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Mycorrhiza

Mycorrhizal associations in Hong Kong Fagaceae

II. The formation of mycorrhizas in *Quercus myrsinaefolia*

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Abstract. A local species of oak (*Quercus myrsinaefolia*) was able to form ectomycorrhizas with a number of fungi already known to form mycorrhizas with *Pinus* and *Eucalyptus* species in Hong Kong. Oak mycorrhizas had the typical radially elongated, epidermal Hartig net seen in other angiosperms. The failure of some fungal isolates to establish mycorrhizal associations with oak indicates a lack of compatibility between fungus and host.

Key words: Oak - *Quercus* - Ectomycorrhiza

Introduction

The genus *Quercus* is distributed widely and dominates various kinds of woody vegetation from relatively mesic to relatively xeric conditions in the northern hemisphere (Matsuda et al. 1989). The territory of Hong Kong is subject to subtropical climatic conditions and supports the growth of nine species of *Quercus*. The "small leaved", evergreen oak *Quercus myrsinaefolia* Bl. is common on the slopes of rocky hills in the southern part of Hong Kong island.

Several investigators have reported that species of *Quercus* form ectomycorrhizas with a range of fungi (Marx 1979; Beckjord and McIntosh 1983, 1984; Dixon et al. 1984, 1985; Mitchell et al. 1984; Beckjord et al. 1985, 1986), and in a preliminary survey of the root systems of the Fagaceae growing in Hong Kong, we noted that the roots of *Quercus* species were frequently short, stout, variously coloured and obviously different from normal roots. Histological examination confirmed that the trees bore ectomycorrhizal roots. The only description we can find of ectomycorrhizal formation in *Quercus* is that of Luppi and Gautero (1967), who examined temperate species of *Quercus* growing in Italy.

Our previous investigations (Tam and Griffiths 1993) indicate that the local species *Q. myrsinaefolia* can form ectomycorrhizas with several fungi under artificial conditions. The objective of this present study was to describe the morphology and structure of synthesized ectomycorrhizas in *Quercus* by means of light microscopy and scanning electron microscopy.

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Materials and methods

Natural oak ectomycorrhizas

Root samples taken from old trees of *Q. myrsinaefolia* growing on the hills were washed thoroughly and mycorrhizal roots were photographed.

Fungal cultures

Cultures of *Pisolithus* sp., *Scleroderma* sp. and *Hymenogaster* sp. were isolated from sporophores growing at the various sites and were maintained on modified Melin:Norkrans (MMN) agar medium (Marx 1969). Other fungal species known to be mycorrhizal were obtained from the American Type Culture Collection (ATCC): *Cenococcum geophilum* (ATCC 38052), *Thelephora terrestris* (ATCC 38058) and *Pisolithus tinctorius* (ATCC 38054); *Rhizopogon roseolus* (388.74) was supplied by the Centraalbureau voor Schimmelcultures at Baarn; all fungi were maintained on MMN agar medium.

Seedlings

Ripe acorns were collected in October from the slopes of the southern hills of Hong Kong island before they fell to the ground. The hard, outer shell was removed and the seeds were placed in shallow trays and allowed to germinate in running tap water. Selected seedlings bearing healthy cotyledons were transferred to paper cups containing a 9:1 mixture of vermiculite and peat moss moistened with distilled water. Two weeks after germination, the seedlings were inoculated with a leached fungal inoculum of all the fungal isolates grown on a nutrient-supplemented peat moss/vermiculite mixture (Marx 1980). Two weeks later, the soil was irrigated with Hoagland's solution. During the following 4 months, seedlings were watered daily with distilled water and the soil was irrigated with Hoagland's solution at 4-week intervals.

Other seedlings were grown in a 1:1 mixture of potting soil (as above) and natural soil collected from the base of the mother tree; control seedlings were grown in an uninoculated potting soil. Both sets of seedlings were watered daily with distilled water and irrigated with Hoagland's solution every 4 weeks. Control seedlings were grown in a 9:1 mixture of vermiculite and peat moss and watered as above.

All seedlings were grown for the first 8 weeks with continuous illumination at 26–28° C in a temperature-controlled propagation unit, after which they were transferred to larger pots in the same unit to allow better development of their root systems.

