

## Mycorrhizal associations in *Pinus massoniana* Lamb. and *Pinus elliottii* Engel. inoculated with *Pisolithus tinctorius*

Paul C. F. Tam

Department of Botany, University of Hong Kong, Pokfulam Road, Hong Kong

**Abstract.** Dichotomous mycorrhizas were induced in *Pinus massoniana* and *Pinus elliottii* seedlings inoculated with *Pisolithus tinctorius* growing under non-axenic conditions. Six months after inoculation, *Pinus massoniana* seedlings exhibited a higher degree of infection, bore more mycorrhizas and had developed more abundant extramatrical mycelium than seedlings of *Pinus elliottii*. Nevertheless, seedlings of *Pinus massoniana* were stunted and exhibited chlorosis of the needles, indicating a possible nutrient deficiency. Histological examination of these pine mycorrhizas showed an ectomycorrhizal association typical of gymnosperms with an intercellular Harting net penetrating between several layers of cortical cells close to the endodermis. However, strong polyphenolic reactions, intracellular hyphae and wall modifications were occasionally observed, indicating that both host-tissue incompatibility and ectendomycorrhizal association can occur in pine species under stressed conditions.

**Key words:** *Pinus massoniana* – *Pinus elliottii* – *Pisolithus tinctorius* – Ectomycorrhizas – Ectendomycorrhizas

### Introduction

*Pinus massoniana* Lamb., ‘Chinese red pine’, is a dominant native conifer which has spread for centuries throughout the temperate regions of southern China and Hong Kong. *Pinus elliottii* Engel., ‘slash pine’, a North American conifer, was introduced into Hong Kong some five decades ago by the Agriculture and Fisheries Department during reforestation programmes. Because of their drought resistance and ability to establish on treeless hill slopes, both pine species were widely planted and are now important woodland components throughout the territory of Hong Kong.

Preliminary investigations by Chan and Griffiths (1988) showed both pine species to be ectomycorrhizal formers with a number of known fungal isolates, in-

cluding the broad-host fungus *Pisolithus tinctorius* (Pers.) Coker & Couch. Several pine species have been reported to develop ectomycorrhizal infections when inoculated with *Pisolithus tinctorius* in semi-axenic conditions of growth in pouches (Fortin et al. 1980; Warrington et al. 1981; Piche and Fortin 1982; Piche et al. 1982, 1983a,b), but little has been reported on ectendomycorrhizal associations under these conditions. However, the prevalence of ectendomycorrhizas in pine species infected by E-strain fungi occurring in forest nurseries has been well documented (Harley and Smith 1983). In this present investigation, the nonaxenic technique (Tam and Griffiths 1993a) was used to synthesize and develop mycorrhizas from two pine species, *Pinus massoniana* and *Pinus elliottii*, inoculated with *Pisolithus tinctorius*. The growth of host seedlings and mycorrhizal development were recorded photographically and mycorrhiza morphology and histology were examined in detail by scanning electron, and both light- and laser scan confocal microscopy.

### Materials and methods

#### Fungal cultures

A culture of *Pisolithus tinctorius* (Pers.) Coker & Couch was obtained from the American Type Culture Collection (ATCC 38054) and maintained on modified Melin-Norkrans (MMN) agar medium.

#### Seedlings

Pine seeds were germinated in petri dishes containing a few pieces of moistened filter paper. Five- to 7-day-old seedlings with no visible fungal contamination were transferred to paper-wicked test tubes and grown as described previously (Tam and Griffiths 1993a). One-month old seedlings were also transferred to paper-sandwiched glass plates. These were inoculated and grown in a compartmented thin-layer-chromatography tank under conditions described previously (Tam and Griffiths 1993a).

## Growth observations

Mycorrhizal development and the growth of the seedlings in the paper-sandwiched glass plates were recorded photographically after 1-month and 6-month periods, respectively, following inoculation. Samples of short roots were removed and fixed in 4% buffered glutaraldehyde and embedded in glycol methacrylate (GMA); 2- to 3- $\mu\text{m}$  sections were cut for light and confocal microscopy. Sections for confocal microscopy were either observed unstained or were stained with 0.1% ethidium bromide in 75% alcohol for 1 min, washed with distilled water and mounted in 50% glycerol. Similar root materials were also processed for scanning electron microscopy as described previously (Tam and Griffiths 1993b).

## Results

### Mycorrhizal development

Seedlings of both pine species produced root systems with numerous first-order lateral roots when sandwiched between chromatography paper and glass plates (Figs. 1, 5). Seven to 14 days after inoculation, lateral roots contacting the hyphae emanating from fungal inocula were induced to form dichotomous short roots which were engulfed by bright yellow hyphal wefts. Two months later, a network of buff-brown extramatrical hyphae was formed which proliferated on the main root and bridged various mycorrhizal apices growing at different locations of the root system (Figs. 2a, b, 6a, b). After 6 months, lateral roots in contact with the fungal inocula were stimulated to form mycorrhizal clusters. Extensive networks of extramatrical hyphae, derived from the mycorrhizal apices and composed of thick mycelial strands and diffuse hyphae, colonized the root systems and interconnected with those of the neighboring seedlings (Figs. 3, 4, 7, 8).

Compared to seedlings of *Pinus elliottii*, the mycorrhizal strands of *Pinus massoniana* were more abundantly produced and the area of the root systems colonized by extramatrical hyphae was much larger; mycorrhizas synthesized in *Pinus massoniana* visibly outnumbered those formed in *Pinus elliottii* (Figs. 4, 8). At the beginning of inoculation, green needles were found on seedling shoots of both pine species. However, after 6 months, most needles, especially those of *Pinus massoniana*, appeared yellow to purple and older needles on the lower portion of the shoot became brown and withered. With regard to the overall growth pattern of seedlings of the two pine species, *Pinus elliottii* exhibited a significant increase in shoot height after 6 months, whereas *Pinus massoniana* showed no shoot height increment even after 5 months. However, seedlings of both pine species after 6 months produced extensive root growth on the chromatography paper.

