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MORPHOLOGICAL VARIATIONS IN DEVELOPING ECTOMYCORRHIZAE OF DRYAS INTEGRIFOLIA AND FIVE FUNGAL SPECIES

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Abstract

A comparative study of ectomycorrhiza formation between the host species Dryas integrifolia and four fungal species belonging to the Basidiomycotina (Laccaria bicolor, L. laccata, Hebeloma cylindrosporum, Paxillus involutus) and one species in the Ascomycotina (Cenococcum geophilum) showed that patterns in ectomycorrhizal development were distinctive enough to characterize each fungal symbiont. Several aspects of hyphal growth on the plant root during colonization were studied in order to demonstrate the usefulness of SEM in observing the ontogeny of ectomycorrhizae. Each fungal species had a different rate of colonization, and varying root and root hair interactions. As a consequence mantle morphology and the overall appearance of mature ectomycorrhizal apices were also characteristic for each fungal species. Results indicate that the mycobiont plays a major role in determining the morphology of mature ectomycorrhizae.

KEY WORDS: Dryas, ectomycorrhizae, roots, scanning electron microscopy.

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Introduction

A mycorrhiza is a symbiotic association between a plant root and a fungus. In a mature ectomycorrhiza, roots become colonized by fungal hyphae to form a mantle, and a Hartig net, a network of fungal hyphae which develop around epidermal, and in some species, cortical cells. Penetration into the root stele is impeded by the endodermis, a layer of cells enveloping the vascular cylinder. The extent of mantle and Hartig net formation depends on both the fungal and plant species involved.

Characterization of mantle morphology of mature ectomycorrhizae has been carried out using the light microscope (7). Although light microscopy allows determination of gross morphology and mantle colour the limited resolution does not permit observation of fine detail. The scanning electron microscope has been used frequently to study the topography of mature ectomycorrhizae (2,3,11,12,15,20, 22) that might be useful in classifying mycorrhizal types (26). The interface between fungal and plant cells (1,5,8,11,16,19,23,24) and inclusions in the plant cells (9,29) have been studied in fractures of mature mycorrhizae using SEM. Some studies have documented the early stages of ectomycorrhizal formation (4,18,19,21,28), but interactions between the fungus and the root from early stages through to full mantle development remain largely unstudied. Although studies of different mycorrhizae formed on a single plant species have been carried out (1,26), SEM has not been used in such studies to follow the morphogenesis of the fungal mantle. In the following study, ectomycorrhizae were produced between Dryas integrifolia, a boreal member of the Rosaceae, and five fungal species. Samples were fixed for SEM at different stages of lateral root development and

fungal colonization to ascertain the role of the fungal symbiont in ectomycorrhiza formation.

Materials and Methods

Dryas integrifolia (Vahl.) seeds collected in the Cambridge Bay district of the Northwest Territories, Canada, were surface sterilized in 10% hydrogen peroxide for 15 min, washed, and germinated on filter paper in a parafilm-sealed petri dish. Seedlings were transferred into growth pouches (6) containing 10 mL. of modified 1/4 strength Crone's mineral solution (10). Forty-eight days after germination, and after lateral roots had appeared, seedlings were inoculated with the following fungi:

Hebeloma cylindrosporum Romagnesi (isolate 75-1 from J.A. Fortin's laboratory);
Cenococcum geophilum Fr. strain CRBF-0074;
Paxillus involutus (Batsch.) Fr . strain CG-9;
Laccaria bicolor (R. Mre) Orton strain CRBF-0101;
Laccaria laccata (Fr.) B.and Br. strain CRBF-0241.

All were previously grown on Melin-Norkrans Modified (MNM) agar medium (12) and introduced randomly as 10 mm diameter plugs into the pouches within 5 mm of lateral roots (17). Each fungal species was introduced separately into ten pouches of *Dryas* seedlings, and ten pouches of seedlings were kept as controls.

Growth Conditions

Seedlings were grown under 5 klux (42 watts/m^2) (105 $\mu E/m^2/sec$) light on a 16 h light/ 8 h dark cycle with a temperature regime of 24C/18C (D/N). High levels of humidity (60-80% R.H.) were maintained. Five mL. of 1/2 strength Crone's mineral solution (10) were added to the pouches weekly. Ectomycorrhizal roots were collected 25 days after the pouches were inoculated. Control roots were collected at the same time from non-inoculated pouches.

Light microscopy

Rate of ectomycorrhizal formation and mantle colour were determined using a Zeiss MC-63 stereophoto- microscope.