Four months after inoculation, seedlings were removed from the soil, their roots washed thoroughly and photographs taken of various stages of mycorrhizal formation. Lateral roots of first-, second- and higher order root clusters were removed for sectioning and scanning electron microscopy. Photographs were also taken of control seedling roots.

Light microscopy

Tissue selected for microscopy was fixed in 4% glutaraldehyde in 0.025 M phosphate buffer (pH 6.8) at 4° C for 10–20 h, washed in buffer and dehydrated for 1 h each in 2-methoxyethanol, ethanol, *n*-propanol and *n*-butanol and embedded in glycol methacrylate. Polymerization was carried out in gelatin capsules at 55° C for 10–15 h. Sections of 2–3 µm were cut with a glass knife and stained in 0.05% toluidine blue in 1% sodium benzoate at pH 4.4.

Scanning electron microscopy

Tissue was fixed as above and post-fixed in 1% (w/v) osmium tetroxide for 1 h. Specimens were washed and dehydrated in a graded series of ethanol, then dried at the critical point, shadowed with platinum/palladium and examined in a Cambridge Stereoscan 150 scanning electron microscope.

Results

Natural oak ectomycorrhizas

A variety of ectomycorrhizas was found associated naturally with this oak species. Some were enveloped by sheets of white rhizomorphs (Fig. 1), others were long, sinuous, pinnately branched and reddish-brown mycorrhizas mixed with scattered, black, club-shaped mycorrhizas bearing radiating hyphae (Fig. 2). Some were yellowish-brown with pinnate branching, often forming mycorrhizal clusters. Some mycorrhizas were black and enveloped by wefts of white, cottony mycelium.

Pot-grown oak seedlings

In uninoculated soil, the control seedlings produced a very long main root with first- and second-order laterals. Four months after germination, many of the older laterals had developed fourth-order rootlets. Roots developing in inoculated soil were frequently modified to form typical ectomycorrhizas, i.e. with restricted root growth, a well-developed fungal sheath or mantle, a Hartig net of intercellular hyphae, and no intracellular infection.

The variations found in each association

Mycorrhizas formed by P. tinctorius. Hyphal growth was extensive and mycelial strands developing from the hyphal mat were bright yellow. The development of lateral roots was pronounced and repeated branching resulted in mycorrhizal clusters (Fig. 3).

Mycorrhizal roots were golden-yellow when young but gold-brown when mature. The main mycorrhizal axis was <6 mm in length and <0.4 mm in diameter.

The mantle surface consisted of loosely interwoven, branched mycelial strands; and emanating hyphae 3–7 µm in diameter bearing clamp connections were also present. Hyphae below the mantle surface were embedded in an amorphous matrix (Fig. 4).

The mantle was 15–25 µm thick and composed of an outer prosenchymatous layer and an inner synenchymatous layer. The para-epidermal Hartig net was uniform and the radial elongation ratio of the epidermal cells was 3:1 (Figs. 5, 6).

Mycorrhizas formed by Scleroderma sp. Hyphal growth resulted in the formation of a white, woolly mycelium from which white threads of mycelial strands developed.

Mycorrhizal roots were seldom clustered but were long and straight and generally pinnately branched; the main axis was <5 mm long and <0.4 mm in diameter (Fig. 7).

The mantle surface consisted of loosely interwoven hyphal strands and bore emanating hyphae 2–4 µm in diameter with clamp connections (Fig. 8).

The mantle was 25–50 µm thick and composed of mixed prosenchymatous and synenchymatous hyphae. The para-epidermal Hartig net was not uniform and thus the radial elongation ratio of the epidermal cells varied from 2:1 to 3:1.