### Scanning electron microscopy

In the early stages of mycorrhiza formation, mantles were formed on the first-order lateral roots behind the

meristemic apex (Fig. 9). Emerging hyphae, derived from the inner mantle enclosing the base of the lateral root, colonized the isodiametric apical cells and a few highly branched hyphae were seen tightly appressed to the apical surface (Fig. 10). The inner mantles of the subapical regions were made up of interwoven hyphae embedded in an amorphous mucilaginous substance (Fig. 11). With increased hyphal ramification on the apical surface, a new mantle composed of interwoven hyphae enclosing the apex and strands of five to seven hyphae grew from the loosely bound outer mantle (Fig. 12).

### Light microscopy

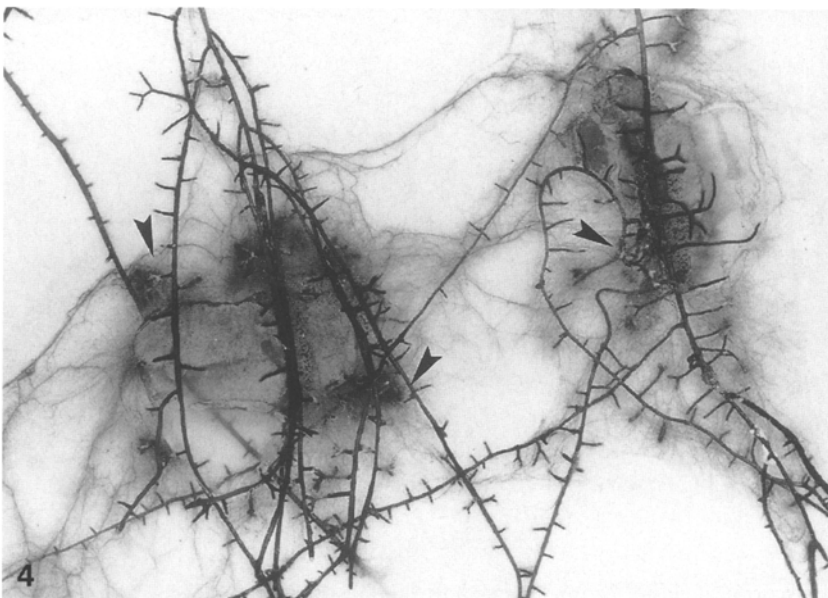
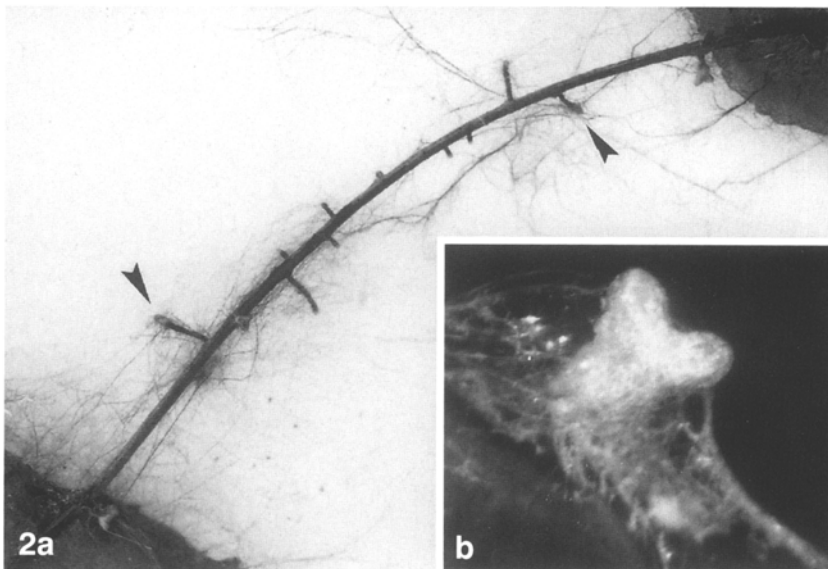
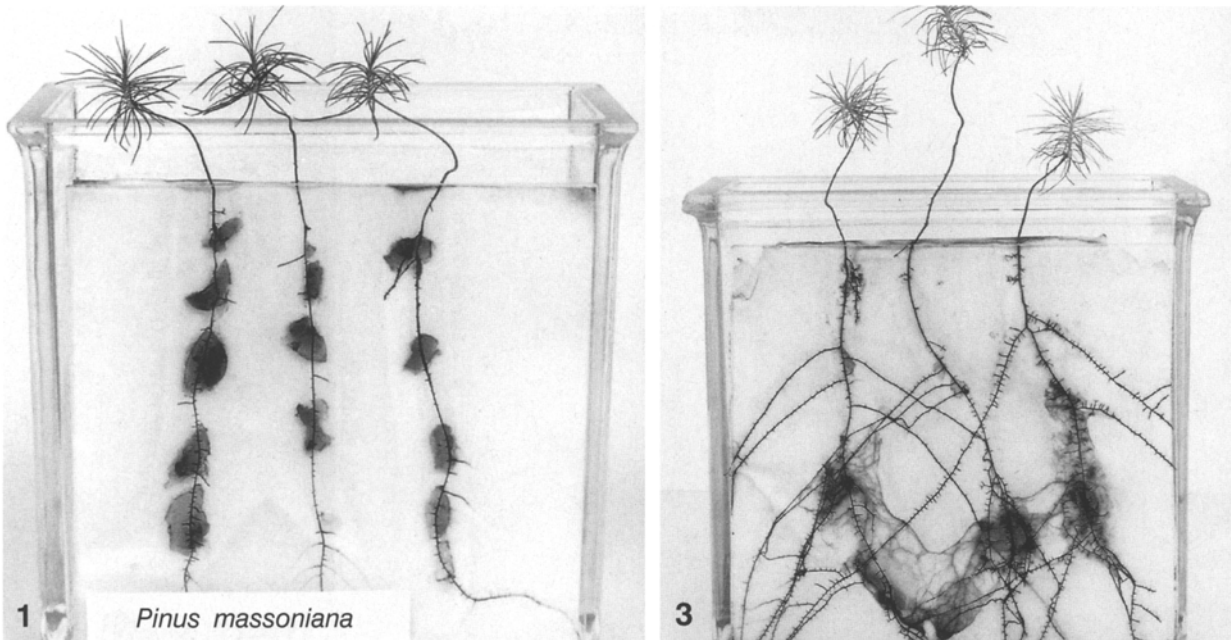
GMA longitudinal sections of synthesized mycorrhizas of both pine species with *Pisolithus tinctorius* stained with toluidine blue showed the histological features of two different types of mycorrhizal association.

