Mycorrhiza (1993) 3:165–170

Mycorrhiza

Mycorrhizal associations in Hong Kong Fagaceae

V. The role of polyphenols

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Abstract. Polyphenols histochemically detected in fresh uninfected roots of *Quercus*, *Castanopsis* and *Lithocarpus* growing in Hong Kong and shown to be condensed tannins were found mainly as intracellular material in the cells of the root cap, the epidermal layer and the endodermis. The cell walls of the outer cortex and the endodermis also contained suberin. Following invasion by compatible ectomycorrhizal symbionts, condensed tannins disappeared from cells of the root cap and the epidermal layer but hyphae were prevented from colonizing the cortex presumably due to suberin barriers. In vitro experiments indicated that a number of broad-host ectomycorrhizal fungi could utilise various polyphenolic compounds, including tannins found in the root exudates of the host trees, with different degrees of efficiency.

Key words: Ectomycorrhizas – Polyphenols – *Quercus* – *Castanopsis* – *Lithocarpus* – Hong Kong

Introduction

Tannins are polyphenolic compounds with relative molecular weights of 500–3000 and a propensity to form complexes with proteins and other macromolecules. There are two major structural groups of tannins: the condensed tannins, which consist of oligomers of catechins (flavan-3-ols) and leucocyandin (flavan-3,4-diols) and are distributed most widely in vascular plants, especially in regions of active growth (Nierenstein 1934), and hydrolysable tannins, which are composed of gallic acid or ellagic acid esterified to a sugar moiety and are restricted to dicotyledenous plants. Several families, including the Fagaceae, retain the capacity to synthesize different types of tannins (Haslam 1981); however, little is known about the kinds of tannins present in seedling roots of the local species of Fagaceae, *Quercus*, *Castanopsis* and *Lithocarpus*, which have been shown to form ectomycorrhizas with various fungal isolates (Tam and Griffiths 1993a). One feature of particular interest that emerged from our studies was the frequently observed change in intracellular polyphenols in the infection zones of sectioned *Quercus* ectomycorrhizas (Tam and Griffiths 1993b, c).

The purpose of this present study was to localize histochemically the polyphenols present in uninfected roots of these species of Fagaceae and to examine in greater detail the changes in polyphenols in the infection zones of *Castanopsis* and *Lithocarpus* ectomycorrhizas by means of light microscopy and confocal microscopy. *Quercus* ectomycorrhizas were also examined by means of transmission electron microscopy. Furthermore, we investigated whether the various mycorrhizal fungi used in our studies could mobilize different kinds of polyphenols in vitro.

Materials and methods

Histochemical localization of polyphenols in uninfected seedling roots

One-month-old seedlings of *Quercus*, *Castanopsis* and *Lithocarpus* were obtained from the paper-wick test tube method (Tam and Griffiths 1993a). Freshly prepared 2,4-dimethoxybenzaldehyde (DMB), used by Mace and Howell (1974) for detecting catechin and its derivatives as a bright red reaction product, was used to localize histochemically the polyphenols present in whole mounts of the first-order lateral roots and in free-hand sections.

Fungal cultures

Local isolates of *Scleroderma* sp. and *Hymenogaster* sp., together with imported isolates of *Pisolithus tinctorius* (ATCC 38054), *Cenococcum geophilum* (ATCC 38052) and *Thelephora terrestris* (ATCC 38058) (obtained from the American Type Culture Collection) were maintained on modified Melin-Norkrans (MMN) agar medium (Marx 1969).

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Mycorrhizal roots

Different mycorrhizas obtained by the paper-sandwiched glass plate method (Tam and Griffiths 1993a) were collected for further histological examination.

Microscopy

Light microscopy. This was carried out as described previously (Tam and Griffiths 1993c) using the following reactions to demonstrate polyphenols in glycol methacrylate (GMA) stained sections:

1. Toluidine blue 0 (0.05%) in 1% sodium benzoate at pH 4.4. The presence of condensed tannins is indicated by blue-green to yellow-green coloration (Sakai 1973).

2. Vanillin-HCl (1 g vanillin in a mixture of 50 ml absolute ethanol and 10 ml conc. HCl). Condensed tannins give a distinctive red colour when aldehyde groups in the vanillin condense with polyphenols (Ling-Lee et al. 1977).

3. DMB reagent (as described above).

4. Lipids stain in Sudan black B (1% solid dissolved in 70% ethanol). GMA sections were stained for 10 s, rinsed in distilled water and mounted in 50% glycerol. Lipids or suberin stained dark blue (Clark 1980).

