INFLUENCE OF *Pisolithus tinctorius* AND MINE SPOIL ON THE GROWTH OF VIRGINIA PINE SEEDLINGS

by

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INTRODUCTION

The importance of mycorrhizae to forest trees is widely accepted and understood. Studies have shown that a prerequisite to healthy and vigorous growth of many trees is the presence of mycorrhizae on their roots. Certain species, especially those of genus Pinus, have an obligate requirement for a mycorrhizal association in order to survive (Marx, 1975a). The beneficial attributes of this symbiotic association to both the tree and the fungus have been extensively researched and discussed by many (Hatch, 1937; Hacskaylo, 1972; Richards, 1974; Marx, 1975b).

Virtually all trees have the capacity to form mycorrhizae, and a nonmycorrhizal tree is the exception rather than the rule. Accordingly, mycorrhizal associations occur in wide abundance throughout the world, as very few natural areas are devoid of indigenous mycorrhiza forming fungi.

Strip-mined areas present a unique exception to the rule of the ubiquity of mycorrhizae. Due to the disruption and mixing of strata during strip mine operations, resulting surface spoils are atypical with respect to their physical, chemical and biological properties. Recently strip-mined sites are often rocky, steeply sloped, and covered with materials of variable weathering rates. Growth-limiting elements for plants, such as sulfur, aluminum and manganese, may be present in toxic amounts. Moreover, essential elements for normal plant growth may be deeply buried, relegating the status of surface mine spoil to that of a biological desert (Marx, 1975a).

In his research on the colonization of mining wastes in Pennsylvania, Schramm (1966) observed that the tree seedlings displaying the best survival and growth were either leguminous species or highly ectomycorrhizal. Since only a few species of ectomycorrhizal fungi were found, Schramm hypothesized that high spoil temperatures limited fungal colonization and population diversity in the spoil material. Marx (1975a) and his associates have been involved with the outplanting of mycorrhizal pines on strip-mine sites from Pennsylvania to Florida since 1973. In most cases, mycorrhizal seedlings survived and grew better than nonmycorrhizal seedlings. Both Schramm (1966) and Marx (1975a) concluded that one of the ectomycorrhizal fungi best adapted to mine spoil was *Pisolithus tinctorius* (Pers.) Coker and Couch, a Basidiomycete.

The value of mycorrhizae to successful afforestation has been demonstrated by Vozzo and Hacskaylo (1971). Problems and failures encountered in reclamation attempts involving afforestation in the past may be overcome not only by the careful selection of tree species capable of adapting to adverse spoil conditions, but by employment of adaptable ectomycorrhizal fungi as well. The containerized seedling system may prove to be a useful regeneration tool, whereby specific tailoring of planting stock can be achieved to aid seedlings in their adaptation to variations of site, climate and other environmental conditions (Tinus et al., 1974).

Tailoring of planting stock means applying the necessary treatment and quality control to seedlings during rearing to better enable the

stock to adapt to its planting site. Typical examples are controlling light and temperature conditions, and using certain fertilizer programs. It is also feasible to consider mycorrhizal inoculation and container substrate mixes in this concept of tailoring seedlings. Balmer (1976) discussed the advantages of the containerized system and noted the growing needs for research and information concerning use of the system on disturbed areas. Marx and Barnett (1974) reported various techniques for inoculating containers with *Pisolithus tinctorius*. They strongly encouraged research on mycorrhizae in the containerized seedling system due to the limited amount of available published information.

The objectives of this study were to determine the influence of: (1) mine-spoil additions to a typical peat:vermiculite containerized seedling substrate and (2) different times of artificial inoculation (before, at, and after seed planting) with the ectomycorrhizal symbiont, *Pisolithus tinctorius*, on the survival and growth of containerized Virginia pine (*Pinus virginiana* Mill.) seedlings. The results of this study will provide insight into the production of mycorrhizal container stock for use in reclaiming mine spoils and other disturbed surface areas.

LITERATURE REVIEW

Mycorrhizae and Mine Spoil

Hacskaylo and Tompkins (1973) have provided a comprehensive bibliography of mycorrhizal literature. Several excellent texts are also available (Sanders et al., 1975; Marks and Kozlowski, 1973; Hacskaylo, 1971; Harley, 1969; Shemakhanova, 1962). Many reports on the widespread associations of mycorrhizal fungal symbionts and host trees have been made, including an extensive compilation by Trappe (1962).

Mycorrhizal associations are the most common form of symbiosis in the plant world, however, not all mycorrhizal associations are equally efficient and beneficial to the symbionts. Since certain mycorrhizal fungi are more adapted to certain sites than others, Marx (1975b) hypothesized that trees in association with adapted fungi will survive and grow better than those with non-adapted fungal symbionts on the same site. Strip-mined sites often present adverse conditions for supporting plant growth and microbial populations. Soil-related factors including nutrient availability, extremes of pH, high temperature, micronutrient toxicity, poor aeration and low levels of organic matter or available water will influence mycorrhizal symbiosis by affecting fungal activity and tree root development.

One species of ectomycorrhizal fungi that is well adapted to mine spoils is *Pisolithus tinctorius*. It has been reported on spoils and adverse sites in the United States by several researchers (Marx, 1976; Medve et al., 1976; Marx, 1975a; Hile and Hennen, 1969; Schramm, 1966;

Lowy, 1964; Lampky and Peterson, 1963). The widespread occurrence of *P. tinctorius* on adverse sites is probably due to its high temperature optimum or range for culture and mycorrhizal synthesis, comparable to surface and subsurface temperatures on mine spoils.

Schramm (1966) recorded midday temperatures on mine spoil ranging from 30°C to 67°C. At 2-3 cm depth, spoil temperature averaged about 42°C. Marx, Bryan and Davey (1970) demonstrated that *Pisolithus tinctorius* formed increasingly more ectomycorrhizae on loblolly pine (*Pinus taeda L.*) as soil temperatures increased to 34°C. *(Thelephora terrestris* (Ehrh.) Fr., one of the most widespread ectomycorrhizal fungi in the United States, did not form mycorrhizae at 34°C, and formed more at 10°C than at 24°C. Marx and Bryan (1971) later reported greater survival of *Pinus taeda* seedlings ectomycorrhizal with *Pisolithus tinctorius* than with *Thelephora terrestris* or controls at 40°C. Furthermore, seedlings ectomycorrhizal with *P. tinctorius* at 40°C grew as well as those at 25°C.

Hacskaylo, Palmer and Vozzo (1965) reported that optimum temperatures for the culture of six other fungi known to be ectomycorrhizal with *Pinus virginiana* ranged from 13°C to 29°C. Results indicate that *Pisolithus tinctorius*, unlike most ectomycorrhizal fungi in that it has higher than average optimum temperatures for culture and synthesis, is more compatible to mine spoil than other fungi.

Marx (1976) presented data and results from several trial plantings of pine seedlings ectomycorrhizal with *Pisolithus tinctorius* and *Thelephora terrestris* on mine spoils in Kentucky, Virginia, Tennessee,

and Georgia. In all cases, survival and growth of seedlings were enhanced due to mycorrhizae, although the *Pisolithus* seedlings far surpassed the *Thelephora* seedlings. Marx reported the ability of *Pisolithus* to rapidly colonize control seedlings on spoils due to airborne basidiospores and from hyphal or rhizomorphic extension through the soil. Planted seedlings originally mycorrhizal with *T. terrestris* had an abundance of *P. tinctorius* mycorrhizae as well after one to two years. Marx (1976) strongly urged the development of mycorrhizal inoculation in reclamation programs, and gave specific attention to the use of *Pisolithus*.

Medve, Hoffman and Gaither (1976) recognized the value of mycorrhizae in mine spoil revegetation success. They noted the scarcity of mycorrhizae in highly fertile soils and their absence in sterilized soils, and they suggested that the practice of heavily fertilizing and sterilizing nursery seed beds would be detrimental to mycorrhizal formation on seedlings. Due to lack of mycorrhizae, seedlings produced under such nursery programs may not be able to survive outplanting on strip-mine spoils. Moreover, heavy fertilizing of spoils may promote good initial growth of nonmycorrhizal seedlings, but would discourage mycorrhizal formation. After discontinuation of fertilizer programs, the lack of essential mycorrhizal roots could account for low survival rates of seedlings in the second and third year of revegetation. The results of Medve et al. (1976) indicated that *Cenococcum graniforme* and *Pisolithus tinctorius* appeared to be appropriate both in their adaptation to mine spoil conditions and as symbionts for mycorrhizal formation.

Reclamation and Virginia Pine

Virginia pine is highly compatible for reclamation of strip mines because of its silvical characteristics and known ectomycorrhizal association with *Pisolithus tinctorius* (Marx and Bryan, 1969). Virginia pine grows well on a wide variety of sites and is especially well adapted to dry, rocky, and otherwise poor sites. The species is very tolerant of high soil acidities, and is also a hearty pioneer of abandoned land and old fields (Folwells, 1965).

