AN ABSTRACT OF THE THESIS OF

Ling-Ling L. Hung for the degree of Doctor of Philosophy in Forest Science presented on August 28, 1984

Title: ECTOMYCORRHIZAL INOCULATION OF DOUGLAS-FIR NURSERY STOCK WITH COMMERCIALLY PRODUCED INOCULUM

Abstract approved: Signature redacted for privacy.
James M. Trappe

Nine species of ectomycorrhizal fungi were grown in liquid media over a pH range of 2-7. Species fell into five major groups: (1) growth significantly best only at the optimal pH, (2) growth increased with increase of pH, (3) significantly best growth spans three pH units, (4) spans four pH units, and (5) spans five pH units. A comparison of four isolates each of Cenococcum geophilum and Laccaria laccata revealed striking infraspecific differences in response to pH. Species also differed in their effect on pH of the medium over the duration of the experiment. Isolates that grow well over a broad pH range would be preferred for inoculation in tree nurseries.

Ectomycorrhizal inoculation of Douglas-fir container-grown seedlings succeeded with commercially produced Laccaria laccata and Hebeloma crustuliniforme in an research greenhouse and/or commercial

nurseries. However, *Pisolithus tinctorius* inoculum was less effective and the most sensitive to inoculum storage. Inoculated seedlings with abundant mycorrhizae generally had significantly more feeder roots than those with poor or no mycorrhizae. Fresh inoculum was the most effective, and this effectiveness declined as storage period increased. In the case of *L. laccata*, mycorrhiza formation increased with increased inoculation rate; the lower the inoculation rate, the sooner the effectiveness declined. The best inoculation rate for seedling growth and mycorrhiza formation differed between nurseries. Storage of inoculum at $2^{o}C$ prolonged inoculum viability for at least two months over that of room-temperature storage. Inoculum from different species or isolates within species responded to storage temperature differently.

Results from ectomycorrhizal inoculation of plug+1 seedlings indicated that inoculated seedlings had significantly higher foliage Zn concentration, and all but *L. laccata*-inoculated seedlings had significantly lower foliage Mn levels as compared to noninoculated controls. After being transplanted from containers to nursery beds and grown there for 17 months, all seedlings had 80% mycorrhizal. *H. crustuliniforme* persisted as a mycorrhizal dominant on seedlings previouly inoculated with this fungus. *L. laccata*-inoculated seedlings had 40% of their feeder roots colonized by *Laccaria* and other 40% by native fungi (*Rhizopogon* and *Thelephora* spp.). All newly formed mycorrhizae of *P. tinctorius*-inoculated and noninoculated seedlings were with fungi native to the nursery bed, not by *P. tinctorius*.

Ectomycorrhizal Inoculation of Douglas-fir Nursery Stock with Commercially Produced Inoculum

by

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This thesis is dedicated to my parents, Tzu-Chao and Hsiu-Chiung Hung.

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Ectomycorrhizal Inoculation of Douglas-fir Nursery Stock with Commercially Produced Inoculum

INTRODUCTION

Container-grown seedlings usually are in better physiological condition and better able to withstand stresses of shipping and planting than bareroot seedlings (Tinus, 1976). However, lack of mycorrhizal inoculum in the sterile container substrate and depression of mycorrhiza formation by high fertilizer applications result in nonmycorrhizal or Thelephora-mycorrhizal container-grown seedlings (Marx et al, 1977; Ruehle, 1980a; Maronek et al., 1981). Marx (1980) indicated that seedlings with Pisolithus tinctorius (Pers.) Coker & Couch mycorrhizae performed better than those with native Thelephora mycorrhizae. Thus, inoculation of container-grown seedlings to ensure optimum mycorrhiza formation can lead to better seedling performance after outplanting.

Container-grown seedlings inoculated with P. tinctorius grow and survive better than noninoculated seedlings on adverse sites such as acid coal spoils and amended borrow pits (Ruehle, 1980b, Marx and Artman, 1979). However, P. tinctorius-colonized container-grown seedlings planted to routine sites often perform no better than

those colonized by native ectomycorrhizal fungi (Marx and Barnett, 1974; Ruehle and Brendemuehl, 1981; Ruehle et al, 1981; Ruehle, 1982). These results indicate the need for nursery inoculation to select fungi ecologically adapted to planting sites.

Several criteria are important in selection of ectomycorrhizal fungi for use in tree nurseries (Trappe, 1977). Among these are the pH optimum and pH tolerance of candidate isolates. An isolate should have the capacity to adapt to the pH of the nursery soil or container substrate as well as the soil at intended planting sites. The widespread problem of acid rain creates a need for fungi that can tolerate recurring addition of H_2SO_4 or HNO_3 to the soil. Efficient production of vegetative inoculum, moreover, requires that the culture substrate be near the optimum pH for the isolate. In Chapter I, testing of several ectomycorrhizal fungi for their pH optimum and ranges of pH tolerance is described.

Southern pine nursery stock has been commonly inoculated in the southeastern U. S. with P. tinctorius (Marx, 1980). However, this fungus has not performed well with conifers in Pacific northwestern nurseries (Alvarez and Trappe, 1983). Some other fungal species, such as Laccaria laccata (Scop.: Fr.) Berk. and Br., and Hebeloma crustuliniforme (Bull.: St. Am.) Quel., have formed abundant mycorrhizae when inoculated on several western conifer species in container systems (Molina, 1980, 1982; Shaw and Molina, 1980; Shaw et al., 1982). Also, inoculation with spores of Rhizopogon vinicolor Smith and R. colossus Smith has succeeded on Douglas-fir

(*Pseudotsuga menziesii* (Mirb.) Franco) seedlings (Castellano, 1984).

Among sources of inocula, pure vegetative inoculum of ectomycorrhizal fungi permits selection of fungi for specific purposes (Mikola, 1973; Trappe, 1977; Marx, 1980; Marx and Kenney, 1982). Researchers have developed several methods to grow vegetative inoculum for research (Marx and Kenney, 1982). However, available and effective commercial inoculum is needed for large-scale nursery inoculation. Industrial production of inoculum can provide high quality control with fast fungal growth and ease of shipping. Butler County Mushroom Farms, Inc., Worthington, Pennsylvania, began inoculum production research on several ectomycorrhizal fungi in 1980. The studies described here were designed to determine whether such inoculum is effective and forms abundant mycorrhizae with container-grown Douglas-fir seedlings. Commercially produced inoculum of several ectomycorrhizal fungi was tested with Douglas-fir seedlings for application rate (Chapter II), shelf life (Chapter III), and performance with container-grown seedlings after they are transplanted to nursery beds (Chapter IV).

The objectives of this research were to (1) select fungi for nursery inoculation in the Pacific Northwest (Chapters I and IV), (2) assess the effectiveness of commercially produced inoculum on Douglas-fir container-grown and plug+1 seedlings for practical application (Chapters II, III, and IV), (3) provide information on application rate and storage life of commercially produced ectomycorrhizal inoculum (Chapters II and III).

CHAPTER I

Growth variation between and within species of ectomycorrhizal fungi in response to pH *in vitro*

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ABSTRACT

Nine species of ectomycorrhizal fungi were grown in liquid media over a pH range of 2-7. Species fell into five major groups: (1) growth significantly best only at the optimal pH, (2) growth increased with increase of pH, (3) significantly best growth spans three pH units, (4) spans four pH units, and (5) spans five pH units. A comparison of four isolates each of Cenococcum geophilum and Laccaria laccata revealed striking infraspecific differences in response to pH. Species also differed in their effect on pH of the medium over the duration of the experiment. Isolates that grew best in vitro at high pH levels did not originate from high pH soils. Other things being equal, isolates that grow well over a broad pH range would be preferred for inoculation in tree nurseries.

INTRODUCTION

Several criteria are important in selection of ectomycorrhizal fungi for use in inoculating soils in tree nurseries (Trappe, 1977). Among these are the pH optimum and pH tolerance of candidate isolates. An isolate should show promise for adapting to the pH of the nursery soil (or container substrate) as well as the soil at the intended planting site. The widespread problem of acid rain creates a need for fungi tolerance to recurring addition of H_2SO_4 to the soil. Efficient production of vegetative inoculum, moreover, requires that the culture substrate be near the optimum pH for the isolate.

All experiments on cardinal pH's for ectomycorrhizal fungi *in vitro* reported to date indicate best growth between pH 3.2 and 6.5, with the optimum for most isolates between pH 4.5 and 5.5 (Table I.1). Broker (1959), however, reported good growth of many species at pH 6.8 and 8.3. Ranges of pH values that support good growth (arbitrarily included in Table I.1 as those within 20% of the growth at optimum pH) vary drastically between species, as well as between isolates within a species. Other things being equal, an isolate that grows reasonably well over a wide range of pH values would be preferable for nursery inoculation to one that grows well only over a restricted range.

In vitro experiments on effects of pH on fungal growth must be interpreted with caution. The results can be affected by duration of growth (Modess, 1941; Norkrans, 1950), nitrogen source (How, 1940), whether iron salts are added before or after autoclaving (Norkrans, 1950), and many other factors. Accordingly, a comparison of results of different workers (Table I.1) may show differences between species or isolates within species that result from treatment, not inherent differences. When experimental conditions are held constant and replication is adequate, real differences between isolates can be revealed. For example, Laiho (1970) found that pH optima could differ by as much as three pH units between different isolates of *Paxillus involutus* and that some isolates grew well over a span of a full pH unit, but others grew well over a few tenths of a unit.

Our study was designed to evaluate response to pH of several ectomycorrhizal fungi that have shown promise for tree nursery

inoculation by other criteria (e.g., growth rate, favorable response by hosts, tolerance to the manipulation required for nursery inoculation). We sought isolates useful not only for routine nursery inoculation but also for potential tolerance to acid rain or acid coal spoils and for adaptability to alkaline soils, which pose localized but difficult reforestation problems in the Pacific Northwest. In light of Laiho's (1970) results with P. involutus, assessing within-species variation for at least selected fungi was also clearly important.

MATERIALS AND METHODS

Nine species of ectomycorrhizal fungi were used in the experiments; four were represented by two or more isolates (Table I.2). Mycelia were grown 4 to 6 weeks on slants of modified Melin-Norkrans' agar (MMN) (Marx, 1969). Inoculum for each flask in the experiments was prepared by scraping the mycelium aseptically from the surface of each of two slants and homogenizing the scraping in 100 ml of sterile distilled water in a blender at low speed for about 25 sec (except for Pisolithus tinctorius mycelium, which tolerated only 10 seconds of blending).

The pH treatments were prepared by adjusting the pH of Melin-Norkrans' nutrient solution (MN) with 1 N or concentrated H_2SO_4 and 1 N NaOH to provide a series from pH 2-7 at about one-unit intervals, as determined with a glass electrode pH meter. Sulfuric acid was used rather than HCl, the traditional acid for

such studies, because of the broader relevance of H_2SO_4 to the problems of acid rain and coal spoils. We elected not to buffer the solutions because buffering in this type of experiment can introduce as many extraneous variables as it may eliminate. For example, phosphate and polycarboxylic buffers and 2-(N-mopholino) ethane-sulfonic acid buffer significantly affect growth of some mycorrhizal fungi, though not necessarily the same isolates (Giltrap and Lewis, 1981; Hung, unpublished data; Michaels, 1982). In addition, changes in pH of the medium over time can provide some clues to effects of pH on metabolism of an isolate. Other workers (Modess, 1941; Norkrans, 1950; Hubsch, 1963) have found that, even with buffering, the pH of the nutrient solution can change by more than a full pH unit between the beginning and the end of a fungal growth experiment.

Six replicate 250 ml Erlenmeyer flasks each received 100 ml of nutrient solution for each pH and fungus combination. The flasks were autoclaved at 121°C for 15 min. Each flask was then inoculated with 1 ml aliquot (containing ca. 0.3 mg) of the previously prepared mycelial homogenate. Inoculated flasks were inoculated at room temperature (ca. 22°C) for 30 days. Mycelia were then filtered out with Whatman glass microfiber filters, oven-dried at 105°C for 24 h, and weighed. Weights were expressed as mg mycelium per ml nutrient solution. Final pH of the filtrate in each flask was determined with a glass electrode pH meter.

In experiment 1, Pisolithus tinctorius and Hebeloma crustuliniforme were each represented by two isolates and the other

seven species by one (Table I.2). All flasks (6 replicates of each of 11 isolates X 6 pH treatments for a total of 396 flasks) were randomly placed on a large-capacity rotary shaker operating at 150 turns/min. Final dry weights and pH's for each species were subjected to analysis of variance with significant differences among means tested by Tukey's honestly significant difference test (HSD) at $P = 0.05$.

In experiment 2, differences among four isolates each of Cenococcum geophilum and Laccaria laccata (Table I.2) were examined. Both species grow well in stationary culture, so flasks were randomly placed in a dark cabinet rather than on the shaker (6 replicates of each of 4 isolates X 6 pH treatments for a total of 144 flasks for each species). The final data were analyzed as a 4 X 6 factorial design, and polynomial regression curves were fitted for each species.

RESULTS AND DISCUSSION

Growth of each isolate in experiment 1 differed significantly with differences in pH; five growth patterns are evident (Table I.3). Suillus lakei tended to have a double peak (at pH 4 and 6), but we included it with isolates having a significantly high growth spanning three pH units, because the double-peak tendency was not pronounced.

The two isolates of Hebeloma crustuliniforme grew in a similar pattern: dry weights increased with pH up to 7. Isolate S-166 grew

particularly rapidly and thus appears to be a good candidate for inoculation on seedlings to be planted in soils with high pH. The two isolates of Pisolithus tinctorius differed in growth pattern. The best growth of isolate S-471 spanned three pH units, with an optimum of pH 4-5 but no growth at pH 2. The best growth of isolate S-431 spanned five pH units, with two optima (pH 4 and 6). S-471 would still be the better candidate for nursery inoculation, because it grew as much as or more than S-431 at all pH's except 2. Of the other species, those with best growth spanning four or five pH units would be good candidates for inoculation, provided they can be grown at acceptable rates and that their other characteristics prove satisfactory for inoculation.

At pH 6 and lower, the mycelium of Piloderma bicolor was bright yellow and that of Pisolithus tinctorius was golden yellow. At pH 7, all three had white mycelia. Reduced pigment production by ectomycorrhizal fungi near one or both extremes of their pH growth range in vitro has been reported for several other ectomycorrhizal fungi (Melin, 1924; Mikola, 1962; Laiho, 1970). Their metabolism is clearly altered in such circumstances, but the nature of the alteration and its effects on the functioning of the fungi are unknown.

The comparisons of different isolates within species (experiment 2, Cenococcum geophilum and Laccaria laccata) revealed significant differences in response to pH. None of the four isolates of C. geophilum grew appreciably at pH 2 (Figure I.1). Growth of isolate A-144 peaked rather strikingly at pH 3 and declined steadily

with further pH increases. Growth of isolate A-145 also increased rapidly from pH 2 to 3, but continued high to pH 7. A-167 showed a pattern similar to A-145, but did not attain the high level until about pH 4.5. A-161 closely resembled A-145 to about pH 5, but its growth then declined with increase of pH.

Diversity among isolates of Laccaria laccata was also pronounced (Figure I.2). Isolate S-22 peaked at near pH 4, S-33 at near pH 4.5, and S-238A at near pH 6. Growth of S-326 continued to increase with increasing pH, but with a striking increase from pH 6 to 7.

For most isolates in experiment 1, the pH of the nutrient solution decreased between the beginning and the end of the experiment in direct proportion to mycelial growth, i.e., the more growth, the greater the reduction in pH (Table I.3). Absolute pH reduction differed between isolates, however. Thus, the maximum growth of the fast-growing Laccaria laccata S-238A (0.782 mg/ml at pH 6) was accompanied by a mean reduction of 2.7 pH units, but the maximum of the slow-growing Amanita muscaria S-230 (0.130 mg/ml, also at pH 6) entailed a mean reduction of 2.6 pH units. The two isolates of Hebeloma crustuliniforme differed drastically from each other in terms of reduction of pH of the medium. S-166 followed the general pattern of greater pH reduction with greater growth. S-260, in contrast, grew strikingly best at the starting pH of 7 (0.708 mg/ml) with a pH drop of 1.7 units by the end of the experiment; the growth at the starting pH 6 was significantly less (0.212 mg/ml), but the pH dropped 2.4 units.