*Ectomycorrhizal association.* Ectomycorrhizas were composed of a uniform mantle, 20–30  $\mu\text{m}$  thick, with an outer layer of prosenchymatous hyphae and an inner layer of synenchymatous hyphae; the intercellular hyphae of the Harting net penetrated three to four layers of cortical cells close to the endodermis. The nuclei appeared large and somewhat convoluted at the nuclear periphery (Figs. 13, 14).

*Ectendomycorrhizal association.* Ectendomycorrhizas were composed of an irregularly thick mantle, 20–50  $\mu\text{m}$ , thicker towards the base of the lateral root. Strong intracellular polyphenolic reactions occurred in the first and second layers of cortical cells, which were in close contact with the inner mantle hyphae, and a limited Hartig net development was observed in some of this region (Fig. 15). In the area of a Hartig net, intracellular hyphae were often observed and the nuclei were either absent or found to be disintegrated inside the cortical cells. Vesicle-like bodies were frequently associated with the inner cortical walls, which were thickly impregnated with polyphenols (Fig. 16).

### Laser scan confocal microscopy

Unstained longitudinal GMA sections showing the outer loosely bound mantle hyphae, closely packed inner mantle hyphae and the Harting net were autofluorescent, while the dark nuclei were conspicuous against the fluorescent polyphenolic background (Fig. 17). The vesicle-like bodies associated with the inner cortical cell wall were strongly autofluorescent, whereas the disintegrated nucleus and the intracellular hyphae were not (Figs. 18, 19). In contrast, sections stained with ethidium bromide showed fluorescent, fan-like nuclei inside the cell enclosed by the illuminated intercellular Hartig net; the cortical walls were unstained and appeared dark (Fig. 20). Stained sections also



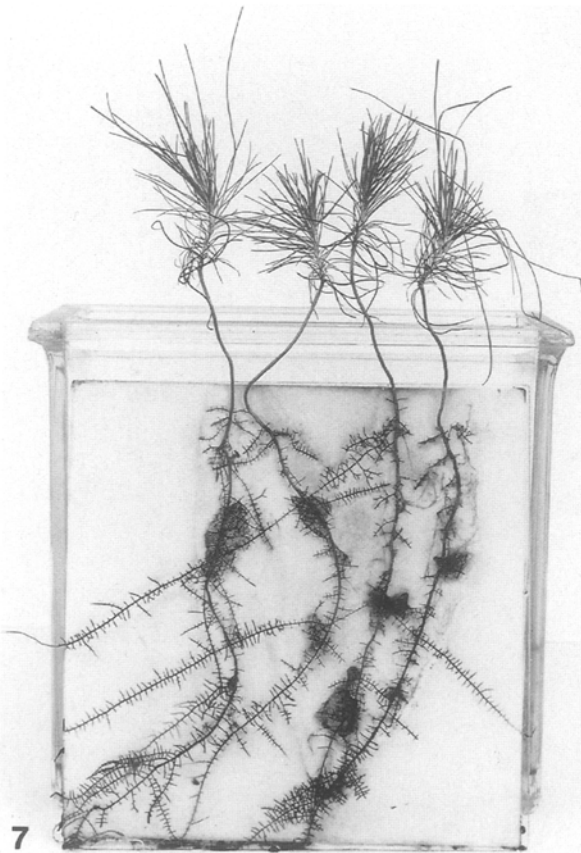
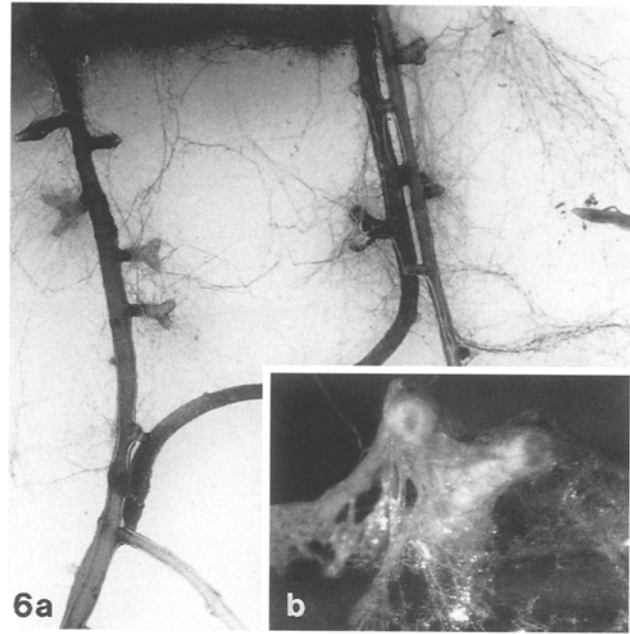
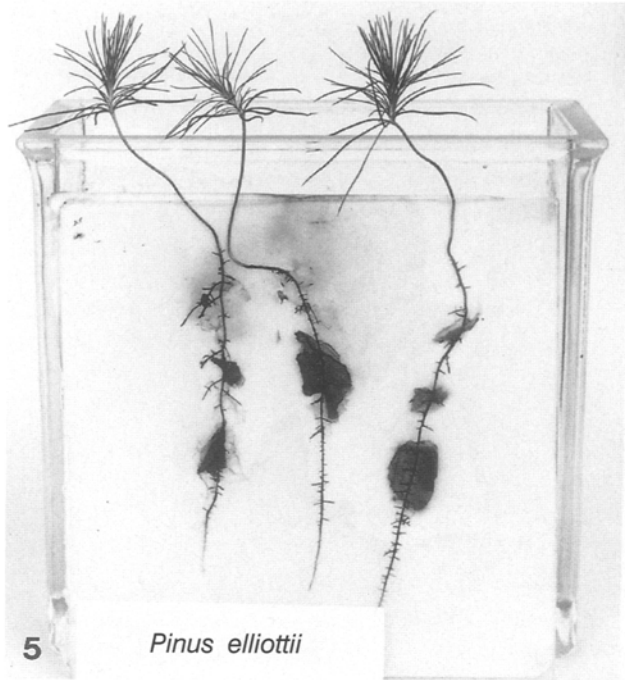
**Figs. 1–4.** External morphology of mycorrhizal associations of *Pinus massoniana* seedlings infected with *Pisolithus tinctorius*

