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Structural features of mycorrhizal associations in two members of the Monotropoideae, *Monotropa uniflora* and *Pterospora andromedea*

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Abstract Species in the subfamily Monotropoideae (family Ericaceae) are achlorophyllous and myco-heterotrophic. They have become highly specialized in that each plant species is associated with a limited number of fungal species which in turn are linked to autotrophic plants. This study provides an updated and comprehensive examination of the anatomical features of two species that have recently received attention with respect to their host-fungal specificity. Root systems of *Monotropa uniflora* and *Pterospora andromedea* collected from the field were characterized by light microscopy and scanning electron microscopy. All roots of both species were associated with fungi, each root having a well-developed mantle, paraepidermal Hartig net, and intracellular "fungal pegs" within epidermal cells. The mantle of *M. uniflora* was multi-layered and numerous outer mantle hyphae developed into cystidia of two distinct morphologies. Large calcium oxalate crystals were present, primarily on the mantle surface. The outer mantle of *P. andromedea* was more loosely organized, lacked cystidia, and had smaller plate-like as well as cylindrical crystals on the surface and between outer mantle hyphae. Fungal pegs in *M. uniflora* originated from inner mantle hyphae that penetrated the outer tangential wall of epidermal cells; in *P. andromedea*, these structures were initiated either from inner mantle hyphae or Hartig net hyphae and penetrated radial walls of epidermal cells. With respect to function, fungal pegs occurred frequently in both host species and, although presumed to be the sites of active nutrient exchange, no direct evidence exists to support this. Differences between these two monotropoid hosts, resulting from the mycorrhizal fungi with which each associates, are discussed.

Keywords *Monotropa* · *Pterospora* · Anatomy · Scanning electron microscopy · Calcium oxalate

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Introduction

All genera in the subfamily Monotropoideae, family Ericaceae, are myco-heterotrophic (Leake 1994) and depend on a close association with specific fungal symbionts to gain carbon from neighbouring autotrophic plants (Cullings et al. 1996; Bidartondo and Bruns 2001). The unique structural characteristics of the mycorrhizal association has placed these in their own category, monotropoid mycorrhiza (Smith and Read 1997). Although monotropoid mycorrhizas share two features, a mantle and an epidermal Hartig net, with angiosperm ectomycorrhizas, the development of an unique feature, a "fungal peg" within epidermal cells, separates the two. Structurally, monotropoid mycorrhizas are also distinct from ericoid and arbutoid mycorrhizas in that these mycorrhiza categories, both in the Ericales, develop intracellular hyphal complexes.

Monotropa uniflora L. is an understorey plant of north temperate forests and it is known to associate with fungal species in the family Russulaceae (Bidartondo and Bruns 2001; Young et al. 2002). Some aspects of the structure of mycorrhizas in this species have been published (Henderson 1919; Campbell 1971; Lutz and Sjolund 1973; Snetselaar and Whitney 1990). All reported the presence of a mantle, Hartig net and the invasion of epidermal cells by fungal hyphae. Lutz and Sjolund (1973) provided detailed information on the ultrastructure of the fungal peg whereas Snetselaar and Whitney (1990) concentrated on the presence of fungal calcium oxalate crystals.

Pterospora andromedea Nutt., a monotypic member of the Monotropoideae, is distributed widely in western North America but also extends into eastern North

America; this plant species appears to form mycorrhizas with species groups in the fungal genus *Rhizopogon,* section *Amylopogon* (Bidartondo and Bruns 2001, 2002).

There is limited structural work published on mycorrhizas of *P. andromedea*, the most detailed being that of Robertson and Robertson (1982) who emphasized the ultrastructure of the fungal peg.

The objectives of this study were to characterize the surface features of mycorrhizas of *M. uniflora* and *P. andromedea* using scanning electron microscopy (SEM) and to determine the structure of the mantle, Hartig net and fungal peg by light microscopy. Anatomical differences occurring between these two monotropoid hosts are explored. A subsequent paper will report the interaction between the fungal peg and host cytoskeleton in *M. uniflora*.

Materials and methods

Plant material

M. uniflora and *P. andromedea* were collected from several sites in a sub-boreal forest ecosystem in central British Columbia, Canada. Details of the sites can be found in Young et al. (2002). For each species, root clusters were excavated with the surrounding soil and stored at 4°C until they were processed for microscopy.

Microscopy

Root clusters 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 sulphonic acid buffer, pH 6.8 at room temperature for 24 h. For light microscopy, samples were washed with buffer, dehydrated in an ascending series of alcohol to 100% and embedded in LR white resin (London Resin, Basingstoke, UK). Sections (1.0–1.5 μm) were stained with 0.05% toluidine blue in 1.0% sodium borate. Some sections were viewed with crossed polars to reveal crystalline structures.

For SEM, samples were rinsed in buffer, post-fixed in 2% aqueous osmium tetroxide for 2 h at 4°C, rinsed with buffer, dehydrated in an ethanol series, critical point dried, mounted on aluminium stubs, coated with gold-palladium and observed with a JEOL JSM-35C scanning electron microscope.

Results

Morphology

Clusters of roots are a characteristic feature of *M. uniflora* (Fig. 1), and the mantle surface of individual roots of *M. uniflora* has numerous cystidia (Fig. 2). Clusters of roots are also a characteristic feature of *P. andromedea* (Fig. 3), but the mantle surface of individual roots of *P. andromedea* contrast with those of *M. uniflora* as they are mostly compact and smooth (Fig. 4). The overall tawny colour of *M. uniflora* roots and the white to mauve or blue-tinged colour of *P. andromedea* roots are due to the presence of a fungal mantle (Figs. 2 and 4, respectively).

