Soil Biol. Biochem. Vol. 12, pp. 575 to 578
Pergamon Press Ltd 1980. Printed in Great Britain

0038-0717/80/1101-0575$02.00/0

SHORT COMMUNICATION

Technique for rapid mycorrhizal colonization of container-grown Douglas-fir by *Hebeloma crustuliniforme*

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(*Accepted* 1 *June* 1980)

The beneficial role of mycorrhizae in forest tree nutrition is well established (Bowen, 1973; Trappe and Fogel, 1977). In addition, the presence of mycorrhizae increases resistance to feeder-root pathogens (Marx, 1973). Current methods for production of mycorrhizal seedlings have been reviewed by Trappe (1977). Problems associated with these techniques include slow growth of fungal inoculum in peat-vermiculite, non-specificity of fungal species when forest soil is used as inoculum, and the occasional failure of inoculation. A consistent and rapid method for production of mycorrhizal seedlings is needed by research scientists and forest nursery personnel.

Our objectives were to develop a technique to promote the rapid mycorrhizal inoculation of container-grown Douglas-fir seedlings; to study the effectiveness of fragmented mycelium as inoculum; and to compare the effectiveness of several inoculation techniques.

A sporocarp isolate of *Hebeloma crustuliniforme* (Bull. ex St. Am.) Qúel (S-166) was obtained from Dr James Trappe (U.S. Forest Service, Corvallis, OR). *H. crustuliniforme* was used because it is a demonstrated rapid mycorrhizal former with Douglas-fir, grows rapidly in culture, and is common in seedling nurseries (Trappe, personal communication). Stock cultures were maintained on modified Melin-Norkrans (MMN) medium (Stevens, 1974) at 25°C, pH 5.8. Three methods were used to produce fungal inoculum.

Method 1 involved the transfer of actively-growing mycelia into 250 ml Erlenmeyer flasks containing 80 ml MMN liquid medium. Inoculated flasks were gently agitated for 3 weeks on a shaker at 25°C. Mycelial pellets were collected by filtration and washed with distilled water in sintered glass crucibles. Half of the mycelium was applied directly without fragmentation. To obtain fragmented mycelia, the remaining mycelium was ground by hand in a tissue homogenizer with MMN for 30 s or 120 s. This suspension of hyphal fragments was then applied directly to seedling roots.

In Method 2, 1 l jars containing 100 g peat-vermiculite (1:1 volume:volume) and 100 ml MMN medium were sterilized in an autoclave and inoculated with *H. crustuliniforme*. Jars were incubated at 25°C with occasional shaking. After 4 months, inoculum was removed, wrapped in cheesecloth, and washed in sterile distilled water. One part of inoculum was mixed with nine parts sterile peat-vermiculite to produce the final planting medium.

Method 3 involved cellulose thimbles. Fungal mycelium was grown on cellulose surfaces moistened with 20 ml MMN. Whatman No. 3 chromatography paper (8 × 12 cm) was shaped into a cylinder, twisted shut at one end and fitted into a 25 × 150 mm test tube. Fungal mycelium from an agar slant was transferred to each thimble and macerated. Once inoculated, thimbles in test tubes were incubated at 25°C for 3–4 weeks. Visible surface growth was apparent after 2 weeks. Before use, thimbles were washed in a slow stream of distilled water to remove excess medium.

Roots of intact container-grown 1-yr-old Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) from the Weyerhaeuser nursey, Rochester, WA, were removed from plastic tubes (2.5 × 16 cm), and washed to remove peat-vermiculite planting mix. The lower 2.5 cm of each root system was excised, since root wrenching stimulated lateral root production (Tanaka *et al.*, 1976). Washed, bare-root seedlings were examined visually with a 40 × dissecting microscope for the presence of mycorrhizas. Less than 1% of the root tips of nursery seedlings were initially mycorrhizal.

Three inoculation techniques were used. In the first technique, approximately 1 mg dry weight of either whole or fragmented mycelium was suspended in 10 ml of dilute MMN mineral solution (without carbohydrates). This suspension was applied directly to root surfaces of seedlings. In the second technique, a 1/10 dilution of the peat–vermiculite grown inoculum was used for planting medium. Cellulose thimbles impregnated with inoculum were wrapped around the bare root system of seedlings and secured with string in the third technique. Uninoculated control seedlings received identical treatments, except that mycelia, media, and thimbles were autoclaved before application.

