THE EFFECT OF ECTOMYCORRHIZAE ON THE UPTAKE OF LEAD BY *PINUS SYLVESTRIS* L. SEEDLINGS

by

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(ABSTRACT)

Clean laboratory technology and pure culture techniques were combined to determine the effect of ectomycorrhizae on the uptake of lead by *Pinus sylvestris* L. seedlings. By culturing the ectomycorrhizal fungus, *Pisolithus tinctorius* (Pers.) Coker and Couch, in liquid Hagem's medium with different concentrations of lead (0, 50, 250 and 500 ng/g sol, it was determined that the fungus readily takes up lead from such and aqueous medium. It was also determined that the pH of the medium controls the amount of lead in solution that can be taken up by the fungus.

A series of growth studies where *Pisolithus* was cultured on Hagem's agar indicated that the growth of the fungus is reduced when the lead concentration in the

medium is approximately 25 ug/g sol. It was estimated that a lead concentration of approximately 1,000 ug/g sol may completely inhibit the growth of the fungus. Furthermore, it was determined that the acetate ion is inhibitory to the growth of Pisolithus.

By culturing mycorrhizal and non-mycorrhizal seedlings of P. sylvestris and analyzing the seedlings' roots, stems and leaves for their lead concentrations, it was determined that ectomycorrhizae facilitate the uptake of lead. Ectomycorrhizal roots can take up more lead from a vermiculite/hydroponic solution mixture because of their greater surface area and exploration of vermiculite surfaces as well as their increased production of lead solubilizing acids. This additional lead found in mycorrhizal roots, however, does not appear to be translocated to the above ground portions of the seedlings. This increased uptake of lead by mycorrhizal roots is expected to take place in natural soil systems as well.

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Introduction

Lead was one of the first metals used by man in early civilizations (Chisolm, 1971). Throughout the history of its use, lead has always been a persistent environmental contaminant. Due to man's recent use of lead as an anti-knock compound in gasoline, it now plays the novel role of being one of the most widely disseminated trace elements in the biosphere (Nriagu, 1978a). Because of its great dispersal, some components of both terrestrial and aquatic ecosystems have been contaminated by lead as much as several thousand fold, as compared to natural lead concentrations found on earth prior to man's use of this metal (Elias and Patterson, 1980). Over the last several decades, a great deal of basic research has determined the biogeochemical pathways of lead. The fact that the biosphere is contaminated with anthropogenic lead is certain, even though there is some disagreement as to the degree of this contamination.

Man's use of lead can be traced back into history more than seven thousand years (Pulsifer, 1888). One of the major factors that determined the wealth of any early civilization was the amount of silver in its treasury.

McDonald (1967) states that silver was used as a currency for trade as early as 4,000 B.C. by the predecessors of the Hittites in Cappadocia (in the eastern part of Asia Minor). Galena, the ore usually mined for silver, consists mostly of lead sulfide with traces of silver and other precious metals. Early, rather crude smelting techniques allowed early man to separate galena into its constituents. Silver was the desired metal and lead was merely an "impurity". Stimulated by a need to improve the efficiency of the smelting process, the ancients developed the process of cupellation, where some of the lead in lead sulfide ores was re-oxidized and lost to the atmosphere while small amounts of silver were left behind. Hofman (1918) states that the Babylonians probably developed the process of cupellation, perhaps as early as 4,000 B.C. Although silver was the desired product in the smelting of lead ores, the process left behind ever increasing stockpiles of metallic lead. Gradually, many different uses were discovered for lead, such as in building construction, paint production, cosmetics, linings for ship hulls, gutters, cisterns, plumbing and, particularly interesting, its use as a bactericidal additive to wine, known as sapa (Patterson, 1980).

Sapa was produced by boiling fruits, herbs and seasonings in solid lead crocks. The mixture was then added to green wine to prevent the wine from spoiling. Since wine was a staple beverage in Greek and Roman cultures, many people, and probably the aristocracy in particular, possessed extremely high body burdens of lead. In fact, some scholars have related the infertility and lunacy of many Roman emperors to outright lead poisoning as a result of consuming large amounts of wine that was treated with sapa (reviewed in Patterson, 1980).

The ever increasing need for silver in the Roman empire stimulated greater exploration and mining for lead ore. During the height of the Roman empire, approximately 2,000 years ago, production of lead may have reached 80,000 metric tons per year (Settle and Patterson, 1980). It has been hypothesized that the eventual exhaustion of the Spanish lead mines (at the expense of a great number of lead-poisoned slaves) led to the ultimate collapse of the Roman empire around 400 A.D., as no new silver was available to replace the silver that was removed from circulation due to hoarding, shipwrecks and other distasters (Patterson, 1972).

During the Middle Ages from 476-1453 A.D., lead production dropped substantially to several thousand metric tons per year (Settle and Patterson, 1980). However, with the discovery of vast silver mines in the New World around 1550 A.D., the Spanish production of lead rivaled that of the Romans. Beginning with the industrial revolution in the mid-eighteenth century, lead production and emissions to the atmosphere increased exponentially. The present level of worldwide atmospheric emissions of lead are estimated by Nriagu (1979) to be approximately 450,000 metric tons per year. These anthropogenic emissions can be broken down into oil and gasoline combustion (61%), primary production of non-ferrous metals (17%), iron and steel production (11%), coal combustion (3.1%), mining (1.8%) and other miscellaneous sources (6.1%). Present natural emissions of lead to the atmosphere are estimated by Nriagu (1979) to be approximately 27,000 metric tons per year, or 16.6 times less than anthropogenic emissions. This estimate of natural emissions of lead to the atmosphere has been questioned by Settle and Patterson (1980), who feel that Nriagu's estimate contains some anthropogenic components. Their estimate of natural emissions of lead (approximately

5,000 metric tons per year) is 90 times less than annual anthropogenic emissions. Today, modern uses of lead include lead storage batteries, chemical pigments, cable, pipe and sheet metal (World Health Organization, 1977). None of these lead products contaminate the environment on a large scale because lead emissions to the environment from the industries that manufacture and use these products are strictly controlled. The production of steel and other metals, the combustion of coal, and particularily the use of alkyllead additives in gasoline, however, have produced widespread environmental lead contamination.

In 1922, T. C. Midgley, Jr. and T. A. Boyd, working for General Motors, discovered that the addition of tetraethyllead greatly increased the octane rating of fuel, allowing high compression engines to burn a cheaper grade of gasoline (Morrison and Boyd, 1977). Since that time, hundreds of thousands of tons of lead per year have been released into the atmosphere as a result of "ethyl" gasoline combustion in automobiles (Environmental Protection Agency, 1977). Lead leaves the exhaust of automobiles in the form of leadbromochloride, adsorbed onto submicron particles (approximately 0.015 um; Harrison

and Laxen, 1981). Through coagulation, these particles eventually reach an approximate diameter of 0.5 um. Depending on automobile driving parameters such as speed and acceleration, larger particles are produced as well. The majority of the large particles, ranging anywhere from 5-3,000 um, settle out of the atmosphere in close proximity to the source of the emissions. The submicron particles, on the other hand, are not as influenced by gravitational settling and can remain airborne within the troposphere for 6-12 days (Nriagu, 1978a). Because of this lengthy time aloft, lead-bearing aerosols have travelled great distances, resulting in the present day global contamination of even very remote ecosystems, expecially in the Northern Hemisphere (Murozumi, et al., 1969; Shirahata, et al., 1980).

No matter what the size of the particles produced, eventually all of these lead-bearing aerosols will find their way back to the earth's surface. By the time these particles have reached their destinations, lead is no longer in its halide form, but instead has been chemically altered through a reaction with acid sulfates to form lead sulfate (Harrison and Laxen, 1981). It is in this form that lead is deposited within terrestrial ecosystems,

either directly on soils or on the surfaces of vegetation. This deposition occurs through two processes: dry deposition, where airborne lead is deposited directly on terrestrial surfaces, and precipitation scavenging, where atmospheric lead is first incorporated into precipitation, which then falls to the ground.

Numerous studies have investigated the deposition of lead on roadside vegetation (as reviewed by Peterson, 1978). These studies show that the size, shape, location and surface texture of leaves determine the number of particles that are deposited on their surfaces. For instance, Little and Wiffen (1977) found that the pubescent leaves of white poplar (Populus alba L.) were approximately eight times more efficient at collecting lead-bearing aerosols than the glabrous leaves of oak (Quercus robur L.).

Although significant amounts of lead can be found on the surfaces of vegetation, this has essentially no effect on the concentration of lead within the plant because foliar uptake of lead appears to be minimal, if existent at all (Arvik and Zimdahl, 1974). The only pathway through which lead can enter into the internal tissues of a terrestrial plant is through the plant's nutrient

absorptive organs, the roots. There may, however, be an indirect relationship between surface deposition and plant internal lead concentrations in that virtually all of the lead deposited on above-ground portions of a plant will eventually find its way to the soil. This transport may occur through the washing of the leaf surfaces by rain (Elias and Croxdale, 1980), through the shedding of plant parts, or through the death of the entire plant.

Soil lead is considered to be fairly immobile (Olson and Skogerboe, 1975). Lead is retained within the soil system and not leached out of an ecosystem through groundwater (Siccama and Smith, 1978). Siccama, et al. (1980) showed that the concentration of lead in soil samples from white pine plantations throughout Massachusetts increased 70% over a sixteen-year period. Because of these phenomena, soils are considered to be the primary sink for atmospheric lead (Nriagu, 1978b).

Soil lead becomes bound to a number of different soil components such as the inorganic clays and hydrous oxides of iron and manganese. The majority of the atmospheric lead that is immobilized by soils, however, is bound to the chemically diverse, organic humus component of soils (Zimdahl and Skogerboe, 1977). In fact, humus production

from decaying vegetation can act as the major binding agent for soil lead because humus and atmospheric lead share the same site of introduction into the soil system (Tyler, 1972). Because of the strong lead binding capacity of many soil components, virtually all of the anthropogenic lead in soils is located in the upper horizons above 15 cm (Motto, et al., 1970). Recent unpublished data (Patterson and Kolbasuk, personal communication) indicate that all atmospheric lead is bound to the upper 2 cm of undisturbed soils.

The fact that lead in soil is tightly bound to different soil components speciously suggests that plant uptake of soil lead is difficult or even impossible. Plants, however, have evolved mechanisms through which other tightly bound ions can be taken up by roots. Many nutrient cations such as calcium, iron, manganese, zinc and copper are bound to soil particles in a manner similar to lead (Brady, 1974). In order to obtain these nutrient cations as well as other necessary nutrients from soils more effectively, most species of plants have evolved symbiotic relationships with fungi in which the fungi inhabit the roots of the higher plants. The fungi provide the higher plants with greater amounts of nutrients and

water in exchange for a source of carbon. This structure, known as a mycorrhiza ("fungus-root") is not some unusual adaptation found in only a few species of higher plants, but is instead extremely widespread and considered ubiquitious among terrestrial plants (Malloch, et al.,1980). Pirozynski and Malloch (1975) even suggest that mycotrophy was the prerequisite for terrestrial plant life. Most workers subdivide the mycotrophic condition into three main groups: ectomycorrhizae, endomycorrhizae and ectendomycorrhizae.

Ectomycorrhizae are usually formed between many different species of basidiomycetous fungi and members of the Pinaceae, Betulaceae, Fagaceae and several other families of woody plants (Malloch, et al., 1980). Ectomycorrhizae consist of an external mat of mycelium called the mantle, from which numerous fungal hyphae extend out into the soil. Within the roots of the higher plant, the hyphae do not penetrate the cortical cells of the host nor do they enter the vascular tissue, but are instead confined to the intercellular spaces between the cortical cells. In cross-section, the cortical cells, with their surrounding hyphae, appear net-like. In fact, this structure is called the Hartig net after Robert

Hartig, who first described it in 1851 (Snell and Dick, 1971). Exchange of nutrients between the two symbionts is believed to occur at this site (Marks and Foster, 1973).

Endomycorrhizae are formed between many different herbaceous and arborescent higher plants and relatively few species of fungi (Hacskaylo, 1972). The vast majority of vascular plants form endomycorrhizae, also know as vesicular-arbuscular (VA) mycorrhizae (Nicolson, 1975). In this symbiosis, the mantle of mycelium found in ectomycorrhizae is lacking, and the fungus actually invades the cortical cells and lives intracellularly, forming vesicles and arbuscules (Harley, 1969). As in ectomycorrhizae, however, hyphae extend out of the root and well into the soil system (Mosse, et al., 1981).

Ectendomycorrhizae are generally thought of as intermediate forms between ectomycorrhizae and endomycorrhizae, sharing many of the features of both groups (Hacskaylo, 1972). Ectendomycorrhizal short roots possess a Hartig net, but there are intracellular hyphae as well. The fungal mantle in ectendomycorrhizal roots, however, is not very well developed. Compared to ectomycorrhizal short roots, ectendomycorrhizal short roots are less frequently dichotomously branched and usually more elongated and slender.

Historically, the greatest interest in terms of affecting nutrient uptake in mycorrhizae has been focused on macronutrients, especially phosphorous. Phosphate uptake has been shown to be greatly increased by mycorrhizae, probably due to the greater efficiency hyphae have for exploring the soil system as compared with the non-mycotrophic roots of other higher plants (Sanders and Tinker, 1973). Phosphate depletion zones around roots can form rapidly due to the low rate of diffusion of phosphate ions in soils (Fitter and Hay, 1981). Growth rates of plant roots and root hairs are not rapid enough to permit the exploration of non-depleted areas of soil. The only exceptions to this inadequacy of root growth by non-mycorrhizal plants are found in some members of the Cyperaceae, Juncaceae and a few other plant families that are strictly non-mycorrhizal but possess a fibrous root system with dense root hairs (Powell, 1975). Fungi, on the other hand, are quite capable of rapidly exploring the soil for nutrients, particularly immobile ions such as phosphate. Rhodes and Gerdemann (1975) showed that mycorrhizal roots exploited the soil for phosphate at a distance seven times greater than non-mycorrhizal roots.

Mycorrhizae have also been shown to increase the uptake rates of micronutrients by plants. Early work by Melin and Nilsson (1955) and Melin, et al. (1958) showed that ^{45}Ca and ^{22}Na are taken up by mycorrhizal fungi and transported to the higher plant. Bowen, et al. (1974) showed that ectomycorrhizal plants of Pinus radiata were as much as three times more efficient at taking up zinc than non-mycorrhizal plants. Endomycorrhizae have been shown to increase the rates of uptake in plants of iron, calcium, copper and manganese (Mosse, 1957; Ross and Harper, 1970). All of these elements are required nutrients for plant growth (Salisbury and Ross, 1978). Very little work, however, has been done on the effect mycorrhizae may have on the uptake of non-nutrient trace elements from the soil. Jackson, et al. (1973) showed that VA mycorrhizae increased the rate of uptake of ^{90}Sr in soybeans as compared to non-mycorrhizal plants. No studies have been made, however, on the uptake of lead by mycorrhizae.

