

Structural characteristics of root–fungus associations in two mycoheterotrophic species, *Allotropia virgata* and *Pleuricospora fimbriolata* (Monotropoideae), from southwest Oregon, USA

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Abstract All members of the Monotropoideae (Ericaceae), including the species, *Allotropia virgata* and *Pleuricospora fimbriolata*, are mycoheterotrophs dependent on associated symbiotic fungi and autotrophic plants for their carbon needs. Although the fungal symbionts have been identified for *A. virgata* and *P. fimbriolata*, structural details of the fungal–root interactions are lacking. The objective of this study was, therefore, to determine the structural features of these plant root–fungus associations. Root systems of these two species did not develop dense clusters of mycorrhizal roots typical of some monotropoid species, but rather, the underground system was composed of elongated rhizomes with first- and second-order mycorrhizal adventitious roots. Both species developed mantle features typical of monotropoid mycorrhizas, although for *A. virgata*, mantle development was intermittent along the length of each root. Hartig net hyphae were restricted to the host epidermal cell layer, and fungal pegs formed either along the tangential walls (*P. fimbriolata*) or radial walls (*A. virgata*)

of epidermal cells. Plant-derived wall ingrowths were associated with each fungal peg, and these resembled transfer cells found in other systems. Although the diffuse nature of the roots of these two plants differs from some members in the Monotropoideae, the structural features place them along with other members of the Monotropoideae in the “monotropoid” category of mycorrhizas.

Keywords Fungal pegs · Hartig net · Mantle · Microscopy · Monotropoid mycorrhiza

Introduction

The subfamily, Monotropoideae (Ericaceae), consists of ten genera, all of which are achlorophyllous mycoheterotrophs (Leake 1994). The fungal symbionts vary according to plant species (Bidartondo 2005), with some hosts showing considerable specificity toward particular fungi (Bidartondo and Bruns 2001, 2002; Taylor et al. 2002). The fungal genera identified as symbionts with members of the Monotropoideae form ectomycorrhizas on autotrophic tree and shrub species (Leake 2004, 2005; Peterson et al. 2004). The unique structural characteristics of the plant–fungus interaction have been used to describe the mycorrhiza category as “monotropoid” (Duddridge and Read 1982; Peterson et al. 2004; Smith and Read 2008). The species studied to date share consistent features: a mantle, a Hartig net confined to the epidermis, “fungal pegs”, hyphae that enter epidermal cells but are then enclosed in finger-like wall projections (Lutz and Sjolund 1973; Duddridge and Read 1982; Massicotte et al. 2005, 2007). Although there is some reference in the literature to fungal pegs developing in

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outer cortical cells (Duddridge and Read 1982), it is clear now that only epidermal cells are invaded by fungal hyphae (Peterson and Massicotte 2004; Bidartondo 2005). Detailed structural studies have only involved *Monotropa hypopitys* L. (Duddridge and Read 1982; Dexheimer and Gérard 1993), *Monotropa uniflora* L. (Lutz and Sjolund 1973; Massicotte et al. 2005), *Pterospora andromedea* Nutt. (Robertson and Robertson 1982; Massicotte et al. 2005), *Sarcodes sanguinea* Torrey (Robertson and Robertson 1982), and to a lesser extent *Pityopus californicus* (Eastw.) H.F. Copel. (Massicotte et al. 2007), and *Monotropastrum humile* (D. Don) H. Hara (Matsuda and Yamada 2003). Kuga-Uetake et al. (2004) were the first to document the interaction between the fungal peg and the plant epidermal cell cytoskeleton in *M. uniflora*. Historically, there has been a lack of structural information concerning plant–fungus interactions for many of the species in the Monotropoideae, as noted by Leake (1994).

