Structure and ontogeny of Betula alleghaniensis – Pisolithus tinctorius ectomycorrhizae

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The ontogeny and ultrastructure of ectomycorrhizae synthesized between Betula alleghaniensis (yellow birch) and Pisolithus tinctorius, a broad host range fungus, were studied to determine the structural modifications in both symbionts during ectomycorrhizae establishment. A number of stages, including initial contact of hyphae with the root surface, early mantle formation, and mature mantle formation, were distinguished. Interactions between hyphae and root hairs were frequent. As a paraepidermal Hartig net developed, root epidermal cells elongated in a radial direction, but wall ingrowths were not formed. Repeated branching of Hartig net hyphae resulted in extensive fine branches and the compartmentalization of hyphal cytoplasm. Nuclei and elongated mitochondria were frequently located in the narrow cytoplasmic compartments, and thickenings developed along walls of cortical cells in primary roots.

Introduction

Most Betula species are known or suspected to be associated with ectomycorrhizal fungi (Trappe 1962; Malloch and Malloch 1981, 1982; Mason et al. 1982; Watling 1984). Atkinson (1975) published a detailed account of the comparative anatomy of in vitro synthesized Betula verrucosa Ehrh. – Amanita muscaria mycorrhizae and field-collected Betula mycorrhizae, and Strullu and Gerault (1977) described anatomical features of Betula pubescens field mycorrhizae. In another study, seedlings of Betula pendula were inoculated in vitro with Pisolithus arhizus (Pers.) Rausch. (= Pisolithus tinctorius), Scleroderma citrinum Pers., and Paxillus involutus to determine if these fungi induced mycorrhizae (Gaie 1977). Further work focussed on in vitro synthesized B. pendula – Astraeus hygrometricus (Pers.) Morg. mycorrhizae where a thick mantle and well-developed Hartig net were described in more detail (Gaie and Heine-mann 1980a). Gaie and Heinemann (1980b) also compared the mycorrhizal potential of 33 species of Betula inoculated with P. arhizus in vitro. Freehand sections of some of the associations revealed extensive Hartig net development with numerous fungal branches. Although the overall morphology was similar, there was variation in the radially elongation of epidermal cells amongst Betula species.

Duddridge (1986a, 1986b) described the incompatible interaction between B. pubescens and Suillus grevillei and reiterated earlier concerns (Atkinson 1975) that sugars included in the medium for in vitro mycorrhizal synthesis may result in anomalies. Recent work by Wilcox and Wang (1987b) contrasted the ectomycorrhizal status of Phialophora finlandia Wang and Wilcox on Betula alleghaniensis with the ectendomycorrhizal behaviour of the same fungus on Pinus resinosa.

Materials and methods

Plant material and mycorrhiza synthesis
Betula alleghaniensis Britton seeds, obtained from the Central Research Forest, Ontario (Latitude 45°24’N, longitude 75°33’W, 70 m asl), were germinated as described by Godbout and Fortin (1983) for Alnus crispa. 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 P. tinctorius (Pers.) Coker and Couch, using the strain 76-1 (Grenville et al. 1985). The mycobiont was grown and introduced into the pouches as described previously (Massicotte et al. 1986).

Growth conditions
Seedlings were grown under 5 klx (68 W/m², 130 E m⁻² s⁻¹) light on a 16:8 h light:dark cycle at 24-18°C day:night temperatures. High levels of humidity (60–80% RH) were maintained using a humidifier. Additional nutrient solution was added to pouches as the paper wick began to dry.

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 up to 2 weeks after
the appearance of a white-beige mantle, and fixed and postfixed using a procedure described previously (Massicotte et al. 1985, 1986). Tissue was then dehydrated in a graded ethanol series and embedded in LR White resin (London Resin Company Ltd.) immediately after dehydration. Thick 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 ectomycorrhizal roots at various stages of development were examined.

**Scanning electron microscopy (SEM)**

Ectomycorrhizae were fixed and postfixed by the same procedure used for light microscopy, followed by a treatment with thio-carbohydrazide and a subsequent postfixation in 1% osmium tetroxide (Kelley et al. 1973). Specimens were then washed in distilled water, dehydrated in a graded series of ethanol, critical-point dried, mounted on aluminum stubs, and observed with a JEOL JSM-35C scanning electron microscope. More than 30 lateral roots at different stages of development were examined.

