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ALTERED FUNGAL MORPHOGENESIS DURING EARLY STAGES OF ECTOMYCORRHIZA FORMATION IN EUCALYPTUS PILULARIS

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Abstract

Scanning electron microscopy (SEM) of Eucalyptus pilularis roots inoculated with Pisolithus tinctorius, under controlled conditions, revealed altered morphogenesis of fungal hyphae in contact with the root surface. These changes occurred prior to the formation of a full fungal mantle and resulted in the formation of a compact fungal layer as a consequence of fusion of proliferating, branching hyphae. Although similar growth patterns have been observed in the inner mantle of fully developed ectomycorrhizae using contrast interference microscopy, this is the first time this feature has been observed during early mantle formation using SEM. Changes in fungal morphology during early stages of colonization may be correlated with recognition between the symbionts, and the subsequent establishment of a symbiotic relationship between compatible partners.

KEY WORDS: Eucalyptus, ectomycorrhiza, mantle, fungal morphogenesis.

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Introduction

Ectomycorrhizae, symbiotic associations between fungi and roots of many forest trees, are characterized structurally at maturity by a mantle - an outer covering of hyphae surrounding the root, and a Hartig net - a network of intercellular hyphae located between host epidermal, and in some cases, cortical cells. Although scanning electron microscopy (SEM) is commonly used in studying the surface features of the mantle of mature ectomycorrhizae (see review by Massicotte et al. 1987), it has been used rarely to document the stages of initial contact between the two symbionts (Warrington et al. 1981; Duddridge 1986a,b; Melville et al. 1987a; Massicotte et al. 1987; Thomson et al. 1988). Observations of the early stages may reveal whether recognition between the two symbionts results in a compatible or incompatible interaction. The present study documents the early stages of symbiosis by examining some aspects of the ontogeny of artificially synthesized ectomycorrhizae formed between Eucalyptus pilularis, an important Australian timber species, and two Australian strains of the broad host range fungus Pisolithus tinctorius.

Materials and Methods

Plant Material

Seeds of Eucalyptus pilularis Smith obtained from A.E.Ashford (University of New South Wales, Australia) were sterilized in 100 mL of 30% H_2O_2 with a drop of Tween 20 for 10 min, rinsed three times in sterile distilled water (Grenville et al. 1986) and germinated on filter paper moistened with sterile distilled water in Petri plates placed on a laboratory bench at room temperature. The first germination occurred within 5 days. Eight days after germination, seedlings with expanded cotyledons were transferred aseptically into plastic growth pouches (Fortin et al. 1980) containing 10 mL of sterilized distilled water.

Fungal Material

Two strains (nos. H-98 and H-53) of Pisolithus tinctorius (Pers.) Coker & Couch obtained from N. Malajczuk (Division of Forest Research, Western Australia) were grown in the dark at 20°C on modified Melin-Norkrans (MMN) agar medium (Marx 1969). Forty-one days after

the seedlings were transferred into the pouches, the mycosymbionts were introduced as 10 mm plugs of mycelium obtained from the periphery of colonies growing on agar (Piché and Fortin 1982). The plugs were placed a few millimeters away from the primary root. The number of plugs per seedling varied from 2-6 depending on the size and complexity of the root system.

Growth Conditions

Seedlings were grown under 5 klx (68 W/m^2) (130 $\mu E.m^{-2}.s^{-1}$) light on a 16 h light : 8 h dark cycle at a temperature of 24:18°C. A humidifier was placed in the growth chamber to maintain 60-80% relative humidity. Modified Melin-Norkrans nutrient solution (Marx and Bryan 1975) was added to the pouches as needed.

Developmental stages

The external morphology of the roots and ectomycorrhizae was examined daily after inoculation using either a hand lens or a stereo binocular dissecting microscope. First order mycorrhizal laterals were collected at 3 and 5 weeks after inoculation. At the time of fixation, developmental stages of the mycorrhizae were recorded by photography with a Zeiss DR photodissecting microscope.

Light Microscopy

Tissue was fixed in 2.5% glutaraldehyde using HEPES buffer (0.1M, pH 6.8) for 3 h at room temperature, rinsed in the same buffer, dehydrated in a graded ethanol series and embedded in LR white resin (London Resin Company Ltd.).