The monotypic genera *Allotropa* (*Allotropa virgata* Torrey and A. Gray, commonly named sugar stick or candy stripe) and *Pleuricospora* (*Pleuricospora fimbriolata* A. Gray, fringed pinesap) are found in mixed and evergreen forests (Wallace 1975) in western North America. Both genera are distributed from British Columbia to California; in addition, *Allotropa* has also been identified in Nevada, Idaho, and Montana (eFloras 2009). *A. virgata* was listed as a “sensitive” species, which has led to some recommendations concerning its management (Wogen and Lippert 1998). Based on previous molecular data and the specificity exhibited by the fungal symbionts shown to be associated with *A. virgata* (*Tricholoma magnivelare* (Peck) Redhead), and *P. fimbriolata* (*Gautieria monticola* Harkn.; Bidartondo and Bruns 2001, 2002; Bidartondo 2005), it is assumed that the same fungal species may be present as fungal symbionts in the plants sampled in this study. No structural studies with *G. monticola* and any plant species were found in the literature. However, Lefevre (2002) provides descriptions of several *T. magnivelare* mycorrhiza associations, including those with *A. virgata*, *Lithocarpus densiflorus*, and *Arbutus menziesii*.

The diffuse roots of both *A. virgata* and *P. fimbriolata* are described as being perennial, slender, and brittle (Wallace 1975) in contrast to roots of some monotropoid species such as *P. andromedea* and *M. uniflora*, which form dense clusters of large robust root tips (Massicotte et al. 2005). Copeland (1938) described the morphology of the root system of *A. virgata* noting that the roots ranged in diameter from 1 to 4 mm. His description of the anatomy from microtome sections was based on a single root tip; no mycorrhizal association was detected on that tip.

The objective of this study was to determine the features and cellular details of *A. virgata* and *P. fimbriolata* mycorrhizas using various forms of microscopy in order to further our understanding of the structural interactions

between members of the Monotropoideae and their associated symbionts.

Materials and methods

Plant material

Root samples from three to five individuals each of *A. virgata* and *P. fimbriolata* were collected on May 2007 and June 2008 from plants growing in a mixed conifer/hardwood forest dominated by old-growth Douglas-fir [*Pseudotsuga menziesii* (Mirbel) Franco] and a sub-canopy of *A. menziesii* Pursh. Lesser amounts of tan oak [*L. densiflorus* (Hook. & Arn.) Rehd.] and golden chinquapin [*Chrysolepis chrysophylla* (Hook.) Hjelmqv.] were also present. Sparsely scattered patches of *Gaultheria shallon* Pursh provided the primary shrub cover. The location was on a north-facing slope above Grayback Creek, about 16 km ESE of Cave Junction, OR, at an elevation of 800 m.

Light microscopy

Root samples were cleaned of soil and individual roots excised and fixed in 2.5% glutaraldehyde in 0.10 M *N*-2-hydroxy-ethylpiperazine-*N*-1-2-ethane sulfonic acid buffer pH 6.8 at room temperature for 4–8 h. For light microscopy of embedded tissue, samples were washed with buffer, dehydrated in an ascending series of ethanol to 100%, and embedded in LR White resin (Canemco, Lachine, Quebec, Canada). Sections 1–1.5 μm thick were cut with glass knives on a Porter-Blum Ultra-Microtome MT-1 (DuPont Co., Newton, CT, USA), heat-fixed onto glass slides, and stained with 0.05% toluidine blue O (Sigma-Aldrich, St. Louis, MO, USA) in 1% sodium borate. For each species, sections of at least ten separate roots obtained from several plants were examined with a Leitz Orthoplan compound microscope (Leica, Mississauga, Ontario, Canada) to which a Nikon Coolpix 4500 digital camera (Nikon, Canada, Mississauga, Ontario, Canada) was attached to capture images.

Fluorescence microscopy

Fixed roots were hand-sectioned with a two-sided razor blade and stained with berberine-aniline blue following the protocol in Brundrett et al. (1988). These were viewed with a Leitz SM-LUX epifluorescence microscope (Leica, Mississauga, Ontario, Canada) using UV and blue light (350–460 excitation wavelength). Images of these sections were captured using the same camera system as for resin-embedded sections.

Transmission electron microscopy

Thin sections (<0.1 μm) of LR White-embedded material were cut with glass knives on a Reichert microtome [model OM-U3; Reichert-Jung (Leica), Wetzlar, Germany], picked up on copper grids, stained with uranyl acetate and lead citrate, and viewed at 100 kV on a Philips CM10 (Philips Electron Optics, Eindhoven, The Netherlands) transmission electron microscope (TEM) interfaced with a digital imaging system.