In experiment 2, all isolates of Cenococcum geophilum behaved similarly in reducing pH of the medium, regardless of differences in growth pattern. The same can be said for all isolates of Laccaria laccata. The two species differed strikingly from each other, however (Figures I.3 and I.4). Only two of the four isolates of each are shown, because the curves overlapped too much for all four to be decipherable. For isolates of C. geophilum, the reduction of medium pH between the start and the end of the experiment became more pronounced as starting pH was increased from 2 to 5 (Figure I.3). By starting pH 7, however, the change from beginning to final pH was negligible, even though growth of A-144 was low and that of A-145 was high at pH 7 (Figures I.1 and I.3). For isolates of L. laccata, in contrast, the higher the starting pH, the more the pH dropped by the end of the experiment - again regardless of growth pattern (Figures I.2 and I.4). Apparently, each species as represented by these isolates responds metabolically to the high pH environment in ways not directly related to growth rate, but the two species do not respond in the same manner.

The reasons for differences between isolates in response to initial pH of the medium or in reductions of medium pH over time might include differences in selective ion uptake and production of organic acids by the mycelium. Further experimentation is required to determine the cause of such pH shifts for any given isolate. Such information could provide important insights into the ecology of these fungi and their interactions with mycorrhizal hosts.

None of the variations in pH reponse between species or isolates within species can be related to the habitat sources of the cultures. For example, none of the isolates that sustained good growth at pH 7 originated from high pH soils. The generality that ectomycorrhizal fungi are acidophilic (Melin, 1924; Modess, 1941) clearly has many exceptions.

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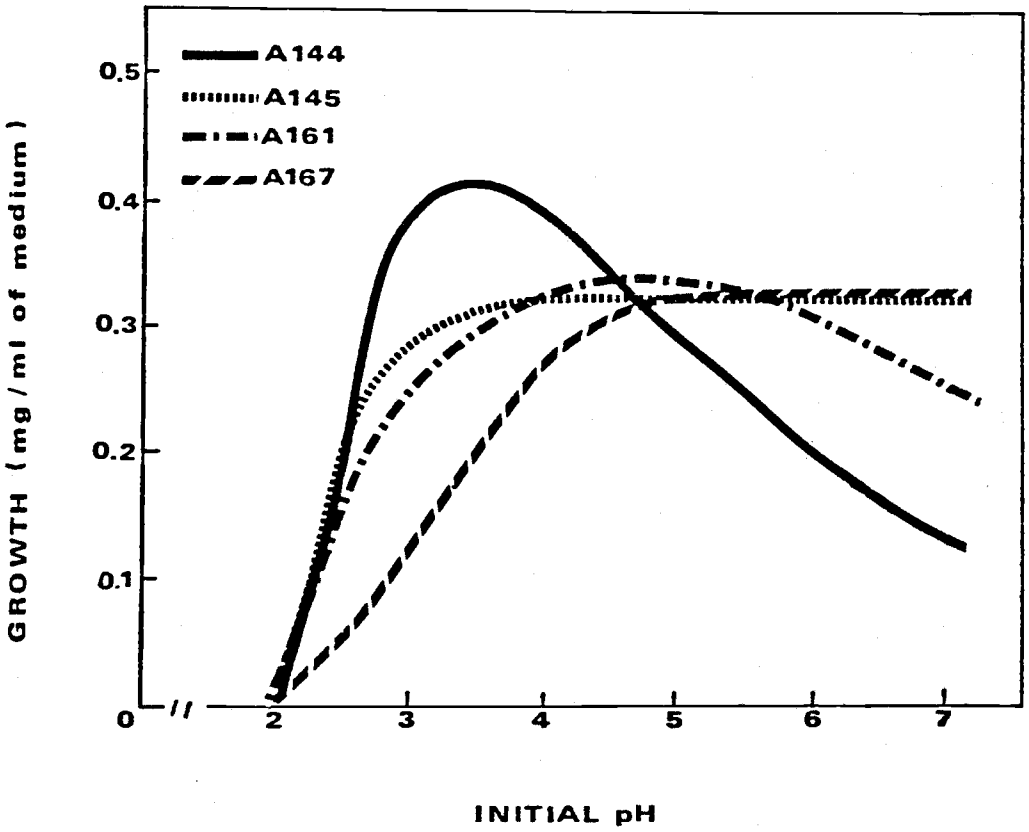

Figure I.1. Mg/ml of Cenococcum geophilum mycelium produced by four isolates in 30 days at different pH's.

A-144 : $Wt = 70.81\ (pH - 2)\ \exp.[-0.63\ (pH - 2)]$, $r^2 = 0.965$.

A-145 : $Wt = 32.68\ \{1-\exp.[-2.03\ (pH - 2)^2]\}$, $r^2 = 0.952$.

A-161 : $Wt = 34.87\ (pH - 2)\ \exp.[-0.36\ (pH - 2)]$, $r^2 = 0.972$.

A-167 : $Wt = 33.47\ \{1-\exp.[-0.43\ (pH - 2)^2]\}$, $r^2 = 0.966$.

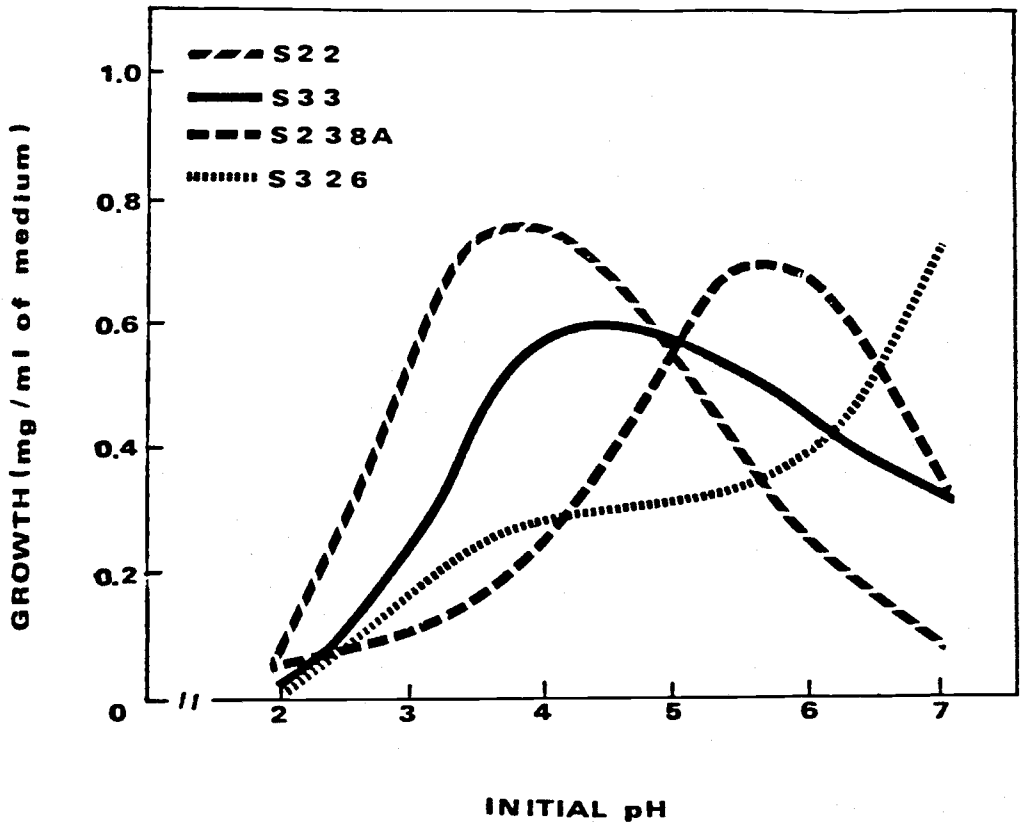

Figure I.2. Mg/ml of Laccaria laccata mycelium produced by four isolates in 30 days at different pH's.

S-22 : $\ln(Wt) = -0.06 + 11.81 \ln(pH) - 3.36\ pH + 0.05\ (pH)^2$, $r^2 = 0.775$.

S-33 : $\ln(Wt) = 0.39 + 17.56 \ln(pH) - 5.99\ pH + 0.23\ (pH)^2$, $r^2 = 0.930$.

S-326 : $\ln(Wt) = 3.01 + 23.67 \ln(pH) - 10.01\ pH + 0.54\ (pH)^2$, $r^2 = 0.991$.

S-238A : $\ln(Wt) = -3.72 - 14.80 \ln(pH) + 8.82\ pH - 0.53\ (pH)^2$, $r^2 = 0.851$.

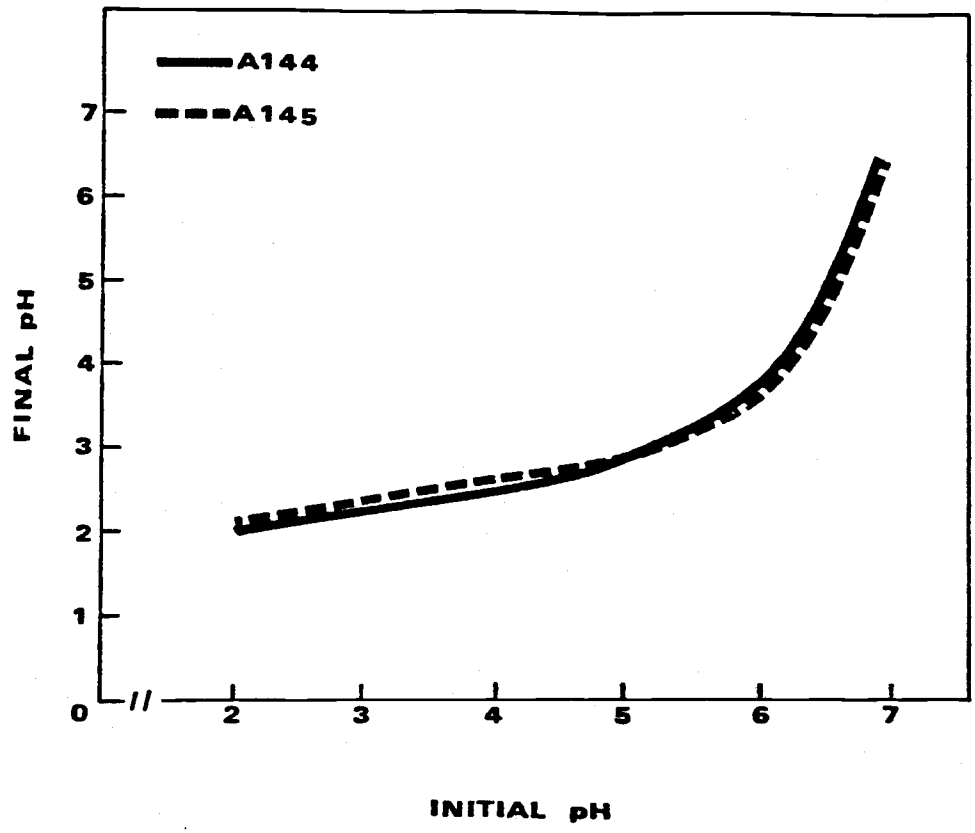

Figure I.3. Final pH (pH_f) over initial pH (pH_i) of media after 30 days mycelial growth of Cenococcum geophilum.

A-144 : $pH_f = 2.34 + 0.13 \exp.[0.14 (pH_i-2)^2]$, $r^2 = 0.989$.

A-145 : $pH_f = 2.56 + 0.08 \exp.[0.15 (pH_i-2)^2]$, $r^2 = 0.986$.

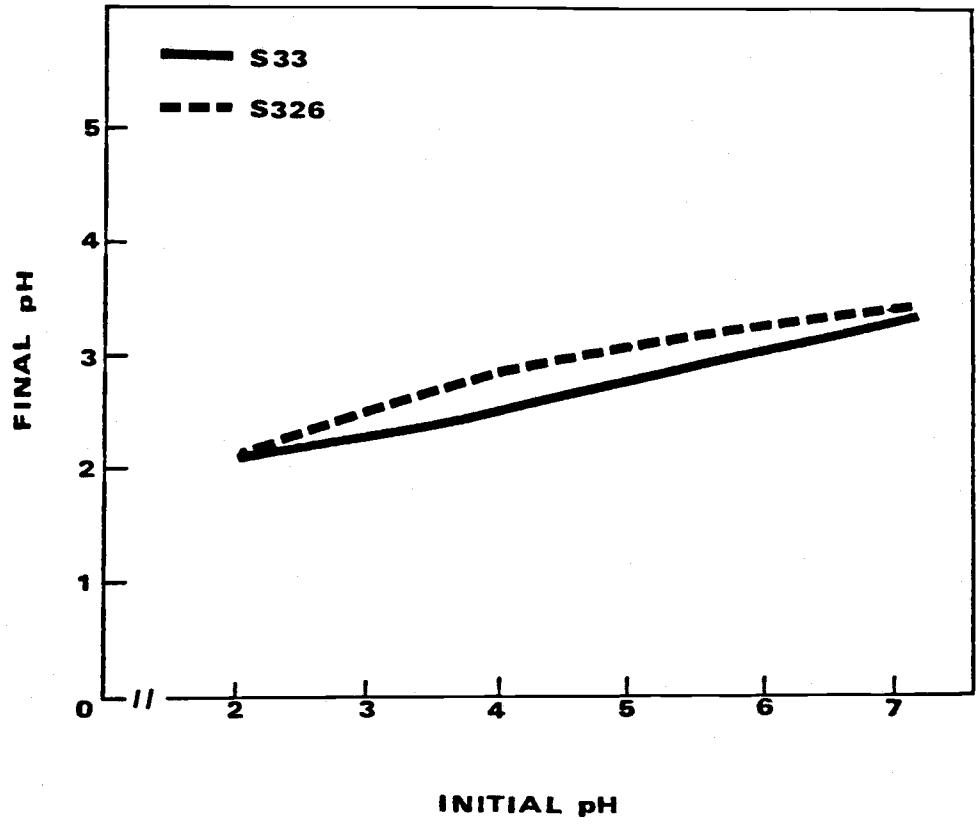

Figure I.4. Final pH (pH_f) over initial pH (pH_i) of media after 30 days mycelial growth of Laccaria laccata.

S-33 : $pH_f = 2.030 + 0.143\ pH_i + 0.007\ (pH_i)^3$, $r^2 = 0.760$.

S-326 : $pH_f = 1.787 + 0.339\ pH_i - 0.017\ (pH_i)^3$, $r^2 = 0.617$.