**Transmission electron microscopy (TEM)**

Ectomycorrhizae were fixed and postfixed by the same procedure used for light microscopy. Tissue was then washed, dehydrated in a graded ethanol series, dehydrated in propylene oxide, and embedded in TAAB EMBED 812 resin. Thin sections were cut with a diamond knife, picked up on copper grids, and stained for 10 min with ethanolic uranyl acetate and 5 min with aqueous lead citrate. Sections were observed in a JEOL 100CX scanning transmission electron microscope at 80 keV, using the transmission mode. For the developmental study, five roots with mature mantles were examined. For each region, electron microscopy was correlated with observations of adjacent sections prepared for light microscopy.

**Results**

**External morphology**

Betula alleghaniensis seedlings grow well in plastic pouches and form many first-order lateral roots (Fig. 1). Hyphae emanating from the fungal plugs induce mycorrhizae on first-order laterals 4-10 days after inoculation. Before a complete mantle is formed (Figs. 7 and 8), scattered hyphae are present on root apices among numerous root hairs (Figs. 2 and 11). This is followed by hyphal proliferation among root hairs (Fig. 12) and aggregation at the apex (Figs. 3-6). Young hyphae growing at the root tip (Figs. 3 and 4) are often a brighter yellow than the surrounding extraradical hyphae, a color also associated with sclerotium initiation (Fig. 9). Just proximal to the region of early fungal aggregation at the apex, a darkening of root tissues often occurs (Figs. 3 and 4) and, consequently, mycorrhizal roots appear darker than uncolonized roots. A very thin mantle is then formed at the apex (Figs. 5 and 13) or along the length of the root (Fig. 6). Underlying dark root cells are still visible beneath the loose mantle. A thick mantle eventually forms (Figs. 7 and 14), followed in some cases by the formation of second-order mycorrhizal laterals (Fig. 8). Sclerotia develop from hyphal strands associated closely with roots (Figs. 9 and 10). Each sclerotium is a lentigeral aggregation of hyphae (Figs. 9 and 10), and both young (Fig. 9) and older stages (Fig. 10) exude droplets that are usually associated with protuberances on the surface (Fig. 10).

**Light microscopy**

Sectioned root apices show that during very early stages of mycorrhiza development, hyphae occur preferentially in regions between root hair papillae in the subapical portion of the root, but are randomly distributed in more apical regions of the root (Fig. 15). Roots at this stage have a sizeable apical meristem, a small root cap, and axially elongated epidermal cells (Fig. 15). At a slightly later stage, hyphae proliferate to form a thin mantle covering the apical and subapical portions of the root (Fig. 16). Root hairs are present in the subapical portion, and some epidermal cells show the beginning of radial elongation, mainly at the root apex (Fig. 16). The apical meristem is well developed, and the small root cap is covered by

![Figs. 1–8. Ectomycorrhizae of Betula alleghaniensis – Pterolitum tinctorius. Fig. 1. Growth pouch showing seedling with a well-developed root system and fungal plug inocula(*). Mycorrhizal roots (arrowheads) are present in the vicinity of the fungal plugs. Figs. 2–6. A sequence in development of early stage mycorrhizal first-order lateral roots. Fig. 2. A few hyphae (arrowheads) are present on the root surface. Fig. 3. Proliferation of hyphae (arrowheads), many of which are perpendicular to the root surface. Fig. 4. Hyphae enveloping the root apex (arrowheads). The subapical portion of the root (double arrowheads) is darker than the remainder of the root. Fig. 5. Hyphae covering the root apex and forming a thin mantle (arrowheads). Fewer hyphae are present in the subapical portion. Fig. 6. Hyphae enveloping the entire root and forming a thin mantle (arrowheads) of loose hyphae. Figs. 7–8. Mature mycorrhizae. Fig. 7. Hyphae forming a thick, compact mantle along the entire root (arrowheads). Fig. 8. Mycorrhizal second-order lateral roots (arrowheads) formed on the mycorrhizal first-order lateral (*). Figs. 9 and 10. Sclerotia of P. tinctorius formed in association with B. alleghaniensis roots. Fig. 9. Early stage of sclerotium (*) initiation along hyphal strands (arrowheads). Exudate (double arrowhead) is present. Fig. 10. Later stage in sclerotium development before melanization. Droplets of exudate (*) are associated with protuberances (arrowheads) on the surface of the sclerotium.