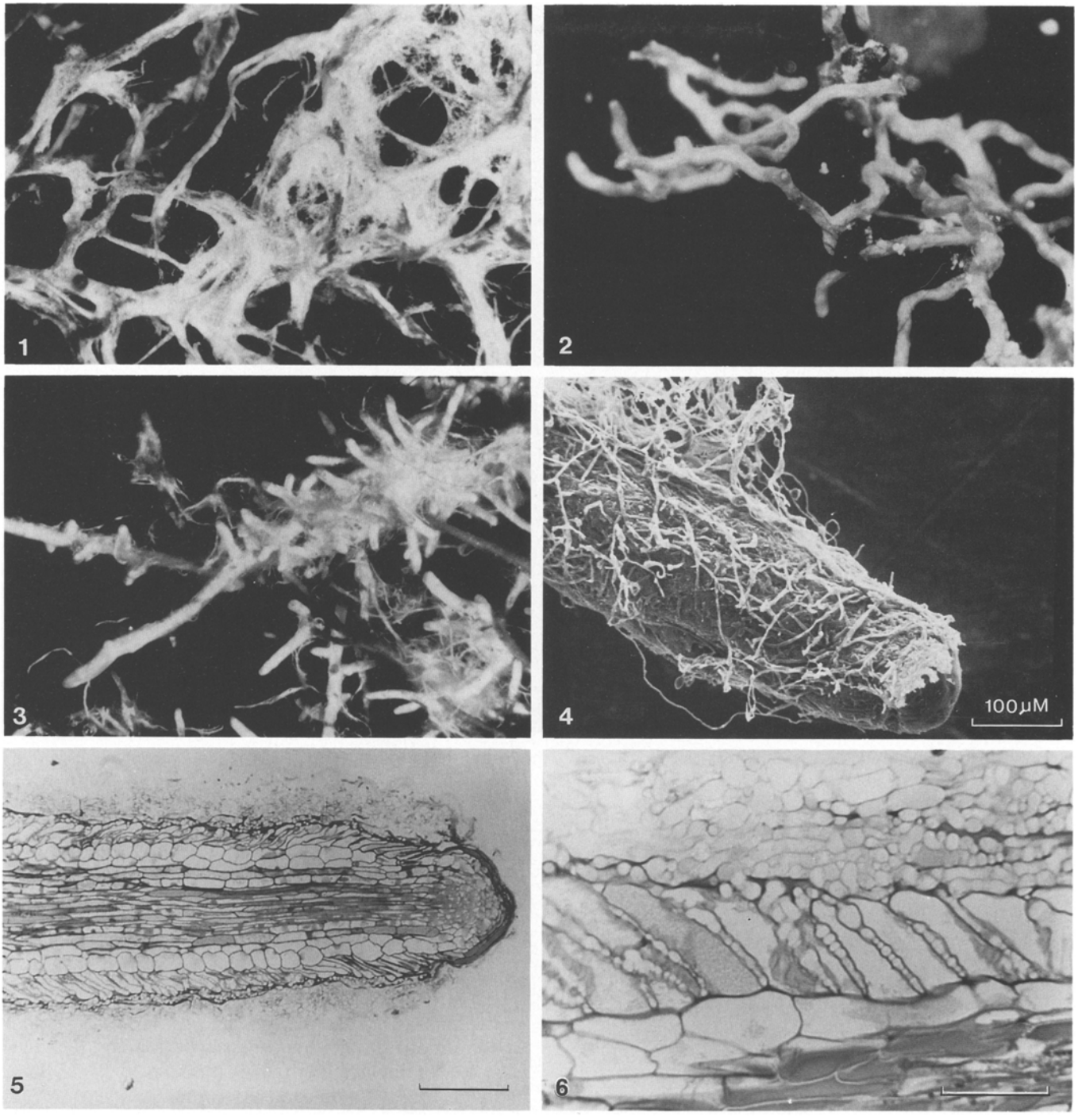
Mycorrhizas formed by Hymenogaster sp. Mycorrhizas were white and cottony and colonization of the growing roots by means of concolorous hyphal strands was quite rapid.

Mycorrhizal roots were generally pinnately branched and the main axis was <4 mm long and <0.5 mm in diameter (Fig. 9).

The mantle surface consisted of an outer zone of loosely interwoven hyphal strands and an inner amorphous hyphal zone of loosely packed hyphae (Fig. 10). Emanating hyphae generally ran parallel to each other, were 2–5 µm wide and bore clamp connections.

