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Mycorrhizal associations in Hong Kong Fagaceae

I. Techniques for the rapid detection and observation of ectomycorrhizas in local genera

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Abstract. A rapid method of screening has been devised whereby the large seeds (acorns) of *Castanopsis*, *Lithocarpus* and *Quercus* growing in Hong Kong can be germinated in the presence of a variety of known mycorrhizal fungi and observations made on the initiation of host/fungal associations. Early stages of Hartig net formation could be detected in bleached roots, and an observation was made on the antibiotic activity of the mycorrhizal fungus, *Pisolithus tinctorius*. The method offers a simple and effective means of assessing the potential of fungi to form mycorrhizal associations under non-sterile conditions.

Key words: Ectomycorrhiza - Screening methods - Fagaceae - Hong Kong

Introduction

The Fagaceae were probably an important element in the oak/laurel forest of Southern China some millenia ago. However, repeated acts of deforestation have now confined them to a few small areas on the Chinese mainland and to a comparative recent regrowth of some genera on the offshore islands, including Hong Kong.

In Hong Kong, the family is represented by the three genera *Castanopsis*, *Lithocarpus* and *Quercus*. Growth of all three is prolific, though no investigation has been carried out to determine whether or not the trees bear mycorrhizas. However, the presence of abundant, larger basidiomycetes in Hong Kong forests (Griffiths 1977), including genera known to be mycorrhiza formers elsewhere, suggests that members of the Fagaceae growing in Hong Kong have mycorrhizal associations similar to those recorded for Fagaceae elsewhere (Newman and Reddell 1987).

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Previous studies on the development of mycorrhizas in tree species have, in the main, been confined to species with small seeds which could easily be surface sterilized. Our attempts to surface sterilize the very large seeds of *Castanopsis*, *Lithocarpus* and *Quercus* were thwarted by the hard seed coats which are impervious to water and, therefore, slow to germinate. In addition, pure culture techniques were impossible due to the presence of intertestal microorganisms attached to the cotyledons. Attempts to control the growth of naturally occurring microorganisms using the technique developed by Griffiths (1965) were also unsuccessful due to the large size of the acorns.

We finally attempted to study mycorrhizal formation with a simple, non-aseptic technique which enhanced germination rates and at the same time offered us a rapid screening system to observe mycorrhizal development.

Materials and methods

Acorns

These ripen from mid-October to mid-November and generally fall to the ground during November. Acorns stored at room temperature and under dry conditions showed low viability and frequently bore extensive fungal contamination, presumably derived from the litter layer. Acorns stored at low temperature and under moist conditions exhibited increased viability.

Seedlings

Ripe acorns were collected from trees of *Quercus myrsinaefolia* Bl., *Castanopsis fissa* Rehd. & Wils., and *Lithocarpus glaber* (Thunb.) Nakai during October and November and stored wet in polyethylene bags at 5° C. Acorns that sank in water were soaked for 2 to 3 days. After removal of the testa, the seeds were allowed to germinate in a shallow tray under running tap water. The germination rates and time required for germination were recorded. Germination rates for intact seeds were obtained elsewhere.

Fungal cultures

Cultures of *Pisolithus* sp., *Scleroderma* sp. and *Hymenogaster* sp. were isolated from sporophores growing at the various sites and 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 obtained from the Centraalbureau voor Schimmelcultures, Baarn and all were maintained on MMN agar medium.

Paper-wick test tube method

Pieces of commercial strength paper towel were rolled into test tubes 15 cm long and 22 mm in diameter. The germinants, selected from the naked seeds, with uncontaminated cotyledons and radicles approximately 1–5 mm long were inserted between the paper and the glass wall of the tube. After moistening the paper with distilled water, the tubes were covered with plastic caps and placed in the dark at room temperature until the first lateral roots emerged. At this stage, seedlings were inoculated close to the lateral roots with 5-mm diameter circular plugs [treated as previously described by Fortin et al. (1980)] of actively growing mycelium of the test fungus growing on MMN medium. The inoculated test tubes were covered with a large transparent polyethylene bag and incubated in a temperature-controlled propagation unit at 26–28° C with a photoperiod of 16 h. The tubes were periodically moistened with distilled water.

Microscopy

Mycorrhizal formation in the test tubes was examined periodically under a stereomicroscope equipped with a camera. Lateral roots enshrouded in wefts of hyphae were photographed and teased out for microscopic examination. The root samples were bleached in 1% sodium hypochlorite to remove any polyphenols and examined under a microscope for mantle and Hartig net formation either unstained or stained with lactophenol/cotton blue.