Mantle surface

SEM revealed that lateral roots were initiated close to the root apex in *M. uniflora* and that each lateral was covered with a well-developed fungal mantle (Fig. 5). In addition, the complex nature of the mantle surface was evident (Figs. 6, 7, 8, 9, 10, 11). Numerous cystidia were visible at low magnification (Figs. 6, 7) and at higher magnification two types of cystidia were apparent (Figs. 8, 9). Sectioned roots showed the structure of these cystidia more clearly (Fig. 9). One type was somewhat fusiform or flask-shaped, often with an apical knob, while the other was elongated with a rough crystalline surface (Fig. 9). Large calcium oxalate crystals were present on the mantle surface (Figs. 8, 10, 11).

The irregular branching pattern of *P. andromedea* roots (Fig. 12) and the heavily colonized nature of the surface of these roots was also evident with SEM (Figs. 13, 15, 16), and rhizomorphs (Fig. 14) were present in the root samples and were often seen emanating from the mycorrhizas. Cystidia were absent but the emanating outer hyphae of the mantle were encrusted with material in which numerous small crystals were embedded (Figs. 15, 16).

Anatomy

M. uniflora

Longitudinal sections of *Monotropa* roots showed that the apical meristem was covered by large, vacuolated root cap cells which in turn were enclosed by mantle hyphae (Figs. 17, 18). The mantle was thinner at the root apex than further back (Fig. 17). At higher magnification, the layered nature of the mantle and the cystidia in the outer mantle were evident (Figs. 19, 20). Hyphae penetrated between epidermal cells to form a paraepidermal Hartig net (Fig. 19). Fungal pegs developed from inner mantle hyphae and penetrated the outer tangential wall of epidermal cells (Fig. 19). In the outer mantle, bacteria were frequently present in the interhyphal spaces which appeared to contain a mucilaginous substance (Fig. 20). The complex branching pattern of outer mantle hyphae (Fig. 21) and inner mantle hyphae (Fig. 22) was evident in paradermal sections of roots. These latter sections also showed the top view of the fungal pegs (Fig. 22) and Hartig net hyphae surrounding epidermal cells (Fig. 23). Examination of the same sections by light microscopy (Fig. 24) and with crossed polars (Fig. 25) showed the presence of calcium oxalate crystals on the surface and within the fungal mantle. Crystals also coat elongated cystidia.

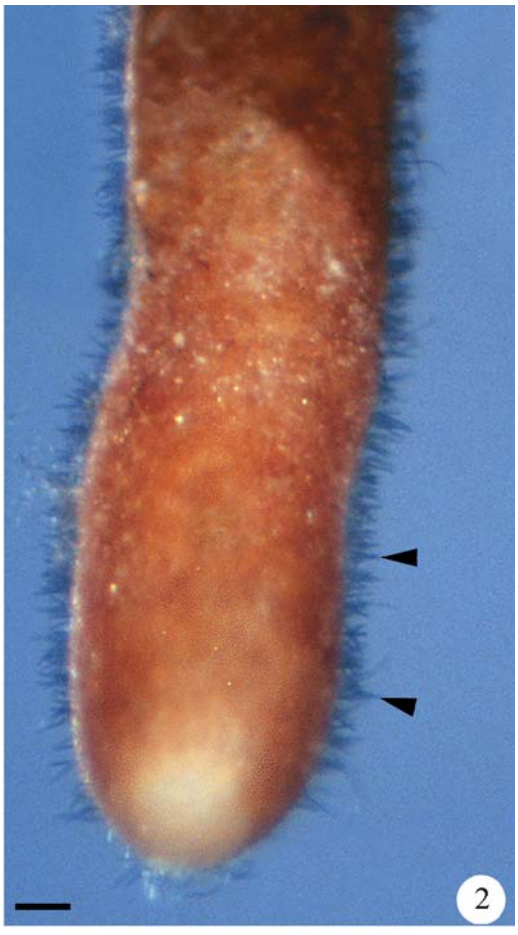


Fig. 1 Young shoots (*) and root cluster (‣) of *Monotropa uniflora*

Fig. 2 Root tip of *M. uniflora* covered with a fungal mantle. Numerous cystidia (‣) are evident. *Scale bar* =100 μm

Fig. 3 Coralloid root mass of *Pterospora andromedea* showing numerous root tips (‣). *Scale bar* =5 mm

Fig. 4 Branched root of *P. andromedea.* The main root apex and lateral roots are covered by a fungal mantle (*). *Scale bar* =100 μm