Numerous studies, such as those reviewed by Zimdahl and Arvik (1973), have shown that plants can take up lead from soils. None of these studies, however, determined whether or not the plants in the field were mycorrhizal.

Furthermore, no laboratory study has cultured mycorrhizal plants in a controlled environment to see if mycorrhizae affect the uptake of lead. This is the purpose of the present study: to determine if ectomycorrhizae affect the uptake of lead by seedlings. In order to avoid the uncertainties of lead studies in natural systems that are caused by anthropogenic contamination, I approached this problem through a laboratory study where lead concentrations in the air could be substantially reduced from present ambient air lead concentrations.

This study was separated into three sets of experiments, each testing a particular aspect of the overall hypothesis. The first step involved determining whether or not a mycorrhizal fungus was capable of taking up lead from an aqueous solution. The second step involved determining whether or not the lead concentrations used in the study were toxic or at least inhibitory to the fungus. The final and most crucial part of this work dealt with the primary hypothesis, that mycorrhizal seedlings can take up greater amounts of lead than non-mycorrhizal seedlings.

Materials and Methods

Clean laboratory technology

In order to carry out research where one is dealing with samples that contain very small amounts of lead (i.e., in the nanogram range), it is necessary to use extreme care in avoiding contamination of these samples from extraneous sources of lead. Since lead is distributed throughout the biosphere, low-level lead investigations must be carried out in a controlled environment, relatively free of lead-bearing particles. Accordingly, this research was carried out in a class 10,000 clean laboratory, under positive pressure (as explained in Zief and Mitchell, 1976), because many of the samples analyzed in this study contained only a few nanograms of lead.

Within the laboratory (Fig. 1), air was constantly filtered through the class 100 high-efficiency-particulate-air filters (HEPA) of the laminar flow hood. This feature insured that, even with the building air shut off, the air within the lab remained filtered. Materials

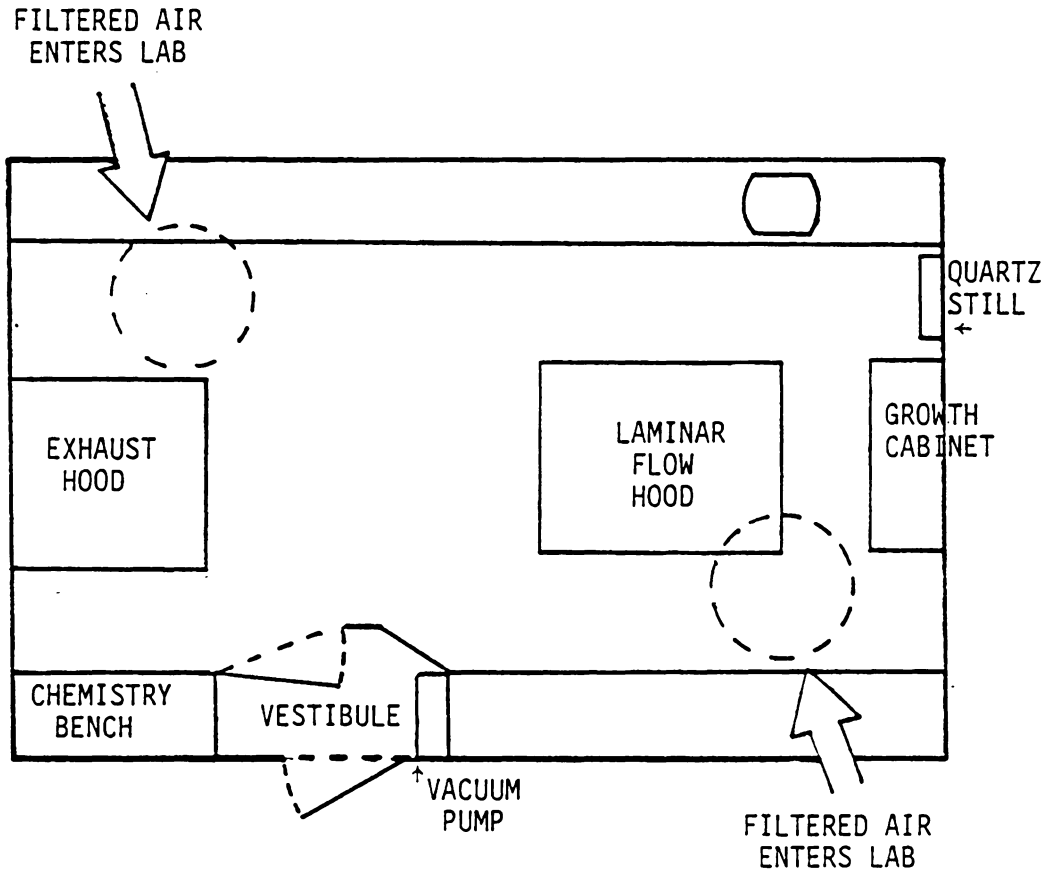


Figure 1. Schematic diagram of class 10,000 clean laboratory.

such as beakers that had completed the acid washing cycle (explained below) were placed in front of the HEPA filters to dry in the cleanest air within the laboratory. Even with these precautions, lead was still present inside the lab. The amount of lead added to the sample by exposure to the air of the clean lab was determined, and this blank value was subtracted from the samples described in this thesis.

Without question, the greatest potential sources of trace element contamination of samples are the workers within the clean lab. Prior to entering the lab, the analysts put on shoes that were stored inside the vestibule of the lab. Upon entering the lab, workers donned synthetic lab coats and hats. As a final precaution, workers wore polyethlene gloves when they handled any sample or labware. All of these precautions helped to minimize contamination to approximately 0.1 ng Pb per sample.

The nitric acid used in the digestion of biotic samples had to be of high purity. For this reason, commercial, double-distilled nitric acid with a concentration of 0.05 ng Pb/g solution (sol) was used. The blank value for the nitric acid was also subtracted

from the samples analyzed in this research. Where such purity was not required, as in the initial acid baths, reagent grade acid was used.

The water used in a laboratory is also a source of lead contamination. Where strict purity was required, I used water that was double-distilled in quartz-glass within the clean lab. This quartz-distilled water (QDW) contained a concentration of 0.05 ng Pb/g sol, which is comparable to the double-distilled nitric acid. Quartz-distilled water was used for rinsing labware throughout the acid washing sequence, as well as for diluting the nitric acid that was used in digestions and acid baths.

With few exceptions, all of the labware used in these experiments was soaked in a series of nitric acid baths to leach lead from their surfaces. The acid bath regimes differ slightly from one material to another, but basically all of the labware went through a set of three acid baths. First, the labware was scrubbed with acetone to remove any adhesives and greases placed on it by the manufacturers. Second, the labware was rinsed with distilled water and placed in a heated (55° C), concentrated nitric acid bath for three days. Polyethylene and polypropylene were placed in 4 N nitric

acid, and Teflon and quartz-glass, which are more acid-resistant, were placed in 16 N nitric acid. At the end of three days, the labware was removed from the baths, rinsed once with distilled water and placed in a heated, dilute (0.01 N) nitric acid solution. After approximately 24 hours, the materials were removed, rinsed once with QDW and placed in the final bath (0.01 N nitric acid) for a period of three days. Finally, the labware was removed from the bath, rinsed three times with QDW and placed on the clean air bench. Once air dried, the labware was placed in acid-washed polyethylene containers that were then sealed and stored.

Fungal uptake of lead in an aqueous medium

To my knowledge, no work has ever been done involving both pure culture work and clean lab technology. Because I dealt with two forms of contamination, trace element contamination and biotic contamination, I decided to limit this study to one particular, easily cultured, ectomycorrhizal fungus, *Pisolithus tinctorius* (Pers.) Coker and Couch, in the family Pisolithaceae of the order Sclerodermatales. *Pisolithus* is very easy to work with in pure culture and grows rapidly on modified Hagem's medium.

Pisolithus has a wide distribution in nature (Marx, 1977), and is known to form ectomycorrhizae with many members of the Pinaceae (Trappe, 1962). The higher plant chosen to form ectomycorrhizae with this fungus was Pinus sylvestris L. (Scotch pine).

A pure culture of Pisolithus tinctorius was obtained from the mycology collection of Dr. O. K. Miller, Jr. (VT 964). This was an isolate shipped to the mycology lab from Dr. D. H. Marx, U.S.D.A., Forest Service, Athens, Georgia. Throughout this reserach, stock cultures of Pisolithus were maintained on petri dishes containing modified Hagem's medium (see Appendix; Palmer, 1971).

For the set of experiments that dealt with lead uptake by Pisolithus grown in liquid medium, it was necessary to prepare Hagem's medium without agar, using QDW. This produced a medium that contained 2.0 ± 0.5 ng Pb/g sol. Two similar experiments were performed. The first tested the hypothesis that Pisolithus can take up lead from solution. The second examined the role of pH in determining the amount of lead taken up by the fungus.

To determine whether or not the fungus was capable of taking up lead from solution, the following design was used. Twenty 250 ml FEP Teflon bottles were divided into

four groups. The groups were a control with no lead added to the medium, and three experimental groups with lead added to the medium to produce calculated concentrations of 50, 250 and 500 ng Pb/g sol. These concentrations were chosen to simulate a range of lead concentrations that could be found in soil moistures from natural and contaminated sites (Patterson, 1980). Within each concentration series, three of the five bottles were inoculated with Pisolithus and two of the bottles remained uninoculated. One of the inoculated bottles from each group was sampled once a week for three weeks and the uninoculated bottles from each group were sampled at one and three weeks. The source of inoculum used was a culture of Pisolithus that was incubated for approximately one month in liquid Hagem's medium, within an acid-washed, FEP Teflon bottle.

To set up the above experiment, two liters of liquid Hagem's medium was prepared in an acid-washed, polypropylene bottle. The medium was autoclaved at 121° C for 15 minutes to insure that all of the ingredients were in solution. Using an acid-washed, polypropylene graduated cylinder, 100 ml of the medium was added to each of the twenty FEP Teflon bottles. A lead spike was

prepared from a commercial standard (10,000 ug Pb/g sol) by diluting the standard to 100 ug Pb/g sol with 0.1 N nitric acid. Lead nitrate was added to the bottles in appropriate amounts using an Eppendorf pipet with an acid-washed tip. To produce the concentration series stated above, the 50 ng Pb/g sol bottles received 50 ul, the 250 ng Pb/g sol bottles received 250 ul and the 500 ng Pb/g sol bottles received 500 ul of the 100 ug Pb/g sol standard.

After the bottles were swirled to distribute the lead spike uniformly, an aliquot of approximately 1 ml was decanted into a tared, FEP Teflon beaker from one bottle of each concentration series so that the lead concentrations within each series could be analytically determined. The beakers with the samples were immediately weighed to determine the sample weight. The samples were then treated with 1 ml of concentrated, double-distilled nitric acid and evaporated to approximately one-half milliliter on a 140° C hot plate under the exhaust hood in the clean lab. The samples were brought up to a working volume of approximately 2 ml by rinsing the beaker three times with dilute (0.1 N) nitric acid. Each rinse was poured into a tared, acid-washed, polyethylene vial

(polycon). The weight of the sample was determined and the polycons were placed in a sample box and stored on the clean air bench, prior to lead analysis. All of the media samples mentioned henceforth were handled in a similar manner.

After an aliquot was removed from one of each of the bottles in a concentration series, the bottles were sealed and autoclaved at 121° C for thirty minutes. The bottles were placed in front of the laminar flow hood in the mycology lab transfer room to cool after they had been sterilized. Once the bottles had cooled, the appropriate bottles aseptically received a portion of Pisolithus from the stock culture. All of the bottles were then taken to the clean lab and placed in polyethylene bags. The bags were sealed and then placed in a 25° C incubator in the mycology lab. At one week intervals, the bags were taken to the clean lab, the bottles to be sampled were removed and the remaining bottles were resealed in the bags and taken back to the incubator.

In order to separate the Pisolithus mycelia from the media for analysis, a filtration apparatus was constructed using a 500 ml polyethylene bottle, polypropylene Buchner funnel and a Nalgene hand pump. A polyethylene connector

was fused to the side of a polyethylene bottle so that the hand pump could be connected and used to evacuate the bottle. To collect the mycelium, an acid-washed, 5.5 cm Whatman 400 cellulose filter was placed in a Buchner funnel. The Buchner funnel was placed on top of the aspirator bottle. The medium was poured into the funnel and pulled across the filter by evacuating the aspirator bottle with the hand pump. An aliquot of the medium was decanted from the aspirator bottle into a tared, FEP beaker. The sample size was then determined by weighing the beaker containing the sample. The medium was digested as explained above. Another aliquot of the medium was poured from the aspirator bottle into a polycon, and the pH of the medium was determined using a Fisher Accument 156 portable pH meter. The mycelium collected on the filter paper was rinsed with approximately 25 ml of QDW three times. It was then picked up with acid-washed forceps, placed into a tared, quartz-glass beaker, and desiccated for approximately 48 hours until all of the moisture was removed from the mycelium. The beaker was then removed from the desiccator and weighed to obtain the dry weight of the mycelium. The mycelium was digested in a manner similar to the medium, using approximately 3 ml

of concentrated, double-distilled nitric acid. All of the media and mycelia samples were handled in this manner.

To see if lead was adsorbed onto the walls of the bottles, the bottles were first rinsed with QDW to remove the remaining drops of Hagem's medium. The insides of the bottles were then leached with 1 ml of concentrated nitric acid. After the bottles had sat on the 55° C hot plate for 24 hours, the acid was poured into a polycon, and the bottle was rinsed again with dilute (0.1 N) nitric acid. The acid rinse was added to the same polycon. The bottle leaches could not be done on the inoculated bottles after several weeks of growth because some of the hyphae were stuck to the sides of the bottles. Therefore, bottle leaches were done on inoculated bottles at the first week of analysis and the uninoculated bottles at the first and third weeks of analysis.

In order to determine the effect of pH on the uptake of lead by Pisolithus in liquid culture, another experiment was performed. Three groups of five FEP Teflon bottles were set up as in the previous experiment. The three groups had different amounts of sodium citrate/citric acid buffer added to the medium to produce pH values of 5.0, 4.0 and 3.0. There were five replicates

in each group. All of the bottles received 500 ul of the 100 ug Pb/g sol standard, to produce a final concentration of 500 ng Pb/g sol. The bottles were autoclaved and inoculated as explained above. At the end of 28 days, the media and mycelia were digested and pH levels of the media were determined.