Paper-sandwiched glass plate method

Infected seedlings which had previously been observed to form ectomycorrhizas readily with compatible mycorrhizal fungi were positioned onto a 20×20 cm glass plate and covered with two or three sheets of Whatman 1 chromatography paper of the same size moistened with water; surface tension secured the seedlings in place. The plates were placed into a compartmented thin-layer chromatography (TLC) tank which contained 250 ml MMN mineral solution. The tanks were covered with a large transparent polyethylene bag and left in a temperature-controlled propagation unit under the conditions described for the paper-wick test tube method. Development of the mycorrhizal seedlings at various stages was observed clearly through the glass plate and periodically photographed. Heavily contaminated chromatography paper and mineral solution were changed at intervals. To speed up the ectomycorrhizal formation of seedlings with relatively fast-growing root systems, pieces of MMN agar medium bearing actively grown mycellium of *P. tinctorius* were introduced to selected areas of growing roots sandwiched between the glass plate and the chromatography papers. These were placed into the TLC tanks containing distilled water instead of mineral solution. Developing roots were sprayed weekly with Hoagland's solution.

Root material was periodically removed and fixed for 20 h in 4% buffered glutaraldehyde at 4° C for histological examination.

Results

The germination of naked seeds of *Lithocarpus* and *Quercus* was significantly higher than intact acorns (Table 1); the difference between the two treatments was less obvious in *Castanopsis*. The naked seeds germinated 3–4 times faster than the intact acorns in all three species.

Variation found in the details of mycorrhizal formation in the three species and the various fungal isolates are given in Table 2. The most rapid association was found with the imported isolate of *P. tinctorius*; this is in marked contrast to the very restricted involvement of the local isolate of *Pisolithus*. *C. geophilum* and *Hymenogaster*, and to a lesser extent *Scleroderma*, were also very compatible symbionts. Hartig net development in these three fungi was achieved both by *C. geophilum* and *Hymenogaster* and to a lesser extent by *Scleroderma*. *T. terrestris* formed a mantle in a relatively short time with all three tree species but *Rhizopogon* failed to form an association in all cases.

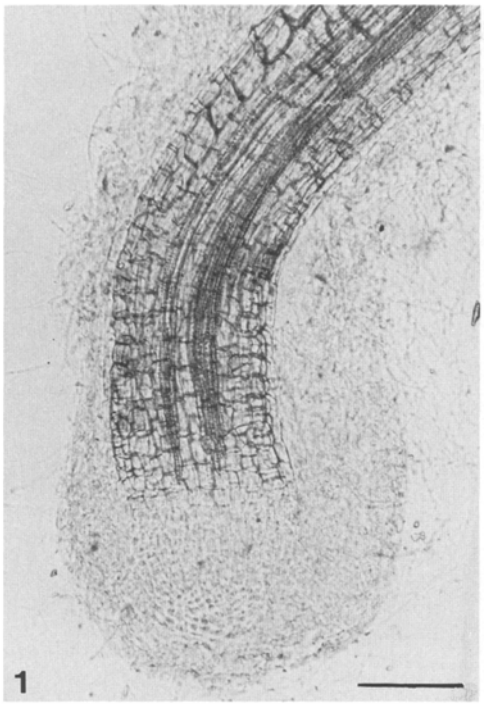
The efficacy of bleached roots to demonstrate mantle and Hartig net formation can be judged from Figs. 1

Table 1. Percentage germination and time lag for germination in three species of Fagaceae

	Percentage germination		Time required for germination (weeks)	
	Intact acorns	Naked seeds	Intact acorns	Naked seeds
Quercus myrsinaefolia	52	92	7–8	2–3
Castanopsis fissa	86	97	4–5	1–2
Lithocarpus glaber	42	83	>16	3–4

Table 2. Details of mycorrhizal formation in three species of Fagaceae with known ectomycorrhizal fungi. + + +, Mycorrhizal synthesis in 1–2 weeks; + +, mycorrhizal synthesis in 2–3 weeks; +, mycorrhizal synthesis in >3 weeks; −, no mycorrhizal formation; M, mantle formation only; HN, Hartig net formation; ATCC, American Type Culture Collection; CBS, Centraalbureau voor Schimmelcultures, Baarn; Pt, *Pisolithus tinctorius*; Tt, *Thelephora terrestris*; Cg, *Cenococcum geophilum*; Rr, *Rhizopogon roseolus*; H, *Hymenogaster* sp.; S, *Scleroderma* sp.; P, *Pisolithus* sp.