Several researchers have reported favorable results with Virginia pine for mine spoil reclamation. It was considered to be the most acid-tolerant of the pines tested by Miles et al. (1973) in Pennsylvania. Plass (1974) evaluated Virginia pine as the pine species least affected by the overall chemistry of twelve spoil materials tested in Kentucky. Schramm (1966) observed the superior resistance of Virginia pine to heat damage on the anthracite spoils of Pennsylvania.

Virginia pine is within the mycorrhizal host range of *Pisolithus tinctorius*. In a study comparing the degree of ectomycorrhizal development by *P. tinctorius* on different conifer hosts, Marx and Bryan (1969) found the greatest association on Virginia pine. Marx, Bryan and Cordell (1976) demonstrated growth increases of approximately 100 percent on Virginia pine nursery seedlings after one season, following soil inoculation and ectomycorrhizal development by *P. tinctorius*. Marx (1976) has found Virginia pine in association with *P. tinctorius* on several eastern mine spoils with soil reaction as low as pH 2.9.

Virginia pine is commercially important as a pulpwood species.

The natural range of Virginia pine is also superimposed over a vast coal mining region. Plass (1973) indicated the importance of genetic variability due to provenance on the growth and survival of Virginia pine on strongly acid spoils. He recommended the establishment of breeding programs to produce genotypes specifically adapted to adverse sites.

Containerized Regeneration

A chief concern in artificial regeneration is the economical production of biologically sound planting stock. Research efforts have been aimed at attaining a high level of quality control in growing containerized seedlings. Greenhouse container stock has been compared to nursery-grown seedlings in several studies. Most researchers agree on the better performance of container stock, due to greater control in the manipulation of environmental conditions to produce seedlings of desired shape, form and physiological state for outplanting (Tinus, 1974b; Kinghorn, 1974; Brix and van den Driessche, 1974).

Balmer (1976) surveyed the status of containerization for disturbed surface areas and mentioned projects underway at several locations. Miller and Budy (1974) recorded high survival rates of containerized Jeffrey pine seedlings planted on poor sites with shallow soil profiles, excessive drainage, steep slopes or little precipitation. Davidson and Sowa (1974) reported good performance of red pine in containers in afforestation trials on Pennsylvania coal spoils. Aldon (1973) achieved high survival success with container grown pinyon pine on spoils in the southwest.

Brix and van den Driessche (1974) discussed the development of seedling quality during the production of container stock, and stressed the importance of drought resistance. Mycorrhizae increase root surface area and thereby facilitate water and nutrient absorption, thus increasing the ability of trees to withstand drought (Hatch, 1937; Safir et al., 1972; Marx, 1975a). Containerized seedlings can achieve these benefits through inoculation and association with mycorrhizal fungi (Bergstrom, 1976; Marx, 1976; Marx and Barnett, 1974).

Along with mycorrhizal inoculation, research on substrate mixes for containers is necessary. Although several commercial mixes are available, a peat and vermiculite substrate is used widely in containerized seedling systems (Tinus, 1974a; Brix and van den Driessche, 1974). Marx and Barnett (1974) tested a topsoil:peat:sand substrate against a commercial peat:vermiculite:perlite mix in their mycorrhizal inoculation study with containerized loblolly pine. Although seedlings grown in the commercial mix were largest, greatest ectomycorrhizal development by *Pisolithus tinctorius* occurred in the containers of soil substrate with low fertility levels. It is well understood that high soil fertility inhibits mycorrhizal development (Hacskaylo and Snow, 1959; Meyer, 1973; Richards, 1974).

Mycorrhizal Synthesis

Mycorrhizal synthesis and fungal culture have been extensively researched (Palmer, 1971; Harley, 1969; Shemakhanova, 1962; Marx and Zak, 1965; Hacskaylo, 1953). Several factors, including nutrition, light, pH, temperature, and moisture have been reported as important

in the culture of mycorrhizal fungi and in the establishment of mycorrhizal symbiosis. Two factors infrequently considered or studied are the timing of application of inoculum and substrate moisture.

It is an accepted principle that mycorrhizal fungi have a dependence on their host trees, especially in soils low in available nutrients. Most mycorrhizal fungi differ from the majority of the lignicolous and cellulose-destroying fungi in that they lack the ability to produce cellulase or have very little capacity, if any to form adaptive enzymes for cellulose decomposition (Harley, 1969; Melin, 1962). It seems unlikely then that mycorrhizal fungi could satisfy their carbohydrate requirement from the humus or litter of forest soils as the lignin and cellulose-destroying fungi do. Consequently, mycorrhizal fungi obtain their needed carbohydrate through symbiosis with plant roots (Hacskaylo, 1972). Hacskaylo (1965) supported this idea by demonstrating the dependence on photosynthesis of Virginia pine seedlings for sporophore formation of *Thelephora terrestris*. Exudates from tree roots, including carbohydrates, amino acids, and organic acids, are among the metabolites that may be utilized by fungi and other microorganisms in the rhizosphere (Hacskaylo, 1973). Vitamins are also important constituents of root exudates (Rovira and Harris, 1961), and most mycorrhizal fungi are heterotrophic for Vitamin B_1 (Palmer, 1971; Melin, 1953). It is necessary that seedlings have healthy root systems capable of providing metabolites to aid the viability and vigor of the mycorrhizal fungi, and to foster subsequent formation of mycorrhizal roots.

Most researchers inoculate experimental substrates at seed planting or soon thereafter. Inoculation prior to planting has been considered in very few studies. Lamb and Richards (1974a) inoculated substrates at seeding and at later intervals, and found that delayed inoculation reduced the proportion of roots that became mycorrhizal. Seedlings from delayed inoculations were phosphorous deficient and soon ceased growth due to suberization of short roots and consequent poor mycorrhizal formation. In a later study, Lamb and Richards (1974b) reported that delayed inoculation resulted in decreased yield or dry matter production. Lamb and Richards (1974a) stressed the necessity for inoculation at seeding, and concluded that survival of mycorrhizal fungi in the soil in the absence of the host may be a critical factor in inoculation programs. Mikola (1973) voiced a similar opinion in his discussion on the utilization of mycorrhizal symbiosis in forestry practice.

Shemakhanova (1962) considered the age of both the seedling and the fungal culture in her mycorrhizal synthesis. Her results showed that two-week-old seedlings inoculated with one-month-old cultures provided the best conditions for mycorrhizal formation. Virtually no mycorrhizae were formed on inoculated one-month-old seedlings, due to root suberization which prevented hyphal penetration. Shemakhanova added that the use of fungal cultures within the age range of two weeks to one month provided results with no significant differences.

Soil moisture is also an important factor to consider in mycorrhizal synthesis. Mexal and Reid (1973) indicated that growth of fungi

over a range of soil moisture tension is often normal in that it is inhibited at very high and very low levels. Similarly, Shemakhanova (1962) discussed findings in which mycorrhizae were least abundant at very high and very low soil moisture retention.

Worley and Hacskaylo (1959) studied the effect of available soil moisture on the mycorrhizal association of Virginia pine. They observed that the incidence of a white mycorrhiza increased with increasing soil moisture, while that of a black mycorrhiza increased with decreasing soil moisture. They also noticed that the effect of amount of available moisture depended on seedling development. Well-developed root systems, favoring mycorrhizal formation by both the white and black fungi, were obtained under favorable moisture conditions.

Typically in aseptic synthesis, many researchers use amounts of moisture (nutrient solutions) that result in extremely high levels of availability, well above field capacity. Although this procedure is a safeguard in that it insures moisture availability throughout the duration of the experiment, the resulting condition would not simulate field conditions. It is important to design vessels for monoxenic mycorrhizal synthesis with a provision for adding sterile water to better control substrate moisture.

MATERIALS AND METHODS

To meet the objectives of this study, the following three experiments were conducted.

EXPERIMENT IA. Monoxenic culture tubes were inoculated with *Pisolithus tinctorius* (Pt) vegetative mycelium either three weeks before seeding, at seeding, or three weeks after seeding of Virginia pine. A non-inoculated control was included. The substrate used was peat:vermiculite (PV) mixture (1:19 v/v). The four treatments were replicated 20 times in a one-way factorial design.

EXPERIMENT IB. The influence of mine spoil ammendments to container substrate was studied by utilizing three mixes. The first was a 1 to 19 (v/v) mixture of peat:vermiculite (PV). The second mix consisted of 3 parts PV to 1 part mine spoil (v/v), designated as PVS3. The third substrate was a 1:1 (v/v) mix of PV to mine spoil, designated as PVS1. Substrates were either inoculated with Pt at seeding for monoxenic synthesis or non-inoculated controls. The six substrate and inoculation treatment combinations were replicated 20 times in a two-way factorial design.