The mantle was 20–50 µm thick and made up of an outer prosenchymatous layer and an inner synenchymatous layer. The para-epidermal Hartig net developed some distance away from the root apex and the radial elongation of the epidermal cells was 2:1.

Mycorrhizas formed by C. geophilum. Typically, this fungus produced a slow-growing mycelial mat of dark coloured, verucose hyphae which were never seen to produce mycelial strands. Mycorrhizas were simple, short, club-shaped structures occasionally with single branches (Fig. 11).



Figs. 1, 2. Naturally occurring mycorrhizas of *Quercus*. Note thickened roots and white rhizomorphs

Fig. 3. External morphology of synthesized ectomycorrhizas of *Pisolithus tinctorius* showing mycorrhizal clusters intermingled with mycelial strands. ×10

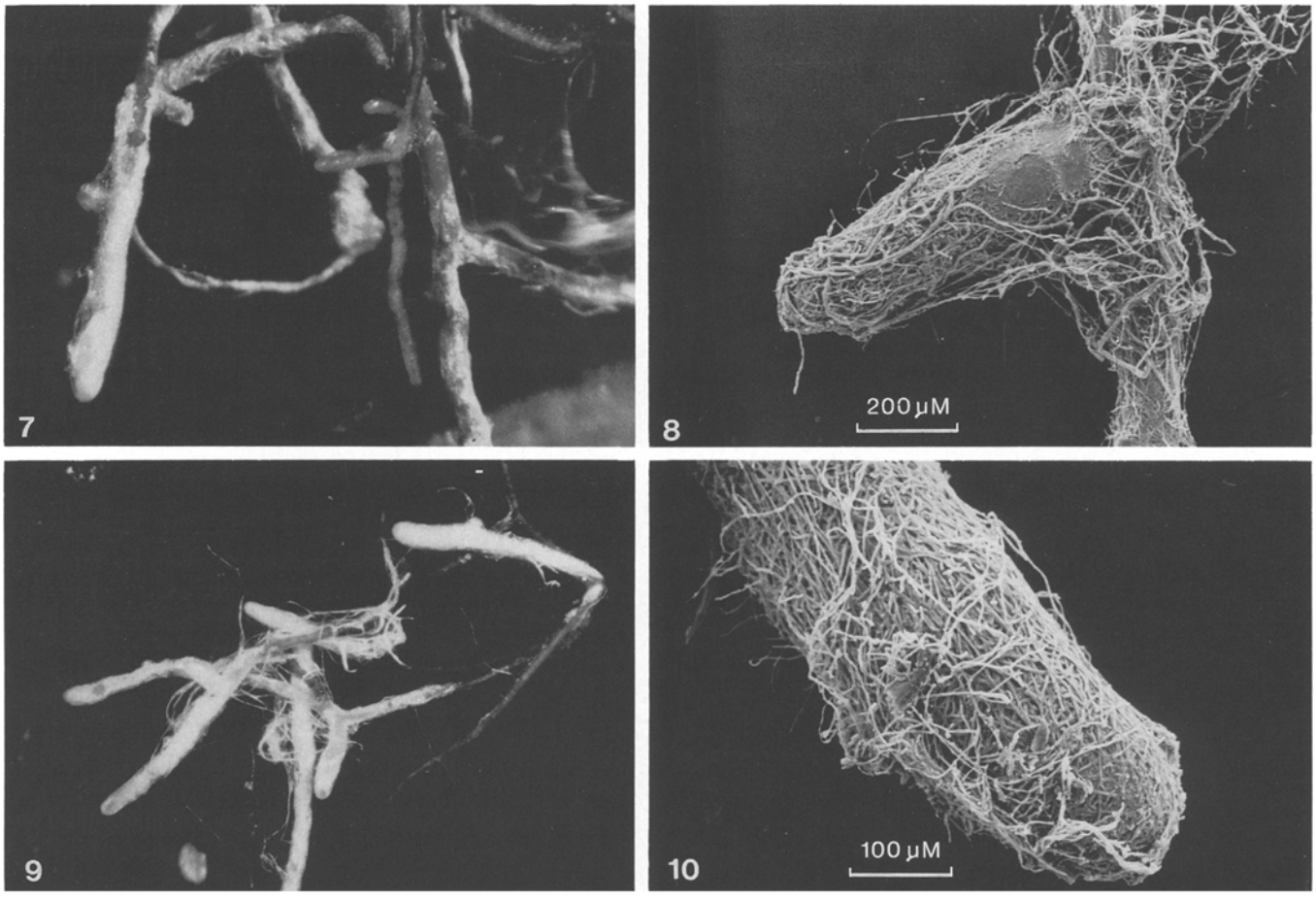
Fig. 4. Scanning electron micrograph of a synthesized ectomycorrhiza of *P. tinctorius* showing loosely interwoven hyphal strands on the compact amorphous matrix. Note the unsheathed root apex

