HARTIG NET STRUCTURE OF ECTOMYCORRHIZAE SYNTHESIZED BETWEEN LACCARIA BICOLOR (TRICHOLOMATACEAE) AND TWO HOSTS: BETULA ALLEGHANIELSIS (BETULACEAE) AND PINUS RESINOSA (PINACEAE)

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ABSTRACT

Hartig net structure and ontogeny were compared in ectomycorrhizae synthesized between the broad host range fungus, Laccaria bicolor and two hosts, Betula alleghaniensis and Pinus resinosa. In B. alleghaniensis, the Hartig net was present in the epidermis of the three ectomycorrhizal types formed, fast-growing first-order laterals with proximal colonization, clavate second-order laterals, and nonclavate second-order laterals. Root hair–fungus interactions occurred in this association. In P. resinosa, the Hartig net developed in epidermal and cortical cell layers of monopodial and dichotomously branched first-order laterals. Short monopodial laterals exhibited a mantle only. Fungal hyphae in the Hartig net exhibited a complex labyrinthine mode of growth in ectomycorrhizae of both tree species.


With the interest in using Laccaria species in field trials, it is important to understand the cytology and morphogenesis of these ubiquitous root–fungus associations. Recent studies of ectomycorrhiza synthesis with monokaryotic and dikaryotic strains of L. bicolor (Kropp and Fortin, 1986; Kropp, McAfee, and Fortin, 1987; Kropp and Fortin, 1988) emphasized that criteria defining compatibility and efficiency between symbionts are needed.

With the exception of Brand and Agerer (1986), who provided a detailed account of field-collected Laccaria amethystina (Bolt. ex Hooker) Murr.—Fagus sylvatica ectomycorrhizae, and Lei and Dexheimer (1988) who demonstrated the ultrastructural localization of ATPase activity in Pinus sylvestris—Laccaria laccata ectomycorrhizae, only general structural features of ectomycorrhizae formed with Laccaria species have been described. This study was undertaken, therefore, to compare Hartig net location, configuration and ontog-
eny in the roots of one angiosperm (*Betula alleghaniensis*) and one gymnosperm (*Pinus resinosa*) using one strain of *L. bicolor*.

**Materials and methods—**Plant material and ectomycorrhizal synthesis— *Betula alleghaniensis* Britt. seeds, obtained from the Central Research Forest, Ontario (45°24′N × 75°33′W, 70 m) and *Pinus resinosa* Ait. seeds, obtained from Petawawa, Ontario (45°58′N × 77°19′W, 120 m) were germinated as described for *Alnus crispa* by Godbout and Fortin (1983).

Seedlings of *B. alleghaniensis* were transferred, 10 days after germination, into growth pouches containing 10 mL of modified Crone’s mineral solution (Lalonde and Fortin, 1972) supplemented with nitrogen (NH₄Cl at 0.30 g/L). Thirty-five days later, seedlings were inoculated with *Laccaria bicolor* (Maire) Orton using the strain CRFB-0101 (Andre Fortin’s laboratory, Univ. Laval, Quebec, Canada). The mycobiont was grown and introduced into the pouches as described previously (Massicotte et al., 1986). Seedlings of *P. resinosa* were transferred, 10 days after germination, into pouches containing 10 mL of modified Melin-Norkrans (MNM) solution (Marx and Bryan, 1975). Thirty days later, seedlings were inoculated using the same *L. bicolor* strain.

**Growth conditions—**Seedlings were grown under 5 klux (68 w/m²) (130 μE/m² sec) light on a 16 hr light–8 hr dark cycle at 24 C day–18 C night temperatures. High levels of humidity (60–80% RH) were maintained using a humidifier. Additional nutrient solution was added to pouches as needed.

External morphology and light microscopy— The external morphology of roots and ectomycorrhizae was examined with a Zeiss DR photodissecting microscope at intervals of 2–3 days after inoculation. Samples were collected from a period up to three weeks after the appearance of a diagnostic purple mantle and fixed using a procedure described previously (Massicotte, Ackerley, and Peterson, 1985; Massicotte et al., 1986). Tissue was then dehydrated in a graded ethanol series and embedded in LR White Resin (London Resin Co.). Sections (1–1.5 μm) were cut with glass knives and stained for light microscopy with 0.05% toluidine blue O in 1% sodium borate. More than 30 samples of each ectomycorrhizal association were examined.