Thick sections 1-1.5 µm were cut with glass knives and stained with 0.05% toluidine blue O in 1% sodium borate. Eighteen first order lateral roots were sectioned.

Scanning Electron Microscopy (SEM)

Tissue was fixed in 4% glutaraldehyde using sodium phosphate buffer (0.07M, pH 6.8) for 3 h and then post-fixed in 1% osmium tetroxide in the same buffer for 1 h. Specimens were then treated with a 1.0% solution of thiocarbohydrazide (Postek and Tucker 1977) for 30 minutes and subsequently post-fixed in 2% osmium tetroxide in distilled water for 1 h. The tissue was then dehydrated using a graded ethanol series, critical point dried, mounted on aluminum stubs using conductive carbon paint, coated in a thin layer of gold-palladium and observed with a JEOL JSM-35C scanning electron microscope at 15kV. More than 50 first order lateral roots of ectomycorrhizae formed with each fungal strain were examined.

Results

Eucalyptus pilularis seedlings grew well in plastic growth pouches (Fig. 1) and produced a root system suitable for inoculation with mycorrhizal fungi 6-8 weeks after germination. Mycorrhizae were initiated 5-10 days following the introduction of the mycosymbiont into the pouches.

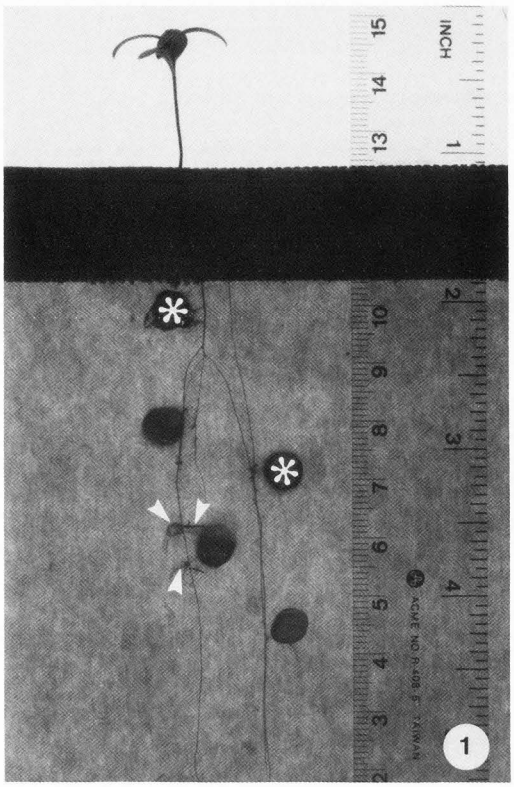
Mycorrhizae, with considerable mantle development, exhibited an Hartig net, a reduced root cap and apical meristem, and a cortex of 2 to 3 cell layers (Fig. 2a) when sectioned for light microscopy. The Hartig net penetrated between the radially-elongated epidermal cells only (Fig. 2b). Scanning electron microscopy revealed that the outer mantle was a complex of individual hyphae and hyphal strands (Fig. 3). The outer mantle region had a coarse texture due to the fairly large interhyphal spaces (Fig. 3).

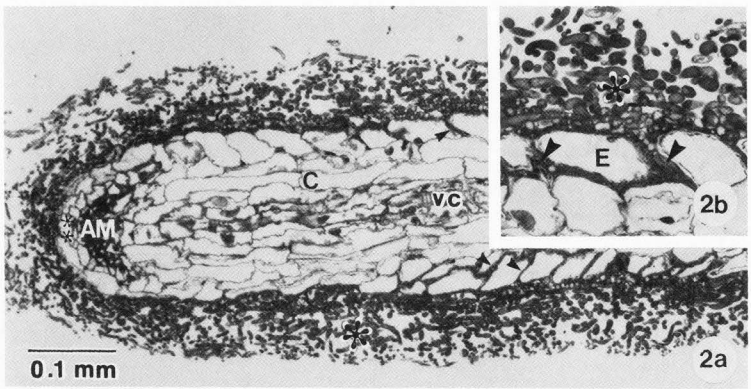
Scanning electron microscopy of very early stages of contact between the two symbionts for ectomycorrhizae formed with either strain of the fungus revealed marked morphological changes in the hyphae growing appressed on the root surface as compared to hyphae not in contact with the root surface. These hyphae were extensively branched, somewhat swollen and appeared to be fused together in places (Figs. 4 & 5). Hyphae showing these morphological changes occurred in the mycorrhizal infection zone, a zone proximal to the apical meristem, when only a few loose hyphae were present above the root surface (Fig. 4). At a stage when more loose hyphae were enveloping the root in the mycorrhizal infection zone, the morphological modifications of the hyphae on the root surface were also evident (Fig. 6). Here again the hyphae on the root surface were branched, swollen and fused in sheets (Figs. 6 & 7). In some cases hyphal tips were multi-branched and swollen (Fig. 8). As the loose hyphae surrounding the root began to form the outer mantle in the mycorrhizal infection zone, morphological changes of hyphae on the root surface were evident proximal to this zone (Figs. 9 & 10). These hyphae appeared to be fused together to form an almost continuous layer and many clamp connections were present (Fig. 10).

