Structural characteristics of root–fungal interactions for five ericaceous species in eastern Canada

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Abstract: A combination of light microscopy (including differential interference contrast) and laser scanning confocal microscopy was used to document the colonization patterns of epidermal cells and details of intracellular hyphal complexes of five native ericaceous hosts: Vaccinium oxycoccos L. (bog cranberry), Ledum groenlandicum Oeder. (Labrador tea), Vaccinium myrtilloides L. (velvet-leaf blueberry), Kalmia angustifolia L. (sheep laurel), and Gaultheria procumbens L. (wintergreen). Colonization patterns, hyphal complex morphology, and the structure of thick-walled epidermal cells varied considerably among hosts. Multiple hyphal connections were observed between adjacent epidermal cells, indicating that one fungal entry point may result in the colonization of more than one epidermal cell. Further field observations combined with fungal isolations from field-collected plants, identification, and reinoculation studies of other species in the large Ericaceae family are required to determine the full range of structural details in ericoid mycorrhizas.

Key words: ericoid mycorrhizas, hair roots, intracellular hyphal complexes, confocal microscopy.

Introduction

The fine roots (hair roots) of many ericaceous species develop mutualistic associations with diverse soil fungi (Perotto et al. 1996; Hambleton and Currah 1997; Monreal et al. 1999; McLean et al. 1999) resulting in the formation of typical ericoid mycorrhizas (Read 1996). Colonization of hair roots involves the penetration of the thickened outer tangential wall of epidermal cells by fungal hyphae followed by the formation of complex intracellular hyphal complexes (coils) confined to epidermal cells (Duclos et al. 1983; Perotto et al. 1995). Surface hyphae may form appressorium-like structures from which narrow-diameter hyphae develop and penetrate the epidermal cell wall (Bonfante-Fasolo and Gianinazzi-Pearson 1979, 1982; Duddridge and Read 1982). The intracellular hyphal complexes that develop in epidermal cells are enclosed by host-derived perifungal membrane and interfacial matrix material (Perotto et al. 1995).
Fig. 1. Root system of *Vaccinium oxycoccos* showing a portion of the rhizome (arrow) with many minute hair roots (arrowheads) attached to adventitious roots. Scale bar = 1.0 cm. **Figs. 2–5.** Differential interference contrast images of hair roots of ericaceous species. All scale bars = 10 μm. **Fig. 2.** A portion of the fungal mantle (*) on *Vaccinium myrtillusoides*. **Fig. 3.** Branching surface hyphae on *Kalmia angustifolia*. **Fig. 4.** Paradermal view showing thick-walled epidermal cells with intracellular hyphal complexes (arrowheads) of *Gaultheria procumbens*. (This figure from Peterson et al. 2004, p. 91, reproduced with permission of NRC Research Press, © 2004 NRC Research Press.) **Fig. 5.** A heavily colonized epidermal cell of *Kalmia angustifolia* showing several points of fungal entry (arrowheads). **Fig. 6.** Surface hyphae of a dark septate endophyte (arrowheads), and another fungal species (double arrowheads) on the surface of a *Gaultheria procumbens* root. Scale bar = 10 μm. **Fig. 7.** Microsclerotia (*) of a dark septate endophyte within epidermal cells of a hair root of *Gaultheria procumbens*. Scale bar = 10 μm.

From the material studied to date, it has been generally concluded that each epidermal cell is colonized separately and that fungal hyphae do not cross from one epidermal cell to the next (Bonfante-Fasolo and Gianinazzi-Pearson 1979; Perotto et al. 1995; Perotto and Bonfante 1998). However, although not commented on, Peterson et al. (1980) illustrate a fungal hypha crossing the wall of two contiguous epidermal cells of a *Rhododendron* sp. hair root.

Hair roots in species of the Ericaceae and Epacridaceae are difficult to prepare for conventional microscopic examination owing in part to the thickened epidermal cell walls (Ashford et al. 1996; Briggs and Ashford 2001) and the fact that field-collected material is variable in age and quality. Laser scanning confocal microscopy (LSCM) of cleared and stained roots provides the opportunity to screen many samples for colonization and to study the colonization process in detail.