EXPERIMENT II. The influence of minespoil substrate and Pt inoculation on Virginia pine seedlings under open-air greenhouse conditions was studied. Substrate types included PV and PVS3. Substrates were either inoculated with Pt at seeding or non-inoculated controls. A two-way factorial design was used, with 20 replications per treatment combination.

The mycorrhizal synthesis procedures recommended by Hacskaylo (1953), Marx and Zak (1965), and Wilcox and Ganmore-Neumann (1975) were used with some modifications developed in this study.

Inoculum Preparation

From a Pt isolate[1], starter plates and three series of transfer plates of vegetative mycelium were cultured on Hagem agar[2]. After the third transfer, plates were incubated approximately one month at room temperature until used in the experiments. Five days before inoculation in Experiments IA and IB, mycelium agar discs were removed from the periphery of plate cultures with a flamed #6 cork hole-borer and placed on plates of fresh Hagem agar. Hyphal expansion proceeded 1-2 mm from the edges of discs, prior to using the discs as inoculum.

For Experiment II, mass inoculum was prepared by placing eight mycelium agar discs from one-month-old cultures of Pt into 2-liter Erlenmeyer flasks containing a mixture of 1520 cc vermiculite, 80 cc ground peat moss and 800 ml modified Melin-Norkrans nutrient solution (MMN) (Marx, 1969). MMN was further modified for inoculum preparation by doubling the amounts of glucose and thiamine (Appendix I). Flasks with substrate and MMN were autoclaved at 121°C for 45 minutes prior

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[1] Isolate 185 of *Pisolithus tinctorius* obtained in 1976 from Dr. D. H. Marx, USDA Forest Service, Athens, Georgia. Originally isolated from loblolly pine ectomycorrhizae in November, 1975.

[2] Formula obtained by personal communication in 1976 from Dr. O. K. Miller, Professor of Botany, Virginia Polytechnic Institute and State University, Blacksburg, Virginia. (See Appendix I.)

to implanting discs. After 15 weeks at room temperature in darkness, sufficient mycelial growth occurred throughout the peat:vermiculite to provide ample inoculum. Uninoculated flasks were made up as control inoculum.

Container Design

To maintain aseptic conditions and yet model a containerized seedling system, glass tubes were used as containers in Experiments IA and IB. The 4.5 x 30 cm tubes totally enclosed the seedlings, and were stoppered with cotton and loosely covered with aluminum foil above and plugged with corks below. Tygon tubes (4 mm diam.) extended through the cotton stoppers to permit additional watering using a sterile hypodermic syringe. The tops of watering tubes were plugged with cotton. In Experiment II, 3 x 15 cm glass tubes were used as containers. Nylon screen discs (1.5 mm mesh) were secured to tube bottoms with an adhesive. Wooden racks were constructed to enclose root systems in darkness.

Container Substrate and Moisture

Tubes in Experiment IA contained 150 cc of PV at pH 5.1 (Marx and Zak, 1965) and 75 ml of MMN. This substrate mixture contained 45 ml of available moisture between one-tenth bar and 15 bars moisture tension, based upon analyses using a pressure membrane extractor. The three substrate types in Experiment IB contained 150 cc per tube of either PV, PVS3, or PVS1, all at pH 5.1, similar to the mine spoil pH (Appendix II). The spoil material was previously passed through a 5 mm mesh sieve. The pressure membrane extractor was used to construct moisture retention curves for each substrate (Appendix III). Using

these data, initial substrate moisture conditions in Experiment IB of 1/10 bar for PV and 1/3 bar for PVS3 and PVS1 were produced by adding 70 ml, 56 ml and 50 ml of nutrient solution to each tube containing the respective substrates. Concentrations of nutrients in the solutions were adjusted so that each tube received the nutrients normally present in 75 ml of MMN. Available moisture between 1/10 and 15 bars tension was 45 ml for all substrates in IB. Tubes for IA and IB containing substrate and MMN were autoclaved at 121°C for 45 minutes.

In Experiment II, the tubes contained 64 cc of either PV or PVS3, plus 20 cc of either control or Pt mass inoculum according to treatment design. Based on pressure membrane extractor data, the addition of 32 ml of MMN to each tube produced initial substrate conditions of 1/10 bar for PV and 1/2 bar for PVS3. Substrate materials and MMN were autoclaved separately prior to placement into the tubes. Glucose and thiamin were deleted from the MMN formula in order to discourage pioneer colonization by contamination organisms. The tubes were mulched with autoclaved perlite to a depth of 1 cm to deter moisture loss.

Seed Preparation and Planting

Stratified Virginia pine seed was surface-sterilized for 45 minutes in 35 percent hydrogen peroxide (Trappe, 1961). Seed were sown on corn meal agar (Bryan and Zak, 1961) in petri plates and incubated at 23°C five days prior to use. Contaminated plates were discarded. At respective sowing times, seeds with radicles elongated approximately 2-5 mm were transferred under sterile conditions from the plates to the glass tubes in Experiments IA and IB. Seeding for Experiment II was

done on an open lab bench. One seed was planted per tube at a depth of about 5 mm.

Inoculation

At the time of inoculation in Experiments IA and IB, three inoculum agar discs of Pt vegetative mycelium were lifted and transferred under sterile conditions to the glass tubes. Inoculum discs were implanted evenly around the seedling, seed or site of seeding (depending on treatment) at a depth of 1 cm. Controls were not inoculated, nor were sterile agar discs implanted, as this has been determined unnecessary by other researchers (Wilcox and Ganmore-Neumann, 1975).

At the time of establishment of Experiment II, 20 cc of either control or Pt mass inoculum were mixed with 64 cc of respective treatment substrates. Autoclaved MMN (less glucose and thiamine) were then added to the tubes as previously described. Prior to inoculation, each flask of mass inoculum was leached with approximately 8 liters of cool running tap water between layers of cheesecloth. Marx and Bryan (1975) indicated that nonassimilated nutrients must be leached from inoculum to deter colonization by pioneer contaminants.

Growth Conditions

The tubes for Experiments IA and IB were randomly placed under Gro-Lux lights (500 ft. candle intensity)[1] on a 12-hour photoperiod at room temperature for seven days. The tubes were then moved to a pre-

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[1] Intensity measured within the seedling foliage canopy using the LI-185 Quantum/Photometer/Radiometer manufactured by Lambda Instruments Corporation, Lincoln, Nebraska.

viously unavailable growth chamber under a 16-hour photoperiod comprised of a mixture of fluorescent (1700 ft. candles) and incandescent (150 ft. candles) light. Thermoperiod was 14 hours with a 25°C day and a 17°C night. Due to a growth chamber malfunction after 33 days, the tubes were placed back under Gro-Lux lights (600 ft. candles) on a 16-hour photoperiod at room temperature for eight days. Thereafter the tubes were transported back to original growth chamber conditions for the remainder of the experiment. Moisture conditions were monitored every 10 to 14 days by weighing randomly chosen tubes. No additional watering was necessary throughout the duration of the experiment.

Experiment II was conducted under open air conditions in a greenhouse. Natural light was supplemented with incandescent light (200 ft. candles intensity) on a 16-hour photoperiod. Temperatures ranged from 10 to 43°C. The tubes were watered with tap water as needed. All experiments proceeded 90 days from seeding. Tube locations were randomized four times at approximately even intervals.

Data Collection

Upon termination of all experiments, survival per treatment was recorded. The extent of fungal colonization by Pt throughout the substrate in each tube was evaluated under 10x magnification, with a binocular scope. Bits of substrate from each tube in Experiments IA and IB were plated under sterile conditions on Hagem agar for re-isolation of Pt and to assess sterility of controls and the presence of contamination. Substrate materials in Experiment II were not plated. Shoot height and diameter at the root collar were determined for 15

randomly chosen seedlings in each treatment cell. After washing the substrate material from roots with a mild detergent solution and several water rinses, percent mycorrhiza was assessed. Percent mycorrhiza was determined directly on two seedlings by counting the number of mycorrhizal short lateral roots as compared to the total number of short laterals (Richards and Wilson, 1963; Marx and Bryan, 1975) under 6x magnification. Percent mycorrhiza on the other 13 seedlings was determined by ocular estimate at 6x magnification. The visible presence of a dense fungal mantle was the criterion for mycorrhizal infection (Theodorou and Bowen, 1965; Marx et al., 1970). Several cross-sections of infected and non-infected roots were examined microscopically for mantle formation and Hartig-net development to confirm mycorrhiza formation. Seedling shoots and roots were placed in an oven for 48 hours at 65°C, and then dry weights were obtained. Analysis of variance was run for each experiment and significant treatment differences were evaluated by Duncan's multiple range test (Sokal and Rohlf, 1969; Barr et al., 1976).

