Development of Ectomycorrhizae on Container-Grown European Larch

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*Container-grown European larch (*Larix decidua *Mill.) were inoculated at sowing with mycelial cultures of eight ectomycorrhizal fungi. Inoculated and uninoculated seedlings were treated with standard and reduced levels of complete fertilizer and fungicides in two experiments. Only seedlings inoculated with* Hebeloma *crustuliniforme and* Laccaria laccata *developed significant ectomycorrhizae. Ectomycorrhizal plants were generally smaller than uninoculated seedlings. Standard fertilization reduced ectomycorrhizal development, stimulated shoot growth, and increased shoot-root ratios. Reduced applications of DCNA (Botran) and benomyl (Benlate) may permit ectomycorrhizal colonization of seedlings by selected fungal isolates.* Tree Planters' Notes 40(2) :12-17; 1989.

The use of ectomycorrhizal fungi as an aid in reforestation has been tested extensively in the nursery, greenhouse, and field in recent years (11, 16). Inoculation of seedlings with fungal isolates indigenous to the outplanting locale is usually beneficial (18). Past studies with container-grown European larch (*Larix decidua* Mill.) revealed erratic colonization by the ectomycorrhizal fungi *Suillus grevillei* (Kl.) Singer, *Suillus cavipes* (Opat.) Smith & Thiers, *Pisolithus tinctorius* (Pers.) Coker & Couch, and *Amanita muscaria* (L.: Fr.) Hooker (2, 3). Moreover, the benefit of ectomycorrhizae to the growth of European larch seedlings has not been clearly demonstrated (3).

Standard cultural practices used in producing container-grown European larch may not be suitable for ectomycorrhizal colonization (3). Factors such as source of fungal inoculum, growth medium fertility, and application of fungicides may significantly influence ectomycorrhizal development on seedlings (11, 16). For example, the frequent use of fungicides to control Botrytis infection of larch may influence ectomycorrhizal colonization (4, 5, 15).

The objectives of this study were to determine if (1) exotic and indigenous ectomycorrhizal fungi could be established on container-grown European larch seedlings through artificial inoculation and (2) standard applications of fertilizer and fungicides reduce seedling ectomycorrhizal colonization.

Materials and Methods

The fungal isolates used in this study (table 1) were collected and stored as stock culture using methods described by Molina (13). Vegetative mycelial inoculum for each isolate was grown aseptically using procedures described by Marx and Bryan (8).

A sterile peat moss—vermiculite–perlite mixture (2:1:1) was the potting medium. Sterilized four-cavity (175-cm^3 capacity) Rootrainers (Spencer—Le Maire Industries, Ltd., Edmonton, Alberta, Canada) were filled with either inoculated or uninoculated growth medium at proportions described below. European larch seeds from Czechoslovakia (NC-9948) were pregerminated and transplanted into container cavities 7 days after inoculation.

Experiment 1. Six fungal inoculation treatments (uninoculated control, *Cenococcum geophilum* (Fr.), *Suillus granulatus* (L.: Fr.) O. Kuntz, *S. grevillei* (Kl.) Singer, *S. luteus* (L.:Fr.) S.F. Gray, and *S. tomentosus* (Kauffm.) Singer, Snell & Dick); two fertilizer levels (standard and reduced); and two fungicide treatments (standard and reduced) were arranged in a split-plot design (fertilizer/ fungicide treatments were main plots and fungus treatments

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Table 1—*Sources and isolation dates of ectomycorrhizal fungus isolates used to inoculate container-grown European larch seedlings*