As background for further controlled laboratory re-synthesis experiments with known fungal endophytes, the colonization pattern in several ericaceous species collected from the field in eastern Canada was examined by LSCM and light microscopy.

**Materials and methods**

**Plant material**

Two or three specimens each of velvet-leaf blueberry (*Vaccinium myrtillusoides* L.), sheep laurel (*Kalmia angustifolia* L.), and wintergreen (*Gaultheria procumbens* L.) were excavated from Ste-Geneviève de Batiscan, approximately 100 km west of Quebec City, Quebec, on 23 September 2001 from typical mineral soils, under mixed woods of eastern white pine (*Pinus strobus* L.), red maple (*Acer rubrum* L.), sugar maple (*Acer saccharum* Marshall), paper birch (*Betula papyrifera* Marshall), and yellow birch (*Betula alleghaniensis* Britton). Two to three specimens each of *Vaccinium oxycoccos* L. (bog cranberry) and *Ledum groenlandicum* Oeder. (Labrador tea) were excavated on 3 October 2001 from a wetland situated 20 km southeast of Guelph, Ontario, from organic soils dominated by mosses, grasses, and sedges. The dominant tree species was tamarack (*Larix laricina* (Du Roi) K. Koch).

Root systems were soaked in water for 1 h before they were carefully washed free of their substrates. Only attached intact samples of hair roots were used. Using a dissecting microscope, 10–15 segments, approximately 3–4 cm in length and with numerous hair roots attached, were collected for each species. Roots were separately processed for root clearing and embedding in resin.

**Root clearing**

Root samples were either preserved in 50% ethanol or processed directly for clearing. For clearing, samples were placed in a glass Petri dish in 10% KOH in a drying oven at approximately 60 °C for up to 20 h. If roots were not cleared, as determined by mounting small samples and observing by microscopy, fresh 10% KOH solution was added and the root samples were left overnight. After this, the KOH solution was removed and roots were rinsed in distilled water for 30 min. A subsample of cleared roots was stained in a 0.05% aqueous solution of trypan blue for 1 h. An additional subsample of cleared roots was acidified in 1% HCl for 5 min and then stained for 24 h at room temperature in the dark in a solution consisting of equal parts of 1.0% aqueous acid fuchsin (w/v), 85% lactic acid, and glycerol. Stained roots were then carefully removed by tweezers, blotted in glycerol, placed on slides in 75% glycerol, and gently covered by a cover glass.

Samples stained with trypan blue were observed by differential interference contrast microscopy with a Leitz Orthoplan light microscope. Acid fuchsin-stained samples were viewed with a BIO-RAD MRC-600 scanning laser confocal microscope interfaced with a Nikon Optiphot-2 upright microscope, and equipped with a krypton/argon gas laser.

**Resin embedding**

Tissue was fixed in 2.5% glutaraldehyde in 0.07 mol/L Sorensen’s phosphate buffer (pH 6.8) overnight, dehydrated in a graded ethanol series, and embedded in LR White Resin (London Resin Company). Sections (1–1.5 μm thick) were cut with glass knives, heat-fixed to microscope slides, and stained for light microscopy with 0.05% toluidine blue O in 1% sodium borate. Five to ten roots were sectioned for each species.

**Results**

The morphology of the sampled root systems was similar for all five species. In general, plants grown in mineral substrates had larger root systems than those grown in organic substrates. Adventitious roots initiated from woody, dark-brown rhizomes branched to form dense clusters of narrow-diameter 2nd, 3rd, and 4th order hair roots (Fig. 1). Only hair roots were examined in this study.