**Results—**External morphology — Seedlings of *B. alleghaniensis* grow rapidly in plastic pouches and produce many first- and second-order lateral roots (Fig. 1). Seedlings of *P. resinosa* grow more slowly and produce fewer laterals (Fig. 5) than *B. alleghaniensis*. Hyphae emanating from the fungal plug (Fig. 1, 5) induce ectomycorrhizae within 4–10 days for *B. alleghaniensis* and 7–12 days for *P. resinosa*. One to two days after the introduction of the
fungal plugs in the pouch, hyphal proliferation occurs and the plug color changes from purple to white. The purple reappears after a fungal mantle forms around the root, usually at the growing root apex. As the ectomycorrhizal apices grow, the purple tip is maintained and proximal regions become white and then brown. Numerous first- (Fig. 3) and second-order (Fig. 2) ectomycorrhizal lateral forms on _B. alleghaniensis_ root systems, most of which show radiating outer mantle hyphae. Occasionally, first-order laterals with a basal, purple fungal mantle that grows acropetally are present (Fig. 4). Outer mantle hyphae are present at the base of these “ectomycorrhizal” laterals while the apex is free of hyphae and appears translucent (Fig. 4). Monopodial first-order (Fig. 6) and dichotomized first-order ectomycorrhizal laterals (Fig. 7) are formed consistently on _P. resinosa_ root systems.

**Light microscopy — Betula alleghaniensis—L. bicolor** — First-order laterals from noninoculated root systems typically have a well-developed apical meristem, a distinct root cap and axially-elongated epidermal and cortical cells (Fig. 8). First-order laterals from inoculated root systems exhibit different degrees of colonization by the fungus (Fig. 9, 10). In some cases, the mycosymbiont forms a thin mantle, mainly in proximal portions of the root with few hyphae at the apex (Fig. 9), whereas in other cases the mantle is thicker and hyphae surround the root apex at least partially (Fig. 10). Epidermal and cortical cells are enlarged radially either in the subapical region (Fig. 9) or in more proximal zones (Fig. 10). Second-order ectomycorrhizal laterals have a smaller meristem than first-order ectomycorrhizal laterals (cf. Fig. 9, 10 with Fig. 11–13) and have a well-developed mantle covering the apex. Small second-order ectomycorrhizal laterals have pronounced radially-elongated epidermal cells with a Hartig net in the proximal portion and a distinct apposition zone closer to the apical meristem where hyphae are in contact with epidermal cells (Fig. 11). Longer second-order ectomycorrhizal laterals exhibit either a typical Hartig net confined to the apical zone (Fig. 12) or a Hartig net along the entire axis (Fig. 13). Laterals of the first type have a clavate morphology with hypha–root hair interactions in the subapical zone where epidermal cells are not radially enlarged (Fig. 12). The mantle is thin and a Hartig net develops in a restricted region of the subapical portion of the root (Fig. 12). Laterals of the second type are of uniform diameter with a well-developed mantle and a Hartig net present along the entire axis, except in the apposition zone (Fig. 13). Root hair–hypha interactions are evident on the first-order laterals bearing second-order ectomycorrhizal laterals (Fig. 14). As well, distinct phi thickenings develop in the second layer of cortical cells of the first-order laterals (Fig. 14).

A sequence of longitudinal sections from the

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Fig. 19–21. Sections of _B. alleghaniensis—L. bicolor_ mycorrhiza. 19. Longitudinal section of root similar to that shown in Fig. 13. A full Hartig net with numerous hyphal branches (arrowheads), many of which are oriented along the long axis of the root, has developed between radially-elongated epidermal cells (e). Vacuolated cortical cells (c) are evident. 20. Longitudinal section of root showing root hair (*)-fungus interaction similar to that shown in Fig. 13. Hyphae have penetrated between (arrowheads) and surround (double arrowheads) root hairs. 21. Transverse sections of two root hairs (rh) surrounded by layers of hyphae, some of which have septa (arrowheads). A clamp connection (double arrowhead) is evident. A third root hair (*) has been sectioned at its tip.

Fig. 22–26. Longitudinal section of _Pinus resinosa—Laccaria bicolor_ ectomycorrhiza. 22. Young first-order lateral with a well-developed meristem (*), and a thin layer of fungal hyphae (arrowheads). A Hartig net has not developed. 23. Older first-order lateral root with a single apical meristem (*), a well-developed mantle (arrowheads), and Hartig net (double arrowheads). A Hartig net (triple arrowheads) has formed between epidermal and cortical cells of the primary root. Zones 1–3 are shown in Fig. 24–26, respectively. 24. Adjacent section to zone 1 indicated in Fig. 23. A compact mantle (m) and the early stages of a Hartig net (arrowheads) are present. Epidermal cells (e) have numerous dark deposits. Cortical cells (c) are evident. 25. Adjacent section to zone 2 indicated in Fig. 23. A glancing section shows the mature Hartig net (arrowheads) up to cells adjacent to the endodermis (*). Epidermal (e), cortical (c) cells, and mantle (m) are evident. 26. Adjacent section to zone 3 indicated in Fig. 23. A typical tangential view of a mature Hartig net consisting of narrow intercellular hyphae (arrowheads). Endodermis (*) and mantle (m) are evident.