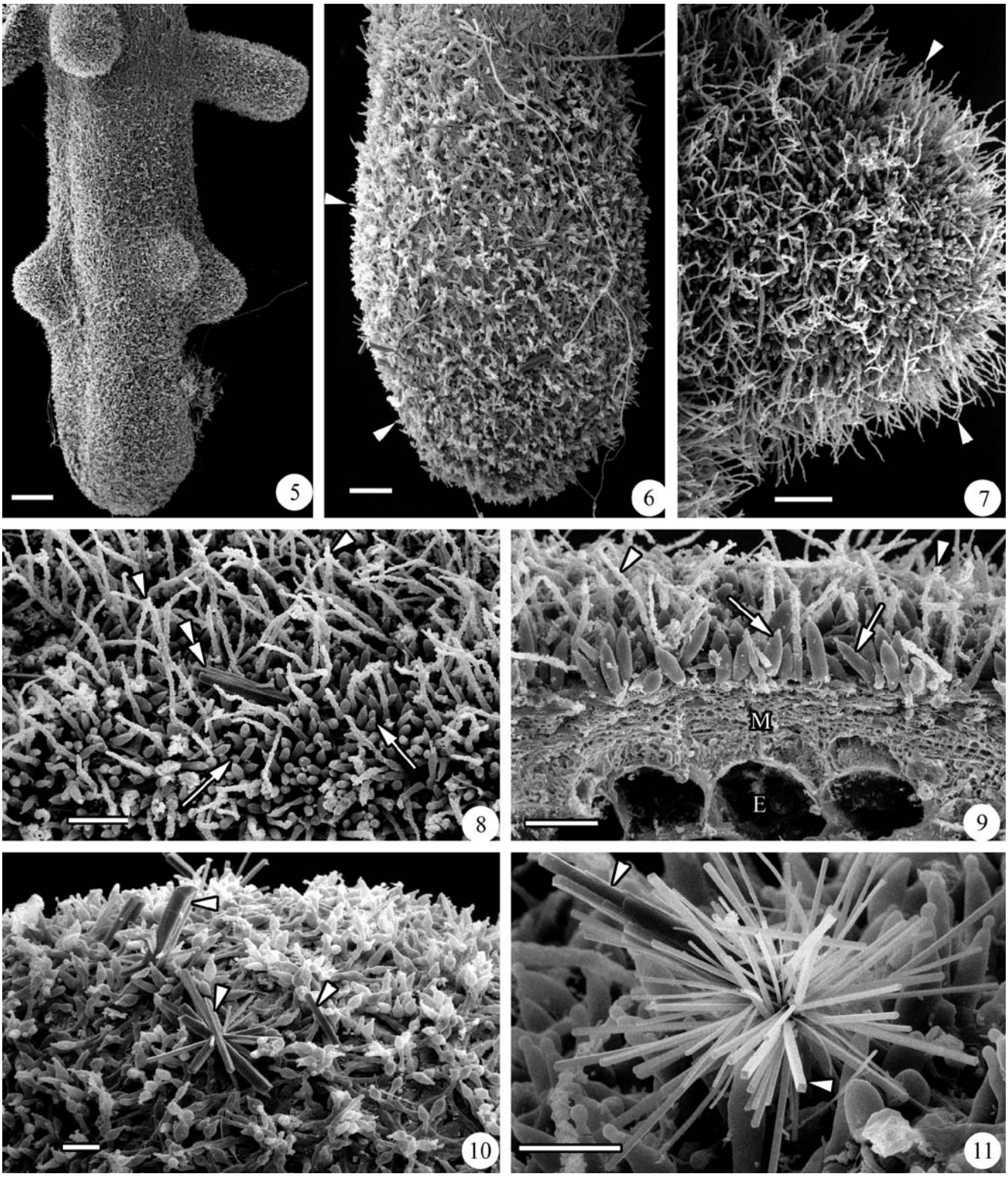


Fig. 5 Scanning electron microscopy (SEM) of *M. uniflora* mycorrhizas. Main root and laterals covered with a fungal mantle. *Scale bar* =200 μm

Fig. 6 SEM of *M. uniflora* mycorrhizas. Mantle surface with numerous cystidia (▹). *Scale bar* =100 μm

Fig. 7 SEM of *M. uniflora* mycorrhizas. Mantle surface with numerous cystidia (▹). *Scale bar* =100 μm

Fig. 8 SEM of *M. uniflora* mycorrhizas. Mantle surface showing elongated cystidia (▹), shorter fusiform/flask-shaped/ampoule-shaped cystidia (⇨), and a large calcium oxalate crystal (▹▹). *Scale bar* =50 μm

Fig. 9 SEM of *M. uniflora* mycorrhizas. Sectioned root showing elongated/cylindrical cystidia (▹), flask-shaped cystidia (⇨), mantle (M) and epidermal cells (*E*). *Scale bar* =50 μm

Fig. 10 SEM of *M. uniflora* mycorrhizas. Calcium oxalate crystals (▹) on the mantle surface. *Scale bar* =25 μm

Fig. 11 SEM of *M. uniflora* mycorrhizas. Calcium oxalate crystals (▹) on the mantle surface. *Scale bar* =25 μm