	Ectomycorrhizal fungi						
	ATCC cultures			CBS culture,	Local isolates		
	Pt	Tt	Cg	Rr	H	S	P
Quercus myrsinaefolia	+ + + M, HN	+ + M	+ M, HN	−	+ + M, HN	+ + M, HN	+ M
Castanopsis fissa	+ + + M, HN	+ + M	+ M, HN	−	+ + M, HN	+ + M	−
Lithocarpus glaber	+ + + M, HN	+ + M	+ M, HN	−	+ M, HN	+ + M	−



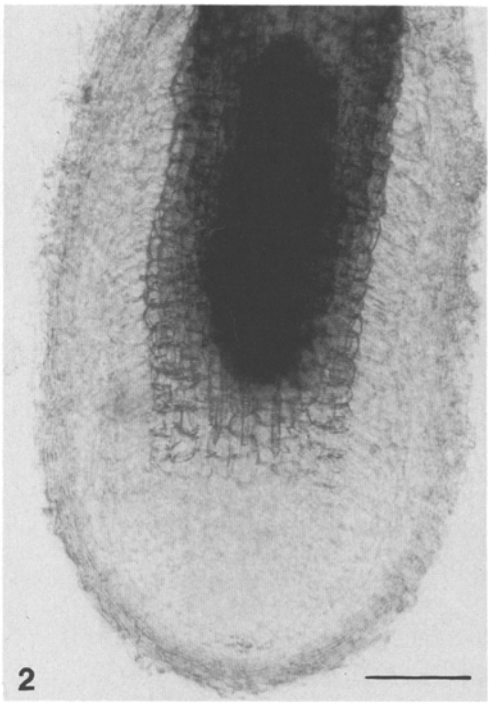


Fig. 1. Whole mount of a bleached mycorrhiza of *Lithocarpus glaber/Thelephora terrestris* showing a mantle but no Hartig net. *Scale bar*, 100 μm

Fig. 2. A whole mount of a bleached mycorrhiza of *L. glaber/Cenococcum geophilum* showing a mantle with a radial elongated epidermal Hartig net. *Scale bar*, 100 μm

and 2; the experimental set up to screen mycorrhizal association is shown in Figs. 3–6.

An interesting feature of the system was the development of zones of antibiosis on the chromatography paper bearing roots of *Castanopsis* and the isolate *P. tinctorius* (Fig. 7). This illustrates the ability of this isolate to restrict hyphal growth by contaminating saprophytic fungi. Details of early mycorrhizal development are shown in Figs. 8 and 9, where hyphae from the original inoculum are shown colonizing newly emerged lateral roots.

Discussion

Based on our observations, acorns retained a high level of viability when stored in the cold under wet conditions. We also noted that naked seeds devoid of their testa gave a higher percentage germination in running water and germinated more rapidly than intact seeds. Removal of the testa removed the layers impervious to water due to the presence of suberin, condensed tannins and lignins (Chalker-Scott and Krahmer 1989), and also removed a source of frequently found polyphenols in the Fagaceae (Haslam 1981), which are known inhibitors of germination (Buta and Lusby 1986).

We have also shown that our method allows rapid screening of potential mycorrhizal fungi under non-aseptic conditions that allow the introduction of a variety of potential symbionts. Furthermore, observations could be made on root colonization, development of a hyphal mantle and, following bleaching of the root, elaboration of a Hartig net.

Our paper-sandwiched glass plate method offers the following advantages over the plastic pouch technique of Fortin et al. (1980):

a) Large seeds can be employed and thus large seedlings with their root systems (measuring up to 25 cm in the case of *Lithocarpus* and *Castanopsis*) can be easily examined.

b) Mycorrhizal development can be photographed sequentially.

c) The large tank provides adequate aeration.