From the remaining five seedlings in each treatment cell, representative mycorrhizae were excised, surface-sterilized in either 100 ppm mercuric chloride (Experiment IA and IB) (Marx, Bryan and Grand, 1970) or 1 percent sodium hypochlorite (Experiment II) (Zak and Bryan, 1963), and placed individually on Hagem agar for re-isolation of Pt. A modification of the reisolation technique of Zak and Bryan (1963) was tested in Experiment II. Rather than subjecting the mycorrhizas to a prolonged sterilization and repeated rinses, mycorrhizas were immersed

in 1 percent sodium hypochlorite for 30-60 seconds, and then transferred directly to agar with flamed forceps. It was assumed that the residual sterilant on the mycorrhiza would prevent growth of surface contaminants, and yet allow subsequent hyphal expansion from within the rootlet. Other mycorrhizas were excised from roots and transferred directly to agar (Pearson and Read, 1973). Thirty milligrams streptomycin were added per liter of Hagem agar in Experiment II for control of bacterial contaminants.

RESULTS

Survival of Virginia pine seedlings in all three experiments was excellent, ranging from 90-100 percent (Table 1). Seedlings of Experiment IA and IB appeared normal in color and vigor. Although Experiment II seedlings were slightly chlorotic with some brown needle tips, survival was 100 percent in each treatment.

Based on subjective assessment using a dissecting scope at 10x, substrate colonization by Pt, and hence success of inoculation ranged from excellent to poor among the treatments in the three experiments (Table 2). In Experiment IA, inoculating at seeding or three weeks after seeding resulted in lesser colonization than when inoculating three weeks before seeding. Hyphal growth decreased considerably in Experiment IB as the ratio of spoil material in the substrate was increased. Colonization of Pt in Experiment II was only slightly decreased in the mine spoil substrate. Several of the inoculated tubes in IA and IB had a strikingly visible presence of hyphae permeating the substrate or fuzzing at the surface. Fungal colonization was not detected in any controls.

Time of inoculation and substrate type had similar effects on the re-isolation of Pt from tube substrates (Table 3). In Experiment IA, Pt was re-isolated from substrates of all tubes inoculated before or at seeding. A lower incidence of re-isolation was encountered in the delayed inoculation (three weeks after seeding). The re-isolated Pt was identified by comparisons with other pure cultures of Pt on the basis of golden brown hyphal color, colony appearance, and clamp connec-

Table 1. Survival of containerized Virginia pine seedlings after 90 days as affected by inoculation with *Pisolithus tinctorius* (Pt) and growth in various substrates.[1]

Inoculation Treatment	Substrate Treatment	Percent Survival
	--Experiment IA--	
Control	PV	100
Pt 3 weeks before seeding	PV	100
Pt at seeding	PV	100
Pt 3 weeks after seeding	PV	95
	--Experiment IB--	
Control	PV	100
Control	PVS3	90
Control	PVS1	95
Pt at seeding	PV	100
Pt at seeding	PVS3	95
Pt at seeding	PVS1	90
	--Experiment II--	
Control	PV	100
Control	PVS3	100
Pt at seeding	PV	100
Pt at seeding	PVS3	100

[1] Based on 20 seedlings per treatment combination.

Table 2. Assessment of colonization by *Pisolithus tinctorius* (Pt) in different substrates in Virginia pine seedling containers 90 days after inoculation.[1]

Inoculation Treatment	Substrate Treatment	Colonization
	--Experiment IA--	
Control	PV	None
Pt 3 weeks before seeding	PV	Excellent
Pt at seeding	PV	Good
Pt 3 weeks after seeding	PV	Poor
	--Experiment IB--	
Control	PV	None
Control	PVS3	None
Control	PVS1	None
Pt at seeding	PV	Good
Pt at seeding	PVS3	Fair
Pt at seeding	PVS1	Poor
	--Experiment II--	
Control	PV	None
Control	PVS3	None
Pt at seeding	PV	Good
Pt at seeding	PVS3	Fair

[1] Overall assessments for 20 containers per treatment combination based on percent substrate permeated by Pt hyphae: 75-100% = Excellent; 50-74% = Good; 25-49% = Fair; 1-24% = Poor; 0% = None.

Table 3. Re-isolation of *Pisolithus tinctorius* (Pt) and assessment of contamination from different substrates from monoxenic Virginia pine seedling containers 90 days after inoculation (Experiments IA and IB).[1]

Inoculation treatment	Substrate treatment	Percent tubes with only Pt re-isolated	Percent tubes with Pt and contaminants	Percent tubes with only contaminants	Percent sterile tubes
		--Experiment IA--			
Control	PV	---	---	10	90
Pt 3 weeks before seeding	PV	100	0	0	0
Pt at seeding	PV	95	5	0	0
Pt 3 weeks after seeding	PV	40	15	10	35
		--Experiment IB--			
Control	PV	---	---	10	90
Control	PVS3	---	---	25	75
Control	PVS1	---	---	10	90
Pt at seeding	PV	95	5	0	0

Table 3. Re-siolation of *Pisolithus tinctorius* (Pt) and assessment of contamination from different substrates from monoxenic Virginia pine seedling containers 90 days after inoculation (Experiments IA and IB).[1] (Continued)

Inoculation treatment	Substrate treatment	Percent tubes with only Pt re-isolated	Percent tubes with Pt and contaminants	Percent tubes with only contaminants	Percent sterile tubes
Pt at seeding	PVS3	65	5	5	25
Pt at seeding	PVS1	15	0	20	65

[1] Based on one isolation from each of the 20 containers per treatment combination.

tions. In Experiment IB, decreasing success of Pt re-isolation occurred as the spoil material percent in the substrate increased. Contamination was evident in IA and IB. Sterility of controls ranged from 75-90 percent. Re-isolation of Pt in Experiment II was not attempted due to the obvious presence of contaminants under open air conditions.

Substrate moisture varied only slightly in all experiments during the study. Moisture retention decreased from initial field capacity conditions of one-tenth to one-third bar, to conditions of one-half to one bar retention in Experiments IA and IB. Additional watering of these monoxenic tubes was unnecessary. In Experiment II, seedling tubes were watered regularly to return substrates to field capacity conditions.

The percent mycorrhiza in each experiment was significantly affected by inoculation with Pt vegetative mycelium (Table 4). All uninoculated controls were completely devoid of mycorrhizae. In Experiments IA and IB, percent mycorrhiza decreased with delayed inoculation time and increasing ratios of spoil material in substrate, respectively. The number of seedlings that formed mycorrhizae decreased similarly as well. Percent mycorrhiza resulting from inoculating at seeding or three weeks before seeding in IA was 70 and 75 percent, respectively, with no significant difference. Percent mycorrhiza was significantly lower (26 percent) in IA due to inoculation three weeks after seeding. Inoculation with Pt in PV resulted in Virginia pine seedlings with the highest percent mycorrhiza (70 percent) in Experiment IB. Percent mycorrhiza of IB seedlings in PVS3 and PVS1 (20 and 10 percent, respec-

Table 4. Percent mycorrhiza of containerized Virginia pine seedlings after 90 days as affected by inoculation with *Pisolithus tinctorius* (Pt) and growth in various substrates.

Inoculation treatment	Substrate treatment	Percent mycorrhiza[1]	Percent seedlings with mycorrhizae[2]	Percent mycorrhiza of infected seedlings[3]
		--Experiment IA--		
Control	PV	0 a	0	0
Pt 3 weeks before seeding	PV	75 b	100	75
Pt at seeding	PV	70 b	100	70
Pt 3 weeks after seeding	PV	26 c	46	56
		--Experiment IB--		
Control	PV	0 a	0	0
Control	PVS3	0 a	0	0
Control	PVS1	0 a	0	0
Pt at seeding	PV	70 b	100	70
Pt at seeding	PVS3	21 c	46	46
Pt at seeding	PVS1	10 a	20	47

Table 4. Percent mycorrhiza of containerized Virginia pine seedlings after 90 days as affected by inoculation with *Pisolithus tinctorius* (Pt) and growth in various substrates. (Continued)

Inoculation treatment	Substrate treatment	Percent mycorrhiza[1]	Percent seedlings with mycorrhizae[2]	Percent mycorrhiza of infected seedlings[3]
		--Experiment II--		
Control	PV	0 a	0	0
Control	PVS3	0 a	0	0
Pt at seeding	PV	22 b	100	22
Pt at seeding	PVS3	24 b	100	24

[1] Percent mycorrhiza is the number of mycorrhizal short roots divided by the total number of short roots, multiplied by 100. Values are means of 15 seedlings per treatment combination. Means followed by the same letter are not significantly different at the .05 level (Duncan's Multiple Range Test).

[2] The percentage of seedlings per treatment combination that had mycorrhizae present on their roots.