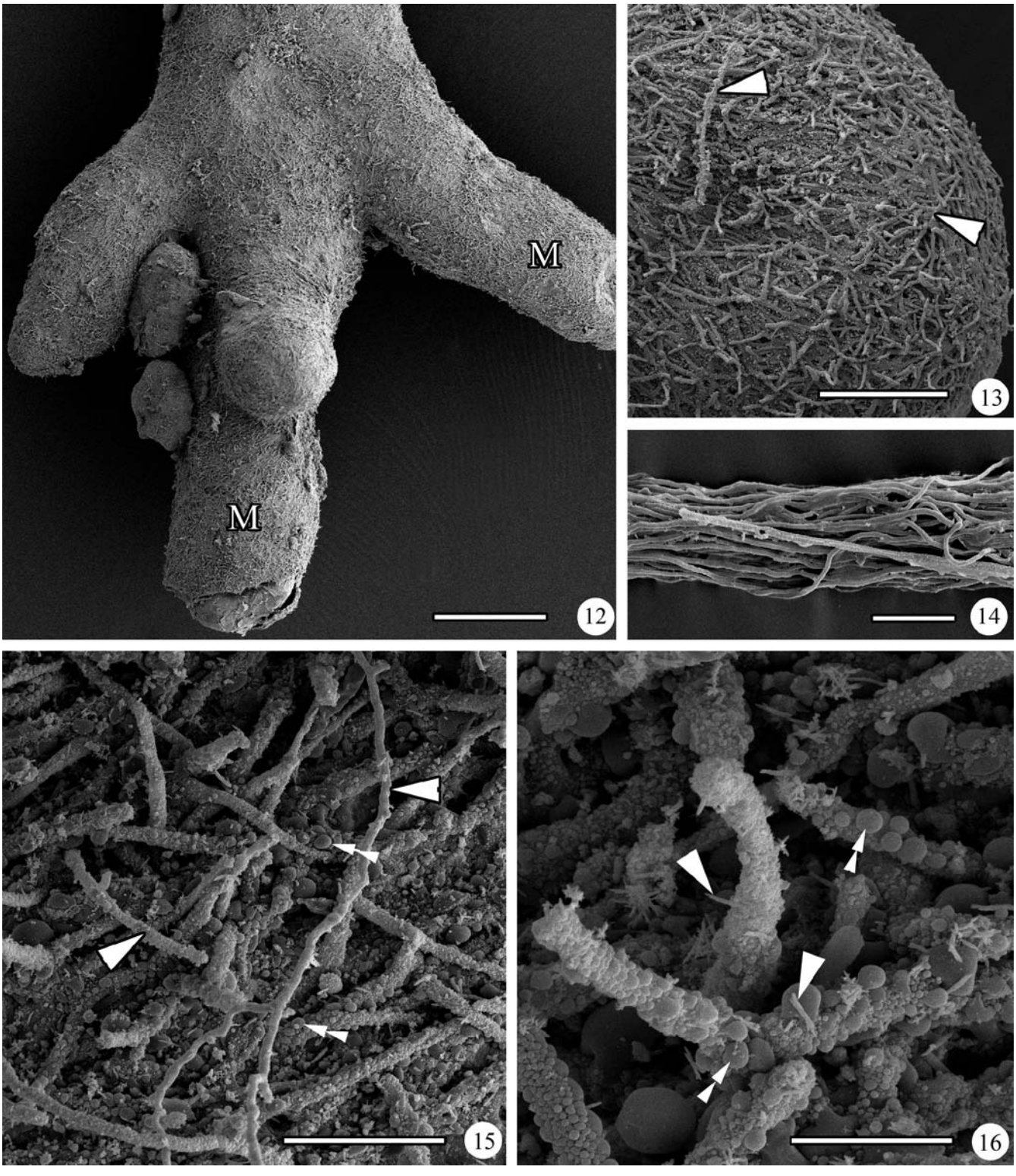


Fig. 12 SEM of *P. andromedea* mycorrhizas. Branched root system showing the compact mantle (M) covering all root tips. *Scale bar* =0.5 mm

Fig. 13 SEM of *P. andromedea* mycorrhizas. Hyphae (▹) on the mantle surface. *Scale bar* =100 μm

Fig. 14 SEM of *P. andromedea* mycorrhizas. A rhizomorph of the fungus associated with mycorrhizas. *Scale bar* =30 μm

Fig. 15 SEM of *P. andromedea* mycorrhizas. Hyphae (▹) on the mantle surface. Emanating hyphae are encrusted with crystalline material (▹▹). *Scale bar* =10 μm

Fig. 16 SEM of *P. andromedea* mycorrhizas. Plate-like (▹▹) and small cylindrical crystals (▹) on outer mantle hyphae. *Scale bar* =10 μm

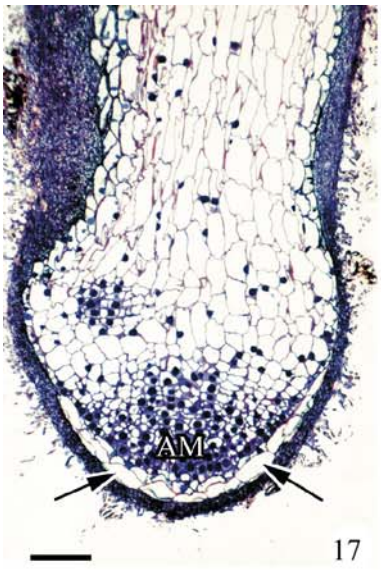


Fig. 17 Light microscopy of *M. uniflora* mycorrhizas. Longitudinal section of a root tip showing the apical meristem (*AM*), enlarged root cap cells (➞). The root is surrounded by a fungal mantle which is thinner around the root apex. *Scale bar* =100 μm

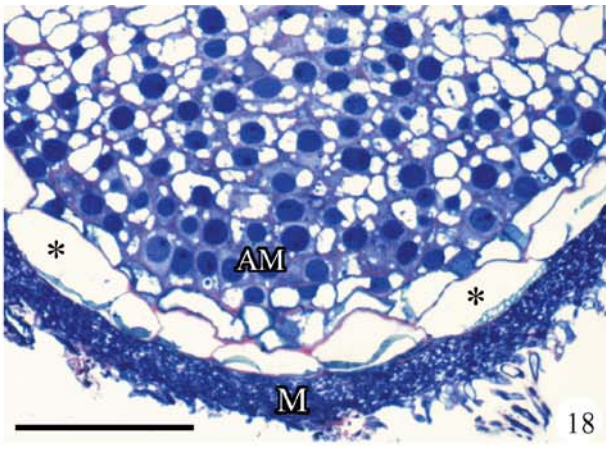


Fig. 18 Light microscopy of *M. uniflora* mycorrhizas. Enlargement of root in Fig. 17 showing the AM, enlarged, vacuolated root cap cells (✻) and the fungal mantle (M). *Scale bar* =100 μm. For abbreviations, see Figs. 2 and 17

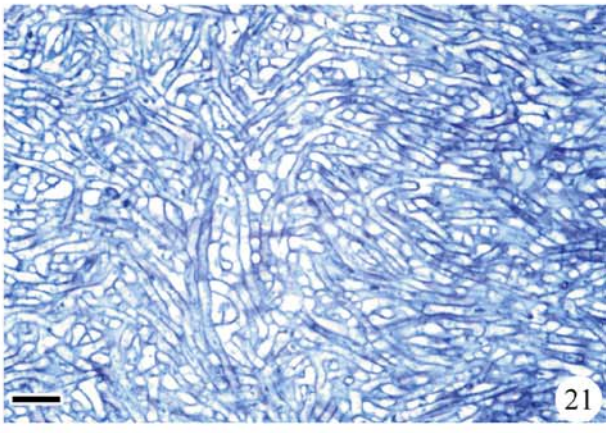


Fig. 19 Light microscopy of *M. uniflora* mycorrhizas. Section of root basipetal to the root apex showing the layered mantle (M), portions of cystidia (‣), E cells, one showing a fungal peg (‣‣). *Scale bar* =20 μm. For abbreviations, see Figs. 2 and 9