Table I.1. Estimated cardinal pH values and ranges of pH's that produced growth within 20% of the maximum for ectomycorrhizal fungi *in vitro*.

	pH			pH range for top 20% growth	Reference
	Mini-mum	Opti-mum	Maxi-mum		
Amarita muscaria (L: Fr.) Pers.: Hook	2.5	4.5	6.8	3.5-5.0	Modess, 1941
	3.0	3.5	6.9	3.5-4.1	Modess, 1941
	--	5.4	7.0	5.0-6.5	Shemakhanova, 1962
A. pantherina (DC.: Fr.) Schum.	2.9	4.5	7.5	4.4-4.6	Modess, 1941
A. porphyria (A.&S.: Fr.) Secr.	2.0	3.9	6.0	3.5-4.4	Modess, 1941
Boletus edulis Bull.: Fr.	2.5	5.6	6.4	5.1-5.6	Modess, 1941
	--	3.5	7.4	3.5-4.2	Shemakhanova, 1962
	--	4.0	7.0	3.6-5.0	Hübsch, 1963
B. luridus Schaeff.: Fr.	--	3.5	7.4	3.5-4.5	Shemakhanova, 1962
B. badius Fr.	--	4.9	--	4.8-5.0	Melin, 1924
B. subtomentosus L.: Fr.	3.5	3.7	5.8	3.5-3.8	Shemakhanova, 1962
	--	4.0	8.0	3.7-4.6	Hübsch, 1963
Cenococcum geophilum Fr.	2.6	4.1	6.9	3.9-4.2	Mikola, 1948
	2.0	4.3	7.4	3.7-4.5	Mikola, 1962
Clitopilus prunulus (Scop.: Fr.) Kumm.	2.5	4.9	7.5	4.4-6.0	Modess, 1941
Fuscoboletinus aeruginascens (Secr.) Pom. & Smith	--	4.0	6.8	3.8-4.5	Hübsch, 1963
Lactarius deliciosus (L.: Fr.) S. F. Gray	3.0	5.4	6.8	5.3-5.9	Hübsch, 1963
Leccinum testaceoscabrum (Secr.) Sing.	--	5.0	6.0	4.8-5.2	Hübsch, 1963
Lyophyllum fumosum (Pers.: Fr.) Kuhn. & Rom. (as *Tricholoma*)	3.0	5.0	7.5	4.8-5.2	Norkrans, 1950
Paxillus involutus (Batsch: Fr.) Fr. isolate 1	2.1	3.2	7.0	3.2-4.2	Laiho, 1970
3	2.0	4.6	6.9	4.2-5.2	Laiho, 1970
4	2.9	4.2	6.4	3.6-4.6	Laiho, 1970
5	2.2	5.0	6.9	3.6-5.0	Laiho, 1970
7	2.2	3.2	6.9	3.2-4.2	Laiho, 1970
8	2.1	3.2	7.3	3.1-3.3	Laiho, 1970
12	2.2	3.2	6.9	3.1-3.6	Laiho, 1970
18	2.0	6.4	7.8	6.0-6.5	Laiho, 1970
24	2.0	3.2	7.4	3.1-4.2	Laiho, 1970
Piloderma croceum Erikss. & Hjortst (as *Corticium bicolor*)	2.2	3.5	7.2	3.3-4.7	Mikola, 1962
Pisolithus tinctorius (Pers.) Coker & Couch	--	6.6	11.0	6.0-7.0	Hung & Chien, 1978
Rhizopogon roseolus (Corda) Hollos	3.5	5.9	6.8	5.4-7.9	Modess, 1941
Suillus bovinus (L.: Fr.) O. Kuntze	2.1	5.5	6.9	4.5-6.0	Modess, 1941
	--	3.8	7.2	3.3-5.0	Shemakhanova, 1962
	--	3.0	6.9	3.0-5.0	Hübsch, 1963
	--	6.0	10.8	5.0-7.0	Hung & Chien, 1978
S. granulatus (L.: Fr.) O. Kuntze	2.5	5.0	7.2	4.8-5.1	Modess, 1941
	--	5.7	--	5.6-6.1	Melin, 1924
	--	3.0	6.8	3.0-4.0	Hübsch, 1963

Table I.1
(continued)

	pH			pH range	
	Mini-mum	Opti-mum	Maxi-mum	for top 20% growth	Reference
S. grevillei (Klotzsch) Sing.	3.6	4.8	6.4	3.8-5.0	How, 1940
(= S. elegans)	--	4.0	6.9	3.8-5.0	Hübsch, 1963
S. luteus (L.: Fr.) S. F. Gray	2.5	5.5	6.8	4.3-5.5	Modess, 1941
	--	5.0	7.2	4.4-6.2	Melin, 1924
	--	4.3	7.1	3.8-5.0	Shemakhanova, 1962
S. placidus (Bon.) Sing.	--	4.0	7.0	3.7-5.0	Hübsch, 1963
S. tridentinus (Bres.) Sing.	--	5.0	6.9	4.7-5.2	Hübsch, 1963
S. variegatus (Swartz.: Fr.) O Kuntze isolate 1939	2.0	6.5	6.9	5.4-6.5	Modess, 1941
1940	2.0	6.0	6.8	5.5-6.4	Modess, 1941
	--	5.0	7.2	4.6-5.5	Melin, 1924
	--	4.0	7.0	2.8-5.0	Hübsch, 1963
Tricholoma flavobrunneum (Fr.) Kumm.	--	5.0	7.0	4.8-5.2	Norkrans, 1950
T. imbricatum (Fr.: Fr.) Kumm.	--	4.0	6.8	3.2-5.0	Norkrans, 1950
T. matsutake (Ho & Imai) Sing.	2.9	4.8	7.7	4.1-5.5	Kuraishi, 1953
	4.0	5.2	6.0	5.0-5.4	Hamada, 1950
T. pardinum Quel. (=T. tigrinum)	3.0	6.8	8.8	4.8-7.2	Rambelli, 1962
T. pessundatum (Fr.) Quel.	2.0	5.0	6.8	4.8-5.3	Norkrans, 1950
T. robustum (A. & S.: Fr.) Rick. isolate 1	--	4.9	7.7	4.7-5.4	Kuraishi, 1953
2	--	4.5	7.7	4.1-4.9	Kuraishi, 1953
3	--	4.9	8.0	4.8-5.5	Kuraishi, 1953
4	--	4.6	7.7	4.5-5.4	Kuraishi, 1953
T. vaccinum (Pers.: Fr.) Kumm.	--	5.0	6.8	4.4-5.3	Norkrans, 1950

Table I.2. Isolates of ectomycorrhizal fungi used in pH experiments.

Species	Number	Origin	Original year of isolation
Amanita muscaria	S-230	Oregon, central Coast Ranges	1976
Cenococcum geophilum	A-144	California, northern Coast Ranges	1974
	A-145	Oregon, west-central Cascade Range	1974
	A-161	Oregon, west-central Cascade Range	1975
	A-167	Washington, west-central Cascade Range	1975
Hebeloma crustuliniforme	S-166	Oregon, central Coast Ranges	1971
(Bull.: St. Am.) Quel.	S-260	Oregon, Blue Mountains	1976
Laccaria laccata (Scop.: Fr.)	S-22	Finland	1964
Berk & Br.	S-33	Georgia	1963
	S-238A	Oregon, southern Cascade Range	1978
	S-326	Oregon, Corvallis	1976
Piloderma bicolor	S-163	Oregon, locality unknown	1968
Pisolithus tinctorius	S-431	Washington, south-central Cascade Range	1978
	S-471	Georgia	1978
Rhizopogon vinicolor Smith	A-153	Oregon, central Coast Ranges	1975
Suillus lakei (Murr.)	A-163	Oregon, central Coast Ranges	1975
Smith & Thiers			
Thelephora americana Lloyd	S-142	Oregon, Corvallis	1965

Table I.3. Mean mycelial dry weight per milliliter nutrient solution of ectomycorrhizal fungi grown 30 days in modified Melin-Norkran's solution initially adjusted to different pH's and change in medium pH between beginning and ending of the experiment.

Growth pattern, species, and isolates	Dry weight* and pH** by initial pH					
pH	2	3	4	5	6	7
SIGNIFICANT PEAK AT OPTIMUM						
Amanita muscaria S-230		b	b	b	a	b
mg/ml	0	.041	.079	.083	.130	.026
Δ pH	0	-.0.13	-0.83	-1.13	-2.62	-0.30
INCREASE WITH INCREASE pH						
Hebeloma crustuliniforme S-166	c	c	b	b	b	a
mg/ml	.004	.017	.357	.374	.449	1.321
Δ pH	-0.02	-0.01	-1.05	-1.83	-2.70	-3.44
H. crustuliniforme S-260			b	b	b	a
mg/ml	0	0	.068	.104	.212	.708
Δ pH	+0.11	-0.07	-0.52	-1.30	-2.36	-1.67
SIGNIFICANT HIGH SPANS THREE pH UNITS						
Laccaria laccata S-238A	c	b	a	a	a	bc
mg/ml	.018	.205	.694	.747	.782	.096
Δ pH	-0.07	-0.13	-1.13	-1.67	-2.68	-0.21
Piloderma bicolor S-163		b	a	a	a	b
mg/ml	0	.493	.627	.744	.570	.353
Δ pH	-0.07	-0.43	-1.32	-2.14	-2.99	-0.59
Pisolithus tinctorius S-471		ab	a	a	b	b
mg/ml	0	.470	.627	.741	.344	.323
Δ pH	-0.11	-0.36	-1.13	-2.25	-2.55	-1.95
Suillus lakei A-163	e	d	ab	bc	a	c
mg/ml	.057	.226	.343	.324	.400	.231
Δ pH	+0.27	-0.18	-1.23	-1.98	-3.06	-1.43
SIGNIFICANT HIGH SPANS FOUR pH UNITS						
Rhizopogon vinicolor A-153		a	a	a	a	b
mg/ml	0	.231	.222	.262	.269	.071
Δ pH	-0.05	-0.37	-1.18	-1.95	-2.55	-1.11
Thelephora americana A-142	b	a	a	a	a	b
mg/ml	.151	.515	.639	.552	.795	.136
Δ pH	-0.20	-0.63	-1.34	-2.11	-3.08	-0.13
SIGNIFICANT HIGH SPANS FIVE pH UNITS						
Cenococcum geophilum A-145	b	a	a	a	a	a
mg/ml	.020	.283	.351	.300	.343	.313
Δ pH	+0.11	-0.19	-0.97	-1.62	-2.54	-0.73
Pisolithus tinctorius S-431	b	ab	a	ab	a	ab
mg/ml	.008	.128	.209	.152	.242	.100
Δ pH	-0.09	-0.15	-0.93	-1.39	-2.71	-1.30

* Weights within lines not sharing a common letter differ significantly (P = 0.05). Initial weight of inoculum (0.003 mg/ml) has been substracted from each weight.
** Δ pH = final pH - initial pH.

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CHAPTER II

Effects of commercially produced *Laccaria laccata* inoculum on container-grown Douglas-fir and ponderosa pine seedlings

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ABSTRACT

Ectomycorrhizal inoculation of container-grown Douglas-fir and ponderosa pine seedlings with Laccaria laccata vegetative inoculum produced by Sylvan Spawn Laboratories succeeded in a research greenhouse but only with Douglas-fir in container nurseries. Inoculated seedlings had more feeder roots than controls. Percentage of L. laccata mycorrhizae increased with increasing inoculation rates. Optimal inoculation rate for seedling growth and L. laccata mycorrhiza formation differed between nurseries. Seedlings inoculated at the optimal rate resulted in best seedling quality and abundant mycorrhiza formation at each nursery. Practical application of this fungus is feasible.

INTRODUCTION

Laccaria laccata (Scop.: Fr.) Berk. & Br. ranges widely over the world in association with many ectomycorrhizal species of angiospermae and gymnospermae. It has formed well developed ectomycorrhizae in pure culture synthesis with species of Eucalyptus (Malajczuk et al., 1982), Larix (Molina and Trappe, 1982a), Picea (Molina and Trappe, 1982a, Thomas and Jackson, 1979), Pinus (Malajczuk et al., 1982; Marx and Davey, 1969; Molina and Trappe, 1982a; Pachlewski and Pachlewska, 1974), and Pseudotsuga (Molina and Trappe, 1982a; Stack and Sinclair, 1975). It also forms arbutoid mycorrhizae with species of Ericaceae (Zak, 1976; Molina and Trappe, 1982b). L. laccata mycelial inoculum in a sphagnum-vermiculite

carrier has produced ectomycorrhizae on numerous container-grown conifers (Molina, 1980, 1982; Molina and Chamard, 1983; Shaw and Molina, 1980; Shaw et al., 1982).

L. laccata also antagonizes Fusarium oxysporum Schlect. emend. Snyd. & Hans. by colonizing primary roots shortly after seed germination, or by inducing phenolic compound accumulation in cortical cells of primary roots (Sylvia, 1983; Sylvia & Sinclair, 1983a, 1983b; Sinclair et al., 1982). All of these features indicate that L. laccata is a good candidate for inoculation in forest nurseries.

Pure vegetative inoculum of ectomycorrhizal fungi in nurseries is useful, because it permits selection of fungi for specific purposes (Mikola, 1973; Trappe, 1977; Marx, 1980; Marx and Kenney, 1982). Available and effective commercial inoculum is needed for large-scale inoculation of nurseries.

Results of preliminary studies indicated that L. laccata inoculum produced by Sylvan Spawn Laboratories was effective with Douglas-fir seedlings in a greenhouse (Genua et al., 1983; Molina, unpublished data). Experiments were then conducted in a greenhouse and in commercial container nurseries to determine (1) effectiveness of Sylvan Spawn inoculum to form mycorrhizae with Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) and ponderosa pine (Pinus ponderosa Dougl. ex Laws.) seedlings and (2) optimum inoculation rate in a research greenhouse and in commercial nurseries.

MATERIALS AND METHODS

Three experiments were designed, the first two each included two tree species, Douglas-fir and ponderosa pine. Stratified Douglas-fir seeds (0.62-1.0) were provided by International Paper Company, Western Forest Research Center at Lebanon, Oregon; seeds of ponderosa pine from near Roseburg, Oregon, were soaked in 30% H_2O_2 (Trappe, 1961) for one hour and then rinsed in running water for another hour. In Experiment 3 only Douglas-fir was used.

Experiment 1: Greenhouse Study

A completely randomized 5 X 8 factorial experiment was established with five harvesting times (8^{th}, 12^{th}, 16^{th}, 20^{th}, and 24^{th} weeks) and eight inoculation rates (0, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256 (v/v, inoculum/container substrate). Each inoculation treatment was replicated with sixty seedlings of each species.

Vegetative inoculum of L. laccata S238A provided by Sylvan Spawn Laboratories, Worthington, Pennsylvania, in polyethylene bags (ca. 7 l capacity each) was leached with running water (Molina, 1982), air-dried overnight, and stored in a cold room (ca. $2^{o}C$) until used.

Steam pasteurized ($80^{o}C$ for 40 min.) vermiculite-peatmoss (V-P) (1:1, v/v) was used as potting substrate. Inoculum was measured according to each treatment, and then thoughly mixed with the proper amount of V-P substrate. "Ray Leach" containers[1] (65 cc capacity) were filled with the mixture and double-sown with stratified Douglas-fir or ponderosa pine seeds. A thin layer of

silica grit was placed over the seeds to hold them in place and reduce growth of moss and algae. All seedlings were randomly placed in racks of 200 cavities each but with only one hundred cavities filled with seedlings, so that no two seedlings were next to each other. Racks were then randomly placed on the bench in the Forestry Sciences Laboratory greenhouse, Corvallis, Oregon, and systematically rotated in position every other week throughout the experiment. Seedlings were grown May-Nov. 1982 under supplemental sodium-vapor light of ca. 11 klx set for a 16-h photoperiod.

All containers were mist-irrigated twice daily until seed germination was complete, then thinned to one germinant per cell. Two weeks after germination, seedlings were fertilized biweekly with soluble 20-19-18 NPK (Peter's peat-lite special) fertilizer and Sequestrene 300 iron chelate, both dissolved in tap water and pipetted into each cell. Each seedling received 1.4 mg of NPK and 0.7 mg Fe for the first five fertilizing times, and 14 mg NPK and 7 mg Fe for the rest of the experiment. Seedlings were mist-irrigated to saturation three times weekly throughout the experiment.

Eight seedlings from each treatment were randomly selected at each harvest time. Shoot height, oven dry weights of shoot and root, and number of lateral roots were recorded for each seedling. Number

[1] Ray Leach "Cone-tainer Nursery", 1787 N. Pine St., Canby, Oregon, USA. Trade or proprietary names are included for information purposes only and do not imply any endorsement by the United States Department of Agriculture.

of feeder roots were counted under a stereomicroscope by mycorrhizal types (L. laccata vs. Thelephora mycorrhizae vs. nonmycorrhizal).

All data were subjected to analysis of variance. If significant interaction occurred, differences among treatment means were then evaluated by Tukey's test at $P \leq 0.05$.

Experiment 2 : Container Nursery Study, 1982

Two nurseries cooperated in this experiment: International Paper Company and Champion International Container Nursery, both at Lebanon, Oregon. A completely randomized block experiment with five inoculation rates (0, 1:4, 1:16, 1:64, and 1:128, v/v) and four blocks was established in each nursery. Thirty seedlings of each tree species were used in each treatment per block per nursery (total of 30 x 5 x 4 x 2 = 1,200 seedlings in each nursery).

The experiment was begun in the Forestry Sciences Laboratory greenhouse with all processes the same as described in Experiment 1. After complete seed germination and thinning (three weeks for Douglas-fir and eight weeks for ponderosa pine), seedlings were moved to the nurseries. Both nurseries grew the seedlings by their normal, operational methods.

Eight seedlings were randomly selected from each treatment per block at the end of 14^{th} week at each nursery to check mycorrhizal status. The rest of the seedlings were harvested at the 24^{th} week, stored in a cold room (ca. $2^{o}C$), and examined within a month. Ten seedlings were randomly selected from each treatment per block. Shoot height, stem diameter at the root collar, oven-dry weights of shoot and root, and number of mycorrhizal and nonmycorrhizal roots

were recorded. Data from different nurseries and different tree species were analyzed separately. Analysis of variance was conducted on all variables; where significant treatment effects occurred, differences among treatment means were then separated by Tukey's test at $P \leq 0.05$.

Experiment 3: Container Nursery Study, 1983

Seedlings in the Champion International nursery were one month behind the nursery's schedule in 1982, so a repeat experiment was set up in this nursery in 1983 for Douglas-fir seedlings. Fresh inoculum of L. laccata S238B was used. This time, the experiment was begun in the nursery, with unsterilized vermiculite-peatmoss (3:2, v/v) as the substrate for used styroblock-type 5 containers (75 cc capacity each, 98 cavities per rack) with one styroblock of seedlings for each treatment per block.