Seedlings were replanted in sterile peat-vermiculite in large plastic tubes (4 × 21 cm), placed in a controlled environment chamber at 25°C on an 18 h photoperiod, and fertilized as necessary with a 1/100 dilution of mineral nutrient solution (Epstein, 1972, p. 39).

At various times, seedlings were examined at ×40 for presence of mycorrhizal roots, which were identified by their characteristic monopodial structure, short-branches, and white mantle of *H. crustuliniforme* (Trappe, 1967).

Roots were also sectioned with a freezing microtome. The sections were stained with 0.05% Trypan blue in lactophenol (Phillips and Hayman, 1970), and examined microscopically (400×). Generally those roots having a distinguishable mantle also had a well-developed Hartig net within the outer 2–3 cortical cell layers. With thimble-inoculated seedlings, mantle formation occurred within several weeks.

Percent mycorrhizal infection was expressed as number of infected root tips divided by total number of root rips per seedling × 100. Approximately 200–500 root tips per seedling were counted, unless the number of tips was greater than 500. In this case, root systems were divided into two halves longitudinally and one of the halves was counted.

Thimbles were inoculated with *H. crustuliniforme* and incubated at 25°C for 1–5 weeks to determine how long to grow the fungus to obtain maximum infection potential. Seedlings were exposed to 1–5 week-old inoculum, using 8 replicates, each week. Thimbles were removed after being in contact with the roots for 7 weeks. Roots were examined to determine percent infection. Liquid cultures of MMN were also inoculated with the fungus to monitor fungal growth and fungal adenosine triphosphate (ATP) content

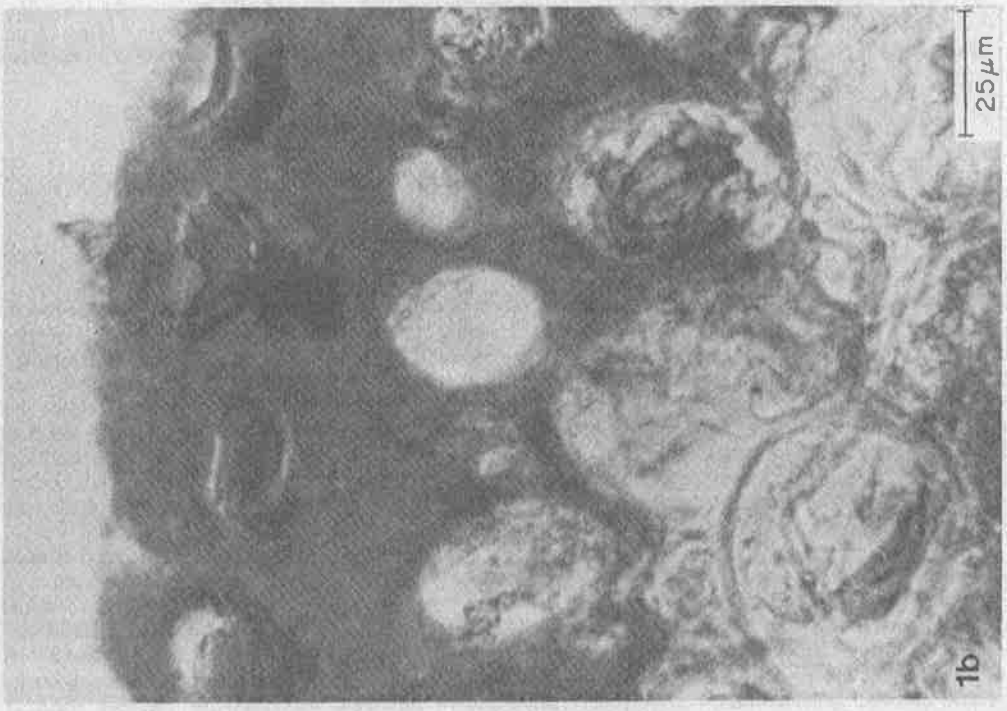

Fig. 1. Photomicrographs of Douglas-fir mycorrhiza 400 × magnification. (a) Douglas-fir + *Hebeloma crustuliniforme*: note extensive mantle formation. (b) Douglas-fir + *Laccaria laccata*: note extensive Hartig net formation.