Scanning electron microscopy

Samples were fixed in 2.5% glutaraldehyde using 0.10 M Hepes buffer (pH 6.8)for 3 h at 25C, and then washed in the same buffer. Samples were post fixed in 2% OsO_4 in Hepes buffer for 2 h at 4C, rinsed in buffer, treated with 1% aqueous thiocarbohydrazide for 1 h, and post fixed again in 2% OsO_4 for 1 h at 4C. The tissue was dehydrated in a graded ethanol series, critical point dried, mounted on stubs, and coated with gold- palladium. Samples were observed with a JEOL JSM 35-C scanning electron microscope at 15 kV. Some mycorrhizal roots were fractured with a Gillette blue razor blade cleaned in acetone, prior to SEM.

Results

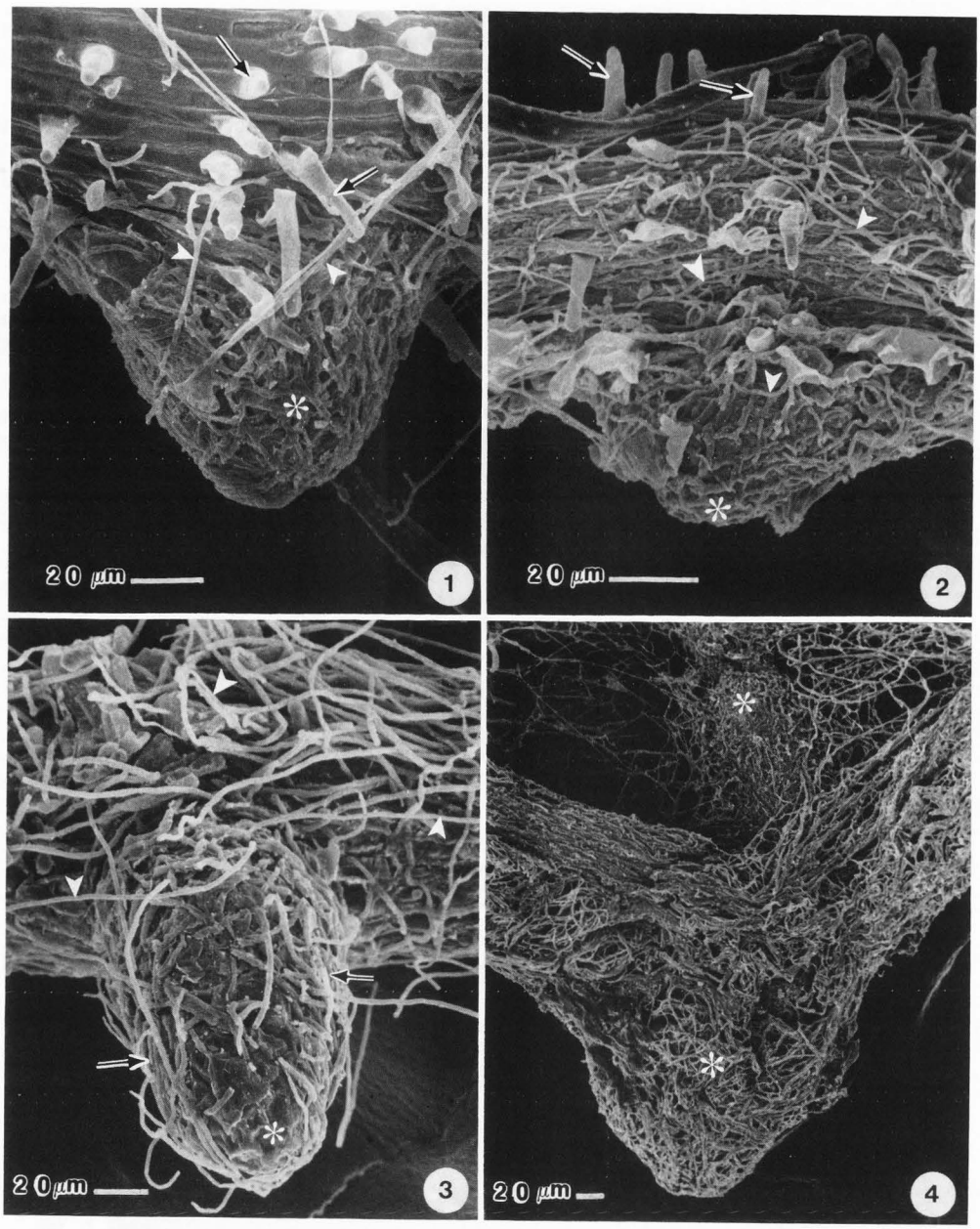
Rate of ectomycorrhizal formation

Although *Dryas integrifolia* seedlings are very small, they grew successfully in plastic growth pouches. Forty-eight days after germination, first order laterals had formed and the root systems were ready for inoculation. Ectomycorrhizae were formed with all of the fungal species used, but the rate of formation varied; *P. involutus* within three days, *H. cylindrosporum* within five days, *L. laccata* and *L. bicolor* within seven days, and *C. geophilum* within twenty-one days. Each growth pouch contained mycorrhizal roots at different stages of development because lateral roots continued to be initiated and subsequently colonized. The first lateral roots to be colonized were closest