Fungal species	Isolate no.	Source and isolation data
Cenococcum geophilum (Fr.)	155	*Quercus alba,* Maryland, USA, isolated in 1973 by E. Hacskaylo from ectomycorrhiza and maintained as stock culture on MMN agar.
Suillus granulatus (L.:Fr.) O. Kuntz	263	*Pinus ponderosa,* Colorado, USA, isolated in 1980 by Z. Cornett from sporocarp tissue and maintained as stock culture on MMN agar.
Suillus grevillei (Kl.) Singer	10	*Pinus resinosa,* Minnesota, USA, isolated in 1984 by M. Palm and E. Stewart and maintained as stock culture on MMN agar.
Suillus luteus (L.:Fr.) S.F. Gray	244	*Pinus nigra,* Maine, USA, isolated in 1977 by W. Otrosina from sporocarp tissue and maintained as stock culture on MMN agar.
Suillus tomentosus (Kauffm.) Singer, Snell & Dick	1	*Pinus resinosa,* Minnesota, USA, isolated in 1984 by M. Palm and E. Stewart and maintained as stock culture on MMN agar.
Pisolithus tinctorius (Pers.) Coker & Couch	293	*Pinus taeda,* Georgia, USA, isolated in 1986 by D. Marx and maintained as stock culture on MMN agar.
Hebeloma crustuliniforme (Bull.) Quel.	166	*Pseudotsuga menziesii,* Washington, USA, isolated in 1981 by C. Bledsoe and maintained as stock culture on MMN agar.
Laccaria laccata (Scop.: Fr.) M.C. Cooke	813	*Pseudotsuga menziesii,* Oregon, USA, isolated in 1985 by R. Molina and maintained as stock culture on MMN agar.

MMN = modified Melin-Norkrans agar medium.

were subplots), with four replications on separate greenhouse benches. Each treatment combination was represented by 20 seedlings per replicate. One part of inoculum was thoroughly mixed with 20 parts of sterilized growth medium.

The standard fertilizer treatment was the operational fertilizer regime for European larch used at our facility, consisting of three weekly applications of Peters 20-20-20 (345 ppm N-P-K after dilution) plus Peters STEM micronutrients (56 ppm after dilution). The reduced fertilizer treatment consisted of three weekly applications of the same fertilizers at one-eighth of the above concentrations. The standard fungicide treatment consisted of weekly applications of benomyl (Benlate) plus DCNA (Botran) (10 g each/10 liters water). The reduced fungicide treatment consisted of biweekly applications of the same fungicides at a rate of 5 g each/10 liters water. Fertilizer and fungicide applications commenced at seedling age 1 week; fertilizer concentration was doubled after age 10 weeks.

Seedlings were grown for 20 weeks in a polyethylene greenhouse with ambient temperature ranging from 22 to 30 °C, and photoperiod of 18 hours supplemented with high-pressure sodium vapor lights (165 µE/m/s). Seedlings were automatically watered with boom sprayers three times weekly.

Experiment 2. Nine fungal treatments (uninoculated control, *Suillus luteus*, *Pisolithus tinctorius*, *Hebeloma crustuliniforme* (Bull.) Quel., *Laccaria laccata* (Scop.: Fr.) M.C. Cooke, and treatments with autoclaved inoculum of the four fungi) were

arranged in a completely randomized design with 16 seedlings per treatment combination and replicate. The autoclaved inocula were included to evaluate any growth regulation by the inoculum substrate (11). The autoclaved inoculum was prepared by placing it in an electric soil sterilizer at 85 °C for 3 hours. One part of inoculum was thoroughly mixed with 10 parts of sterilized growth medium. All seedlings were grown under the reduced fertilizer/reduced fungicide regime described in experiment 1. Experiment 2 was conducted concurrently with experiment 1 under the same environmental conditions.

For both experiments, all seedlings were harvested after 20 weeks, and ectomycorrhizal colonization, shoot height and dry weight, stem caliper, root area index, and dry weight were measured. Ectomycorrhizal colonization was characterized on a subsample of 5 seedlings from each treatment replication, using standard techniques (11). To confirm ectomycorrhizal

Table 2—*Ectomycorrhizal colonization and growth of containerized European larch seedlings inoculated with 5 fungal species and grown under 4 regimes of fertilization and fungicide application (experiment 1)*