Scanning electron microscopy

Samples fixed as for light microscopy were rinsed in buffer and post-fixed in 2% aqueous osmium tetroxide for 2 h at

4°C, rinsed in water and dehydrated in an ethanol series to 100% prior to critical point drying. Specimens were then mounted on aluminum stubs using two-sided sticky tape, coated with gold–palladium, and viewed with a Hitachi S-570 (Nissei-Sangyo, Tokyo, Japan) scanning electron microscope. Images were captured with a digital camera.

Results

Allotropia virgata

Emergent shoots of *A. virgata* were often found in clusters or small groups (1 in Fig. 1); shoots were erect, pink-

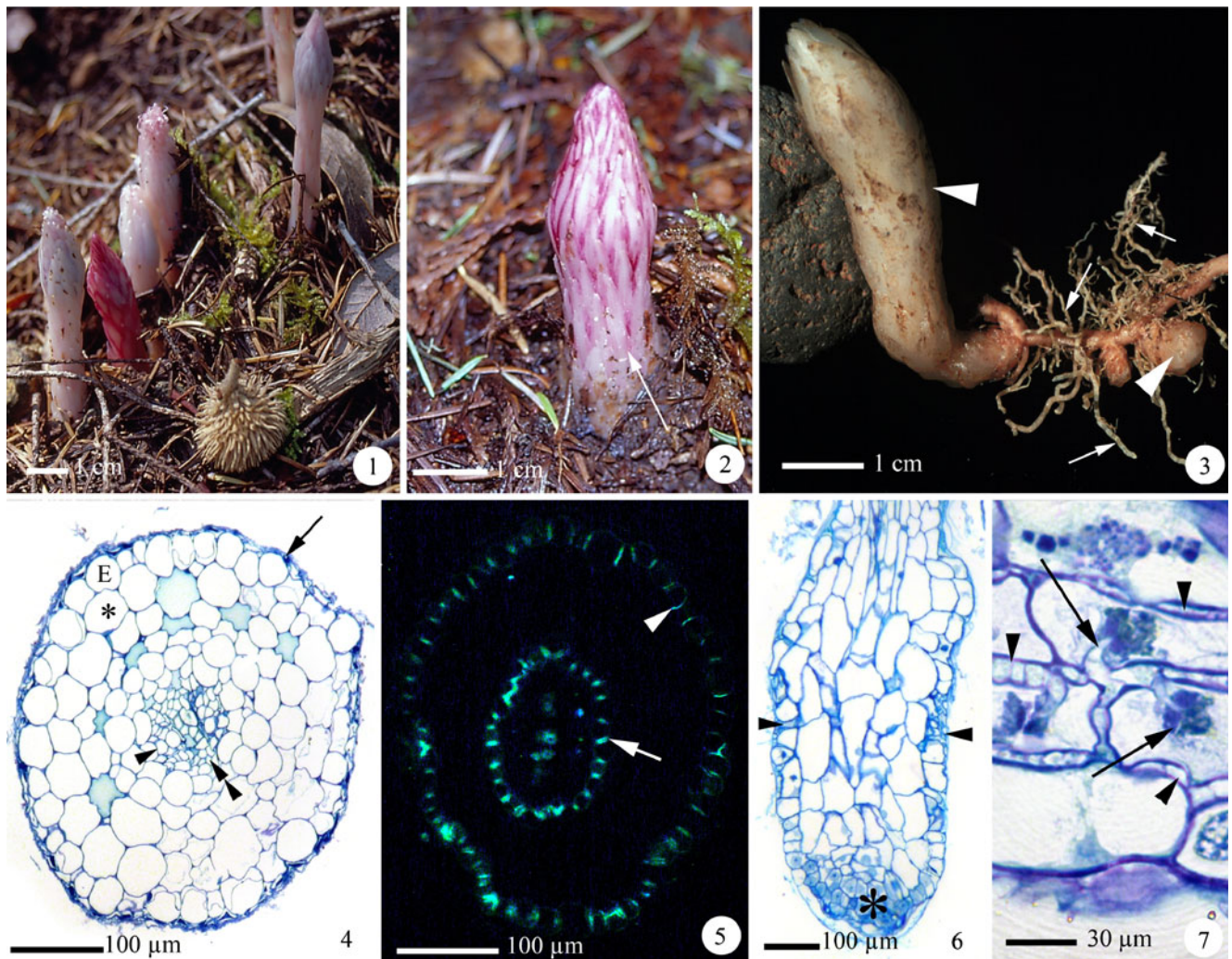


Fig. 1 Field samples of *Allotropia virgata* (1–3). Cluster of shoots at different stages of emergence, from Oregon, USA (1). Single emerging shoot (arrow) with scale-like leaves, from Vancouver Island, BC (2). Excavated rhizome with a portion of the root system (arrows) and shoot buds (arrowheads) (3). Resin-embedded (4, 6, and 7) and fixed free-hand sectioned (5) *Allotropia virgata* mycorrhizas. 4 Transverse section showing collapsed epidermis (arrow), exodermis (E), cortex (asterisk), and vascular cylinder with xylem (arrowhead)

and phloem (double arrowhead). 5 Hand section stained with berberine-aniline blue and viewed with blue-UV light showing Casparian bands in the exodermis (arrowhead) and endodermis (arrow). 6 Longitudinal section of root showing small apical meristem (asterisk) and some fungal colonization (arrowheads) in the sub-apical region. 7 Oblique longitudinal section of root showing Hartig net (arrowheads) and fungal pegs (arrows)