to the fungal inoculum; colonization then spread at varying rates along the root system from the point at which first infection occurred. Colonization in pouches was 100% for all fungal species except *C. geophilum*, which was 20%.

Early mantle development on young laterals

Early infection of first and second order lateral roots emerging from a previously colonized older root resulted in various patterns of mantle formation depending on fungal species. *Laccaria laccata* rapidly formed a compact, interwoven, white mantle around the root apex at the earliest stage of lateral emergence, even when few hyphae were present on the parent root (fig.1). Colonization by *L. bicolor* was similar to that of *L. laccata*, but more rapid.

Hebeloma cylindrosporum hyphae spread gradually to the emerging lateral root in a random fashion, but did not form a well established mantle until the lateral had grown 2-3 mm from the main root, even when a large number of hyphae were present on the main root (fig.2). *Cenococcum geophilum* followed a similar developmental pattern to that of *L. laccata*, but the hyphae were arranged in a more regular pattern (fig.3), grew more slowly, and were black. *Paxillus involutus* rapidly formed a loose, superficial, brown mantle around emerging lateral roots (fig.4).



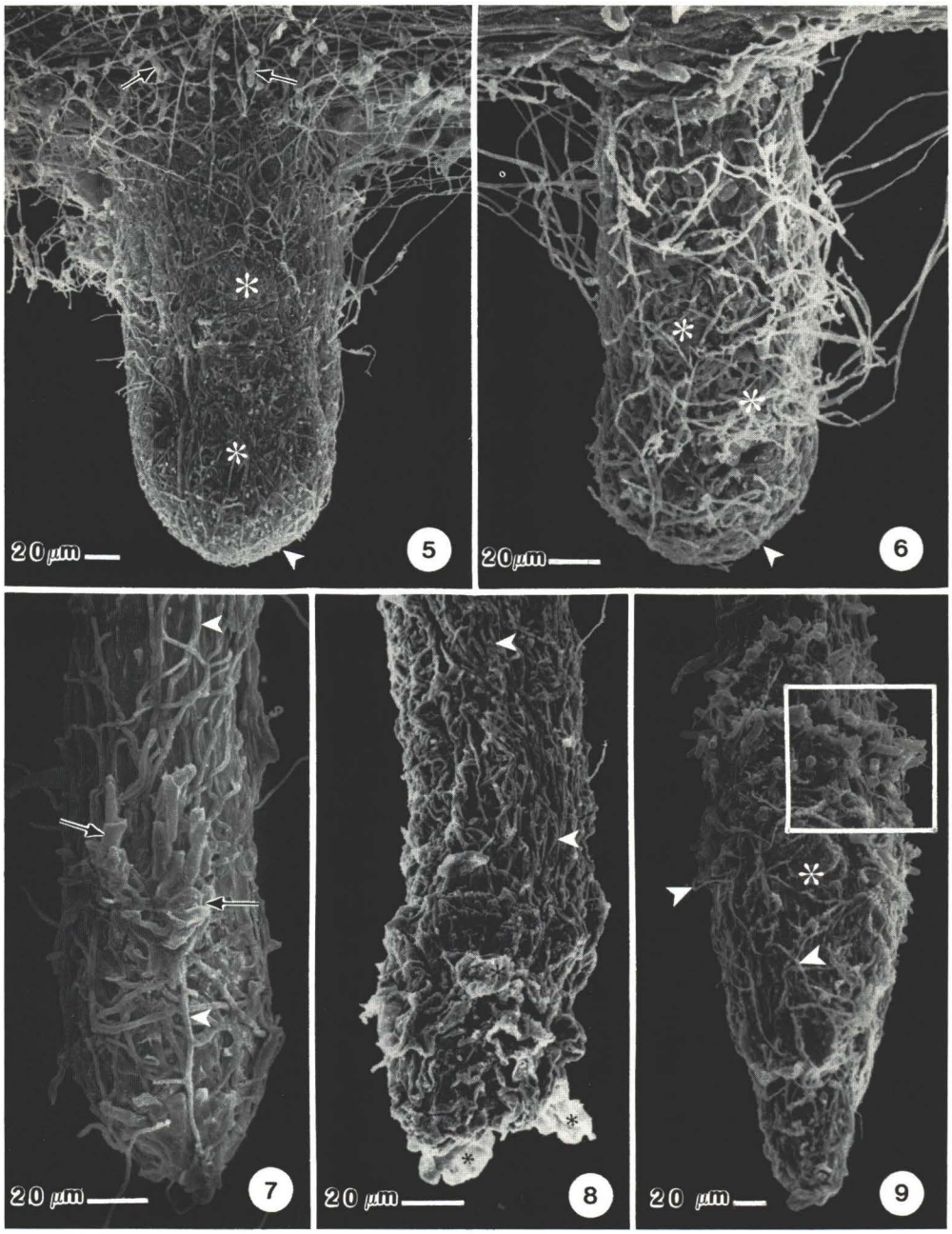
Figures 1-4. Early mantle formation on young lateral roots of Dryas integrifolia.