Lead-induced growth inhibition of Pisolithus

Three growth studies were performed to determine whether or not the lead concentrations used in this research were inhibitory to the growth of Pisolithus. The first growth study used concentrations of lead in the medium of 0.01, 0.1, 1.0 and 10 ug Pb/g sol to determine if these levels were inhibitory to the growth of the fungus. The second growth study extended the range of the first growth study to 100 ug Pb/g sol. Since the growth of Pisolithus was reduced at 100 ug Pb/g sol, a third and final growth study was performed using concentrations of lead in the medium between 10 and 100 ug Pb/g sol. It was hoped that these concentrations would produce a gradation of growth reduction in the fungus.

To set up the first growth study, one liter of Hagem's medium was prepared with agar and autoclaved to insure

complete dispersal of the nutrients. Six Teflon bottles received 100 ml of the above medium. Lead nitrate was then added in appropriate amounts to produce concentrations of 0.01, 0.1, 1.0 and 10 ug Pb/g sol within the bottles. One bottle received 100 ul of dilute (0.1 N) nitric acid and one bottle received nothing except the medium. Both of these bottles were controls. All of the bottles were autoclaved at 121° C for 30 minutes. After cooling sufficiently, the media were transferred to sterile petri dishes. The polystyrene petri plates were not acid washed because of their lack of resistance to concentrated acids and because once they were removed from their sterile packages, they could not be re-autoclaved. Contamination was not a major concern in these experiments because the fungi were not analyzed directly for their lead concentrations. Four plates were poured from the medium within each of the Teflon bottles. After the agar had solidified, each plate aseptically received a plug of Pisolithus (cut with an 8 mm cork borer) from a stock plate of the fungus. After the plates were inoculated, they were sealed and stacked in the 25° C incubator.

The colony diameter of each plate was measured after two weeks of growth. The diameter of each plate was read

four times, each from a different position across the plate. The colony diameters from the different groups were compared with each other using the ANOVA procedure of the Stastical Analysis System (SAS). Duncan's Multiple Range Test was used to separate the groups that showed a statistically significant difference between their mean colony diameters.

To extend the range of this first growth study one order of magnitude to 100 ug Pb/g sol, it became necessary to buffer the medium. This was so because our most concentrated lead standard was 10,000 ug Pb/g sol, and 1 ml of this standard would have to be added to 100 ml of Hagem's to produce a medium with 100 ug Pb/g sol. From prior experience, I knew that 1 ml of dilute nitric acid would lower the pH of the agar medium to the point that the agar would not solidify. Therefore, the second growth study was set up using a sodium citrate/citric acid buffer. Four FEP Teflon bottles received 50 ml of double strength Hagem's medium. Fifty milliliters of QDW was added to one of the bottles. In the other three bottles, 20.5 ml of 0.1 M citric acid and 29.5 ml of 0.1 M sodium citrate were added to produce a pH of 5.0. One of the bottles with buffered medium received no further

treatment, while another bottle received 1 ml of dilute nitric acid. Each of these three bottles was a different control. The final bottle received 1 ml of the 10,000 ug Pb/g sol standard, producing a concentration of 100 ug Pb/g sol within the medium. All of the bottles were then autoclaved, and four petri plates were poured from each bottle. The plates were inoculated with Pisolithus plugs as stated previously. The plates were sealed and placed in the 25° C incubator. Colony diameters were measured after two weeks of growth.

Since there appeared to be reduced growth of the fungus in the second growth study, a final growth study was performed where the lead concentrations of the media were intermediate between those used in the two previous studies. Eight Teflon bottles received 50 ml of double strength Hagem's medium. One of the bottles received 50 ml of QDW, and the remainder of the bottles received 50 ml of the sodium citrate/citric acid buffer solution, to produce a pH of 5.0. One of the bottles with the buffered medium received no further treatment and one other bottle received 1 ml of dilute nitric acid. These three bottles were controls. The final five bottles received 100, 250, 500, 750 and 1,000 ul of the

10,000 ug Pb/g sol standard, respectively. This produced lead concentrations in the media from 10 ug/g sol to 100 ug/g sol. These concentrations should have produced a gradation of growth inhibition in the fungus. After the bottles were autoclaved, four plates were poured from each bottle. The plates were inoculated and the colony diameters were determined after two weeks of incubation. A linear regression was performed on the colony diameters to estimate the concentration of lead that would completely inhibit the growth of Pisolithus.

Lead uptake by mycorrhizal and non-mycorrhizal seedlings

The last phase of this research involved culturing both mycorrhizal and non-mycorrhizal seedlings of Pinus sylvestris and comparing the amounts of lead they took up from a hydroponic solution. These plants were grown in polypropylene growth vessels especially designed for this experiment. The growth vessels were produced by cutting off the tops and boring a hole in the sides of conventional polypropylene bottles. After these vessels were acid washed, cotton plugs were inserted into the holes on the sides of the bottles to allow for gas exchange. Approximately 6 gm (70 ml) of sieved

($\geq$ 2.5 mm^2) vermiculite was added to each bottle. The bottles were covered with aluminum foil and stored in the clean lab until they were needed.

Pinus sylvestris seeds of the variety "Scotland" were obtained commercially from the F. W. Schumacher Company. Seeds were surface sterilized in a 50% Chlorox solution for 15 minutes. The seeds were then rinsed in sterile, distilled water and placed in an autoclaved, polypropylene bottle with sterile distilled water. They were soaked at room temperature for 48 hours. The water was aseptically drained off and the seeds were cold stratified at a temperature of 2° C for at least 15 days, as recommended in Seeds of Woody Plants in the U. S., (Department of Agriculture, Forest Service, 1974). Cold stratification is necessary to break the dormancy of Scotch pine seeds because the enbryos are often underdeveloped when the seeds are shed from the cones (Suszka, 1976). After sufficient cold stratification, the seeds were placed in petri plates containing water agar which was prepared with QDW. The plates were placed under plant growth lamps in the clean lab growth cabinet, until the seeds had successfully germinated (7-14 days).

The growth cabinet within the clean lab was constructed by lining a shelf with one-eighth inch thick polyethylene sheets. Banks of three General Electric "Gro-N-Sho" plant growth lights were placed in the top of the cabinet. A small fan was mounted on the outside of the cabinet to circulate air within the cabinet and dissipate the heat produced by the growth lamps. With the air conditioning turned on in the lab, the cabinet's temperature averaged 25 ± 5° C. Using a standard commercial timer, the growth lamps were set on a 10 hour day/14 hour night cycle.

Without the air conditioning on, the growth cabinet would heat up beyond 35° C. This occurred at one point in the study, and the pine seedlings were moved to a growth chamber in the mycology lab for a period of six days. The temperature in the mycology lab growth chamber was 20 ± 2° C. Since the growth bottles were sealed, no lead contamination of the plants was observed from moving them outside the clean lab. If lead contamination had occurred, the lead concentrations of the leaves would have been much higher than the values I observed. At the end of six days, the air conditioning was turned on in the building and the plants were moved back to the clean lab.

To synthesize mycorrhizae, Scotch pine seedlings with roots approximately 6 cm long were aseptically placed on agar petri dishes containing Hagem's medium. A rectangular plug of Pisolithus was cut from a stock culture and placed on top of a pine root so that the mycelium was in direct contact with the root and the surrounding Hagem's agar. Studies with Pinus resinosa Ait. have shown that Pisolithus can invade the primary root of pine seedlings and subsequently form mycorrhizae (Wilcox, 1982). The plates with the inoculated seedlings were placed in the clean lab growth cabinet for approximately one week until the fungus had grown into the agar. The plug of mycelium was lifted off the root, and the seedling was ready to be replanted in a hydroponic growth vessel. Uninoculated seedlings were handled in a similar manner except, of course, they were not exposed to the fungus.

To determine whether or not the mycorrhizal condition had an effect on lead uptake in Scotch pine seedlings, two very similar experiments were undertaken. The first tested the general trend, and the second used replicate samples to determine the statistical significance between the means of the lead concentrations in the two groups (mycorrhizal versus non-mycorrhizal seedlings).

The first experiment involved growing nine mycorrhizal and nine non-mycorrhizal pines in growth vessels containing hydroponic solution with different amounts of added lead. Three media concentration series were prepared by adding lead nitrate to U.S.D.A. (Maryland) hydroponic medium (see Appendix; Douglas, 1976), prepared with QDW. One series, the control, had no added lead but instead received 100 ul of dilute nitric acid per 100 ml of hydroponic solution. The second series received 50 ul of a 1,000 ug/g sol lead standard per 100 ml of hydroponic solution, producing a solution concentration of 500 ng Pb/g sol. Dilute nitric acid (50 ul) was also added to this series so that all three groups of hydroponic media would have the same amount of added nitric acid. The third and final series received 100 ul of the same lead standard per 100 ml of hydroponic solution, producing a solution concentration of 1,000 ng Pb/g sol. Three mycorrhizal and three non-mycorrhizal growth vessels from each concentration series received 40 ml of the appropriate hydroponic solution. The vessels were covered with aluminum foil and autoclaved for 30 minutes at 121° C.

After sterilization, the vessels were set in front of the laminar flow hood of the mycology lab transfer room for 24 hours before the seedlings were transplanted in them so that the lead in the hydroponic solution could equilibrate with the vermiculite (Zimdahl and Skogerboe, 1977). Following this time period, mycorrhizal and non-mycorrhizal seedlings were aseptically placed in their respective growth vessels. Polyethylene sheets were soaked in a 50% Chlorox solution for 24 hours, rinsed in sterile QDW and placed over the tops of the growth vessels. The sheets were held in place with washed rubber bands. The growth vessels with their pine seedlings were placed in the clean lab growth cabinet. A mycorrhizal and non-mycorrhizal plant from each concentration series was sampled at two-week intervals for six weeks.

In order to determine the lead concentration of the soil moisture within the hydroponic vessels, a device was constructed so that the vermiculite could be squeezed hard enough that the soil moisture dripped out. This device was constructed from a 50 ml polycon. The soil-moisture-piston-polycon (SMPP), as it became known, was packed with vermiculite after the pine seedling had been removed. The SMPP was placed on top of an acid-washed, Teflon filter

and the soil moisture was squeezed directly into a tared, FEP Teflon beaker. The beaker was weighed to obtain the sample size. Two milliliters of concentrated nitric acid was added and the soil moisture was digested in the same manner as the Hagem's medium. Soil moistures were obtained whenever a seedling was sampled.

At the proper sampling interval, six plants were individually removed from their respective growth bottles. The vermiculite was loosened around the plant with a polyethylene strip and the entire plant was removed intact using acid-washed, stainless-steel forceps. Most of the pieces of vermiculite that were attached to the roots were rinsed off with QDW. The remaining pieces of vermiculite were removed with forceps. Each seedling was placed on another polyethylene strip and cut into root, stem and leaves with an acid-washed scalpel blade. The plant parts were placed in tared, quartz-glass beakers and desiccated to dryness. Dry weights were obtained and the plant parts were digested in hot, concentrated nitric acid. The digestion of the leaves was facilitated by covering the beaker's top with a TFE Teflon watchglass. The leaves were then refluxed in the hot nitric acid for approximately two hours before the samples were

evaporated, diluted to a working volume and transferred to polycons. A three-way factorial ANOVA was performed on the data, using the mean square of the three-way interaction as the error mean square. The three factors tested were time, amount of lead added to the hydroponic medium and the presence or absence of mycorrhizae.

The second experiment involving mycorrhizal and non-mycorrhizal Scotch pine seedlings was performed in a similar manner, although the design was somewhat different. Three mycorrhizal and three non-mycorrhizal plants were placed in hydroponic growth vessels that had received 40 ml of hydroponic solution containing 1,000 ng Pb/g sol. After the plants had grown in the clean lab growth chamber for six weeks, the plants were removed, and their roots, stems and leaves were desiccated and digested. The lead concentrations of each group's (mycorrhizal versus non-mycorrhizal) roots, stems and leaves were averaged and a one-way factorial ANOVA was performed on the data. Soil moistures were also extracted, but instead of acidifying and digesting the solutions, their pH levels were measured.

All of the digested samples were analysed for lead on the HGA-2100 graphite furnace of a Perkin-Elmer 460 Atomic

Absorption Spectrophotometer. The wavelength was set at 283.3 nm and the deuterium arc background corrector was used to eliminate background interferences. A series of lead standards was prepared by diluting a stock 10,000 ug Pb/g sol atomic absorption standard. These standards ranged from 5-150 ng Pb/g sol. They were used to produce a curve from which the lead concentrations of the samples could be calculated.

Because of the small amount of plant tissue produced in the previous two experiments, it was necessary to digest the entire roots (mycorrhizal or not) of the pine seedlings in order to determine their lead concentrations. To verify the formation of mycorrhizae with Pisolithus and Scotch pine using the technique explained above, several seedlings were grown in a lead-free solution within hydroponic vessels in the same manner as described previously. After growing for six weeks, the seedlings were removed from the growth vessels and the roots were fixed in FAA, stained with saffranin and fast green and sectioned (10 um) in order to observe the formation of the Hartig net. The Hartig net is usually assumed to be present if a functional mycorrhizal root has been formed (Marks and Foster, 1973).

Results

Figure 2 shows the uptake of lead by Pisolithus grown in liquid Hagem's medium containing different amounts of added lead. Clearly, the mycelial lead concentrations in the experimental groups increased over time. The first week's analyses are not plotted because little growth of the fungus had occurred (< 1 mg dry weight of mycelia). The media were not buffered in this experiment and the pH levels of the media dropped throughout the sampling period (Fig. 3). The initial pH levels reflect the amount of lead (in nitric acid) that was added to the growth bottles to produce the calculated media concentrations. The pH levels of the media within the non-inoculated bottles remained at their initial levels.

Table 1 shows the lead concentrations in the media of the four groups. The zero week samples were taken immediately after the lead spikes were added to the media and the bottles were shaken. Apparently, some of the lead in the bottles was no longer in solution. Table 2 shows the estimated amounts of lead that were leached from the sides of the Teflon bottles. These data indicate that

some of the lead in the media was absorbed by the fungus, and some of the lead was adsorbed onto the walls of the bottles.

Figure 4 shows the mean lead concentrations of the mycelia of Pisolithus grown in liquid Hagem's medium, buffered to pH 3.0, 4.0 and 5.0 with sodium citrate/citric acid buffer. The differences in the mycelial lead concentrations are significant at the 0.05 level. The pH levels of the media in the three groups remained unchanged throughout the 28 day growth period of this experiment. The final media concentrations of the three groups are shown in Figure 5. The lead concentration in the medium buffered to 5.0 was higher than the other two groups of media ($p < 0.01$).