[3] The percent mycorrhiza per treatment combination of only those seedlings that had mycorrhiza present on their roots.

tively) were significantly different, although percent mycorrhiza of seedlings in PVS1 was not significantly different from the three non-mycorrhizal substrate controls.

In Experiment II, percent mycorrhiza was affected by Pt inoculation, but not by substrate type. Percent mycorrhiza for seedlings inoculated in PV and PVS3 (22 and 24 percent, respectively) was not significantly different (Table 4). All inoculated seedlings were mycorrhizal in this experiment, and all uninoculated control seedlings were completely devoid of mycorrhiza. Pioneer colonization by other mycorrhizal fungal species did not occur.

Mycorrhizas formed in all experiments were similar in color and morphological diversity. Using a dissecting scope at 6x, several morphological types were observed, including monopodial, bifurcate, double bifurcate, and corralloid. The mycorrhizas appeared golden brown in color with a thick cotton-like mantle. Numerous golden brown hyphae and rhizomorphs radiated outward from the mycorrhizas, permeating substrate particles. Sclerotia were present on many mycorrhizal roots, with rhizomorphic attachment. The hard blackish-brown sclerotia appeared as ellipses, approximately 1 x 2 mm. From microscopic examination of sections, a thick fungal mantle was readily discernible, as was a well-developed Hartig net extending to the endodermis. The Hartig net averaged two hyphal cells in thickness. Several clamp connections were observed on the loose hyphae surrounding the mantle. Uninfected and control roots displayed occasional dichotomous branching, but they were suberized and lacked fungal association. This was

verified by microscopic observation.

Attempts to re-isolate Pt from mycorrhizae in Experiments IA and IB were unsuccessful. The mercuric chloride (100 ppm) surface steriland apparently annihilated the fungus as well as the surface contaminants. Slight success was achieved in Experiment II using 1 percent sodium hypochlorite as the surface sterilant and adding 30 mg/liter streptomycin to the agar. Although some contaminants appeared, characteristic golden brown hyphae extended out from a few mycorrhizal tips. Clamp connections were discernible with microscopic examination. After several re-isolations were attempted, one pure culture Pt isolate was obtained.

In many instances, the growth of Virginia pine seedlings was significantly influenced by Pt inoculation, substrate type, or both. In Experiment IA (Table 5; Appendix IV), inoculation significantly increased height growth and stem diameter at the root collar, although differential timing of inoculation was of no consequence. Dry weights of shoots and roots were highest for seedlings inoculated at seeding or three weeks before seeding. All seedlings in IA showed poor morphological balance as evidenced by the shoot/root ratios. Seedlings inoculated three weeks before seeding had a mean ratio of 4.0, significantly higher than the other treatments which averaged 3.0 to 3.1. Root systems were poorly developed in each treatment.

In Experiment IB, there was no Pt inoculation effect on shoot/root ratio or stem diameter of seedlings (Appendix V). Height growth and dry weight of shoots and roots were significantly increased by inocu-

Table 5. Effect of timing of inoculation with *Pisolithus tinctorius* on the growth and ectomycorrhizal development of containerized Virginia pine seedlings after 90 days in monoxenic culture (Experiment IA)[1].

Time of Inoculation	Height (cm)	Diameter[2] (mm)	Root Dry wt. (mg)	Shoot Dry wt. (mg)	Shoot/Root (Dry wt.)
Control	5.2 a	0.69 a	15 a	45 a	3.0 a
3 weeks before seeding	6.4 b	0.80 b	16 a	66 b	4.0 b
At seeding	6.2 b	0.77 b	21 b	63 b	3.0 a
3 weeks after seeding	6.2 b	0.76 b	18 a	55 c	3.1 a

[1]Values are means of 15 seedlings per treatment. Means followed by the same letter are not significantly different at the .05 level (Duncan's Multiple Range Test).

[2]Diameter measured at root collar.

lation (Table 6). Substrate type had no significant effect on seedling height, stem diameter and dry shoot weight (Appendix V). Dry root weight was significantly lower for seedlings in PVS3 (Table 7). Consequently, the seedlings grown in PVS3 has significantly greater shoot/root ratios (4.7) than seedlings grown in PV (3.0) and PVS1 (3.7). Shoot/root ratios were high for all seedlings in IB, since root branching and development was poorer than shoot development.

Analysis of variance indicated a signficant effect on seedling height, stem diameter and dry shoot weight due to the combination of inoculation and substrate treatments in IB (Appendix V). Examination of the growth data from the combination of inoculation and substrate treatments revealed that no one combination produced seedlings that were consistently highest for all or several growth parameters measured (Table 8).

Inoculation with Pt caused significantly greater dry shoot weight and shoot/root ratios in Experiment II. Stem diameter and dry root weight of inoculated seedlings were significantly lower than the non-inoculated controls (Table 9). There was no inoculation effect on seedling height (Appendix VI). Substrate type affected several growth variables. Stem diameter and dry weight of shoots and roots were significantly greater for seedlings in PVS3, whereas seedlings in PV had greater shoot/root ratios (Table 10).

Analysis of variance in Experiment II indicated significant effects on stem diameter and the dry weight of shoots and roots due to inoculation and substrate treatment combination (Appendix VI). Dry weights and diameters of all seedlings grown in PVS3 were greater

Table 6. Effect of inoculation with *Pisolithus tinctorius* (Pt) on the height and dry weight of containerized Virginia pine seedlings after 90 days in monoxenic culture (Experiment IB)[1].

Inoculation Treatment	Height (cm)	Root Dry wt. (mg)	Shoot Dry wt. (mg)
Control	5.6 a	14 a	51 a
Pt	6.1 b	18 b	63 b

[1]Values are means of three substrate treatments (PV, PVS1 and PVS3; 15 seedlings per treatment). Means followed by the same letter are not significantly different at the .05 level (Duncan's Multiple Range Test).

Table 7. Effect of substrate on dry root weight and shoot/root ratio of containerized Virginia pine seedlings after 90 days in monoxenic culture (Experiment IB)[1].

Substrate Treatment	Root Dry wt. (mg)	Shoot/Root (Dry wt.)
PV	18 a	3.0 a
PVS3	13 b	4.7 b
PVS1	16 a	3.7 a

[1]Values are means of two inoculation treatments (inoculated with *Pisolithus tinctorius* and noninoculated control; 15 seedlings per treatment). Means followed by the same letter are not significantly different at the .05 level (Duncan's Multiple Range Test).

Table 8. Effect of combination of inoculation with *Pisolithus tinctorius* (Pt) and substrate type on the growth of containerized Virginia pine seedlings after 90 days in monoxenic culture (Experiment IB)[1].

Inoculation Treatment	Substrate Treatment	Height (cm)	Diameter[2] (mm)	Root Dry wt. (mg)	Shoot Dry wt. (mg)	Shoot/Root (Dry wt.)
Control	PV	5.2 a	0.69 a	15 ab	44 a	3.1 a
Pt	PV	6.2 bc	0.77 bc	21 c	63 cd	3.0 a
Control	PVS3	5.6 ab	0.80 c	11 a	52 ab	4.7 b
Pt	PVS3	6.6 c	0.73 ab	15 ab	67 d	4.7 b
Control	PVS1	5.9 abc	0.74 abc	15 ab	58 bc	3.9 ab
Pt	PVS1	5.5 ab	0.78 bc	18 bc	58 bc	3.4 b

[1]Values are means of 15 seedlings per treatment combination. Means followed by the same letter are not significantly different at the .05 level (Duncan's Multiple Range Test).

[2]Diameter measured at root collar.

Table 9. Effect of inoculation with *Pisolithus tinctorius* (Pt) on stem diameter, dry weight and shoot/root ratio of greenhouse-reared containerized Virginia pine seedlings (Experiment II).[1,2]

Inoculation Treatment	Diameter[3] (mm)	Root Dry wt. (mg)	Shoot Dry wt. (mg)	Shoot/Root (Dry wt.)
Control	1.24 a	116 a	120 a	1.0 a
Pt	1.17 b	102 b	135 b	1.3 b

[1]Values are means of two substrate treatments (PV and PVS3; 15 seedlings per treatment). Means followed by the same letter are not significantly different at the .05 level (Duncan's Multiple Range Test).

[2]90-day data.

[3]Diameter measured at root collar.

Table 10. Effect of substrate on the stem diameter, dry weight and shoot/root ratio of greenhouse-reared containerized Virginia pine seedlings (Experiment II).[1,2]

Substrate Treatment	Diameter[3] (mm)	Root Dry wt. (mg)	Shoot Dry wt. (mg)	Shoot/Root (Dry wt.)
PV	1.13 a	85 a	106 a	1.3 a
PVS3	1.28 b	133 b	148 b	1.1 b

[1]Values are means of two inoculation treatments (inoculated with *Pisolithus tinctorius* and noninoculated control; 15 seedlings per treatment). Means followed by the same letter are not significantly different at the .05 level (Duncan's Multiple Range Test).