purple, and each consisted of a single axis with overlapping scale-like leaves (2 in Fig. 1). The typical white and pink-striped appearance, giving the species the common name of candy cane or sugar stick, is shown in Fig. 1 (2). Underground rhizomes (Luoma 1987) often had developed one or more non-pigmented shoots and many adventitious roots, some with lateral branches (3 in Fig. 1). Portions of the root system appeared phenolized and darkened. Root systems and soil released a characteristic strong odor, typical of the fungus *T. magnivelare*, as plants were being excavated and cleaned. Root systems were often intermingled with those of several other angiosperm species, most notably *A. menziesii* and *L. densiflorus*.

Sectioned roots showed that they consist of a simple anatomy: an epidermis (often collapsed at distances back

from the root apex), four to five rows of cortical cells, and a vascular cylinder consisting of a few xylem tracheary elements and phloem (4 in Fig. 1). A distinct endodermis and exodermis, both with Casparian bands, were present (5 in Fig. 1). Longitudinal sections showed sporadic colonization confined to the epidermis (6 in Fig. 1). Paradermal sections in regions of colonization showed the presence of a Hartig net also confined to the epidermis and branches of Hartig net hyphae forming fungal pegs that are initiated on the radial walls (7 in Fig. 1).

Pleuricospora fimbriolata

Emergent shoots of *P. fimbriolata* were cream-colored to slightly pink (8 and 9 in Fig. 2), and the root systems were