Fig. 27–29. Longitudinal section of _P. resinosa—L. bicolor_ ectomycorrhiza. 27. Elongated first-order lateral root similar to that shown in Fig. 6. A single apical meristem (*), a well-developed mantle (arrowheads), and a Hartig net (double arrowheads) are evident. Zones 4, 5 are shown in Fig. 28, 29, respectively. 28. Portion of root shown in zone 4 indicated in Fig. 27. A well-developed mantle (m) and mature Hartig net have developed. Some Hartig net hyphae have enlarged and developed branches (arrowheads). Endodermis (*), cortex (c), and epidermis (e) are evident. 29. Junction between first-order lateral and primary root in a zone similar to that indicated by zone 5 in Fig. 27. A glancing section of the Hartig net shows many enlarged and branched hyphae (double arrowheads). Endodermis (arrowheads) is also seen.
apex to proximal regions on a first-order ectomycorrhizal lateral (Fig. 15–18) shows conspicuous changes in the mode of growth and morphology of the mycosymbiont during Hartig net formation. In the apposition zone, hyphae aggregate to form a loose mantle (Fig. 15) and, immediately proximal to this, inner mantle hyphae begin to grow between epidermal cells (Fig. 16); hyphal branching and septa are present. Proximal to this region, the mantle is more compact, especially in the inner portion, and the Hartig net hyphae are enlarged, as compared to outer mantle hyphae, and have numerous branches (Fig. 17). The mature Hartig net seen in glancing section at the base of first-order lateral roots reveals a complex branching pattern (Fig. 18) with enlarged hyphae and ultimate fine branches smaller in diameter than the outer mantle hyphae. The inner mantle forms a continuum with the Hartig net hyphae and is compact at this level. In this plane, the small fungal branches appear to be oriented primarily in the radial direction (Fig. 18), whereas in comparable longitudinal sections taken from a second-order lateral root similar to that shown in Fig. 13, epidermal cells are radially-elongated and hyphal branches are oriented primarily along the long axis of the root (Fig. 19). As in Fig. 18, the Hartig net abuts the outer cortical layer (Fig. 19). Associations between B. alleghaniensis–L. bicolor show many root hair–hypha interactions (Fig. 20, 21). Hyphae often penetrate between root hair cells, form a thick mantle around the root and a mantlike structure around root hairs (Fig. 20, 21). Hyphae aggregated around root hairs are not enlarged or branched (Fig. 21) as those in the Hartig net. Some hyphal separation is, however, present (Fig. 21).

Pinus resinosa–L. bicolor—Short first-order laterals formed on inoculated root systems have a thin layer of fungal hyphae but lack a Hartig net (Fig. 22). Longer first-order laterals have a thicker and more developed fungal mantle than short first-order laterals and a fully developed cortical Hartig net confined to a short region in the proximal area of the root (Fig. 23). First-order ectomycorrhizal laterals that have elongated further develop a full Hartig net over a greater length of the root but maintain a similar apical zone where the Hartig net is not fully formed (Fig. 27). The size of the meristem is approximately similar in these three root types (cf. Fig. 22, 23, and 27). In the zone preceding the full cortical Hartig net, hyphae penetrate between epidermal cells (Fig. 24) and eventually cortical cells (Fig. 25). Dense globular deposits are often present in the epidermal cells surrounded by hyphae (Fig. 24, 25, 28). Numerous small hyphal branches are present in different views of the Hartig net (Fig. 25, 26). In an older portion of a cortical Hartig net, enlarged, vacuolated hyphae with numerous branches are evident (Fig. 28). At the junction between a first-order lateral and the primary root, enlarged hyphal and fungal branches, sometimes juxtaposed against the inner limiting cell layer, probably the endodermis, are evident (Fig. 29).

Dichotomously-branched first-order ectomycorrhizal lateral roots have a similar graduation of fully developed Hartig net in proximal portions and developing Hartig net closer to the apex of each root branch (Fig. 30). The apical meristem of each branch appears smaller than in monopodial axes (cf. Fig. 30 with Fig. 27). The morphology of the Hartig net is more obvious in older portions of the ectomycorrhiza where the hyphal cytoplasm is not as dense (Fig. 31, 32). For instance, in a portion of the root at the base of the two axes, numerous hyphal branches are present (Fig. 31). In an older portion, on one axis of a longer dichotomous root (not shown), enlarged hyphae, some of which have grown around dark deposits, are obvious at all levels of penetration (Fig. 32).