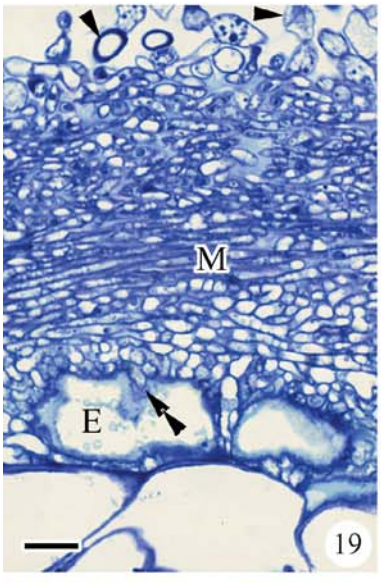


Fig. 20 Light microscopy of *M. uniflora* mycorrhizas. Outer mantle with sections of cystidia (‣) and bacteria (‣‣) in interhyphal spaces and on the mantle surface. *Scale bar* =20 μm

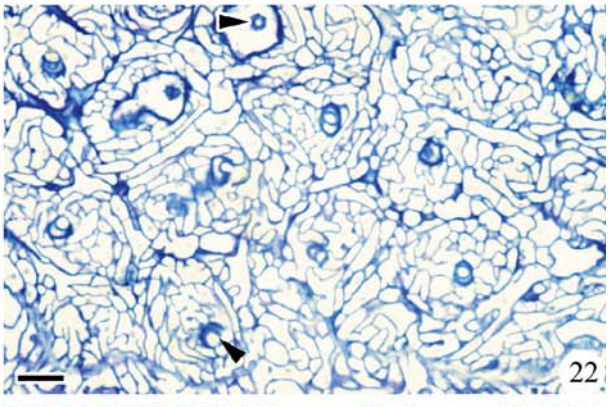


Fig. 21 Paradermal sections of mycorrhizas. Section close to the surface of the mantle showing the complex nature of fungal hyphae. *Scale bar* =20 μm

Fig. 22 Paradermal sections of mycorrhizas. Section immediately above the root epidermis showing top views of hyphal pegs (‣) and the complex configuration of fungal hyphae. *Scale bar* =20 μm

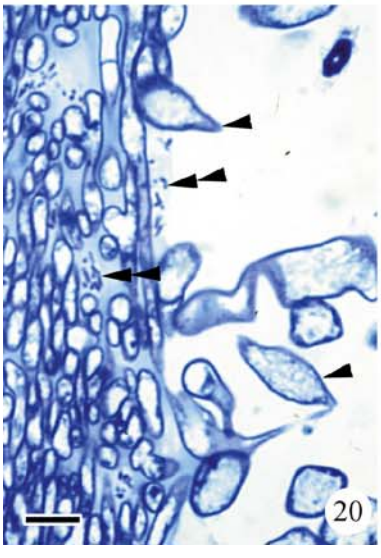
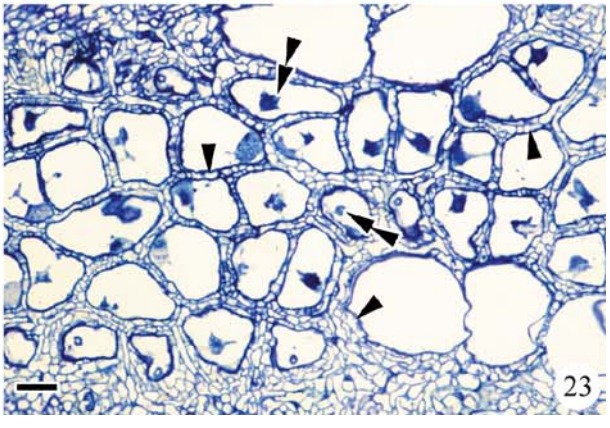


Fig. 23 Paradermal sections of mycorrhizas. Section through the epidermis showing the Hartig net (‣) and portions of fungal pegs (‣‣). *Scale bar* =30 μm



P. andromedea

Roots, including apices, were covered with a mantle (Fig. 26) that consisted of a loosely organized outer mantle and a compact and layered inner mantle (Fig. 27). Hartig net hyphae of wide diameter were present between epidermal cells but, as in *M. uniflora,* these did not penetrate between cortical cells (Figs. 28, 29). Fungal pegs originated either from Hartig net hyphae or from inner mantle hyphae and penetrated the radial wall of epidermal cells (Figs. 28, 29). Dark vacuolar deposits, presumably phenolic in nature, were present in most epidermal cells (Fig. 29). Examination of the same section by light microscopy (Fig. 30) and with crossed polars (Fig. 31) showed that numerous crystalline deposits were present on the mantle surface and occasionally within the mantle.

Discussion

The present structural examination of *M. uniflora* shows that the mycorrhizal association consists of the basic monotropoid features (i.e. a mantle, Hartig net, and fungal pegs).

Similar characteristics have been published for *Monotropa hypopitys* (Kamienski 1882; Duddridge and Read 1982; Duddridge 1985; Dexheimer and Gérard 1993) and *M. uniflora* (Henderson 1919; Campbell 1971; Lutz and Sjolund 1973; Snetselaar and Whitney 1990). Although Lutz and Sjolund (1973) included some SEM photomicrographs of *M. uniflora* roots, they did not describe detailed features of the mantle; instead, their study concentrated on the ultrastructure of the fungal peg. These authors refer only to hyphal projections extending outward from the mantle but make no reference to actual cystidia (although the flask-shaped cystidia are evident in at least one micrograph). More recently, Snetselaar and Whitney (1990) studied mycorrhizas of *M. uniflora,* and their SEM photomicrographs showed two types of cystidia similar to those observed in our samples. These were referred to only briefly and the authors suggested that the flask-shaped cystidia might be related to their "pink" mycorrhizas and the more filamentous (cylindrical) type to the "yellow" form of mycorrhizas. They did not refer to both types occurring together. Nevertheless, the presence of two cystidial types suggests that the fungal symbionts in both studies may be closely related species or at least of the same genus.