Fig. 1. Laccaria laccata. A young mantle (*) has formed. Few hyphae (arrowheads) and root hairs (arrows) are evident on the primary root.

Fig. 2. Hebeloma cylindrosporum. The lateral root apex (*) is free of hyphae but a few hyphae are present at the base of the lateral (arrowheads). Root hairs (arrows) are evident.

Fig. 3. Cenococcum geophilum. Coarse, unbranched hyphae (arrowheads) are present on the primary and lateral root. A young mantle (arrows) has formed along the base of the lateral, but the apex (*) has few hyphae.

Fig. 4. Paxillus involutus. A dense mantle (*) has formed over the surface of two lateral roots.



Figures 5-9. Mantle development on elongated lateral roots of Dryas integrifolia.

Fig. 5. Laccaria bicolor. A dense pseudoparenchymatous mantle (*) has formed. The ectomycorrhizal apex (arrowhead) is rounded. Intact root hairs (arrows) are present on the primary root.

Fig. 6. Laccaria laccata. A loose, interwoven mantle (*) is present. The apex (arrowhead) is rounded.

Fig. 7. Cenococcum geophilum. Branching, coarse hyphae (arrowheads) are present at this stage of mantle formation. Sloughed cap cells (arrows) are evident. The apex is pointed.

Fig. 8. Paxillus involutus. Hyphae of the outer mantle (arrowheads) are usually oriented parallel to the root axis. Sloughed root cap cells (*) are present at the pointed apex.

Fig. 9. Hebeloma cylindrosporum. The mantle has developed just behind the root apex, forming a swollen region (*). Small randomly arranged hyphae (arrowheads) are present. The apex is very pointed. The area indicated by the box is illustrated in figure 13.

Hyphae of at least two different sizes and hyphal strands were present. The apex of the root often acquired a bulbous shape when a large number of hyphae were present (fig.4).

Early hyphal interactions on long laterals

Most of the ectomycorrhizae formed with Laccaria spp. did not elongate more than 10 mm., and all developed a rounded apex. Laccaria bicolor rapidly formed a dense, solid pseudoparenchymatous mantle with a purple hue that encased the entire lateral root (fig.5). Occasionally, lateral roots broke through the mantle. Ectomycorrhizae formed with L. laccata were similar in shape to those formed with L. bicolor, but the mantle had a looser, more random weave (fig.6). In early stages of ectomycorrhiza formation with C. geophilum on longer lateral roots, hyphal growth was towards the apex and parallel to the root axis (fig.7). When the hyphae reached the apex they formed many lateral branches (fig.7). Paxillus involutus hyphae followed a similar pattern, but with less hyphal branching at the apex (fig.8). The longer ectomycorrhizal laterals formed with H. cylindrosporum showed a slight swelling of the root behind the apex after formation of a loose, random weave of fungal hyphae. The hyphae did not proliferate until the root apex was colonized (fig.9).

Root hair/ hyphal interactions

In C. geophilum, hyphae diverged around root hairs, following the natural contours of the root, and occasionally branched at obstructions to acropetal growth (fig.10). Hyphae of L. laccata formed a tight, random weave around the root that usually circumvented root hairs (fig.11). Fungal hyphae branched when in contact with root hairs (fig.11). A fracture of a L. laccata / D. integrifolia mycorrhiza showed the base of a root hair in contact with the Hartig net hyphae and surrounded by a complete mantle (fig.12).