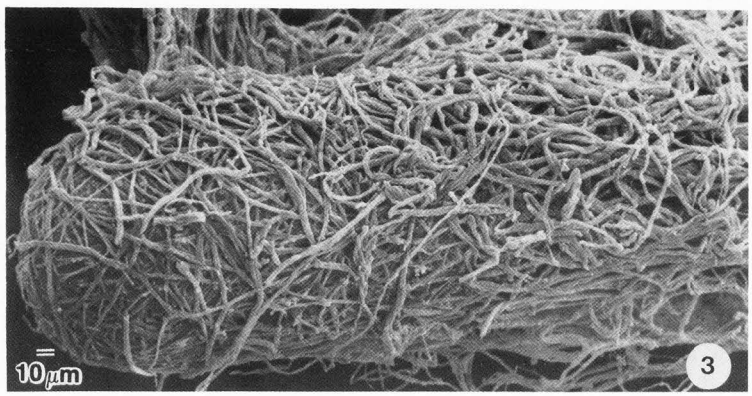
Discussion

The early stages of mantle formation, penetration of the root, and Hartig net formation, are the subject of much debate. A common theme is the morphogenetic change in fungal growth pattern in the Hartig net and/or the inner mantle (Nylund et al. 1982; Nylund and Unestam 1982; Duddridge and Read 1984; Kottke and Oberwinkler 1986, 1987; Blasius et al. 1986; Melville et al. 1987b; Ashford et al. 1988). Mangin (1910) described `palmettes' - fan-like branchings of the fungus within the Hartig net. This unconventional pattern of hyphal growth in the Hartig net has been described subsequently as a randomized labyrinthine structure (Nylund et al. 1982; Nylund and Unestam 1982; Melville et al. 1987a,1987b), an anastomosing complex of hyphae (Duddridge and Read 1984) and a highly ordered coenocytic, transfer cell-like structure (Kottke and Oberwinkler 1987). Nomarski interference contrast micrographs by Agerer (1986) and Brand and Agerer (1986) document similar morphological changes of the inner mantle of mature field ectomycorrhizae. This study presents the first direct evidence in the form of scanning electron micrographs of marked morphological change of hyphae on the root surface in early stages of mantle formation.

This change occurs initially in the mycorrhizal infection zone and most likely proceeds acropetally following root growth. The formation of a loose hyphal weft (Nylund and Unestam 1982; Piché et al. 1983; Malajczuk et al. 1984; Duddridge and Read 1984; Blasius et al. 1986; Massicotte et al. 1986) is not a prerequisite to hyphal modification. It has been suggested that morphological change in hyphal growth is the first visible sign of