Laser scan confocal microscopy. Fresh mycorrhizal roots were washed thoroughly to remove any adhering particles and the sheath at the tip was carefully cut open and moved aside by a fine forcep. The preparation was mounted in 50% glycerol and examined under the confocal microscope (Bio-Rad MRC600) using an Argon ion laser as a light source at 488 nm with a blue, high-sensitivity filter. The exposed infection zones were scanned and photographed at eight different levels of focus in 0.3-μm steps.

Transmission electron microscopy (TEM). Mycorrhizal root tips were cut into 1–2 mm segments and fixed in 4% glutaraldehyde in 0.025 M phosphate buffer (pH) at 4° C for 10–12 h, and post-fixed at 1% osmium tetroxide in the same buffer for 2 h. After fixation, the root segments were washed, dehydrated in a graded series of ethanol and embedded in low-viscosity resin (Spurr 1969) which was polymerized overnight at 65° C. Ultrathin sections were cut with glass knives on a Reichert OMU3 ultramicrotome. Sections were collected on formvar-coated copper grids, stained with lead citrate and uranyl acetate and examined with a JEOL 100SX electron microscope.

Preparation of root exudates from Castanopsis fissa seedlings

About 200 germinants with seed-coats removed were placed on perforated plastic plates with their emergent radicles pointing downward and the plates were suspended in enamel trays containing distilled water. Large transparent polyethylene bags pierced with small holes were used to cover these preparations to maintain moist conditions and to allow for gaseous exchange. The trays were placed in a temperature-controlled propagation unit at 26–28° C with a photoperiod of 16 h. The water containing the root exudate was collected once a week and stored at 5° C and the trays were refilled with distilled water. After about 1 month, the bulked root exudate solution was suction-filtered and the filtrate was concentrated in vacuo at 40° C to a small, measured volume. The concentrated brown, viscous liquid contained polyphenols and root mucilage, and the total amount of polyphenols in this exudate was determined by the method described below.

Preparation of media supplemented with root exudate and known polyphenols

MMN mineral solution (supplemented with 0.1% glucose as a starter carbohydrate) was used as the basal medium. Screw-capped conical flasks (100 ml) containing 25 ml of the basal medium were autoclaved and divided into four lots, each consisting of 15 flasks inoculated with two 5-mm diameter circular plugs cut from the margins of actively growing test mycorrhizal fungi: *P. tinctorius*, *C. geophilum*, *Scleroderma* sp. and *Hymenogaster* sp. The flasks were incubated at 25° C in the dark until mycelia were visible radiating from the plugs.

Tannic acid (a hydrolysable gallotannin), gallic acid (a gallotannin precursor) and catechin (a condensed tannin precursor) were dissolved separately in basal medium at a concentration of 2.5 mg/ml and the solutions were Millipore-filtered to maintain sterility. Root exudate equivalent to 2.5 mg/ml tannic acid determined as described below was also Millipore-filtered. Aliquots (1 ml and 0.5 ml) of each of the above solutions was pipetted aseptically into the liquid fungal cultures to final polyphenol contents in the liquid medium of 0.1 mg/ml and 0.05 mg/ml, respectively. Liquid fungal cultures without the addition of polyphenols were kept as controls. The initial and subsequent total polyphenol contents in each liquid culture were determined by removing aseptically 1-ml aliquots to a set of test tubes for colorimetric determinations. The procedure was repeated at fortnight intervals. At the end of 4 weeks, the mycelium of each culture was filtered and washed thoroughly with distilled water. The pH of the filtrate was measured and the mycelium of each culture was dried at 80° C for dry weight determination. The disappearance of polyphenol in the medium was determined from the difference between the optical densities of the initial (ODi) and final (ODf) total polyphenol contents, and was expressed as a percentage of the initial content, $\frac{\mathrm{ODi}-\mathrm{ODf}}{\mathrm{ODi}} \times 100$.

Determination of total polyphenol content

A sample (1 ml) of exudate was added to a test tube and diluted to 7 ml with distilled water. Folin-Ciocalteu phenol reagent (Sigma F9252, stock solution diluted 1:1 with distilled water) (0.5 ml) was added, followed by 1 ml of a saturated sodium carbonate solution after exactly 3 min. The content of the tube was made up to 10 ml with distilled water and mixed thoroughly. After exactly 30 min, the absorbance was measured at 760 mm. A calibration curve was prepared from known concentrations of tannic acid. The test was performed in duplicate for each sample.