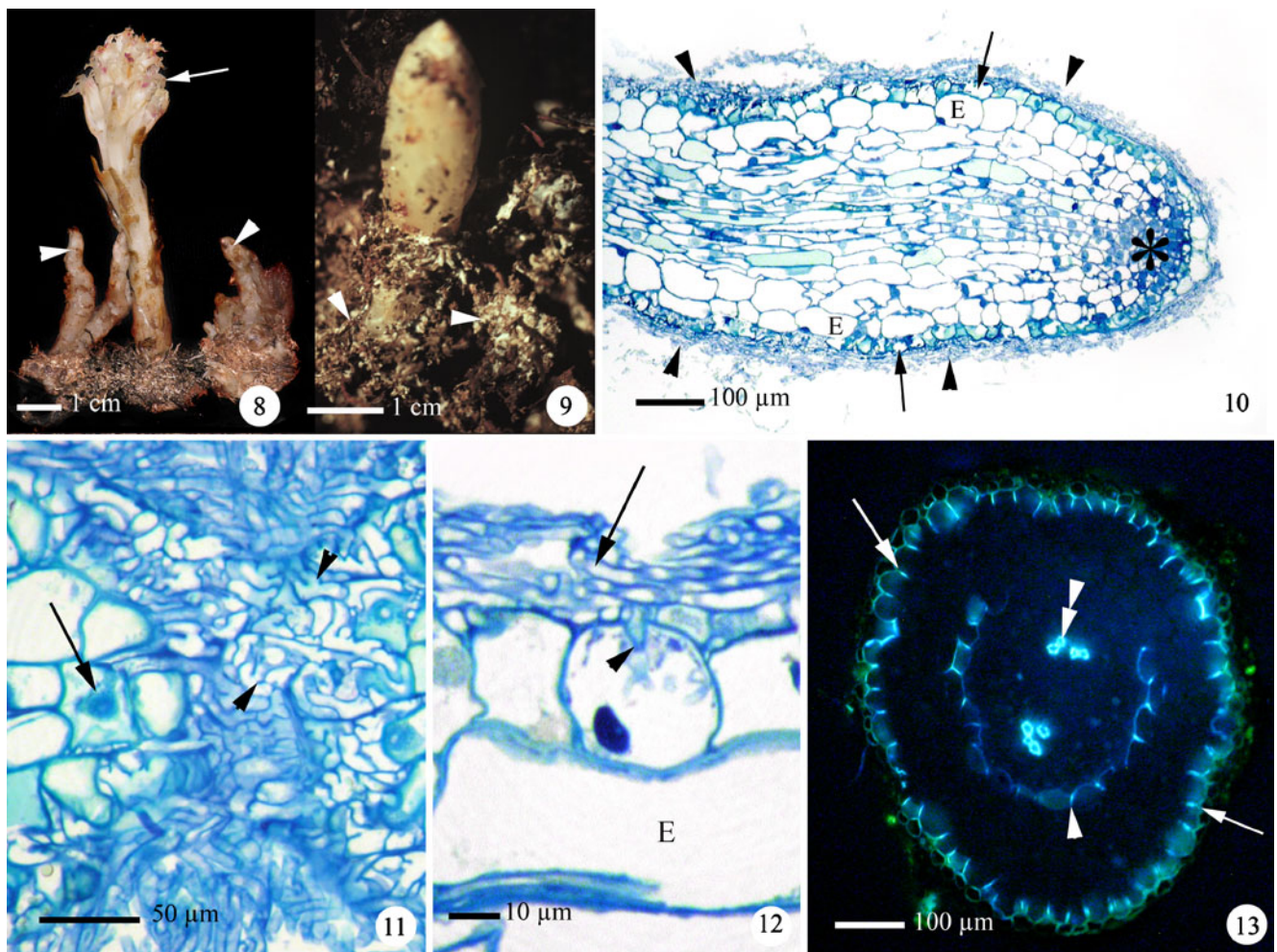


Fig. 2 8 Field samples of *Pleuricospora fimbriolata* collected in Oregon, USA. A shoot with an inflorescence (arrow) and several younger emerged shoots (arrowheads) attached to a root system. 9 An emerged shoot. Surface litter has been removed to show the fungal mat (arrowheads). Light microscopy of resin-embedded (10–12) and free-hand sectioned (13) *Pleuricospora fimbriolata* mycorrhizas. 10 Longitudinal section of a root tip showing apical meristem (asterisk), epidermis (arrows), exodermis (E), and a well developed fungal mantle (arrowheads). 11 Paradermal section

showing labyrinthine branching of inner mantle hyphae (arrowheads). A fungal peg (arrow) is evident in one epidermal cell. 12 Longitudinal section showing a fungal peg (arrowhead) that has formed along the outer tangential wall of an epidermal cell. The mantle (arrow) consists of several layers of hyphae. E exodermis. 13 Hand section stained with berberine and viewed with blue-UV light showing Casparian bands in the exodermis (arrows) and endodermis (arrowhead). Tracheary elements in the xylem (double arrowhead) also fluoresce

often associated with surrounding fungal mats (9 in Fig. 2). The overall underground systems were similar to those of *A. virgata*, consisting of rhizomes with adventitious roots (8 in Fig. 2). Most roots were extensively colonized, although more intermittent colonization was apparent on some apices. As with *A. virgata*, roots of neighboring trees and shrubs occurred intermingled with roots of *P. fimbriolata*.