Fig. 5. Longitudinal section of a synthesized ectomycorrhiza of *P. tinctorius* with an unsheathed apex. *Scale bar*, 100 μm

Fig. 6. Enlarged portion of a synthesized ectomycorrhiza of *P. tinctorius* showing the structure of the mantle and Hartig net. *Scale bar*, 25 μm

The mantle surface was composed of tightly packed verucose, dark hyphae without clamp connections (Fig. 12).

The mantle was 5–10 μm thick, usually located near the root apex, and composed of thick-walled melanized synenchymatous hyphae. The remains of non-functional root hairs were frequently observed at the base of the mycorrhizas. The para-epidermal to peri-epidermal Hartig net was not uniform and had a radial elongation ratio of epidermal cells from 2:1 to 3:1.



Figs. 7–10. External morphology (×8) and scanning electron micrograph of ectomycorrhizas synthesized with *Scleroderma* sp. **(Figs. 7, 8)** and with *Hymenogaster* sp. **(Figs. 9, 10)**

Mycorrhizas formed by an unidentified, soil-borne ascomycete. These mycorrhizas, developed from unseen hyphae present in natural soil, were simple, short, club-shaped and bore radiating, smooth, black hyphae (Fig. 13). No mycelial strands were observed.

The mantle surface was made up of a smooth, tightly packed matrix of hyphae bearing straight hyphae each 4–6 µm in diameter and without clamp connections.

The mantle was 15–25 µm thick and consisted of two layers of synenchymatous hyphae, an outer layer of thick-walled hyphae and an inner layer of thin-walled, darkly stained hyphae. The para-epidermal Hartig net was uniform and the radial elongation ratio of the epidermal cells was 3:1 (Fig. 14).

Mycorrhizas formed by an unidentified, soil-borne basidiomycete. Mycorrhizas were long, slender, smooth, dark brown and sinuous with frequent, short side branches. Mycelial strands were not seen.

The mantle surface consisted of loosely interwoven hyphae each 2–4 µm in diameter and bearing clamp connections.

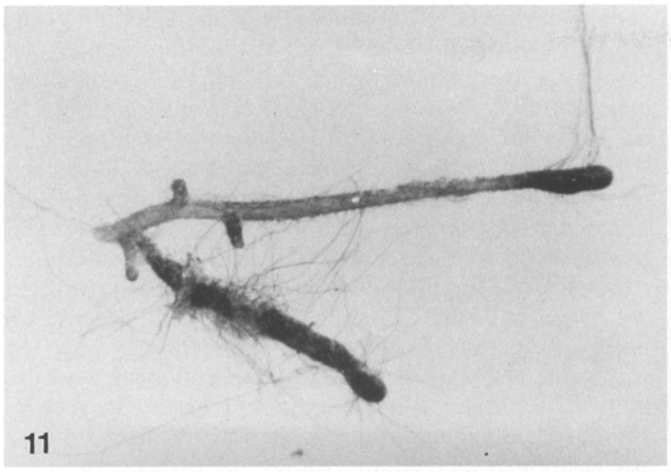
The mantle was 23–30 µm thick and made up of thick-walled synenchymatous hyphae. The para-epidermal Hartig net was uniform and the epidermal cells had a radial elongation ratio of 2:1 (Figs. 15, 16).