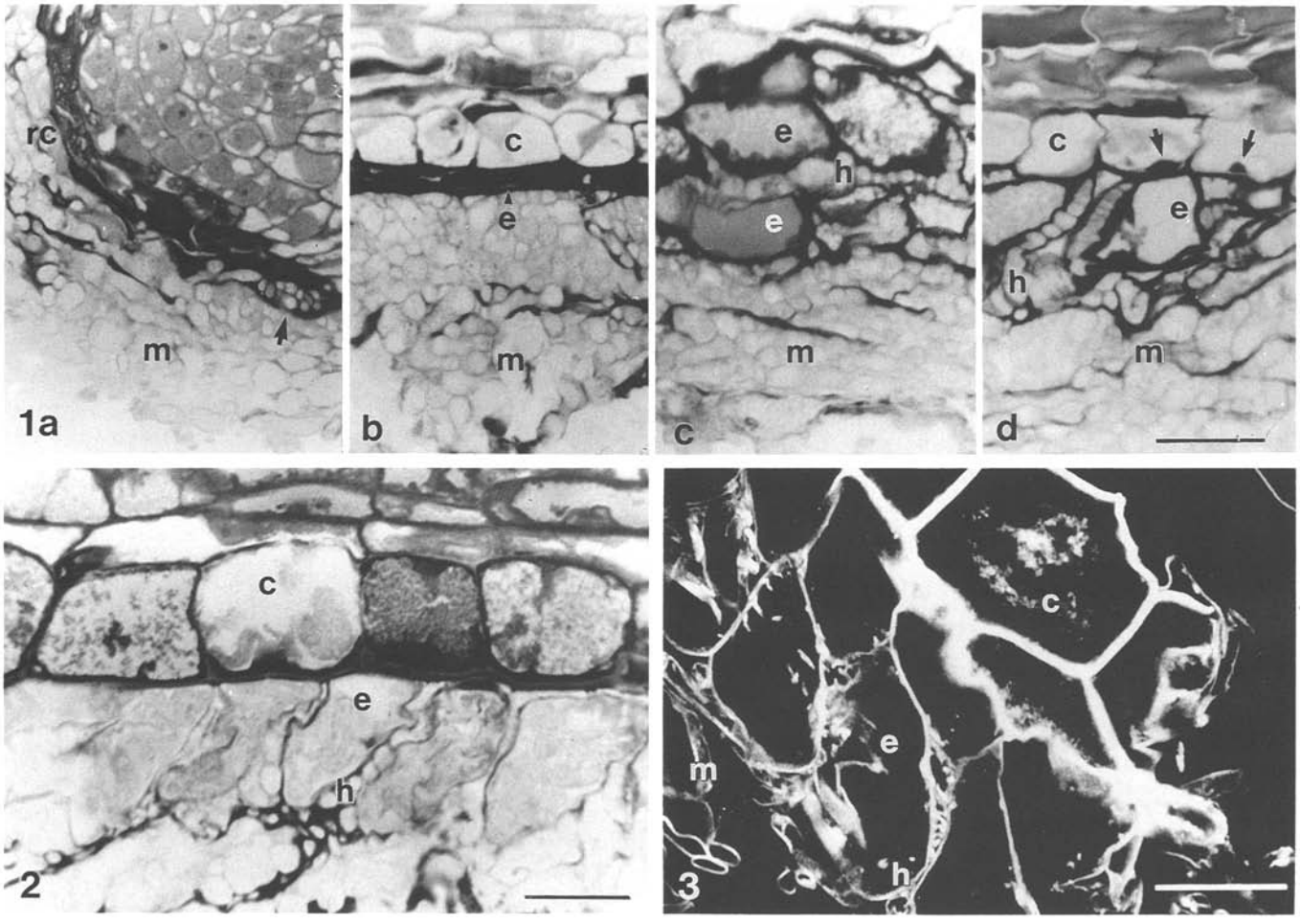
Results

Fresh, uninfected first-order roots of *Quercus*, *Castanopsis*, and *Lithocarpus* gave a distinct red coloration with DMB reagent. Staining was most intense behind the root tip.

Microscopy

In transverse sections of fresh roots, the staining reaction was confined to the epidermal layer, the root hairs and to the cells immediately adjacent to the endodermis; cortical cells remained unstained.