Snetselaar and Whitney's study (1990) concentrated on the localization and nature of crystals found within the mantle. It is clear from our observations and those of Snetselaar and Whitney (1990) that the mantle of field-collected *M. uniflora* mycorrhizas is very complex in structure. It is multi-layered, compact, with abundant cystidia of two morphological types in the outer mantle, as well as numerous crystals on and within the mantle. The cystidia are of particular interest because of their frequency and dimorphism. Although the term cystidium is defined as a sterile body occurring at any surface of a basidioma (Hawksworth et al. 1983), the term has been used in the mycorrhizal literature in reference to distinctive hyphal structures that emanate from the outer mantle hyphae of some ectomycorrhizas (Agerer and Weiss 1989; Kernaghan et al. 1997; Massicotte et al. 2000). The fusiform or flask-shaped cystidia characterized in our study were similar but not identical to the ampoule-shaped cystidia of *Russula brevipes* (probably on the host *Abies lasiocarpa*) described by Kernaghan et al. (1997), and to those for *M. uniflora* mycorrhizas formed with members in the family Russulaceae described by Martin (1986). Kernaghan et al. (1997) also mention seeing cylindrical cystidia although they suggest these were rare and no pictures are presented of this type in their publication. The flask-shaped cystidia also resemble those illustrated for an unknown fungal symbiont on roots of *Quercus robur* L. (Edwards and Gessner 1984). Other ectomycorrhizas with two distinct cystidial types have been described for some (although not all) members in the genus *Russula*. The two morphologies comprise a variety of forms of knob-bearing and awl-shaped cystidia, and include species such as *Russula aeruginea, R. atroglauca, R. medullata, R. vesca* and others (Agerer et al 1996–2002).

The function of cystidia in the mantles of these mycorrhizas remains unknown. When cystidia are abundant and predominate on the mantle surface, other indeterminate emanating hyphae and/or rhizomorphs are often less abundant, or sometimes absent. It is frequently cited that mutualistic fungi, by extending into the soil, facilitate water and nutrient uptake to the host. Several questions arise. Do short cystidia-like hyphae function in a similar manner to emanating hyphae, contributing to nutrient uptake? Do they modify the absorption properties at the fungal-soil interface, or are they modified swollen hyphae of strictly morphological interest? These questions should be explored.

With respect to the fungal symbionts of *M. uniflora*, sequencing of a portion of the ribosomal DNA of a subsample of tips collected from our sites suggests that, in this geographical region, *M. uniflora* is associating uniquely with fungi in the family Russulaceae (Young et al. 2002). Bidartondo and Bruns (2001) identified the fungal symbiont of several populations of *M. uniflora* occurring in two mountain ranges in Oregon as being *Russula brevipes*.

The nature of the crystals and their possible functions have been discussed in detail by Snetselaar and Whitney (1990) for field-collected *M. uniflora* roots. Solubility in hydrochloric acid but not acetic acid, combined with X-ray microanalysis, indicated that the crystals were calcium oxalate. The crystals observed in our samples were similar morphologically to those observed by Snetselaar and Whitney (1990); although presumed to also be calcium oxalate, their composition has not been determined. Calcium oxalate crystals occurring on hyphae of ectomycorrhizal fungi have sometimes been associated with soil weathering and the release of phosphorus and iron from compounds such as rock phosphates and iron hydroxy phosphates (Smith and Read 1997). Presumably a similar process could be occurring in *M. uniflora* mycorrhizas;

however, the external mycelium of *M. uniflora* appears to have limited access to the surrounding soil, access normally considered an essential component for weathering processes.

Bacteria were consistently localized on the surface and in interhyphal material within the mantle of *M. uniflora* mycorrhizas, a feature that is not uncommon for ectomycorrhizas (Schelkle et al. 1996) and tuberculate mycorrhizas (Massicotte et al. 1992). In the latter situation, the bacteria (a *Bacillus* sp.) fix atmospheric nitrogen which may be made available to the host species (Li et al. 1992).

The Hartig net is paraepidermal in nature, typical for most angiosperm ectomycorrhizas (Smith and Read 1997), and consists of hyphae of wide diameter. The confinement of the Hartig net to the epidermis may be due to the presence of wall modifications to the outer cortical cell layer as in many angiosperms (Massicotte et al. 1987, 1993), but this has not been determined. In agreement with Lutz and Sjolund (1973) and Snetselaar and Whitney (1990), our observations indicate that fungal pegs originate from inner mantle hyphae that penetrate the outer tangential wall of epidermal cells. The majority of epidermal cells along the longitudinal axis of individual roots appear to be invaded by hyphae to form fungal pegs. Since the epidermal cell wall elaborates in finger-like projections around the fungal peg (Lutz and Sjolund 1973; Snetselaar and Whitney 1990), it has been suggested that nutrient exchange may occur at these sites. There is, however, no direct evidence for this.

With respect to *P. andromedea,* our results show that a mantle, paraepidermal Hartig net and intracellular fungal pegs are characteristic features of this mycorrhizal association; these observations support those of Robertson and Robertson (1982). The mantle of *P. andromedea* is simpler in morphology compared to that of *M. uniflora* in that cystidia are lacking and the outer mantle appears to be more loosely organized giving rise to many emanating hyphae. The lack of cystidia correlates with the fact that *P. andromedea* is known to associate perhaps exclusively with fungi within, or closely related to, the genus *Rhizopogon* (Bidartondo and Bruns 2001, 2002). These fungal species are not known to have cystidia when associated with a variety of hosts (Massicotte et al. 1999, 2000).