Figs. 11–14. SEM of B. alleghaniensis first-order lateral roots at different stages of interaction with P. tinctorius hyphae. Fig. 11. A few hyphae (arrowheads) are present and numerous root hairs (double arrowheads) are evident. Fig. 12. Proliferation of hyphae (arrowheads), which interact with the root hairs on the root surface. Fig. 13. Hyphae have formed a thin mantle (arrowheads) on the root surface. Some root hairs are still evident (double arrowheads). Fig. 14. Hyphae have covered the root, forming a compact mantle. Root hairs are not evident.

Figs. 15–20. Light microscopy of stages in the ontogeny of ectomycorrhizae formed between B. alleghaniensis and P. tinctorius. All are longitudinal sections of first-order lateral roots. Fig. 15. Root similar to that in Fig. 12, showing hyphae (arrowheads) between root hairs (*) in the subapical region and in other regions of the root. The root apical meristem (AM) is well developed. Fig. 16. Root similar to that shown in Fig. 13. Hyphae have proliferated to form a thin mantle (arrowheads) covering the apical and subapical portions of the root. Some of the epidermal cells (e) are beginning to elongate radially. The apical meristem (AM) is well developed, and root hairs (*) are present in the subapical portion. Fig. 17. Later stage showing the development of a thick mantle (*), radially elongated epidermal cells (e), mainly in the apical portion, and a reduced apical meristem (AM). Fig. 18. Later stage showing a thick mantle (*), radially elongated epidermal cells (e) along the entire axis of the root, and a very small apical meristem (AM). Fig. 19. Higher magnification of apical portion of root similar to that in Fig. 18, showing radially elongated epidermal cells (e), a well-developed mantle (*), and darkly staining materials (arrowheads) in the inner mantle. Fig. 20. Higher magnification of a portion of root proximal to that in Fig. 19, showing the inner mantle hyphae (arrowheads) adhering to radially elongated epidermal cells (e). The apical meristem is at the right of the picture. A, apposition zone.
fungal hyphae (Fig. 16). At a later stage of mycorrhiza development, the mantle thickens considerably and epidermal cells either in the apical portion of the root only (Fig. 17) or along the entire axis (Fig. 18) elongate radially. The apical meristem of older stages of mycorrhizae is very small (Figs. 17 and 18).

The apposition zone (pre-Hartig net zone) in the subapical portion of well-developed mycorrhizal roots contains inner mantle hyphae tightly apposed to (Fig. 19), and in some cases within, the groove between epidermal cells (Fig. 20). In both cases, a fairly thick mantle with darkly staining material is evident (Figs. 19 and 20). A sequence of transverse sections taken at three different levels in the Hartig net zone shows the onset of penetration by inner mantle hyphae between epidermal cells (Fig. 21), deeper penetration by hyphae between cells (Fig. 22), and, finally, the completion of the Hartig net up to the first cortical layer (Fig. 23). In the mature Hartig net, longitudinal sections show clearly the multibranched hyphae, which are primarily oriented radially (Fig. 24). In early stages of Hartig net development, the hyphae (Figs. 21 and 22) have dense cytoplasm, whereas in older stages, they are more vacuolated (Figs. 23 and 24). Paradermal sections taken at three different levels from the outer mantle to the epidermal layer in a mature Hartig net zone illustrate the structural complexity of the mantle and the Hartig net (Figs. 25–27).

The outer mantle is composed of narrow, cylindrical hyphae that aggregate to form a dense covering (Fig. 25). In the inner mantle, where hyphae are apposed to the root surface, hyphae are enlarged and have branched to form a pattern reminiscent of a jigsaw puzzle (Fig. 26). Dense deposits have been incorporated into the mantle at this level (Fig. 26). At the level of epidermal cells, hyphae are no longer cylindrical and show numerous branches, convolutions, and septa (Fig. 27).