In ectomycorrhizae formed by H. cylindrosporum, root hairs in contact with hyphae appeared to degenerate (fig.13). Eventually, a mantle covered the apical portion of the lateral root and enclosed degenerated root hairs and epidermal cells (fig. 14). This mantle consisted of a thin, compact inner layer of hyphae, a loose amorphous outer layer, and a cortical Hartig net (fig.14). Root hairs of D. integrifolia retained their shape when interacting with C. geophilum and Laccaria spp. during the early stages of infection. Mature mantles were free of protruding root hairs. In L. bicolor intact root hairs remained on the main root at the base of the mycorrhizal lateral, where few hyphae were present (fig.5). All root hairs that had been contacted by P. involutus were collapsed (fig. 15).

Morphology of mature mycorrhizal apices

Although apical morphology of mature ectomycorrhizae shared some common general characteristics, it was unique for each fungal species. Laccaria bicolor formed a dense mantle of interwoven hyphae encased in a matrix which enveloped the root (fig.16). The pseudoparenchymatous outer mantle had a purplish hue and the root apex was always rounded (fig.16). Laccaria laccata ectomycorrhizae had a similar rounded apex, but the outer mantle hyphae were in a looser, random weave with fewer anastomoses (fig.17). This ectomycorrhiza was white. The hyphae of both Laccaria species had abundant clamp connections. Hebeloma cylindrosporum produced a white, cottony mantle with many clamp connections which adhered tightly to the root surface (fig.18). At the apex, which was always pointed,the hyphae were present in a thin, randomly woven layer and were often covered with mucilage, probably produced by root cap cells (fig.18). Paxillus involutus produced an ectomycorrhiza with a loose, bulky, brown mantle with hyphae of two different diameters in a random weave. Hyphal strands were common (fig.19). The mantle and many individual hyphae collapsed and often revealed a bulbous root apex. Cenococcum geophilum ectomycorrhizae had a dense, black mantle and a bulbous apex (fig.20). Surface hyphae were fused into a pseudoparenchyma with a convoluted pattern. Single, straight hyphae, originating from a swollen hyphal base at the mantle surface, radiated outward from the outer mantle (fig.20).

Discussion

Root systems of D. integrifolia were inoculated with five different fungi and analyzed at various stages of development in order to determine patterns of colonization and maturation in the respective ectomycorrhizae. Fungal hyphae were stimulated to grow when they contacted growing root apices, and each species had a unique colonization pattern. By documenting specific sites of fungal activity and recognizing differences in fungal 'behavior' patterns it is possible to identify critical stages in the establishment of ectomycorrhizal symbioses, and to use SEM to study the incompatibility of particular host and fungal species. Similar studies have been undertaken using SEM for other

plant-fungus associations (1,5,18,21, 28). These papers, however, do not show progression of changes in the developing mycorrhiza.