**Fig. 1.** One-month-old inoculated *Pinus massoniana* seedlings sandwiched between a few pieces of chromatography paper and a glass plate.  $\times 1/3$

**Fig. 2. a** A network of extramatrical hyphal strands link various mycorrhizal apices (arrows).  $\times 5$ . **b** Detailed structure of dichotomous mycorrhizal roots enshrouded by hyphal weft.  $\times 40$

**Fig. 3.** Mycelial fans developed from mycorrhizal laterals proliferate and interconnect the neighboring root systems.  $\times 1/3$

**Fig. 4.** Detailed structure of the mycelial fan composed of a network of thicker hyphal strands and diffuse hyphae bridging various mycorrhizal apices (arrows).  $\times 2$



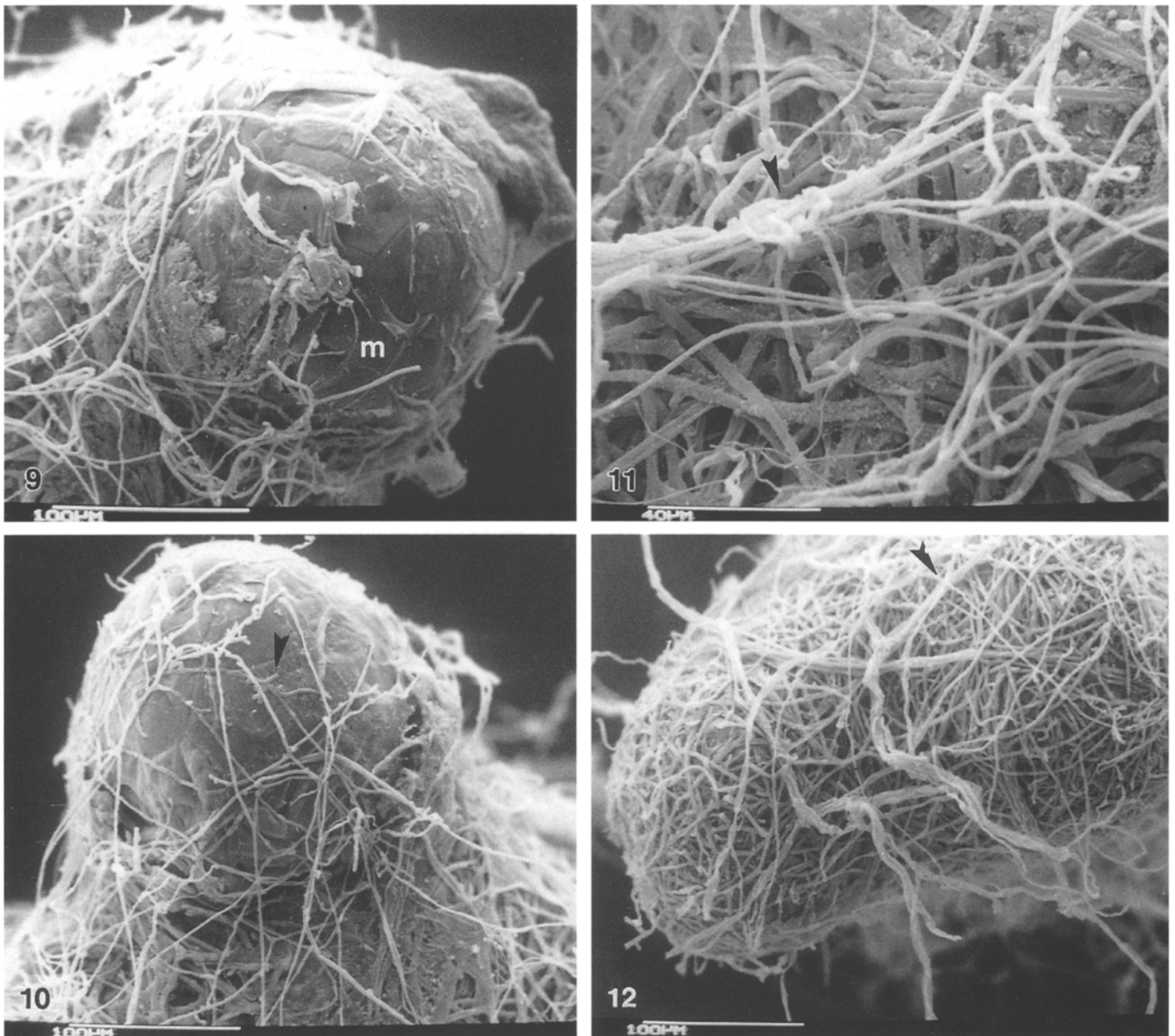
**Figs. 5–8.** External morphology of mycorrhizal associations of *Pinus elliotii* seedlings infected with *Pisolithus tinctorius*