GMA sections of uninfected roots stained with toluidine blue showed distinct blue/green reactions in



Figs. 1–3. Longitudinal sections of mycorrhizal roots. rc, Root cap cell; m, mantle; e, epidermal cell; h, Hartig net; c, outer cortical cell. **Fig. 1a–d.** Glycol methacrylate sections of *Lithocarpus/Thelophora* mycorrhizal root stained with toluidine blue. *Bar* = 10 μm. **a** Blue/green-stained polyphenols of the root cap cells. Note intracellular hyphae (*arrow*). **b** Apposition zone; epidermal cells in contact with hyphal mantle contain unaltered blue/green-stained polyphenols. **c** Hartig net zone; epidermal cells radially enlarged and polyphenols in the form of aggregates lining the inner walls. **d** Vacuolated epidermal cells; walls impregnated with dark blue stained polyphenols lining the inner walls. Patches of dark blue stained polyphenols (*arrows*) are present in the cortical cells. **Fig. 2.** GMA section of *Castanopsis/Pisolithus* stained with toluidine blue. Conspicuous polyphenolic compounds stained purple to blue in the outer cortical cells with much thickened suberized walls. **Fig. 3.** Whole mount of mycorrhizal root of *Castanopsis/Pisolithus* scanned by confocal microscopy at the level where the autofluorescent polyphenolic compounds accumulate on the outer cortical cell walls and inside the cortical cells

cells of the root cap, the epidermal layer and the endodermis. Sections stained in either vanillin/HCl or DMB reagent gave positive red coloration in similar regions. After staining with Sudan black B, a dark blue colour was observed in the cell walls of the outer cortex and the endodermis, but not in the walls of the epidermis.

In GMA sections of mycorrhizal roots of *Lithocarpus/Thelophora*, the blue/green polyphenolic materials in the root cap cells were replaced by intracellular hyphae (Fig. 1a). Similar polyphenols occurring in the inner mantle region were frequently fragmented. In the apposition zone, where the inner mantle hyphae were in contact with the epidermis, the intracellular polyphenols seemed to be unaffected (Fig. 1b). However, in the Hartig net zone, where the hyphae had just penetrated intercellularly, the epidermal cells became radially enlarged and some of the light blue-stained cells containing the homogenous polyphenols had formed dark blue intracellular aggregates which lined the inner walls (Fig. 1c). In the Hartig net zone where the intracellular hyphae had reached the outer cortical walls, some of the epidermal cells became highly vacuolated and thicker dark blue materials impregnated the inner walls. In the same samples, dark blue patches were also seen attached to the inner walls of the cortical cells at the point where the Hartig net hyphae were in contact with the outer cortical walls (Fig. 1d).

The changes in polyphenols appeared more dramatic in the outer cortical cells of the *Castanopsis/Pisolithus* association, where variable forms of purple-blue aggregates were seen (Fig. 2), while in the corresponding cells of the uninfected root only light blue-stained homogenous polyphenols were present. Similar my-