An extensive mantle (Fig. 28) and Hartig net (Fig. 29) may develop in the root hair zone of mycorrhizal roots. Hartig net hyphae between root hair cells show numerous fine branches (Fig. 29). Primary roots at the base of first-order mycorrhizal lateral roots develop φ thickening in the 2nd cortical layer (Figs. 30 and 32) or in the 2nd and 3rd layer of cortical cells (Figs. 31 and 33). In the 2nd cortical layer, φ thickenings form on radial walls (Fig. 32), whereas in the 3rd layer, intercellular spaces can be completely surrounded (Fig. 33) in a manner similar to that seen in angular collenchyma; φ thickenings lack any internal substructure (Fig. 47).

Transmission electron microscopy

The apposition zone (pre-Hartig net zone) contains hyphae adjacent to cap cell remnants, to electron-dense deposits, and to epidermal cells (Fig. 34). Epidermal cells are either slightly pulled apart, with fibrillar material between (Fig. 35), or appear unaltered (Fig. 36). In some cases, the groove between epidermal cells is filled with electron-dense deposits (Fig. 36). Electron-lucent regions around hyphae suggest some degradation of the electron-dense material (Figs. 34–36). Hyphae apposed to epidermal cells have large nuclei, endoplasmic reticulum cisternae, mitochondria (Figs. 35–37), and vacuoles, some with dense deposits (Fig. 37). Epidermal cells with a large central vacule have few organelles, including mitochondria and endoplasmic reticulum (Figs. 34 and 35). Electron-dense material is prevalent between hyphae in the mantle (Fig. 37).

Inner mantle and Hartig net hyphae in the early Hartig net zone possess nuclei and vacuoles with dense deposits (Fig. 38). One (Fig. 40) or two nuclei (Fig. 39) as well as mitochondria and endoplasmic reticulum are present in each compartment of hyphae that penetrate partially between epidermal cells. Depending on the plane of section, some Hartig net hyphae appear branched (Fig. 40).

In the mature Hartig net zone, very few organelles appear between adjacent hyphal branches in transverse view (Fig. 41). A longitudinal view of hyphal branches, however, shows numerous elongated mitochondria (Figs. 42 and 43), often oriented longitudinally between contiguous branches, and an occasional nucleus (Fig. 42). Endoplasmic reticulum cisternae and small vacuoles are also distributed in the compartments formed by hyphal branching (Fig. 43).

Figs. 21–23. Transverse sections of portions of root similar to that in Fig. 18 at different stages of Hartig net formation. Fig. 21. Inner mantle hyphae (arrowheads) located partly between epidermal (e) cells. Fig. 22. Later stage showing hyphae (arrowheads) between epidermal (e) cells. Hyphae are cytoplasmic at this stage. Fig. 23. Later stage with hyphae (arrowheads) between epidermal (e) cells up to the outer cortical cell layer (c). Hyphae are compact and vacuolated. Fig. 24. Longitudinal section of a root similar to that in Fig. 18, showing mature Hartig net hyphae with numerous branches (arrowheads). e, epidermal cells. Figs. 25–27. Paradermal sections at various levels in the mantle, and Hartig net in a root similar to that in Fig. 18. Fig. 25. Compact outer mantle with cylindrical and intertwining hyphae. Septa (arrowheads) are present. Fig. 26. Compact inner mantle with swollen and branched hyphae (arrowheads). Some dark deposits (double arrowheads) are incorporated in the puzzelike mass of hyphae. Fig. 27. Inner mantle and Hartig net zone showing multibranched hyphae (*), septa (arrowheads), and epidermal cells (e).