Ten seedlings were randomly selected at the end of 14^{th}, 18^{th}, and 24^{th} weeks. Variables recorded were the same as in Experiment 2, except that percentage of mycorrhizae was only estimated to the nearest 10% for the 24^{th} week harvest, because of extensive Thelephora colonization. Statistical analysis was the same as for Experiment 2; each harvest was analyzed separately.

RESULTS

Experiment 1: Greenhouse Study

Douglas-fir : Seedlings had abundant L. laccata mycorrhiza formation at the first harvest (8 weeks), with 48% for 1:256, 63% for 1:128, 74% for 1:64, 90% for 1:32, 91% for 1:16, and 99% for 1:8 and 1:4. Significant harvest time x inoculation interactions occurred on all variables measured except root dry weight and total number of lateral roots which also did not differ significantly within harvest times (Table II.1). All variables with significant time x inoculation interaction differed significantly only at the last harvest (24^{th} week) (Table II.2).

At 24^{th} week, inoculated seedlings tended to be taller, had heavier shoot and total weights, and more total feeder roots than noninoculated control seedlings (Table II.2). Within the inoculation treatments, no significant differences were detected for seedling dry weights, shoot:root ratio, total number of lateral roots, and percentage of L. laccata mycorrhizae. Seedlings inoculated at the rate of 1:128 had 79% of the feeder roots colonized by L. laccata and seedling growth equaled that of other inoculations (Table II.2).

Ponderosa pine : Seedlings inoculated at the higher rates (1:4 and 1:8) had abundant L. laccata mycorrhiza formation at the 8^{th} week (50% and 37%, respectively). At lower inoculation rates, mycorrhiza formation was sporadic. Significant harvest time x inoculation interaction occurred only on shoot height, shoot:root ratio and percentage of L. laccata mycorrhizae. Significant effects of inoculation rates occurred on dry weights (shoot, root, and

total), and total number of feeder roots (Table II.3). However, no significant differences were detected among treatment means within each harvest time.

At the 24th week, inoculated seedlings had 70% or more of their feeder roots colonized with L. laccata and control seedlings had none. Otherwise, no significant differences appeared among treatments.

Experiment 2: Container Nursery Study, 1982

At the 14th week, inoculated Douglas-fir and ponderosa pine seedlings had abundant L. laccata mycorrhizae at both nurseries, regardless of inoculation rate. Noninoculated seedlings were free of mycorrhizae. At the 24th week, more than 50% of the feeder roots of all ponderosa pine seedlings were severely contaminated with Thelephora spp. whereas less than 7% of the Douglas-fir feeder roots were contaminated. So, data were collected only from Douglas-fir. Inoculated Douglas-fir had primordia and small fruiting bodies of L. laccata on their root plugs at all inoculation rates at each nursery.

International Paper Company : During the experiment, one block of seedlings was moved to another greenhouse, so only three blocks were used for analysis. Analysis of variance indicated significant block effects on all variables except for shoot:root ratio. Treatment significantly affected all variables except for shoot:root ratio and percentage of Thelephora mycorrhizae.

Inoculated seedlings averaged 73.8% to 80.2% of their feeder roots colonized by L. laccata mycorrhizae as compared to no Laccaria on noninoculated seedlings; all seedlings had some Thelephora mycorrhizae (Table II.4). Seedlings inoculated at the rate of 1:64 had significantly more feeder roots than noninoculated seedlings. Inoculation did not significantly affect total number of feeder roots or percentages of mycorrhizae (Laccaria, Thelephora, and total)(Table II.4). In general, the best treatment was at the 1:64 application rate.

Champion Nursery: Analysis of variance indicated a significant block effect occurred on all variables except for percentages of mycorrhizae (Laccaria, Thelephora, and total). Laccaria inoculation significantly improved percentages of Laccaria mycorrhizae and total mycorrhizae over noninoculated controls. In general, percentage of feeder roots with Laccaria mycorrhizae increased with increased inoculation rate (Table II.5) as did seedling root and total weight.

Experiment 3: Container Nursery Study, 1983

Percentage of feeder roots colonized by Laccaria mycorrhizae increased with increased inoculation rate at all harvests. Seedlings receiving the two lower inoculation rates (1:128 and 1:64) were not as heavy as controls at 18th week on root, and on all weights at 24th week (Tables II.6 and II.7). Seedlings at the 18th week had only L. laccata mycorrhizae. By the 24th week, all seedlings had at least 46% Thelephora mycorrhizae.

DISCUSSION

Laccaria laccata vegetative inoculum produced by Sylvan Spawn Laboratories was effective with Douglas-fir seedlings under research greenhouse and container nursery operations and with ponderosa pine under greenhouse conditions. This first report of successful *L. laccata* mycorrhiza inoculation by commercially produced inoculum under normal cultural practices indicates that this fungus can be used operationally in commercial nurseries.

In nearly all cases, inoculated seedlings had more feeder roots than noninoculated controls. Mycorrhizal fungi produce hormones and growth factors which alter root physiology and affect the morphogenesis of roots (Slankis, 1973). Marx *et al.* (1970) reported that loblolly pine seedlings with ectomycorrhizae of either *Pisolothus tinctorius* (Pers.) Coker & Couch or *Thelephora terrestris* (Ehrh.) Fr. had more feeder roots than those without ectomycorrhizae under aseptic conditions. Graham and Linderman (1980) reported that in an aseptic synthesis Douglas-fir seedlings inoculated with *Cenococcum geophilum* Fr. , *Hebeloma crustuliniforme* (Bull.: St. Am.) Quel. or *L. laccata* stimulated lateral root formation whereas *P. tinctorius* inhibited it as compared to noninoculated controls. Yang and Wilcox (1984), using a culture tube technique, showed that inoculated *Pinus resinosa* Ait. seedlings with *Suillus subluteus* (Peck) Snell or an E-strain fungus (BDG) had more total short roots than control or *P. tinctorius*-inoculated seedlings. In a container system, Douglas-fir seedlings with abundant mycorrhiza formation

(>80%) with either L. laccata or H. crustuliniforme had significantly more feeder roots than those with no mycorrhizae or poor mycorrhiza formation with P. tinctorius (4.0%) (Chapter IV).

In our study, percentages of L. laccata mycorrhizae generally increased with increased inoculation rates, although at 24 weeks all inoculation treatments produced 65% or more colonization of feeder roots by Laccaria except where Thelephora was competing strongly for the root system. Marx et al. (1982), using different inoculation rates (i.e. 1:33, 1:17, and 1:8) to test vegetative inoculum of P. tinctorius in several different container nurseries, had similar results: percentage of short roots and seedlings with P. tinctorius mycorrhizae increased as the inoculation rate increased. However, the differences between different inoculation rates was much more striking in our 1983 experiment than in 1982. This appears to be related to heavy competition by Thelephora sp. in 1983. In 1982, Thelephora contamination was negligible, even by the 24-week harvest. In 1983, Thelephora contamination was negligible at the 18-week harvest, and the percentage of feeder roots colonized by Laccaria reached as high as 56% at the highest rate of application (Table II.6). Between the 18-week and 24-week sampling, however, Thelephora colonization "exploded", increasing during that 6 week period from 0% to 76% of the feeder roots colonized in noninoculated controls (Tables II.6 and II.7). In inoculation treatments, the percentage of feeder roots colonized by Laccaria increased little or even decreased between the 18-week and 24-week samplings (Tables II.6 and II.7). By the 24-week harvest, Laccaria colonization was

roughly inversely propotional to Thelephora colonization. Our experience with ponderosa pine in the container nursery suggests that it may be even more susceptible to Thelephora colonization than is Douglas-fir. Because the percentage of Thelephora mycorrhizae on Douglas-fir seedlings was inversely correlated with percentage of Laccaria mycorrhizae, L. laccata would seem able to compete well with the Thelephora. The ultimate goal of mycorrhiza inoculation is plantation performance; whether or not Laccaria-Thelephora combination produces better field results than either alone remains to be determined.

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Table II. 1. Summary from analysis of variance table of Douglas-fir seedlings inoculated with different rates of *Laccaria laccata* inoculum, grown in a greenhouse and harvested at different times.

	Shoot Height	Dry Weight			Shoot: Root Ratio	Total Feeder Roots	Lateral Roots	*L. laccata* Mycorrhizae (%)
		Shoot	Root	Total				
Time (T)	**	**	**	**	**	**	**	**
Rates (R)	**	**	NS	NS	**	**	NS	**
Interaction (T X R)	**	**	NS	**	**	*	NS	**

* - significantly different at $P \leq 0.05$.
** - significantly different at $P \leq 0.01$.
NS - not significantly different ($P > 0.05$).

Table II. 2. Mean Douglas-fir seedling responses to *Laccaria laccata* inoculation at different rates in a greenhouse after 24 weeks growth.

Rates	Shoot Height (cm)	Dry Weight (mg)			Shoot: Root Ratio	Total Feeder Roots	Lateral Roots (no.)	*L. laccata* Mycorrhizae (%)
		Shoot	Root	Total				
0	8.4c*	363b	275a	637b	1.29b	131c	30a	0.0b
1:256	9.8bc	551a	356a	906a	1.57ab	290ab	29a	65.73a
1:128	12.2a	626a	312a	939a	2.05a	359a	29a	78.79a
1:64	11.0ab	603a	333a	936a	1.80ab	281ab	34a	82.94a
1:32	10.2bc	599a	336a	935a	1.81ab	304ab	30a	84.07a
1:16	10.5ab	531a	309a	840a	1.73ab	296ab	28a	82.98a
1:8	11.4ab	629a	312a	941a	2.01a	303ab	32a	83.68a
1:4	9.6bc	547a	311a	858a	1.78ab	206bc	28a	86.57a

* - Means in the same column not sharing a common letter differ significantly by Tukey's test at $P \leq 0.05$.

Table II. 3. Summary from analysis of variance table of ponderosa pine seedlings inoculated with different rates of *Laccaria laccata* inoculum, grown in a greenhouse and harvested at different times.

	Shoot Height	Dry Weight			Shoot: Root Ratio	Total Feeder Roots	Lateral Roots	*L. laccata* Mycorrhizae (%)
		Shoot	Root	Total				
Time (T)	**	**	**	**	**	NS	**	**
Rates (R)	*	*	**	**	NS	**	NS	**
Interaction (T X R)	*	NS	NS	NS	**	NS	NS	**

* - significantly different at $P \leq 0.05$.
** - significantly different at $P \leq 0.01$.
NS - not significantly different ($P > 0.05$).

Table II. 4. Mean growth responses and mycorrhizal status of Douglas-fir container-grown seedlings inoculated with different rates of Laccaria laccata inoculum, grown in a greenhouse of International Paper Company for 24 weeks (1982).

Rates	Shoot Height (cm)	Stem Diameter (mm)	Dry Weight (mg)			Shoot: Root Ratio	Total Feeder Root	Mycorrhizae (%)		
			Shoot	Root	Total			Laccaria	Thelephora	Total
0	12.7ab*	2.12a	568b	509ab	1077ab	1.14a	256b	0.0b	6.3a	6.3b
1:128	13.3ab	2.06a	588ab	449bc	1036ab	1.35a	318ab	73.8a	2.8a	76.7a
1:64	14.0a	2.15a	666a	529a	1195a	1.27a	367a	80.1a	3.7a	83.8a
1:16	12.7ab	1.95a	523b	416c	939b	1.28a	288ab	78.8a	3.2a	82.0a
1:4	11.8b	2.00a	496b	444bc	940b	1.12a	305ab	80.2a	2.7a	82.9a

* - Means in the same column not sharing a common letter differ significantly by Tukey's test at $P \leq 0.05$.

Table II. 5. Mean growth responses and mycorrhizal status of Douglas-fir container-grown seedlings inoculated with different rates of Laccaria laccata inoculum, grown in a Champion commercial nursery greenhouse for 24 weeks (1982).

Rates	Shoot Height (cm)	Stem Diameter (mm)	Dry Weight (mg)			Shoot: Root Ratio	Total Feeder Root	Mycorrhizae (%)		
			Shoot	Root	Total			Laccaria	Thelephora	Total
0	6.6a*	1.15a	180a	214b	394b	0.86a	90b	1.2c	0.3a	1.5c
1:128	6.7a	1.17a	192a	235b	427ab	0.83a	173a	74.6b	0.0a	74.6b
1:64	6.9a	1.21a	186a	228b	411ab	0.82a	161a	76.6b	0.0a	76.6b
1:16	7.2a	1.24a	205a	246ab	451ab	0.84a	170a	80.3ab	0.0a	80.3ab
1:4	7.1a	1.29a	208a	275a	483a	0.77a	192a	87.9a	0.1a	88.0a

* - Means in the same column not sharing a common letter differ significantly by Tukey's test at $P \leq 0.05$.

Table II. 6. Mean growth responses and mycorrhizal status of Douglas-fir container-grown seedlings inoculated with different rates of Laccaria laccata grown in a greenhouse of Champion commercial nursery for 18 weeks (1983).

Rates	Shoot Height (cm)	Stem Diameter (mm)	Dry Weight (mg)			Shoot: Root Ratio	Total Feeder Root	L. laccata Mycorrhizae (%)
			Shoot	Root	Total			
0	15.5a*	1.60a	556abc	355a	911abc	1.57b	256b	0.8c
1:128	15.0a	1.49a	518bc	294b	812bc	1.77ab	344a	7.2b
1:64	14.3a	1.60a	481c	293b	774c	1.64ab	347a	30.8b
1:16	15.0a	1.60a	622a	356a	978a	1.74ab	380a	48.9a
1:4	14.8a	1.53a	593ab	334ab	927ab	1.80a	385a	56.2a

* - Means in the same column not sharing a common letter differ significantly by Tukey's test at $P \leq 0.05$.

Table II. 7. Mean growth responses and mycorrhizal status of Douglas-fir container-grown seedlings inoculated with different rates of Laccaria laccata grown in a greenhouse of Champion commercial nursery for 24 weeks (1983).

Rates	Shoot Height (cm)	Stem Diameter (mm)	Dry Weight (mg)			Shoot: Root Ratio	Mycorrhizae (%)		
			Shoot	Root	Total		Laccaria	Thelephora	Total
0	17.7a*	2.53a	948a	690a	1637a	1.39a	0.0d	75.9a	75.9a
1:128	15.5a	2.44a	776bc	574bc	1350bc	1.37a	19.1c	74.4a	93.4a
1:64	15.0a	2.42a	719c	540c	1255c	1.34a	30.3bc	59.7a	90.0a
1:16	17.4a	2.50a	924ab	666ab	1590ab	1.39a	38.8ab	59.4a	98.1a
1:4	16.3a	2.47a	922ab	750a	1671a	1.24a	48.8a	46.6a	95.3a

* - Means in the same column not sharing a common letter differ significantly by Tukey's test at $P \leq 0.05$.

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CHAPTER III

Time and temperature affect commercially produced ectomycorrhizal inoculum in storage

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ABSTRACT

Eight isolates of three ectomycorrhizal fungi were tested for effect of inoculum storage on mycorrhiza formation with Douglas-fir. Factors tested were inoculation rate (from 1:256 to 1:4), storage temperature (2°C vs. 21°C), and storage time (up to 1 year). Initial growth chamber tests showed that fresh inocula of the three available isolates of Laccaria laccata and the one isolate of Hebeloma crustuliniforme formed abundant mycorrhizae (>76.3% of the feeder roots) with container-grown Douglas-fir seedlings. However, only one out of the four isolates available of Pisolithus tinctorius was effective.

In general, fresh inocula of L. laccata and H. crustuliniforme were the most effective; their effectiveness remained high for a month of storage, then declined rapidly for a short period, then slowly to the point of no mycorrhiza formation. The effectiveness declined more rapidly with lower inoculation rates. Storage at 2°C prolonged inoculum viability for at least two months over that of 21°C storage. Inoculum from different species or isolates within a species responded to storage temperature differently. P. tinctorius inoculum was the most sensitive: one-month storage drastically reduced its effectiveness. The difference between 2°C and 21°C storage was more obvious in H. crustuliniforme than in either isolate of L. laccata.