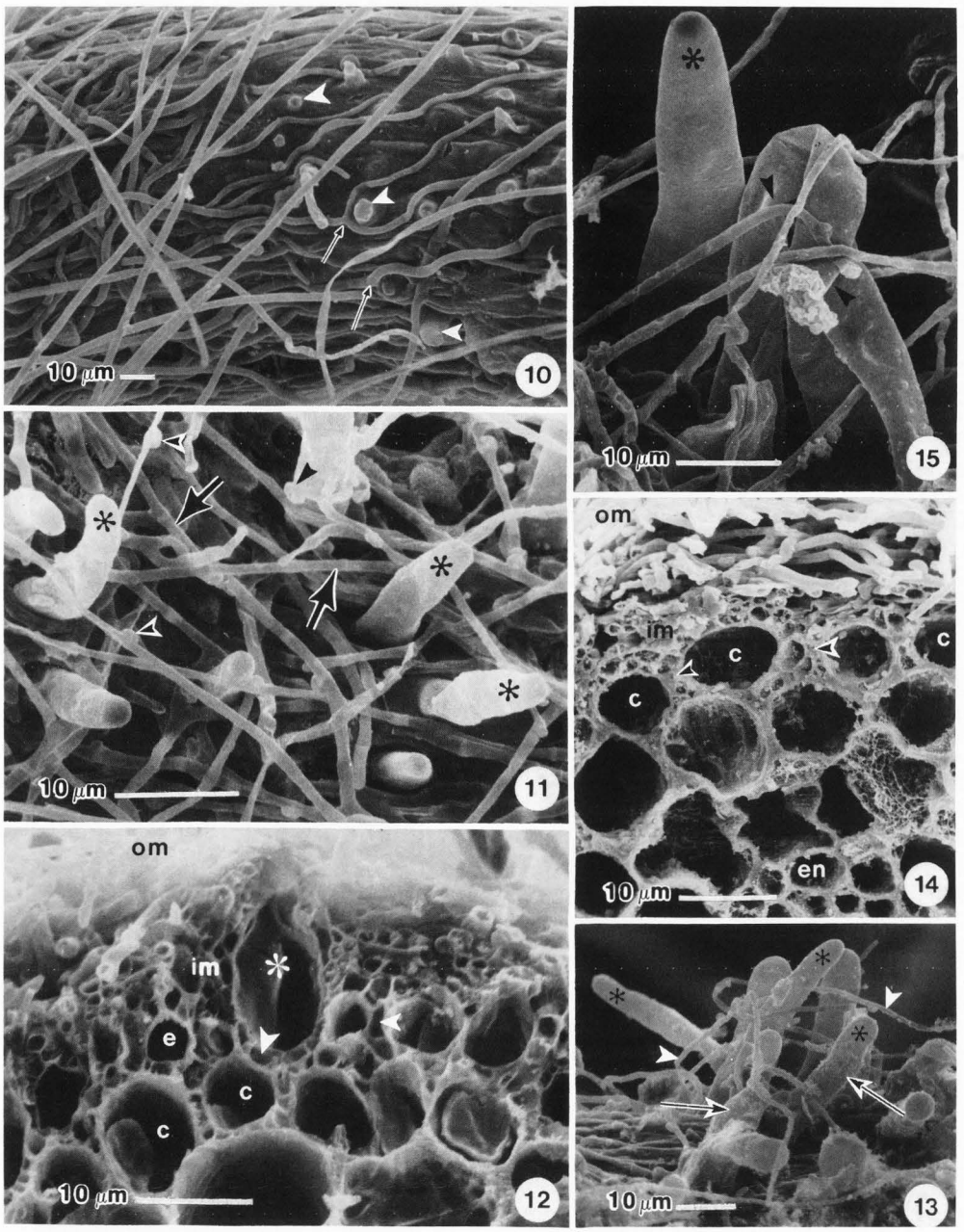
With the exception of H. cylindrosporum, lateral roots of all species studied rapidly developed a mantle when fungal hyphae were in contact with the root apex (1,18). This could indicate that substances produced in the root cap play a vital role in triggering mantle formation. In ectomycorrhizae of D. integrifolia formed by H. cylindrosporum, however, fungal proliferation occurred in a region just basal to the root cap, suggesting the possibility of at least two distinct types of ectomycorrhizal colonization. The mature ectomycorrhizae formed between D. octopetala and Hebeloma alpinum and H. marginatulum did not show this feature (2,3). The apparent difference between host species may actually be a consequence of the age of the mycorrhizae examined.

A well-developed mantle was nearly always present on recently emerged laterals of D. integrifolia when in the presence of L. bicolor, L. laccata, P. involutus and C. geophilum. This indicates that mantle formation was extremely rapid once fungal hyphae contacted the apex. Observation of longer laterals in the early stages of colonization by C. geophilum showed that mantle formation by this species is preceded by a branching of the hyphae at the apex. This may, in turn, give rise to the stellate configuration of some apical hyphae reported for C. geophilum in other mycorrhizae (15,19).

Interaction between fungal hyphae and root hairs may precede intercellular penetration of the hyphae to form the Hartig net. Degradation of root hairs by hyphae, and inhibition of root hair formation by the fungal mantle has been suggested for other ectomycorrhizae (1,4,5,18,20), although rarely documented directly. In mycorrhizae of D. integrifolia / H. cylindrosporum (fig.14) the absence of epidermal cells beneath the mantle may be a consequence of root hair degradation. This has been supported by a light microscopic examination of transverse sections through the same zone (14). The root hairs in ectomycorrhizae formed between D. integrifolia and P. involutus were collapsed when in contact with fungal hyphae. Root hairs were present within the Hartig net and mantle of D. integrifolia/L. laccata mycorrhizae, but the Hartig net was broader between the epidermal cells than between cortical cells. This may be due to the partial collapse of hair cells. Root hairs were not visibly degraded by C. geophilum, but were diminished in the presence of full mantle formation (13). The rounded apices of L. laccata and L. bicolor, and the bulbous apices of P. involutus and C. geophilum, may be an indication of inhibited root extension, whereas the pointed apex shown by the mycorrhizae of H. cylindrosporum suggests continued root elongation. In D. integrifolia/H. cylindrosporum, by the time the apex becomes rounded, the ectomycorrhiza may be quite old and less active (14). Renewed axial growth of the root beneath the mantle of C. geophilum (13) and L. bicolor (unpublished data) can result in the fracturing of the mantle and emergence of a mantle-free root apex. The long, radiating hyphae of C. geophilum, and the hyphal strands of P. involutus may improve exploitation of the soil for nutrients and increase the efficiency of mineral transport (15). The shell-like mantles of C. geophilum and L. bicolor may protect the root from bacterial and nematode invasion, and reduce water loss during drought.