The first growth study in which Pisolithus was grown on Hagem's medium with agar (unbuffered) shows that no growth inhibition occurred (decrease in mean colony diameter), even at the highest lead concentration of 10 ug/g sol (Fig. 6). The mean colony diameters were not significantly different at the 0.05 level. The second study where the lead concentration in the buffered medium was raised to 100 ug/g sol showed several effects (Fig. 7). First, the sodium citrate/citric acid buffer

reduced the growth of the fungus significantly ($p < 0.01$). Second, the medium that contained lead also showed a significant reduction in growth compared to both of the other buffered groups. Third, adding 1 ml of dilute nitric acid (0.1 N) does not effect the growth of Pisolithus. The last growth study confirmed the findings of both the previous growth studies. In this final growth study, all of the buffered media showed significant growth reduction when compared to the unbuffered control (Fig. 8). Furthermore, no difference in growth of the fungus was observed in the 10 ug Pb/g sol group when it was compared with the two groups of media that were buffered but had no added lead. Also, the 100 ug Pb/g sol group showed a reduction in fungal growth when compared with the 10 ug Pb/g sol group. The bars above the histograms of mean colony diameters show which groups in the concentration series differ from each other ($p < 0.05$). The linear regression performed on the mean colony diameters shows that complete inhibition of Pisolithus would be expected to occur at approximately 1,000 ug Pb/g sol (Fig. 9). This assumes that the observed linear response between the lead concentrations in the media and reductions in mean colony diameters is

not affected by lead concentrations beyond 100 ug/g sol. Since I have no direct evidence for this, the complete inhibition of the growth of Pisolithus at 1,000 ug Pb/g sol remains a rough estimate.

The first experiment involving the growth of mycorrhizal and non-mycorrhizal seedlings of Pinus sylvestris (Figs. 10, 11 and 12) shows that up to four weeks into the experiment, there is little difference between the lead concentration of mycorrhizal and non-mycorrhizal roots. From four to six weeks, however, the lead concentrations of the mycorrhizal roots increase rapidly, whereas the lead concentrations of the non-mycorrhizal roots do not. This phenomenon is observed in the roots grown in the control hydroponic solution with no added lead and the experimental group with a lead concentration of 500 ng/g sol. This trend does not seem to occur from four to six weeks in the roots grown in the hydroponic solution with a lead concentration of 1,000 ng/g sol, perhaps due to the relatively high concentration of lead in this experimental group. Using the mean square of the three-way interaction as the error term in the ANOVA procedure, the effect of the fungal symbiont on lead uptake in the roots was significant at

the 0.1 level. The concentrations of lead in the stems and leaves of mycorrhizal and non-mycorrhizal plants were not significantly different by the end of the six week growth period. The effect of sampling time (2, 4 and 6 weeks) was significant at the 0.05 level. In other words, the lead concentrations of the roots increased over time in all groups of hydroponic solution and for both mycorrhizal and non-mycorrhizal seedlings. The lead concentrations of the roots from the three groups of hydroponic media (no added lead, 500 and 1,000 ng Pb/g sol) were not significantly different. Therefore, adding lead in the above concentrations to the hydroponic solution/vermiculite mixture did not affect the lead concentrations of the pine roots. Apparently, the untreated vermiculite has some lead adsorbed onto its surfaces, probably as a result of mining and manufacturing practices. Attempts to acid-wash the vermiculite and remove this lead proved unsuccessful. The lead concentrations of the soil moistures from the different groups are shown in Table 3. Even though no trend is shown by these values, one can see that most of the added lead is not in the soil solution.

For the second experiment in which replicates (n=3) of mycorrhizal and non-mycorrhizal pine seedlings were analyzed for their lead concentrations, the mean lead concentrations of the two different kinds of roots were statistically different at the 0.05 level (Fig. 13). The stems and leaves of the mycorrhizal and non-mycorrhizal plants were not significantly different. However, the roots of both groups, analyzed together, contained more lead (Fig. 14) than the stems and leaves of both groups ($p < 0.01$). Therefore, little or no lead taken up by the roots is translocated to the above ground portions of the seedlings, at least during the early stages of seedling growth. The pH levels of the soil moistures in the growth vessels containing mycorrhizal and non-mycorrhizal seedlings were 5.6 and 6.5, respectively.

The mycorrhizal seedlings that were fixed, stained and sectioned showed the beginnings of Hartig net formation (Fig. 15), as well as a thick mantle. These sections show that the inoculation procedure used in this study to synthesize mycorrhizae was successful.

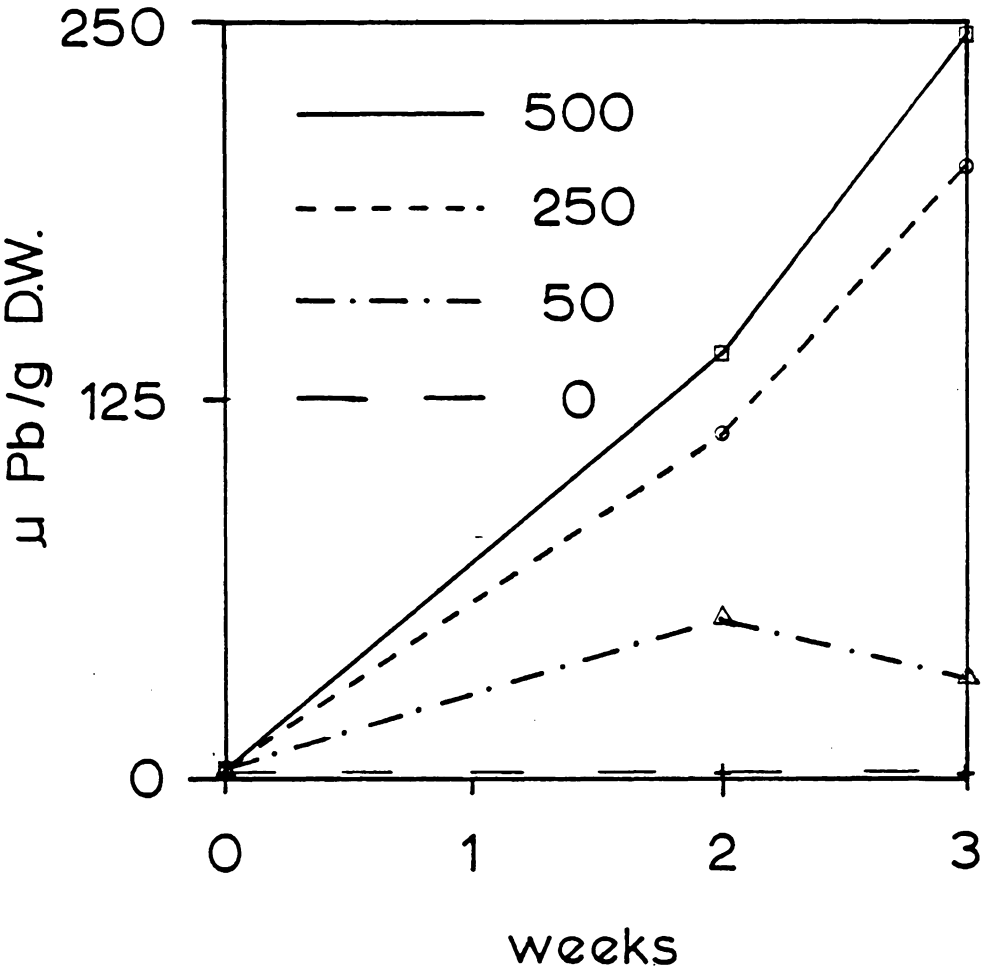


Figure 2. Lead concentrations of mycelia over time of Pisolithus tinctorius cultured in liquid Hagem's medium with different concentrations of lead (0, 50, 250 and 500 ng/g sol).

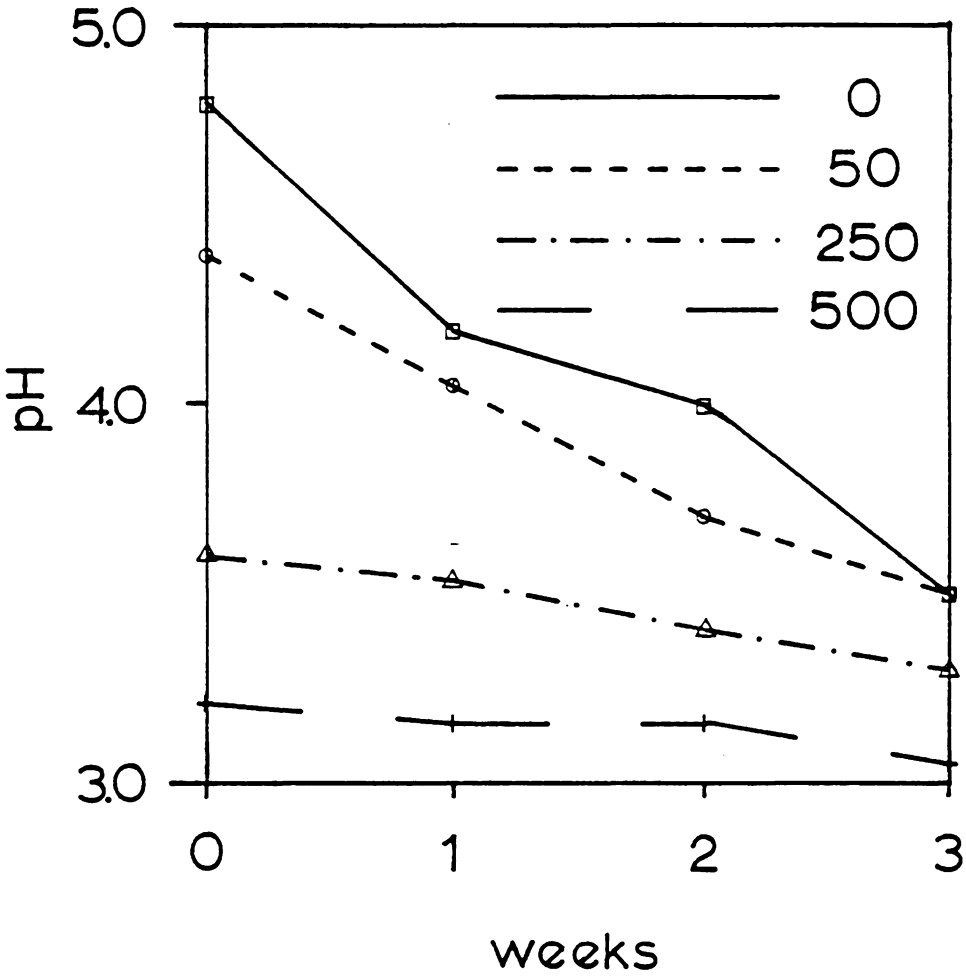


Figure 3. pH levels over time of Hagem's medium in which Pisolithus tinctorius was cultured with different concentrations of lead (0, 50, 250 and 500 ng/g soil).

Table 1. Lead concentrations of Hagem's medium (ng/g sol) from bottles inoculated with P. tinctorius (In) and not inoculated (Un) for concentration series of 0, 50, 250 and 500 ng Pb/g sol.

Calculated lead concentration ng Pb/g sol

time (weeks)	0		50		250		500	
	In	Un	In	Un	In	Un	In	Un
0	2.2	-	45	-	160	-	300	-
1	1.6	2.4	49	41	110	120	220	250
2	2.4	-	30	-	170	-	-	-
3	1.4	.42	23	41	170	190	370	450

Table 2. Extimated amounts of lead in nanograms leached from FEP Teflon bottles with Hagem's medium inoculated with P. tinctorius (In) and not inoculated (Un) for concentration series of 0, 50, 250 and 500 ng Pb/g sol.

Concentration series (ng/g sol added lead)

time (weeks)	0		50		250		500	
	In	Un	In	Un	In	Un	In	Un
1	5.6	8.5	23	25	56	37	45	8.5
3	-	0	-	17	-	39	-	31

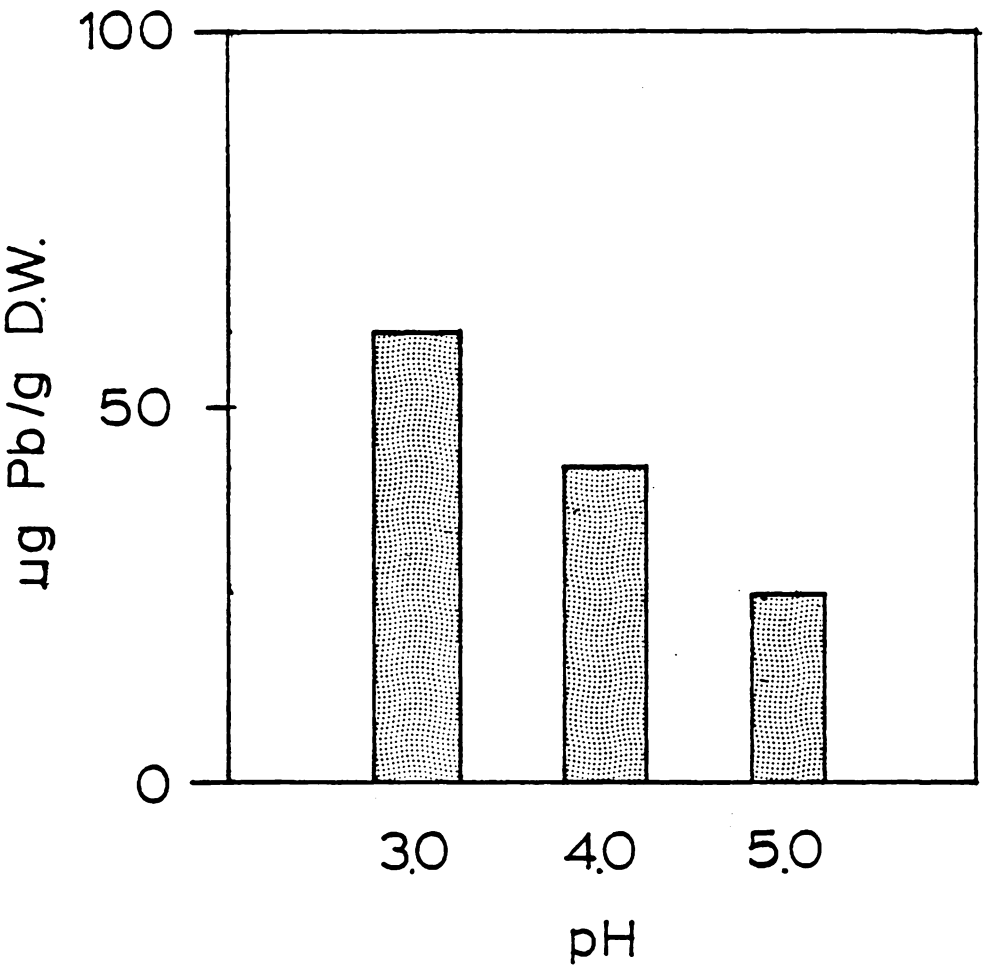


Figure 4. Mean lead concentrations of P. tinctorius mycelia cultured in liquid Hagem's medium for 28 days with 500 ng/g sol added lead buffered to 3.0, 4.0 and 5.0 with sodium citrate/citric acid buffer; (n=5), ($p < 0.05$).