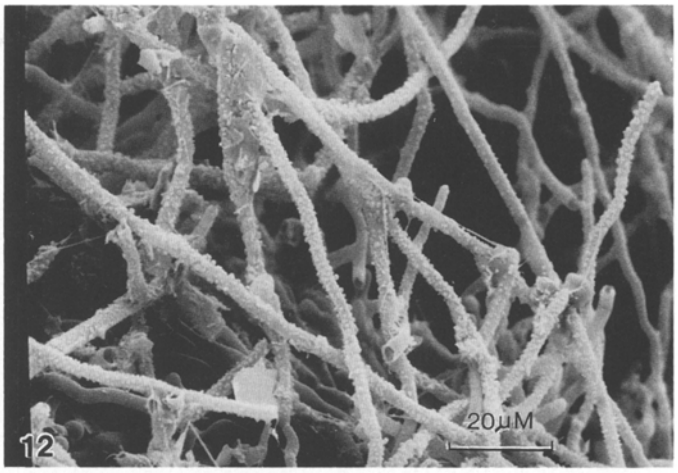
Ephemeral mycorrhizas. Some known ectomycorrhizal fungi became closely associated with growing seedling roots of *Quercus* and exhibited early stages of mycorrhizal formation during the first 4 weeks. However, within a 4-month period the hyphae disappeared and the seedling roots remained uninfected.

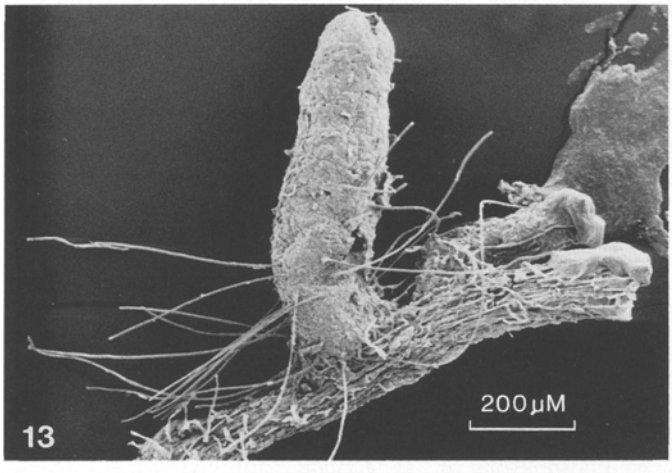
1. *T. terrestris.* A number of feeder roots in contact with the inoculum were colonized by a thick weft of white mycelial strands. The mantle surface consisted of loosely bound emanating hyphae, 2–3 µm in diameter and bearing clamp connections. The mantle was 40–50 µm thick and composed of a uniform synenchymatous layer. Neither a para-epidermal Hartig net nor radial elongation of the epidermal cells was observed.

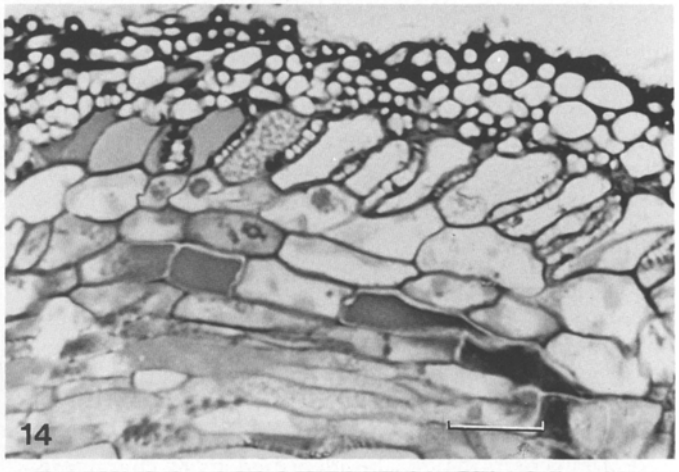
2. *R. roseolus.* Some feeder roots in contact with the inoculum were colonized by a loose weft of creamy-coloured hyphae.