**Light microscopy**

Differential interference contrast microscopy revealed variable colonization patterns. Some hair roots exhibited a compact fungal “mantle” (Fig. 2) whereas others showed a loose arrangement of branching hyphae on the root surface (Fig. 3). Many hair roots examined by light microscopy did...
not show obvious external colonization. In all segments examined, internal colonization was restricted to epidermal cells. The thickness of the epidermal wall and the shape of epidermal cells varied among hosts and with the location along the root axis (Figs. 4, 5). Intracellular hyphal complexes were variable in shape and complexity (Figs. 4, 5), sometimes revealing several fungal entry points (Fig. 5). Darkly pigmented, thick-walled hyphae (Fig. 6) and
Figs. 8–13. Light microscopy of sectioned ericaceous hair roots embedded in LR White Resin. All scale bars = 10 μm. Fig. 8. Transverse section of *Kalmia angustifolia* root showing copious amounts of mucilage and soil debris (arrows). Several epidermal cells are colonized (arrowhead). One layer of cortical cells (C), an endodermis (E), and a very narrow vascular cylinder (VC) are present. Fig. 9. Transverse section of *Vaccinium oxycoccos* root showing enlarged colonized epidermal cells (arrows). One layer of cortical cells (C), an endodermis (E), and a narrow vascular cylinder (VC) are present. Fig. 10. A somewhat oblique section of *Gaultheria procumbens* root showing several colonized epidermal cells, one with an obvious entry point (arrow). A loose mantle (arrowheads) is present. Epidermal cell walls are thin. Fig 11. A longitudinal section of *Ledum groenlandicum* root showing unevenly thickened epidermal cells with intracellular hyphal complexes (*). Narrow hyphae (arrowheads) occupy a region adjacent to the thickened portion of the cell wall. Fig. 12. Epidermal cells in a paradermal section of *Ledum groenlandicum* root showing evenly thickened walls, intracellular hyphal complexes (*), and narrow hyphae (arrowheads) occupying a region adjacent to the thickened portion of wall. Fig. 13. Epidermal cells in a paradermal section of *Kalmia angustifolia* root. Cell walls are evenly thickened, multilamellate and have several “gaps” (arrowheads). The intracellular hyphal complexes (*) have partially degenerated.
microsclerotia (Fig. 7) having a typical appearance of structures formed by dark septate endophytes were frequently observed in cleared hair roots.

Sections of resin-embedded roots of *K. angustifolia* and *V. oxyccocos* illustrate the simple anatomy of hair roots in the Ericaceae. A layer of enlarged, thick-walled epidermal cells encloses a single layer of cortical cells, an endodermis, and a very small vascular cylinder (Figs. 8, 9). Frequently, the root surface was covered with mucilage (Fig. 8) and usually a thin mantle was present (Figs. 9, 10). Several uncolonized epidermal cells were typically seen in an area of cells examined at any point along the root axis (Fig. 8). Hyphae that had penetrated the outer tangential wall of epidermal cells were occasionally observed in sectioned material (Fig. 10). Variation in the pattern of epidermal cell wall thickening was evident. For example, hair roots of *G. procumbens* (Fig. 10) had epidermal cells with thin walls whereas hair roots of *L. groenlandicum* had epidermal cells with thickened outer tangential walls (Fig. 11). Root epidermal cells of *K. angustifolia* had rather evenly thickened walls (Fig. 13). Some thickened epidermal cell walls had “gaps” (Figs. 12, 13) surrounding hyphae growing within them, possibly the result of enzymatic activity by the fungal hyphae. In some species, the epidermal cell wall appeared to be layered, with fungal hyphae embedded in the innermost layer (Fig. 11, 12) that was always separated from the intracellular hyphal complex by a thin, dense layer.