Figs. 28 and 29. Sections of B. alleghaniensis – P. tinctorius ectomycorrhizae in the root hair zone similar to the zone indicated by the double arrowheads in Fig. 16. Fig. 28. Transverse section showing a thick mantle (*), in some cases surrounding root hairs (arrowheads). Fig. 29. Longitudinal section showing a root hair (arrowheads) partially covered with a compact mantle. Numerous hyphal branches (double arrowheads) of Hartig net hyphae are evident between root hair cells. c, cortical cells; e, epidermal cells. Figs. 30–33. Transverse sections of portions of primary root taken at the base of first-order mycorrhizal lateral roots. Fig. 30. φ thickenings (arrowheads) developed along the radial walls of the second cortical cell layer. A thin mantle (double arrowheads) is present at the base of the first-order lateral root. Fig. 31. Additional φ thickenings (arrowheads) in the 3rd layer of cortical cells. A thin mantle (double arrowheads) is present at the base of the first-order lateral root. Fig. 32. Section adjacent to that shown in Fig. 30, showing well-developed φ thickenings (arrowheads) in the 2nd cortical layer and the initiation of additional thickenings (double arrowheads). Fig. 33. Section adjacent to that shown in Fig. 31, showing φ thickenings (arrowheads) in the 2nd and 3rd layer of cortical cells.

Figs. 34–36. TEM of B. alleghaniensis – P. tinctorius ectomycorrhizae: apposition zone (pre-Hartig net zone) of a root similar to the one indicated by zone A in Fig. 20. Fig. 34. Portion of root showing hyphae in contact with remnants of root cap cell (RC), electron-dense deposits (*), and epidermal cells (E). Mitochondria (M) and nuclei (N) are evident. Fig. 35. Higher magnification of a portion of Fig. 34. Fibular material (black arrowheads) and electron-lucent material (white arrowheads) are present adjacent to the hyphal walls. The region indicated by the asterisk is a site of a fungal hypha as shown in adjacent sections. Mitochondria (M), endoplasmic reticulum (ER), and a large nucleus (N) are evident in one fungal hypha, whereas mitochondria (M) and some endoplasmic reticulum (ER) are present in the epidermal cell (E). Fig. 36. Hypha adjacent to electron-dense deposits (*) between epidermal cells (E). An elongated nucleus (N) is evident in the hypha.
Proximally to the mature Hartig net, hyphal compartments still exhibit one (Fig. 44) or two nuclei (Fig. 45) in the cytoplasm, which is less dense than at the mature stage. Hyphal branches forming the Hartig net are still present, but numerous vacuoles, mitochondria, and electron-dense deposits, presumably polyphosphates, are characteristic of this stage (Fig. 46). Fungal nuclei are consistently associated with microtubules, but it is in the older zone that these are more easily distinguished (Fig. 44).

Discussion

Results of this investigation on early development and ultrastructure of peripheral layers of the root–fungus interface in mature *B. alleghaniensis*–*P. tinctorius* ectomycorrhizae confirm many previous findings, but also reveal a number of new features.

A useful marker of early hyphal aggregation in apical and subapical regions of the root is a change in color of the *P. tinctorius* hyphae to a brighter yellow. This color shift is similar to that described during sclerotium initial formation in this study and in previous studies of sclerotium initiation and field (Dennis 1980). Similarly, a recent descriptive study on *Laccaria bicolor* mycorrhizae (Massicotte et al. 1989b) showed that white hyphae became blue when in contact with root apices, and it was speculated that pigment synthesis might be triggered by root exudates. Nylund and Unestam (1982) also noticed, using carbohydrate-free medium, that hyphae of *Piloderma croceum* Erikss and Hjortst were colorless and showed poor growth, but when growing in the vicinity of *Picea abies* roots, they soon turned yellow. The triggering of pigment synthesis in ectomycorrhizal fungal hyphae is still poorly understood.

Early morphological changes of ectomycorrhizae have rarely been recorded in the literature (Massicotte et al. 1987a), making comparisons between associations very difficult. Darkening of the *B. alleghaniensis* first-order laterals in subapical regions, concomitant with early fungal aggregation at the apex, was also noted on *Eucalyptus pilularis* roots, using the same *P. tinctorius* strain (Massicotte et al. 1987c). Although more observations of this type are needed, it is tempting to speculate that such precocious darkening may be a manifestation of an early host defense reaction.