The crystals that are present, although taking several shapes, are much smaller and simpler in structure than those observed by us and by Snetselaar and Whitney (1990) for *M. uniflora* mycorrhizas. Nevertheless, they are widespread, occurring along emanating hyphae as well as on and within the mantle, not unlike those of *Rhizopogon* spp. associated with other hosts (Massicotte et al. 1999, 2000). Some of the deposits, because of their non-angular amorphous shape may, in fact, be organic in nature rather than crystalline. The only other structural study of *P. andromedea* showed that, in sectional view, the mantle appeared multi-layered with considerable electron-dense interhyphal material (Robertson and Robertson 1982). Both our study and theirs showed that fungal pegs are initiated by Hartig net hyphae that penetrate radial walls of epidermal cells and subsequently become surrounded with host cell wall. Robertson and Robertson (1982) also suggested that these pegs are probable sites of nutrient exchange.

Although both *Monotropa* and *Pterospora* exhibit similar structural features of monotropoid mycorrhizas, their fungal symbionts appear to interface very differently with the rhizosphere and perhaps with their autotrophic hosts. It is assumed that myco-heterotrophic plants in general obtain fixed carbon from autotrophic plants by means of their shared fungi. Interestingly, we did not observe obvious fungal "bridges" with *M. uniflora* even though some fungal species in the genus *Russula* have been reported to possess rhizomorphs. These rhizomorphs are often described as being rare or infrequent, or present on only some of the mycorrhizas examined (Agerer et al 1996–2002). In contrast, *P. andromedea* had numerous possibilities for fungal connections with autotrophic plants via abundant emanating hyphae and rhizomorphs produced by its *Rhizopogon* mycobiont. *M. uniflora* may have had rhizomorphs that were not observed; however, a striking two-layered array of cystidia suggests that a very close physical association between mycorrhizas of *M. uniflora* and roots of adjacent autotrophic plants might be required for carbon transport through fine hyphae. The differences in crystal formation as documented for *M. uniflora* and *P. andromedea*, as well as their contrasting external anatomical features, could imply some degree of functional difference between these two hosts.

The present study has extended the structural understanding of mycorrhizas in two myco-heterotrophic species in the Monotropoideae and has raised several questions with respect to function and differences associated with crystal structure and external features; the study of other species in this group is desirable. A

Fig. 24 Section of a *M. uniflora* mycorrhiza viewed with light microscopy showing the location of calcium oxalate crystals. *Scale bars* =30 μm

Fig. 25 The same section of a *M. uniflora* mycorrhiza as in Fig. 24 viewed with crossed polars showing the location of calcium oxalate crystals (‣). *Scale bars* =30 μm

Fig. 26 Light microscopy of *P. andromedea* mycorrhizas. Longitudinal section of a main root tip (✻) and a lateral (*L*), both of which are covered by a mantle (M). *Scale bar* =100 μm

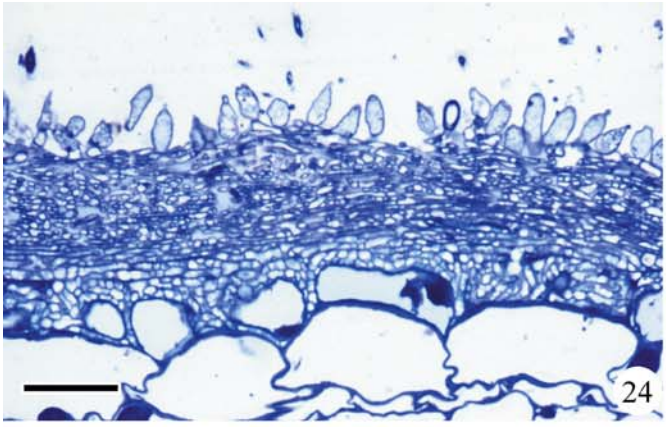
Fig. 27 Light microscopy of *P. andromedea* mycorrhizas. Loosely organized outer mantle (*OM*) and more compact inner mantle (*IM*). Epidermol and cortical (*C*) cells are evident. *Scale bar* =50 μm. For other abbreviations, see Figs. 2 and 9

Fig. 28 Light microscopy of *P. andromedea* mycorrhizas. Mantle (M), Hartig net (➞) and fungal pegs (‣) within epidermal cells. *Scale bars* =20 μm

Fig. 29 Light microscopy of *P. andromedea* mycorrhizas. Mantle (M), Hartig net (➞) and fungal pegs (‣) within epidermal cells. *Scale bars* =20 μm

Fig. 30 Light microscopy of *P. andromedea* mycorrhizas. Section viewed with light microscopy showing the location of crystals on the surface and within the M. *Scale bars* =50 μm

