal fungi can be divided ecologically into three groups. Some fungi colonize the host relatively early in its life, some tend to be associated with mature specimens of the host (Dighton and Mason, 1985), and some do not form mycorrhizae with the host in question. Ectomycorrhizal fungi such as *Pisolithus tinctorius*, *Scleroderma aurantium*, and some species of *Suillus* (e.g. *S. luteus*) are often found as primary colonizers of disturbed sites (Schramm, 1966; McCreight and Schroeder, 1982; Thompson and Medve, 1984). Fungi commonly found with mature hosts include species of *Amanita* (Mason et al., 1983), *Piloderma bicolor*, and some species of *Suillus* (Trappe, 1962).

We used pure culture synthesis in the present work to compare the effectiveness of early stage, late stage, and nonmycorrhizal species of ectomycorrhizal fungi in promoting growth of *Pinus densiflora* D. Don under conditions of copper stress. In order to evaluate the intrinsic, or nonadaptive metal tolerance, fungi were not collected from sites known to have high soil copper levels. Of the fungi used, *Pisolithus tinctorius* is considered to be a colonizer of young seedlings (Marx, 1977), while *Piloderma bicolor*, *Suillus granulatus* and *Suillus pictus* are generally collected in association with more mature trees (Trappe, 1962). *Boletinellus merulioides* (Schw.) Murrill has not synthesized mycorrhizae with members of the genus *Pinus*, but is consistently associated with *Fraxinus americana* L. in North America (Snell and Dick, 1970) However, attempts to synthesize mycorrhizae between *Fraxinus* and *Boletinellus* have not been successful (Cotter 1987). In previous axenic culture experiments, we determined that *P. tinctorius* and *B. merulioides* were more copper tolerant than *S. pictus* and *P. bicolor* (Gruhn and Miller, previous chapter). We also found that

isolates of *S. granulatus* varied in their ability to grow in copper amended media. As a result, this work attempts to correlate the *in vitro* copper tolerance with the tolerance of the five fungi in the mycorrhizal association. The three isolates of *S. granulatus* were compared under the same conditions. Finally, in order to determine if a heavy metal toxin can alter the competitive abilities of mycorrhizal fungi, we compared the growth response of *P. densiflora* in the presence of copper inoculated with *P. tinctorius*, *P. bicolor* and *S. pictus* alone or in pairs.

MATERIALS AND METHODS

The fungi used in this experiment were tissue isolates maintained at 4°C on Hagem's agar (Hagem, 1910) as part of the Virginia Polytechnic Institute and State University mycology culture collection. They included *Pisolithus tinctorius* (Pers.) Coker & Couch (VT389), *Piloderma bicolor* (Pk.) Julich (VT987), *Suillus pictus* Peck (Smith & Thiers) (OKM22174), *Suillus granulatus* (OKM22298, OKM22366 and OKM22233) and *Boletinellus merulioides* (Schw.) Murrill. The *Suillus* isolates were associated with *Pinus densiflora* in Korea and had been collected by O.K. Miller, Jr. The *Boletinellus* was collected under *Fraxinus americana* by V. Cotter. (Note: The *S. granulatus* isolate used in the first chapter was OKM22298.)

Experiment 1: Single inoculations with isolates of *Suillus pictus, Piloderma bicolor, Suillus granulatus, Boletinellus merulioides* and *Pisolithus tinctorius*

Fungi were grown in 250 ml Erlenmeyer flasks containing 100 ml of Palmer's defined liquid medium (Palmer, 1971) (PDM) for 4 weeks in the dark at 23C. Cultures were ground for 5 seconds in a Waring blender set at speed 5, and 10 ml of the resulting suspension were inoculated into sterile 24.5 x 3 cm screw top glass tubes. Tubes contained 11.25 g of perlite, passed over a 2 mm sieve to remove small particles, to which had been added 30 ml of PDM containing copper as copper sulfate. Final copper concentrations in the tubes were 0, 15, and 30 μg/g (= parts per million) in solution after addition of the mycorrhizal fungus in 10 ml of media. These levels, much higher than the organisms would be likely to encounter in most natural ecosystems, were chosen in order to determine if the mycorrhizal association has the potential to tolerate atypical environmental extremes of metal stress. Perlite has no cation exchange sites, so the copper added initially could be

expected to remain in solution for the duration of the experiment unless it was taken up by the fungus or the tree. Tubes were maintained in the dark at room temperature to allow the fungi to become established. After 14 days, a sterile seedling of *P. densiflora* with a radical length of 5-10 mm was introduced into each tube. Sterile seedlings were obtained by immersing seeds in a 10% solution of sodium hypochlorite for 10 minutes, rinsing in sterile deionized water, and germinating on 1.5% water agar. After approximately 14 days at room temperature and indirect sunlight, seedlings were planted into the culture tubes and the portion of the tubes filled with perlite was covered with aluminum foil. The experiment consisted of two replications with three trees per replication. Tubes were placed in one of two environmental growth chambers with a constant temperature of 21°C and 16 hrs of light per day. For 4 of the 16 hours, the trees received only fluorescent light while the remaining 12 hours included incandescent light. Tubes in replication 1 were placed in a reach-in chamber and received 163 $\mu E/m^2/s$ photosynthetically active radiation (PAR) with both types of lights and 148 $\mu E/m^2/s$ with fluorescent bulbs only. Tubes in replication 2 were in a walk-in growth chamber and received 54 and 47 $\mu E/m^2/s$, respectively. PAR was reduced approximately 8% through the glass tubes.

The height of the first true leaves was measured through the glass tubes approximately every 15 days from the base of the cotyledons beginning 52 days after planting. The experiment was concluded after 6 months when it was observed that seedling height had not increased in the majority of the treatments during the previous 30 day period. The following parameters were used to evaluate the effect of the treatments: shoot height, shoot and root fresh and dry weights, root tips per root dry weight, and percent mycorrhization of the root system (mycorrhizal root tips/total root tips). Root tips per root dry weight was used to give an estimate of branching of the root system, which is inhibited by copper in *Pinus* (Heale and Ormrod, 1982).

To evaluate the amount of copper ionically bound to the root systems, fresh roots were rinsed in distilled water and placed in 25 ml of pH 2.5 deionized water (pH obtained with sulfuric acid) for 1 hr on a rotary shaker. The amount of copper in the water was measured using Atomic Absorption Spectroscopy. Data are presented as μg copper in 25 ml water per gram dry weight of root tissue.

Samples of mycorrhizal roots were removed for light microscopy to confirm the presence of a mantle and Hartig net. Roots were stored in FAA (5 ml acetic acid/5 ml formalin/90 ml 50% EtOH), dehydrated in a TBA series and embedded in paraffin. Sections made on a rotary microtome were stained with safranin and fast green.

Plants were dried to a constant weight at 45°C. For elemental analysis of dried shoots and roots, samples were pooled from each replication in the 0 and 15 μg/g copper treatments, ground with a mortar and pestle, and ashed in a muffle furnace for 6 hours at 450°C. The ash was dissolved in 12N HCl and brought to 1.2N with deionized water. Samples were analyzed on the ICP Plasma Emission Spectrophotometer at the Virginia Polytechnic Institute and State University Soil and Plant Analysis Laboratory. Results are presented both as the amount of nutrient in μg/g dry weight (= ppm) and on a mass uptake basis (μg/g x total dry weight).

Statistical separations were performed using Duncan's New Multiple Range Test (Duncan, 1975) at P = .05.

Experiment 2: Single inoculations with three isolates of *Suillus granulatus*

Tissue isolates from three different populations of *Suillus granulatus* were grown in liquid PDM 90 days. The isolates were OKM22366, OKM22233 and OKM22298; OKM22298 was used in Experiment 1. In previous axenic culture experiments, we demonstrated that the *in vitro* tolerance of OKM22233 was significantly greater than that of the other two isolates. Cultures were ground and inoculated into culture tubes as described above to achieve final copper concentrations of 0, 7.5, 15, and 30 μg/g. Sterile seedlings of *P. densiflora* were introduced 9 days after addition of the fungi. Trees were grown in a walk-in growth chamber and received 245 $\mu E/m^2/s$ PAR with both fluorescent and incandescent bulbs, and 225 with fluorescent alone. The experiment consisted of four replications, with a single tree per treatment in each replication.

Seedlings were harvested after 109 days, and growth was evaluated as in Experiment 1 with the following exceptions. Two trees from each treatment at 0, 7.5 and 15 μg/g copper were selected for nutrient analysis and analyzed separately. The percentage of bifurcate, quadrifurcate and octifurcate mycorrhizal rootlets as a percentage of total mycorrhizal rootlets was compared for all

of the treatments. Stem cross sections were made directly below the cotyledons and examined microscopically to determine the ratio of vascular tissue to ground tissue.

Experiment 3: Multiple inoculations

Selection of fungi was based on stimulation of macroscopically different morphologies of pine short roots. *S. pictus, P. bicolor,* and *P. tinctorius* were the same strains used in the first experiment. The treatments in this experiment included all possible combinations of the three ectomycorrhizal fungi alone or in pairs. The fungi, grown in liquid culture as described above, were inoculated into the culture tubes at differing times, the fungus with the slowest growth in pure culture was added to the tubes first so that it did not become overrun by a faster growing fungus. After 38 days in liquid, 15 ml of a ground culture of *S. pictus* was added to the top of sterile perlite filled culture tubes containing 30 ml of Palmer's media. Tubes were incubated in the dark at room temperature. After three days, *P. bicolor* was introduced. *P. tinctorius* was added after 10 days at the time the sterile pine seedlings were planted into the tubes. Addition of 15 ml instead of 10 ml of fungal suspension led to slightly different conditions in the tubes; the final copper levels in the tubes after addition of the fungi were 0, 13, and 27 μg/g intstead of the 0, 15 and 30 μg/g used in the previous two experiments. Tubes were placed in a walk-in growth chamber with conditions identical to those in Experiment 2. Each treatment was replicated four times.

Trees were harvested after 123 days, and data were collected as previously described. Replicates were pooled for nutrient analyses. Fungi were reisolated from the culture tubes onto Hagem's agar to determine if both mycorrhizal fungi were viable in the case where two had been inoculated.

To evaluate the potential of any of the three species used to inhibit the growth of the others *in vitro*, 8 mm disks were removed from the leading edge of actively growing cultures on PDM. The disks were placed on fresh media, 5 cm apart from another mycorrhizal fungus. Cultures were examined weekly for signs of inhibition.

RESULTS

Experiment 1: Single inoculations with isolates of *Suillus pictus, Piloderma bicolor, Suillus granulatus, Boletinellus merulioides* and *Pisolithus tinctorius*

All of the fungi formed mycorrhizal short roots with *Pinus densiflora* except *B. merulioides*. Dichotomously branched roots were observed in inoculated seedlings; significantly greater numbers were seen in roots colonized by *B. merulioides* and very few were observed in *P. bicolor*. All fungi except *B. meruliodes* produced a mantle around the short roots and Hartig net between the cortical cells. *B. merulioides* produced a loose weft of mycelium around the short roots, but no Hartig net. As a result, the association cannot be defined as being mycorrhizal (Wilcox, 1983). A biochemical basis for the dichotomy in *B. merulioides* is discussed in the following chapter (Gruhn, Gruhn and Miller).

There were no significant differences in tree growth between the two replications, despite the disparity in light levels, so the data was pooled for analysis. The trees grown at 30 μg/g copper were severely stunted even with the addition of the mycorrhizal fungi, therefore the majority of the results reported will deal with the 0 and 15 μg/g copper treatments.

Based on the percentage of the root system that was colonized by the fungus, the ability of the mycobiont to form the mycorrhizal association was significantly impaired due to copper in *S. granulatus* at both 15 and 30 μg./g copper, and in *S. pictus* at 30 μg/g copper (Table 1). The percent colonization of the root system by *P. bicolor* and *P. tinctorius* was not reduced by the addition of copper.

At both 0 and 15 μg/g copper, *S. pictus* was the most effective mycorrhizal symbiont for *Pinus densiflora* under the conditions used in this study (Table 1). At both copper levels, shoot height and fresh weight were significantly greater than in the uninoculated trees. Dry weights followed the same trends as fresh weights, but are not presented in the tables as the numbers were so small as to induce error. Trees inoculated with *P. bicolor* also showed enhanced shoot and root dry weights when compared to the nonmycorrhizal plants although the increases were not as marked as those with *S. pictus*. At 15 μg/g copper *S. granulatus*, *B. merulioides*, and *P. tinctorius* also significantly improved tree growth over that of the uninoculated trees. Only trees inoculated with *P. tinctorius* did not decrease the amount of root branching in the presence of 15 μg/g copper.

When roots were exposed to deionized water at pH 2.5, the amount of copper lost per gram root dry weight was significantly greater from the nonmycorrhizal root systems than in any of the inoculated trees, including those inoculated with *B. merulioides*.

The same trends in root nutrient levels are apparent in both μg/g and mass uptake calculations (Tables 2 and 3, respectively) but are more significant in the latter. When μg/g (ppm) calculations are examined, a false picture of plant nutrient uptake may be presented, since very small plants may show high levels of nutrients on a dry weight basis. Nutrient uptake can be more realistically viewed if μg/g dry weight of nutrients is multiplied by total plant dry weight. At 0 μg/g copper, *S. pictus* significantly increased root phosphorus and magnesium levels over those in nonmycorrhizal trees. Calcium levels were also higher but not significantly so. Trees inoculated with *P. bicolor* also showed increased levels of all three nutrients. In addition, root calcium was increased by inoculation with *S. granulatus*, and the presence of *B. merulioides* enhanced both phosphorus and magnesium levels in the host.

At 15 μg/g copper, the presence of all of the fungi significantly improved root phosphorus and magnesium levels. Trees inoculated with *S. pictus*, *P. bicolor*, and *P. tinctorius* also had significantly higher levels of root copper than did nonmycorrhizal trees.

Nutrient levels appear higher in uninoculated than in inoculated plants on a μg/g basis (Table 4), but this is probably an artifact traceable to the significantly smaller uninoculated plants. Shoot phosphorus and potassium levels were increased on a mass uptake basis by both *S. pictus* and *P. bicolor* at both 0 and 15 μg/g copper (Table 5). *Piloderma bicolor* also increased shoot copper at 0 μg/g and copper and calcium at 15 μg/g media copper on a mass weight basis. Shoot nutrient levels in trees inoculated with the other fungi were not significantly different from the controls, with the exception of higher potassium and calcium levels in plants inoculated with *B. meruliodes* and higher calcium levels in those with *P. tinctorius*.

Experiment 2: Single inoculations with three isolates of *Suillus granulatus*

All of the isolates formed mycorrhizae with *P. densiflora*, based on the presence of a mantle and Hartig net. None of the mycorrhizal roots were unifurcate. Copper significantly altered the

ratio of short roots branching into two, four or eight branches. In most cases, copper induced higher degrees of branching (Table 6).

None of the *Suillus* isolates used in this study significantly improved seedling growth over that of the uninoculated controls (Table 6), but all of the growth parameters measured were consistently higher in the mycorrhizal seedlings regardless of copper level or fungal isolate. This suggests that the observed differences would probably be significant if more plants had been included in the experiment or if it had been run longer. The proportion of vascular tissue to ground tissue in the stems significantly decreased in all of the mycorrhizal seedlings compared with nonmycorrhizal seedlings when copper was added to the growth media (Table 6).

In the roots, nutrient levels were not significantly increased by mycorrhization at 0 μg/g copper (Tables 7 and 8). At 7.5 μg/g copper all nutrients except copper and iron were higher in the mycorrhizal plants, and at 15 μg/g, all nutrients measured were higher in the mycorrhizal plants. At 15 μg/g copper, seedlings inoculated with OKM22233 had significantly higher levels of phosphorus, potassium, and calcium than the control trees. Root copper levels were also higher in these plants, but not significantly so.

Comparison of shoot nutrient levels shows that at 0 μg/g copper all of the fungi significantly improved the P, K, and Ca status of the host plant, while levels of Mg, Fe and Cu were no greater than in nonmycorrhizal seedlings (Tables 9 and 10). These differences remained at 7.5 and 15 μg/g copper but were not significant. There were no differences in shoot nutrient levels among the mycorrhizal treatments.