**Fig. 5.** One-month-old inoculated *Pinus elliotii* seedlings sandwiched between a few pices of chromatography paper and a glass plate.  $\times 1/3$

**Fig. 6. a** A network of extramatrical hyphal strands linking various mycorrhizal apices. **b** Detailed structure of dichotomous mycorrhizal roots enshrouded by hyphal weft.  $\times 40$

**Fig. 7.** Proliferation of mycelial strands linking neighboring root systems.  $\times 1/3$

**Fig. 8.** Detailed structure of the mycelial strands and mycorrhizal cluster (*arrow*) contacting the fungal inoculum



**Figs. 9–12.** Scanning electron micrographs showing sequence of sheath formation on the apical region of *Pinus massoniana* by *Pisolithus tinctorius*

**Fig. 9.** Mantle formed on the lateral root behind the apex consisting of isodiametric cells covered by a layer of mucilage-like material (*m*)

**Fig. 10.** Hyphae with clamp connections constituting a loose net on the apical surface and some highly branched hyphae (*arrow*) firmly cemented to the apical cells

**Fig. 11.** Enlarged portion of the mantle showing the interwoven hyphae embedded in mucilage-like material and loosely connected hyphal strands, branched and ornamented (*arrow*)

**Fig. 12.** The root apex completely enshrouded with new mantle and extramatrical hyphal strands (*arrow*) emanating from the mantle

showed that intercellular hyphae had broken through the thinner modified wall and entered into the highly vacuolated cell lumen, where the brightly illuminated intracellular hyphae were also visible (Fig. 21). Wall modifications included wall thickenings, disruption, associated bodies of various shapes and vesicle-like bodies (Fig. 22).

## Discussion

Seedlings of *Pinus massoniana* and *Pinus elliottii* were grown under identical conditions. Dichotomous mycorrhizas, which are a characteristic feature of ectomycorrhizal associations of conifer species, were induced. These results indicate the host compatibility of these two pine species to the mycobiont *Pisolithus tinctorius*.



However, 6 months after inoculation, the growth and mycorrhizal development in seedlings of the two species varied. More mycorrhizas were induced and a greater quantity of extramatrical mycelium proliferated in seedlings of *Pinus massoniana* than *Pinus elliotii*, implying that the degree of host compatibility of the same fungus may vary with the plant species, even within the same genus. After 6 months, *Pinus massoniana* seedlings were stunted and many needles had withered and dropped, indicating a nutrient deficiency. This can be attributed to enhanced mycorrhizal infection of the root system and diversion of photosynthate from the host to the mycosymbiont for mycelial growth. Reductions in plant dry weight due to mycorrhizal infections have been reported previously (Reid et al. 1983; Cairney et al. 1989; Dosskey et al. 1990), and a negative correlation between the degree of fungal development in terms of extramatrical mycelium and the growth of the host plants, as demonstrated in the present study, was also shown by Colpaert et al. (1992). Extramatrical mycelial strands serve as channels for the translocation of water and mineral nutrients (Read and Boyd 1986), and mycelial networks linked to root systems of the same or another plant species may have physiological and ecological roles (Read 1976; Allen 1991).

The formation of dichotomous mycorrhizas in these two pine species is similar to those described previously (Piche et al. 1982, 1983a), and amorphous mucilage-like material secreted by the host root may be responsible for an initial step in ectomycorrhizal attachment or may act as a recognition factor (Piche et al. 1983a).

Duddridge and Read (1984a,b) suggested that one of the most striking features of the host-fungus interface in ectomycorrhizal associations is the apparent absence of cytoplasmic reaction to the presence of intercellular fungus. In the present study, mycorrhizal material also showed a regular intercellular Hartig net enclosing the host cytoplasm with large nuclei but without an aggressive intracellular polyphenolic response, indicating some mutualistic feature of ectomycorrhizal association. Marks and Foster (1973) and Marx (1972) suggested that the presence of polyphenols or the availability of soluble sugars in the cortical cells contacting mantle and Hartig net hyphae in ectomycorrhizas inhibits fungal cellulase production, thus restricting intracellular penetration. However, in the present study, strong intracellular polyphenolic reactions were also observed at the host-fungus interface even with limited Hartig net development. This reaction indicates a hypersensitive response by the host towards the incompatible mycorrhizal fungus (Malajczuk et al. 1984; Giovannetti and Lioi 1990) and is analogous to the host response towards pathogens. The ectomycorrhizal fungus was demonstrated to have the ability to penetrate highly modified walls into the senescing cortical cells and produce ectendomycorrhizas. This ectendomycorrhizal condition may have been induced by the limited carbohydrate supply from the host arising from stunted shoot growth and defoliation, itself a re-

sult of the high degree of mycorrhizal colonization by extramatrical hyphae. The production of cellulase is positively correlated with the glucose content of the root (Melin 1948, 1953), and lytic enzymes produced by the mycosymbiont may lead to wall destruction and intracellular hyphal formation as well as wall modifications such as vesicle-like bodies. Similar wall bodies have been observed in mycorrhizal roots of *Pinus mugo* (Wills and Cole 1978), and modified wall structures such as papillae and wall ingrowth occurred in *Picea abies* (Nylund et al. 1982; Kottke and Oberwinkler 1986), while wall protuberances were observed in natural pine species (Duddridge and Read 1984a). All such wall modifications are probably brought about by host-wall/fungus interactions.