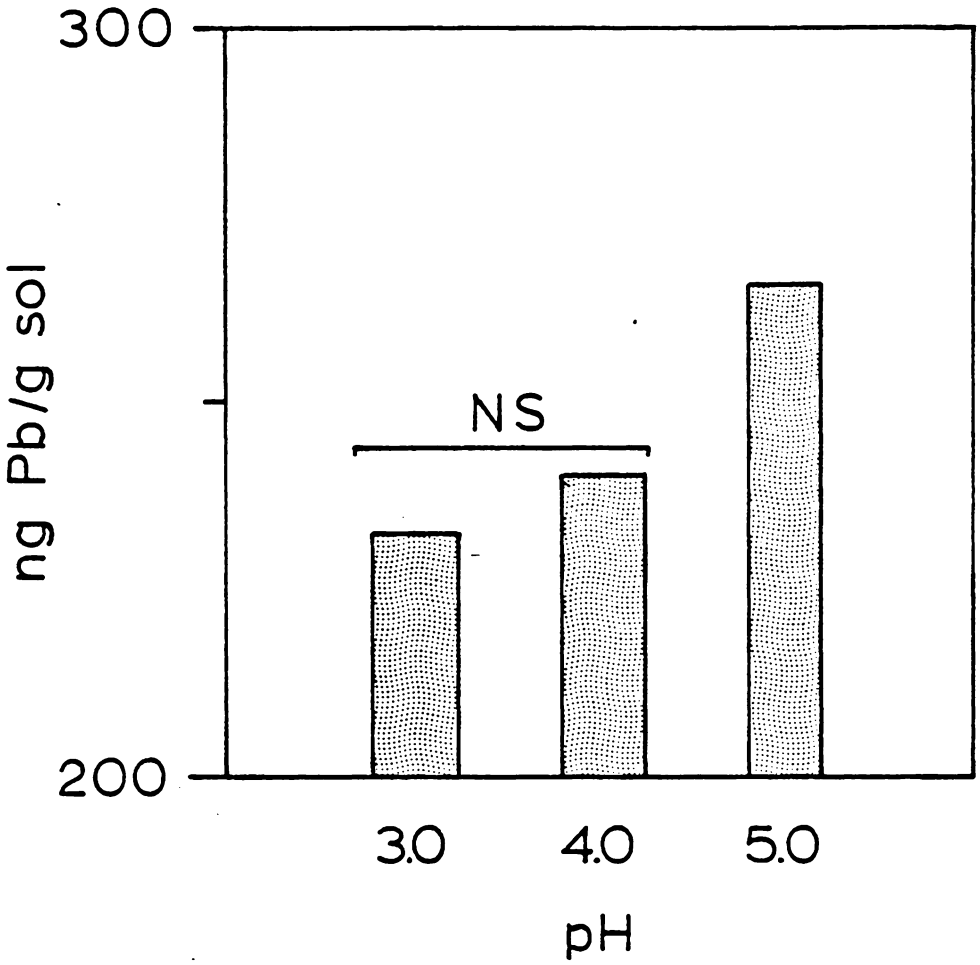


Figure 5. Mean lead concentrations (after 28 days of growth by P. tinctorius) of liquid Hagem's medium with 500 ng/g sol added lead buffered to 3.0, 4.0 and 5.0 with sodium citrate/citric acid buffer; (n=5), (NS=not significant), ($p < 0.01$).

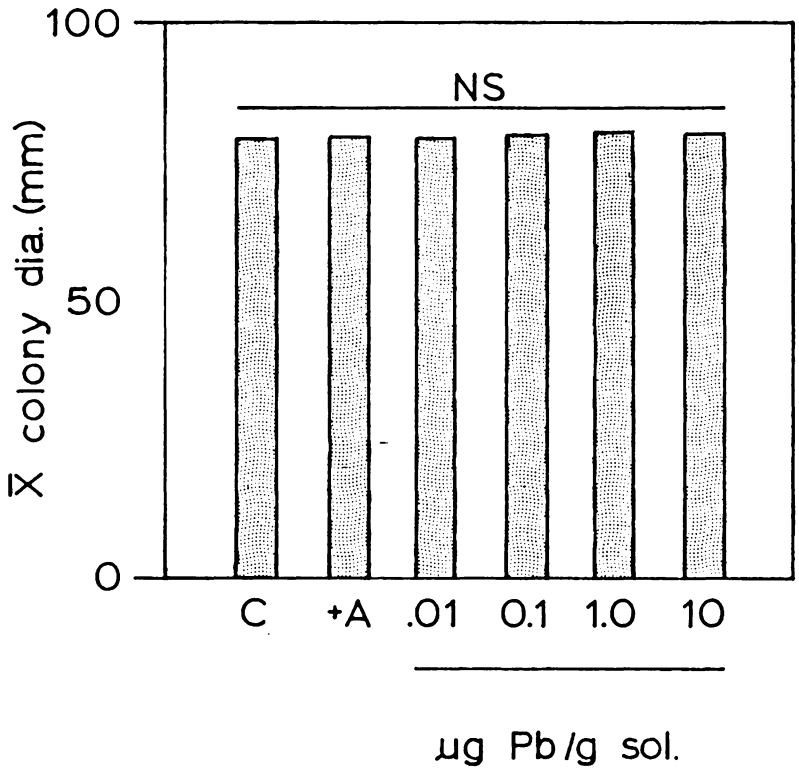


Figure 6. Mean colony diameters of P. tinctorius after 14 days growth at 25° C in unbuffered Hagem's medium. (C) control, (+A) with 100 ul of 0.1 N nitric acid, (.01-10) with different concentrations of lead in medium; (n=4), (NS=not significant), ($p < 0.05$).

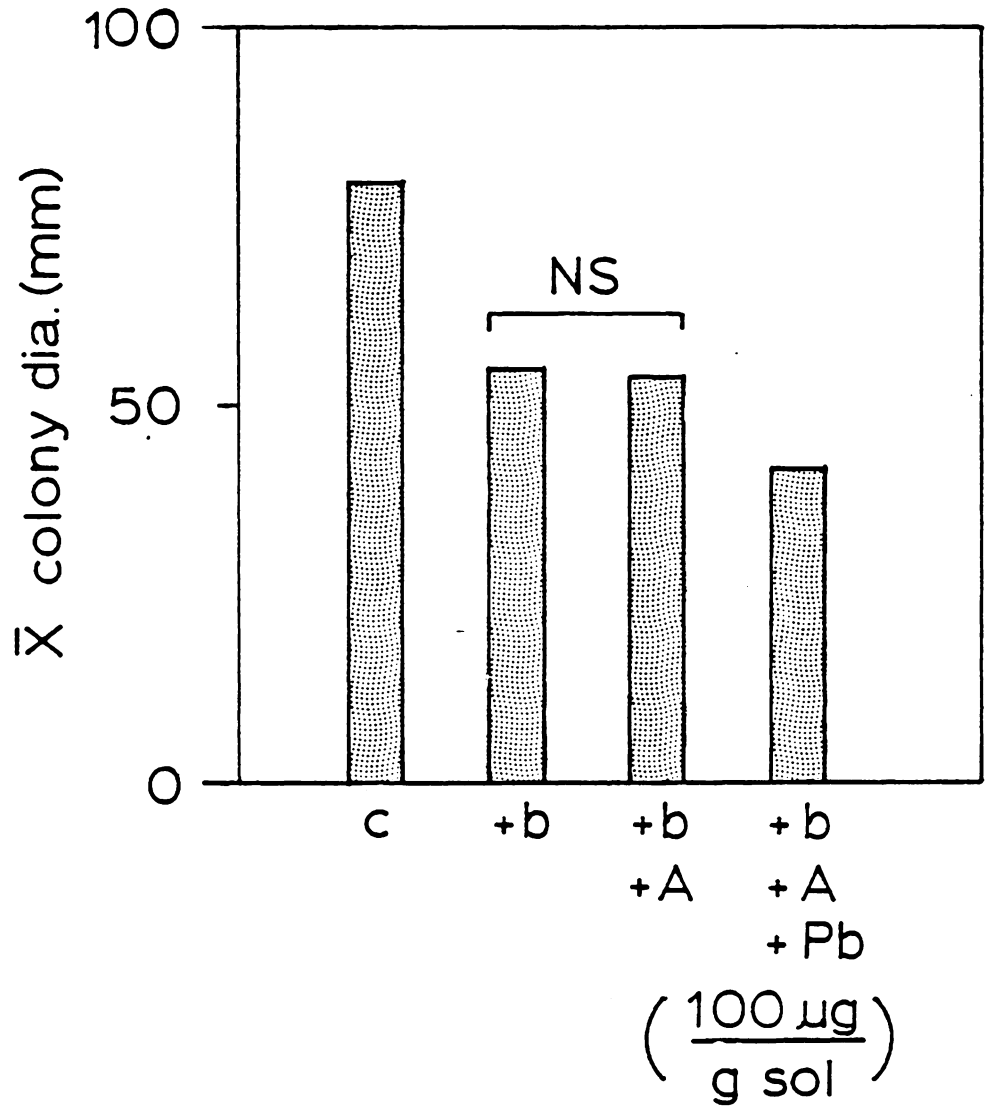


Figure 7. Mean colony diameters of P. tinctorius after 14 days growth at 25° C in buffered (except control) Hagem's medium. (C) control, (+b) with sodium citrate/citric acid buffer, (+A) with 1 ml of 0.1 N nitric acid, (+Pb) with added lead. (n=4), (NS=not significant), ($p < 0.01$).

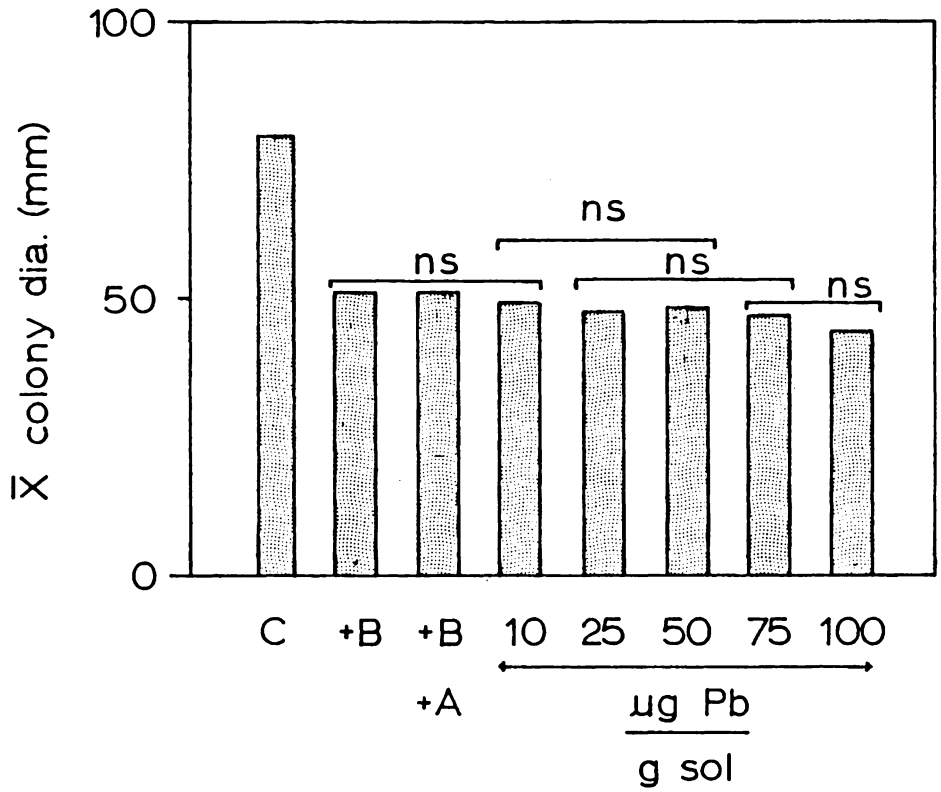


Figure 8. Mean colony diameters of P. tinctorius after 14 days growth at 25° C in buffered (except control) Hagem's medium. (C) control, (+B) with sodium citrate/citric acid buffer, (+A) with 1 ml 0.1 N nitric acid, (10-100) with different concentrations of lead in medium. (n=4), (NS=not significant), ($p < 0.05$).

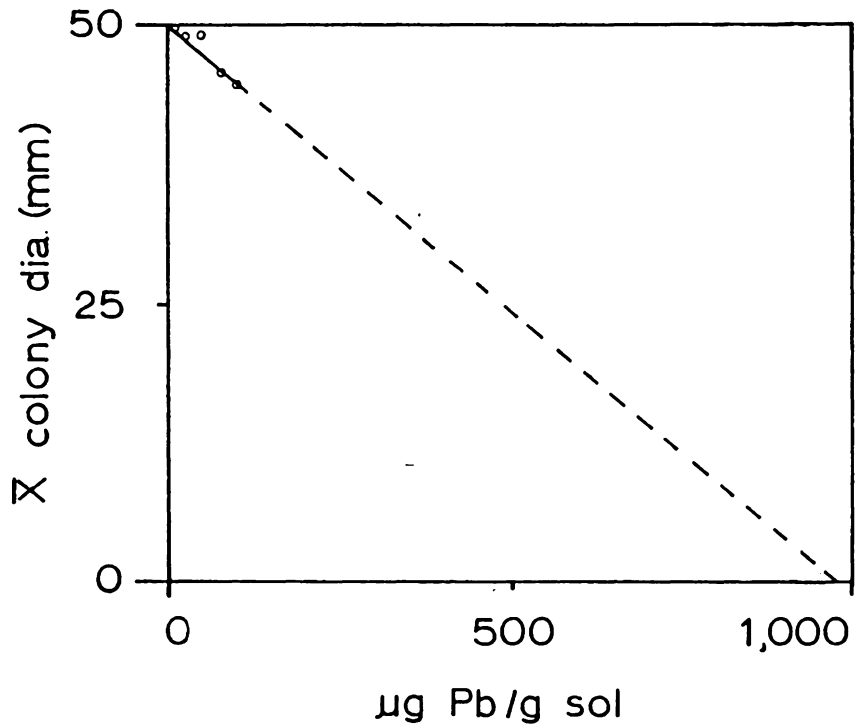


Figure 9. Linear regression of mean colony diameters from third growth study (Fig. 8) plotted against calculated lead concentrations of the medium. (r^2 = 0.94).