Longitudinal sections showed that roots of this species also had a simple anatomy; when colonized by the fungal symbiont, roots possessed a characteristic mantle that surrounded the root apex, as well as a Hartig net restricted to the epidermis (10 in Fig. 2). The mantle was multilayered, with the inner layer showing a labyrinthine branching pattern (11 in Fig. 2) and compact arrangement of hyphae (12 in Fig. 2). Fungal pegs, initiated by inner mantle hyphae adjacent to the outer tangential wall of epidermal cells, were present in all roots sampled (11 and 12 in Fig. 2). Suberized Casparian bands were present in the endodermis and exodermis (13 in Fig. 2), the latter possibly acting to restrict the Hartig net to the epidermis. Scanning electron microscopy showed the extent of the mantle and loose extraradical mycelium (14 in Fig. 3) as well as the presence of clamp connections on some extraradical hyphae (15 in Fig. 3). *A. virgata* mycorrhizas had similar mantle features and extraradical hyphae bearing clamp connections (not shown). Ultrastructure showed that fungal pegs were encased in plant-derived wall material that branched extensively (16 in Fig. 3); similar features were observed in *A. virgata* (not shown).

Discussion

This is the first structural study that describes in detail the interaction between the fungal symbionts and two myco-

heterotrophic species, *A. virgata* and *P. fimbriolata*. Results show that, consistent with other members of the Monotropoideae (Peterson et al. 2004; Smith and Read 2008), a mantle, epidermal Hartig net, and fungal pegs characterize these plant–fungal symbiotic associations. This combination of mycorrhizal structural features places them in the general description of “monotropoid” mycorrhizas (Duddridge and Read 1982; Peterson et al. 2004).

Roots of *A. virgata* had limited colonization compared to *P. fimbriolata* and other members of the Monotropoideae such as *M. uniflora* and *P. andromedea* (Massicotte et al. 2005). This might reflect either the nature of the habitat and time of year that roots were collected or the interaction between this plant species and its known fungal symbiont, *T. magnivelare*. Lefevre (2002) also found that *A. virgata* roots had thin, often inconspicuous, patchy white mantles and noted that Hartig net palmetti were rarely observed. Field collected *Tricholoma matsutake* (S. Ito & S. Imai) Singer (a closely related fungal species)–*Pinus densiflora* Siebold & Zucc. ectomycorrhizas showed signs of incompatibility, including limited extraradical hyphae and the deposition of phenolic compounds and necrosis (Gill et al. 2000).

Although considerable ultrastructural information is available concerning the nature of the interface between the fungal peg and epidermal cell cytoplasm in several members of the Monotropoideae (Lutz and Sjolund 1973; Duddridge and Read 1982; Robertson and Robertson 1982), the function of the fungal pegs is still in question. It has been suggested, since the formation of the plant-derived wall ingrowths resembles that of transfer cells in other systems (Gunning and Pate 1969), that the fungal pegs might be involved in nutrient mobilization from the Hartig net into epidermal cells via this mechanism (Lutz and Sjolund 1973; Duddridge and Read 1982; Robertson