Fertilizer treatment	Fungicide treatment	Fungus treatment	Ectomycorrhizal short roots (%)	Height (cm)	Stem diameter (mm)	Shoot dry weight (g)	Root dry weight (g)	Shoot–root ratio	Root area index (cm^2)
Standard	Standard	Control	1[a]	29.8	3.5	1.66	0.44	3.7	28.9
		Cenococcum geophilum	7	29.5	3.4	1.56	0.44	3.6	32.6*
		Suillus granulatus	3	28.1	3.3	1.46	0.37*	4.0	29.0
		Suilllus grevillei	3	30.0	3.3	1.57	0.40	3.9	28.6
		Suillus luteus	2	29.6	3.4	1.68	0.44	3.8	31.5
		Suillus tomentosus	1	29.1	3.2	1.40	0.37*	3.8	26.8
Standard	Reduced	Control	5	30.5	3.4	1.97	0.43	4.6	32.7
		Cenococcum geophilum	18	28.5	3.3	1.68	0.40	4.2	28.8*
		Suillus granulatus	5	27.9	3.2	1.58	0.39	4.1	28.3*
		Suillus grevillei	7	30.4	3.4	1.91	0.47	4.1	32.3
		Suillus luteus	9	28.2	3.1	1.67	0.45	3.7	30.8
		Suillus tomentosus	5	30.0	3.3	1.84	0.45	4.1	28.3*
Reduced	Standard	Control	5	17.4	2.4	0.73	0.41	1.8	34.5
		Cenococcum geophilum	17	14.6	2.2	0.62	0.40	1.6	33.0
		Suillus granulatus	18	14.8	2.2	0.56	0.30*	1.9	27.3*
		Suillus grevillei	8	18.6	2.5	0.76	0.41	1.9	33.0
		Suillus luteus	13	18.8	2.6	0.84	0.53*	1.6	39.4*
		Suillus tomentosus	15	15.4	2.3	0.65	0.33*	2.0	27.8*
Reduced	Reduced	Control	8	17.2	2.4	0.76	0.33	2.3	26.0
		Cenococcum geophilum	7	19.3	2.6	0.81	0.38	2.1	30.1*
		Suillus granulatus	16	17.5	2.3	0.65	0.31	2.1	27.2
		Suillus grevillei	9	17.0	2.3	0.66	0.38	1.7*	31.8*
		Suillus luteus	9	15.6	2.2	0.66	0.33	2.0	27.5
		Suillus tomentosus	7	16.7	2.2	0.66	0.30	2.2	25.2

*Significantly different ($P \leq 0.05$) from respective control.

[a]*Thelephora terrestris* ectomycorrhizae developed on control seedlings following contamination.

development, sample root segments were sectioned and stained to confirm presence or absence of Hartig net (1). Seedling root area index (a two-dimensional estimate of root system size) was measured with a LI-COR LI-3000 portable area meter. All data were subjected to analysis of variance, and differences among means were compared by the least significant difference test ($P < 0.05$).

Results

Experiment 1. Root systems of control seedlings were infected with *Thelephora terrestris*. Ectomycorrhizae formed by each of the test isolates did not differ significantly from the control (table 2). Ectomycorrhizal colonization was significantly lower on seedlings supplemented with standard fertilizer treatments compared to reduced fertilizer. The two rates of fungicide application had no significant effect on ectomycorrhizae. Ectomycorrhizal infection, even at these low rates, reduced seedling growth, but only root dry weight, shoot-root ratio, and root area index were significantly affected.

Fertilizer treatment significantly influenced seedling growth. Seedlings supplemented with standard fertilizer were nearly twice the size of those receiving the reduced fertilizer rate. However, root dry weight and root area index were unaffected by fertilizer, presumably because of the restricted root volume of the containers, resulting in a shoot-root ratio of about 2.0 for the reduced-fertilizer treatment compared to about 4.0 for the highly fertilized seedlings. Fungicide application rate did not significantly affect seedling growth.

Experiment 2. Ectomycorrhizal infection by *Hebeloma crustuliniforme* (63%) and *Laccaria laccata* (24%) was significantly greater than infection by *Thelephora* ectomycorrhizae on control seedlings (table 3). Ectomycorrhizal colonization generally decreased seedling size as in experiment 1. The *Hebeloma* and *Laccaria* isolates significantly reduced shoot growth, but only *L. laccata* reduced root growth. Consequently, the shoot-root ratio of *H. crustuliniforme*—infected seedlings was significantly lower than that of the control.

Discussion

The ectomycorrhizal colonization of container-grown European larch in experiment 1 was relatively low compared to other tests with larch seedlings (2, 13). Previously, Molina (13) reported

Table 3—*Ectomycorrhizal colonization and growth of containerized European larch seedlings inoculated with viable and autoclaved cultures of 4 fungal species and grown under a reduced fertilization and fungicide regime (experiment 2)*