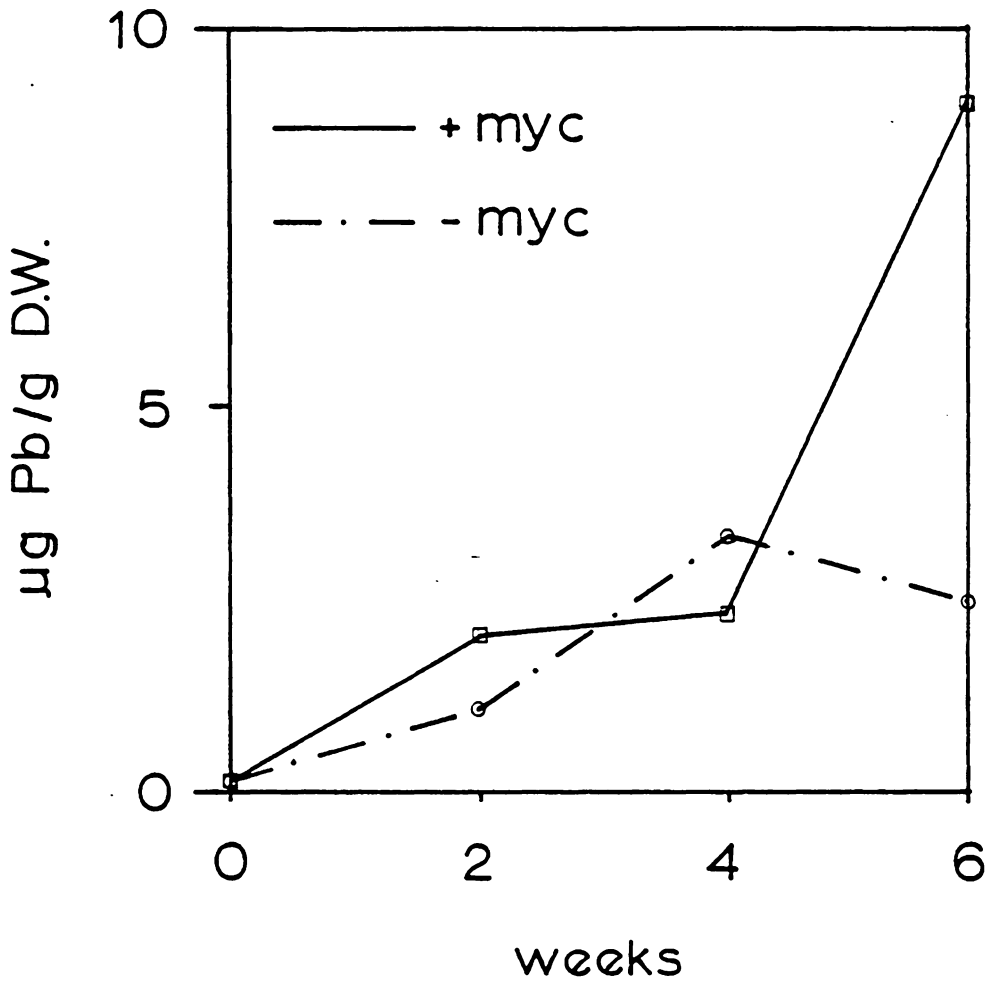


Figure 10. Lead concentration of mycorrhizal (+myc) and non-mycorrhizal (-myc) roots of Pinus sylvestris seedlings over time, grown in vermiculite and hydroponic solution with no added lead.

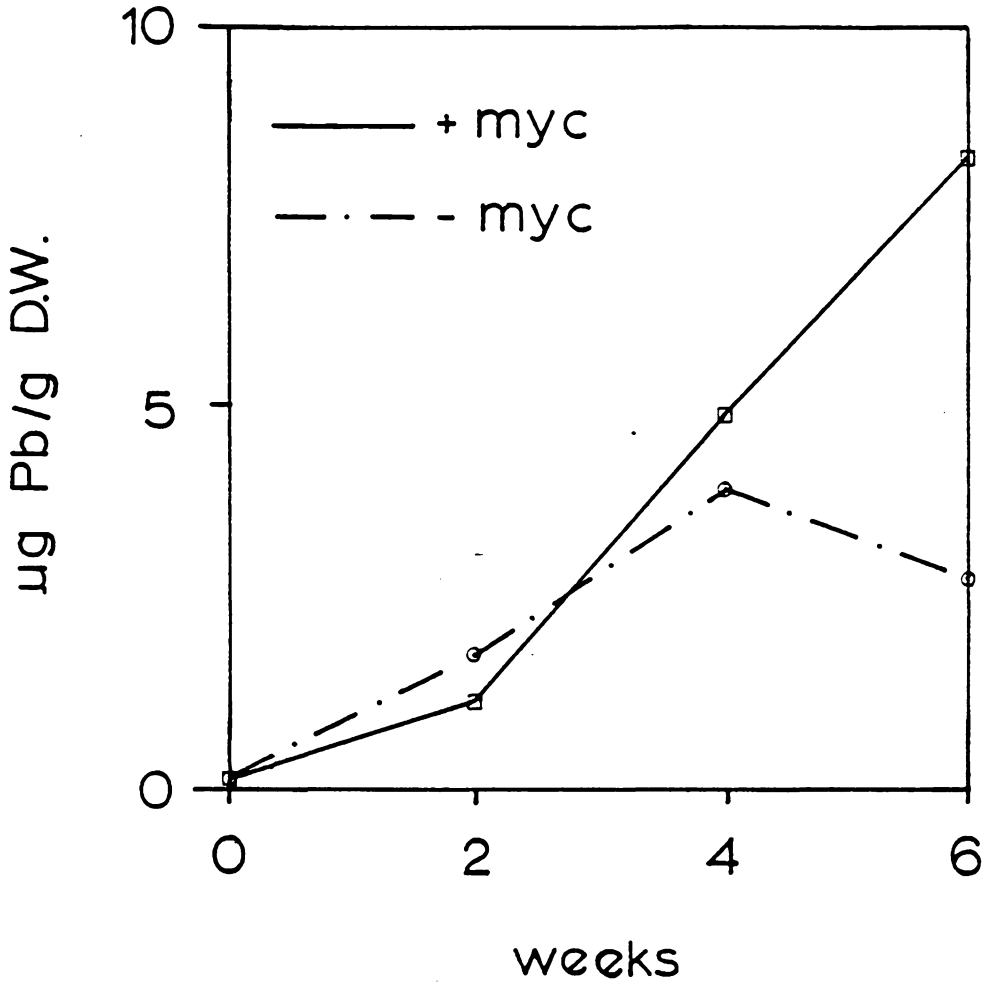


Figure 11. Lead concentrations of mycorrhizal (+myc) and non-mycorrhizal (-myc) roots of P. sylvestris seedlings over time, grown in vermiculite and hydroponic solution with lead concentration of 500 ng/g sol.

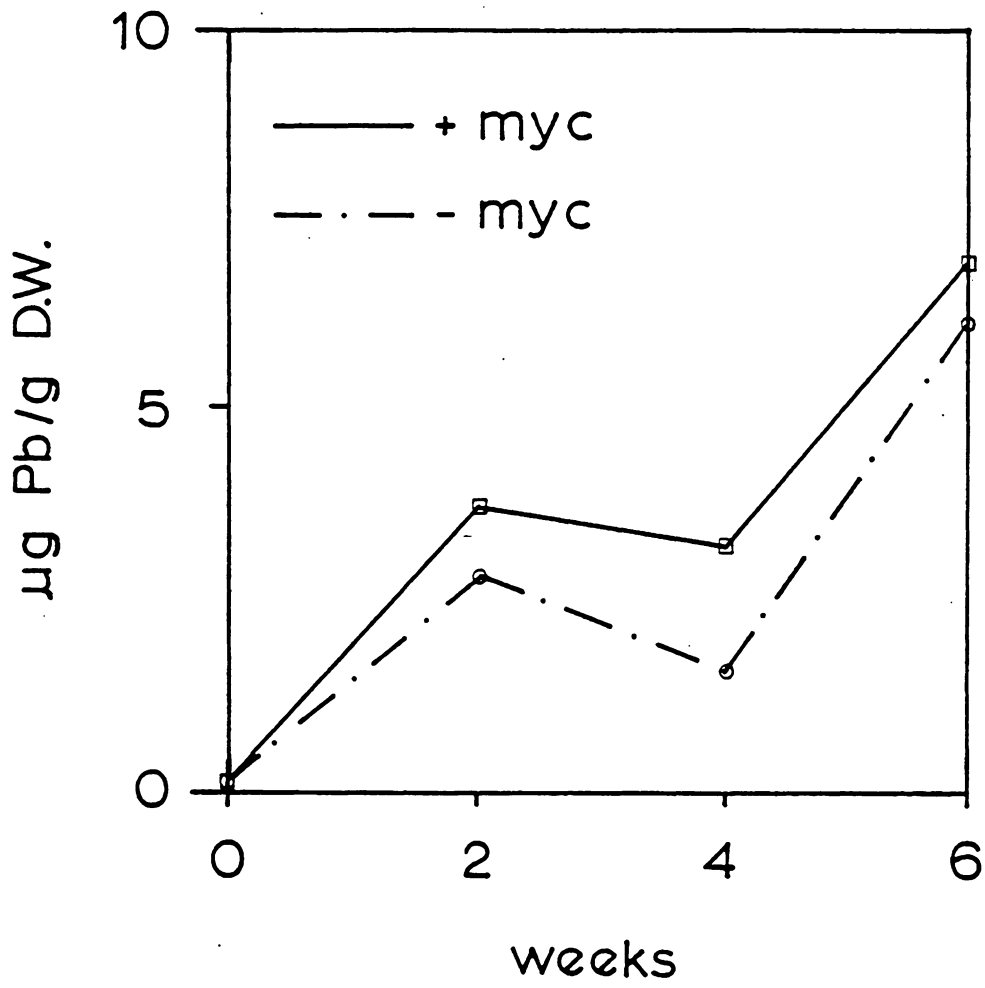


Figure 12. Lead concentrations of mycorrhizal (+myc) and non-mycorrhizal (-myc) roots of P. sylvestris seedlings over time, grown in vermiculite and hydroponic solution with lead concentration of 1,000 ng/g sol.

Table 3. Lead concentration of soil moisture (ng/g sol) from hydroponic growth vessels containing mycorrhizal (+myc) and non-mycorrhizal (-myc) seedlings grown in vermiculite and hydroponic solution containing lead concentrations of 0, 500 and 1,000 ng/g sol.

Calculated lead concentration (ng Pb/g sol)

time (weeks)	0		500		1,000	
	+myc	-myc	+myc	-myc	+myc	-myc
2	2.8	2.6	0	4.0	8.6	4.3
4	1.9	3.0	2.1	2.6	2.5	2.2
6	9.4	11	16	4.2	11	6.4

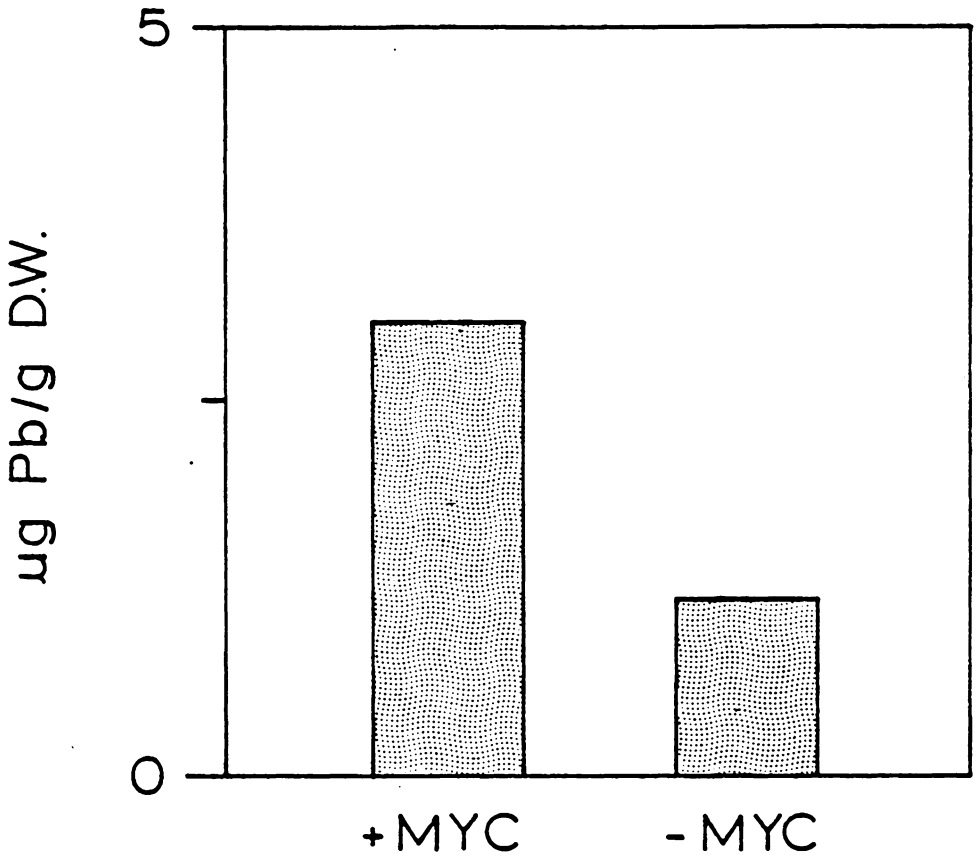


Figure 13. Mean lead concentrations of mycorrhizal (+MYC) and non-mycorrhizal (-MYC) roots of P. sylvestris seedlings grown for six weeks in vermiculite and hydroponic solution with lead concentration of 1,000 ng/g sol; (n=3), ($p < 0.05$).