Experiment 3: Inoculations with two isolates

When colonies of the mycorrhizal fungi used in this experiment were paired solid media, there appeared to be antagonism by *Piloderma bicolor* toward *Suillus pictus* and *Pisolithus tinctorius*. *S. pictus* did not grow around the *P. bicolor* colony despite the fact that it is a much faster growing fungus (Figure 1). The growth of *P. tinctorius* was slowed but not completely inhibited by *P. bicolor* (Figure 2). When *S. pictus* and *P. tinctorius* were plated together, *S. pictus* was completely overrun by the faster growing *P. tinctorius* (Figure 3).

At the end of the synthesis experiment ectomycorrhizae were formed in all cultures. All fungi were viable when the growth medium from the single inoculations was plated onto solid media. However, when the combination of *S. pictus* and *P. bicolor* was plated, only *P. bicolor* grew out. The combination of *S. pictus* and *P. tinctorius* and the combination of *P. bicolor* and *P. tinctorius* both yielded only *P. tinctorius*.

As in the first experiment, *S. pictus* was most effective in improving host growth at all three copper levels (Table 11). *Piloderma bicolor* and *Pisolithus tinctorius* also improved host growth at all copper levels but less so than *S. pictus*. In addition, the two fungal combinations which included *S. pictus* also improved host growth at 0, 13, and 27 μg/g copper. The combination of *P. bicolor* and *P. tinctorius* did not significantly improve host growth at 27 μg/g copper. The only substantial decrease in mycorrhization due to copper was in *S. pictus*. Percent mycorrhizal short roots was decreased 46% in the trees grown in 27 μg/g copper.

S. pictus formed exclusively bifurcate mycorrhizal short roots (Figure 4), and *P. bicolor* formed primarily unifurcate short roots (Figure 5). Colonization of the root system by *P. tinctorius* led to a ratio of 1 quadrifurcate root to 1.3 bifurcate roots in a given root system (Figure 6). Short roots colonized by *S. pictus* appear smooth; those by *P. bicolor* have a dense mantle of bright yellow hyphae; and those by *P. tinctorius* are covered with brown hyphae. As in the first experiment, there was a trend towards the higher branching orders with the addition of copper to the growth media.

Seedlings inoculated with both *S. pictus* and *P. bicolor* combined the branching patterns of both fungi. In trees grown at 0 μg/g copper, the percentage of unifurcate mycorrhizal short roots was 100% greater than in the trees inoculated with *S. pictus* alone, and the percentage of bifurcate short roots was 65% greater than in trees inoculated with *P. bicolor* alone (Table 11). When bifurcate short roots in this combination were compared with those in trees colonized by *S. pictus* alone, they were much longer and were covered with a dense mantle of bright yellow hyphae. With increasing copper in the growth medium, the percentage of unifurcate mycorrhizal roots increased and the percentage of bifurcate short roots decreased in this combination. In the trees inoculated with *S. pictus* and *P. tinctorius*, the macroscopic appearance of the short roots was similar to those

colonized by *P. tinctorius*. In addition, the ratio of quadrifurcate to bifurcate short roots is 1 to 1.9, which is very similar to that noted in trees colonized by *P. tinctorius* alone (Table 11). Copper did not alter the ratios in this combination. Finally, mycorrhizal short roots of trees inoculated with both *P. bicolor* and *P. tinctorius* also had the macroscopic appearance of roots colonized by *P. tinctorius* alone. However, there were 64 to 77% more unifurcate and 76 to 89% fewer bifurcate short roots than in trees colonized by *P. tinctorius* alone (Table 11). The percentage of branching patterns in this combination was not appreciably altered by copper in the growth medium.

Root phosphorus levels were increased at all copper levels by all of the mycorrhizal fungi examined (Tables 12 and 13). In this experiment, roots of seedlings colonized by *S. pictus* and the combination of *S. pictus* and *P. bicolor* had consistently lower phosphorus levels than any of the other mycorrhizal seedlings, irrespective of copper level. Mycorrhizal fungi enhanced levels of all of the other nutrients examined, including copper. At 13 μg/g copper, *P. tinctorius* and the combination of *P. tinctorius* and *P. bicolor* were the most effective at enhancing root nutrient levels.

All of the ectomycorrhizal fungi improved shoot phosphorus levels at all three copper levels (Tables 14 and 15). However, this improvement was most significant at 13 and 27 μg/g copper. Enhanced potassium and magnesium in the shoot were also noted at all copper levels, while calcium levels were significantly improved only at the highest copper level. Iron uptake was decreased by mycorrhizal colonization at 0 μg/g copper, but no differences were observed at 13 and 27 μg/g. Overall, *S. pictus* enhanced shoot nutrient levels to a greater extent than any of the other fungi. At all three copper levels, *P. tinctorius* significantly increased copper uptake to the shoot, and at 13 and 27 μg/g copper the combinations which included this fungus also showed significantly greater shoot copper levels than the uninoculated trees.

Experiment 1 - Single inoculations

Table 1: Effect of five ectomycorrhizal fungi on growth and mycorrhization of *Pinus densiflora* in copper amended media (n = 6).

Fungus	Media Copper (μg/g)	Shoot Height (cm)	Shoot FW[1] (mg)	Root FW (mg)	Tips/ Root DW[2]	Copper Loss[3] (μg/g)	% Myco[4]
Suillus pictus	0	4.3a[5]	276a	154a	6075b	0e	9de
	15	4.3a	258ab	143a	4764b-d	12de	12c
	30	2.2de	96cd	70a	2160d-f	23cd	0e
Piloderma bicolor	0	3.8ab	202a-c	122a	5582b	1e	37bc
	15	3.1a-d	133cd	150a	3398c-f	17c-e	56ab
	30	2.0de	107cd	128a	2069ef	23cd	29cd
Suillus granulatus	0	3.7a-c	170bc	70a	6575ab	3e	54ab
	15	3.5a-c	150cd	86a	2976d-f	24cd	10de
	30	1.9de	117cd	115a	1993f	22cd	10de
Boletinellus merulioides	0	3.1a-d	140cd	138a	8729a	0e	-
	15	2.2de	107cd	155a	4043b-f	18c-e	-
	30	1.9de	110cd	93a	2151d-f	31a-c	-
Pisolithus tinctorius	0	3.0b-d	146cd	80a	4669b-e	0e	47bc
	15	2.9b-d	157b-d	105a	6096b	27b-d	74a
	30	2.6c-e	130cd	68a	2902d-f	33a-c	58ab
None	0	2.9bcd	103cd	60a	6092b	0e	-
	15	2.0de	100cd	22a	2757d-f	44ab	-
	30	1.6e	50d	34a	2554d-f	47a	-

[1] FW = Fresh weight

[2] Tips/root DW = total number of root tips/root dry weight (g).

[3] Copper loss = amount of copper lost into 25ml of pH 2.5 water (μg/g) when fresh root system immersed for 1 hour/root dry weight (g).

[4] Percent mycorrhization = number of mycorrhizal root tips/total number of root tips.

[5] Numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Experiment 1 - Single inoculations

Table 2: Effect of five ectomycorrhizal fungi on root nutrient levels of *Pinus densiflora* in copper amended media (n = 6). (μg/mg root dry weight = ppm)

Media Copper	Fungus	P	K	Ca	Mg	Fe	Cu
0 μg/g	*Suillus pictus*	276b[1]	95a	449b	169a	59a	7a
	Piloderma bicolor	545a	144a	559b	207a	113a	9a
	Suillus granulatus	318ab	186a	1727a	224a	99a	23a
	Boletinellus merulioides	377ab	83a	464b	179a	31a	10a
	Pisolithus tinctorius	265b	84a	616b	151a	37a	26a
	None	234b	99a	505b	173a	63a	14a
15 μg/g	*Suillus pictus*	324bc	136a	604ab	174b	106a	32ab
	Piloderma bicolor	596a	226a	604ab	217ab	25a	32ab
	Suillus granulatus	229c	93a	996a	188ab	84a	32ab
	Boletinellus merulioides	500ab	242a	474b	241a	15b	19b
	Pisolithus tinctorius	260c	78a	686ab	179ab	32a	54a
	None	381bc	238a	572ab	162b	108a	33ab

[1]For a given copper level, numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Experiment 1 - Single inoculations

Table 3: Effect of five ectomycorrhizal fungi on root nutrient levels of *Pinus densiflora* in copper amended media (n = 6). (μg/g root dry weight x root dry weight (g) = mass uptake)

Media Copper	Fungus	P	K	Ca	Mg	Fe	Cu
0 μg/g	*Suillus pictus*	6.7abc[1]	2.3a	10.9ab	4.1a	1.4a	0.2a
	Piloderma bicolor	9.7a	2.6a	10.0ab	3.7ab	2.0a	0.2a
	Suillus granulatus	2.7cd	1.6a	14.8a	1.9bc	0.9a	0.2a
	Boletinellus merulioides	8.1ab	1.8a	10.0ab	3.9ab	0.7a	0.2a
	Pisolithus tinctorius	2.6cd	0.8a	6.0ab	1.5c	0.4a	0.3a
	None	1.8d	0.8a	4.0b	1.4c	0.5a	0.1a
15 μg/g	*Suillus pictus*	5.8c	2.4ab	10.8ab	3.1b	1.9a	0.6a
	Piloderma bicolor	13.8a	5.3a	14.0a	5.0a	0.6b	0.7a
	Suillus granulatus	2.7d	1.1b	11.9ab	2.3b	1.0b	0.4ab
	Boletinellus merulioides	10.6b	5.1a	10.1ab	5.1a	0.3b	0.4ab
	Pisolithus tinctorius	3.2cd	1.0b	8.4b	2.2b	0.4b	0.7a
	None	1.4d	0.9b	2.1c	0.6c	0.4b	0.1b

[1]For a given copper level, numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Experiment 1 - Single inoculations

Table 4: Effect of five ectomycorrhizal fungi on shoot nutrient levels of *Pinus densiflora* in copper amended media (n = 6). (μg/g shoot dry weight = ppm)

Media Copper	Fungus	P	K	Ca	Mg	Fe	Cu
0 μg/g	*Suillus pictus*	552a[1]	1043a	161a	210a	10a	1.9b
	Piloderma bicolor	531a	911a	170a	247a	9a	2.4ab
	Suillus granulatus	530a	802a	212a	228a	12a	2.5ab
	Boletinellus merulioides	492a	643a	214a	257a	11a	1.8b
	Pisolithus tinctorius	443a	698a	131a	208a	12a	2.7ab
	None	622a	870a	132a	256a	36a	3.4a
15 μg/g	*Suillus pictus*	502b	1066a	230a	210b	13b	4.0a
	Piloderma bicolor	505b	948a	155a	228b	10b	4.4a
	Suillus granulatus	459b	759a	244a	191b	72a	4.8a
	Boletinellus merulioides	452b	656a	212a	245ab	9b	3.9a
	Pisolithus tinctorius	433b	590a	285a	227b	8b	3.5a
	None	772a	881a	168a	299a	22b	4.7a

[1]For a given copper level, numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Experiment 1 - Single inoculations

Table 5: Effect of five ectomycorrhizal fungi on shoot nutrient levels of *Pinus densiflora* in copper amended media (n = 6). (μg/g shoot dry weight x shoot dry weight = mass uptake)

Media Copper	Fungus	P	K	Ca	Mg	Fe	Cu
0 μg/g	*Suillus pictus*	2.4bc[1]	4.6ab	.75a	.95ab	.05a	.008a-c
	Piloderma bicolor	2.8a	4.8a	0.9a	1.3a	.05a	.012a
	Suillus granulatus	0.9d	1.3c	0.4a	0.4b	.02a	.005c
	Boletinellus merulioides	1.6cd	2.1c	0.7a	0.8ab	.04a	.006a-c
	Pisolithus tinctorius	1.5cd	2.4bc	0.5a	0.7ab	.04a	.009a-c
	None	1.3d	1.9c	.3a	.5b	.07a	.007a-c
15 μg/g	*Suillus pictus*	1.7ab	3.6ab	0.8ab	0.7abc	.05ab	.014ab
	Piloderma bicolor	2.2a	4.2a	0.7b	1.0a	.04ab	.019a
	Suillus granulatus	0.5c	0.8c	0.3c	0.2c	.07a	.005b
	Boletinellus merulioides	1.5bc	2.2bc	0.7b	0.8abc	.03b	.013ab
	Pisolithus tinctorius	1.8ab	2.5bc	1.1a	0.9ab	.03b	.014ab
	None	0.7bc	0.8c	0.2c	0.3bc	.02b	.004b

[1]For a given copper level, numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Experiment 2 - Comparison of *Suillus granulatus* isolates

Table 6: Effect of three isolates of *Suillus granulatus* on growth and mycorrhization of *Pinus densiflora* in copper amended media (n = 4).

Isolate	Media Copper (μg/g)	Shoot FW[1] (mg)	Root FW (mg)	% Vasc.[2]	Tips/ Root DW[3]	% Myco.[4]	% Bi[5]	% Quad[5]	% Oct[5]
OKM22366	0	300a[6]	163a	38ab	14344a	65a	48bcd	48b	16a
	7.5	265a	160a	37ab	9476bc	35a-e	15c-e	32bc	52a
	15	170a	145a	34ab	2577ef	7d-f	66ab	33bc	0a
	30	97a	40a	28b	1080f	10d-f	0e	100a	0a
OKM22233	0	203a	188a	44a	12410ab	57ab	46bcd	28bc	33a
	7.5	248a	170a	43a	7502cd	29b-f	59a-c	27bc	15a
	15	150bc	157a	38ab	3842d-f	7d-f	100a	0c	0a
	30	95a	90a	30b	722f	0f	-	-	-
OKM22298	0	245a	175a	42a	12260ab	41a-d	70ab	29bc	3a
	7.5	245a	153a	45a	6039c-e	8d-f	100a	0c	18a
	15	145a	183a	37ab	2997ef	21d-f	50b-d	47b	0a
	30	77a	103a	36ab	1669ef	46a-c	6de	14bc	44a
None	0	180a	205a	35ab	9986a-c	-	-	-	-
	7.5	195a	113a	42a	3594d-f	-	-	-	-
	15	128a	98a	37ab	1912ef	-	-	-	-
	30	132a	110a	39ab	1275f	-	-	-	-

[1]FW = Fresh weight

[2]Percent vascular tissue = (xylem area + phloem area)/stem area.

[3]Tips/Root DW = Total number of root tips/root dry weight.

[4]Percent mycorrhization = mycorrhizal root tips/total root tips.

[5]Percent bifurcate, quadrifurcate and octifurcate mycorrhizal root tips determined by dividing by the number of mycorrhizal short roots in two, four or eight branches by the total number of mycorrhizal roots. *S. granulatus* did not produce unifurcate mycorrhizal roots.