Fungus treatment	Ectomycorrhizal short roots (%)	Height (cm)	Stem diameter (mm)	Shoot dry weight (g)	Root dry weight (g)	Shoot–root ratio	Root area index (cm^2)
Control	1	18.5	2.3	0.67	0.30	2.2	26.5
Suillus luteus	4	15.3*	2.2	0.61	0.28	2.2	23.1*
Pisolithus tinctorius	5	15.1*	2.2	0.57*	0.27	2.1	25.1
Hebeloma crustuliniforme	63*	14.0*	2.0*	0.50*	0.29	1.7*	25.7
Laccaria laccata	24*	16.0*	2.1*	0.57*	0.26*	2.2	19.9*
Suillus luteus (dead)	1	15.9*	2.2	0.63	0.30	2.1	24.9
Pisolithus tinctorius (dead)	1	16.0*	2.2	0.58*	0.26*	2.2	23.9
Hebeloma crustuliniforme (dead)	1	15.9*	2.1*	0.53*	0.23*	2.3	22.0*
Laccaria laccata (dead)	1	16.9	2.3	0.70	0.28	2.5	22.9*

*Significantly different ($P \leq 0.05$) from the control.

that *Laccaria laccata* and *Cenococcum geophilum* Fr. formed abundant ectomycorrhizae on western larch (*Larix occidentalis* Nutt). In our experiment 2, ectomycorrhizal colonization by *Laccaria laccata* and *Hebeloma crustuliniforme was* more abundant compared to the uninoculated seedlings, under the prevailing environmental conditions. Growth media inoculum density was greater in experiment 2 than in experiment 1. Thus, fungal propagule density and/or viability was probably adequate for the *Laccaria* and *Hebeloma* isolates. The poor colonization of European larch by *Suillus* and *Cenococcum* isolates may be a result of inoculum with low viability (13), incompatible host-fungus combinations (7, 18), or greenhouse cultural conditions that inhabited ectomycorrhizal symbiosis (11).

Autoclaving inoculum of all four species resulted in significant decreases of some seedling growth variables compared to the uninoculated control (table 3). This occurrence is not unusual because autoclaving inoculum and vermiculite may release fungal toxins, toxic levels of manganese, or other breakdown products (personal communication, J.G. lyer, University of Wisconsin—Madison).

The reduced application of the fungicides benomyl and DCNA in this study, even at low concentrations, did not significantly suppress ectomycorrhizal development of European larch. Applications of captan, zineb, thiram, benomyl, and chlorothalonil have reportedly both increased and decreased ectomycorrhizal development of conifers under various cultural conditions (5, 9, 15, 17). The effect of fungicides seems to be specific to individual tree and fungal species. Due to the widescale problem with *Botrytis* infection of European larch, it would be difficult to produce healthy seedlings without frequent use of fungicides (4).

Ectomycorrhizal colonization of container-grown seedlings under routine greenhouse conditions rarely increases seedling growth (12, 14). Seedling size was reduced in our study even at low rates of ectomycorrhizal colonization. The fungal symbiont may utilize up to 20% of the host photosynthate, thereby reducing seedling size (12). The reduction in fertilizer applications in this study stimulated ectomycorrhizal development by some fungal isolates, but seedling size was not improved. Molina and Chamard (14) and Langlois and Fortin (6) concluded that large ectomycorrhizal conifer seedlings could be obtained by carefully selecting fungal isolates and precisely adjusting fertilizer applications.

Cultural regimes currently used for producing container-grown European larch seedlings are apparently not suitable for ectomycorrhizal colonization by most fungi tested in this study. The higher-fertility regimes and restricted rooting volume of the containers in this study produced excessive shoot growth with no proportional increase in root mass or ectomycorrhizal colonization. Wenny and Dumroese (19) recently reported a successful production regime for container-grown western larch whereby macronutrient fertilization is stopped after week 9. This low-fertility regime may also improve ectomycorrhizal development of larch seedlings (10). Fertilization and pesticide application regimes for producing container-grown European larch should be modified to encourage ectomycorrhizal development of seedlings (2). Moreover, further tests are needed to identify additional isolates of ectomycorrhizal fungi that are compatible with European larch.