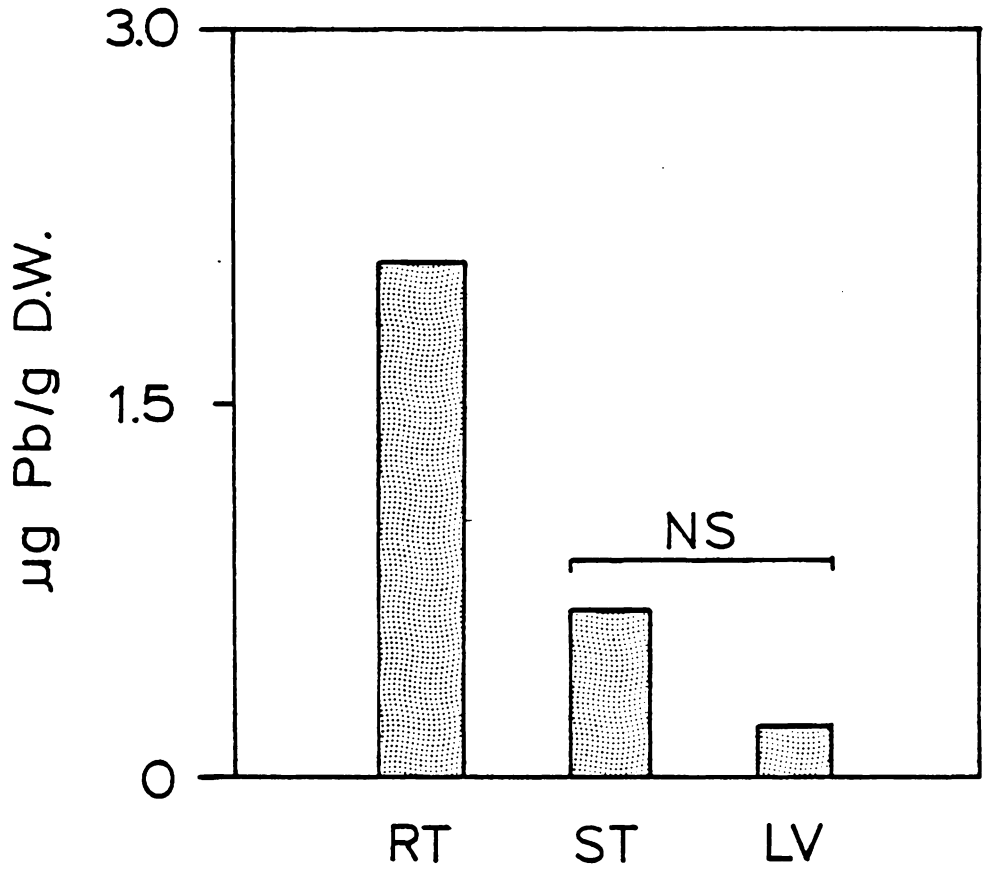
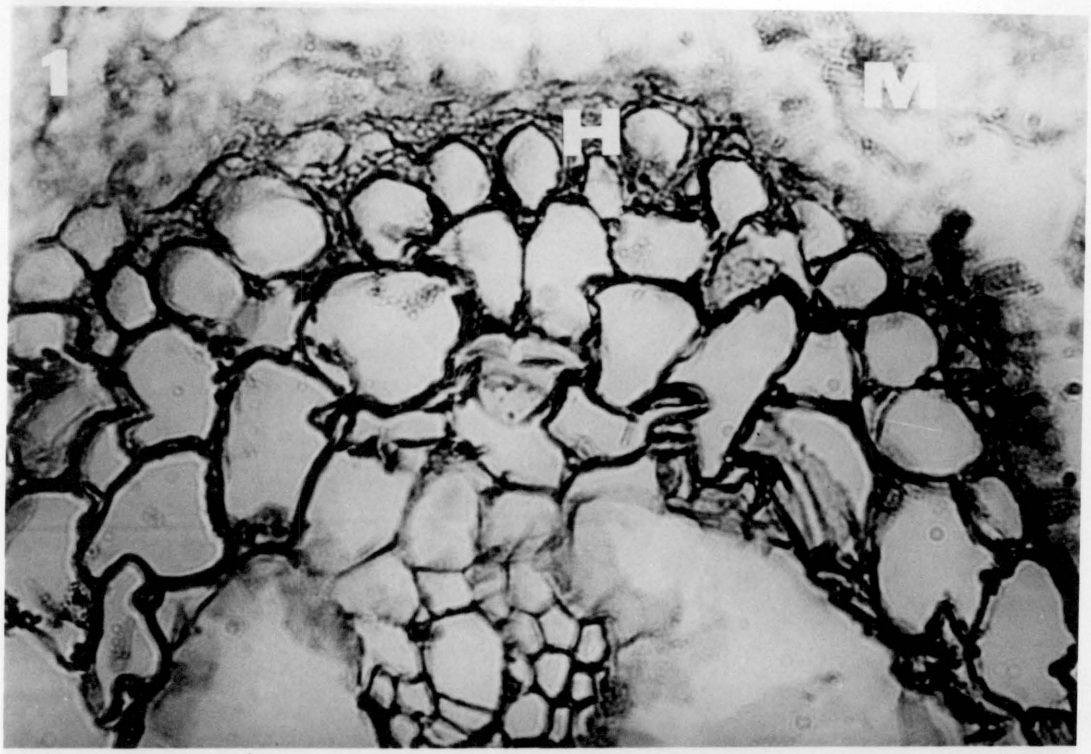


Figure 14. Mean lead concentration of roots (RT), stems (ST) and leaves (LV) from mycorrhizal and non-mycorrhizal seedlings (grouped together) of P. sylvestris grown for 6 weeks in vermiculite and hydroponic solution with lead concentration of 1,000 ng/g sol; (n=6), (NS=not significant), ($p < 0.05$).

Figure 15. (1 and 2) Cross-sections of ectomycorrhizal root of P. sylvestris, inoculated with P. tinctorius. (M) mantle (H) Hartig net. Total magnification=1760 x.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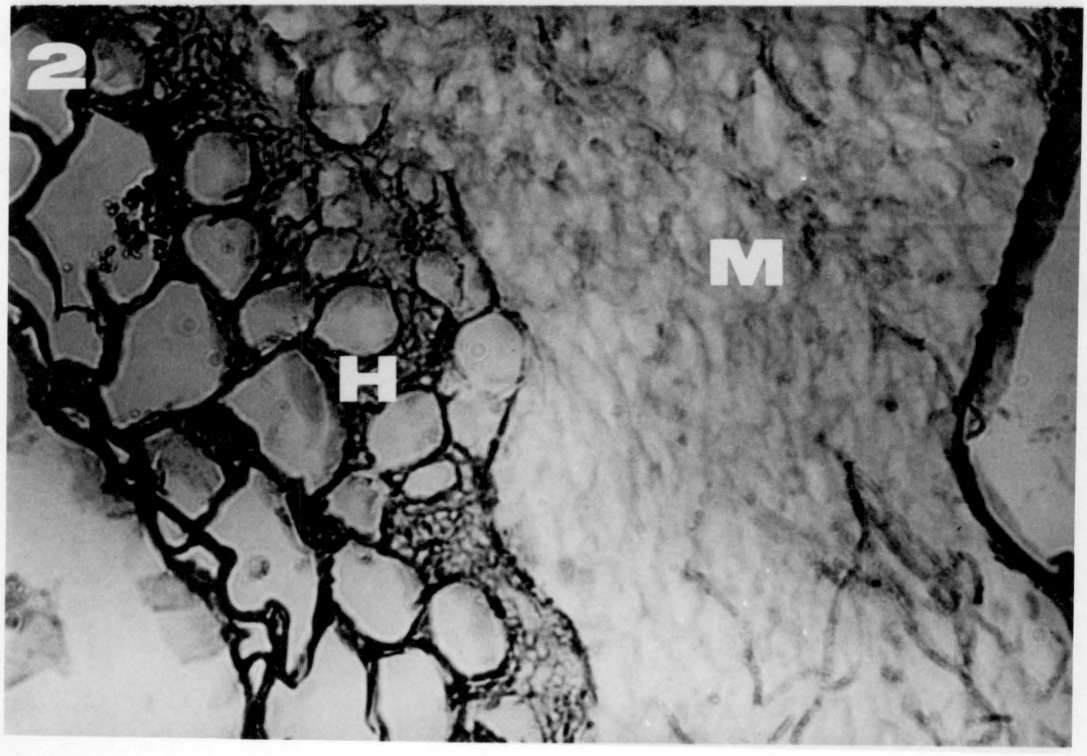
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Discussion

Figure 2 shows that Pisolithus took up lead from the culture medium. The greatest amount of uptake occurred in the medium containing the greatest concentration of lead. The amount of lead added to the medium was probably the major factor that produced the observed mycelial lead concentrations, but pH may have played a role in determining the lead concentration within the medium. When a solution containing lead is placed into a bottle, some of the lead will adsorb onto the surface of the bottle. Ramamoorthy and Kushner (1975) found that 20% of the lead added to their culture medium was adsorbed onto the surface of a Pyrex flask. When collecting natural waters for lead analyses, Patterson and Settle (1976) recommend the acidification of the sample to prevent significant amounts of lead from depositing on the inside surface of the collection vessel. Unfortunately, the low pH that results from such an acidification (< 1) would have completely inhibited the growth of the fungus.

As the pH level in the medium dropped (Fig. 3), more lead would go into solution from the walls of the bottle.

Unfortunately, bottle leaches could not be performed on the inoculated bottles for the reason mentioned above. The media lead concentrations (Table 1) do not show a marked reduction, as might be expected if the fungus were rapidly absorbing lead from the media. Quite possibly, as the hyphae took up lead from the media, lead adsorbed on the sides of the bottles went into solution to re-establish an equilibrium.

Apparently, Pisolithus does not possess some mechanism for preventing the uptake of non-nutrient elements, such as lead. Lead may follow the same pathway of uptake as calcium or some other nutrient element. The ionic radius of lead (1.18-1.32 A), is close to the ionic radii of several nutrient elements such as calcium (0.99-1.06 A), potassium (1.33 A), and sodium (0.95-1.0 A) (Nriagu, 1978b). Presumably, the enzyme(s) responsible for uptake of calcium (or perhaps potassium or sodium) cannot discriminate between the nutrient ion and the non-nutrient lead ion. In solution, these elements are all cations. Because their electrical charges are all similar, they could all be taken into fungal cells because of the electronegative charges within the cells. Most cells produce an internal electronegative charge through the

efflux of hydrogen ions. This phenomenon greatly enhances the uptake of nutrient cations. The pH of the medium in which the fungus was growing in the unbuffered experiment dropped in all cases (Fig. 3) as the fungus excreted acids into the medium. Fungi are known to excrete many different organic acids, such as oxalic acid and citric acid (Alexopoulos, 1962). By excreting these acids into the medium, they may be maintaining slightly electronegative charges within their cells.

The excretion of acids and the lowering of the pH may have also enhanced fungal uptake of lead by increasing the solubility of lead in the medium. Figure 4 shows that the fungus took up more lead when the pH of the medium was lowest (3.0 > 4.0 > 5.0), even though all three groups of media had the same amount of added lead. Babich and Stotzky (1979) showed that lower pH levels increased the toxicity of lead to the fungi they studied. Even though my study did not deal with toxic concentrations of lead, pH inversely affected the concentration of lead within the fungus.

The most likely effect pH had in increasing the mycelial concentrations of lead would have been the increased solubility of lead at lower pH levels. However,

the enzyme(s) responsible for the uptake of lead may possibly have greater activities at lower pH levels. Unfortunately, very little is known about the uptake kinetics of lead by fungi (Ross, 1975),

The effect that pH played on the final lead concentrations within the medium in the buffered uptake study is not clear. Figure 5 shows the mean concentrations of lead in the media of the buffered uptake study. The lead concentration was highest in the medium buffered to 5.0 (270 ng Pb/g sol), which was significantly different ($p < 0.01$) from the lead concentrations in the media buffered to 4.0 (240 ng Pb/g sol) and 3.0 (230 ng Pb/g sol). The lead concentrations in the media buffered to 4.0 and 3.0 were not significantly different from each other. Presumably, the lead concentration should have been highest in the medium buffered to 3.0, and it may well have been the highest initally. One possible explanation is that more lead was taken out of solution by the fungus growing in the media with the lowest pH level.

The data of Figure 6 indicate that the addition of as much as 10 ug Pb/g sol had no effect on the growth of Pisolithus. Figure 7, on the other hand, shows that

adding 100 ug Pb/g sol inhibits the growth of the fungus. This figure also shows that the sodium citrate/citric acid buffer inhibits the growth of the fungus as well, even though Smith (1982) found, using the same buffer, that the pH optimum for the growth of Pisolithus is 5.4. My data seem to indicate that lower pH levels would keep more nutrients in solution and allow for increased growth of the fungus.

The final growth study (Fig. 8) not only confirms the results of the previous growth studies, but it also shows a linear decrease in growth of Pisolithus following an increase in the lead concentration of the medium. Using linear regression (Fig. 9), I estimate 1,000 ug /g sol as the concentration of lead in the medium that would completely inhibit the growth of Pisolithus. This estimate differs greatly from the value reported by McCreight and Schroeder (1982), who found that 60 ug Pb/ml completely inhibited the growth of Pisolithus. In their study, lead was added to Hagem's medium as lead acetate. Their control medium with sodium acetate also produced a substantial growth reduction in Pisolithus. Their explanation of the reduction in the growth of the fungus in the control medium centered around the possibility that

metabolized acetate increased the pH of the medium beyond the optimum for growth of Pisolithus. However, when I was determining which buffer I should use to regulate the pH of the medium used in these growth studies, I first tried a 0.2 M sodium acetate/acetic acid buffer. A control medium containing the buffer but no added lead showed complete growth inhibition of Pisolithus, even after three months of incubation. Another experiment using the sodium acetate/acetic acid buffer in liquid Hagem's medium showed that the pH of the medium did not rise above 5.0. No growth occurred in the liquid Hagem's buffered with sodium acetate/acetic acid although a control flask containing the same medium without the buffer showed luxuriant growth of the fungus. These facts tend to refute the explanation of McCreight and Schroeder (1982) and instead seem to indicate that the acetate ion itself is toxic to Pisolithus. Even though I have no direct evidence for the complete inhibition of Pisolithus at a lead concentration of 1,000 ug/g sol, my data show that the growth of the fungus is not arrested at 60 ug/ml. Further growth studies using higher concentrations of lead in the medium must be performed to validate my estimate of the lead concentration that is completely inhibitory to Pisolithus.

The actual concentration of lead that is completely inhibitory to the growth of Pisolithus may well be higher or lower than my estimate. All of the lead added to a nutrient medium does not remain in solution. Ramamoorthy and Kushner (1975) showed that bivalent lead is strongly sequestered by various compounds that are commonly used in nutrient media. Therefore, all of the lead added to a medium is not available to the fungus, and under different conditions from mine, lower amounts of lead in solution could be inhibitory to the growth of Pisolithus. Since lower pH levels increased the uptake of lead in liquid culture, they might be expected to increase the toxicity of lead in Pisolithus (Babich and Stotzky, 1979).