[2]90-day data.

[3]Diameter measured at root collar.

than for those in PV. Furthermore, noninoculated seedlings in PVS3 had significantly greater stem diameters and dry root weights than Pt inoculated seedlings in PVS3 (Table 11).

Root systems of the seedlings in Experiment II were better developed and weighed more than those in IA and IB, hence shoot/root ratios were lower. Inoculation with Pt resulted in significantly greater shoot/root ratios for seedlings in both substrates (1.4 and 1.3 for PV and PVS3, respectively), as compared to non-inoculated seedlings (1.1 and 1.0 for PV and PVS3, respectively).

Table 11. Effect of combination of inoculation with *Pisolithus tinctorius* (Pt) and substrate type on the growth of greenhouse-reared containerized Virginia pine seedlings (Experiment II).[1,2]

Inoculation Treatment	Substrate Treatment	Height (cm)	Diameter[3] (mm)	Root Dry wt. (mg)	Shoot Dry wt. (mg)	Shoot/Root (Dry wt.)
Control	PV	4.8 ab	1.14 a	82 a	92 a	1.1 a
Pt	PV	4.7 ab	1.12 a	87 a	122 b	1.4 b
Control	PVS3	5.3 a	1.33 b	149 b	149 c	1.0 a
Pt	PVS3	4.8 ab	1.22 c	117 c	148 c	1.3 b

[1]Values are means of 15 seedlings per treatment combination. Means followed by the same letter are not significantly different at the .05 level (Duncan's Multiple Range Test).

[2]90-day data.

[3]Diameter measured at root collar.

DISCUSSION

The use of mycorrhizal inoculation and natural substrates is feasible in the production of containerized seedling stock. The results of this study and of Marx and Barnett (1974) suggest that some production techniques are sound and straightforward, while others require extensive research. Consideration in this study was given to the timing of application of fungal inoculum to container substrates, and to amending substrates with mine spoil. The need for attention to other considerations including substrate fertility, inoculum density and control of lighting was ascertained as a result of this study.

From the production standpoint, it is not economically feasible to produce container stock under monoxenic conditions in growth chambers, as was done in Experiments IA and IB. These experiments were conducted solely to determine the effects of differential timing of inoculation with *Pisolithus tinctorius* and additions of mine spoil to substrate on the growth and ectomycorrhizal development of Virginia pine seedlings under controlled conditions. As the results indicated, both delays in inoculation and increases in spoil percentage in substrate decreased percent mycorrhiza, but were not detrimental to seedling growth. Based on an overall assessment of the data from IA and IB, inoculation before or at seeding, and the addition of mine spoil to container substrates is recommended.

The benefits of mycorrhizae to seedling survival and growth are well understood. A seedling with all of its short roots converted to

mycorrhizae should survive and grow better than one with lesser or no mycorrhizae at all. Thus, the inoculum density used in containerized seedling production should be sufficient to result in a total conversion of short roots to mycorrhizae. The inoculum density used in this study should be increased in order to achieve that goal. Further research is needed concerning the use of mine spoil in containers, due to the lack of conclusive data in Experiment IB. It is reasonable to add natural materials such as mine spoil to containers to aid seedlings in their adaptation to future outplanting sites (Tinus et al., 1974). More research is needed, including outplanting studies, to determine sound containerized seedling production prescriptions.

The fact that Pt was re-isolated from the inoculated tubes in IA and IB, and that Pt was not present in the control tubes indicated that inoculation techniques were sound. However, more care should be given to deter contamination, as was the case in this study. Perhaps a greater problem encountered was control of light conditions.

Low light levels cause shallow and poorly developed root systems of most forest trees (Spurr and Barnes, 1973). Conversely, as light intensity approaches full sunlight, photosynthetic production increases. Increased translocation of photosynthate to the roots thus results in better root growth and development. Similarly, mycorrhizal development increases with increasing light intensity (Hacskaylo and Snow, 1959). The growth chamber used in this study provided 1850 foot candles of light, or about 21 percent full sunlight conditions. Under temporary Gro-lux lights, intensity ranged from 500-600 foot candles, or less than 10 percent full sunlight. It seems possible that both root

development and percent mycorrhiza were hindered in this study, since the shade intolerant Virginia pine seedlings did not receive light of a sufficient intensity for normal photosynthetic production. This assumption explains the poor root growth and consequent highly imbalanced shoot/root ratios encountered in Experiments IA and IB.

It was apparent from Table 2 that substrate colonization by Pt and inoculation success were strongly affected by substrate type in Experiment IB. Additions of mine spoil to the PV container substrate decreased the hyphal growth of Pt throughout the substrate in IB. Due to poor inoculation success in PVS1, percent mycorrhiza was significantly lower than in PV or PVS3. The PV substrate is highly conducive to fungal colonization due to high aeration resulting from its platey expanding structure. Conversely, the weights per unit volume of PVS3 and PVS1 were more than twice that of PV. The addition of fine-textured mine spoil to PV resulted in PVS3 and PVS1 substrates with considerably lower levels of aeration. Poor aeration may have inhibited colonization of *Pisolithus tinctorius*. Colonization inhibition was probably not related to soil moisture, since moisture retention for all substrates was essentially uniform through the duration of IA and IB.

The results of Experiment II, the greenhouse study, provided information and insight into the production of mycorrhizal container stock in spoil-amended substrate. The results showed that seedlings grown in PVS3 had greater diameter growth and dry weight production than those in PV, regardless of inoculation treatment. The problems of poor substrate aeration and low light intensity were not experienced in the greenhouse. Shoot/root ratios approached unity (Table 11) while

root growth and development were enhanced by near full sunlight conditions. The greater seedling growth in PVS3 substrate as compared to PV was probably due to nutrient status. Vermiculite is typically an inert, infertile substance. The peat component of PV is primarily of organic nature, lacking in inorganic nutrients. Comparatively, the mine spoil material used in the study had low to medium availability of the basic inorganic nutrients needed by plants (Appendix II). As the nutrients from the MMN solution were depleted in the containers, both the seedlings and Pt relied on the nutrients stored in the substrate material. Consequently, seedlings in PV had minute quantities of nutrient elements, while those in PVS3 had a larger nutrient supply. Eventually, all seedlings became chlorotic, probably due to malnutrition. With a greater amount of available nutrients, PVS3 seedlings had larger stem diameters and dry weight production than PV seedlings.

Substrate colonization by Pt was expected to be greater in Experiment II than in IA or IB, due to better aeration conditions in the greenhouse as compared to monoxenic culture tubes. Actually, subjective assessments of hyphal colonization were similar for similar substrate types among the experiments (Table 2). Less-than-expected colonization in the greenhouse was probably due to substrate nutrition and the method of inoculation.

A lesser amount of MMN solution was added to containers in Experiment II than in IA and IB. Glucose and thiamine were also deleted from the nutrient formula to deter pioneer contamination. Some competition by pioneer microorganisms undoubtedly occurred, and thus the

growth of Pt was consequently limited. Furthermore, mycorrhizal fungi have a dependence on their plant hosts for carbohydrates and vitamins (Melin, 1953; Hacskaylo, 1973). In the absence of glucose and thiamine, Pt was probably in a lag-phase of growth in the container substrates. After the establishment of seedling root systems, Pt was probably better able to obtain nourishment to enable its growth throughout the substrates and eventual infection of roots.

The method of inoculum preparation and inoculation in Experiment II also probably caused less-than-expected substrate colonization by Pt as compared to IA and IB. Important considerations in inoculum preparation in II were contamination, hyphal density, and inoculum density. Contaminated inoculum cultures must be discarded. Furthermore, cultures must be incubated long enough to provide a high density of fungal hyphae throughout the culture PV substrate. Since some hyphal material is washed out of the inoculum when it is leached of non-assimilated carbohydrates, inoculum cultures should be incubated longer to make up for the loss, or greater densities of inoculum should be mixed into container substrates. Research on both the techniques for mass inoculum preparation and for container inoculation is necessary before practical production of mycorrhizal container stock can become widespread.

The use of dichotomous branching of short roots on *Pinus* spp. as a criterion for ectomycorrhizal infection has been reported often in the literature. Several researchers have cautioned against the use of this criterion in assessing percent mycorrhiza (Grand, 1968; Riffle,

1973). Several non-mycorrhizal forked roots were observed on seedling roots in this study, based on microscopic examination. Slankis (1971) suggested that the forking of non-mycorrhizal roots was caused by excreted fungal metabolites such as indoleacetic acid. The presence of a dense fungal mantle and Hartig-net development is necessary to conclude that a root is ectomycorrhizal. Several workers have used the criterion of the presence of a dense fungal mantle in their visual assessment of percent mycorrhiza (Theodorou and Bowen, 1965; Marx et al., 1970).