[6]Numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Experiment 2 - Comparison of *Suillus granulatus* isolates

Table 7: Effect of three isolates of *Suillus granulatus* on root nutrient levels in *Pinus densiflora* in copper amended media (n = 4). (μg/g root dry weight = ppm)

Media Copper	Fungus	P	K	Ca	Mg	Fe	Cu
0 μg/g	OKM22366	946a[1]	382a	442a	293a	153a	10.0a
	OKM22233	674a	315a	607a	257a	76a	25.6a
	OKM22298	1011a	474a	791a	466a	162a	6.6a
	None	802a	272a	464a	312a	94a	5.9a
7.5μg/g	OKM22366	1774a	1946a	389a	538a	81a	21.7a
	OKM22233	892b	531ab	608a	286b	417a	31.3a
	OKM22298	990b	605ab	664a	308b	60a	21.8a
	None	594b	289b	476b	172b	85a	25.5a
15 μg/g	OKM22366	824b	613c	319ab	176ab	190a	33.4b
	OKM22233	1249a	709b	438a	294a	120ab	49.7a
	OKM22298	845b	762a	265b	224ab	48b	30.9b
	None	751b	479d	343ab	163b	98ab	35.7ab

[1] For a given copper level, numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Experiment 2 - Comparison of *Suillus granulatus* isolates

Table 8: Effect of three isolates of Suillus granulatus on root nutrient levels of *Pinus densiflora* on copper amended media (n = 4). (μg/g root dry weight x root dry weight = mass uptake)

Media Copper	Fungus	P	K	Ca	Mg	Fe	Cu
0 μg/g	OKM22366	15.5a[1]	6.3a	7.3a	4.8a	2.5a	.16a
	OKM22233	14.3a	6.6a	1.34a	5.5a	1.6a	.59a
	OKM22298	14.9a	6.9a	11.7a	6.8a	2.4a	.10a
	None	17.0a	15.8a	9.9a	6.6a	2.0a	.13a
7.5 μg/g	OKM22366	27.4a	30.6a	5.7a	8.3a	1.2a	.32a
	OKM22233	16.9a	10.1a	12.0a	5.5a	7.3a	.06a
	OKM22298	18.5a	10.4a	13.2a	5.3a	1.3a	.41a
	None	6.3b	3.1a	5.0a	1.8b	0.9a	.27a
15 μg/g	OKM22366	16.0a	11.9ab	6.1a	3.4a	3.7a	.65a
	OKM22233	20.2a	11.5a	7.0ab	4.8a	1.9b	.81a
	OKM22298	21.2a	19.1a	6.7a	5.6a	1.2b	.78a
	None	8.3a	5.0b	3.5a	1.9a	0.9b	.36a

[1]For a given copper level, numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Experiment 2 - Comparison of *Suillus granulatus* isolates

Table 9: Effect of three isolates of Suillus granulatus on shoot nutrient levels in *Pinus densiflora* in copper amended media (n = 4). (μg/g shoot dry weight = ppm)

Media Copper	Fungus	P	K	Ca	Mg	Fe	Cu
0 μg/g	OKM22366	723ab[1]	1320ab	162a	323a	38a	4.9bc
	OKM22233	776ab	1116b	245a	346a	49a	2.7a
	OKM22298	982a	1597a	171a	376a	65a	4.1ab
	None	633b	450c	124a	387a	59a	2.1c
7.5 μg/g	OKM22366	796a	1617a	292a	374a	33a	4.6c
	OKM22233	864a	1235ab	570a	400a	28a	6.7a
	OKM22298	849a	1200ab	265a	386a	33a	6.0ab
	None	858a	1042b	208a	357a	33a	5.0bc
15 μg/g	OKM22366	1035a	547b	152a	424a	46a	7.4a
	OKM22233	889b	1255a	210a	376a	81a	7.9a
	OKM22298	787c	682b	183a	360a	100a	7.0a
	None	995a	917ab	132a	370a	41a	6.4a

[1]For a given copper level, numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Experiment 2: Comparison of *Suillus granulatus* isolates

Table 10: Effect of three isolates of *Suillus granulatus* on shoot nutrient levels of *Pinus densiflora* in copper amended media (n = 4). (μg/g shoot dry weight x shoot dry weight = mass uptake)

Media Copper	Fungus	P	K	Ca	Mg	Fe	Cu
0 μg/g	OKM22366	30a[1]	57a	7a	13a	1.6b	.11ab
	OKM22233	25ab	38ab	9a	11a	1.5b	.16a
	OKM22298	34a	55a	6a	13a	2.2a	.14ab
	None	19b	14b	4b	12a	1.8ab	.06b
7.5 μg/g	OKM22366	31a	64a	12a	15a	1.3a	.18b
	OKM22233	31a	45ab	19a	14a	1.0a	.24a
	OKM22298	29a	44ab	10a	13a	0.8a	.20ab
	None	25a	31b	6a	10a	1.0a	.15b
15 μg/g	OKM22366	29a	15a	4ab	12a	1.3b	.21a
	OKM22233	26a	37a	6a	11a	2.4ab	.24a
	OKM22298	26a	23a	6.1a	12a	3.3a	.23a
	None	25a	23a	3b	9a	1.0b	.16a

[1]For a given copper level, numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Experiment 3 - Interactions between species

Table 11: Effect of three ectomycorrhizal fungi alone and in combination on growth and mycorrhization of *Pinus densiflora* in copper amended media (n = 4).

Fungus	Media Copper (μg/g)	Shoot Height (cm)	Shoot FW[1] (mg)	Root FW (mg)	Tips/ Root DW[2]	% Myco[3]	% Uni[4]	% Bi[4]	% Quad[4]	% Oct[4]
Suillus	0	4.4a[5]	340a	188a-c	4003a-c	24ef	0a	98a	3e	0a
pictus	13	3.9a-d	318ab	128b-e	4769ab	24ef	0a	83ab	23c-e	0a
	27	3.0e-i	260a-d	170a-c	4550ab	13f	0a	100a	0e	0a
Piloderma	0	3.9a-d	265a-c	235a	2547b-d	41c-e	86a	14ef	0e	0a
bicolor	13	3.6a-f	228b-e	118b-f	2548b-d	36c-e	93a	7f	0e	0a
	27	2.4i	145ef	113c-f	1023d	30e-f	71a	29d-f	0e	0a
Pisolithus	0	3.2c-i	253a-d	187a-c	4956ab	82c	8c	47b-f	37a-c	8a
tinctorius	13	2.8f-i	227b-e	177a-c	3886bc	83a	7c	51a-e	38a-c	4a
	27	2.7g-i	175c-f	118b-f	1639cd	65a-c	9c	23d-f	57a	11a
S. pictus	0	4.4ab	260a-d	127b-e	3776bc	30ef	60ab	40c-f	0e	0a
x P. bicolor	13	4.0a-c	230b-e	110c-f	3066b-d	21ef	69a	25c-e	6e	0a
	27	3.1c-i	163d-f	115b-e	1802cd	41c-e	79a	21d-f	0e	0a
S. pictus	0	3.6a-f	257a-d	180a-c	6400a	76ab	19c	47ab	25c-e	9a
x P. tinctorius	13	3.5c-h	265a-c	163a-d	4204a-c	69ab	12c	47b-f	30cd	11a
	27	3.4c-h	245a-d	153a-d	3071b-d	56a-d	12c	57b-d	22c-e	9a
P. bicolor	0	3.5b-g	263a-d	210ab	3982a-c	79ab	23c	72a-c	4e	0a
x P. tinctorius	13	3.8a-e	313ab	200a-c	3047b-d	67ab	31bc	50b-e	9de	9a
	27	3.1d-i	147ef	70d-f	3367b-d	76a-c	25c	75a-d	0e	0a
None	0	2.8f-i	128f	40ef	3694bc	-	-	-	-	-
	13	3.1d-i	133ef	33f	3554bc	-	-	-	-	-
	27	2.6hi	113f	25f	1896cd	-	-	-	-	-

[1] FW = fresh weight [2]Tips/Root DW = total number of root tips/root dry weight.

[3]% mycorrhization = mycorrhizal root tips/total root tips.

[4]% bi-, quad- and octifurcate mycorrhizae determined by dividing the number of mycorrhizal root tips involved in a 2, 4 or 8 branched structure by the total number of mycorrhizal root tips.

[5]Numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Experiment 3 - Interactions between species

Table 12 - Effect of three ectomycorrhizal fungi alone and in combination on root nutrient levels of *Pinus densiflora* in copper amended media. (μg/g root dry weight = ppm)

Media Copper	Fungus	P	K	Ca	Mg	Fe	Cu
0 μg/g	*Suillus pictus*	551	373	373	207	22.5	9.4
	Piloderma bicolor	785	201	439	263	40.4	15.1
	Pisolithus tinctorius	625	520	394	243	26.1	12.9
	S. pictus x P. bicolor	729	170	774	257	36.4	28.1
	S. pictus x P. tinctorius	885	239	433	282	26.5	2.6
	P. bicolor x P. tinctorius	1214	647	367	301	28.7	8.0
	None	750	553	319	304	101	17.7
13 μg/g	*Suillus pictus*	638	448	276	236	31.6	28.4
	Piloderma bicolor	884	496	488	280	43.4	56.2
	Pisolithus tinctorius	1392	733	455	346	30.7	70.0
	S. pictus x P. bicolor	693	270	757	257	37.3	59.7
	S. pictus x P. tinctorius	1174	534	296	313	27.5	69.0
	P. bicolor x P. tinctorius	1171	627	246	256	30.4	65.1
	None	770	498	598	280	48.8	83.8
27 μg/g	*Suillus pictus*	617	407	347	230	34.4	42.0
	Piloderma bicolor	998	711	282	228	28.8	43.2
	Pisolithus tinctorius	978	530	332	267	16.2	107.7
	S. pictus x P. bicolor	972	428	655	298	47.1	80.7
	S. pictus x P. tinctorius	737	381	390	226	20.8	69.5
	P. bicolor x P. tinctorius	851	435	580	348	39.5	75.5
	None	482	440	228	196	48.15	31.1

Replicates were pooled for analysis and statistical separations were not possible.

Experiment 3 - Interactions between species

Table 13: Effect of three ectomycorrhizal fungi alone and in combination on root nutrient levels of *Pinus densiflora* in copper amended media (n = 4). (μg/g root dry weight x root dry weight = mass uptake)

Media Copper	Fungus	P	K	Ca	Mg	Fe	Cu
0 μg/g	*Suillus pictus*	15.0c[1]	10.2b	10.2a	5.6bc	0.6b	.26bc
	Piloderma bicolor	24.1b	6.2c	13.5a	8.1ab	1.2a	.46a
	Pisolithus tinctorius	14.8c	12.3b	9.3a	5.8bc	0.6b	.31b
	S. pictus x P. bicolor	13.1cd	3.1d	13.9a	4.6cd	0.7b	.51a
	S. pictus x P. tinctorius	25.7b	6.9c	12.6a	8.2ab	0.8b	.08d
	P. bicolor x P. tinctorius	36.0a	19.2a	10.9a	8.9a	0.9b	.24bc
	None	5.3d	3.9cd	2.2b	2.1d	.71b	.12cd
13 μg/g	*Suillus pictus*	12.1cd	8.5c	5.2cd	4.5c	0.6bc	.5b
	Piloderma bicolor	15.7c	8.8c	8.7bc	5.0c	0.8abc	1.0b
	Pisolithus tinctorius	45.0a	23.7a	14.7a	11.2a	1.0a	2.3a
	S. pictus x P. bicolor	10.4cd	4.1cd	11.4b	3.9cd	0.6c	.9b
	S. pictus x P. tinctorius	30.0b	13.6b	7.6c	8.0b	0.7abc	1.8a
	P. bicolor x P. tinctorius	34.0b	18.2b	7.1c	7.4b	0.9ab	1.9a
	None	4.2d	2.7d	3.3d	1.5d	0.3d	.5b
27 μg/g	*Suillus pictus*	104.9ab	69.2a	59.0a	39.1a	5.8a	7.1ab
	Piloderma bicolor	119.8a	85.3a	33.8a	27.4ab	3.5abc	5.2ab
	Pisolithus tinctorius	114.9a	62.3ab	39.0ab	31.4ab	1.9c	12.7a
	S. pictus x P. bicolor	111.8a	49.2ab	75.3a	34.3ab	5.4ab	9.3ab
	S. pictus x P. tinctorius	112.4a	58.1ab	59.5a	34.5ab	3.2abc	10.6ab
	P. bicolor x P. tinctorius	59.6ab	30.5ab	40.6ab	24.4ab	2.8bc	5.3ab
	None	12.1b	11.0b	5.7b	4.9b	1.2c	0.8b

[1]For a given copper level, numbers followed by the same letter letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Experiment 3 - Interactions between species

Table 14: Effect of three ectomycorrhizal fungi alone or in combination on shoot nutrient levels of *Pinus densiflora* in copper amended media (μg/g shoot dry weight)

Media Copper	Fungus	P	K	Ca	Mg	Fe	Cu
0 μg/g	*S. pictus*	502	913	144	294	10.0	1.78
	P. bicolor	463	885	109	271	16.2	2.28
	P. tinctorius	423	633	162	200	8.8	2.36
	S. pictus x P. bicolor	414	939	125	255	6.2	3.5
	S. pictus x P. tinctorius	397	1150	105	233	9.1	2.14
	P. bicolor x P. tinctorius	400	931	101	217	10.7	1.91
	None	629	813	220	329	34	3.94
13 μg/g	*S. pictus*	522	1310	158	265	5.9	2.6
	P. bicolor	340	79	360	120	17	13.1
	P. tinctorius	390	974	103	202	6.5	3.9
	S. pictus x P. bicolor	471	1108	262	238	13.5	5.17
	S. pictus x P. tinctorius	444	979	115	222	6.7	3.13
	P. bicolor x P. tinctorius	459	1084	109	204	6.3	3.4
	None	662	1042	137	317	10.1	4.71
27 μg/g	*S. pictus*	512	1015	107	222	9.7	3.08
	P. bicolor	398	783	54	230	6.2	3.4
	P. tinctorius	399	790	56	199	4.9	2.84
	S. pictus x P. bicolor	458	1023	139	282	6.1	4.64
	S. pictus x P. tinctorius	430	1012	98	247	5.9	2.98
	P. bicolor x P. tinctorius	479	884	123	283	10.4	5.6
	None	541	742	63	244	20.7	2.73

Replicates were pooled for analysis and statistical separations were not possible.

Experiment 3 - Interactions between species

Table 15: Effect of three ectomycorrhizal fungi alone or in combination on shoot nutrient levels of *Pinus densiflora* in copper amended media (n = 4). (μg/g shoot dry weight x shoot dry weight = mass uptake)

Media Copper	Fungus	P	K	Ca	Mg	Fe	Cu
0 μg/g	*Suillus pictus*	36.0a[1]	65.5a	10.3a	21.1a	.72b	.13b
	Piloderma bicolor	27.7b	52.9a	6.5b	16.2b	.97a	.14b
	Pisolithus tinctorius	24.8bc	37.1b	9.5a	11.7cd	.52cd	.14b
	S. pictus x P. bicolor	23.2bc	52.6a	7.0b	14.3bc	.35d	.20a
	S. pictus x P. tinctorius	23.4bc	67.9a	6.2b	13.7bcd	.54bcd	.13b
	P. bicolor x P. tinctorius	22.5bc	52.5a	5.7b	12.2bcd	.60bc	.11b
	None	18.7c	24.3b	6.5b	9.8d	1.01a	.11b
13 μg/g	*Suillus pictus*	43.4bc	30.5bc	18.8c	16.1c	2.2ab	1.9d
	Piloderma bicolor	50.4b	28.3c	27.8b	16.0c	2.5a	3.2b
	Pisolithus tinctorius	84.5a	44.5a	27.6b	21.0a	1.9bc	4.3a
	S. pictus x P. bicolor	34.1c	13.3d	37.3a	12.7d	2.9bc	1.8bc
	S. pictus x P. tinctorius	74.8a	34.0b	18.9c	20.0ab	1.8cd	4.4a
	P. bicolor x P. tinctorius	79.3a	42.5a	16.7c	17.3bc	2.1bc	4.4a
	None	23.1d	14.9d	17.9c	8.4e	1.5d	2.5cd
27 μg/g	*Suillus pictus*	160.4a	105.8a	90.2ab	59.8a	8.9a	10.9abc
	Piloderma bicolor	143.1a	101.9a	40.4c	32.7bc	4.1c	6.2bc
	Pisolithus tinctorius	171.2a	92.8ab	58.1bc	46.7abc	2.8c	18.8a
	S. pictus x P. bicolor	158.0a	69.6ab	106.4a	48.4ab	7.7ab	13.1ab
	S. pictus x P. tinctorius	180.6a	93.3ab	95.6ab	55.4ab	5.1bc	17.0a
	P. bicolor x P. tinctorius	124.8ab	63.8ab	85.1ab	51.0ab	5.8ab	11.1abc
	None	54.2b	49.5b	25.7c	22.1a	5.4bc	3.5c

[1]For a given copper level, numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Figure 1: *Suillus pictus* (left) and *Piloderma bicolor* on Palmer's media, O ppm copper.