Exactly how lead produces its toxic effect on fungi at the molecular level is presently unknown. Vallee and Ulmer (1972) list a wide variety of possible biochemical effects that lead may have on different types of cells. Due to its affinity for sulfur, lead has been found to complex with the sulfhydryl group(s) of many amino acids. Lead binding to the sulfhydryl group(s) of enzymes may inhibit their activity. This mechanism is thought to be the primary mode of action of several metallic ions in producing fungal toxicity (Somers, 1959). Although the

mechanism is not understood, lead also has been found to bind firmly to cellular and subcellular membranes and subsequently affect their integrity. Lead poisoned human erythrocytes, for instance, leak potassium, which may be a result of the effect(s) lead has on the cellular membrane. Zlochevskaya (1968) found that uracil was released from cells of Aspergillus niger after treatment with lead. Cell walls have been shown to accumulate most of the lead taken up by bacterial cells (Tornabene and Edwards, 1972). Lane, et al. (1978) showed that lead produced a reduction in elongation of Triticum coleoptiles. When they supplied calcium to the cells, they noted a lessening of the lead-induced inhibition of coleoptile elongation. They state that this last observation would indicate a direct competition between ions of calcium and lead for cell wall uptake sites. Substitution of lead for calcium in membranes could produce the "leaking" of cellular constituients as a result of membrane damage (Somers, 1961) because calcium is known to be an important factor in maintaining membrane integrity (Epstein, 1972). Plant cells that are deficient in calcium (especially plant root cells) cannot maintain normal cell homeostasis. Calcium deficiency could be produced in cells as lead replaces calcium on membrane sites through competitive uptake.

Figures 10, 11 and 13 show that from four to six weeks of growth, mycorrhizal roots of Pinus sylvestris took up more lead from the medium than non-mycorrhizal roots. Numerous characteristics of mycorrhizae could help explain this phenomenon. The soil moisture data (Table 3) show that lead added in solution is quickly bound to the surfaces of vermiculite. Vermiculite has a very high net negative charge due to the substitution of aluminum for silicon in its tetrahedral structure (Brady, 1974). This high negative charge binds cations such as lead to the surfaces of vermiculite and effectively removes the cations from solution. The pH of the soil moisture in the mycorrhizal growth vessels (5.6) was significantly lower than the pH of the soil moisture in the non-mycorrhizal growth vessels (6.5). This again is probably a result of acidic compounds excreted by the fungus. Many fungi are known to release acidic compounds such as oxalic and citric acid into the soil solution and thereby solubilize nutrients from parent rock material (Henderson and Duff, 1963). Routien and Dawson (1943) found that mycorrhizae increased the rate of carbon dioxide produced by short roots. In solution, the carbon dioxide would form carbonic acid which would lower the pH of the soil

moisture film surrounding the mycorrhizal root. This reduction in pH would also solubilize some of the lead bound to the vermiculite surfaces. Once a cation is in solution in excess, that is the root is not taking up all of the element in solution, the major factor controlling root uptake is the surface area of the absorbing organ. Mycorrhizae, with their associated wefts of mycelial strands and hyphae, exhibit greater surface area than non-mycorrhizal roots (Reid and Woods, 1969; Skinner and Bowen, 1974). The nutrient absorbing areas of mycorrhizal short roots remain active for a longer period of time than non-mycorrhizal roots (Bowen and Theodorou, 1967). Therefore, as long as lead is in solution, mycorrhizal roots will present a greater amount of active surface area to absorb lead.

The lead that is tightly bound to the surfaces of vermiculite is brought into solution through cation exchange. Cation exchange occurs when one equivalent of a positively charged ion replaces one equivalent of another cation on the surfaces of a negatively charged soil mycelle. The exchange reaction for vermiculite and lead (adapted from Bunzl, 1974) is:

$$[\text{verm.}]Pb^{+2} + 2H^{+} \text{ <-----> } [\text{verm.}]2H^{+} + Pb^{+2}$$

An excess of lead in solution would drive the reaction to the left and an excess of hydrogen ions would drive the reaction to the right. Routien and Dawson (1943) suggest that mycorrhizae increase the absorption of cations by adding to the root's supply of exchangable hydrogen ions. As a plant root exchanges hydrogen ions for lead, depletion zones for lead would be formed around the root. Mycorrhizal roots with mycelial strands and hyphae are able to explore the soil medium more rapidly than non-mycorrhizal roots (Bowen, 1973). Since hyphae are much smaller in diameter than roots or root hairs (2-4 um versus > 10 um), hyphae can grow through the small pores between pieces of vermiculite and exploit them for their bound lead. When I removed the pine seedlings from their hydroponic vessels, I noticed that the mycorrhizal roots had far more pieces of vermiculite attached to them than the non-mycorrhizal roots. In fact, some of the mycorrhizal roots had completely surrounded pieces of vermiculite with their mycelia. Through cation exchange, the hyphae could absorb lead from the pieces of vermiculite they surrounded and perhaps transfer the element to the plant root.

Unfortunately, in these studies, I could not determine where lead is sequestered in a mycorrhizal root. Lead could be bound to hyphal membranes, the fungal mantle, within the Hartig net, or in some component of the higher plant root. Studies with non-mycorrhizal plants have shown that most of the lead taken up by them is sequestered in the roots and not translocated to the above ground portions (Tso and Fisenne, 1968; Athalye and Mistry, 1972; Jones, et al., 1973). My data (Fig. 14) show that mycorrhizal plants probably exhibit the same phenomenon. Other studies on lead uptake in non-mycorrhizal plants have shown that lead is specifically sequestered in the cell wall components of plant roots (Lane, et al., 1978; Malone, et al., 1974). An interesting continuation of this research could involve the separation of a mycorrhizal root into its components to see if the fungal portion of the mycorrhiza acts as a barrier to the transfer of lead to the higher plant root. The above experiment may need to be performed with radioactive lead (^{210}Pb) because handling of the mycorrhizae and separating it into its components could introduce substantial contamination into the sample.

The hydroponic solution used in this study contained monocalcium phosphate (0.4 g/l) and calcium sulfate (0.4 g/l; see Appendix). Therefore, calcium was probably not limiting to the growth of the plant. Simon (1978) found a relationship between the amount of exchangable lead to calcium and the degree of lead tolerance in plant populations growing near lead spoils. The more calcium in the soil, the greater the degree of lead tolerance in the plants. This may be a result of calcium raising the pH of the soil moisture or competition for uptake sites on root surfaces between these two chemically similar cations. Again, it would be interesting to repeat these experiments and adjust the amount of calcium in the solution to see if the effect of ectomycorrhizae on the uptake of lead would be enhanced. One could buffer the pH of the soil moisture with something other than calcium to test the effect of pH on the uptake of lead by mycorrhizal and non-mycorrhizal roots. Furthermore, my studies were only of six weeks duration. Previous experiments using the hydroponic growth vessels with the same amount of vermiculite and hydroponic solution as used in this research, showed that the seedlings could grow for approximately three months without additional water. As the hyphae of mycorrhizal

roots grow into more of the vermiculite medium in search of nutrients and water, the differences in the lead concentrations between mycorrhizal and non-mycorrhizal roots may become more pronounced over time.

This study shows that mycorrhizal roots take up more lead than non-mycorrhizal roots grown in an artificial system; but how does this study relate to a natural system? In terrestrial ecosystems, most of the lead in soils is anthropogenic and the majority of anthropogenic lead is confined to the humus-rich upper layers of soil. Humus is a very efficient complexing agent of metallic ions such as lead. It helps to keep lead available for plant uptake rather than having lead form insoluble precipitates (Stevenson, 1972). Cation exchange can occur on humus just as it can on clays such as vermiculite. Since mycorrhizal roots inhabit the upper soil horizons, they could easily exchange hydrogen ions for lead from the surfaces of humus. The production of organic acids in the rhizosphere by organisms such as bacteria is known to facilitate cation uptake. Some evidence indicates that mycorrhizae support larger populations of bacteria in the rhizosphere (mycorrhizosphere) than non-mycorrhizal plants (Barea, et al., 1975). The greater amounts of exuded

acids produced by mycorrhizal fungi and the bacteria they help support would increase the solubilization of lead from organic chelates such as humus in the upper soil horizons. This phenomenon would not be limited to organic substrates, but would also occur in mineral soils that contain lead. In his lengthy article on plant colonization studies of coal spoils in Pennsylvania, Schramm (1966) shows graphic evidence of how Pisolithus in particular obtains mineral nutrients from inorganic soils and passes these elements to its higher plant host. Schramm was able to trace rhizomorphs of Pisolithus from the mantle of mycorrhizal pine roots down into the interlamellar cracks of blocks of shale. He was able to split the blocks of shale and directly observe the growth of the fungus into the substrate. The rhizomorphs, with their associated wefts of hyphae, formed an irregular open net between the cracks of shale. More than likely, the acids secreted by the numerous hyphae of the fungus were able to solubilize elements directly from the shale and pass them on to the host seedling. In this way, seedlings were able to grow in this extremely harsh environment.

Pisolithus seems to be one of the most versatile fungi for forming ectomycorrhizal associations with seedlings in

harsh environments. As mentioned previously, Pisolithus is cosmopolitan in distribution. In fact, although the fungus can be found in forest communities, many of the reported sightings of Pisolithus have occurred in harsh environments such as coal spoils, eroded copperbasins and burrow pits, where the mycorrhizal seedlings were growing on substrates which contain little or no organic matter (Marx, 1977). Marx and Bryan (1975) describe seeing large numbers of Pisolithus basidiocarps (as many as 300 per hectare) under young pines growing on coal spoils in the northestern and Appalachian regions of the United States. Since the pioneer seedlings on these sites are growing on soils with no cover, the surfaces of the soil reach temperatures that thermally stress these plants (Schramm, 1966). One of the major reasons why Pisolithus is able to live and form mycorrhizae in such communities lies in its ability to withstand broad temperature fluctuations. Lamb and Richards (1974) found that Pisolithus is capable of continuous growth at 40° C. They also found that Pisolithus basidiospores could successfully germinate in a medium buffered to low pH levels. Marx and Kenny (1982) state that Pisolithus ectomycorrhizae on pine and oak have been observed on coal spoils ranging in pH from 2.6 to

8.4. The great adaptability of this fungus has prompted commercial use of Pisolithus inoculum in forestry applications, especially in reforestation of disturbed ecosystems.

Went and Stark (1968) proposed that, in the tropical forests of the Amazon, mycorrhizae directly transfer nutrients from litter (which the mycorrhizae may help break down) to the higher plants. The "direct mineral cycling theory" helps explain how such a lush growth of vegetation can be produced on such infertile soils as those found in tropical rain forests. The majority of nutrients that are tied up in the litter are rapidly absorbed by plants through mycorrhizae, before substantial amounts of the elements are leached out of the soil and removed from the system through the ground water. Although the direct cycling theory has not been expanded to incorporate temperate forest systems, I think the biogeochemical implications for the direct cycling of lead would be immediately clear should the theory be validated for non-tropical forests. Since all atmospheric lead is introduced into a plant community through the litter layer, mycorrhizae could have direct access to this lead if Went and Stark's theory is correct. Even if the fungal

component of mycorrhizae is not responsible for the decomposition of plant litter, the bacterial flora the mycorrhizae support may be capable of breaking down plant debris and solubilizing lead which would then be accessible to the mycorrhizae.

Terrestrial ecosystems, especially in the northern hemisphere, are becoming increasingly contaminated with lead as a result of the activities of man. Unfortunately, man's use of this element has produced such widespread contamination that, even if anthropogenic emissions of lead to the atmosphere were to cease immediately, soils have sufficient concentrations of lead that mobilization and uptake of lead by plants, expecially mycorrhizal plants, can be expected to continue for decades. The effect(s) that progressively higher root concentrations of lead may have on plants is presently unclear. The possible effects may be limited to plant roots, where most of the lead taken up by plants is sequestered. However, as the lead concentrations of roots increase beyond what could possibly be sequestered there, greater amounts of lead may be translocated to the above ground portions of the plants. If sufficient amounts of lead are translocated to the above ground portions of plants,

photosynthesis may be affected (Bazzaz, et al., 1975; Miles, et al., 1971). The only way to determine what effects increased lead uptake may have on plants is to continue studies in a controlled, clean lab environment that reproduces ambient lead concentrations that existed prior to man's use of this metal. A controlled atmosphere where lead concentrations are very low produces the only realistic control for studies that attempt to determine the effects of lead on plants.

Summary

The data presented herein indicate that mycorrhizae facilitate the uptake of lead by Pinus sylvestris roots. Their ability to do so is apparently a function of the greater surface area and exploration of soil surfaces as well as the increased production of lead-solubilizing acids exhibited by mycorrhizal roots. In the natural world, mycorrhizal roots can be expected to take up greater amounts of lead than non-mycorrhizal roots from a variety of lead-complexing soil components for the previously mentioned reasons. In addition, the greater numbers of other microorganisms found in the mycorrhizosphere can be expected to aid in solubilizing lead in natural soil systems. Although mycorrhizal roots of Scotch pine took up more lead from the vermiculite/hydroponic solution mixture than non-mycorrhizal roots, this additional lead was apparently not translocated to the above ground portions of the seedlings, at least not in the six-week period of this study. Further research is needed to determine if this increased uptake of lead by mycorrhizal roots becomes more

pronounced over greater periods of time and whether or not the increased concentrations of lead in mycorrhizal roots can eventually affect metabolic processes in the above ground portions of plants.

Additional data in this study indicate that Pisolithus tinctorius readily takes up lead from an aqueous solution. The major factor that determines the amount of lead taken up by the fungus is the concentration of lead within the medium. Furthermore, the major factor that determines how much lead that is added to a nutrient medium remains in solution (and is available for uptake by the fungus) is the pH level of the medium. Lastly, data from the growth studies in this research show that, contrary to the literature, the growth of Pisolithus is not completely arrested at a lead concentration in the medium of 60 ug/g sol, but is more than likely completely arrested at a lead concentration much higher than that, probably approaching 1,000 ug/g sol.

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Appendix

Table A1: Formula for modified Hagem's medium (Palmer, 1971).

Agar (when used)	15.0 g
Malt extract	5.0 g
Glucose	5.0 g
Potassium phosphate	0.5 g
Magnesium sulfate	0.5 g
Ammonium chloride	0.5 g
Iron chloride (1% sol)	0.5 ml
Biotin (5.0 ug/ml) and Thiamine HCl (1.0 mg/ml)	0.5 ml
Water (QDW)	1,000 ml

Table A2: Formula for U. S. D. A. (Maryland) Hydroponic medium (Douglas, 1976).

Potassium nitrate	0.53 g
Ammonium sulfate	0.09 g
Monocalcium phosphate	0.22 g
Magnesium sulfate	0.40 g
Calcium sulfate	0.44 g
Trace elements	0.02 g
Water (QDW)	1,000 ml

Trace elements

Iron sulfate	113 g
Manganese sulfate	7.5 g
Copper sulfate	3.5 g
Sodium tetraborate	85 g
Zinc sulfate	3.5 g

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