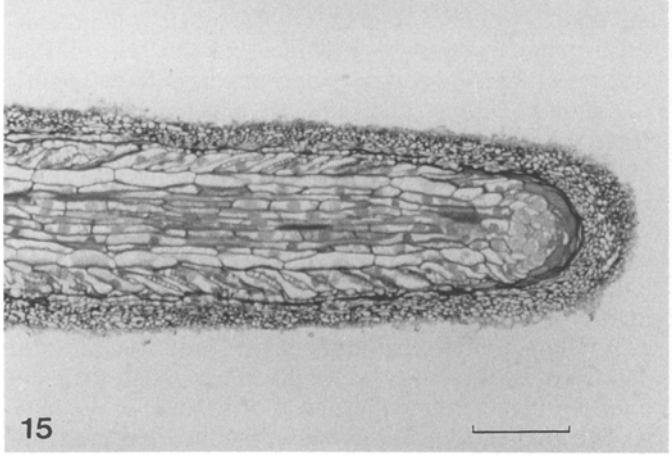
3. Local *Pisolithus sp.* Only a few emergent laterals in contact with the inoculum were colonized by a thin weft of bright yellow hyphae.











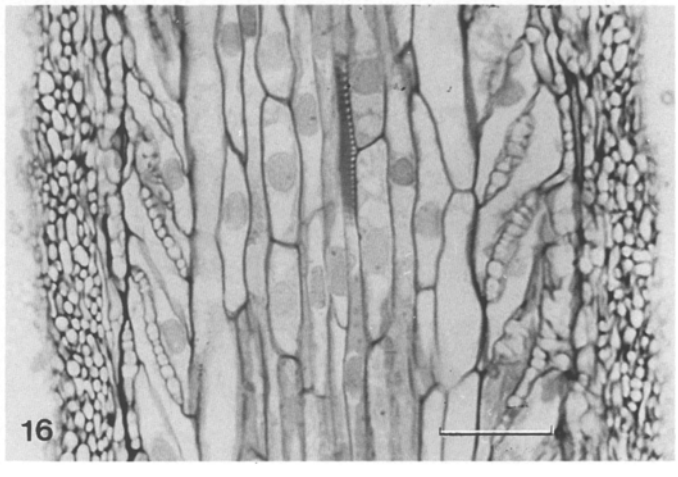


Fig. 11. External morphology of synthesized ectomycorrhiza of *Cenococcum geophilum* showing black, club-shaped tips with radiating hyphae. ×10

Fig. 12. Scanning electron micrograph showing detailed structure of verucose hyphae of *C. geophilum*

Fig. 13. Scanning electron micrograph of a synthesized ectomycorrhiza of an unidentified ascomycete

Fig. 14. An enlarged portion of a longitudinal section of synthesized ectomycorrhiza shown in Fig. 13, illustrating an outer thick-walled and an inner thin-walled synenchymatous mantle. *Scale bar*, 25 µm

Fig. 15. Longitudinal section of synthesized ectomycorrhiza of an unidentified basidiomycete. *Scale bar*, 100 µm

Fig. 16. An enlarged portion of Fig. 15 showing the structure of the mantle and the Hartig net. *Scale bar*, 25 µm

In all three examples, the partially colonized roots showed no radial enlargement and, therefore, no sections were cut. Table 1 summarizes the level of infection in *Quercus* seedling roots by recording the percentage infection of root tips by the various fungi.

Discussion

Our results indicate that *Q. myrsinaefolia* is associated with different fungi under natural conditions and capable, under our experimental conditions, of forming my-

Table 1. Percentage infection of *Quercus* root tips by mycorrhizal fungi after 4 months in artificial soil

Fungi	Infection (%)[a]
Exotic cultures	
Pisolithus tinctorius	70–80
Cenococcum geophilum	5–10
Thelephora terrestris	0
Rhizopogon roseolus	0
Local isolates	
Hymenogaster sp.	30–40
Scleroderma sp.	20–30
Pisolithus sp.	0
Natural soil fungi	10–20
Uninoculated control	0

[a] Values in each case based on three seedlings for each treatment

corrhizal associations with some local Hong Kong fungi as well as with two imported species known to form mycorrhizas with a wide range of trees. The fungi employed in this study were also successful in forming mycorrhizas with local species of *Pinus* and with exotic species of *Pinus* and *Eucalyptus* (Chan and Griffiths 1988, 1991).