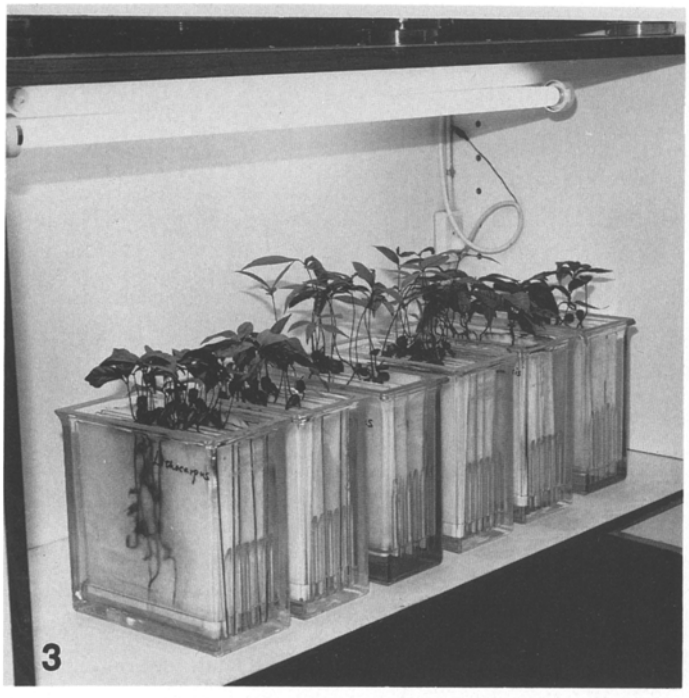
d) Contaminated absorptive paper can be removed easily.

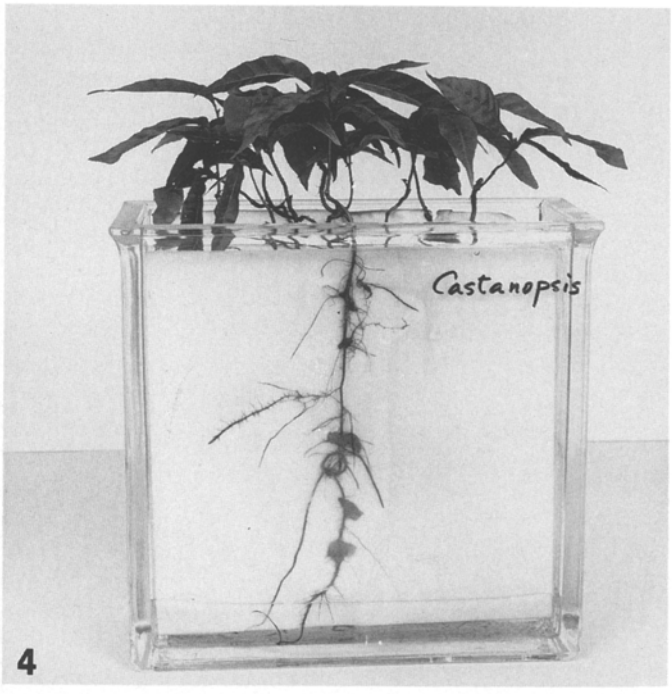
e) Fungal inocula can be introduced to all and every part of the root system of the seedling.

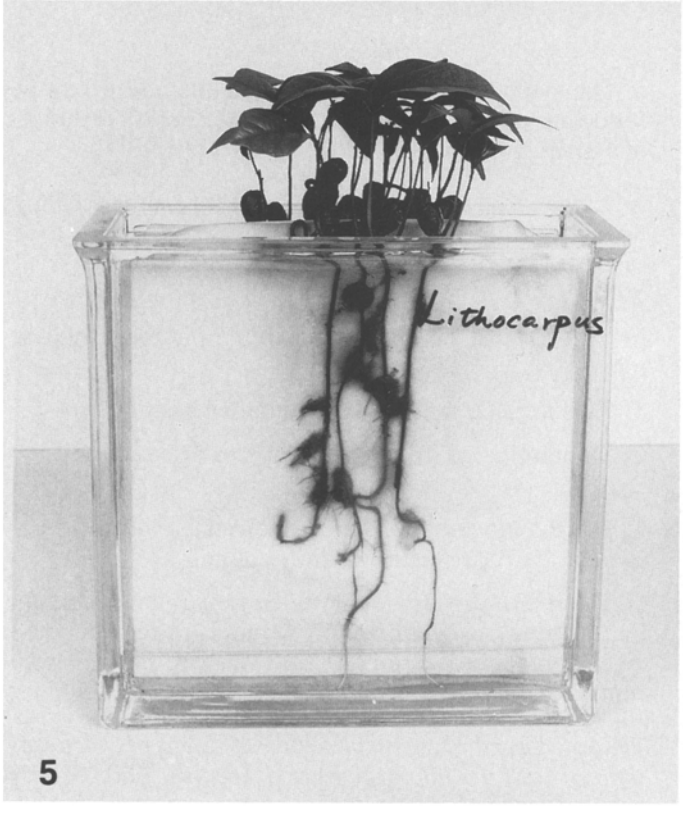
f) The use of distilled water in the tank reduces contamination and mycorrhiza formation is often enhanced when roots are occasionally sprayed with nutrient solution.

