# A Technique for Isolating the Mycorrhizal Fungus *Laccaria laccata* from Conifer Seedlings

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**ABSTRACT**

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A protocol involving an extensive washing sequence and modified Melin-Norkrans' medium containing gentamicin sulfate, achromycin V, and benomyl was developed for isolating the mycorrhizal fungus *Laccaria laccata* from greenhouse- and field-grown conifer seedlings. Isolation rates of 54 and 76% were attained from field-grown seedlings of *Pinus resinosa* and *Pseudotsuga menziesii*, respectively.

Ectomycorrhizal fungi are necessary for survival and healthy growth of conifers in nature. Fumigation to eliminate pathogenic fungi from nursery soils also eliminates ectomycorrhizal fungi. Methods for introducing mycorrhizal fungi into previously fumigated soil in commercial conifer nurseries have been under investigation in the United States since the early 1960s (5). To evaluate the effectiveness of such mycorrhizal introductions, the identities of the fungi forming mycorrhizae in the nursery crop must be verified. The only positive confirmation available at present is to isolate the fungal symbiont from the host roots and to compare it with known cultures used as inoculum.

This report describes a selective medium and isolation protocol for recovery of the fungal symbiont *Laccaria laccata* (Scop. ex Fr.) Berk. & Br. from various conifer species. Preliminary reports have been published (1,2).

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## MATERIALS AND METHODS

**Greenhouse studies.** The inoculum of *L. laccata* used in these studies was Sylvan Spawn (Sylvan Spawn Laboratories Inc., Worthington, PA). The spawn was incorporated at a ratio of 1:8 (v/v) into sterilized 1:1 (v/v) peat-perlite potting mix in 125-ml Hillson root trainers (Spencer-Lemaire Industries Ltd., Edmonton, Alberta, Canada). Seeds of various coniferous species were surface-sterilized for 30 min in 30% hydrogen peroxide (7) and planted 4 mm deep in the spawned mix. After 10 mo of growth in the greenhouse, seedlings were removed from the trainers and mycorrhizal roots were processed for recovery of the fungal symbiont via the following basic protocol.

Roots were washed under running cold water for 1–12 hr, depending on the amount of soil on individual seedlings. Root pieces 1–3 cm long were selected and placed for 3–5 min in an ultrasonic bath containing a 1% (w/v) detergent solution (Dreft, Proctor & Gamble, Cincinnati, OH) (10). Five root pieces with 10–20 representative mycorrhizal feeder roots each were placed in a Histoprep capsule (Fisher Scientific, Pittsburgh, PA) with open-mesh top and bottom. Capsules were placed in a beaker of 1% detergent solution (100 ml of solution per capsule) and agitated on a mechanical stirrer for 3–5 min, then placed in an open-mesh sieve and rinsed under cold running water for 7–10 min. Individual root pieces were removed from the capsules under a laminar flow hood and placed for 10–20 sec in a solution of 30% hydrogen peroxide, then rinsed in a series of six to 10 petri dishes of sterile distilled water, 30–60 sec per wash (6,8,9). Mycorrhizal roots then were removed aseptically and placed (one root piece per dish) onto an agar medium in petri dishes. The basic medium used was modified Melin-Norkrans' medium (MMN) (4). Cultures were incubated at 23 C for up to 6 wk.

**Field studies.** In a commercial nursery near Lewisburg, PA, replicated plots 1.3 m square were located and permanently staked in the seedbeds. A layer of soil about 13 mm thick was removed from the surface of each plot, Sylvan Spawn was spread on the exposed bed at a rate of 330 $cm^3/m^2$, and the soil was replaced. The bed surfaces were smoothed, immediately machine sown, and mulched with a sterilized mixture of soil, vermiculite, and sawdust. *Pseudotsuga menziesii* (Mirb.) Franco was sown in beds previously fumigated with methyl bromide-chloropicrin (Dowfume MC-2). *Pinus resinosa* Ait. was sown in nonfumigated beds. Check plots were treated the same, except no mycorrhizal spawn was added to the beds. After seed germination, the beds were shaded with wood-lath snow fence. Twelve months after germination, seedlings were removed from all plots. Mycorrhizal roots were processed via the described protocol (modified to include 10 distilled water rinses with the final rinse water changed for every root piece) and then plated onto amended MMN.