SEM is effective for observing the early stages of fungal colonization of the root surface, improving the resolution of hyphal and mantle detail significantly over that of the light microscope. Once sites of early colonization have been identified using SEM, samples can then be processed for light and transmission electron microscopy (18). Categorization of developing mantle behavior can increase our understanding of how the mycorrhizal organ adapts to different ecological situations and may provide a means for selecting more effective symbionts for particular environmental conditions. Characteristics such as rate of infection, orientation of hyphae , root hair interaction, zone of hyphal proliferation, hyphal strands, and degree of extramatrical hyphae should be measured. Superficial characteristics of the mature ectomycorrhizal apex are determined largely by the fungal species, and each is unique. Trappe (27) suggested characterizing the various features in order to identify unknown fungal species. These features are largely consistent when one fungal species forms an ectomycorrhiza on different plants (30). For example, descriptions of mature apices of ectomycorrhizae established between various hosts and either C. geophilum (13,15,19) or L. laccata (1,6) are similar to what was found for D. integrifolia in this study.

A comprehensive SEM survey of ectomycorrhizae accompanied by descriptions of each using consistent



Figures 10-15. Interaction of hyphae with root hairs in Dryas integrifolia.

Fig. 10. Cenococcum geophilum. Root surface near apex showing root hair initials (arrowheads) and fungal hyphae, some of which have diverged around root hair initials (arrows).

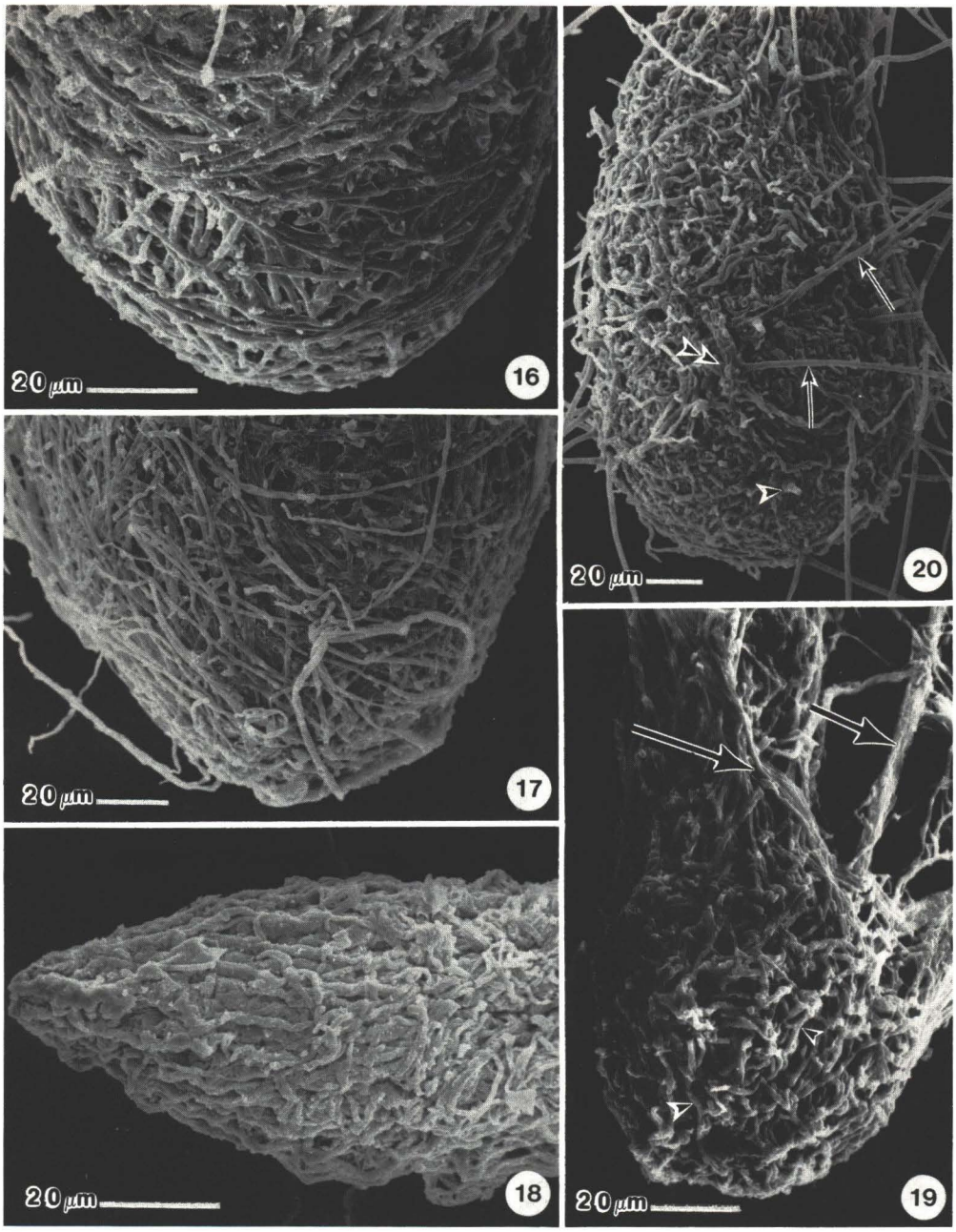
Fig. 11. Laccaria laccata showing emergent root hairs (*) and surrounding hyphae (arrows). Clamp connections (arrowheads) are present.