Table 1. Relationship between method of inoculation with *Hebeloma crustuliniforme* and development of Douglas-fir mycorrhizae. Values are means of root counts of 5 or 10 seedlings per treatment

Inoculation method	% Mycorrhizal root tips per seedling	Average No. of root tips per seedling		Evaluation time (weeks)
		Non-mycorrhizal	Mycorrhizal	
Thimble-grown mycelium	39	406	266	6
Peat-vermiculite grown mycelium	15	518	88	17
Whole mycelium	4	566	24	14

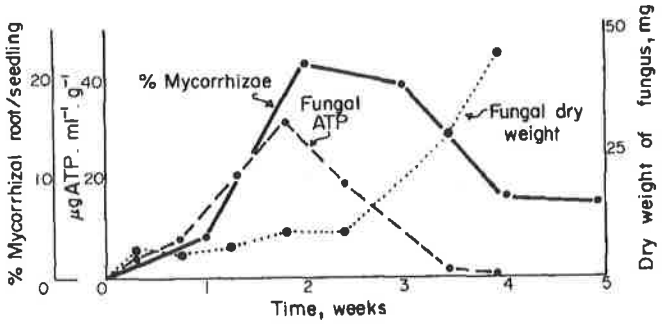

Fig. 2. The infection potential of *Hebeloma crustuliniforme* grown on cellulose thimbles reaches a maximum when the fungus is grown on thimbles for 2–3 weeks. Fungal infection potential, measured as % mycorrhiza per seedling, was determined for 16 seedlings at each sampling. Fungal ATP and fungal dry weight values are means of 8 determinations at each sampling.

for 4 weeks. Increase in fungal growth was determined by dry weight accumulation. ATP content was measured by a luciferin–luciferase analysis using a SAI Technology photometer and a modified technique of Dhople and Hanks (1973).

When roots were examined 4 months after inoculation, extensive fragmentation (120 s homogenization) of inoculum had completely inhibited fungal growth. Partial fragmentation (30 s homogenization) markedly reduced fungal ability to colonize the roots (20% of the seedlings formed mycorrhizae). In the intact mycelial treatment, 50% of the seedlings had mycorrhizal roots. Control seedlings were not mycorrhizal.

An increase in mycorrhizal development was found in those seedlings receiving the thimble inoculum (Table 1). Thimble-inoculated seedlings became mycorrhizal more rapidly than did other seedlings. Mantle and Hartig net initiation began as early as 10–14 days after inoculation. However, root tips were evaluated quantitatively 6 weeks after inoculation, when mycorrhizal development was extensive. A well-developed mantle and a less-extensive Hartig net were characteristic of *H. crustuliniforme* (Fig. 1a). The number of root tips per seedling did not differ substantially among treatments. Several other fungal species were used with the thimble technique, *Laccaria laccata* (Scop. ex Fr.) Berk. and Br. being the most successful. In contrast to *H. crustuliniforme*, *L. laccata* formed a more extensive Hartig net (Fig. 1b). *Amanita muscaria* Fr. and *Paxillus involutus* (Batsch. ex Fr.) Fr. grew well on thimbles and subsequently on roots. *Cenococcum graniforme* (Sow.) Ferd. and Winge grew well on thimbles, but did not infect the roots.

Infection potential of the fungus reached a maximum at 2–3 weeks growth on the thimble, then declined rapidly (Fig. 2). Since fungal biomass continued to increase throughout the 4-week period, it did not correlate with fungal infection potential. Fungal ATP content peaked at 2 weeks of growth in liquid culture. Ausmus (1973) suggested that fungal ATP content is an indicator of fungal metabolic activity. Our results suggest that fungal ATP content may also be an indicator of fungal infection potential. Since older inactive hyphae formed a greater proportion of total dry weight as fungal cultures aged, dry weight was a poor indicator of fungal infection potential. ATP content was a better indicator of infection potential, suggesting that younger actively-growing hyphae produced mycorrhizas more effectively than did a large biomass of older hyphae.

We conclude that cellulose thimbles are an efficient method of inoculating containerized Douglas-fir seedlings with mycorrhizas. Thimbles allowed rapid production of fungal inoculum within 2–3 weeks, and subsequent infection of Douglas-fir roots occurred by 4–6 weeks. Fungal ATP levels were good indicators of the infection potential of *H. crustuliniforme*.

Acknowledgements—This work was supported by a National Science Foundation grant DEB 77-24907 to Drs Bledsoe and Edmonds. We thank Ms May Solomonson for technical assistance.

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