Attempts to re-isolate the fungal symbiont from mycorrhizal rootlets of Virginia pine seedlings met with little success. The 100 ppm mercuric chloride sterilant utilized in Experiments IA and IB apparently killed both *Pisolithus tinctorius* and surface contaminants. Using a 1 percent sodium hypochlorite sterilant in Experiment II, slight success was achieved as Pt was re-isolated in three of thirty attempts. Although streptomycin (30 mg/l) was added to the Hagem agar, some contamination still occurred. Nevertheless, the characteristic golden brown hyphae of *Pisolithus tinctorius* extended from three mycorrhizal tips and one sclerotium in association with a mycorrhiza. Typical clamp connections were observed. After several transfers, only one pure culture isolate of Pt was obtained. Direct-plating of mycorrhizas without surface sterilization resulted in Pt hyphal growth, but contamination was rampant. Pearson and Read (1973) reported similar results with direct-plating, but noted good re-isolation success using a maceration procedure.

As seen with the greenhouse seedlings, mycorrhizal inoculation and

the use of natural material such as mine spoil is feasible in the production of containerized seedling stock. Provided a sufficient density of inoculum is added to each container, pioneer colonization by other mycorrhizal fungi should not be a problem. Under the inoculation conditions in Experiment II, which are evaluated as minimal with respect to inoculum density, all inoculated seedlings were mycorrhizal with Pt, and all uninoculated controls were devoid of any mycorrhizae.

Future research should be designed to consider the aspects presented in this study concerning the production of mycorrhizal containerized seedlings. Furthermore, the utility of these seedlings should be determined in outplanting studies. Mycorrhizal containerized seedlings that have grown in mine spoil amended substrates may be valuable in the reclamation of strip mine spoils or other drastically disturbed sites.

SUMMARY AND CONCLUSIONS

This study was designed to determine the influence of mycorrhizal symbiosis and the use of mine spoil-amended substrates in the production of containerized seedlings for strip mine reclamation. *Pisolithus tinctorius* was chosen due to its ability to withstand harsh spoil conditions, especially high soil temperatures. Virginia pine was used because of its silvical characteristics and commercial value.

Seedlings were inoculated at different times, and different ratios of mine spoil material to artificial peat:vermiculite substrate were tested. These experiments took place under monoxenic conditions in a growth chamber. An open-air greenhouse study was also established, testing inoculation and one-spoil mixture against controls. All experiments proceeded 90 days, after which seedling growth data were recorded and percent mycorrhiza assessed. Using the criterion of the presence of a fungal mantle, supplemented by microscopic observation for Hartig-net development, mycorrhizal infection was determined on the basis of short mycorrhizal roots as a percentage of the total number of short lateral roots.

The monoxenic synthesis results indicated that inoculation with *Pisolithus* at seeding or three weeks before seeding significantly increased percent mycorrhiza and seedling dry weight as compared to the control or delayed inoculation (three weeks after seeding). Mine spoil amendments to a peat:vermiculite container substrate significantly decreased percent mycorrhiza on the seedlings. The mine spoil had no detrimental effect on seedling growth.

In the greenhouse study, all seedlings inoculated with *Pisolithus* were mycorrhizal, and all non-inoculated controls were completely devoid of mycorrhizae. Dry weights and stem diameters of all seedlings grown in spoil-amended substrates were greater than for those in only peat:vermiculite. Furthermore, stem diameters and dry root weights were greater for the non-inoculated seedlings than for the seedlings inoculated with *Pisolithus* in the spoil-amended containers.

The use of both mycorrhizal inoculation and natural substrates such as mine spoil is feasible in containerized seedling production. Higher levels of inoculum than those used in this study are recommended, with a moderate addition of mine spoil to artificial container substrates. Mycorrhizal symbiosis and the containerized system could have valuable utility in the reclamation of strip mines and other drastically disturbed sites.

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Appendix I. Growth media for culture of ectomycorrhizal fungi.

Hagem Agar

Bacto agar	15.0 gm
Malt extract	5.0 gm
Glucose	5.0 gm
KH_2PO_4	0.5 gm
$MgSO_4 \cdot 7H_2O$	0.5 gm
NH_4Cl	0.5 gm
$FeCl_3$ (1% soln.)	0.5 ml
H_2O	to 1000 ml

Modified Melin-Norkrans Nutrient Solution

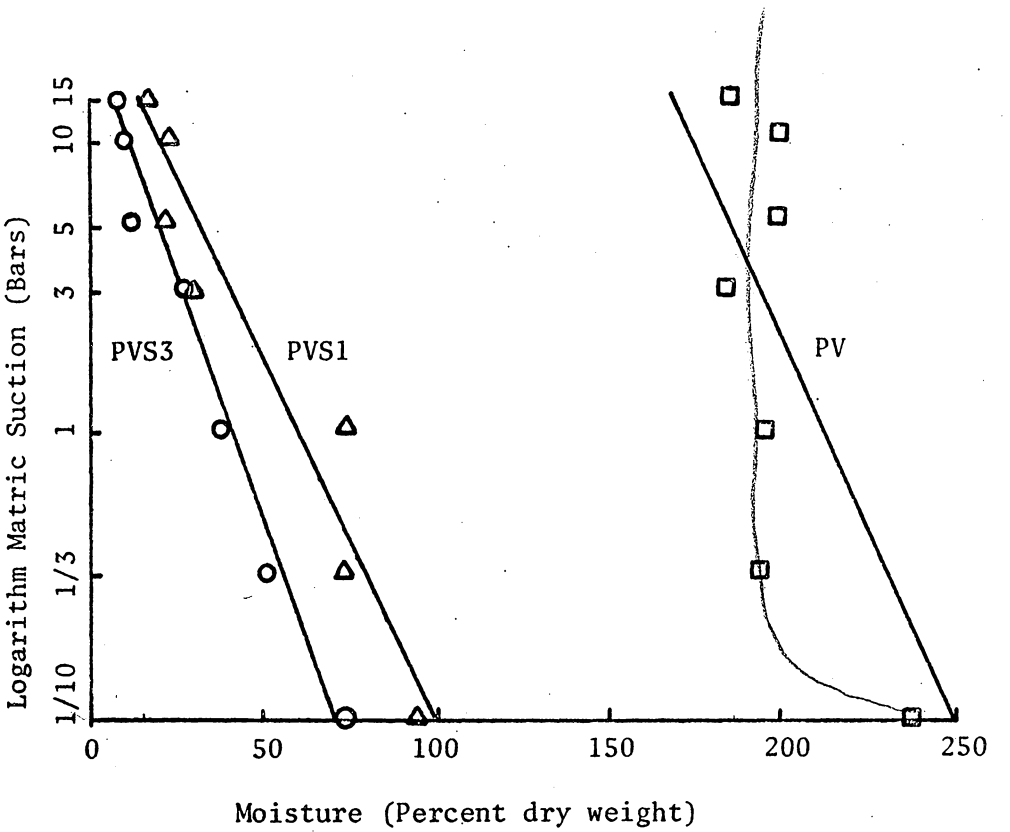
$CaCl_2$	0.05 gm
NaCl	0.025 gm
KH_2PO_4	0.50 gm
$(NH_4)_2HPO_4$	0.25 gm
$MgSO_4 \cdot 7H_2O$	0.15 gm
$FeCl_3$ (1% soln.)	1.20 ml
Thiamin	50 µg
Glucose	5.0 gm
H_2O	to 1000 ml

Appendix II. Characterization of mine spoil material.

The spoil material used in this study was obtained from the Pocahontas No. 10 seam of an active strip-mine operated by the Bluestone Mining Company near Keystone, West Virginia. The spoil from this operation was less than one year old. Large rocks were hand-picked from the spoil after loading. Physical and chemical properties of the material are as follows:

Physical		Chemical	
Sand	47.5%	pH	5.1
Silt	39.3%	NO_3	5.2 ppm
Clay	13.2%	P	17.6 ppm
Texture Class	Loam	K	54.0 ppm
		Ca	413.6 ppm
		Mg	117.6 ppm
		Zn	6.0 ppm
		Mn	16.0 ppm
		% organic matter	2.7

Appendix III. Soil moisture characteristic curves for container substrates.



Appendix IV. Anova tables for Experiment IA.