INTRODUCTION

Cultured mycelium is desirable for ectomycorrhizal inoculation of nurseries, because it enables a high degree of control of the quality of the inoculum (Mikola, 1973; Marx, 1977; Marx, 1980; Marx and Kenney, 1982). For commercial applications, inoculum with some degree of storage shelf life would be desirable to give the nursery flexibility in timing the inoculation. Few reports are available on inoculum storage, however. Maronek and Hendrix (1980) reported that storage of laboratory-produced Pisolithus tinctorius (Pers.) Coker & Couch mycelial inoculum at 3°C for 6 days drastically reduced its ability to form mycorrhizae with Norway spruce (Picea abies (L.) Karst.), eastern hemlock (Tsuga canadensis (L.) Carr.), and Austrian pine (Pinus nigra Arn.) seedlings. Marx et al. (1982) emphasized that duration of inoculum storage must be tested for commercially produced inoculum.

The objective of our study was to determine how long commercially produced inoculum can be stored and still remain effective in relation to different inoculation rates, storage temperatures, fungal species, and isolates within species.

MATERIALS AND METHODS

Experiment 1

A completely randomized, unbalanced factorial experiment was designed with three factors (temperature, inoculation rate, and storage period). Laccaria laccata (Scop.: Fr.) Berk. and Br. S238A inoculum in a vermiculite/peatmoss substrate was produced and shipped air freight by Sylvan Spawn Laboratories, Worthington, Pennsylvania to Corvallis, Oregon. On receipt of inoculum, one experiment was initiated with eight different inoculation rates (0, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, v/v, inoculum/potting substrate) to test its effectiveness. Then, the inoculum was put into polyethylene bags, half to be stored in a cold room (ca. 2°C), and half in a dark cabinet at room temperature (ca. 21°C). After 1, 2, 3, 4, 5, 6, 9, and 12 months, respectively experiments were set up for fifteen treatments for each storage period: 2 temperatures X 7 rates factorial plus 1 control. Prior to each experiment, a few particles of vermiculite from each bag were aseptically transferred onto modified Melin Norkran's (MMN)(Marx, 1969) agar plates to check the existance of desirable and contaminating fungi.

One part vermiculite and one part peatmoss (v/v) (V-P) was steam pasteurized (80°C, 30 min) as substrate for seedling growth. Because no significant differences were detected on mycorrhiza formation and seedling growth from using leached vs. nonleached inoculum (Hung, unpublished data), and because inoculum of most isolates showed no residual glucose as determined by Test-Tape

(glucose enzymatic test trips, Eli Lilly and Co. Indianapolis), inoculum was not leached. Proper amounts of inoculum and V-P were thoroughly mixed and thirty "Ray Leach" Super cell containers (165 ml) were filled for each treatment. Control treatment received the V-P mixture only. Stratified Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) seeds were double-sown in each container. A thin layer of silica grit was applied to hold seeds in place and reduce growth of moss and algae.

All tubes were then randomly assigned to the 98-tube racks, but only 49 cells were filled, so no two seedlings were next to each other. All seedlings were grown in a walk-in growth chamber in the Forest Research Laboratory, Oregon State University, Corvallis, with 16-h photoperiod, light provided by fluorescent lamps with intensity 2,000 fc at the seedling's level, and temperature 25±2°C/day, 16±2°C/night. Seedlings were watered three times a week throughout the experiment. After seed germination, seedlings were thinned to one germinant per tube. Liquid fertilizer N-P-K 20-19-18 (Peter-lite special) with iron chelator Sequestrene 300 was pipetted every other week as follows : 1.4 mg NPK and 0.7 mg Fe first three times; 2.8 mg NPK and 0.7 mg Fe fourth and fifth times; 14 mg NPK and 7 mg Fe for the rest of the experiment. To eliminate effects of location in the growth chamber, all racks were systematically rotated in position weekly throughout the experiment.

After six months growth, ten seedlings were randomly selected from the original thirty of each treatment to assess mycorrhiza formation and seedling growth. Seedling height, stem diameter at the

root collar, oven-dry weights of shoot and root were recorded. Number of feeder roots were counted under a stereomicroscope (8 X) by mycorrhizal type (L. laccata mycorrhizal vs. nonmycorrhizal).

Data were subjected to analysis of variance, and polynomial regression curves were fitted for percentage of mycorrhizae over storage period.

Experiment 2

This experiment was a completely randomized unbalanced factorial with fungi, temperature and storage period as factors. Seven fungal isolates were used : Hebeloma crustuliniforme (Bull.: St. Am.) Quel. S166, L. laccata S238B and T813, and four isolates of P. tinctorius, S216, 146-246, S270, and BCMF. Inoculation rate was 1:8 (v/v). When the inocula arrived from Sylvan Spawn Laboratories, the first part of the study was established to test the effectiveness of fresh inoculum of each isolate. Then, half of each remaining inoculum was stored at room temperature and half in a cold room as described in Experiment 1. After storage of 1, 2, 4, 6, 9, and 12 months, respectively sequential experiments with fifteen treatments (2 temperatures X 7 fungal isolates plus 1 control) was set up for each storage period with 15 seedlings per treatment.

This experiment was similar to Experiment 1, except smaller "Ray Leach" containers (65 ml capacity each) were used for this study, and each rack (200 seedlings capacity) only held 100 seedlings to minimize cross contamination. Seed sowing, thinning, seedling growth conditions, fertilization, harvesting and data collection and analyses were the same as mentioned in Experiment 1.

RESULTS

No contamination occurred in the growth chamber: noninoculated controls remained completely nonmycorrhizal.

Experiment 1

Seedling growth

Due to the relatively low light intensity, all seedlings were slender, with mean shoot heights of 17.1 cm and stem diameters at the root collar of 1.83 mm. Because growth of controls (noninoculated) seedlings differed significantly between different times in which new batches of seedlings were sown, seedling growth data were analyzed within inoculum storage treatments as the difference between inoculation treatment and control treatment. All means showed in tables were adjusted by using average of all noninoculated seedlings. A significant three-way interaction (inoculation rate x temperature x storage time) occurred for stem diameter and shoot and root dry weights; all two-way interactions were significant for shoot height (Table III.1). However, no general trends could be discerned (Tables A.1, A.2, A.3, and A.4). Seedlings inoculated with fresh inoculum were significantly heavier in shoot weight and lighter in root weight than those with stored inoculum (618 mg vs. 536 mg, and 231 mg vs. 281 mg, respectively) (Tables A.3 and A.4).

Mycorrhiza formation

Again, a significant three-way interaction (inoculation rate x temperature x storage time) occurred (Table III.1). Seedlings inoculated with fresh inoculum had significantly more mycorrhizae

overall than did those inoculated with stored inoculum (86.42% vs. 29.12%) (Table A.5). Although the cubic response surface was significant from regression analysis, adjusted coefficient of multiple determination ($\hat{r}^2$) for cubic fit did not differ significantly from that of quadratic. So, all data were fitted for quadratic regression curves. Because curves for inoculation rates 1:32, 1:64, and 1:16 closely overlapped, only the 1:16 curve is shown in Figure III.1.

Mycorrhiza-forming effectiveness of inoculum decreased with increased in storage time (Figures III.1 and III.2). Differences among inoculation rates increased with increasing storage time, especially between the highest and lowest rates (Figure III.1). Inoculum stored for 4 months or more at cold temperature was more effective than that stored at room temperature, and that difference increased with increasing storage time (Figure III.2).

Experiment 2

Pisolithus tinctorius

All inocula had abundant mycelia and grew well from vermiculite particles aseptically transferred to MMN agar. Only isolate 144-246 contained 0.1% residual glucose. Isolate 216 was contaminated with Penicillium spp.. Seedlings treated with fresh inoculum of isolates 146-246, S216, or S270 formed no mycorrhizae after six months in the growth chamber. Those receiving fresh inoculum of isolate BCMF had 30.8% feeder-root colonization by P. tinctorius. However, its mycorrhiza-forming ability was significantly reduced to $<1.0\%$ when inoculum was stored for one or more months at either temperature

treatment. No mycorrhizae occurred on seedlings treated with 4-month-stored inoculum, so the study on P. tinctorius was terminated and data analyzed separately as seven treatments (2 temperatures X 3 storage times plus time 0).

Seedlings had average height of 14.3 cm and stem diameter of 1.7 mm. Storage time significantly affected shoot and root dry weights (Tablea III.2 and A.6). Seedlings inoculated with fresh inoculum formed significantly more mycorrhizae than the mean of those receiving stored inoculum (30.8% vs. 1.0%) (Tables III.2 and A.6).

Hebeloma crustuliniforme and Laccaria laccata

Seedling growth

For the same reasons as in Experiment 1, data were analyzed within inoculum storage treatments as the difference between inoculated and noninoculated treatment. A significant storage time main effect occurred on shoot height and stem diameter (Table III.3), but no trend could be perceived (Table A.7). Stem diameter of seedlings inoculated with fresh inoculum was significantly greater than for those receiving stored inoculum (1.77 mm vs. 1.69 mm) (Table III.8). A significant fungus x time x temperature interaction was detected on shoot and root dry weights (Table III.3), but, again no trend could be drawn from data. Seedlings inoculated with fresh inoculum had significantly heavier shoot weight and lighter root weight than those receiving stored inoculum, and different fungi responded differently (Table A.8).

Mycorrhiza formation

A significant fungus x time x temperature interaction occurred (Table III.3). Seedlings receiving fresh inoculum had significantly more mycorrhizae than those receiving stored inoculum, regardless of fungal isolates and storage temperature (83.62% vs. 26.32%)(Table A.9).

Different fungi responded to storage period and temperature differently. In general, mycorrhiza-forming ability was reduced with increased storage periods. Inoculum stored at 21°C lost its viability sooner than that stored at 2°C (Figure III.3). The difference in H. crustuliniforme mycorrhiza formation between 2°C and 21°C temperature storage increased with increasing storage period. The two isolates of L. laccata differed less in this way than did the Hebeloma isolate (Figure III.3).

DISCUSSION

The general trend of inoculum effectiveness and storage period is as follows: fresh inoculum was the most effective, its effectiveness remained high until one month and declined rapidly for a short period, then slowly to the point of no mycorrhiza formation. In Experiment 1, at the lower inoculation rate, the effectiveness of L. laccata S238A declined faster than that at the higher rate, and this difference was most striking when the highest and lowest rate were compared (Figure III.1). Storage at 2°C prolonged L. laccata S238A inoculum effectiveness for at least 3 months. The difference

between temperature storage was very pronounced after inoculum was stored for more than three months (Figure III.2).

In Experiment 2, fungal isolates and storage temperature interacted with inoculum storage period on inoculum effectiveness. Different species and isolates within a species responded differently on storage temperature and period. Regarding inoculum effectiveness, H. crustuliniforme was the most sensitive species tested to storage temperature, and L. laccata S238B was more sensitive than isolate T813 to temperature. Storage at 2°C prolonged inoculum effectiveness of H. crustuliniforme and L. laccata T813 for at least three months over that at 21°C storage, whereas effectiveness of L. laccata S238B was prolonged two months (Figure III.3).

Fresh inocula of H. crustuliniforme and L. laccata formed abundant mycorrhizae (>76.3%) with Douglas-fir seedlings. P. tinctorius inocula differed from the others. Although inocula of all isolates of P. tinctorius had abundant hyphae and grew well on nutrient agar plates, only isolate BCMF formed mycorrhizae with container-grown Douglas-fir seedlings under experimental conditions. The reason for failure of P. tinctorius inoculum to form mycorrhizae with Douglas-fir might include microbial contamination (isolate 216), or residue glucose (isolate 144-246). This fungus is sensitive and tolerates little disturbance. Hung and Trappe (1983) indicated that P. tinctorius was the only fungus of several studied that could not stand homogenization for more than 10 sec., while others (including H. crustuliniforme and L. laccata) withstood it for 25

sec. Marx et al. (1982) reported that leaching, drying and several days storage of inoculum destroyed the external hyphae of P. tinctorius inoculum. Maronek and Hendrix (1980), reported that seedlings inoculated with stored ($3^{o}C$, 6 days) P. tinctorius inoculum had 74% fewer total numbers of P. tinctorius mycorrhizal seedlings on Norway spruce and 96% on eastern hemlock as compared to those with fresh inoculum. Although we used nonleached inoculum to minimize the disturbance, one month storage of inoculum of BCMF reduced its mycorrhiza-forming ability (Table A.6). Thus, the inoculum of P. tinctorius should be carefully handled and we cannot recommend storage.

From our study, hyphae of L. laccata and H. crustuliniforme grew from vermiculite particles after aseptic transfer onto MMN agar plates. This indicates these fungi were still alive in the vermiculite. Although Pantidou et al. (1983) reported that a few conidia formed on a 30-day-old L. laccata culture on PDA, scanning electron microscopy of vermiculite particles from stored inoculum revealed no conidia or spore-like structures.

Fresh and stored inoculum clearly had different physiological and growth phases. According to Cochrane (1958), mycelial growth of fungi can be qualitatively divided into three stages : (1) lag phase, where no apparent growth occurs, (2) linear phase, with rapid and approximately linear growth, and (3) decline phase, with no growth or a decline in dry weight due to autolysis. Mycelium of fresh inoculum was fully grown and probably at its physiologically active linear growth phase. Storing inoculum at room temperature

would keep mycelia growing until it depleted the available nutrients and went into the decline phase. Cold temperature slows fungal metabolism and mycelial growth rate, thus prolonging inoculum viability.

If rich nutrients are given to stored inoculum, hyphae inside vermiculite particles might use these nutrients to begin new growth and remain physiologically active, as happened in plating vermiculite particles from stored inoculum onto MMN agar. However, in container systems the inoculum is mechanically disturbed by mixing with soil substrate, and the fungi are nutrient-deprived, especially for a carbon source, for four to six weeks between seeding and first feeder root formation. These delays might force the fungi to senesce, become physiologically inactive, and reduce or lose their mycorrhiza-forming ability. Further experimentation is required to determine the cause of this reduction in mycorrhiza-forming ability.

CONCLUSIONS AND RECOMMENDATIONS

Fresh inocula of Laccaria laccata and Hebeloma crustuliniforme produced by Sylvan Spawn Laboratories formed abundant mycorrhizae with Douglas-fir seedlings. However, inoculum of Pisolithus tinctorius were less effective, with only one of four isolates forming as much as 30.8% mycorrhizae. In general, mycorrhiza-forming ability of fungi tested decreased with increased storage time. The lower was the inoculation rate, the sooner was the inoculum

effectiveness decline. Storage at 2°C of inoculum prolonged the mycorrhiza-forming ability for at least two months.

Different fungal species and different isolates within a species responded to inoculum storage differently. P. tinctorius was the most sensitive fungus in our study. Its mycorrhiza-forming ability decreased significantly after inoculum storage of one month, and was totally lost after four-months of storage. Thus, storage of P. tinctorius inoculum is not recommended. H. crustuliniforme was more sensitive to storage temperature than either isolates of L. laccata tested.

We recommended:

1. Use of fresh inoculum whenever possible, especially for sensitive fungi such as P. tinctorius.
2. Storage of inoculum either at room or cold temperature for only a short term (less than two months) and prevention of its exposure to direct sun (cold storage preferable).
3. If longer storage is needed, inoculum should be cold-stored, and higher inoculation rates (i.e. 1:4 or 1:8) used to ensure good mycorrhiza formation.