Literature Cited

1. Dixon, R.K.; Garrett, H.E.; Cox, G.S.; Sander, I.E. 1984. Inoculation of three isolates of ectomycorrhizal fungi. I. Inoculation success and seedling growth relationships. Forest Science 30:364-372.
2. Goebl, von Friederike. 1974. Mykorrhiza Versuche bei Paperpotsaemlingen. I. Impfungen von Laerche. Zentralblatt fuer das Gesamte Forstwesen 91:74-87.
3. Heazel, T.E. 1983. Techniques for mycorrhizae inoculation of European larch. Rep. A291 (April 18, 1983). Appleton, WI: The Institute of Paper Chemistry. 35 p.
4. James, R.L. 1984. Biology and management of *Botrytis* blight. In: Murphy, P.M., ed. Proceedings, 1983 Intermountain Nurseryman's Association. Gen. Tech. Rep. INT-168. Ogden, UT: U.S. Department of Agriculture, Forest Service, Intermountain Forest and Range Experiment Station: 39-43.
5. Kelly, W.D. 1982. Effect of triadimefon (Bayleton) on ectomycorrhizae of loblolly and slash pines in Alabama. Forest Science 28:232-236.
6. Langlois, C.G.; Fortin, J.A. 1981. Mycorrhizal development on containerized seedlings. In: Scarratt, J.B.; Glerum, C.; Plexman, C.A., eds. Proceedings, Canadian containerized seedling symposium; 14-16 September 1981; Toronto. COJFRC Symp. Proc. O-P-10. Sault Ste. Marie, ON: Canadian Forestry Service, Great Lakes Forest Research Centre: 183-202.
7. Marx, D.H. 1981. Variability in ectomycorrhizal development and growth among isolates of *Pisolithus tinctorius* as affected by source, age, and reisolation. Canadian Journal of Forest Research 11:168-174.
8. Marx, D.H.; Bryan, W.C. 1975. Growth and ectomycorrhizal development of loblolly pine seedlings in fumigated soil infested with the fungal symbiont *Pisolithus tinctorius*. Forest Science 21:245-254.
9. Marx, D.H.; Rowan, S.J. 1981. Fungicides influence growth and development of specific ectomycorrhizae on loblolly pine seedlings. Forest Science 27:167-176.
10. Marx, D.H.; Hatch, A.B.; Mendicino, J.F 1977. High soil fertility decreased sucrose content and susceptibility of loblolly pine roots to ectomycorrhizal infection by *Pisolithus tinctorius*. Canadian Journal of Botany 55:1569-1574.
11. Marx, D.H.; Ruehle, D.S.; Kenney, D.S.; Cordell, C.E.; Riffle, JW.; Molina, R.I.; Pawuk, W.H.; Navaratil, S.; Tinus, R.W.; Goodwin, O.C. 1984. Commercial vegetative inoculum of *Pisolithus tinctorius* and inoculation techniques for development of ectomycorrhizae on bare-root seedlings. For. Sci. Monogr. 25. [Suppl. to Forest Science 3(3)]. Washington, DC: Society of American Foresters. 101 p.
12. Molina, R. 1982. Use of the ectomycorrhizal fungus *Laccaria laccata* in forestry. I. Consistency between isolates in effective colonization of containerized conifer seedlings. Canadian Journal of Forest Research 12:469-472.
13. Molina, R. 1980. Ectomycorrhizal inoculation of containerized western conifer seedlings. Res. Note PNW-357. Portland, OR: U.S. Department of Agriculture, Forest Service, Pacific Northwest Forest and Range Experiment Station. 10 p.
14. Molina, R.; Chamard, J. 1983. Use of the ectomycorrhizal fungus *Laccaria laccata* in forestry. II. Effects of fertilizer forms and levels on ectomycorrhizal development and growth of containerized Douglas-fir and ponderosa pine seedlings. Canadian Journal of Forest Research 13:89-95.
15. Pawuk, W.H.; Ruehle, I.E.; Marx, D.H. 1980. Fungicide drenches affect ectomycorrhizal development of container-grown *Pinus palustris* seedlings. Canadian Journal of Forest Research 10:61-64.
16. Shaw, C.G., III; Molina, R.; Walden, J. 1982. Development of ectomycorrhizae following inoculation of containerized Sitka and white spruce seedlings. Canadian Journal of Forest Research 12:191-195.
17. Theodorou, C.; Skinner, M.F. 1976. Effects of fungicides on seed inoculation of basidiospores of mycorrhizal fungi. Australian Forestry Journal 7:53-58.
18. Trappe, J.M. 1977. Selection of fungi for ectomycorrhizal inoculation in nurseries. Annual Review of Phytopathology 15:203-222.
19. Wenny, D.L.; Dumroese, R.K. 1987. A growing regime for containerized western larch seedlings. Bull 43. Moscow, ID: University of Idaho Forest, Wildlife, and Range Experiment Station. 16 p.