## RESULTS AND DISCUSSION

In isolation attempts with greenhouse-grown *Pseudotsuga menziesii* involving several hundred isolations and numerous variations of the described isolation protocol, *L. laccata* was recovered only once on unamended MMN. Cultures were consistently overgrown by various fungi and/or bacteria. Therefore, MMN was amended with various concentrations of benomyl (Benlate). Benomyl at 1 mg/L of medium greatly reduced contaminating species of *Penicillium* and *Trichoderma*, but bacteria overgrew all other organisms. *L. laccata* was not recovered in 140 attempts on this medium. When MMN was amended with gentamicin sulfate (Garamycin) (3) at a concentration of 10 mg/L of medium or achromycin V at a concentration of 23.6 mg/L of medium, growth of bacteria was inhibited but species of *Trichoderma* and *Penicillium* overgrew the cultures. *L. laccata* was recovered only once in 178 attempts. When MMN was amended with both benomyl and gentamicin sulfate, bacterial contamination was decreased and *L. laccata* was recovered in eight of 442 attempts. When MMN was amended with benomyl (1 mg/L), gentamicin sulfate (10 mg/L), and achromycin V (23.6 mg/L), *L. laccata* was recovered in 80 of 230 attempts from *P. menziesii*, 33 of 230 attempts from *P. resinosa*, 11 of 221 attempts from *P. sylvestris* L., and in lower percentages from *Larix leptolepis* (Sieb. & Zucc.) Gord., *Picea glauca* (Moench) Voss, *Pinus banksiana* Lamb., *P. nigra* Arnold, *P. strobus* L., *P. thunbergiana* Franco, and *P. virginiana* Mill.

The frequency of recovery of *Laccaria laccata* from greenhouse-grown seedlings on amended MMN was equal to or greater than frequencies of recovery reported previously (6). Therefore, the wash-rinse portion of the protocol was refined as described previously for application to field-grown seedlings. *L. laccata* was recovered in 76 and 54% of attempted isolations from field-grown seedlings of *Pseudotsuga menziesii* and *Pinus resinosa*, respectively. In no case was the fungus recovered from seedlings grown in uninoculated plots.

*L. laccata* was rarely recovered on unamended MMN no matter how the roots were washed and rinsed. On the other hand, low rates of recovery were obtained on amended MMN unless great care was taken in washing and rinsing the root pieces. We attribute the increased rate of recovery from field-grown seedlings to increased care in washing and especially to changing the rinse water with each root piece—the major differences between the protocols used for the greenhouse-grown and the field-grown seedlings. It also was important to maintain the cultures for sufficient time; *L. laccata* emerged in some cases 5 wk after placing treated mycorrhizae on the medium. The combination of amended medium and careful wash-rinse protocol is a simple method that requires relatively few isolations and provides consistent recovery of *L. laccata* from inoculated conifer seedlings.

Single isolates of *Hebeloma crustuliniforme* (Bull. ex Sanit Amans) Quél., *Rhizopogon ellenae* Smith, *R. vinicolor* Smith, and *Suillus granulatus* (L. ex Fr.) O. Kuntz and four isolates of *Pisolithus tinctorius* (Pers.) Cok. & Couch were grown on MMN or MMN amended as described. The growth rate and appearance of each isolate on the two media were identical. Thus the amended medium may be useful for recovery of fungal symbionts from various ectomycorrhizal associations. Some other ectomycorrhizal fungi will be inhibited; however, growth of *Cenococcum graniforme* (Sow.) Ferd. & Winge (one isolate) was prevented by adding achromycin V to the medium.


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