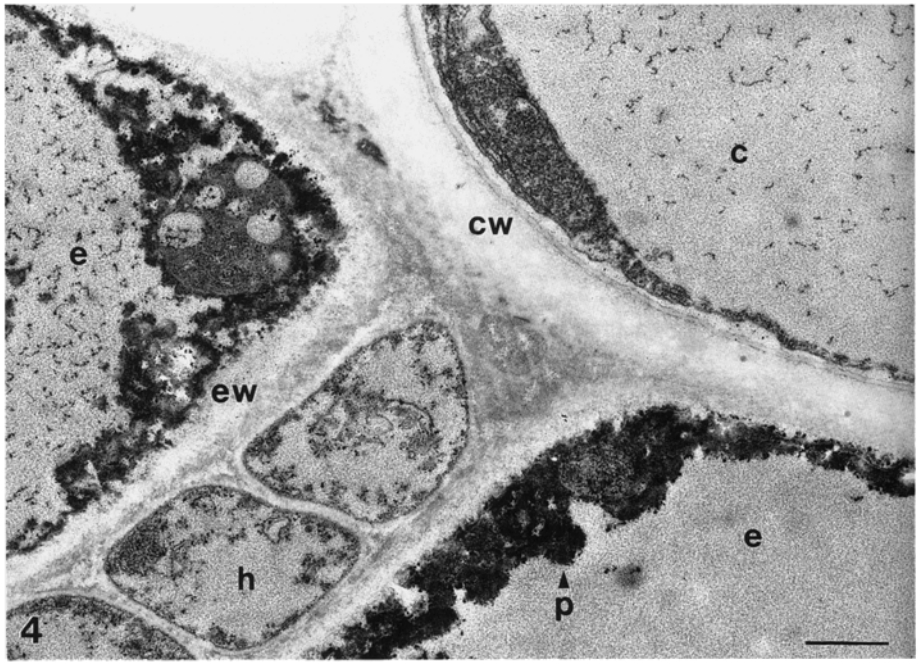


Fig. 4. Electron micrograph of the interface between Hartig net (*h*) and the adjacent host epidermal cells (*e*) in *Quercus/Pisolithus* association. Note electron-opaque epidermal wall (*ew*) and darkly stained polyphenols (*p*) and electron-lucent cortical wall (*cw*). Other symbols as in Figs. 1–3. *Bar* = 2 μm

corrhizal sections stained with Sudan black B gave a dark blue reaction with the thick outer cortical walls and the polyphenolic aggregates.

A fresh, whole mount of *Castanopsis/Pisolithus* short root scanned under confocal microscopy illustrated vividly the polyphenol changes in vivo. This method of visualization precludes the possibility that the polyphenolic reactions seen are associated with the various organic solutions used in histological preparation. Diffuse, autofluorescent polyphenols distinguishable from the distinctly lined autofluorescent intercellular hyphae and epidermal cell walls were clearly observed near the Hartig net region. The suberized outer cortical walls were strongly fluorescent and the autofluorescent aggregates were clearly seen accumulated along the outer cortical walls as well as inside the cortical cells (Fig. 3).

Electron micrographs of a mycorrhizal root of *Quercus/Pisolithus* gave further evidence that intracellular polyphenols were broken down by the invading hyphae. At the Hartig net region, there was evidence of a modification of host cell walls with the formation of an electron-opaque matrix; this was in marked contrast to the unmodified electron-lucent (suberized) outer cortical walls. In the epidermal cells, the darkly stained polyphenolic granules masked the organelles and the plasmalemma, while in the outer cortical cells these granules appeared to be smooth and were seen adjacent to the plasmalemma (Fig. 4).

Polyphenol mobilisation

The comparative rates of polyphenol mobilisation by the four ectomycorrhizal fungi are shown in Tables 1 and 2. Mycelial dry weight data indicates that there was no apparent toxic effect of the polyphenols on mycelial growth of the four fungi. In both cases, the final pH was similar and the disappearance of polyphenols was faster at the lower concentration. In general, the results show that the test fungi could mobilize gallic acid, tannic acid, and catechin comparatively more easily than polyphenols present in the root exudate, and these were almost completely utilized after 28 days by *C. geophilum*; this fungus thus appeared to be the most efficient polyphenol mobilizer.

Discussion

The polyphenols found in the roots of *Quercus*, *Castanopsis* and *Lithocarpus* are condensed tannins and their precursors are catechins. Nierenstein (1934) suggested that tannins may serve as biotic defence polymers due to their fungitoxic and antipredatory properties and that they could protect the plants against abiotic stresses due to their water-binding and antioxidant abilities. Recent research suggests condensed tannins have a structural role much like that of lignin (Pizzi and Cameron 1986), and they may also be involved in suberin structure (Cottle and Kolattukudy 1982). Because catechins are structurally similar to lignin and suberin phenolic precursors, Chalker-Scott and Krahmer (1989) postulated that they could become part of the cell wall by oxidative polymerization.

These polyphenolic compounds are mainly distributed intracellularly in the cells of the root cap, epidermis and endodermis, while suberin is found in the cell walls of the outer cortex and endodermis in uninfected roots of Fagaceae, and probably constitute defence barriers against fungal invasion. However, in mycorrhizal roots, mycorrhizal fungi apparently successfully overcame the outer chemical barrier and decomposed the polyphenols of the root cap and root hairs (the

Table 1. Comparative mobilization of polyphenols (0.05 mg/ml) in vitro by *Pisolithus tinctorius*, *Cenococcum geophilium*, *Scleroderma* sp. and *Hymenogaster* sp. in still liquid cultures, 28 days after the addition of polyphenols. GA, Gallic acid; TA, tannic acid; CT, catechin; RT, root exudate; FC, fungal control