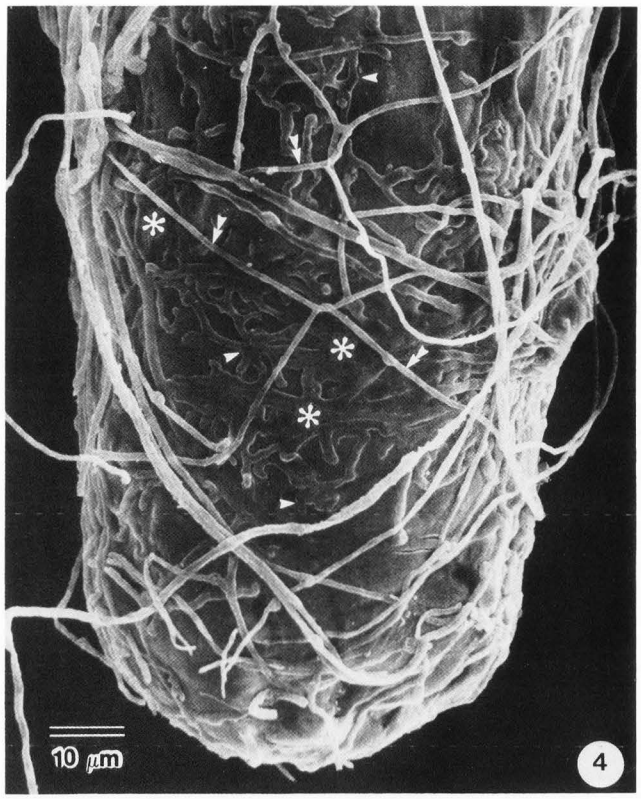




Figs. 1-3. Ectomycorrhizae synthesized between Eucalyptus pilularis and Pisolithus tinctorius. Fig. 1. Growth pouch system showing plugs of mycelium (*) of strain no. H-53 and mycorrhizal laterals (arrowheads). Fig. 2a. Longitudinal section of a first order mycorrhizal lateral formed with strain no. H-98 showing a small root cap (**), apical meristem (AM), cortex (C), vascular cylinder (VC), Hartig net (arrowheads) and mantle (*).
Fig. 2b. Enlargement of part of Fig. 2a showing the paradermal Hartig net (arrowheads), mantle (*) and epidermal cells (E).
Fig. 3. Scanning electron micrograph of a mature ectomycorrhiza formed with strain no. H-53.

physiological change and marks the commencement of symbiosis (Nylund et al. 1982). The trigger for the change in growth form remains unknown. It may be either thigmotropic, chemotropic or both. Piché et al. (1983) suggested that hyphal growth along the root surface is stimulated by root exudates. Thus root exudates may also play a role in stimulating the morphogenetic change in hyphal growth. At this stage the fungus undergoes prolific branching and eventually appears to fuse to form a compact fungal layer over the root surface. Since these changes are similar to those that occur in the Hartig net, morphological change on the root surface may be a prerequisite to Hartig net formation and perhaps even to penetration.