Wilcox (1983) suggested that the ectomycorrhizal condition is not absolute and can be influenced towards the ectendomycorrhizal condition by either associant; similarly, ectomycorrhizal fungi can also change from beneficial to harmful under some conditions of stress. The shift of pine mycorrhizas infected with *Pisolithus tinctorius* from a mutualistic, beneficial ectomycorrhizal association to a harmful, saprophytic- or parasitic-like ectendomycorrhizal association, probably the result of the plant host losing control of the mycobiont, is clearly evident in this present study.

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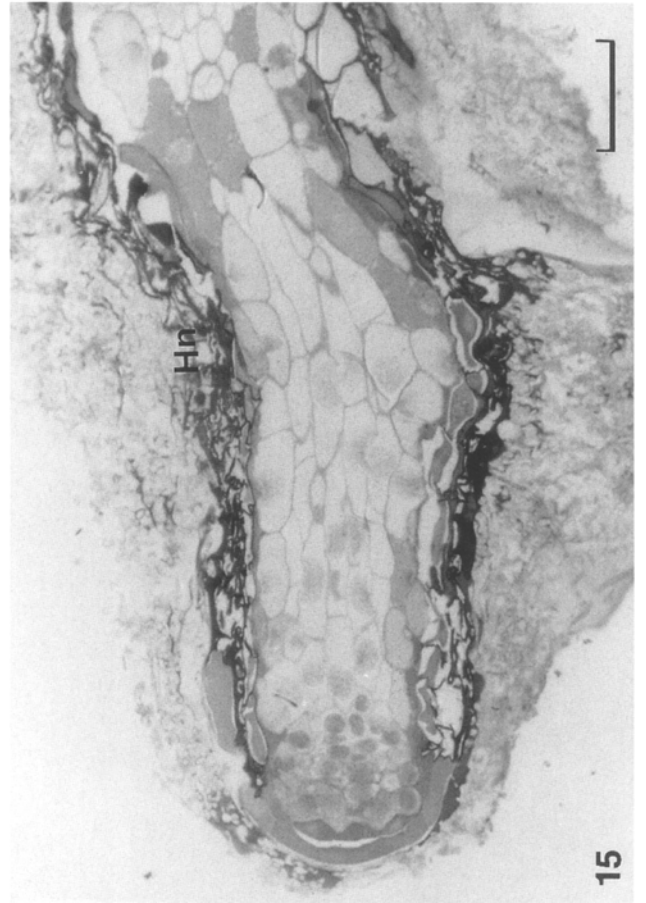
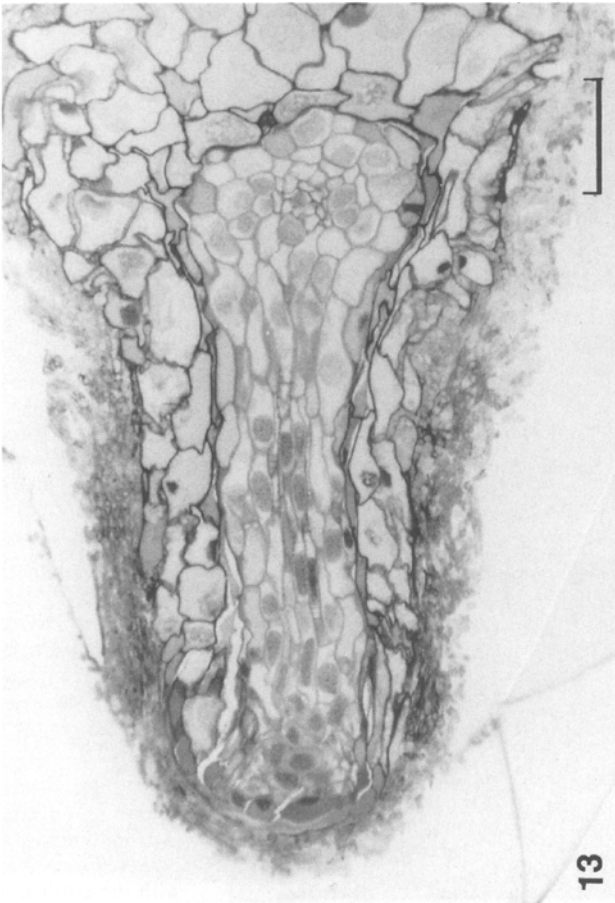
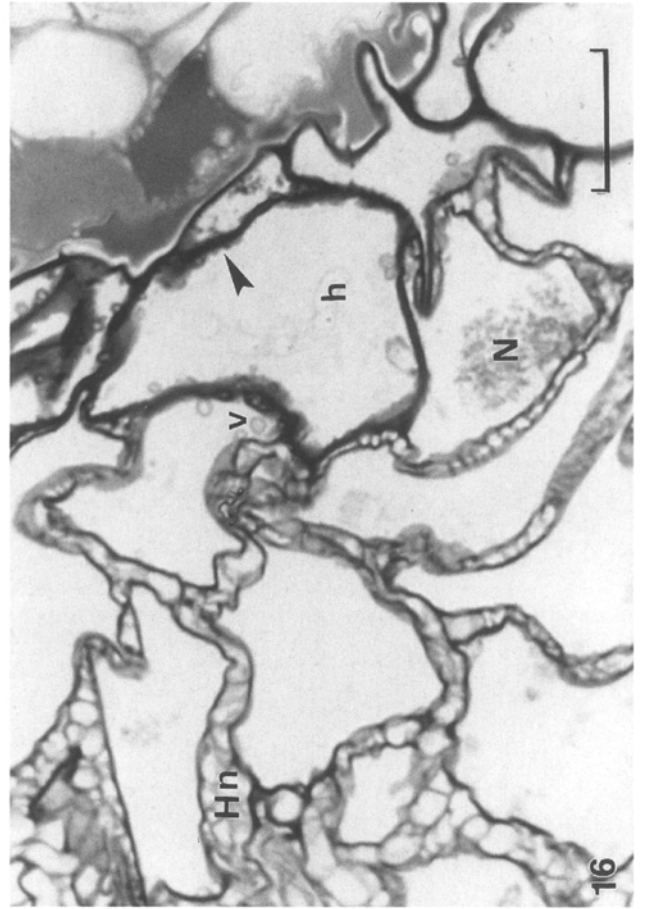
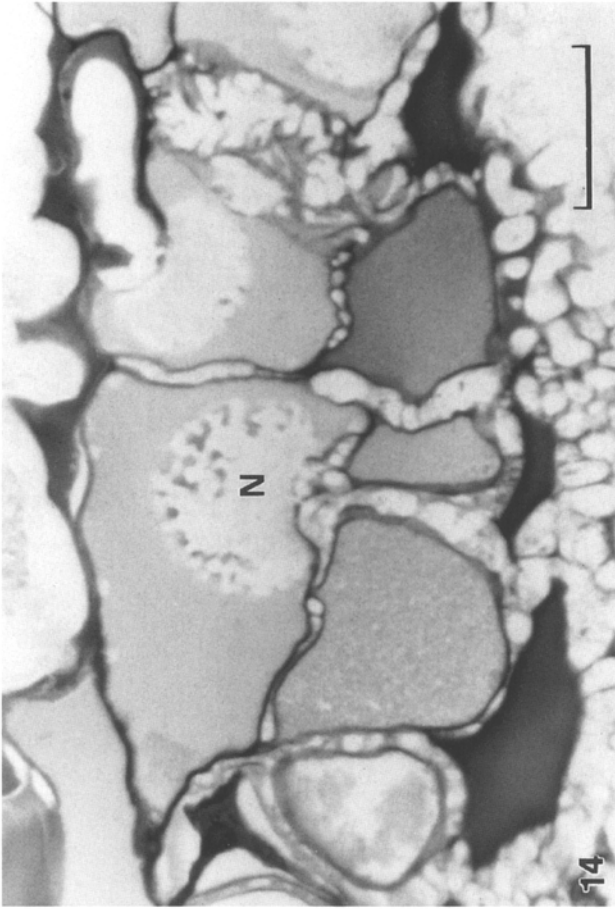
**Figs. 13–16.** Light micrographs of longitudinal glycol methacrylate (GMA) sections of synthesized mycorrhizal root *Pinus elliotii*/*Pisolithus tinctorius*