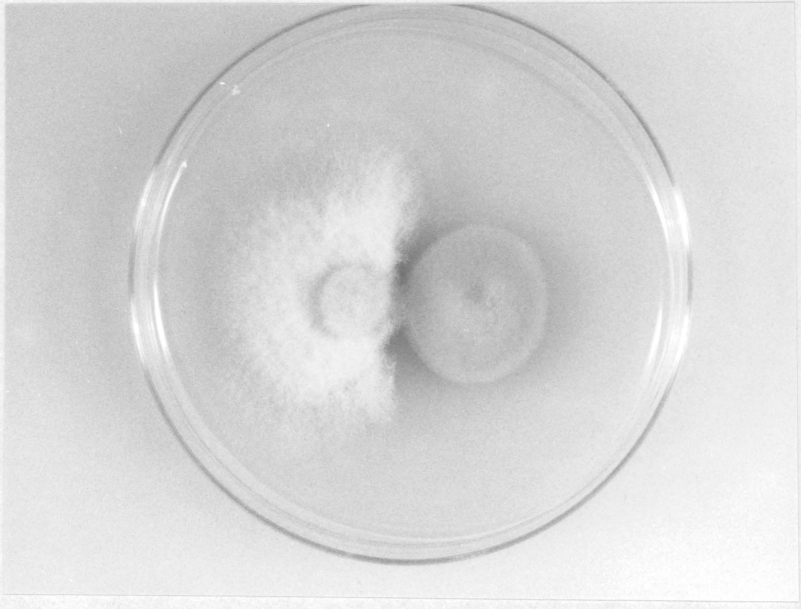


Figure 2: *Piloderma bicolor* (left) and *Pisolithus tinctorius* on Palmer's media, 0 ppm copper.

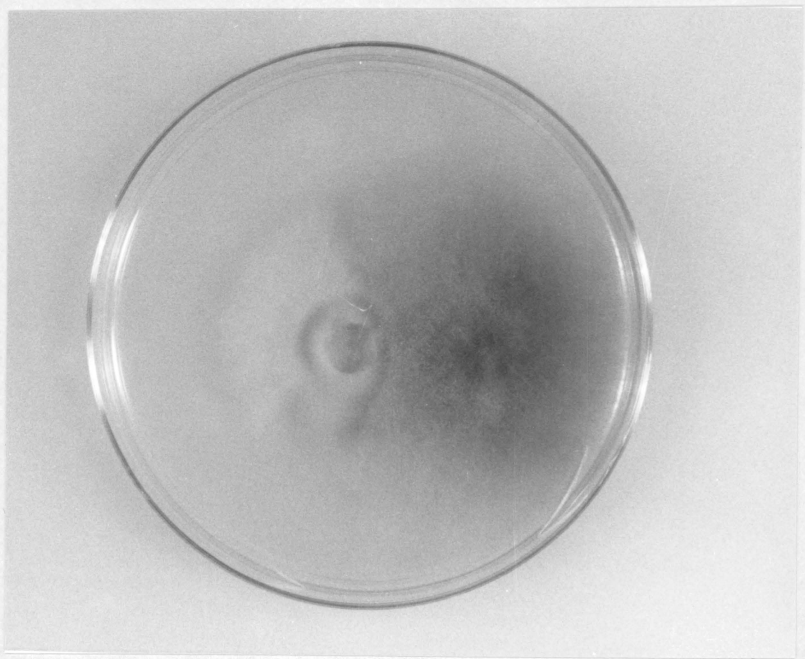


Figure 3: *Suillus pictus* (left) and *Pisolithus tinctorius* on Palmer's media, 0 ppm copper.

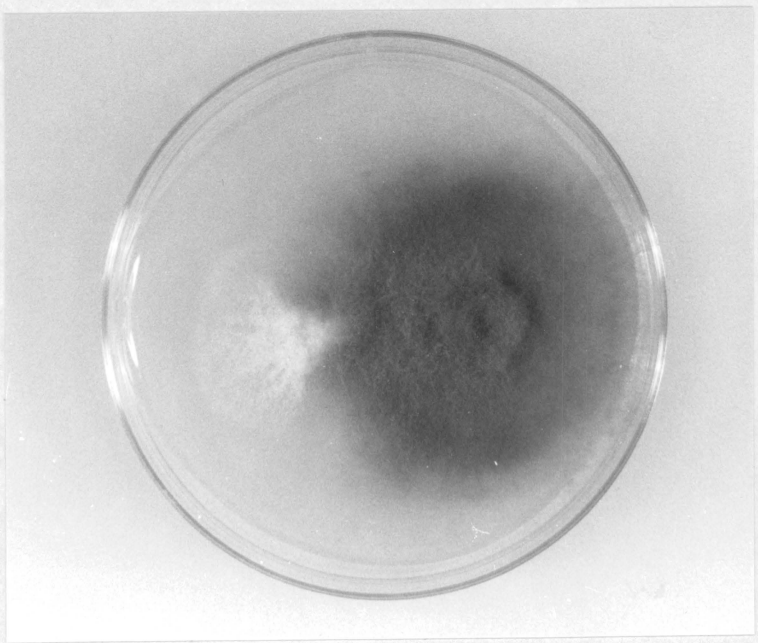


Figure 4: Short root of *Pinus densiflora* colonized by *Suillus pictus*.

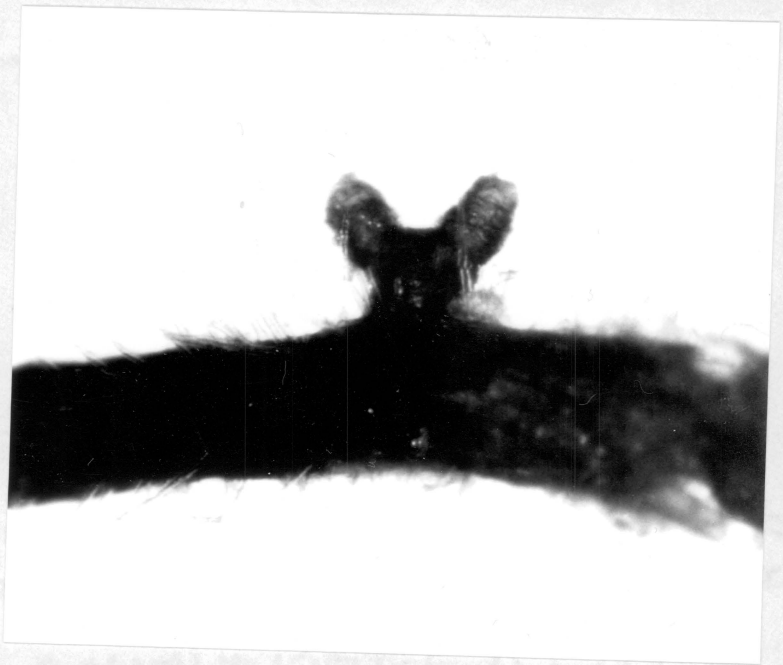


Figure 5: Short root of *Pinus densiflora* colonized by *Piloderma bicolor*.

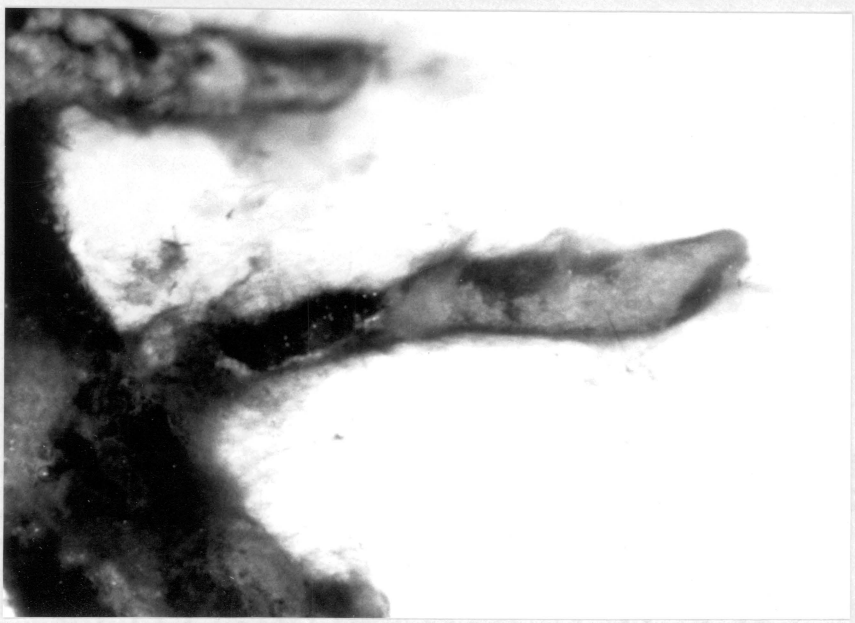
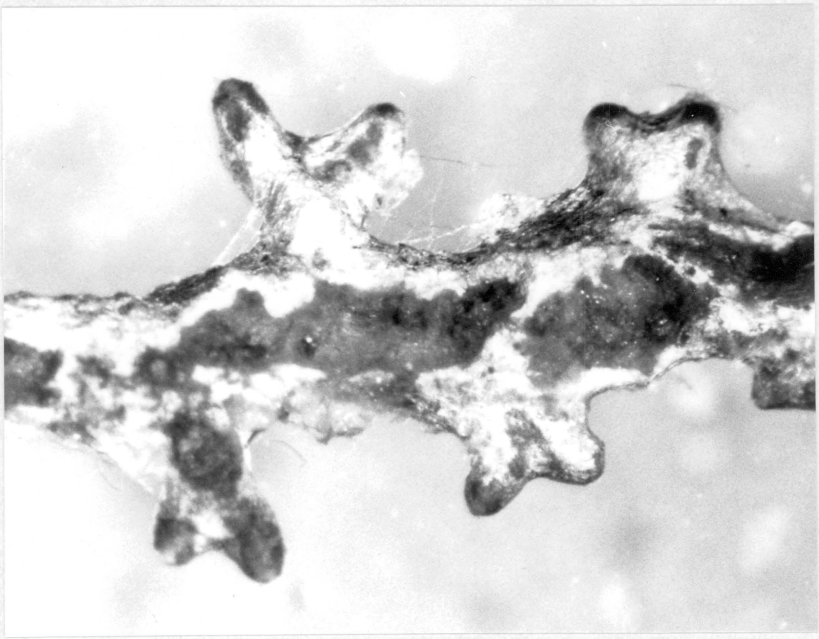


Figure 6: Short roots of *Pinus densiflora* colonized by *Pisolithus tinctorius*.



DISCUSSION

Tree growth under conditions of copper stress appears to be ameliorated most by the mycorrhizal fungus which most effectively stimulates growth under nonstressed conditions. In this work, *Suillus pictus* was the most effective mycorrhizal symbiont at both copper stressed and unstressed conditions, despite the fact that *Pisolithus tinctorius* exhibited significantly greater *in vitro* copper tolerance. These results confirm the suggestion of Denny and Wilkins (1987), that the degree of compatibility between host and fungus is more significant in improvement of host growth under conditions of metal stress than is the metal tolerance of a particular fungal isolate. We have previously demonstrated that *S. pictus* binds copper to hyphal cell walls, and this is apparently an effective mechanism for copper tolerance in association with the host plant. The increase in copper lost from nonmycorrhizal roots when compared to mycorrhizal roots under acidic conditions also suggests some sort of nonionic binding to hyphal cell walls or surfaces in all of the fungi examined including *Boletinellus merulioides*, which did not form mycorrhizae with *Pinus densiflora*. Hyphae of *B. merulioides* did grow in loose wefts around the short roots, however.

Despite the high sensitivity of *Piloderma bicolor* to copper *in vitro*, this fungus proved almost as effective as *S. pictus* in stimulating host growth in the presence of copper. This lack of correlation between *in vitro* metal tolerance and improvement of host growth has also been demonstrated in mycorrhizal birch at high zinc (Brown and Wilkins, 1985b) and nickel levels (Jones and Hutchinson, 1988a).

All of the mycorrhizal fungi examined tended to enhance shoot and root nutrient levels, but only *Pisolithus tinctorius* consistently increased shoot and root copper levels as well. We have previously demonstrated that copper is probably associated with polyphosphate bodies in the hyphae of this fungus. The parallel uptake of copper and phosphorus by *P. tinctorius* indicates copper may be bound to polyphosphate bodies as a mechanism of detoxification. Jones and Hutchinson (1988b) have suggested a similar explanation for the parallel distribution they observed between nickel and phosphorus in mycorrhizal roots of beech. Polyphosphates tend not to be normal metabolites in microorganisms but are thought to accumulate under conditions of age or nutritional imbalance (Harold, 1966). The nutritional imbalance created by copper stress could be

responsible for induction of polyphosphate bodies in *P. tinctorius* and the observed relationship between phosphorus and copper uptake.

Metal tolerant plants are characterized by higher metals in roots and the same or lower levels in shoots (Baker, 1981) when compared with nontolerant plants. All of the fungi in this study enhanced root copper levels but with the exception of those plants colonized by *P. tinctorius*. Copper levels in the shoot were generally the same as in nonmmycorrhizal plants. However, shoot copper levels may not be indicative of plant response in pine, as other workers have demonstrated that needle levels of copper in *Pinus resinosa* did not increase in response to copper in the growth medium (Heale and Ormrod, 1982).

The decrease in percent vascular tissue due to copper in trees inoculated with *Suillus granulatus* was correlated with a decrease in mycorrhizal colonization. This suggests that this fungus is producing factors conducive to the formation of xylem and phloem. Nonmycorrhizal plants did not show similar decreases in percent vasculature, indicating that the decrease was not due to copper acting directly on the tree. Many mycorrhizal fungi, including *S. granulatus*, have been shown to produce plant hormones such as auxins and cytokinins (Slankis, 1973). It is probable that reduced fungal colonization, due to copper, is producing a decrease in hormone production which leads to the observed reduction in vascular tissue. It might be hypothesized that the percent vasculature would not decrease due to copper in trees colonized by *P. tinctorius* or *P. bicolor* where percent mycorrhization is not affected by the metal.

The different ratios of unifurcate, bifurcate, and quadrifurcate mycorrhizal short roots when the mycorrhizal fungi were inoculated in combination compared to a single fungus alone, suggests that both fungi were probably present in the combinations *S. pictus* x *P. bicolor* and *P. bicolor* x *P. tinctorius*. The combination of *S. pictus* x *P. tinctorius* was probably colonized solely by *P. tinctorius*.

The relative competitive abilities of the mycorrhizal fungi studied in this experiment were not altered by the addition of copper, unless copper levels were so high as to eliminate one of the two mycorrhizal fungi. The highest copper level used in this study significantly impaired the ability of

S. pictus to form mycorrhizae. When *S. pictus* and *P. bicolor* were combined on the same root system, colonization by *S. pictus* ceased at the highest copper level.

It appears that many ectomycorrhizal fungi have the ability to improve the growth of their hosts under conditions of heavy metal stress. Ectomycorrhizal fungi and their hosts are often found on acid soils (Meyer, 1973) where exposure to heavy metal ions would be greater than on alkaline soils (Adriano, 1986). This suggests that ectomycorrhizal fungi may have evolved intrinsic mechanisms of metal tolerance which could improve host plant growth. The early stage mycorrhizal fungus used in these experiments, *P. tinctorius*, was no more successful in stimulating seedling growth under metal stress than the late stage *S. pictus*. Thus, it is not the successional stage of the mycorrhizal fungus nor the metal tolerance of a particular isolate that helps the tree grow when exposed to metal stress. Rather, it is the degree to which a mycorrhizal fungus is compatible with the host plant. Compatibility cannot be measured by the percentage of the root system colonized by a particular fungus. A fungus such as *S. pictus* with a low ability to stimulate the formation of mycorrhizal rootlets may produce less of a carbohydrate drain on the host while still enhancing nutrient uptake.