From our studies we propose a model which treats the inner mantle and the Hartig net as a related complex separated from the outer mantle. This is based on the morphological similarity between the inner mantle and the Hartig net. Also, both are in direct contact with the root surface and therefore probably play a similar physiological role in the functioning of the symbiosis. The outer mantle, on the other hand, at least in its inner portion is an aggregation of hyphae held together by interhyphal `cement'. Recently, it has become apparent that interhyphal `cement' may act as a barrier to apoplastic transport between the external environment and the inner mantle - Hartig net complex (Ashford et al. 1988). Our model necessitates that the outer mantle hyphae be younger, or at least of a similar age, to those of the inner mantle. If the outer hyphae are less cytoplasmic than inner mantle hyphae they may have suffered from the fixation process or microbial activities at the root-soil interface or may simply be less active and therefore more vacuolated. Recent observations of distinct morphological zones within the mantles of mycorrhizae formed between Eucalyptus pilularis and Hydnangium carneum (Moore, unpublished data) in which the hyphae of the inner mantle are enlarged and quite compact and the outermost hyphae are loosely aggregated and smaller in diameter, support our proposed model. Zones within the mantle are also described for 17 types of spruce ectomycorrhizae from the field (Haug and Oberwinkler 1987; Haug et al. 1986). However these zones vary between mycorrhizal types. Mycorrhizae whose inner mantles consist of loose hyphae of smaller diameter than the outer hyphae appear to be exceptions to our model.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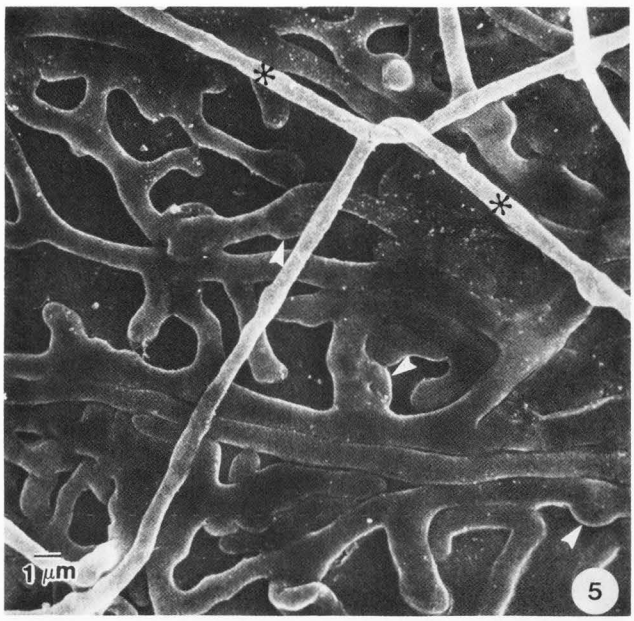
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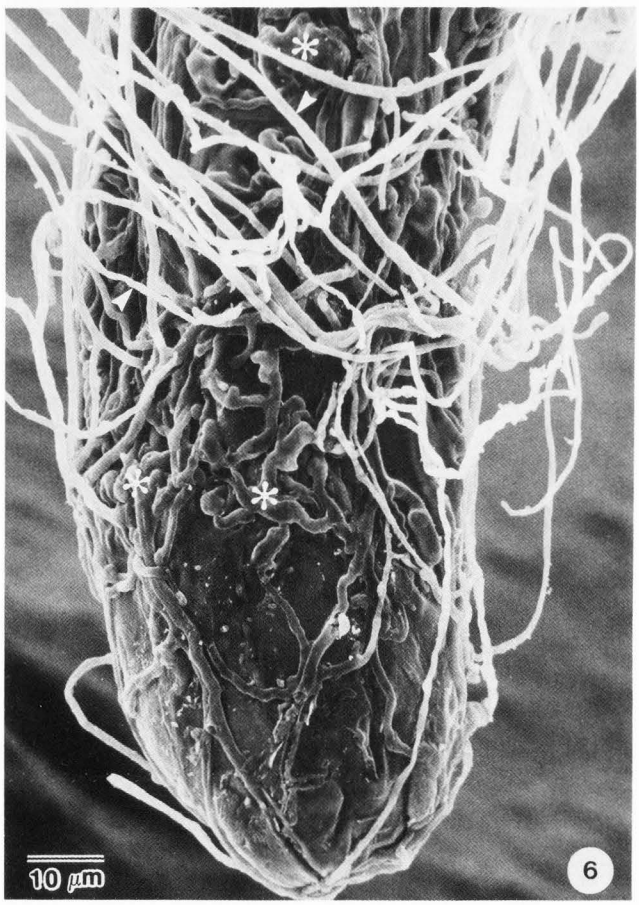
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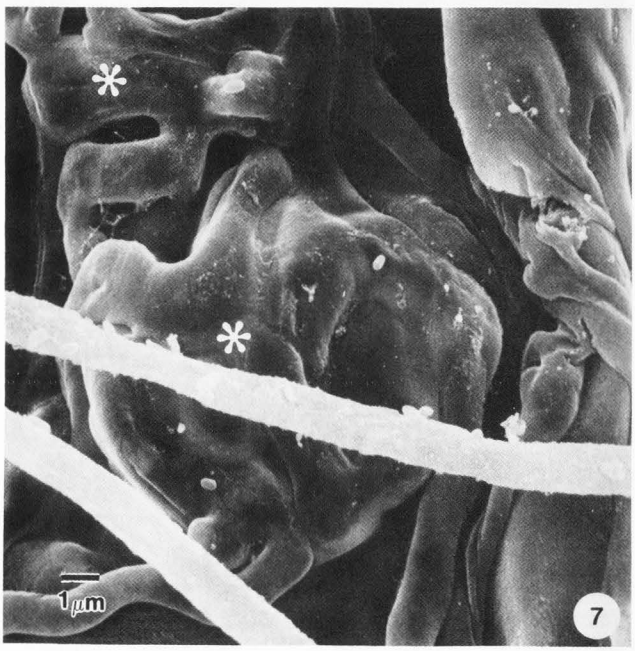
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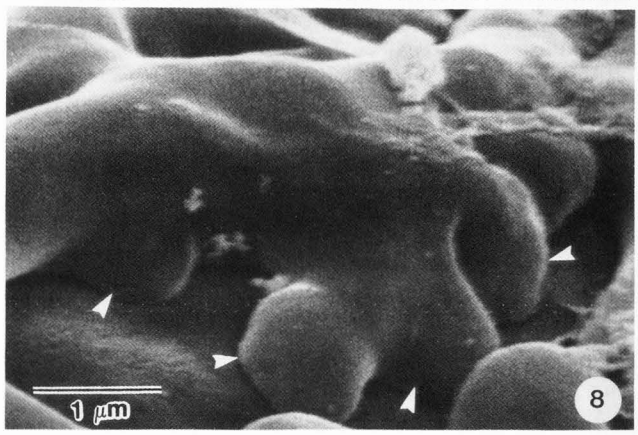
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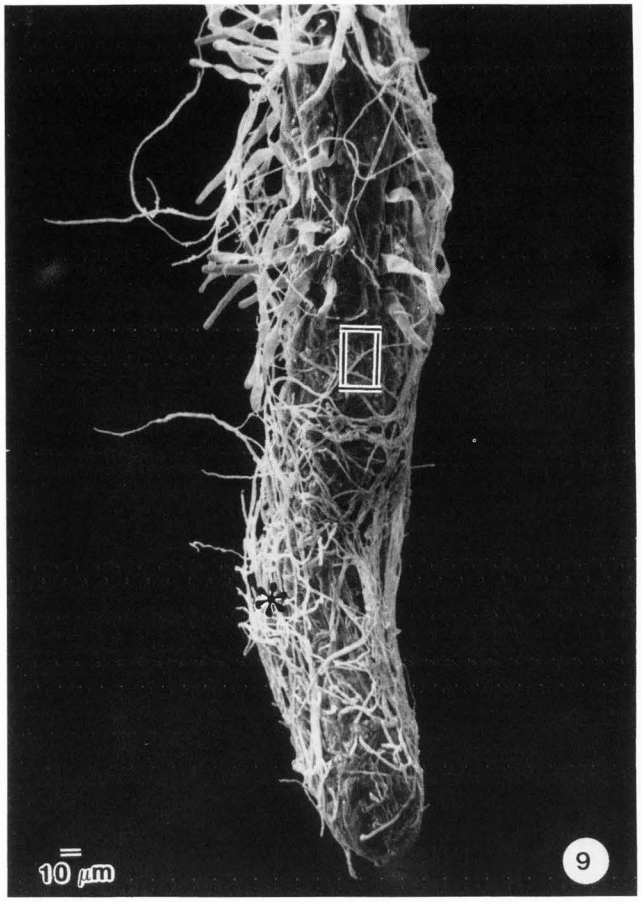