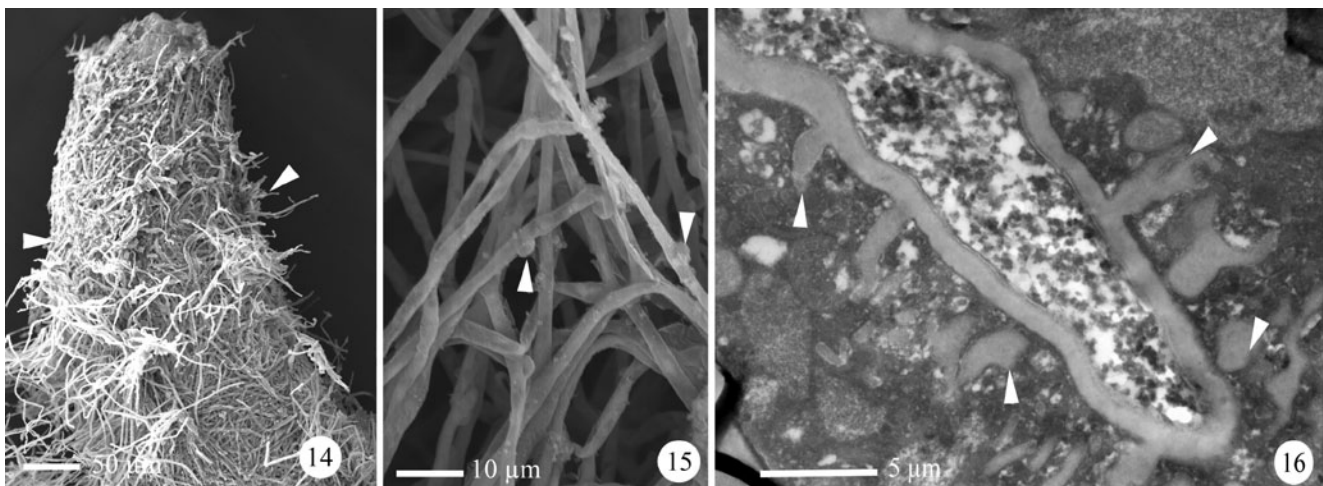


Fig. 3 Scanning electron microscopy of *Pleuricospora fimbriolata* mycorrhizas (14, 15). 14 Low magnification of a root with mantle and surface hyphae (arrowheads). 15 Higher magnification showing loose

arrangement of hyphae with clamp connections (arrowheads). 16 Transmission electron microscopy of *Pleuricospora fimbriolata* mycorrhiza showing a fungal peg with surrounding wall ingrowths (arrowheads)

and Robertson 1982). An additional means of nutrient transfer could involve the tip of the fungal peg that opens at some stage in its development (Lutz and Sjolund 1973; Robertson and Robertson 1982).

In the Monotropoideae, the fungal symbionts differ depending on the plant species, but all develop fungal pegs; this suggests that the plant genome controls the development of the nutrient transfer interface. The positioning of the fungal pegs in relation to the epidermal cell wall shows some differences. For example, *M. uniflora* (Massicotte et al. 2005), *M. hypopitys* (Duddridge and Read 1982), *M. humile* (Matsuda and Yamada 2003), and, from this study, *P. fimbriolata*, all develop fungal pegs restricted to the outer tangential epidermal cell walls. However, in *S. sanguinea* (Robertson and Robertson 1982), *P. andromedea* (Robertson and Robertson 1982; Massicotte et al. 2005) and *A. virgata*, as shown in this study, fungal pegs occur along the radial walls. This level of plant control over the colonization and developmental process supports the view that increased plant control is an important trend in mycorrhizal evolution (Brundrett 2002). Whether the orientation of pegs impacts their functioning or efficiency is unknown.

Future work with monotropoid species could further our understanding of how fungal symbioses facilitate the functioning of mycoheterotrophic plants. As well, developmental studies from seed germination to seedling establishment, using experimental approaches such as those described for other members of the Monotropoideae (Bruns and Read 2000; Leake et al. 2004), would extend our understanding of how these species establish themselves in natural habitats. To date, experiments, using seed of both *A. virgata* and *P. fimbriolata* enclosed in cloth packets buried in forest soils in habitats where these species occur, have failed to result in seed germination (Bidartondo and Bruns 2005).