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Boletinellus merulioides alters root morphology of Pinus densiflora without mycorrhizal formation

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ABSTRACT

Boletinellus merulioides is a member of the Boletaceae, an ectomycorrhizal fungal family. Although this fungus is consistently associated with *Fraxinus americana*, it has never been proven to be mycorrhizal. Using pure culture synthesis, seedlings of *Pinus densiflora*, a nonhost tree, were inoculated with cultures of *B. merulioides*. Shoot weights were not enhanced by inoculation, but root weights were significantly increased and roots were highly branched. Short roots were dichotomously branched, lacked root hairs, and had an internal morphology similar to that of ectomycorrhizal rootlets. A loose weft of fungal hyphae surrounded the short roots, but the fungus did not penetrate the epidermis. In inoculated seedlings, root weight and branching were enhanced at higher glucose levels. Culture filtrates from liquid cultures of *B. merulioides* and *Pisolithus tinctorius* were assayed using High Performance Liquid Chromatography (HPLC). Both fungi produced measureable quantities of indole-3-acetic acid (IAA), the production of which was enhanced at high media glucose. These results suggest that IAA is the compound responsible for the dichotomization observed in the short roots of *P. densiflora*.

Key words: Ectomycorrhizae, *Boletinellus merulioides*, *Pisolithus tinctorius*, IAA, HPLC, morphology

INTRODUCTION

Fungi in the genus *Boletinellus* are commonly associated in nature with members of the angiosperm genus *Fagus* (Snell and Dick, 1970). In the southeastern United States *Boletinellus merulioides* (Schw.) Murrill is consistently collected near *Fagus americana* L. (Cotter, 1987). *B. meruliodes* is a member of the Boletaceae, a family whose members are considered by some authors to be exclusively mycorrhizal (Miller, 1983). However, the ecological role of this fungus is still unclear; the ability to form a mycorrhizal association has not yet been demonstrated in pure culture synthesis (Cotter and Miller, 1985).

Ectomycorrhizal roots differ from nonmycorrhizal roots in both morphology and anatomy. They are swollen, lack root hairs, and in pines, are commonly dichotomously branched (Hatch, 1937). Auxins are probably the compounds responsible for the dichotomous branching induced by an ectomycorrhizal symbiont (Palmer, 1954). Auxins cause cell elongation in differentiating cells; indole-3-acetic acid (IAA) is the main auxin found in plants (Goodwin and Mercer, 1983). Both culture filtrates from ectomycorrhizal fungi and IAA induce the formation of dichotomous short roots in pine (Slankis, 1973). In addition, a large number of ectomycorrhizal forming fungi produce auxins (Moser, 1959; Ek, Ljungquist and Stenstrom, 1983), and some have been specifically shown to produce IAA (Strzelczyk, Sitek and Kowalski, 1977; Frankenberger and Poth, 1987). Slankis (1973) has suggested that the auxin produced by ectomycorrhizal fungi may stimulate the formation of short roots receptive to subsequent colonization by the fungus. Exudates from some nonmycorrhizal Basidiomycetes, Zygomycetes and Fungi Imperfecti can also induce dichotomous branching (Turner, 1962). Thus, it is not possible to determine an ecological role for a fungus solely on the basis of either auxin production or the induction of dichotomous branching in pine roots. Cytokinins, another class of plant hormones, may also stimulate dichotomy in pine roots (Gogala, 1971).

High glucose concentrations in pure culture synthesis studies have been shown to lead to abnormal mycorrhizal morphologies, and may also cause mycorrhizal fungi to form a mantle and a Hartig net, the two characteristic features of the mycorrhizal association, with a nonhost plant (Duddridge and Read, 1984; Duddridge, 1985).

The present studies used pure culture synthesis to examine the effect of *B. merulioides* on the growth of *Pinus densiflora* D. Don, Japanese Red Pine, at low and high glucose levels. Attempts are made to correlate the morphological changes observed with *in vitro* IAA production by the fungus. IAA production in *B. merulioides* was compared with that in *Pisolithus tinctorius* Coker & Couch. This fungus forms the mycorrhizal association with *P. densiflora* (see previous chapter) and produces IAA in pure culture (Frankenberger and Poth, 1987).

MATERIALS AND METHODS

Experiment 1 - Effect on *P. densiflora* at 36 mM glucose

The culture of *Boletinellus merulioides* was a tissue isolate taken from a fruiting body collected under *Fraxinus americana* by Dr. V. Cotter. It was maintained on Hagem's agar (Hagem, 1910) at 4°C. The fungus was grown for 30 days in liquid culture in the defined medium of Palmer (Palmer, 1971) (PDM) at 23°C in the dark. Cultures were ground in a Waring blender at speed 5 and 10 ml of the resulting suspension was added to 25 x 4 cm screw top glass tubes. The tubes contained 11.25g of sterile coarse perlite, sifted through a 2mm screen to remove small particles, and 30ml of PDM. PDM has a glucose concentration of 5g/l (= 36mM). Tubes were incubated in the dark at room temperature. After two weeks, a sterile seedling of *Pinus densiflora* was introduced into each tube. The portion of the tubes containing the perlite was covered with aluminum foil and they were placed in a growth chamber at 21°C where they received 16 hours of light per day. Seedlings received fluorescent light alone for 4 of the 16 hours which provided 148 $\mu E/m^2/s$ of photosynthetically active radiation (PAR) and incandescent plus fluorescent light for the remaining 12 hours. The PAR during this period was 163 $\mu E/m^2/s$. Seedlings were harvested after 180 days. Growth was evaluated based on the following parameters: shoot and root fresh and dry weights and total numbers of root tips. The last value was used to give an estimate of branching of the root system. Samples of short roots from the inoculated trees were fixed in FAA (formalin/acetic acid/ethanol), dehydrated in a tertiary butyl alcohol and ethanol, paraffin embedded, sectioned on a rotary microtome and stained in safranin/fast green. Dry weights were measured after drying to a constant weight at 45°C.

Experiment 2 - Effect of increasing glucose

Boletinellus meruliodes was grown for 5 weeks in liquid Hagem's media (Hagem, 1910) at 20C in the dark. Cultures were inoculated as previously described by adding 10 ml of ground culture to glass tubes containing vermiculite/peat moss (110:10, v:v) saturated with 70 ml of liquid Hagem's media containing 0 or 10g of glucose per liter. The final glucose concentration in a tube was either 0.63g/l (= 4mM) or 10g/l = (72mM).

Experiment 3 - Effect of glucose on IAA production *in vitro*

Cultures of *Boletinellus merulioides* and *Pisolithus tinctorius* were grown on solid PDM for 28 days at 20C in the dark. Liquid cultures were initiated by removing two 0.8 cm disks from the leading edge of the agar cultures and placing these in 250 ml erlenmeyer flasks containing 50 ml of PDM containing glucose at 500 mg/l (= 3.6mM) or 10 g/l(= 72mM). Flasks were incubated at 23C in the dark for 4 weeks. Cultures filtrates were Seitz-filtered and used without further modification for HPLC analysis. Fungi were dried at 45°C and assayed for total chitin (Vignon et al., 1986).

Chromatography was performed by AVG on an HP1090 liquid chromatograph (Hewlett-Packard, Palo Alto, CA USA) equipped with an HP79846A autosampler, a 960 Polychrom diode array detector (Varian, Walnut Creek, CA, USA), and a Hitachi F1000 fluorescence detector (Hitachi LTD, Toyko, Japan) connected in series. The UV detector was operated at 229nm and 282nm: the fluorescence detector wavelengths were 220nm excitation and 350nm emission. Quantitation was done on a Nelson 6000 data system (Nelson Analytical, Cupertino, CA, USA). The 250 x 2.1mm ID 5 μm Vydac C18 column and guard column (Vydac, Hesperia, CA, USA) were at ambient temperature. The eluent was 18% acetonitrile and 0.1% trifluoroacetic acid in water and was used at a flow rate of 0.2 ml/min. The chromatography was performed isocratically. Standards were prepared with media containing glucose at 10 g/l (= 72mM) by successive dilutions of a 1 mg/ml solution of IAA in 10% acetonitrile in deionized water. 40 μl of each standard or sample was injected onto the column.

Statistical separations for all three experiments were performed using Duncan's New Multiple Range Test (DNMRT) at P = .05 (Duncan, 1975).

RESULTS & DISCUSSION

Inoculation with *Boletinellus meruliodes* had no effect on shoot fresh or dry weight of *Pinus densiflora* (Table 1). However, root weights were significantly enhanced by the presence of the fungus. This was primarily due to an increase in branching of the short roots, as evidenced by the significant differences in total root tips and root tips per root weight when the inoculated and un-inoculated seedlings were compared (Table 1). Roots of *P. densiflora* rarely dichotomized without the presence of *B. merulioides*, suggesting that the substances emanating from the fungus trigger the

dichotomy. The glucose levels used in this experiment did not induce dichotomy. Dichotomy in *Pinus pinaster* is apparently possible without the addition of exogenous growth regulators or a mycorrhizal fungus (Faye, Rancillac and David, 1980), but this is not the case for *P. strobus* (Piche et al., 1982) or *P. densiflora*. Uninoculated seedlings exhibited relatively long, narrow short roots which were covered with root hairs. In contrast, inoculated seedlings had dichotomously branched short roots which lacked root hairs (Figure 1). These were very similar in appearance to the nonmycorrhizal short roots of *P. sylvestris* exposed to exudates from the mycorrhizal fungus *Suillus variegatus* (Slankis, 1973). Longitudinal sections through the dichotomously branched short roots induced by *B. merulioides* showed a loose mantle of fungal tissue surrounding the root. Each apex of the dichotomous branch had a dome shaped apex covered with a thin layer of tangentially flattened cells. The cortex was narrow and composed of enlarged, highly vacuolated cells. The cells in both of the apical meristems had very large nuclei. These observations are similar to those described by Piche et al. (1982) for the apices of *P. strobus* mycorrhizal short roots colonized by *Pisolithus tinctorius*. However, each apex of a dichotomous branch of *P. densiflora* conforms to the description of the young apical meristem prior to dichotomizing. This would suggest that further growth and branching from these meristems is possible. Figure 1 demonstrates that many of the dichotomous short roots did continue to elongate and branch.

When the growth of *P. densiflora* inoculated with *B. merulioides* was compared at low and high glucose levels, high glucose appeared to stimulate the enhanced root development induced by the fungus (Table 2). The differences between high and low glucose were not as dramatic as those between the inoculated and uninoculated plants. High glucose can induce abnormal development of mycorrhizae formed by *Suillus grevillei* and *Larix kaempferi* and can also induce the fungus to form the mycorrhizal association with a nonhost tree (Duddridge, 1985). However, in our study it does not appear that the high glucose stimulated the morphological changes in the roots but only enhanced them. High glucose may stimulate growth of *B. merulioides*, or it may cause the fungus to produce more of the compound it is presumably excreting to trigger the dichotomy.

Both *B. merulioides* and *P. tinctorius* produced detectable amounts of IAA under the conditions used (Table 3 and Figure 2). Fluorescence data were selected for quantitation due to the

greater selectivity and sensitivity for indole compounds. Based on a signal to noise ratio of 4, the minimum detectable quantity of IAA by fluoresence was approximately 0.01 μmole/l. Identification of IAA was based on retention time, fluorescence/UV absorption ratios and on-line UV spectral data. Data are presented as μmoles/l IAA per mg fungal chitin. Both fungi are apparently able to synthesize IAA without tryptophan (TRP) as a nitrogen source. Ho (1986) has also demonstrated IAA production by *P. tinctorius* in a non-TRP supplemented medium. TRP is required for IAA production in most of the ectomycorrhizal fungi that have been studied although some can produce IAA without this compound (Moser, 1959). IAA production in both fungi was stimulated by the addition of glucose to the growth medium but the increase was not statistically significant in the case of *B. merulioides.* These results conflict with those of Gay (1986) who found that increasing glucose concentrations between 0 and 55.5 mM led to a decrease in IAA production by the mycorrhizal *Hebeloma heimale.* However, the difference may be due to the fact that IAA production in *H. hiemale* requires TRP (Gay, 1986; Gay and Debaud, 1987) and a non-TRP requiring pathway for IAA synthesis in *P. tinctorius* and *B. merulioides* responds differently to glucose. Our results do suggest that the increase in stimulation of root branching in *P. densiflora* by high glucose was due to increased IAA production by *B. merulioides.*

In the HPLC chromatograms, there were a number of unidentified peaks that had either fluoresence or UV absorption. On-line UV spectral data were used to obtain further information about these peaks. The peaks resulting when the culture filtrate from *B. merulioides* was analyzed are shown in Figure 3. Similar peaks were seen in filtrates from *P. tinctorius.* The peak purity parameters for each species seen in the chromatograms are presented in Table 4. The purity parameter is an average wavelength weighted by the square of absorbance calculated over a specified range of wavelengths (Sheehan, 1989). It can be used as a tool for comparison with known standards. Although the peak purity parameter for Peak #3 was essentially identical to the peak purity parameter for kinetin, their spectra did not overlay exactly (Figure 5). Peak #2 is very probably another indole compound, but neither this nor the question of kinetin production by *B. merulioides* can be resolved without further study.

The ecological significance of IAA production by *B. merulioides* is unclear. Since it does not appear to be an ectomycorrhizal symbiont of *Pinus*, the hypothesis that IAA production stimulates dichotomous rootlets conducive to colonization by the fungus is unlikely. Production of diffusible compounds by ectomycorrhizal fungi has been shown to enhance the growth of endomycorrhizal trees (Levisohn, 1953; Levisohn, 1956). It is possible that the auxin produced by *B. merulioides* may stimulate the growth of *Fraxinus americana*, with which it appears to be consistently associated (Cotter, 1987). *B. merulioides* may in turn be living at least partially on exudates from *F. americana* roots, the production of which would be enhanced by a more actively growing plant. This hypothesis is currently being experimentally tested.

Table 1: Effect of *Boletinellus merulioides* on growth of *Pinus densiflora* in Pure Culture at 36mM Glucose

Inoculum	Total Root Tips	Tips per RFW	Tips per RDW	Shoot FW (mg)	Root FW (mg)	Shoot DW (mg)	Root DW (mg)
None	37b[1]	723b	6092b	86a	60b	33a	8a
B. merulioides	191a	1375a	8792a	140a	138a	42a	22a

[1]Numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Table 2: Effect of *Boletinellus merulioides* on growth of *Pinus densiflora* at 4 and 72mM glucose.

Glucose mM	Total Root Tips	Tips per RFW	Tips per RDW	Shoot FW (mg)	Root FW (mg)	Shoot DW (mg)	Root DW (mg)
4	243b[1]	1234b	9739a	515a	215b	102a	25b
72	447a	1264a	8790a	593a	333a	105a	51a

[1]Numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Table 3: Effect of glucose on *in vitro* indole-3-acetic acid (IAA) production in *Boletinellus merulioides* and *Pisolithus tinctorius*.

Fungus	Glucose (mM)	μmoles IAA/mg chitin
Boletinellus merulioides	3.6	.008b[1]
	72.0	.024b
Pisolithus tinctorius	3.6	.048b
	72.0	.160a

[1]Numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Table 4: Peak Purity Parameters for Species Seen in HPLC Chromatograms
UV 282nm

Peak	Purity Parameter (nm)
IAA Standard	228.0
Kinetin Standard	264.7
Sample Peak 1	252.9
Sample Peak 2	231.7
Sample Peak 3	264.8
Sample Peak IAA	227.2

Figure 1: Short roots of *Pinus densiflora* grown in perlite with 36mM glucose and *Boletinellus merulioides*.

Figure 2: IAA Identification (Fluoresence Data) - *Pisolithus tinctorius* grown at 72 mM glucose.