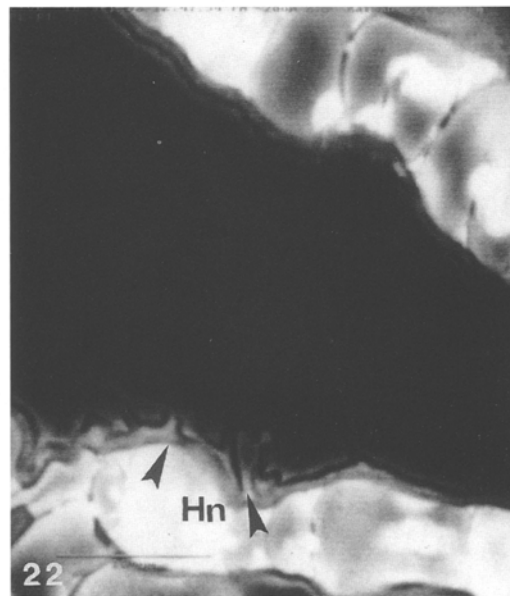
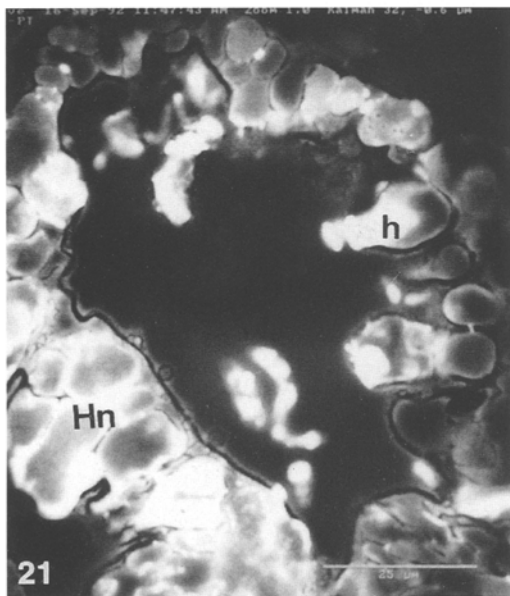
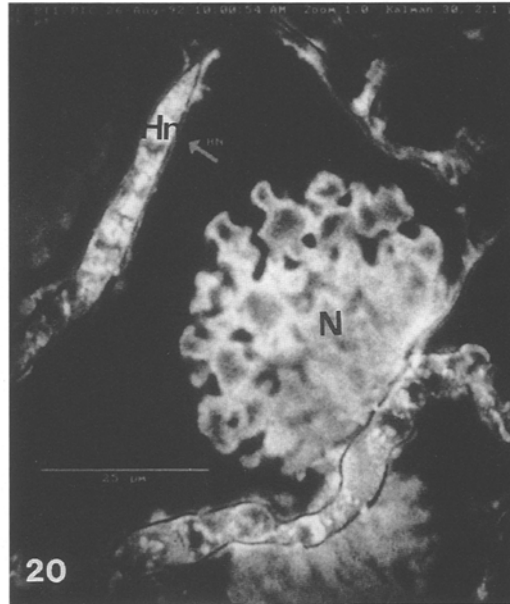
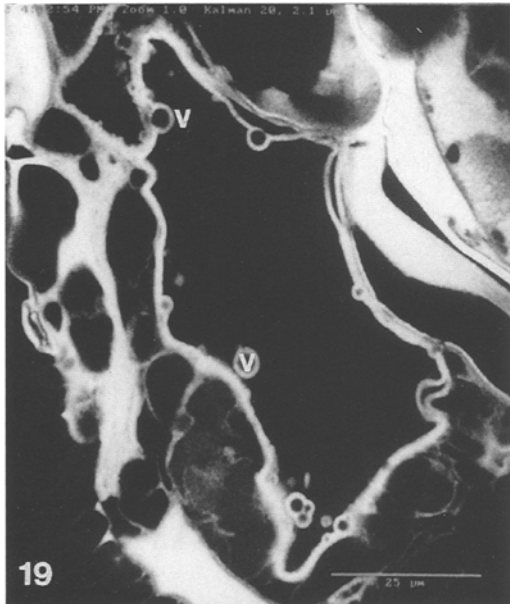
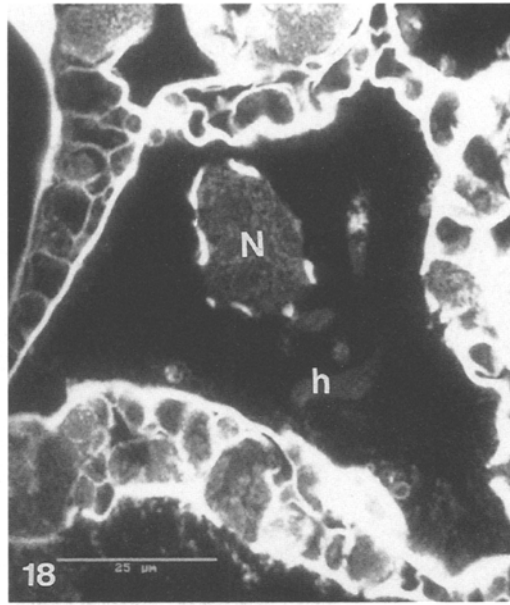
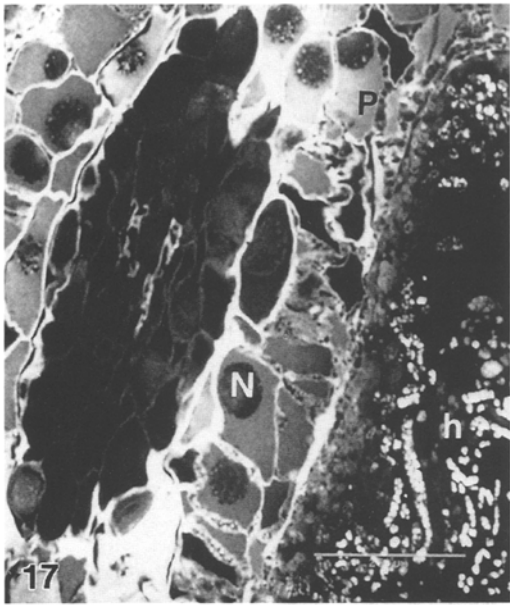
**Fig. 13.** Mycorrhizal root composed of outer prosenchyma and inner synenchyma and intercellular Hartig net extending through several layers of cortical cells close to the endodermis; *bar* = 50  $\mu$ m