**Laser scanning confocal microscopy**

LSCM was particularly useful to show the variation in surface colonization and the differences in colonization patterns of epidermal cells (Figs. 14–20). Although surface hyphae were often difficult to detect with light microscopy, they were very evident with LSCM (Figs. 14, 15, 18). These hyphae generally had a narrower diameter than the intracellular hyphal complexes (Fig. 18). Two main patterns of epidermal cell colonization were observed: loosely arranged intracellular hyphal coils (Figs. 14–18) and compact intracellular hyphal coils occupying most of the host cell volume (Figs. 19, 20). In some epidermal cells, a fungal hypha appeared to develop in the peripheral region of the epidermal cell prior to the formation of the intracellular coil (Fig. 18). Hyphal connections between contiguous epidermal cells were frequently observed in roots with loosely arranged intracellular coils (Figs. 14, 16). Because of their very narrow diameter, these could be demonstrated more convincingly by inverting the contrast of collected images (Figs. 15, 17). Hyphal connections between contiguous epidermal cells were not as prevalent when compact hyphal coils were present (Fig. 19), but again by inverting the contrast, narrow connecting hyphae could be demonstrated (Fig. 20).

**Discussion**

The order Ericales consists of five families with over 9400 species, many of which form ericoid mycorrhizas with a diverse assemblage of fungal endophytes. In spite of this, structural aspects of mycorrhizas have been studied for few species. In the family Ericaceae, the majority of structural studies have involved *Calluna vulgaris* (Bonfante-Fasolo and Gianinazzi-Pearson 1979, 1982; Bonfante-Fasolo et al. 1987; Perotto et al. 1995, 1996), *Vaccinium* spp. (Bonfante-Fasolo et al. 1981; Dalpé 1986), *Erica carnea* (Duclos et al. 1983), and *Rhododendron* spp. (Peterson et al. 1980; Duddridge and Read 1982; Douglas et al. 1989). Structure of hair roots from field-collected material has not been reported for any of the species included in the present study although ultrastructural details of *G. procumbens* epidermal cells colonized in vitro with *Peziza ericae* = *Hymenoscyphus ericae* = *Rhizoscyphus ericae* (Zhang and Zhuang 2004) have been published (Bonfante-Fasolo et al. 1984).

Two types of root–fungal interactions were observed in epidermal cells of hair roots of the species studied. The most common type had an initial colonization of the periphery of epidermal cells, often in very close proximity to the cell wall, followed by branching and coiling of the hypha to form a loosely organized intracellular hyphal complex. A recent account by Villarreal-Ruiz et al. (2004), showed that a fungus of the *H. ericae* aggregate formed ectomycorrhizas with *Pinus sylvestris* and hyphal coils (complexes) in epidermal cells of *Vaccinium myrtillus* similar to the most common type observed in this study. The second type observed here had dense intracellular hyphal coils occupying most of the epidermal cell volume. This is typical of the colonization pattern shown by hair roots of *Vaccinium vitis-idaea* colonized by *H. ericae* (Vrålstad et al. 2002). Loose versus compact colonization patterns occurring in epidermal cells may reflect different fungal endophytes; however, this was not established.

Observations with LSCM clearly showed that hyphae of very narrow diameter often connected intracellular hyphal complexes between contiguous epidermal cells (see also Peterson et al. 2004). This observation is contrary to the conclusion reached previously that each epidermal cell in ericoid mycorrhizas is colonized by a separate fungal entry and that there is no lateral spread of the fungus (Bonfante-Fasolo et al. 1984; Perotto and Bonfante 1998). Differences in results may be due to the fact that these connecting hyphae are of very narrow diameter, and therefore could easily be missed by conventional light microscopy and transmission electron microscopy (TEM). Additionally, it is possible that our systems may be colonized by fungi that are able to produce different fungal structures, such as intercellular connections. Interestingly, Peterson et al. (1980) also illustrated, using TEM, a fungal hypha crossing adjacent epidermal cell walls in a *Rhododendron* sp. hair root. To further explore these findings, fungal isolation from natural systems, taxonomic assessment, and reinoculation could be coupled with LSCM and TEM to determine if these lateral hyphal morphologies are a common feature in ericoid systems.