Fungus	Polyphenols in medium	Disappearance (%) of polyphenols after		Final pH	Mycelium dry weight
		14 days	28 days		
P. tinctorius	GA	19	22	3.6	10.3
	TA	5	15	3.3	10.2
	CT	13	28	3.2	9.6
	RE	14	14	3.4	9.3
	FC	0	0	3.1	9.6
C. geophilum	GA	65	89	3.2	13.0
	TA	82	94	2.9	13.5
	CT	67	90	2.9	13.1
	RE	11	39	3.3	15.1
	FC	0	0	3.4	14.6
Scleroderma sp.	GA	2	22	3.9	9.6
	TA	2	28	3.5	10.0
	CT	29	55	3.2	11.0
	RE	7	28	3.5	9.6
	FC	0	0	3.1	10.4
Hymenogaster sp.	GA	12	29	4.0	8.9
	TA	11	57	3.5	9.6
	CT	36	52	3.4	9.7
	RE	9	18	3.7	9.9
	FC	0	0	3.2	9.6

Table 2. Comparative mobilization of polyphenols (0.1 mg/ml) in vitro by *P. tinctorius*, *C. geophilum*, *Scleroderma* sp., *Hymenogaster* sp. in still liquid cultures, 28 days after the addition of polyphenols

Fungus	Polyphenols in medium	Disappearance (%) of polyphenols after		Final pH	Mycelium dry weight
		14 days	28 days		
P. tinctorius	GA	12	13	3.6	10.8
	TA	12	12	3.2	11.3
	CT	18	21	3.2	10.7
	RE	13	13	3.3	11.9
	FC	0	0	3.1	9.6
C. geophilum	GA	63	87	3.1	14.5
	TA	68	88	3.0	15.4
	CT	63	92	2.9	16.0
	RE	6	26	2.9	17.8
	FC	0	0	3.0	14.5
Scleroderma sp.	GA	2	22	3.9	9.7
	TA	2	23	3.5	10.3
	CT	11	34	3.4	10.6
	RE	2	19	3.8	9.6
	FC	0	0	3.2	10.5
Hymenogaster sp.	GA	2	15	4.1	8.9
	TA	11	44	3.5	10.7
	CT	15	34	3.4	9.9
	RE	12	15	3.8	10.0
	FC	0	0	3.3	9.8

fragmentary residues of which were seen by light and electron microscopy). It was further demonstrated that the same mycorrhizal fungi in vitro were able to mobilize various kinds of tannins and their precursors, possibly through the polyphenoloxidase system.

The changes in polyphenols occurring in advance of fungal penetration were vividly illustrated by light and laser scan confocal microscopy. These changes are evidently induced by the Hartig net hyphae. Furthermore, the detailed change at the interface was seen in electron micrographs where the host epidermal cell wall had lost its integrity and formed an electron-opaque matrix embedding the Hartig net. The polyphenolic granules lining the modified inner walls probably strengthen wall structure and prevent hyphae from intracellular invasion.

Suberin can also function as a barrier to fungus penetration (Bell 1981). It was found particularly in the inner walls of cortical cells and this may be accounted for by the presence of fungi in the Hartig net. Suberin was also reported in the outer cell walls of *Eucalyptus* roots by Ling-Lee et al. (1977), who suggested that this wall characteristic could be responsible for limiting the ectomycorrhizal fungi to the epidermal layer. Alteration of polyphenols in the cortical cell layer as the result of Hartig net hyphal attachment to the outer cortical cell walls was also observed. These cell wall phenomena are analogous to disease resistance responses in plants where the host cells are induced to produce chemical and structural mechanisms that block fungal penetration of host cells. However, in the case of the symbioses demonstrated in ectomycorrhizas, the interactions between the phytobionts and the mycobionts are delicately controlled and maintain the continuous interdependent relationship.

It was also found that roots of *Castanopsis* seedlings exuded a large quantity of polyphenols. In the natural environment, these exuded polyphenols of the rhizosphere may exert an allelopathic effect on other plant species and, at the same time, act as a chemical screen to select compatible mycorrhizal fungi able to withstand the toxicity of these polyphenols. This may result in communities in which pure stands of *Castanopsis* live in association with a selection of compatible mycorrhizal fungi; such pure stands are found in areas of the Tai Po Kau country park located in the northern part of Hong Kong.

Acknowledgement. The authors wish to express their thanks to Mr. Jason W. T. Tam for technical assistance in cutting ultrathin sections and using the confocal microscope.

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