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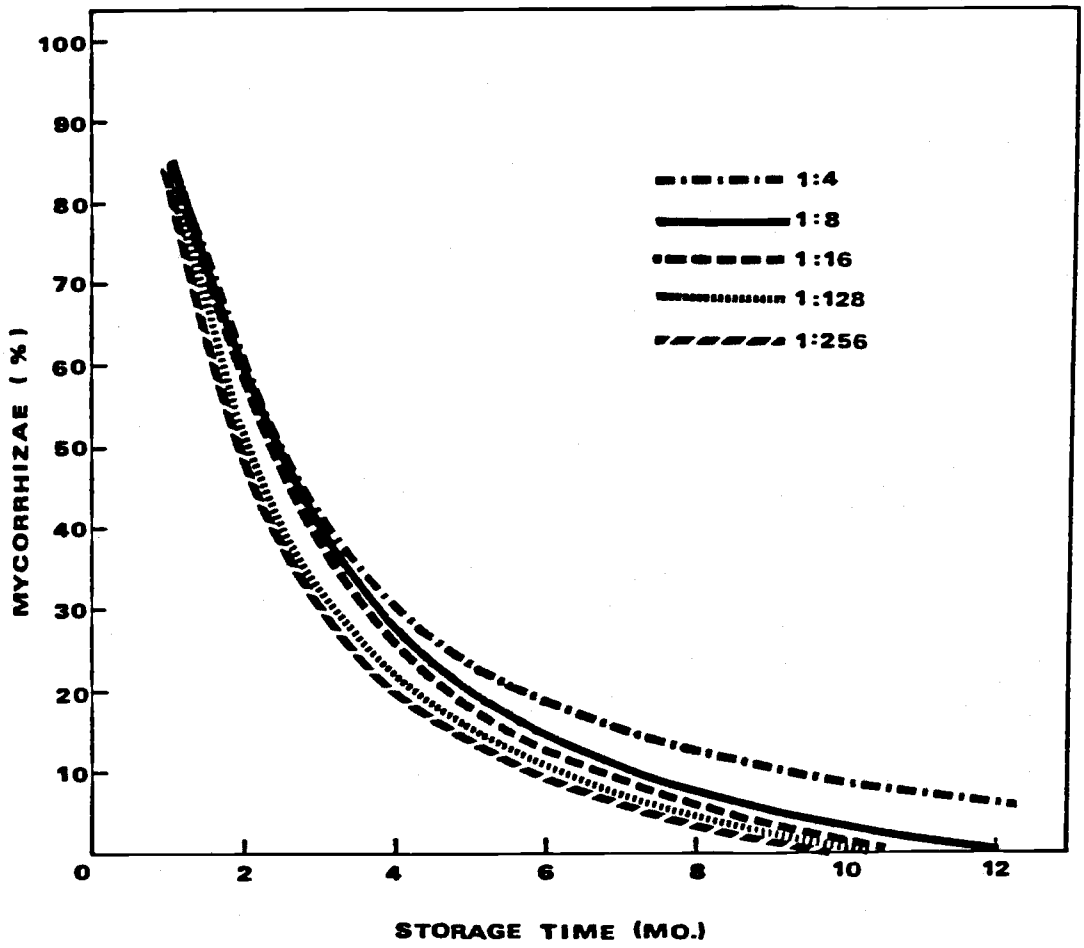

Figure III.1. Relationship between Laccaria laccata mycorrhiza formation and inoculum storage time with different inoculation rates.

1:4, $Y = -7.49 + 169.96\ (1/X) - 78.40\ (1/X^2)$, $r^2 = 0.934$.

1:8, $Y = -15.16 + 193.53\ (1/X) - 97.10\ (1/X^2)$, $r^2 = 0.912$.

1:16, $Y = -17.19 + 193.04\ (1/X) - 90.04\ (1/X^2)$, $r^2 = 0.913$.

1:128, $Y = -15.64 + 167.02\ (1/X) - 69.97\ (1/X^2)$, $r^2 = 0.961$.

1:256, $Y = -14.60 + 150.08\ (1/X) - 52.27\ (1/X^2)$, $r^2 = 0.959$.

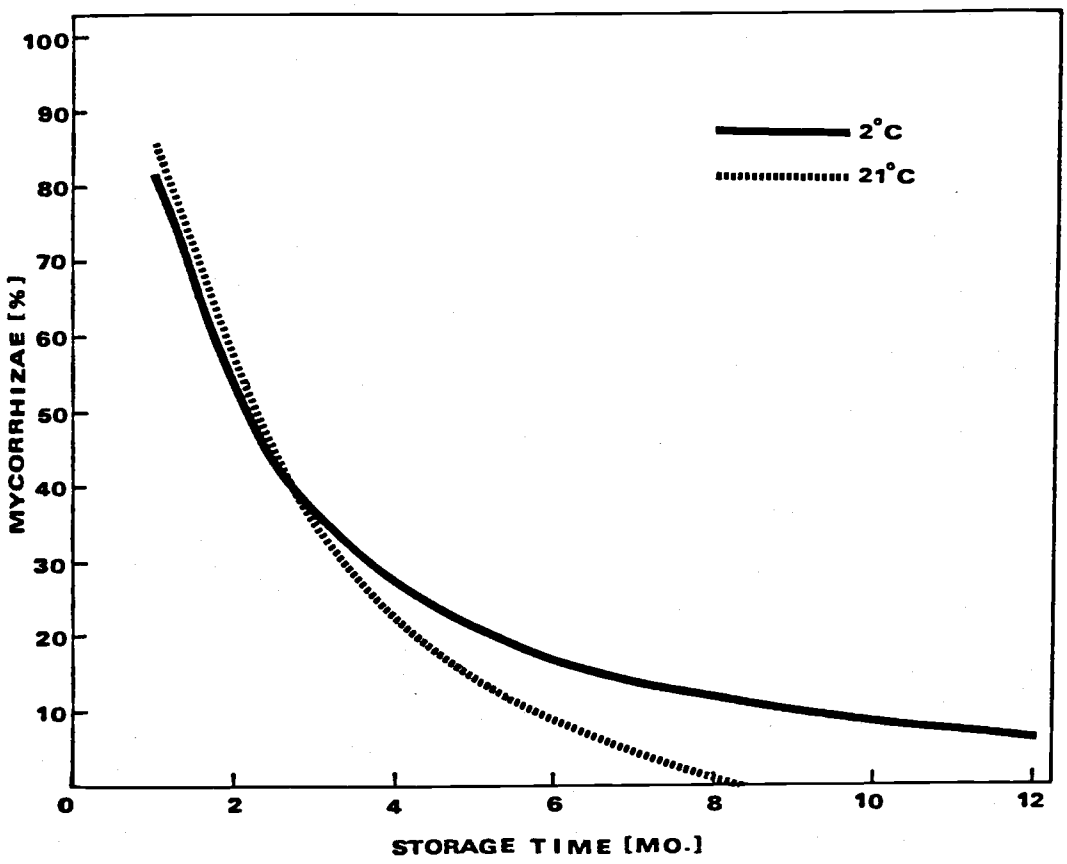

Figure III.2. Relationship between Laccaria laccata mycorrhiza formation and inoculum storage time with inoculum stored at different temperatures.

$2^{o}C$: $Y = -5.46 + 145.98\ (1/X) - 59.07\ (1/X^2)$, $r^2 = 0.914$.

$21^{o}C$: $Y = -23.41 + 207.59\ (1/X) - 98.60\ (1/X^2)$, $r^2 = 0.918$.

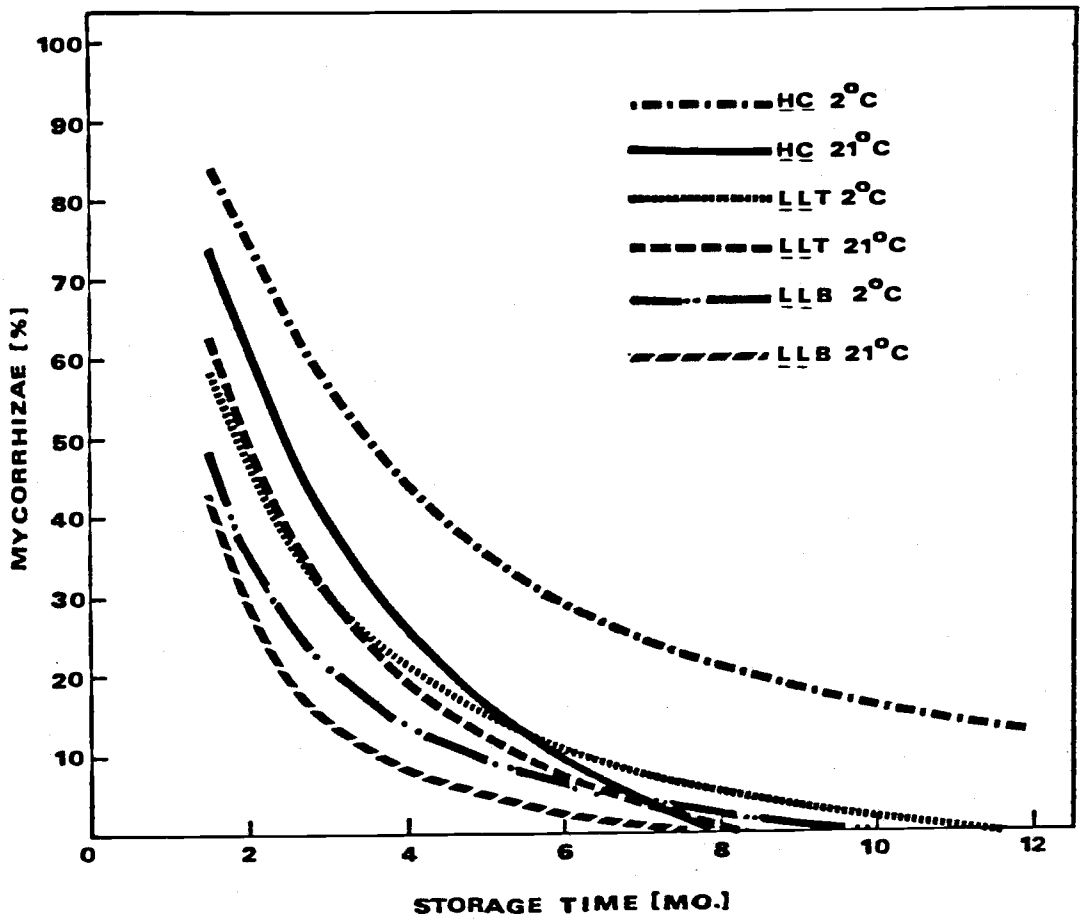

Figure III.3. Relationship between mycorrhiza formation of Douglas-fir seedlings and inoculum storage time for different fungal inoculum. (HC: Hebeloma crustuliniforme, LLT: Laccaria laccata T813, LLB: Laccaria laccata S238B.)

HC 2°C: $Y = -6.38 + 239.31\ (1/X) - 156.02\ (1/X^2)$, $r^2 = 0.928$.

HC 21°C: $Y = -28.22 + 250.23\ (1/X) - 145.37\ (1/X^2)$, $r^2 = 0.952$.

LLT 2°C: $Y = -9.60 + 96.70\ (1/X) - 15.93\ (1/X^2)$, $r^2 = 0.986$.

LLT 21°C: $Y = -8.28 + 58.56\ (1/X) + 26.75\ (1/X^2)$, $r^2 = 0.984$.

LLB 2°C: $Y = -12.76 + 152.75\ (1/X) - 70.28\ (1/X^2)$, $r^2 = 0.977$.

LLB 21°C: $Y = -19.94 + 175.30\ (1/X) - 76.92\ (1/X^2)$, $r^2 = 0.977$.

Table III. 1. Summary from analysis of variance table of 6-month-old container-grown Douglas-fir seedlings inoculated with Laccaria laccata mycelial inoculum stored at different temperatures for different periods (values are P values of corresponding F statistics).

Source of Variation	d.f.	Shoot Height (cm)	Stem Diameter (mm)	Dry Weights (mg) Shoot	Dry Weights (mg) Root	Mycorrhizae (%)
Trt (1): 0 vs. 2 x 8	1	0.998	0.764	0.000**	0.000**	0.001**
Trt (2): Temp (C)	1	0.167	0.002**	0.000**	0.001**	0.001**
Trt (3): Time exc. 0 (T)	7	0.000**	0.000**	0.000**	0.000**	0.001**
Trt (4): C X T	7	0.026*	0.000**	0.014*	0.001**	0.001**
Den (D)	6	0.364	0.000**	0.012*	0.000**	0.001**
D X Trt (1)	6	0.411	0.529	0.102	0.018*	0.675
D X Trt (2): D X C	6	0.036*	0.246	0.681	0.241	0.002**
D X Trt (3): D X T	42	0.001**	0.000**	0.002**	0.001**	0.000**
D X Trt (4): D X C X T	42	0.096	0.022**	0.026*	0.003**	0.000**

*, ** significant at 5% and 1% level, respectively.

Table III. 2. Summary from analysis of variance table of 6-month-old container-grown Douglas-fir seedlings inoculated with *Pisolithus tinctorius* mycelial inoculum which stored at different temperature for different periods. (values are P-values of corresponding F statistics).

Source of Variation	d.f.	Shoot Height (cm)	Stem Diameter (mm)	Dry Weights (mg) Shoot	Dry Weights (mg) Root	Mycorrhizae (%)
Trt(1): 0 vs 2x3	1	0.165	0.501	0.149	0.167	0.000**
Trt(2): Temp (C)	1	0.310	0.905	0.699	0.997	0.496
Trt(3): Time (T)	2	0.542	0.595	0.010**	0.001**	0.813
Trt(4): C x T	2	0.424	0.529	0.666	0.404	0.813

** significant at 1% level.

Table III. 3. Summary from analysis of variance table for 6-month-old container-grown Douglas-fir seedlings innoculated with different ectomycorrhizal inoculum which stored at different temperatures for different periods (values are P-values of corresponding F statistics).

Source of Variation	d.f.	Shoot Height (cm)	Stem Diameter (mm)	Dry Weights(mg) Shoot	Dry Weights(mg) Root	Mycorrhizae (%)
Fungi (F)	2	0.465	0.774	0.989	0.080	0.000**
Trt (1): 0 vs. 2 x 6	1	0.500	0.002**	0.002**	0.424	0.000**
Trt (2): Temp. (C)	1	0.463	0.950	0.726	0.476	0.000**
Trt (3): Time (exclude 0)(T)	5	0.000**	0.000**	0.000**	0.000**	0.000**
Trt (4): C x T	5	0.468	0.283	0.771	0.951	0.000**
(F) x Trt (1)	2	0.314	0.787	0.001**	0.000**	0.115
(F) x Trt (2): F x C	2	0.431	0.283	0.077	0.112	0.000**
(F) x Trt (3): F x T	10	0.242	0.219	0.476	0.349	0.000**
(F) x Trt (4): F x T x C	10	0.098	0.224	0.000**	0.031*	0.038*

*, ** significant at 5% and 1% level, respectively.

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CHAPTER IV

Ectomycorrhizal inoculation of Douglas-fir plug+1 seedlings with commercially produced inoculum

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ABSTRACT

Ectomycorrhizal inoculation of Douglas-fir plug+1 seedlings succeeded with Laccaria laccata and Hebeloma crustuliniforme, with 83.0% and 90.2%, respectively, of the feeder roots colonized by inoculated fungi at the end of the container growing phase. Pisolithus tinctorius-inoculated seedlings, in contrast, were only 4.0% mycorrhizal. L. laccata- or H. crustuliniforme-inoculated seedlings had significantly more mycorrhizal and total feeder roots than P. tinctorius-inoculated or noninoculated seedlings. Inoculated seedlings had significantly higher foliage Zn level, and all but L. laccata-inoculated seedlings had significantly lower foliage Mn level as compared to noninoculated controls.

After being transplanted from containers to nursery beds and growth in the beds for 17 months, all seedlings were 80% mycorrhizal. H. crustuliniforme persisted as a mycorrhizal dominant on seedlings initially inoculated with this fungus. L. laccata-inoculated seedlings had 40% of their feeder roots colonized by Laccaria and another 40% by native fungi (Rhizopogon and Thelephora spp.). All mycorrhizae of P. tinctorius-inoculated and noninoculated seedlings were formed with fungi native to the nursery bed.

INTRODUCTION

"Plug+1" seedlings, initially grown in containers for one season and then transplanted into bareroot nursery beds in late summer, are one of the newer seedling types used for reforestation. They characteristically have bushy tops and mop-like, fibrous root systems. They survive and grow well on typical Pacific northwestern sites, and have cost:benefit ratios competitive with other seedling types (Hahn, 1984). Mycorrhiza formation increases root-soil interfaces, water and nutrient uptake of hosts, and host tolerance to environmental stresses such as extremes of temperature, moisture, pH, and toxicants (Harley and Smith, 1983; Maronek, 1981; Trappe, 1977). Mycorrhiza inoculation of container-grown seedlings may help the seedling overcome the environmental stress during transplanting and increase seedling survival and growth thereafter. Our study was established with commercially produced mycelial inoculum of Hebeloma crustuliniforme (Bull.: St. Am.) Quel. S166, Laccaria laccata (Scop.: Fr.) Berk & Br. S238A, and Pisolithus tinctorius (Pers.) Coker & Couch S216 to determine for Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) plug+1 seedlings, (1) the effectiveness of inoculum to form mycorrhizae, (2) differences among fungal species in effects on seedling growth and mycorrhiza development, and (3) persistence of the inoculated fungus after transplanting.

MATERIALS AND METHODS

Inoculation

The study was a completely randomized block with four inoculation treatments: H. crustuliniforme, L. laccata, or P. tinctorius vegetative inocula vs. noninoculated controls, each with three replicated blocks. Two hundred seedlings were used in each treatment per block (total of 200 x 4 x 3 = 2,400 seedlings).