*Pisolithus tinctorius* forms a Hartig net with *B. alleghaniensis* that is restricted to the epidermis, supporting earlier observations (Atkinson 1975; Gaie 1977; Gaie and Heinemann 1980a, 1980b). The presence of k thickenings in the 2nd cortical cell layer from the epidermis is therefore not necessarily correlated with hyphal invasion, as it is in *Dryas integrifolia*–*Hebeloma cylindrosporum* mycorrhizae (Melville et al. 1987a, 1987b). Wilcox and Wang (1987a) showed, however, that invasion of root tissue by *Chloridium paucisporum* Wang and Wilcox, an ectendomycorrhizal fungus on *B. alleghaniensis*, is limited by the k thickenings present in the 2nd inner cortical cell layer. It may therefore be critical to consider a range of possible root–fungal associations in order to assess relationships between structural features such as k thickenings of roots and fungal colonization.

The interface between Hartig net hyphae and root cells in *B. alleghaniensis*–*P. tinctorius* ectomycorrhizae does not involve wall modifications of epidermal cells, as seen earlier in the mycorrhizae formed between *Alnus crispa* and a host genus-specific fungus, *Alpova diplophloeus* (Massicotte et al. 1986). Wall ingrowths were also absent from *Alnus rubra*–*Alpova diplophloeus* and *Eucalyptus pilularis*–*P. tinctorius* ectomycorrhizae (Massicotte et al. 1989a, 1989b). Only a few other root–fungus interfaces have been described in any detail (Atkinson 1975; Ashford and Allaway 1982; Duddridge and Read 1984a, 1984b, 1984c; Kotke and Oberwinkler 1986a; Melville et al. 1988), and no clear pattern relating to epidermal wall ingrowth formation is emerging.

The most dramatic alteration of symbionts in the interface region involves the fungus as it forms the Hartig net and gives rise to a complex system of branches (labyrinthine growth). This morphogenetic change has been noted in a large number of ectomycorrhizal systems (Mangin 1910; Atkinson 1975; Nyland and Unestam 1982; Nylund et al. 1982; Blasius et al. 1986; Kotke and Oberwinkler 1986a, 1986b, 1987; Melville et al. 1987a, 1988). In the *B. alleghaniensis*–*P. tinctorius* ectomycorrhizae synthesized in this study, the distance between fungal branches is barely wide enough to accommodate...
date the elongated mitochondria. The physiological consequences of this labyrinthine structure need to be investigated, since it is not clear where bidirectional transfer of nutrients takes place. Kottke and Oberwinkler (1987) postulated that the tips of these fine branches are the sites of mutual exchange, whereas Lei and Dexheimer (1988), using an indirect technique of enzyme localization, indicated that adjacent fungal walls between the fungal branches should also be considered. We do not see why exchange should be restricted to hyphal tips, since the interface is apoplastic and there has not been any demonstration of an apoplastic barrier in any of the hyphal walls in this region. Tracers such as cellufluor should be used to test the permeability of walls in the interface (see Ashford et al. 1989).

Observations of fungal nuclei in this Hartig net were confined to the larger fungal branches. Contrary to Kottke and Oberwinkler (1987), who found up to five nuclei in a fungal compartment of Picea abies—Amanita muscaria mycorrhizae, only one or two nuclei were observed with B. alleghanensis—P. tinctorius mycorrhizae, confirming our previous findings on A. rubra—A. diplophloeus (Massicotte et al. 1989a). Clearly, more observations on mitoses and migrational patterns of fungal nuclei are needed before the concept of “coenocytic organization” (Kottke and Oberwinkler 1987) can be generalized.

Until now, the general feature used to confirm ectomycorrhizal associations, has been the presence of a Hartig net (Harley and Smith 1983). The modification of fungal morphology as the hyphae form the Hartig net appears to be a constant feature, since it has been observed in numerous associations with basidiomycetes (see Blasius et al. 1986; Kottke and Oberwinkler 1986b; Melville et al. 1987a), ascomycetes (Miller and Miller 1984; Wilcox and Wang 1987b; H. B. Massicotte, unpublished data), and zygomycetes (Walker 1985). Recently, Jacobs et al. (1989), using SEM, described marked changes in morphology of hyphae on the root surface prior to mantle formation. This morphogenetic change, as well as the change in Hartig net hyphae, may be a fairly reliable compatibility criterion. These changes are likely to be accompanied by qualitative and quantitative changes in gene expression and protein synthesis.

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