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References

- Bidartondo M (2005) Tansley review. The evolutionary ecology of myco-heterotrophy. *New Phytol* 167:335–352
- Bidartondo MI, Bruns TD (2001) Extreme specificity in epiparasitic Monotropoideae (Ericaceae): widespread phylogenetic and geographical structure. *Molec Ecol* 10:2285–2295
- Bidartondo MI, Bruns TD (2002) Fine-level mycorrhizal specificity in the Monotropoideae (Ericaceae): specificity for fungal species groups. *Molec Ecol* 11:557–569
- Bidartondo MI, Bruns TD (2005) On the origins of extreme mycorrhizal specificity in the Monotropoideae (Ericaceae): performance trade-offs during seed germination and seedling development. *Molec Ecol* 14:1549–1560
- Brundrett MC (2002) Coevolution of roots and mycorrhizas of land plants. *New Phytol* 154:275–304
- Brundrett MC, Enstone DE, Peterson CA (1988) A berberine-aniline blue fluorescent staining procedure for suberin, lignin, and callose in plant tissue. *Protoplasma* 146:133–142
- Bruns TD, Read DJ (2000) *In vitro* germination of non-photosynthetic, myco-heterotrophic plants stimulated by fungi isolated from the adult plants. *New Phytol* 148:335–342
- Copeland HF (1938) The structure of *Allotropa*. *Madroño* 4:137–153
- Dexheimer J, Gérard J (1993) Application de quelques techniques cytochimiques à l'étude des interfaces des ectendomycorhizes de Monotrope (*Monotropa hypopitys* L.). *Acta Bot Gallica* 140:459–472
- Duddridge JA, Read DJ (1982) An ultrastructural analysis of the development of mycorrhizas in *Monotropa hypopitys* L. *New Phytol* 92:203–214
- eFloras (2009) <http://www.efloras.org>. Accessed 10 November 2009. Missouri Botanical Garden, St. Louis, MO & Harvard University Herbaria, Cambridge, MA
- Gill WM, Guerin-Laguette A, Lapeyrie F, Suzuki K (2000) Matsutake—morphological evidence of ectomycorrhiza formation between *Tricholoma matsutake* and host roots in a pure *Pinus densiflora* forest stand. *New Phytol* 147:381–388
- Gunning BES, Pate JS (1969) 'Transfer cells'—Plant cells with wall ingrowths specialized in relation to short distance transport of solutes—their occurrence, structure and development. *Protoplasma* 68:107–133
- Kuga-Uetake Y, Purich M, Massicotte HB, Peterson RL (2004) Host microtubules in the Hartig net region of ectomycorrhizas, ectendomycorrhizas, and monotropoid mycorrhizas. *Can J Bot* 82:938–946
- Leake JR (1994) Tansley review no 69. The biology of myco-heterotrophic ('saprophytic') plants. *New Phytol* 127:171–216
- Leake JR (2004) Myco-heterotroph/epiparasitic plant interactions with ectomycorrhizal and arbuscular mycorrhizal fungi. *Curr Opin Plant Biol* 7:422–428
- Leake JR (2005) Plants parasitic on fungi: unearthing the fungi in myco-heterotrophs and debunking the 'saprophytic' plant myth. *Mycologist* 19:113–122
- Leake JR, McKendrick SL, Bidartondo M, Read DJ (2004) Symbiotic germination and development of the myco-heterotroph *Monotropa hypopitys* in nature and its requirement for locally distributed *Tricholoma* spp. *New Phytol* 163:405–423
- Lefevre CK (2002) Host associations of *Tricholoma magnivelare*, the American matsutake. Ph.D. dissertation, Department of Forest Science, Oregon State University, Corvallis, OR
- Luoma DL (1987) Synecology of the Monotropoideae within Limpy Rock Research Natural Area, Umpqua National Forest, Oregon. MS thesis, Oregon State University, Corvallis
- Lutz RW, Sjolund RD (1973) *Monotropa uniflora*: ultrastructural details of its mycorrhizal habit. *Am J Bot* 60:339–345
- Massicotte HB, Melville LH, Peterson RL (2005) Structural features of mycorrhizal associations in two members of the Monotropoideae, *Monotropa uniflora* and *Pterospira andromedea*. *Mycorrhiza* 15:101–110
- Massicotte HB, Melville LH, Tackaberry LE, Peterson RL (2007) *Pityopus californicus*: structural characteristics of seed and seedling development in a myco-heterotrophic species. *Mycorrhiza* 17:647–653
- Matsuda Y, Yamada A (2003) Mycorrhizal morphology of *Monotropastrum humile* collected from six different forests in central Japan. *Mycologia* 95:993–997
- Peterson RL, Massicotte HB (2004) Exploring structural definitions of mycorrhizas, with emphasis on nutrient-exchange interfaces. *Can J Bot* 82:1074–1088
- Peterson RL, Massicotte HB, Melville LH (2004) Mycorrhizas: anatomy and cell biology. NRC, Ottawa

- Robertson DC, Robertson JA (1982) Ultrastructure of *Pterospora andromedea* Nuttall and *Sarcodes sanguinea* Torrey mycorrhizas. *New Phytologist* 92:539–551
- Smith SE, Read DJ (2008) Mycorrhizal symbiosis, 3rd edn. Academic, London
- Taylor DL, Bruns TD, Leake JR, Read DJ (2002) Mycorrhizal specificity and function in myco-heterotrophic plants. In: van der Heijden MGA, Sanders I (eds) Mycorrhizal ecology, ecological studies, vol. 157. Springer, Berlin, pp 375–413
- Wallace GD (1975) Studies of the Monotropoideae (Ericaceae): taxonomy and distribution. *Wasmann J Biol* 33:1–87
- Wogen NS, Lippert JD (1998) Management recommendations for *Allotropa virgata* Torrey & Gray. US Department of Interior, Bureau of Land Management, Portland