Fungal inocula in vermiculite-peatmoss were provided by Sylvan Spawn Laboratories, Worthington, Pennsylvania in polyethylene bags (ca. 7 l capacity each). Inoculum of each species was wrapped separately with doubled cheesecloth and leached with cold running tap water to remove unused nutrients. Excess water was removed by gently squeezing the inoculum. Inoculum was then spread on paper, air-dried overnight, and used the following day. Before leaching the inoculum, a few vermiculite-peatmoss particles of each fungal species from each bag were aseptically transferred to modified Melin-Norkran's (MMN) (Marx, 1969) agar plates to ensure the viability of the desirable fungus and check for contaminations.

Steam pasteurized (80°C, 40 min.) vermiculite:peatmoss (V-P) (1:1, v/v) served as soil mixture. The inoculation rate was 1:6 (v/v). V-P mixture was spread in a layer over brown paper; proper amounts of inoculum were then sprinkled evenly onto the V-P mixture and throughly mixed in. "Ray Leach" containers (65 cc capacity each) were then filled with the mixture. For noninoculated controls, containers were filled with V-P mixture only. Stratified Douglas-fir seeds were double-sown in each tube and covered with a layer of

silica grit to hold them in place.

Container Growing Phase

The study was installed in a greenhouse of International Paper Company, Western Forest Research Center at Lebanon, Oregon on May 27, 1982. Seedlings were grown under nursery's routine culture practices.

At the end of the first growing season (October), fifteen seedlings were randomly selected from each treatment per block (total 15 x 4 x 3 = 180 seedlings) for growth measurement and mycorrhizal status determination. Shoot height, stem diameter at root collar, and dry weights of shoot and root were recorded. Feeder roots were counted under a stereomicroscope (8 X) according to their mycorrhizal status. Foliage of the seedlings from the same treatment and block were grouped, analyzed for macro- and micro-elements (N, P, K, Ca, Mg, Mn, Fe, Cu, B, Zn, and Al) by the Plant Analysis Laboratory, Department of Horticulture, Oregon State Univerity, Corvallis.

All data were subjected to analysis of variance; where significant treatment effects occurred, differences among treatment means were separated by Scheffe's multiple pairwise comparison at $P \leq 0.05$.

Transplanting Phase

The remaining seedlings (185 per treatment per block) were manually transplanted into transplant beds in a completely randomized design in fall, 1982. Three nursery beds each with four 4'x16' plots were used as blocks; fungal treatments were randomly

assigned into each plot. Some chemical properties of this transplant bed soil (loam) were: NO_3^--N, 24.9 ppm; NH_4^+-N, 8.8 ppm; extractable P, 20 ppm; K, 226 ppm; Ca, 19.1 meq/100 g; Mg, 5.9 meq/100 g; total N, 0.11%; organic matter, 2.4%; with soil pH 6.0 (analysis by Soil Testing Laboratory, Oregon State University, Corvallis.).

In Spring 1983, terminal bud break was recorded weekly until all seedlings broke their terminal buds. Data were analyzed as split-plot, with fungal treatment as main plot and time (week) as subplot.

Thirty seedlings from each fungal treatment per block were randomly lifted in Spring, 1984 for growth measurement and mycorrhizal status determination. Poor drainage of one nursery bed cause a serious root rot problem, only seedlings from the other two beds were lifted. Shoot height, stem diameter at the root collar, fresh weights of shoot and root were recorded for each seedling. Mycorrhizal status was estimated under a stereomicroscope (8 X) to the nearest 10% according to their morphological types.

RESULTS

Container Growing Phase

At the conclusion of the container growing phase, all seedlings appeared healthy and had set buds. L. laccata-inoculated seedlings had L. laccata mycelial mats and primordia among their root systems. Also, seedlings inoculated with H. crustuliniforme had profused mycelia that tightly bound the root system together.

Analysis of variance indicated significant treatment effects on number of feeder roots (mycorrhizal, nonmycorrhizal, and total), and percentage of mycorrhizae. Neither fungal treatment nor block significantly affected seedling growth.

Inoculation of H. crustuliniforme and L. laccata succeeded: seedlings had 90.2% and 83.0%, respectively, of their feeder roots colonized (Table IV.1). However, only 6 out of 45 seedlings examined showed P. tinctorius mycorrhizae with a mean of only 4% of the feeder roots colonized (Table IV.1). Seedlings inoculated with H. crustuliniforme and L. laccata had significantly more mycorrhizal and total feeder roots, and fewer nonmycorrhizal roots than noninoculated or P. tinctorius-inoculated seedlings.

Significant differences were detected only for foliage Mn and Zn concentrations. Inoculated seedlings had significantly higher foliage Zn level, and all but L. laccata-inoculated seedlings had significantly lower foliage Mn level, as compared to noninoculated controls (Table IV.2).

Transplanting Phase

Significant fungal treatment x harvest time interaction was detected ($P \leq 0.001$) on bud break data. Buds of Hebeloma-inoculated seedlings tended to break before those of the other treatments. All terminal buds burst within five weeks, regardless of fungal treatment (Figure IV.1).

In spring, 1984, seedlings appeared healthy, with mean height of 33.0 cm, stem diameter 8.45 mm, shoot fresh weight 33.86 g, and root fresh weight 27.43 g. All newly formed feeder roots were 80% mycorrhizal. H. crustuliniforme persisted as a mycorrhizal dominant on seedlings previously inoculated with this fungus, only two seedlings examined had Rhizopogon mycorrhizae (<10%). Seedlings originally inoculated with L. laccata had a mean of 40%, 20%, and 20%, respectively, of their feeder roots colonized by Laccaria, Thelephora, and Rhizopogon. Most of the Laccaria mycorrhizae appeared near the top of the root systems. P. tinctorius-inoculated seedlings had no P. tinctorius mycorrhizae, and their mycorrhizal status was similar to noninoculated controls: a mean of 60% of feeder roots colonized by Thelephora, 20% by Rhizopogon; some had a very few Laccaria mycorrhizae near the top of the root systems (Table IV.3).

DISCUSSION

Vegetative inocula of L. laccata and H. crustuliniforme formed abundant ectomycorrhizae with Douglas-fir seedlings during the container growing phase, but P. tinctorius inoculum was less effective. These results coincide with previous studies (Chapter III).

Mycorrhizal fungi produce hormones and other growth regulators which might alter root physiology and morphogenesis (Slankis, 1973). Feeder root production of Douglas-fir seedlings was affected by mycorrhizal inoculation: the higher was the mycorrhizal percentage, the more was the total number of feeder roots (Table IV.1). Different mycorrhizal fungi affected seedlings differently. L. laccata and H. crustuliniforme stimulated feeder root production, but P. tinctorius did not. Graham and Linderman (1980) reported that Cenococcum geophilum Fr., L. laccata or H. crustuliniforme stimulated Douglas-fir lateral root formation in aseptic culture as compared to uninoculated controls, whereas P. tinctorius inhibited it. They also suggested a relationship of ethylene to lateral root formation and ectomycorrhizae establishment.

Most studies on differences in micronutrient concentration between mycorrhizal and nonmycorrhizal plants are on vesicular-arbuscular (VA) mycorrhizae; zinc and copper have been consistantly higher (Harley and Smith, 1983); and manganese consistantly lower (Mosse, 1973) in mycorrhizal plants. Our inoculated Douglas-fir plug+1 seedlings at the end of the container growing phase had significantly higher foliage zinc concentrations than controls, and

all but L. laccata-inoculated seedlings had significantly lower manganese concentrations (Table IV.2). Bowen et al. (1974) reported that metabolically mediated absorption of zinc by ectomycorrhizae of Pinus radiata D. Don was considerablly greater than that of nonmycorrhizal short roots. They related this to greater absorbing surface of ectomycorrhizae and greater absorbing power/cm^2 of surface with mycorrhizae.

Seedlings of all our treatments grew well after transplanting to nursery beds: mean shoot height increased from 14.7 to 33.0 cm and stem diameter at the root collar from 2.0 to 8.5 mm in 17 months.

Bledsoe et al. (1982) reported that new roots of container-grown Douglas-fir seedlings inoculated with L. laccata and H. crustuliniforme, were colonized by native mycorrhizal fungi within five months of outplanting. Feeder roots of our H. crustuliniforme-inoculated seedlings were mostly colonized by H. crustuliniforme after 17 months in the nursery beds (Table IV.3). This fungus evidently competes strongly with native fungi such as Rhizopogon and Thelephora spp., so its operational application in nurseries seems feasible. L. laccata-inoculated seedlings had a mean of 40% of their feeder roots colonized by Laccaria and another 40% by native fungi (Rhizopogon and Thelephora spp.)(Table IV.3). L. laccata thus did not compete as well as H. crustuliniforme with Rhizopogon and Thelephora. P. tinctorius-inoculated seedlings had few mycorrhizae before transplanting, so colonization of their newly formed feeder roots by native fungi was predictable.

Surface sterilized Laccaria, Hebeloma and Thelephora ectomycorrhizae from this study had relatively high nitrogenase activity as assayed by acetylene reduction, apparently because of free-living, N_2-fixing bacteria embedded in the mycorrhizal mantle (Li and Hung, unpublished data). Further research is needed to study the relationships between host plants, mycorrhizal fungi, and free-living N_2-fixing bacteria.

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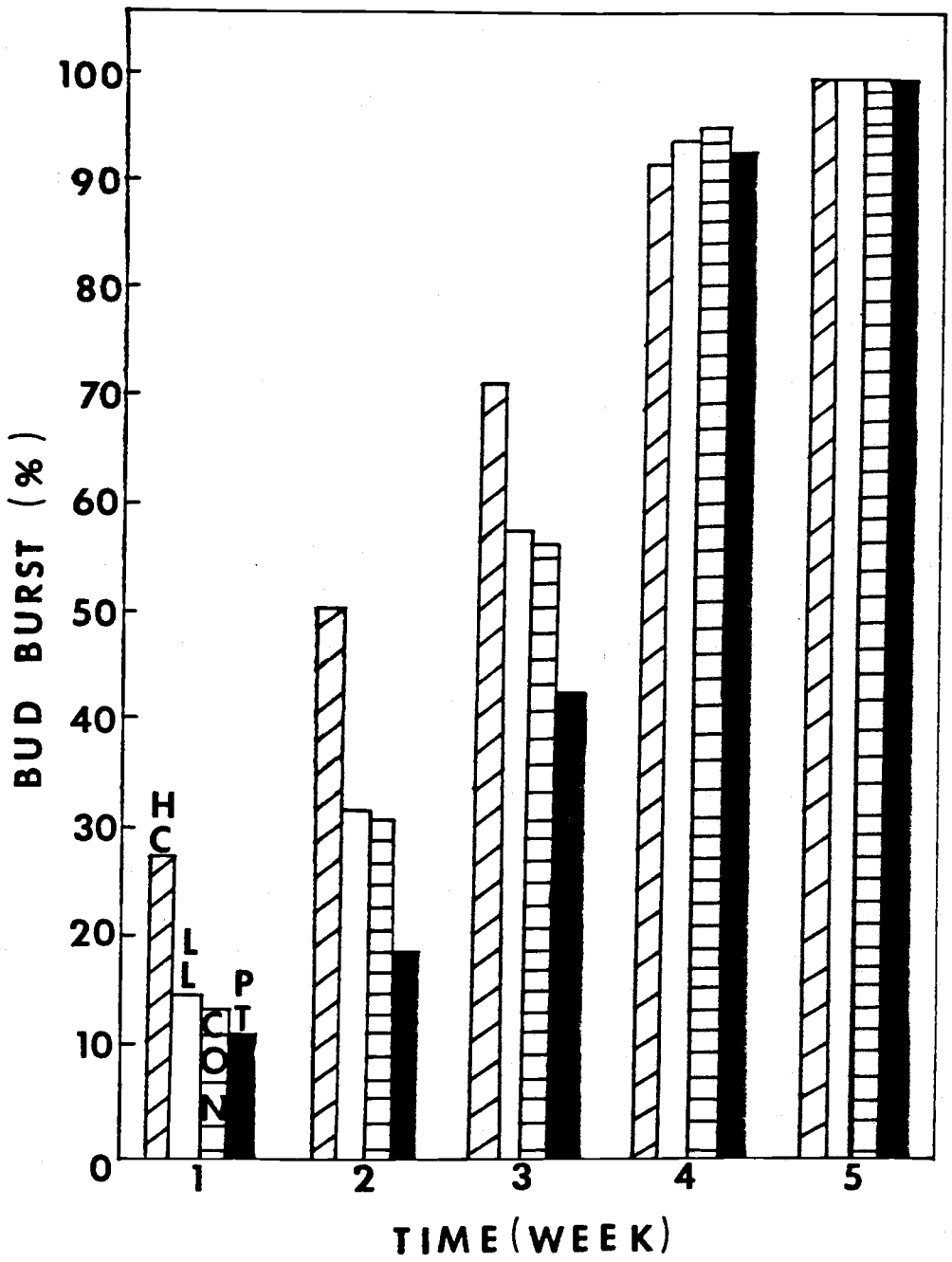

Figure IV.1. Mean percentage of terminal bud burst of Douglas-fir plug+1 seedlings previously inoculated with different mycorrhizal inoculum vs. noninoculated controls. HC: Hebeloma crustuliniforme, LL: Laccaria laccata, CON: Control, PT: Pisolithus tinctorius.

Table IV. 1. Mean growth responses and mycorrhizal status of Douglas-fir seedlings inoculated with different fungal inocula, grown in a greenhouse for 4.5 months.

Treatment	Shoot Height (cm)	Stem Diameter (mm)	Dry Weight (mg)			Shoot: Root Ratio	Number of Feeder Root			Mycorrhizae (%)
			Shoot	Root	Total		Myc	Nonmyc	Total	
Control	15.3a*	1.89a	420a	223a	644a	1.97a	0b	175a	176b	0.0c
Laccaria laccata	14.3a	1.91a	385a	236a	620a	1.67a	234a	43b	277a	83.0b
Hebeloma crustuliniforme	14.6a	2.10a	414a	248a	662a	1.69a	264a	28b	292a	90.2a
Pisolithus tinctorius	14.6a	1.86a	441a	253a	693a	1.84a	9b	170a	186b	4.0c

* - Means in the same column not sharing a common letter differ significantly by Scheffe's test at $P \leq 0.05$.
Myc - mycorrhizal feeder root.
Nonmyc - nonmycorrhizal feeder root.

Table IV. 2. Mean foliage nutrient levels of container-grown Douglas-fir seedlings inoculated with different fungal inocula, grown in a greenhouse for 4.5 months.

Treatment	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	Mn* (ppm)	Fe (ppm)	B (ppm)	Cu (ppm)	Zn* (ppm)	Al (ppm)
Control	1.59	0.64	1.53	0.51	0.15	299a	102	107	51	54b	30
Laccaria laccata	1.51	0.64	1.61	0.35	0.11	231ab	90	98	70	97a	26
Hebeloma crustuliniforme	1.41	0.57	1.44	0.44	0.12	205b	108	98	57	87a	38
Pisolithus tinctorius	1.45	0.61	1.41	0.47	0.12	200b	102	105	52	96a	41

Means in the same column not sharing a common letter differ significantly by Scheff's test at $P \leq 0.05$.
* - significant treatment effect occurred in this variable.

Table IV. 3. Mean mycorrhizae percentage of Douglas-fir plug+1 seedlings after transplanting and growth in nursery beds for 17 months.

Treatment	Mycorrhizal Types				
	Hebeloma	Laccaria	Pisolithus	Rhizopogon	Thelephora
Control	--	--	--	20	60
Hebeloma crustuliniforme	70	--	--	10	--
Laccaria laccata	--	40	0	20	20
Pisolithus tinctorius	--	--	--	20	60

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SUMMARY AND CONCLUSIONS

Results from Chapter I indicates that all fungi tested except Amanita muscaria had a broad pH tolerance range (at least 3 pH units). However, slow mycelial growth of Cenococcum geophilum, inconsistant results from inoculating seedlings with vegetative inocula of Rhizopogon and Suillus species (Molina, unpublished data), and poor performance of Pisolithus tinctorius-inoculated seedlings in the field (Castellano and Trappe, unpublished data) preclude recommendation of vegetative inoculum of these fungi for Pacific Northwestern nurseries at present.