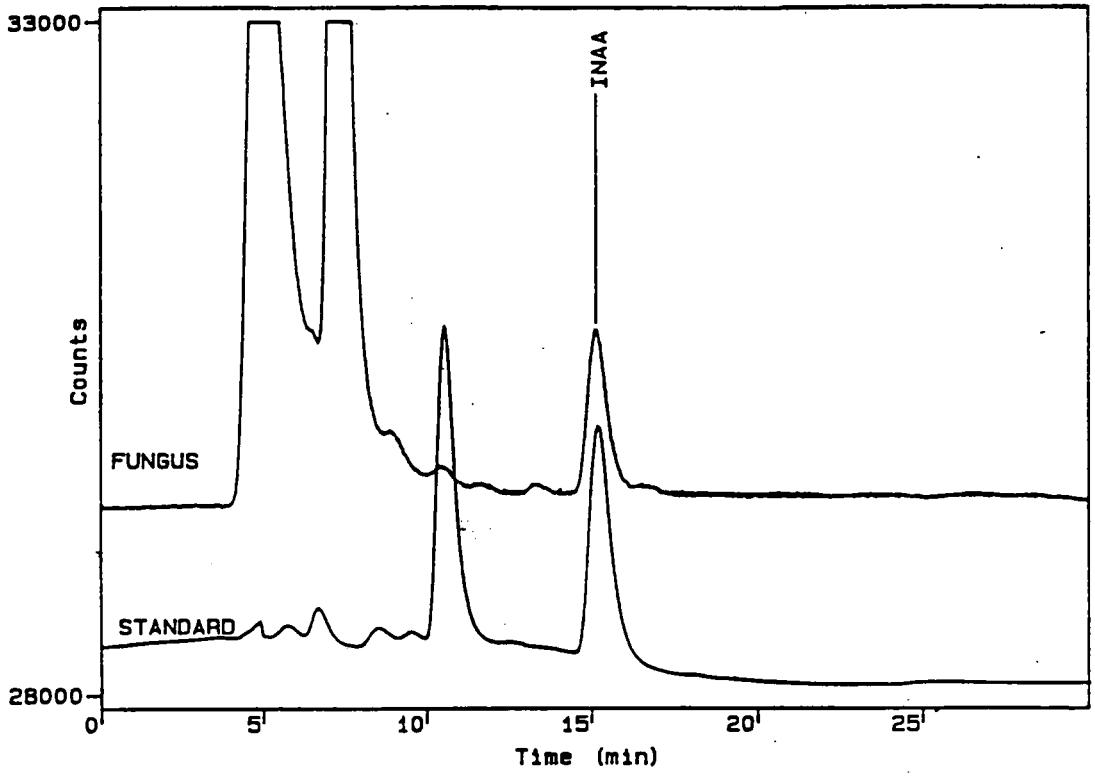


Figure 3: IAA Identification (Fluoresence and UV Data) - *Boletinellus merulioides* grown at 72 mM glucose.

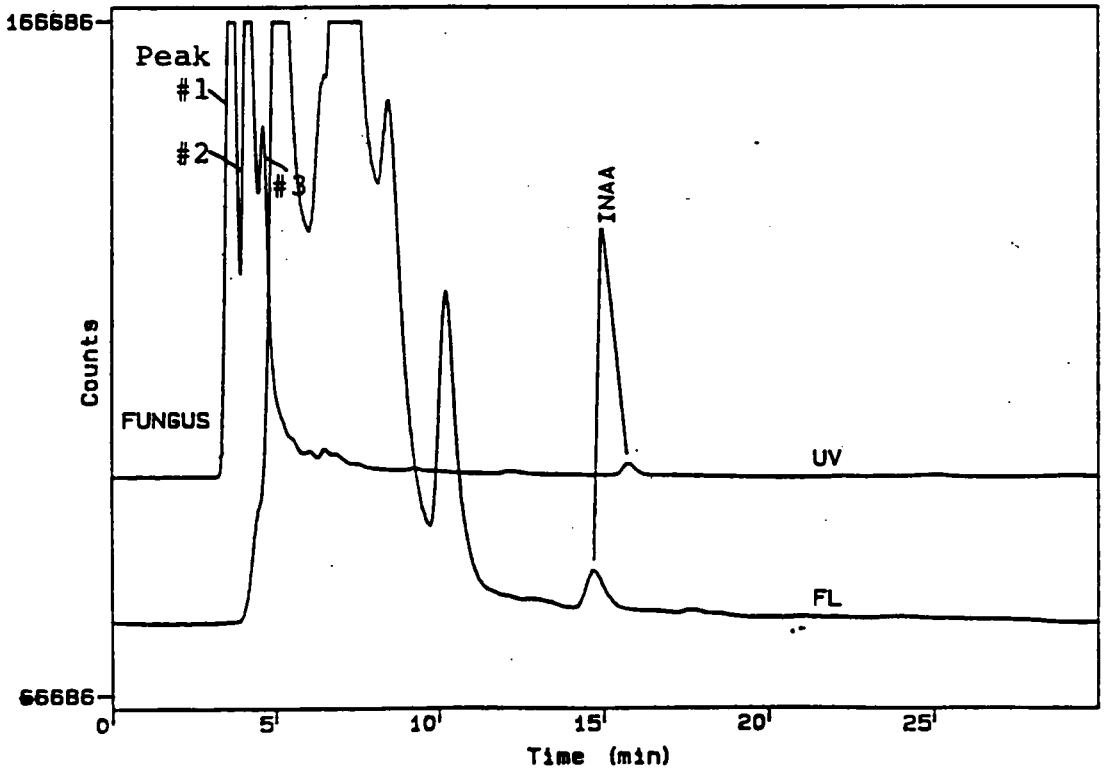
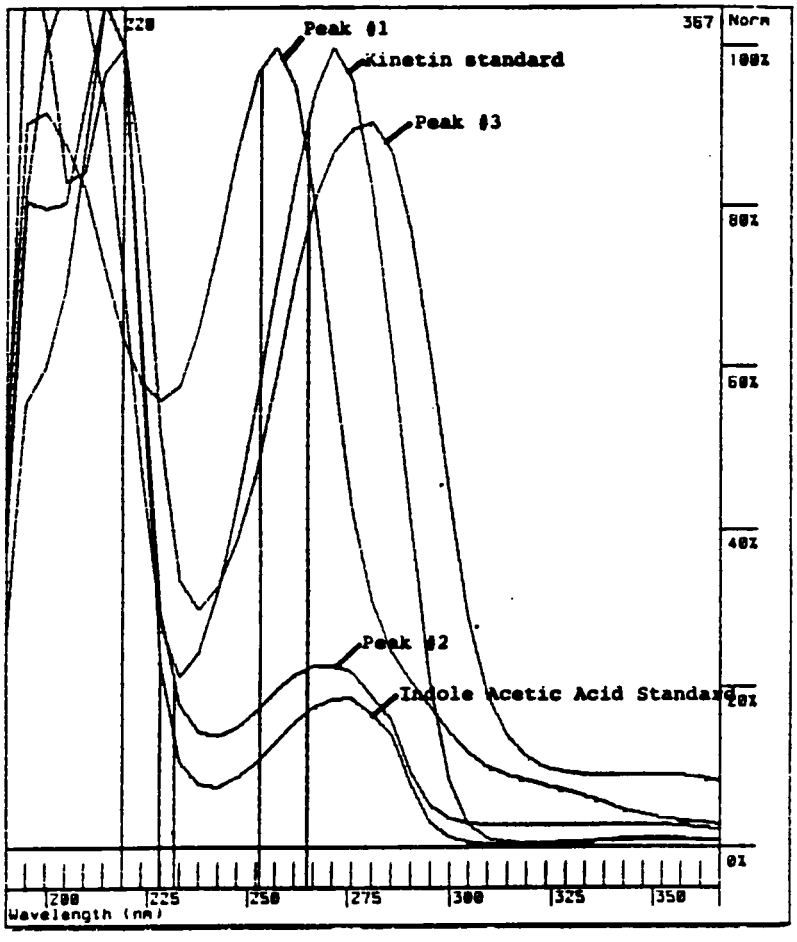


Figure 4: On-line UV Spectral Data



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Effect of copper on the interaction between Glomus diaphanum and Allium cepa: Growth and photosynthetic relationships

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ABSTRACT
The influence of copper on growth and photosynthesis was studied in vesicular-arbuscular (VA) mycorrhizal and nonmycorrhizal onions (*Allium cepa L.*) cultured at low and moderate levels of soil phosphorus (P). Copper sulfate and potassium phosphate (KH_2PO_4) were added to the soil to achieve 0 and 40 μg/g copper and 5 and 50 μg/g phosphorus. Percent VA-mycorrhizal (*Glomus diaphanum* Morton et Walker) colonization could be reduced by the addition of either copper or phosphorus. In plants grown in moderate P soil, mycorrhizal colonization resulted in increased levels of shoot P, calcium, and magnesium. Potassium, iron and copper were not increased in mycorrhizal plants when compared with nonmycorrhizal plants, even at high soil copper levels. Plants grown in low P soil were so small without mycorrhizal colonization that comparisons between mycorrhizal and nonmycorrhizal plants were not possible. Photosynthetic rate was not correlated with VA mycorrhizal colonization, shoot P content, leaf chlorophyll content, or soil copper at either low or high relative humidity. These results suggest that the presence of VA mycorrhizal fungi has neither a positive nor a negative effect on plant growth when exposed to phytotoxic levels of copper.

Key words: Endomycorrhizae, copper, photosynthesis, nutrients

INTRODUCTION

Colonization of plants by vesicular-arbuscular (VA) or endomycorrhizal fungi promotes growth by enhancement of phosphate uptake in low phosphorus (P) soils and can also stimulate uptake of metalliferous micronutrients such as copper and zinc where these elements are deficient (Benson and Covey, 1976; Lambert, Baker and Cole, 1979; Swaminathan and Verma, 1979; Timmer and Leyden, 1980; Gildon and Tinker, 1983b). However, high levels of heavy metals in the soil may decrease or even eliminate colonization of the host by VA mycorrhizal fungi (McIlveen

and Cole, 1978/79; Gildon and Tinker, 1983a). Increased levels of P may have a similar effect (Koide, 1985; Gruhn, Roncadori and Kormanik, 1987). At least one study has demonstrated that if VA mycorrhizae are able to colonize host plants in the presence of phytotoxic levels of heavy metals, they may decrease host growth by increasing metal uptake and transport to the shoot under conditions of low pH (Killham and Firestone, 1983).

In the research reported here, copper amended soil was used to better understand the response of VAM fungi to toxic levels of metals. Mycorrhizal and nonmycorrhizal onions were grown at low or moderate soil phosphorus and low or high copper. The aim was to determine if VA mycorrhizae actually stimulate metal uptake and thereby toxify the host plant or if they lead to reduced growth by producing an additional carbon drain on the host under conditions of metal stress. The specific parameters evaluated were VA mycorrhizal colonization, photosynthetic rate, plant growth and shoot nutrient levels in order to assess the influence of phosphorus or copper stress and VAM on carbon metabolism.

MATERIALS AND METHODS

The study consisted of three separate experiments conducted within one year of each other.

Experiment 1

Fungal and Plant Material

The fungus used in all three experiments was *Glomus diaphanum* Morton et Walker. It had been isolated from a coal spoil pile in southwestern Virginia and maintained on a mixture of *Paspalum notatum* var. *saure* Parodi (Bahia grass) and onion *Allium cepa* L.. In the experiments the host plant was onion, *A. cepa* var. *Danver's Yellow Globe*. Onion was selected both because it is very sensitive to heavy metals and because it readily becomes colonized by VA mycorrhizal fungi (Manjunath and Bagyaraj, 1981). In addition, a growth response can be obtained with the introduction of low levels of inoculum (Powell, 1981). Seeds were neither sterilized nor pregerminated before planting.

Soil and Experimental Design

Soil was a cherty silt loam in the Fredrick series, A horizon, (pH 5.5), obtained from an abandoned apple orchard and had an available phosphorus level of approximately 5 μg/g determined by extraction with ammonium fluoride (Bray and Kurtz, 1945). It was sterilized before planting with methyl bromide (Dowfume MC-2, Dow Chemical Co., Midland, MI 48640) applied at a minimum rate of 1.36 kg/800 liter of soil for 48 hours under a plastic film. After venting for 1 week, the soil was stored in metal cans. Soil was amended with copper sulfate and/or potassium phosphate (KH_2PO_4) to achieve copper and phosphorus levels of 0 and 40, 5 and 50 μg/g, respectively. Dry crystals were dissolved in water and mixed with soil by hand. (Note: Copper levels in this chapter are not comparable to the levels used previously where plants were grown in perlite. Perlite, unlike soil, has no cation exchange sites and hence, no ability to bind copper ions). An equal volume of coarse perlite was mixed with the soil prior to filling 8 cm plastic pots that had been sterilized with sodium hypochlorite. Mycorrhizal treatments received a root-soil inoculum consisting of approximately 20 azygospores. Roots of the stock culture were chopped into 5mm segments and 50ml of the mixture was added to the soil surface of each pot. Nonmycorrhizal treatments received soil alone. Onion seeds were planted in four groups directly on the surface of the inoculum and were covered with 60 ml of sifted perlite (coarse particles removed). Each pot was covered with a square of cheesecloth until the seedlings germinated. Pots were arranged randomly in the greenhouse on inverted glass petri dishes and watered whenever the soil appeared dry. After 14 days, seedlings were thinned to 4 per pot by cutting off the tops of the excess plants.

The experiment began on February 23, 1988 and ended after 79 days. Shoot and root fresh weights were determined by weighing all four plants from one pot together and dividing by four. Shoots were dried to a constant weight at 45°C before weighing to determine dry weight. Root length was determined as the maximum length to which at least seven roots of the root systems of the four plants extended. Whole root systems were stained in 1% trypan blue in lactic acid (Philips and Hayman, 1970), and percent mycorrhization was determined by the gridline-intersect method (Giovannetti and Mosse, 1980).

Experiment 2

The soil copper and phosphorus levels in this experiment were 0, 20 and 40, 5 and 50 μg/g, respectively. The mycorrhizal inoculum consisted of 10 azygospores per pot added in 5 ml of tap water. Azygospores were collected by centrifugal floatation (Jenkins, 1964). To obtain a spore-free filtrate for nonmycorrhizal controls, spores were kept in tap water for 30 minutes at room temperature, filtered through a 45 μm sieve; 5 ml of the suspension was added to each control pot. Plants were fertilized with 5 ml of a 1 g/l NH_4NO_3 solution 20 days after planting. The experiment was initiated on July 13, 1988 and terminated after 96 days. During the growth period the greenhouse was covered with a shade cloth which reduced photosynthetically active radiation (PAR). Photosynthetic rates were measured between 2:00 and 4:00 PM with the Li-Cor Portable Photosynthesis System (LI-6000) two days prior to harvest. Soil was drenched in the morning prior to taking measurements.

Experiment 3

Soil copper and phosphorus levels were 0 and 40, 10 and 40 μg/g. Pot size was 10 cm and seedlings were thinned to 2 per pot. Mycorrhizal treatments were initiated using 5 g of root-soil inoculum containing approximately 20 azygospores. Nonmycorrhizal controls received 2 ml of a filtrate made by mixing 100 ml of soil from the fungal culture with 200 ml of water and filtering through Whatman #1 paper. Pots were not covered with cheesecloth after planting. Seedlings were fertilized with 5 ml of a 15mM NH_4NO_3 solution at 10, 17, and 24 days after planting. Seeds were planted on December 4, 1988, and plants were harvested after 98 days. Photosynthesis was measured 93 days after planting, and measurements were taken from 10:30AM until 12:30PM. Two measurements were taken per leaf, the first when the relative humidity (RH) in the cuvette was 50%. For the second measurement, the cuvette remained closed on the leaf and RH was lowered by increasing the flow rate through the desiccant. When RH had dropped to 25%, photosynthesis measurements were started again. The Li-Cor monitored a 4 μg/g drop in CO_2 at each RH level. Measurements were made under an artificial light which gave a PAR of 400 $\mu E/m^2/s$. Chlorophyll a and b levels were determined from 4 cm leaf segments (Jeffrey and Humphry, 1975). The leaves were the same as those on which photosynthesis had been measured and they were frozen prior to analysis.

For analysis of phosphorus, potassium, calcium, magnesium, iron and copper, dried shoots were ground with a mortar and pestle and ashed in a muffle furnace for 5 hours at 475°C. The ash was dissolved in 12N HCl and brought to 1.2N with deionized water. Analysis used Plasma Emission Spectroscopy at the Virginia Polytechnic Institute and State University Soil Testing and Plant Analysis Laboratory.