ANOVA FOR HEIGHT (IA)

Source	DF	Sum of Squares	Mean Square	F Value
Model	3	12.99733333	4.33244444	4.94
Error	56	49.10266667	0.87683333	
Corrected Total	59	62.10000000		

Source	DF	Anova SS	F Value	PR > F
Inoculation treatment	3	12.99733333	4.94	0.0042

ANOVA FOR STEM DIAMETER (IA)

Source	DF	Sum of Squares	Mean Square	F Value
Model	3	0.09683333	0.03227778	5.65
Error	56	0.32000000	0.00571429	
Corrected Total	59	0.41683333		

Source	DF	Anova SS	F Value	PR > F
Inoculation treatment	3	0.09683333	5.65	0.0020

ANOVA FOR DRY SHOOT WEIGHT (IA)

Source	DF	Sum of Squares	Mean Square	F Value
Model	3	0.00468954	0.00156318	11.26
Error	56	0.00777308	0.00013880	
Corrected Total	59	0.01246261		

Source	DF	Anova SS	F Value	PR > F
Inoculation treatment	3	0.00468954	11.26	0.0001

ANOVA FOR DRY ROOT WEIGHT (IA)

Source	DF	Sum of Squares	Mean Square	F Value
Model	3	0.00034424	0.00011475	5.35
Error	56	0.00120122	0.00002145	
Corrected Total	59	0.00154546		

Source	DF	Anova SS	F Value	PR > F
Inoculation treatment	3	0.00034424	5.35	0.0027

ANOVA FOR SHOOT/ROOT (DRY WEIGHT) (IA)

Source	DF	Sum of Squares	Mean Square	F Value
Model	3	8.46105861	2.82035287	4.33
Error	56	36.48580683	0.65153226	
Corrected Total	59	44.94686544		

Source	DF	Anova SS	F Value	PR > F
Inoculation treatment	3	8.46105861	4.33	0.0083

ANOVA FOR PERCENT MYCORRHIZA (IA)

Source	DF	Sum of Squares	Mean Square	F Value
Model	3	58348.33333333	19449.44444444	57.62
Error	56	18902.40000000	337.54285714	
Corrected Total	59	77250.73333333		

Source	DF	Anova SS	F Value	PR > F
Inoculation treatment	3	58348.33333333	57.62	0.0001

Appendix V. Anova tables for Experiment IB.

ANOVA FOR HEIGHT (IB)

Source	DF	Sum of Squares	Mean Square	F Value
Model	5	19.47022222	3.89404444	3.56
Error	84	91.78933333	1.09273016	
Corrected Total	89	111.25955556		

Source	DF	Anova SS	F Value	PR > F
Inoculation treatment	1	6.40000000	5.86	0.0177
Substrate treatment	2	3.79822222	1.74	0.1821
Inoculation * substrate	2	9.27200000	4.24	0.0176

ANOVA FOR STEM DIAMETER (IB)

Source	DF	Sum of Squares	Mean Square	F Value
Model	5	0.11291667	0.02258333	3.76
Error	84	0.50433333	0.00600397	
Corrected Total	89	0.61725000		

Source	DF	Anova SS	F Value	PR > F
Inoculation treatment	1	0.00802778	1.34	0.2508
Substrate treatment	2	0.02466667	2.05	0.1346
Inoculation * substrate	2	0.08022222	6.68	0.0020

ANOVA FOR DRY SHOOT WEIGHT (IB)

Source	DF	Sum of Squares	Mean Square	F Value
Model	5	0.00502113	0.00100423	7.82
Error	84	0.01079029	0.00012846	
Corrected Total	89	0.01581142		

Source	DF	Anova SS	F Value	PR > F
Inoculation treatment	1	0.00272999	21.25	0.0001
Substrate treatment	2	0.00050387	1.96	0.1471
Inoculation * substrate	2	0.00178728	6.96	0.0016

ANOVA FOR DRY ROOT WEIGHT (IB)

Source	DF	Sum of Squares	Mean Square	F Value
Model	5	0.00089171	0.00017834	5.64
Error	84	0.00265689	0.00003163	
Corrected Total	89	0.00354859		

Source	DF	Anova SS	F Value	PR > F
Inoculation treatment	1	0.00051720	16.35	0.0001
Substrate treatment	2	0.00033925	5.36	0.0064
Inoculation * substrate	2	0.00003526	0.56	0.5749

ANOVA FOR SHOOT/ROOT (DRY WEIGHT) (IB)

Source	DF	Sum of Squares	Mean Square	F Value
Model	5	45.85731189	9.17146238	5.60
Error	84	137.50279537	1.63693804	
Corrected Total	89	183.36010725		

Source	DF	Anova SS	F Value	PR > F
Inoculation treatment	1	1.15764098	0.71	0.4028
Substrate treatment	2	43.37115701	13.25	0.0001
Inoculation * substrate	2	1.32851389	0.41	0.6677

ANOVA FOR PERCENT MYCORRHIZA (IB)

Source	DF	Sum of Squares	Mean Square	F Value
Model	5	56004.40000000	11200.88000000	46.05
Error	84	20430.00000000	243.21428571	
Corrected Total	89	76434.40000000		

Source	DF	Anova SS	F Value	PR > F
Inoculation treatment	1	25401.60000000	104.44	0.0001
Substrate treatment	2	15301.40000000	31.46	0.0001
Inoculation * substrate	2	15301.40000000	31.46	0.0001

Appendix VI. Anova tables for Experiment II.

ANOVA FOR HEIGHT (II)

Source	DF	Sum of Squares	Mean Square	F Value
Model	3	3.76183333	1.25394444	2.20
Error	56	31.98400000	0.57114286	
Corrected Total	59	35.74583333		

Source	DF	Anova SS	F Value	PR > F
Inoculation treatment	1	1.70016667	2.98	0.0900
Substrate treatment	1	0.44150000	2.52	0.1178
Inoculation * substrate	1	0.62016667	1.09	0.3019

ANOVA FOR STEM DIAMETER (II)

Source	DF	Sum of Squares	Mean Square	F Value
Model	3	0.40304105	0.13434702	14.09
Error	56	0.53378252	0.00953183	
Corrected Total	59	0.93682357		

Source	DF	Anova SS	F Value	PR > F
Inoculation treatment	1	0.05895054	6.18	0.0159
Substrate treatment	1	0.30683511	32.19	0.0001
Inoculation * substrate	1	0.03725540	3.91	0.0530

ANOVA FOR DRY SHOOT WEIGHT (II)

Source	DF	Sum of Squares	Mean Square	F Value
Model	3	0.03279711	0.01093237	24.01
Error	56	0.02550206	0.00045539	
Corrected Total	59	0.05829917		

Source	DF	Anova SS	F Value	PR > F
Inoculation treatment	1	0.00353818	7.77	0.0072
Substrate treatment	1	0.02596963	57.03	0.0001
Inoculation * substrate	1	0.00328930	7.22	0.0095

ANOVA FOR DRY ROOT WEIGHT (II)

Source	DF	Sum of Squares	Mean Square	F Value
Model	3	0.04307404	0.01435801	44.96
Error	56	0.01788449	0.00031937	
Corrected Total	59	0.06095852		

Source	DF	Anova SS	F Value	PR > F
Inoculation treatment	1	0.00300136	9.40	0.0033
Substrate treatment	1	0.03487174	109.19	0.0001
Inoculation * substrate	1	0.00520094	16.29	0.0002

ANOVA FOR SHOOT/ROOT (DRY WEIGHT) (II)

Source	DF	Sum of Squares	Mean Square	F Value
Model	3	1.55350344	0.51783448	13.64
Error	56	2.12642051	0.03797179	
Corrected Total	59	3.67992395		

Source	DF	Anova SS	F Value	PR > F
Inoculation treatment	1	1.32199410	34.82	0.0001
Substrate treatment	1	0.22938162	6.04	0.0171
Inoculation * substrate	1	0.00212772	0.06	0.8137

ANOVA FOR PERCENT MYCORRHIZA (II)

Source	DF	Sum of Squares	Mean Square	F Value
Model	3	8089.73333333	2696.57777778	17.15
Error	56	8802.66666667	157.19047619	
Corrected Total	59	16892.40000000		

Source	DF	Anova SS	F Value	PR > F
Inoculation treatment	1	8073.60000000	51.36	0.0001
Substrate treatment	1	8.06666667	0.05	0.8216
Inoculation * substrate	1	8.06666667	0.05	0.8216

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INFLUENCE OF *Pisolithus tinctorius* AND MINE SPOIL ON THE GROWTH OF VIRGINIA PINE SEEDLINGS

by

Thomas Kuzmic

(ABSTRACT)

Containerized Virginia pine seedlings were inoculated with *Pisolithus tinctorius* and grown monoxenically in various substrates consisting of peat:vermiculite amended by mine spoil. Significantly better seedling growth and ectomycorrhizal development occurred when substrates were inoculated three weeks before or at seeding, rather than three weeks after seeding or not at all. Low levels of mine spoil substrate amendments did not affect seedling growth, but did result in significantly lower ectomycorrhizal development. A concurrent open-air greenhouse study showed that mine spoil amendments aided seedling growth more than inoculation did. Mycorrhizal infection was not affected by spoil substrate. Recommendations for the production of mycorrhizal container stock were presented.