We also demonstrated that *Q. myrsinaefolia* can form mycorrhizas with an unidentified ascomycete and an unidentified basidiomycete, indicating that the broad spectrum of ectomycorrhizal associations possible with our local species of oak is shared with other oak species such as those referred to in the Introduction.

Ectomycorrhizas can be identified from characteristics of the sheath, Hartig net and associated hyphae (Trappe 1967; Zak 1973). The ectomycorrhizas formed with *Q. myrsinaefolia* were all characterized by a well-organized sheath consisting of interwoven hyphae associated with extrametrical mycelial strands or with radiating hyphae. Moreover, in sectioned material they all demonstrated a regular Hartig net between the epidermal cells, similar to that documented by Luppi and Gautero (1967) and Brundrett et al. (1990) in natural ectomycorrhizas in *Quercus* species. The pattern was the same as that formed in *Eucalyptus* (Chilvers 1968; Chan and Griffiths 1991) but different from ectomycorrhizas formed in *Pinus*, where the Hartig net penetrated several layers of cortical cells (Piche et al. 1981; Chan and Griffiths 1988). Hartig net formation observed in *Q. myrsinaefolia* was predominantly para-epidermal, except for that seen in the ectomycorrhiza formed by *C. geophilum*, where some peri-epidermal insertion was also observed. The para-epidermal Hartig net usually had a radial elongation two to three times larger than that measured in uninfected epidermal cells, indicating that the contact surface between the host and the mycobionts had been increased in the interests of more rapid exchange of nutrients and metabolites. In all oak ectomycorrhizas examined here, the Hartig net was confined to the epidermal layer and the mycobionts did not penetrate the cortical region.

A common phenomenon observed in sectioned mycorrhizal roots stained with toluidine blue was the presence of polyphenolic compounds in the epidermal cells which were often absent from the mature Hartig net zone. The intercellular epidermal cell walls, and more conspicuously the outer cortical cell walls, were thickened and impregnated with polyphenols as a result of the direct contact of the hyphae. Polyphenols have been observed in the cap, epidermal and endodermal cells of uninoculated, short roots in *Pinus* by Piche et al. (1981), as well as in similar regions of our uninfected *Quercus* roots, and it may be that some polyphenols present in the epidermal cells of the mycorrhizal root were mobilized during fungal penetration and then resynthesized within the cell walls to prevent intracellular penetration and further invasion of the cortical region. The mobilization of polyphenols by our ectomycorrhizal fungi is still being investigated and will be reported on later.

Godbout and Fortin (1985) stressed that ectomycorrhizas formed by congeneric fungi share similar, distinctive characters. The results obtained with *Pinus* and *Eucalyptus* (Chan and Griffiths 1988, 1991) and with the *Quercus* species investigated here illustrate that ectomycorrhizas formed by the same fungus have similar morphological and structural characters independent of the host plant.

Q. myrsinaefolia can form a variety of ectomycorrhizas with some compatible fungal symbionts both in its natural environment and in inoculated, artificial soil, resulting in a characteristic mantle and a para-epidermal Hartig net. *T. terrestris*, *R. roseolus* and a local species of *Pisolithus* which did not penetrate the host roots or which did not develop a Hartig net were considered to be incompatible or "superficial ectomycorrhizas", to use the term of Malajczuk et al. (1984, 1987). It is not surprising that *R. roseolus* was incompatible with oak as isolates of *Rhizopogon* have been reported to be specific to conifers (Harley and Smith 1983; Torres et al. 1991). However, the broad host-range mycorrhizal fungi such as *T. terrestris* and the local isolates of *Pisolithus* also failed to form ectomycorrhizas, possibly due to their incompatibility with the host roots; other factors such as the growth characteristics of fungi, the concentration of inoculum and the growth conditions may also have been involved. To date, little is known of the mechanism of compatibility between the mycosymbiont and the host root. However, in our experiments *P. tinctorius* exhibited the highest percentage of infection within the same growth period, indicating that it was the most compatible mycorrhizal fungus with *Q. myrsinaefolia* of those observed. Therefore, it was selected for further investigations of its effect on the infection, growth, and nutrient uptake of oak seedlings. The results will be reported in the next paper in this series.

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