Laccaria laccata and Hebeloma crustuliniforme, both common ectomycorrhizal fungi in the Douglas-fir region, are easy to isolate, have fast mycelial growth with a broad pH tolerance range and wide host range, withstand inoculation manipulations, and form abundant mycorrhizae with seedlings under high fertility conditions. Thus, they are good candidates for nursery inoculation. Results of this research indicate that commercially produced vegetative inocula of these two fungi effectively form mycorrhizae with Douglas-fir containerized seedlings in a greenhouse and/or container nurseries. Moreover, Hebeloma crustuliniforme competes very well with native mycorrhizal fungi such as Rhizopogon and Thelephora spp.. Thus, ectomycorrhizal inoculation with these two fungi in container nurseries is feasible. Future research should emphasize seedling

performance of Laccaria- or Hebeloma-colonized containerized seedlings after outplanting.

Some considerations for management of ectomycorrhizal inoculation in container nurseries are:

(1) Use fresh inoculum whenever possible; prevent exposure of inoculum to direct sun.

(2) Use inoculum without leaching.

(3) Apply at rates of 1:64 or 1:128, adequate for forming abundant mycorrhizae.

(4) If native fungi (e.g. Thelephora spp.) are present, use higher inoculation rates to assure that most feeder roots become colonized by the inoculated fungus before the natives invade the containers.

(5) If inoculum must be stored, store it in a cold room and use a higher inoculation rate (e.g. 1:4, 1:8) than needed for fresh inoculum to assure abundant mycorrhiza formation.

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APPENDIX

Table A.1. Adjusted means* of shoot height (cm) of 6-month-old container-grown Douglas-fir seedlings inoculated with Laccaria laccata mycelial inoculum which stored at different temperatures for different peirods.

Temperature	Month of Storage	Inoculation Rate 1:4	1:8	1:16	1:32	1:64	1:128	1:256	$\bar{X}$
	0	17.0	17.8	15.7	17.7	16.8	17.1	17.4	17.1
2°C	1	16.7	16.1	16.6	15.8	17.4	17.3	17.1	16.7
	2	16.8	17.7	17.7	16.8	17.5	16.8	16.8	17.2
	3	19.2	19.0	17.5	17.1	17.1	17.5	18.9	18.0
	4	16.8	17.5	16.6	15.5	16.3	15.9	16.0	16.4
	5	15.1	16.5	17.1	15.5	17.5	17.7	16.6	16.6
	6	14.3	15.2	15.8	14.7	15.2	17.1	16.0	15.5
	9	17.6	16.5	16.5	16.4	17.1	16.6	18.2	17.0
	12	20.2	18.7	21.7	19.9	19.6	20.0	19.0	19.9
	$\bar{X}$	17.1	17.1	17.4	16.5	17.2	17.4	17.3	17.1
21°C	1	16.6	17.0	14.5	15.4	17.6	17.3	16.8	16.5
	2	17.3	16.8	17.8	17.4	18.0	16.6	16.9	17.2
	3	17.9	18.4	18.0	17.6	17.6	16.9	19.0	17.9
	4	15.8	17.1	16.4	17.0	16.6	16.6	16.2	16.5
	5	18.3	17.4	13.5	16.6	17.8	16.3	13.2	16.2
	6	16.3	15.0	15.4	17.0	17.4	15.8	16.3	16.2
	9	15.9	16.4	15.1	15.1	15.0	16.5	15.0	15.6
	12	20.1	19.3	21.9	19.4	19.0	19.6	17.9	19.6
	$\bar{X}$	17.3	17.2	16.6	17.0	17.4	16.9	16.4	17.0
	$\bar{\bar{X}}$	17.2	17.2	17.0	16.7	17.3	17.2	16.9	17.1

* Means are adjusted by using average height (17.1 cm) of all noninoculated seedlings.

Table A.2. Adjusted means* of stem diameter (mm) of 6-month-old container-grown Douglas-fir seedlings inoculated with Laccaria laccata mycelial inoculum which stored at different temperatures for different peirods.

Temperature	Month of Storage	Inoculation Rate 1:4	1:8	1:16	1:32	1:64	1:128	1:256	$\bar{X}$
	0	1.88	1.75	1.80	1.81	1.90	1.87	1.80	1.83
2°C	1	2.04	1.96	1.96	1.88	1.96	1.85	1.86	1.93
	2	1.69	1.85	1.79	1.89	1.78	1.88	1.73	1.80
	3	1.95	2.02	1.74	1.81	1.82	1.78	1.78	1.84
	4	1.89	1.83	1.69	1.78	1.94	1.83	1.74	1.81
	5	1.87	1.80	1.76	1.57	1.75	1.82	1.74	1.76
	6	1.99	1.88	1.83	1.76	1.68	1.78	1.79	1.82
	9	1.99	1.89	1.68	1.78	1.81	1.73	2.00	1.84
	12	2.07	1.91	2.14	1.98	1.89	1.84	1.84	1.95
	$\bar{X}$	1.94	1.89	1.82	1.81	1.83	1.81	1.81	1.84
21°C	1	1.86	2.01	1.67	1.68	1.92	1.88	1.77	1.83
	2	1.93	1.83	1.88	1.76	2.04	1.58	1.78	1.83
	3	1.87	1.88	1.79	1.79	1.76	1.77	1.72	1.80
	4	1.61	1.79	1.65	1.85	1.84	1.71	1.78	1.75
	5	1.66	1.62	1.52	1.55	1.71	1.56	1.48	1.59
	6	2.04	1.95	2.08	1.93	1.93	1.85	1.77	1.94
	9	1.84	1.63	1.70	1.72	1.79	1.66	1.78	1.73
	12	1.99	1.97	2.06	1.95	1.94	1.96	1.90	1.97
	$\bar{X}$	1.85	1.84	1.79	1.78	1.87	1.75	1.85	1.81
	$\bar{\bar{X}}$	1.90	1.87	1.81	1.80	1.85	1.78	1.83	1.83

* Means are adjusted by using average diameter (1.78 mm) of all noninoculated seedlings.

Table A.3. Adjusted means* of shoot dry weight (mg) of 6-month-old container-grown Douglas-fir seedlings inoculated with Laccaria laccata mycelial inoculum which stored at different temperatures for different peirods.

Temperature	Month of Storage	Inoculation Rate 1:4	1:8	1:16	1:32	1:64	1:128	1:256	$\bar{X}$
	0	618	605	544	627	617	649	666	618
2°C	1	625	581	574	618	643	599	558	599
	2	531	576	627	531	562	570	539	562
	3	606	599	525	555	516	532	538	553
	4	547	600	508	535	592	532	544	551
	5	441	503	521	389	487	561	482	483
	6	582	546	521	545	544	565	551	551
	9	599	562	520	564	516	453	566	540
	12	654	532	654	549	572	532	501	571
	$\bar{X}$	573	562	556	536	554	543	535	551
21°C	1	576	557	507	489	641	591	570	562
	2	566	557	582	576	591	476	586	562
	3	570	591	552	543	536	502	527	546
	4	461	519	466	551	546	577	500	517
	5	472	525	342	417	483	387	327	422
	6	560	544	573	619	529	489	470	541
	9	537	441	421	424	466	452	459	457
	12	557	562	616	548	542	543	521	555
	$\bar{X}$	537	537	507	521	542	502	495	520
	$\bar{\bar{X}}$	555	550	532	528	548	522	515	536

* Means are adjusted by using average (505 mg) of all non-inoculated seedlings.

Table A.4. Adjusted means* of root dry weight (mg) of 6-month-old container-grown Douglas-fir seedlings inoculated with Laccaria laccata mycelial inoculum which stored at different temperatures for different peirods.

Temperature	Month of Storage	Inoculation Rate 1:4	1:8	1:16	1:32	1:64	1:128	1:256	$\bar{X}$
	0	267	241	217	217	199	207	269	231
2°C	1	379	343	319	348	373	314	284	337
	2	205	273	311	263	274	275	206	258
	3	299	305	230	290	278	284	270	280
	4	291	272	254	268	287	249	268	270
	5	265	283	284	242	272	261	254	266
	6	358	320	285	326	298	293	287	310
	9	307	283	282	277	286	254	286	282
	12	307	286	298	295	279	274	267	286
	$\bar{X}$	301	296	283	289	293	275	265	286
21°C	1	324	378	300	274	359	331	363	333
	2	258	272	258	242	305	203	245	254
	3	285	304	299	244	238	276	258	272
	4	231	233	207	255	241	238	245	236
	5	230	235	220	219	247	224	219	228
	6	372	352	358	332	301	297	273	326
	9	268	256	233	243	269	262	269	257
	12	312	289	302	278	298	285	295	294
	$\bar{X}$	285	290	272	261	282	265	271	275
	$\bar{\bar{X}}$	293	293	278	275	288	270	268	281

* Means are adjusted by using average (279 mg) of all non-inoculated seedlings.

Table A.5. Mean mycorrhizae percentage of 6-month-old container-grown Douglas-fir seedlings inoculated with Laccaria laccata mycelial inoculum which stored at different temperatures for different peirods.

Tempe-rature	Month of Storage	Inoculation Rate 1:4	1:8	1:16	1:32	1:64	1:128	1:256	$\bar{X}$
	0	86.4	86.4	87.8	84.9	89.2	85.7	84.5	86.4
2°C	1	80.2	80.1	81.9	80.5	75.2	81.4	85.6	80.7
	2	47.2	60.7	62.1	57.2	63.2	62.0	59.0	58.8
	3	49.3	36.6	23.9	28.0	26.8	30.8	27.0	31.8
	4	26.6	15.6	20.6	24.9	23.3	37.8	30.7	25.6
	5	23.9	33.0	29.6	26.0	29.9	21.2	15.1	25.5
	6	11.8	7.4	3.1	2.6	1.2	1.3	1.7	4.2
	9	32.9	25.7	21.7	25.1	24.6	13.2	15.2	22.6
	12	6.3	5.3	5.0	4.1	2.2	2.3	2.8	4.0
	$\bar{X}$	34.8	33.1	30.9	31.1	30.8	31.2	29.6	31.7
21°C	1	86.0	78.9	85.8	87.7	86.8	79.6	78.4	83.3
	2	82.2	80.2	81.0	78.3	76.7	53.9	53.0	72.2
	3	31.6	25.2	31.2	26.1	20.2	21.1	20.4	25.1
	4	9.5	14.4	12.2	13.5	8.4	9.7	7.0	10.7
	5	21.6	11.5	5.6	7.5	15.7	3.8	3.8	9.9
	6	21.4	9.7	5.3	7.3	2.1	2.0	1.2	7.0
	9	11.3	3.2	4.7	3.0	4.9	2.6	1.3	4.4
	12	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	$\bar{X}$	33.0	27.9	28.2	27.9	26.8	21.6	20.6	26.6
	$\bar{\bar{X}}$	33.9	30.5	29.6	29.5	28.8	26.4	25.1	29.1

Table A.6. Adjusted means* of seedling growth and mean mycorrhizae percentage of 6-month-old container-grown Douglas-fir seedlings inoculated with Pisolithus tinctorius mycelial inoculum which stored at different temperatures for different periods.

Month of Storage	Shoot Height (cm)		Stem Diameter (mm)		Dry Weight (mg) Shoot		Dry Weight (mg) Root		Mycorrhizae (%)	
0	13.5		1.8		500		297		30.8	
	COLD	ROOM	COLD	ROOM	COLD	ROOM	COLD	ROOM	COLD	ROOM
1	14.6	13.4	1.7	1.8	389	396	223	293	0.0	4.8
$\bar{X}$	14.0		1.8		393		258		2.4	
2	14.5	14.2	1.7	1.7	472	454	276	296	0.0	1.6
$\bar{X}$	14.4		1.7		463		286		0.8	
4	14.5	14.7	1.7	1.7	471	512	306	314	0.0	0.0
$\bar{X}$	14.5		1.7		492		310		0.0	
$\bar{X}$	14.5	14.1	1.7	1.7	444	454	268	301	0.0	2.1
$\bar{\bar{X}}$	14.3		1.7		449		285		1.0	

* Means are adjusted by using averages (14.5 cm, 1.8 mm, 474 mg, and 269 mg, respectively) of all noninoculated seedlings.

Table A.7. Adjusted means* of shoot height (cm) and stem diameter (mm) of 6-month old container-grown Douglas-fir seedlings inocultated with different ectomycorrhizal inoculum which stored at different temparatures for different periods.

Temperature	Month of Storage	*Hebeloma crustuliniforme* S166		*Laccaria laccata* T813		*Laccaria laccata* S238B		$\bar{X}$	
		Ht	Dim	Ht	Dim	Ht	Dim	Ht	Dim
	0	14.0	1.74	14.7	1.81	14.9	1.76	14.5	1.77
2°C	1	15.0	1.72	13.9	1.68	13.4	1.65	14.0	1.68
	2	15.2	1.74	14.4	1.76	15.7	1.72	15.1	1.74
	4	14.5	1.43	14.9	1.40	14.4	1.41	14.6	1.42
	6	16.6	1.76	16.7	2.09	16.6	1.96	16.6	1.94
	9	14.4	1.49	14.0	1.51	14.4	1.52	14.3	1.51
	12	14.7	1.79	14.0	1.73	14.9	1.73	14.5	1.75
	$\bar{X}$	15.0	1.66	14.6	1.70	14.9	1.67	14.9	1.67
21°C	1	14.0	1.56	14.4	1.69	14.6	1.68	14.3	1.64
	2	16.1	1.80	15.3	1.79	15.1	1.76	15.5	1.78
	4	14.4	1.31	12.8	1.31	15.0	1.42	14.1	1.35
	6	16.0	1.82	16.2	2.15	15.2	1.76	15.8	1.91
	9	13.6	1.47	15.8	1.78	14.1	1.54	14.5	1.60
	12	15.6	1.80	14.3	1.72	13.8	1.71	14.6	1.75
	$\bar{X}$	14.9	1.63	14.8	1.74	14.6	1.64	14.8	1.67
	$\bar{\bar{X}}$	15.0	1.64	14.7	1.72	14.8	1.72	14.8	1.69

* means are adjusted by using averages (14.7 cm and 1.64 mm) of all noninoculated seedlings.

Table A.8. Adjusted means* of shoot and root dry weight (mg) of 6-month-old container-grown Douglas-fir seedlings inoculated with different ectomycorrhizal inoculum which stored at different temparatures for different periods.

Temperature	Month of Storage	Hebeloma crustuliniforme S166		Laccaria laccata T813		Laccaria laccata S238B		$\bar{X}$	
		Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
	0	366	139	463	200	484	227	438	189
2°C	1	409	162	304	121	316	126	343	136
	2	446	205	402	190	477	237	442	211
	4	335	200	375	208	311	198	340	202
	6	442	221	428	197	514	269	461	229
	9	357	186	336	182	358	186	350	185
	12	425	222	423	232	412	216	420	223
	$\bar{X}$	402	199	378	188	398	205	393	198
21°C	1	302	111	358	132	329	167	330	136
	2	457	208	476	220	459	220	464	216
	4	353	206	277	189	337	215	322	204
	6	475	215	467	228	432	196	458	213
	9	326	184	428	195	300	162	351	181
	12	432	214	401	230	400	211	411	218
	$\bar{X}$	391	190	401	199	376	195	389	195
	$\bar{\bar{X}}$	397	194	390	193	387	200	391	196

* means are adjusted by using averages of shoot and root dry weight (389 mg, and 200 mg, respectively) of all noninoculated seedlings.

Table A.9. Mean percentage of mycorrhizae of 6-month-old contaner-grown Douglas-fir seedlings inoculated with different ectomycorrhizal inoculum which stored at different temperatures for different periods.

Temperature	Month of Storage	Hebeloma crustuliniforme S166	Laccaria laccata T813	Laccaria laccata S238B	$\bar{X}$
	0	89.5	82.0	76.3	83.6
20°C	1	77.7	71.2	69.2	72.7
	2	68.8	34.0	49.4	50.7
	4	55.6	18.0	17.2	30.3
	6	22.0	0.3	5.3	9.2
	9	24.5	1.4	9.2	11.7
	12	6.2	0.0	0.0	2.1
	$\bar{X}$	42.5	20.8	25.1	29.4
21°C	1	75.4	76.4	77.7	76.5
	2	68.5	32.0	53.2	51.2
	4	16.7	1.7	14.8	11.1
	6	0.1	0.0	0.1	0.1
	9	0.7	0.2	0.1	0.3
	12	0.0	0.0	0.0	0.0
	$\bar{X}$	26.9	18.4	24.3	23.2
	$\bar{\bar{X}}$	34.7	19.6	24.7	26.3