Fig. 31 Light microscopy of *P. andromedea* mycorrhizas. The same section as in Fig. 30 viewed with crossed polars showing the location of crystals (‣) on the surface and within the M. *Scale bars* =50 μm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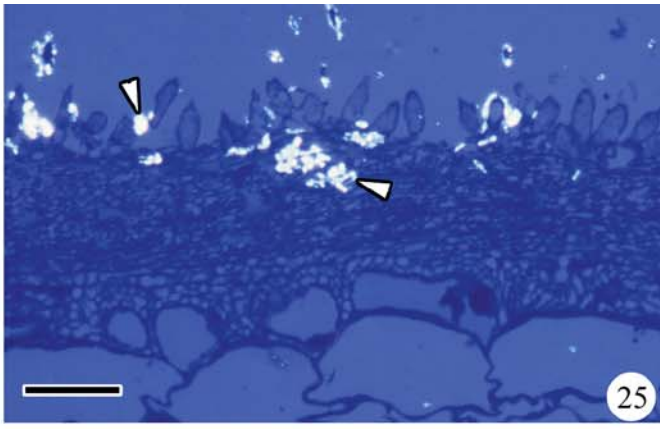
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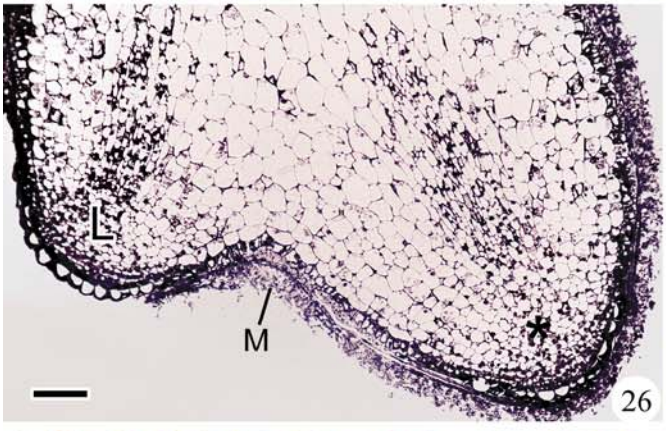
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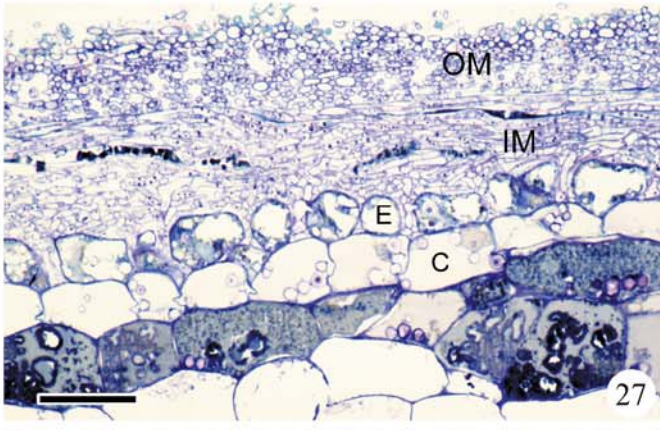
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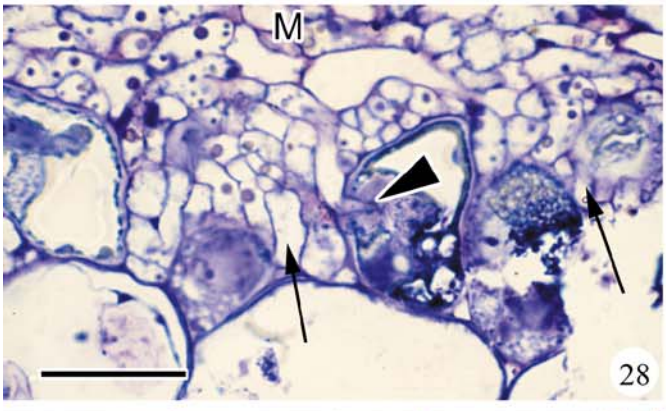
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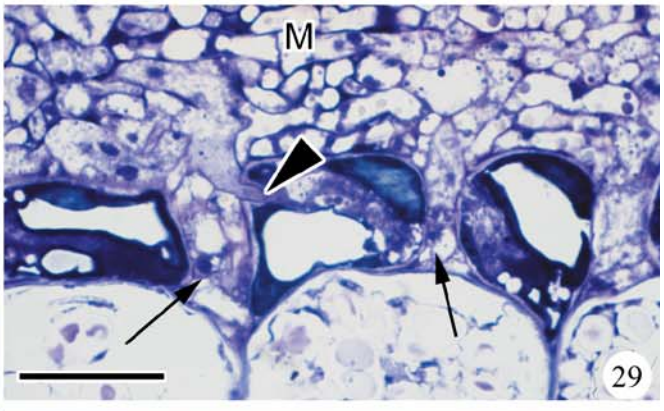
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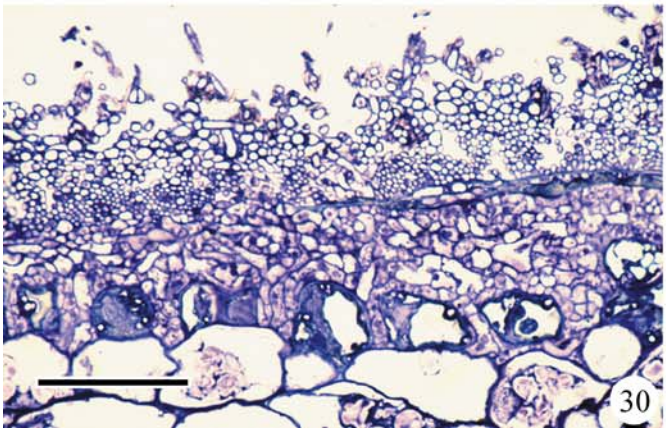
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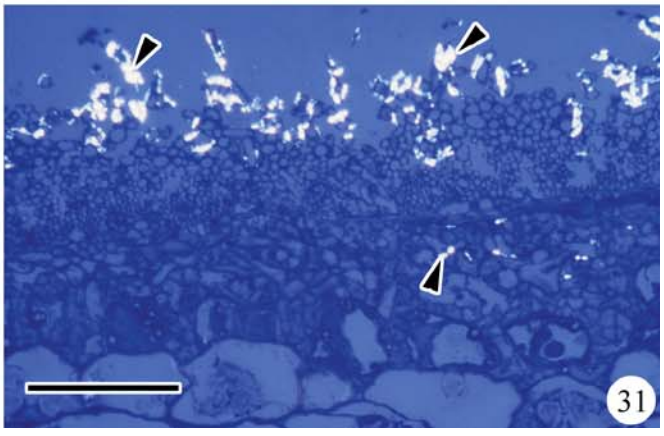
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subsequent paper will consider aspects of fungal peg formation in *M. uniflora.*

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