Dark septate endophytes were also ubiquitous in the five ericaceous species studied, regardless of whether the habitat consisted of a rather dry forest soil or a moist acidic soil. This is expected, since dark septate endophytes are widespread among angiosperms (Jumpponen and Trappe 1998) and have been isolated from ericoid hair roots in a variety of habitats (Perotto et al. 1996; Hambleton and Currah 1997; Vrålstad et al. 2002). In spite of the frequent occurrence of
dark septate endophytes, their function(s) in natural ecosystems has not been determined.

The structure and chemical composition of thickened epidermal cell walls in hair roots of species of the Ericaceae (Peterson et al. 1980; Peretto et al. 1990) and Epacridaceae (Ashford et al. 1996; Briggs and Ashford 2001; Cairney and Ashford 2002) are known to be complex and may play a role in the establishment of a mutualistic association in these species (Cairney and Ashford 2002). In the present study, the occurrence of fungal hyphae both within the thickened, dense portion of epidermal cell walls and within an inner, lightly stained layer of cell walls was often observed. The

Figs. 14–20. Laser scanning confocal microscopy images of ericaceous plant roots. All scale bars = 10 µm. Fig. 14. *Vaccinium oxycoccos* root with some surface hyphae (arrow) and showing narrow diameter hyphae (arrowheads) connecting intracellular hyphal complexes between adjacent epidermal cells. (This figure from Peterson et al. 2004, p. 93, reproduced with permission of NRC Research Press, © 2004 NRC Research Press.) Fig. 15. Reverse image of Fig. 14 showing the narrow diameter fungal hyphae (arrowheads) connecting intracellular hyphal complexes of adjacent epidermal cells. Fig. 16. *Vaccinium oxycoccos* root showing connections between hyphal complexes of adjacent epidermal cells. Septa can be seen on many hyphae. (This figure from Peterson et al. 2004, p. 93, reproduced with permission of NRC Research Press, © 2004 NRC Research Press.) Fig. 17. Reverse image of Fig. 16 showing the very small diameter hyphae linking the intracellular hyphal complexes between epidermal cells. Fig. 18. *Ledum groenlandicum* root showing fungal colonization of epidermal cells with hyphae (arrows) emanating from them. Distinct peripheral hyphae (arrowheads) and hyphal complexes (*) are present in most cells. Fig. 19. *Gaultheria procumbens* root showing dense intracellular hyphal complexes within epidermal cells. Linkages between cells are not obvious. Fig. 20. A reverse image of a portion of *Gaultheria procumbens* root showing very narrow hyphae (arrowheads) connecting adjacent intracellular hyphal complexes.
former may indicate the entry of saprophytic fungi into this apoplastic space as epidermal cells senesce, and the subsequent utilization of wall materials for their growth. The occurrence of narrow-diameter fungal hyphae in an inner layer of cell wall was observed in cells that appeared to contain viable intracellular hyphal complexes. It is unclear if these hyphae are involved in forming the hyphal complexes or if they represent a separate fungal endophyte. Molecular approaches have shown that several fungal species can be present in the same root system of C. vulgaris collected from the field (Perotto et al. 1996). Berch et al. (2002) showed that at least five putative species and two polyphyletic assemblages of fungi produce ericoid mycorrhizas with several ericoid and epacrid hosts, including G. shal- lon. Most of these fungi were ascomycetes, but isolates closely related to the basidiomycete, Sebacina vermifera, were also isolated from roots of G. shal- lon. Although we did not determine the fungal species in the roots of the ericoid species examined, it is reasonable to suggest that roots of the five host species may harbour a diverse assemblage of fungi.

The present study demonstrates the excellent resolving power of confocal microscopy, combined with light microscopy, to document the structure of ericoid fungal colonization in different species originating from a range of natural habitats. In addition to the presence of ericoid mycorrhizas and variations in fungal colonization patterns, lateral cell to cell fungal connections were repeatedly observed in all systems studied. These are believed to be formed by “true” ericoid fungi; however, we cannot exclude the possibility that other endophytic fungi may be involved. LSCM and TEM combined with syntheses approaches mentioned previously, might provide greater resolution on root–fungal morphologies in ericaceous plants.

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References