Fig. 12. Laccaria laccata. Fracture of ectomycorrhiza showing a root hair (*) surrounded by inner mantle (im). Hartig net hyphae (arrowheads) are present. om- outer mantle; e - epidermal cells; c - cortical cells.

Fig. 13. Hebeloma cylindrosporum. Detail of root hairs shown in box on fig.9. Hyphae (arrowheads) have contacted root hairs (*), some of which have collapsed (arrows).

Fig. 14. Hebeloma cylindrosporum. Fracture of ectomycorrhiza showing outer mantle (om); inner mantle (im), Hartig net (arrowheads), cortical cells (c), and endodermis (en).

Fig. 15. Paxillus involutus. Root hairs (*) showing collapse where hyphae have become attached (arrowheads).



Figs. 16-20. Apices of mature Dryas integrifolia ectomycorrhizae.

Fig. 16. Laccaria bicolor. A rounded apex of an ectomycorrhiza with a tightly interwoven mantle.

Fig. 17. Laccaria laccata showing a rounded apex and interwoven mantle hyphae.

Fig. 18. Hebeloma cylindrosporum. Apex is pointed and the outer mantle hyphae have collapsed onto the root surface.

Fig. 19. Paxillus involutus. Note bulbous apex, hyphae (arrowheads) and hyphal strands (arrows).

Fig. 20. Cenococcum geophilum. The apex is swollen, and is covered by a dense mantle showing stellate patterns (double arrowheads). An initial (arrowhead) of the radiating coarse hyphae (arrows) is evident close to the apex.

terminology (19) needs to be established. Since collections of light micrographs of ectomycorrhizal morphology are currently being compiled by various researchers (7,25) for taxonomic purposes, these should be correlated with scanning electron micrographs to provide a means for identification of unknown fungi in the field.

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Discussion with Reviewers

Reviewer I: Is there any evidence that the differences observed between D. integrifolia/H. cylindrosporum mycorrhizae and the other associations studied could result in differences in the relative ability of H. cylindrosporum to promote host growth?
Authors: Evidence indicates that lateral root growth continues after colonization by H. cylindrosporum. In this case elongation of roots may be enhanced, whereas in Laccaria spp. the numbers of lateral roots may be increased (unpublished data). Different fungal species may promote plant growth in different ways.
Reviewer I: Is it safe to assume that patterns of infection development and ectomycorrhizal morphology of 73-day-old seedlings grown in growth pouches will be representative of ectomycorrhizal associations of older plants growing in soil?
Authors: While in vitro growth conditions do not reflect those in the field, an understanding of development under controlled conditions is necessary before the complex nature of an in vivo microenvironment can be studied.
Reviewer III: Do the authors have any information on the meristem development before and after colonization of host roots by ectomycorrhizal fungi?
Authors: A previous study of Dryas integrifolia/Hebeloma cylindrosporum ectomycorrhizae deals with the subject of meristem development (Melville, et al. Botanical Gazette, in press).
Reviewer IV: What sort of results would be predicted if the mycosymbiont plays a significant role in ectomycorrhizal development. Alternatively, what results would support a primary determining role by the host root?

Authors: Once specific fungal species are characterized then ectomycorrhizal root morphology would allow identification of fungal species in the field. It may also be possible to predict symbiotic compatibility. If ectomycorrhizal characteristics such as morphology and ultrastructure were similar for ectomycorrhizae formed by one plant then the plant might be considered to have a determining role. If such were the case identification of the mycosymbiont would be difficult.