**DISCUSSION**—Using the growth pouch system, it was easy to monitor the colonization of both B. alleghaniensis and P. resinosa roots by the strain of L. bicolor used, because of the distinct purple color to the hyphae as they came in contact with a root. Although hyphae growing in culture medium were purple, they lost the color two or three days after transfer of
inoculum plugs into pouches and then regained the color after they came in contact with a root. It is possible that pigment formation is triggered by sugars either from the culture medium or from root exudates. If root exudates trigger pigment synthesis, one could then use the change in color as a marker to indicate root-fungus interaction. Experimental work is required to test the effects of various components of root exudate on pigment synthesis.

Based on synthesis of Betula alleghanensis—L. bicolor ectomycorrhizae in growth pouches, three morphological types were recognized. One type is shown by fast-growing first-order laterals which developed a purple mantle in proximal regions but not at the root apex. Although the mantle and a Hartig net developed acropetally, a distinct zone, free of hyphae and mantle was present at the apex. The fungus did not keep up with the activity of the meristem and therefore these roots provided an ontogenetical sequence from the earliest stage of hypha contact through to mature mantle and Hartig net formation.

The second and third types were observed on second-order laterals which grew more slowly than first-order laterals. The typical clavate type was formed when hyphae interacted with a lateral that had already elongated, whereas the uniformly thick ectomycorrhizal type was formed when hyphae interacted with a very young root primordium. These two types have been described for a number of ectomycorrhizae synthesized in pouches: Alnus crispa—Alpova diplophloeus (Massicotte et al., 1986), Eucalyptus pilularis—Pisolithus tinctorius (Massicotte, Peterson and Ashford, 1987), Alnus rubra—Alpova diplophloeus (Massicotte, Peterson, and Melville, 1989). These types were not obvious, however, in Dryas integrifolia—Hebeloma cylindrosporum mycorrhizae (Melville, Massicotte, and Peterson, 1987). Although no quantitative assessment was done, it is possible that fast-growing fungi such as Hebeloma reach more second-order lateral roots at an early stage of development, thereby forming uniform ectomycorrhizae. Fewer clavate-type ectomycorrhizae would therefore form as compared to root systems colonized by slow-growing fungi.

The clavate-type ectomycorrhiza exhibited an interesting developmental sequence as seen in section. In addition to the typical Hartig net and mantle configuration found in the apical portion, root hairs were colonized in the subapical portion. In this region, intercellular hyphae mimicked Hartig net formation. More proximally, root hairs were not colonized by hyphae, and epidermal cells elongated axially.

There seemed to be a threshold of epidermal cell and root hair differentiation beyond which no interaction with hyphae occurred. A number of ectomycorrhizae exhibit root hair—fungus interactions (see Massicotte, Melville, and Peterson, 1987). In the case of Picea mariana—Pisolithus tinctorius (Thomson, Melville, and Peterson, 1989), a mantelike structure is formed around the root hair, indicating that factors responsible for hypha aggregation are present in root hairs.

The strain of L. bicolor used in this study formed a Hartig net with distinct fungal branches (or palmettes) between epidermal cells of first- and second-order ectomycorrhizal lateral of B. alleghanensis and between epidermal and cortical cells in monopodial and dichotomously branched first-order ectomycorrhizal lateral of P. resinosa. Very young monopodial stages of P. resinosa had a fungal mantle but lacked a Hartig net. The ultimate fungal branches of the Hartig net, seen in section, were a fraction of the complex labyrinthine system developing between epidermal cells (B. alleghanensis) and between epidermal and cortical cells up to the endodermis (P. resinosa) and was similar to descriptions in previous studies (see Brand and Agerer, 1986; Atkinson, 1975; Nylund and Unestam, 1982; Blasius et al., 1986; Kottke and Oberwinkler, 1986a, b; Melville et al., 1987, 1988). These fungal branches originated from multiple growing points of inner mantle hyphae and proceeded to develop inward in the root, more or less radially, depending on the system and the plane of section. It was not clear if this intercellular growth of hyphae was a random growth process or was influenced by overall cell shape, plasmodesmata distribution, physical and chemical properties of cell walls, and other barriers such as suberin and lignin in the exodermis (Peterson, 1988). Organelle distribution and compartmentalization may be correlated with the ultimate architecture of the Hartig net (Kottke and Oberwinkler, 1987; Massicotte, Peterson, and Melville, 1989).

Descriptions of Hartig net morphogenesis, using different genotypes of the same fungal species, mutants or monokaryotic and dikaryotic strains of fungal species are needed to elucidate the critical steps in the establishment of this complex interface between the two symbionts. An approach correlating structural events with biochemical changes may be possible using molecular techniques, such as those used by Hilbert et al. (1987) with Eucalyptus globulus Labill.—Pisolithus tinctorius. It is likely that the profound growth modifications of the hyphae as they form the Hartig net are the
result of symbiosis-specific genes, although this has never been demonstrated.

LITERATURE CITED