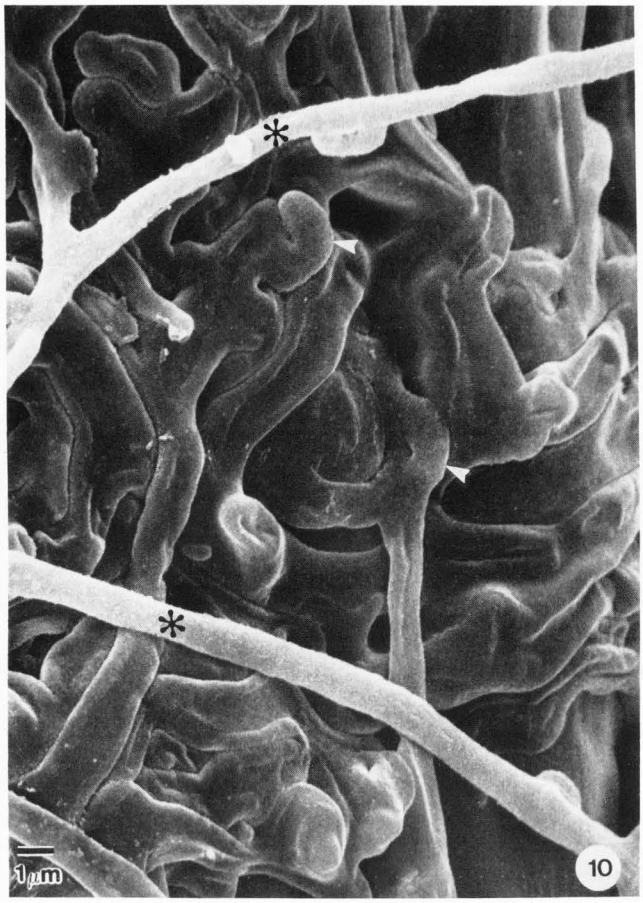

Figs. 4-8. Early stages in the formation of ectomycorrhizae between Eucalyptus pilularis and strain no. H-53 of Pisolithus tinctorius.
Fig. 4. Hyphae are branching (arrowheads) and appear to be fusing (*) on the root surface. Loose hyphae (double arrowheads) are present above the root surface.
Fig. 5. Enlargement of part of Fig. 4. Numerous clamp connections (arrowheads) can be seen amongst the branching hyphae on the root surface. Loose hyphae (*) are present above the root surface.
Fig. 6. Changes in fungal morphogenesis (*) occur in the mycorrhizal infection zone beneath loose hyphae (arrowheads).
Fig. 7. Enlargement of one of the regions asterisked in Fig. 6 showing the fusion of branching hyphae into a fungal layer (*).
Fig. 8. Same root as in Figs. 6,7. Example of morphogenesis of hyphae on the root surface. Note the many lobes (arrowheads) of the branching hypha.

Figs. 9,10. Early stages in the formation of ectomycorrhizae between Eucalyptus pilularis and strain no. H-98 of Pisolithus tinctorius.
Fig. 9. Early mantle (*) formation of an ectomycorrhiza formed with strain no. H-98. Changes in fungal morphogenesis (inside box) occur proximal to the formation of the mantle.
Fig. 10. Enlargement of area inside box in Fig. 9. Morphogenesis of hyphae on the root surface. The branched hyphae now form a more or less continuous layer in this region. Loose hyphae (*) are present above the root surface. Clamp connections and developing clamp connections (arrowheads) exist amongst the fusing hyphae.

Modification of the fungal symbiont during the early stages of contact with the root surface perhaps reflects the result of a positive recognition between the two symbionts and therefore may represent a fundamental step in the establishment of the symbiotic association. Further studies on this early stage of infection are urgently needed.

Acknowledgements

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Discussion with Reviewers

R. Molina: A full discussion of compatibility between host and fungus should include non-recognition or incompatibility. Thus, it is important to compare reactions of a particular fungus with both compatible and incompatible hosts. Have you attempted surface colonization of Pisolithus tinctorius on an incompatible host and looked for morphological changes upon surface contact?

Authors: We have attempted, in previous studies, to synthesize ectomycorrhizae between a number of Alnus species and fungal species that do not colonize these species. We have not, however, studied the very earliest stages using SEM. Some of this work is being repeated with this in mind.

R. Molina: Do you think that the differentiated inner mantle hyphae may act in nutrient exchange similarly to the Hartig net hyphae?
Authors: It is possible that inner mantle hyphae, particularly in ectomycorrhizae with paraepidermal Hartig nets, are as important as Hartig net hyphae in nutrient exchange. This has not been demonstrated experimentally.

G.S. Ellmore: How do the hyphal changes you find in the early stages of symbiosis compare to the well documented pattern of hyphal growth in the inner mantle of mature ectomycorrhizae?
Authors: Our data suggest that these early stages lead rapidly to the establishment of a structure typical of the inner mantle of a mature mycorrhiza. This has not been observed directly, however, since outer mantle hyphae mask these events.

G.S. Ellmore: How similar do you feel the initial contact stage of Pisolithus tinctorius is to that of pathogenetic fungi which produce appressoria?
Authors: Although there is some similarity, especially in the dramatic increase in hyphal diameter, the growth of Pisolithus tinctorius and probably other ectomycorrhizal fungal hyphae by a number of growing points is unique.

Reviewer IV: I fail to see a clear structural and functional comparison between an inner mantle and a Hartig net as the authors suggest. I believe that the Hartig net is a morphologically more complex and biochemically more active structure than to be simply compared with the inner mantle. Specific ultrastructural features (transfer cell-like organization) and enzymatic activities (ATPase activities) give to the Hartig net different and distinctive function and form. Please comment.
Authors: In this ectomycorrhizal system we suggest that the innermost layer of the mantle only is comparable to the Hartig net based on morphological similarity and on the position of both structures in direct contact with the root cells. Cytological and cytochemical analysis correlated with physiological studies are needed to further test our model.