Statistical separations for all experiments were performed using Duncan's New Multiple Range Test (DNMRT) (Duncan, 1975). Numbers followed by the same letter are not significantly different at $P = .05$.

RESULTS AND DISCUSSION

Experiment 1

By the end of the experiment, soil P levels had dropped to 4 and 20 $\mu g/g$ in the low and moderate P treatments respectively. In the nonmycorrhizal treatment, copper significantly reduced plant growth only at the moderate soil P level (Table 1). Shoot and root fresh weights and shoot dry weight were decreased by 34, 53, and 52% when compared with plants grown in soil not amended with copper. At both P levels, copper reduced total root length and the root to shoot ratio.

Within the mycorrhizal treatment, only the root fresh weight of plants grown at the moderate P level was decreased by the addition of copper. As with the nonmycorrhizal plants, copper did not affect the weights of plants grown at low soil P. Root length and the root to shoot ratio were decreased by copper at both P levels as in the nonmycorrhizal plants. Mycorrhizal colonization was not inhibited by soil copper but was reduced 37% at the moderate P level. However, this apparent reduction due to P is probably due to the larger root system in these plants. They may have the same fungal biomass as in the low P-grown plants but because of the larger root system, the grid-line intersect method recorded proportionally less fungi. When copper was added, the root system became much smaller and the percentage of VA mycorrhizal colonization rose again. Although other authors have demonstrated a reduction in colonization due to heavy metals (McIlveen and Cole, 1978/79; Gildon and Tinker, 1983a), all host-fungus combinations do not respond

identically to the same abiotic stresses (Sanders et al., 1977; Kahn, 1981; Daft and Hogarth, 1983; Jensen, 1983). It is likely that the response of onion to *G. diaphanum* may not be the same as the response of soybean to *G. mosseae* (McIlveen and Cole, 1978/79) or that of onion to *G. mosseae* (Gildon and Tinker, 1983a). However, we may have missed a decrease in colonization due to copper by using the grid-line intersect method of evaluating mycorrhization.

When the nonmycorrhizal and mycorrhizal treatments are compared, VA mycorrhizae significantly increased plant growth in the low P soil. Shoot and root fresh weights and shoot dry weight were increased by 52, 45 and 68% due to mycorrhizal colonization. VA colonization enhanced plant growth even in the presence of copper. Root length and root to shoot ratio were not altered by VA mycorrhizae.

Experiment 2

Plants grew so poorly at 40 μg/g copper that the data are not presented in Table 2. Neither copper nor VA mycorrhizae significantly affected plant growth or photosynthesis in this experiment. Within the mycorrhizal treatment, copper did not affect the colonization of *A. cepa* by *G. diaphanum*. However, colonization was completely inhibited at the moderate level of soil P. A combination of low irradiance and high P in the greenhouse can lead to extremely low colonization in onion (Son and Smith, 1988). It is probable that the combination of the reduced irradiance in the greenhouse and the low inoculum prevented colonization by the mycorrhizal fungi in the moderate P, high copper treatments.

Experiment 3

Soil P levels had dropped to 5 and 20 μg/g in the low and moderate P soils by the end of the experiment.

Nonmycorrhizal plants in low P soil and mycorrhizal plants in the low P soil plus copper did not grow well enough to be accurately measured. Among nonmycorrhizal plants grown at the moderate P level, addition of copper reduced growth in all of the parameters examined (Table 3). A similar response to copper was observed in mycorrhizal plants. At the moderate phosphorus level, addition of copper decreased root fresh weight by 71%, shoot dry weight by 47% and mycorrhizal colonization by 65%.

Within the nonmycorrhizal plants, copper neither affected the rate of photosynthesis at high or low RH nor did it alter the level of chlorophyll in the leaves (Table 4). Among the mycorrhizal treatments at 50% RH, the photosynthetic rate of plants in soil with moderate P plus copper was significantly less than that in all other treatments (Table 4). However, the decrease probably was not due to copper since the photosynthetic rate was not significantly different from that in the plants grown at 0 μg/g copper. Conductance was also decreased in the mycorrhizal plants at the moderate P level when compared with all of the other treatments. Conductance in the mycorrhizal plants at the moderate P level was 52% lower than that in mycorrhizal plants grown at low P. At a RH of 25% or less, photosynthesis and conductance were significantly less in the mycorrhizal plants grown at moderate soil P than in any of the other treatments. When compared with mycorrhizal plants grown at low P, photosynthesis and conductance were 42 and 63% lower in the plants grown at the moderate P level. Differences in photosynthesis could not be correlated with differences in leaf chlorophyll amount.

Although several workers have demonstrated higher rates of photosynthesis due to mycorrhizal colonization (Allen et al., 1981; Harris, Pacovsky and Paul, 1985), more recent work has shown no difference in photosynthetic rates between mycorrhizal and nonmycorrhizal plants (Fredeen and Terry, 1988). The latter authors suggest that the increases previously noted were due to enhanced leaf area due to improved plant P status in mycorrhizal plants. In our study, we did not observe differences in photosynthesis between mycorrhizal plants at 10 μg/g P and nonmycorrhizal plants at 40 μg/g P. The decrease in photosynthesis due to mycorrhizal colonization at 40 μg/g P could represent parasitic growth of the fungus at the moderate P level. Bethlenfalvay, Brown and Pacovsky (1982) found that levels of P as low as 10 μg/g P were required for mutualistic growth of *G. fasciculatum* in soybean. At higher P levels the fungus became parasitic on the host photosynthate and decreased host growth. Parasitic growth of *G. diaphanum* in onion could be responsible for the observed decreases in photosynthesis in this study.

Levels of P, potassium (K), calcium (Ca) and magnesium (Mg) were slightly reduced in nonmycorrhizal plants when grown in copper amended soil (Table 5). Although shoot copper was increased in the high phosphorus treatment in copper amended soil, the dramatic decreases in

copper and iron in mycorrhizal plants due to the addition of phosphorus indicates extreme interactions between phosphorus and these two metals. The formation of phosphate salts of copper may be limiting uptake of the metal by the plant (Dolar and Keeney, 1971). Lambert, Baker and Cle (1979) demonstrated that the addition of phosphorus reduced the copper and zinc concentration in mycorrhizal plants but had no effect when the plants are not colonized. Mycorrhizal plants grown at moderate P levels without copper had significantly higher amounts of P, K, Ca, and Mg than did mycorrhizal plants grown in low P soil or grown in the moderate P soil with copper. Mycorrhizal colonization did not affect the amount of copper in the shoot, even when the plants were grown at 40μg/g copper. Lack of increased copper uptake to the shoots in copper amended soil has also been demonstrated in onions colonized by *G. mosseae* (Gildon and Tinker, 1983a) and in alfalfa colonized by *G. caledonium* in unamended soil (Nielsen and Jensen, 1983). Increased shoot copper levels due to VA mycorrhizae has only been demonstrated in the bunchgrass *Ehrharta calycina* colonized by *G. fasciculatum* watered with a pH 3 solution (Killham and Firestone, 1983).

The moderate P level used in this study is not particularly high (Bray and Kurtz, 1945) and may not be adequate for optimum growth of onion. Chapman (1966) considers low, moderate and high levels of bulb phosphorus in onion to be 1700, 2600 and 7400 μg/g respectively. Based on the phosphorus values obtained shown in Table 5, nonmycorrhizal plants were severely phosphorus stressed at both P levels.

At 40 μg/g P, mycorrhizal plants had higher levels of P, Ca, and Mg in the shoots than nonmycorrhizal plants, while the amounts of K, Fe and Cu did not differ. The first three elements are moved into the plant by a diffusion gradient through the apoplast while uptake of the latter three depends on active transport (Barber, 1984). This suggests improved water relations in mycorrhizal versus nonmycorrhizal plants, which has been noted in a number of species including onion (Sieverding, 1981; Nelsen and Safir, 1982; Levy, Syvertsen and Nemec, 1983). Since uptake mechanisms for heavy metals differ fundamentally from those for P, it is logical that an improvement in P nutrition would not necessarily correlate with toxification by heavy metals in a contaminated soil.

GENERAL DISCUSSION

The presence of *Glomus diaphanum* in the roots of *Allium cepa* neither helps the host plant deal with phytotoxic levels of soil copper nor does it lead to a reduction in plant growth over that induced by copper alone. Our results indicate that nonmycorrhizal onions exposed to copper are no less vigorous than mycorrhizal onions. *G. diaphanum* is able to improve the phosphorus status of mycorrhizal onions grown at low and moderate phosphorus levels and at phytotoxic levels of copper without a correlative increase in copper uptake. Although copper may decrease the level, colonization by *G. diaphanum* is not completely eliminated even by 40 μg/g copper added to the soil.

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Table 1: Experiment 1 - Effect of copper and *Glomus diaphanum* on growth of *Allium cepa* at two phosphorus levels (n = 6).

VAM	P (μg/g)[1]	Cu (μg/g)	Shoot FW (mg)	Root FW (mg)	Shoot DW (mg)	Root Length (cm)	Root/Shoot Ratio	Colo (%)[2]
0	5	0	130d[3]	145cd	6c	7.9a	1.10a	0
0	5	40	120d	105d	6c	4.2f	0.87b	0
0	45	0	465a	480a	42a	8.7a	1.00ab	0
0	45	40	305bc	225bc	20bc	6.6cd	0.75cd	0
+	5	0	275c	265b	19bc	7.0bcd	0.99abc	82a
+	5	40	305bc	250bc	22b	6.2de	0.86bc	76a
+	45	0	425ab	415a	39a	7.5bc	1.00ab	52b
+	45	40	390abc	220bc	32ab	5.2ef	0.58d	73a

[1]Extractable phosphorus (Bray and Kurtz, 1945).

[2]Percentage of the total root system in which VAM structures can be observed

[3] Numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Table 2: Experiment 2 - Effect of copper and *Glomus diaphanum* on growth and photosynthesis of *Allium cepa* at two phosphorus levels (n = 6).

VAM	P (μg/g)[1]	Cu (μg/g)	Shoot FW (mg)	Root FW (mg)	Root Length (cm)	Root/Shoot Ratio	Colo (%)	Photosyn. (μmolCO_2/m^2/s)
0	5	0	205c[2]	96c	6.7a	0.47a	0	-
0	5	20	239c	147c	5.8c	0.61a	0	-
0	50	0	677ab	331ab	7.1a	.59a	0	13.8a
0	50	20	732a	407a	7.1a	.56a	0	10.4a
+	5	0	376bc	207bc	5.8a	.57a	71a	12.5a
+	5	20	377bc	240abc	5.8a	.59a	75a	11.8a
+	50	0	Plants did not become mycorrhizal					
+	50	20	Plants did not become mycorrhizal					

[1]Extractable phosphorus (Bray and Kurtz, 1945).

[2]Numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Table 3: Experiment 3 - Effect of copper and *Glomus diaphanum* on growth of *Allium cepa* at two phosphorus levels (n = 6).

VAM	P (μg/g)[1]	Cu (μg/g)	Shoot FW (mg)	Root FW (mg)	Shoot DW (mg)	Root/Shoot Ratio	Colo (%)[2]
0	40	0	1065ab[3]	891b	94ab	1.52a	0
0	40	40	638b	310b	51bc	0.57a	0
+	10	0	779ab	337b	47bc	0.46a	57a
+	40	0	1864a	1716a	128a	1.11a	48a
+	40	40	846ab	498b	68bc	0.53a	17b

[1]Extractable phosphorus (Bray and Kurtz, 1945)

[2]Percentage of the total root system in which VAM structures can be observed.

[3]Numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Table 4: Effect of copper and *Glomus diaphanum* on photosynthesis and leaf chlorophyll levels in *Allium cepa* at two phosphorus levels (n = 6).

VAM	P (μg/g)[1]	Cu (μg/g)	50% RH Photosyn. (μmol/m^2/s)	50% RH Cond. (cm/s)	25% RH Photosyn. (μmol/m^2/s)	25% RH Cond. (cm/s)	Chl a (mg/ml)	Chl b (mg/ml)
-	40	0	19.7a[2]	.38ab	17.5a	0.54b	15.8c	3.7b
-	40	40	19.6a	.39ab	19.0a	1.36ab	18.7abc	4.9ab
+	10	0	20.7a	.50a	20.5a	1.68a	22.1a	5.8a
+	40	0	15.1ab	.24b	11.8b	0.63b	21.4ab	5.5a
+	40	40	9.4b	.18b	8.6b	0.54b	17.7bc	5.1a

[1]Extractable phosphorus (Bray and Kurtz, 1945).

[2] Numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

Table 5: Effect of copper and *Glomus diaphanum* on shoot nutrient uptake in *Allium cepa* at two phosphorus levels (n = 6).

VAM	P (μg/g)[1]	Cu (μg/g)	Nutrients (μg/g) P	K	Ca	Mg	Fe	Cu
0	40	0	1430b[2]	20250a	8460b	2710a	288a	17a
0	40	40	1530b	15560a	8450b	3110a	637a	55a
+	10	0	3490b	21040a	14000a	4130a	901a	47a
+	40	0	5780a	25170a	13480ab	4230a	157a	21a
+	40	40	1880b	14580a	12610ab	3890a	242a	49a

[1]Extractable phosphorus (Bray and Kurtz, 1945).

[2] Numbers in the same column followed by the same letter are not significantly different at P = .05 according to Duncan's New Multiple Range Test.

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A survey of vesicular-arbuscular mycorrhizal fungi on mine spoils in southwest Virginia and central North Carolina

ABSTRACT

Solitary herbaceous plants growing directly on refuse piles from coal, metal and arsenic mines were sampled to determine colonization by vesicular-arbuscular (VA) mycorrhizal fungi. Mining had ceased at all sites at least 15 years prior to sampling. Associated soil was examined for fungal spores, and assayed for pH and heavy metal content. VA fungi colonized 68% of the root systems examined, and colonization ranged from 5 to 71%. Spores were collected in 39% of the samples. *Glomus clarum* was the most commonly collected VA mycorrhizal fungus. Both roots colonized by VA fungi and VA spores were present when metals in the soil were at potentially phytotoxic levels. This suggests that it is not an absence of VA mycorrhizal fungi that is limiting plant establishment on abandoned mines in southwest Virginia and central North Carolina.

Key words: Endomycorrhizae, heavy metals, mine spoils, establishment

INTRODUCTION

Colonization of herbaceous plants by vesicular-arbuscular (VA) mycorrhizal or endomycorrhizal fungi enhances nutrient uptake, primarily of phosphorus (Tinker, 1978). Many angiosperms are probably obligately VA mycorrhizal at the soil phosphorus levels of their natural ecosystems (Janos, 1987). VA mycorrhizae can also stimulate uptake of metalliferous micronutrients (Benson and Covey, 1976; Lambert, Baker and Cole, 1979; Swaminathan and Verma, 1979; Timmer and Leyden, 1980; Gildon and Tinker, 1983b). However, high levels of heavy metals in the soil have been shown to decrease or even eliminate colonization of host roots by VA mycorrhizal fungi (McIlveen and Cole, 1978/ 79; Gildon and Tinker, 1983a). In addition, *in vitro* germination of VA mycorrhizal spores may be inhibited by zinc, copper and manganese, although there are differences between isolates (Hepper and Smith, 1976; Daniels and Duff, 1978; Hepper, 1979).

Spoils created by mining tend to be variable in pH, physical and chemical characteristics, have low cation exchange capacities, and may often contain phytotoxic levels of heavy metals (Hutnik and Davis, 1973). The presence of VA mycorrhizal relationships is important in allowing successful recolonization and growth of herbaceous plants on disturbed soils (Aldon, 1975; Daft and Hacksaylo, 1976; Lambert and Cole, 1980)

The present study was conducted to characterize the VA fungal flora of mine spoils in Virginia and North Carolina and to determine if the chemical characteristics of the spoil material were inhibiting spore numbers and root colonization by these fungi.