We noted during mycorrhizal development between *Castanopsis* and *P. tinctorius* that a zone of inhibition developed around the actively growing fungal colony. Such anti-fungal activity against various pathogenic fungi was previously reported for this fungus by Kope and Fortin (1990).

The system described in this paper has advantages with large-seeded trees where chemical sterilisation of seeds to produce aseptic cultures is difficult or impossible. Furthermore, mycorrhizal symbionts grow in the presence of naturally occurring fungi present on the cotyledons and conditions are thus less artificial than in an aseptic system. Our system can be routinely adopted for







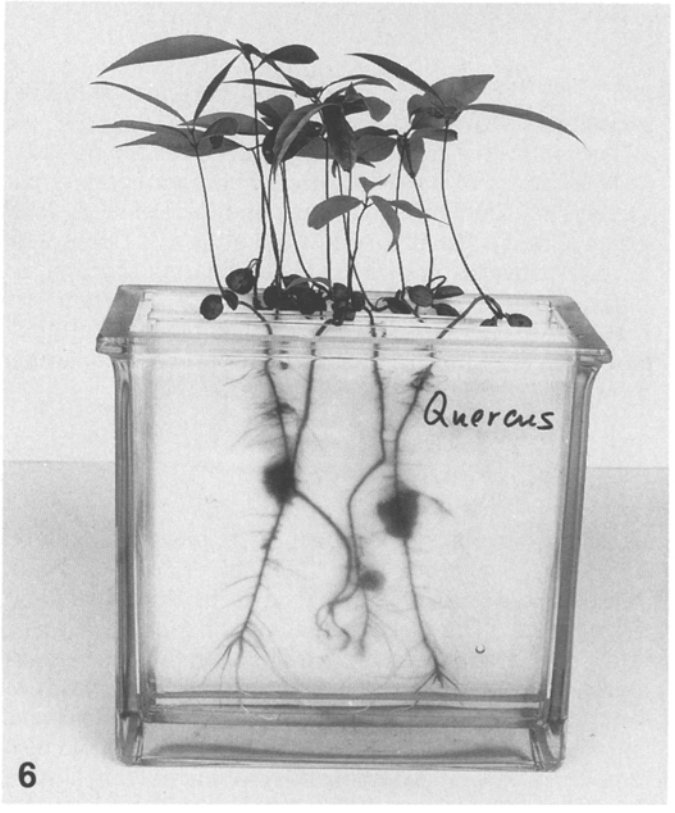


Fig. 3. An array of thin-layer chromatography tanks containing seedlings sandwiched between chromatography papers and glass plates growing in an illuminated temperature-controlled propagation unit. ×0.1

Fig. 4. Seedlings of *Castanopsis fissa* infected with *Pisolithus tinctorius* forming mycorrhizas. ×0.25

Fig. 5. Seedlings of *L. glaber* infected with *P. tinctorius* forming mycorrhizas. ×0.25

Fig. 6. Seedlings of *Quercus myrsinaefolia* infected with *P. tinctorius* forming mycorrhizas. ×0.25

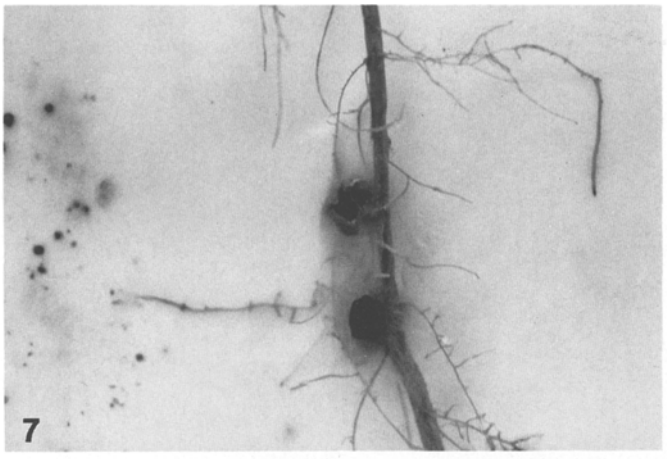


Fig. 7. Mycorrhizas of *C. fissa* showing antifungal properties. ×0.7

Fig. 8. Mycorrhizas of *L. glaber*. ×1.4

Fig. 9. Mycorrhizas of *Q. myrsinaefolia*. ×1.4

screening potential mycorrhizal symbionts and early stages of the association can be studied with ease; it is also a useful tool in physiological studies of mycorrhizal symbiosis.

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