**Fig. 14.** Enlarged portion of the intercellular Hartig net and conspicuous, convoluted nuclei (N) inside cortical cells; *bar* = 25  $\mu$ m

**Fig. 15.** Mycorrhizal root with thick mantle. Note strong, dark polyphenolic reaction (*arrow*) and limited Hartig net (Hn) development; *bar* = 50  $\mu$ m

**Fig. 16.** Enlarged portion of the cortical cells showing intercellular Hartig net (Hn), disintegrated nucleus (N) and intracellular hyphae (h). Note polyphenol-impregnated cell walls (*arrow*) and vesicle-like bodies (v); *bar* = 25  $\mu$ m





**Figs. 17–22.** Laser scan confocal micrographs of GMA sections of synthesized mycorrhizal root *Pinus elliotii*/*Pisolithus tinctorius*. **Figs. 17–19** are unstained sections and **Figs. 20–22** are stained sections

**Fig. 17.** Mycorrhiza showing fluorescent outer mantle hyphae (*h*), polyphenol-containing cells (*P*), cortical walls and Hartig net (*Hn*) and dark nuclei (*N*)

**Fig. 18.** Detailed structure of the mycorrhiza showing autofluorescent cortical walls in contrast to dimly illuminated intracellular hyphae (*h*) and the deformed nucleus (*N*)

**Fig. 19.** Detailed structure of cortical cells showing fluorescent, vesicle-like bodies (*v*) attached to the walls

**Fig. 20.** Detailed structure of the mycorrhiza showing fluorescent intercellular Hartig net (*Hn*) hyphae confined to dark cortical walls and a fluorescent fan-shaped nucleus (*N*)

**Fig. 21.** Detailed structure of the mycorrhiza showing fluorescent intercellular hyphae (*h*) breaking through disrupted cortical walls and entering into the cell lumen as intracellular hyphae (*h*)

**Fig. 22.** Detailed structure of wall modifications showing various-shaped bodies (arrows) associated with the cortical walls



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