MATERIALS AND METHODS

Solitary herbaceous plants growing directly on refuse piles of abandoned coal, metal and arsenic mines were sampled for study in September, 1985; March, 1986 and June, 1986. The mine sites and material mined are listed in Table 1. Entire plants were collected along with the associated soil. Roots were cleared and stained with acid fuchsin in lactic acid (Kormanik et al., 1980). Percent mycorrhizal colonization was determined using the grid-line intersect method (Giovannetti and Mosse, 1980). Metals were extracted from air-dried soil using diethylenetriaminepentaacetic acid (DPTA) (Lindsay and Norvell, 1978) and assayed using atomic absorption spectrometry. Phytotoxic levels of metals were considered to be twice the highest level reported by Lindsay and Norvell for manganese (Mn), copper (Cu) and zinc (Zn) and three times for iron (Fe). These values were Mn = 120 μg/g (= ppm), Cu = 5 μg/g, Zn = 23 μg/g, and Fe = 61 μg/g. Soil pH was determined after equilibrating an equal volume of air-dried soil with deionized water. Spores were extracted from 150cc of soil in a 50% sucrose solution using centrifugal flotation (Jenkins, 1964).

RESULTS

VA colonization of the root systems sampled ranged from 5 to 71% (Tables 2, 3 and 4). Of the 50 plants sampled, 34 or 68% were colonized by VA mycorrhizae. Healthy spores were collected in 20 of the 50 samples (39%). If the reclaimed site is not included, spores were collected in 44% of the samples. *Glomus clarum* Nicolson & Schenck was the most common species collected

(9 of 20 samples). Other VA mycorrhizal species found on the spoils included *Gigaspora margarita* Becker & Hall, *Glomus diaphanum* Morton and Walker, *Glomus mosseae* (Nicol. & Gerd.) Gerdemann & Trappe, and 5 unidentified *Glomus* species. In addition, *G. clarum* was probably the species found sporulating in the roots of several of the species of *Agrostis*, making a total of 12 observations for this species. Roots were already stained when the spores were observed, so records of the additional 12 observations for *G. clarum* were based only on ecology, spore size, and spore shape. Most VA mycorrhizal species do not sporulate within the host roots. *Andropogon virginicus* was the primary colonizer of most of the mine spoils sampled. Colonization of the roots ranged from 5 to 49% and 90% of the collections of this species examined had either colonized roots or healthy appearing spores in the associated soil. Finally, neither colonization, spore numbers nor species could be correlated with soil pH, or potentially phytotoxic levels of Mn, Cu, Zn or Fe in the soil.

Table 1: Locations of mine sites in Virginia and North Carolina (mining had ceased at all sites at least 15 years prior to sampling)

Site #	Location	Material(s) Mined
1	Austinville, Virginia	Lead, Zinc (reclaimed)
2	Davison Co., North Carolina	Silver, Lead
3	Rowan Co., North Carolina	Copper, Gold
4	Wise Co., Virginia	Coal
5	Wythe Co., Virginia	Manganese
6	Wythe Co., Virginia	Manganese
7	Tazewell Co., Virginia	Coal
8	Floyd Co., Viginia	Arsenic

Table 2: VA mycorrhizal Status of Herbaceous Species on Mine Spoils in southwestern Virginia and central North Carolina
Sites 1 and 2

Site	pH	Host Plant	% Colo	Spore #	VA Species	Toxic Metal(s)
1	6.6	*Festuca ovina*	21	0	-	Zn
	6.7	*F. ovina*	28	0	-	Zn
	6.1	*F. ovina & Agrostis sp.*	24	0	-	Zn
	7.0	*F. ovina & Agrostis sp.*	21	0	-	Zn
	7.1	*F. ovina*	5	0	-	Zn
2	6.8	*Aristida sp.*	0	0	-	Zn, Cu
	6.5	*Andropogon virginicus*	0	9	*Glomus sp. #1*	Zn, Cu
	6.7	*Abutilon theophrasti*	0	0	-	Zn, Fe
	6.7	composite	15	47	*Gigaspora margarita*	none
	3.6	*Aristida dichotoma*	5	0	-	none
	6.5	*Panicum depauperatum*	0	4	*Glomus sp. #1*	Zn
	6.2	*Agrostis sp.* & composite	3	0	*Glomus clarum* (in roots)	Zn
	6.7	*A. virginicus*	50	0	-	Fe
	4.9	*A. virginicus*	0	2	*Glomus sp. #1*	none
	3.7	*A. virginicus*	8	0	-	Mn
	5.3	grass	25	0	-	none
	6.8	*A. virginicus*	24	0	-	none
	3.7	*A. virginicus*	0	0	-	Mn
	6.9	herbaceous perennial	0	0	-	none

Table 3: VA mycorrhizal status of herbaceous species on mine spoils in southwestern Virginia and central North Carolina
Sites 3, 4 and 5

Site	pH	Host Plant	% Colo	Spore #	VA Species	Toxic Metal(s)
3	3.6	*A. virginicus*	0	0	-	Cu
	3.6	*A. virginicus*	0	19	*G. margarita*	Zn, Cu, Fe
	5.5	*A. virginicus*	0	960	*G. margarita*	Zn, Cu
	3.9	*A. virginicus*	20	0	-	Zn, Cu
	3.9	*A. virginicus*	0	0	*G. clarum* (in roots)	Cu, Zn
	3.7	*A. theophrasti*	0	0	-	Cu
	4.0	*Digataria spicata*	71	28	*G. margarita*	ND[1]
				3	*Glomus mosseae*	
	6.2	*A. virginicus*	5	6	*Glomus sp. #2*	ND
	5.0	*A. virginicus*	49	0	-	ND
	3.4	*A. virginicus*	0	0	-	ND
4	4.5	*Digitaria sanguinalis*	60	50	*G. clarum*	Fe
	3.9	*Phytolacca americana*	0	1	*G. clarum*	ND
	3.1	*A. virginicus*	35	2	*G. clarum*	none
	3.5	*A. virginicus*	14	0	-	none
	3.4	*A. virginicus*	23	10	*G. clarum*	none
5	3.7	*A. virginicus*	5	1	*Glomus sp #2*	Mn
	3.8	*A. virginicus*	5	0	-	none
	4.0	*Salix sp.*	5	8	*G. clarum*	Mn
				3	*G. mosseae*	

[1]ND = not determined.

Table 4: VA mycorrhizal status of herbaceous species on mine spoils in southwestern Virginia and central North Carolina
Sites 7 and 8

Site	pH	Host Plant	% Colo	Spore #	VA Species	Toxic Metal(s)
6	4.6	*Agrostis perennis & Danthonia spicata*	45	53	*G. mosseae*	none
	4.7	grass	7	0	-	none
	3.8	*A. virginicus*	10	95	*Glomus diaphanum*	copper
7	9.1	*Hieracium pratense*	ND	0	-	ND
	4.5	*A. virginicus*	ND	8	*G. clarum*	ND
				102	*G. diaphanum*	
8	5.0	*A. perennis*	18	0	-	ND
	6.3	*A. virginicus*	0	50	*Glomus sp. #3*	ND
	4.4	*A. virginicus*	48	0	*Glomus sp. #4*	ND
	4.1	grass	0	0	-	ND
	6.5	*A. perennis*	20	0	-	ND
	6.2	*A. perennis*	31	0	*G. clarum* (in roots)	ND
	6.2	*Aster undulatus*	30	0	*G. clarum* (in roots)	ND
	4.7	*Eupatorium perfoliatum & A. undulatus*	0	0	-	ND
	4.1	*A. virginicus*	20	2	*Glomus sp. #5*	ND

[1]ND = not determined

DISCUSSION

Colonization of herbaceous plants by VA mycorrhizal fungi as well as viable appearing spores were present on mine spoil piles, even with potentially toxic levels of heavy metals in the surrounding soil. Since high metals have been shown to be deleterious to some VA fungi (Hepper and Smith, 1976; McIlveen and Cole, 1978/79; Gildon and Tinker, 1983a; Siqueira, Hubbell and Mahmud, 1984), this may suggest heavy metal tolerant ecotypes of either the fungi or the host plants or both. A heavy metal tolerant strain of *Glomus mosseae* has been demonstrated (Gildon and Tinker, 1981; Gildon and Tinker, 1983) and there have been numerous reports of metal tolerant ecotypes of green plants (e.g. Antonovics, Bradshaw and Turner, 1971). On the other hand, the plant roots may be exploiting pockets of low metal substrate, which were not measured by the technique used here.

Regardless of the reasons for the presence of VA mycorrhizal fungi on these toxic sites, it appears that their absence is not a limiting factor for plant growth on the mine spoils examined.

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Conclusions

The results of the preceding six chapters support the hypothesis that ectomycorrhizal and endomycorrhizal fungi have differing ways of responding to heavy metal stress. Ectomycorrhizal fungi are commonly found in association with plants in the Pinaceae, order Coniferales, which has many members found on acid soils where heavy metals may be mobile. This work demonstrates that ectomycorrhizal fungi have intrinsic mechanisms which increase their tolerance to heavy metal stress. However, they are a taxonomically and ecologically diverse group of organisms and consequently, have differing responses to heavy metals.

The copper containing enzyme tyrosinase, which catalyzes the production of melanin, appears to be involved in the copper tolerance of some ectomycorrhizal fungi. Ectomycorrhizal fungal hyphae that are naturally melanin pigmented show greater *in vitro* copper tolerance that those fungi which develop darkly pigmented hyphae in response to copper stress. When copper was added to an enzyme reaction mixture from *Pisolithus tinctorius* grown without additional copper, the specific activity of tyrosinase was greatly enhanced. A similar increase was not observed when extracts from *Suillus granulatus* were examined. However, when the fungi were grown in the presence of high amounts of copper, addition of copper to the reaction mixture increased the activity of tyrosinase from both fungi. *Pisolithus tinctorius* is a naturally melanin- pigmented fungus while *Suillus granulatus* has white to cream colored hyphae which darken in response to copper in the growth medium. The polyamine content of two fungi which have natually dark hyphae did not change dramatically when these fungi were grown under copper stressed conditions. However, polyamine content of those fungi which develop hyphal pigment was significantly decreased when they were

exposed to copper. This suggests a difference in the natural pigment versus the pigment produced in response to stress. Polyamine production can be stimulated by changes in ionic concentration outside the cell, and these results indicate that naturally darkly pigmented hyphae may be better able to maintain normal processes when exposed to copper stress.

Ectomycorrhizal hyphae bind copper, however, binding occurs at a number of different sites depending on the fungus involved. Ultrastructural evidence shows that *Suillus pictus* binds copper almost exclusively to its cell walls; in *Suillus granulatus*, the metal is found to the exterior of the cell wall in the hyphal sheath; *Pisolithus tinctorius* not only binds it to the exterior of the wall but sequesters it inside the plasmalemma, probably in association with polyphosphate bodies. In the fungus *Piloderma bicolor*, no copper was found in association with the fungal hyphae. The most copper tolerant of these fungi *in vitro* was *P. tinctorius* followed by one isolate of *S. granulatus*, *S. pictus*, two additional isolates of *S. granulatus*, and the least tolerant was *P. bicolor*.

The significant metabolic changes which a fungus undoubtedly undergoes when it forms the mycorrhizal association with a host plant suggests that *in vitro* copper tolerance is probably not correlated with tolerance of the mycorrhizal association. This was found to be the case in our examination of the tolerance of Japanese Red Pine (*Pinus densiflora*) when it was inoculated with four species of ectomycorrhizal fungi and one nonmycorrhizal fungus. The ectomycorrhizal fungi were the same as were used to establish the sites of copper binding, the nomycorrhizal fungus was *Boletinellus merulioides*. *Boletinellus merulioides* is associated with American Ash (*Fraxinus americanus*), but has not been proven to be mycorrhizal. The fungus which most promoted seedling growth at both low and moderate copper levels was *Suillus pictus*, not *Pisolithus tinctorius*, which was the most tolerant fungus *in vitro*. This indicates, as has been previously suggested, that it is the degree of compatibility between host and fungus that is most important in promoting host plant growth under conditions of heavy metal stress, rather than the metal tolerance of a particular fungal strain. In addition, it demonstrates that a late successional stage mycorrhizal fungus, such as *S. pictus*, can successfully colonize the roots of a young seedling and promote seedling growth. One reason for the increased stimulation of tree growth by *S. pictus* may be that it does not heavily colonize the host roots. The percentage of the root system colonized by *S. pictus*

is approximately one-fourth of that colonized by *P. tinctorius*. *S. pictus* apparently perfoms the functions of nutrient uptake and metal binding while producing a minimal carbon drain on the host plant. At higher copper levels, *P. tinctorius* was a more effective symbiont than *S. pictus*; suggesting that the cytoplasmic sequestration of copper which was observed ultrastructurally is a more effective mechanism under conditions of extreme stress. Ecologically, *P. tinctorius* is often found on harsh sites such as abandoned mines in the absence of other fungi, but it is rarely found in undamaged ecosystems. Our results indicate that it may be a poor competitor except under conditions of extreme stress. *P. tinctorius* was the only fungus tested which increased uptake of copper to the shoot. This was correlated with an increase in phosphorus and implicates polyphosphate bodies as possible factors in copper tolerance in this fungus.

Ectomycorrhizal fungi increased levels of phosphorus, calcium and magnesium in the host seedlings, but generally did not alter levels of iron, copper, and potassium with respect to the controls. This suggests an indirect role of the fungi in nutrient uptake. Nutrients such as P, Ca, and Mg are moved into a plant by a diffusion gradient at the apoplast and their increase may indicate improved water relations of the host plants. Fe, Cu and K enter the plant via active processes and the fact that their levels are not increased may imply that ectomycorrhizal fungi do not influence their metabolic pathways of uptake. In each of the four ectomycorrhizal associations examined, copper decreased the percentage of short roots colonized by the fungus and tended to cause higher orders of branching. That is, if the fungus normally produced bifurcate short roots with a minimum of quadrifurcate roots, the presence of copper would induce a higher percentage of quadrifurcate roots. Most likely this is due to the previously documented effect of excess copper in inhibiting cell divisions in the host roots. This might tend to limit the number of short roots available for colonization by the fungus and thus, the roots that are colonized experience higher levels of branching.

When three different isolates of *Suillus granulatus* were compared under conditions of copper stress, no differences in growth of the host plant were observed. This is in contrast to experiments done *in vitro*, where one of the isolates showed much greater tolerance than the other two. As with the other associations, copper decreased the percent colonization of the root system, and a

correlative decrease in stem vascular tissue was observed. This decrease in vasculature was not due to copper, since it was not observed in control plants. These data support the hypothesis that one reason for the enhanced uptake of elements via the transpiration stream in mycorrhizal plants may be increased amounts of vascular tissue.

Boletinellus merulioides did not form mycorrhizae on the roots of *P. densiflora*, but did stimulate dichotomous branching of the root system. The branching could be increased when the tree- fungus combination was grown under conditions of high glucose. When liquid grown cultures of *B. merulioides* were assayed for the plant growth promoting hormone indole-3-acetic acid (IAA), it was found to be produced in greater quantities in cultures grown at high glucose levels. This indicates that the compound responsible for the dichotomous branching in *P. densiflora* is probably IAA. If IAA is universally produced by ectomycorrhizal fungi, as has been indicated by many other studies, it could be responsible for the increased vascular tissue due to increased mycorrhization noted in the *S. granulatus* treatments.

Endomycorrhizal fungi do not appear to be directly involved in the response of the host plant to copper. By improving the phosphorus status of the host plant, endomycorrhizal fungi indirectly enhance the ability of the host to grow under conditions of copper stress. Copper does not appear to dramatically limit colonization of the root system by the endomycorrhizal partner, nor do endomycorrhizal fungi nonselectively transfer copper to the host plant as they take up phosphorus. A survey of endomycorrhizal fungi on abandoned mines also indicated that despite the low numbers of vascular plants on the mines and the high levels of toxic metals, the plants that are present are well colonized by endomycorrhizal fungi. In addition, numerous viable spores can be extracted from the rhizosphere of these plants.

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