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Mycorrhiza

Mycorrhizal associations in Hong Kong Fagaceae

III. The ontogeny of mycorrhizal development, growth and nutrient uptake by *Quercus myrsinaefolia* seedlings inoculated with *Pisolithus tinctorius*

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Abstract. The broad host range ectomycorrhizal fungus *Pisolithus tinctorius* (Pers.) Coker and Couch was grown in association with seedlings of a local species of oak *Quercus myrsinaefolia* Bl. and the ontogeny of mycorrhizas examined microscopically. Development was similar to that seen previously in *Eucalyptus*. No significant growth stimulation occurred in infected seedlings but their uptake of N, P and K was enhanced. Nutrient uptake by seedlings bearing natural mycorrhizas with unidentified fungi was less efficient. The results indicate that *P. tinctorius* has a potential for application in reafforestation programmes in Hong Kong and southern China.

Key words: Oak – *Quercus myrsinaefolia* – *Pisolithus tinctorius* – Ectomycorrhiza

Introduction

Pisolithus tinctorius has a worldwide distribution and is one of the few species of ectomycorrhizal fungi ecologically adapted to adverse sites with obvious potential application in afforestation and reforestation programmes throughout the world (Marx 1977). The fungal isolate *P. tinctorius* (Pers.) Coker and Couch has been introduced to Hong Kong in our laboratory, where we demonstrated its ability to form ectomycorrhizas with local species of pine and exotic species of pine and *Eucalyptus* (Chan and Griffiths 1988, 1991). More recently, it was shown that the same fungus can readily establish ectomycorrhizal associations with three genera of local Fagaceae, *Quercus*, *Castanopsis* and *Lithocarpus* (Tam and Griffiths 1993a); it also exhibited a high degree of infection with pot-grown *Quercus myrsinaefolia* seedlings (Tam and Griffiths 1993b). The production of ectomycorrhizal oak seedlings infected with *P. tinctorius* has been widely reported by many authors (Marx 1979; Ruehle 1980; Beckjord and McIntosh 1984; Kwong 1984; Mitchell et al. 1984; Dixon et al. 1985; Pope and Chaney 1985; Beckjord et al. 1986; Parker et al. 1986). Most of these authors reported mycorrhizal stimulation of growth in oak seedlings and an enhanced uptake of mineral nutrients.

This present paper examines the ontogeny of mycorrhizal development in a local species of oak, *Q. myrsinaefolia* and analyses in detail how the ectomycorrhizal infection affects the growth and physiology of seedlings with respect to nutrient uptake.

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Materials and methods

Fungal culture

P. tinctorius (Pers.) Coker and Couch (ATCC 38054) was obtained from the American Type Culture Collection and maintained on a modified Melin:Norkrans agar medium (Marx 1969).

Seedlings

These were grown in an artifical soil as described previously (Tam and Griffiths 1993b). Controls were grown in uninoculated soil. One batch of seedlings was inoculated with an actively growing culture of *P. tinctorius* and a second batch grown in artifical soil supplemented with natural soil from beneath the parent oak tree.

Microscopy

Normal as well as mycorrhizal roots were fixed in 0.025 M phosphate buffer (pH 6.8) at 4° C for 10–12 h and washed in the same buffer, dehydrated through a series of alcohols and embedded in glycol methacrylate. Polymerization was carried out in gelatin capsules at 55° C for 10–15 h. Sections 2–3 μm thick were cut with a glass knife, stained with 0.05% toluidine blue in 1% sodium benzoate at pH 4.4, and examined in a light microscope. Unstained sections were examined in a laser scan confocal microscope (Bio-Rad MRC 600) using an Argon ion laser as a light source at 488 nm with a blue high sensitivity filter.

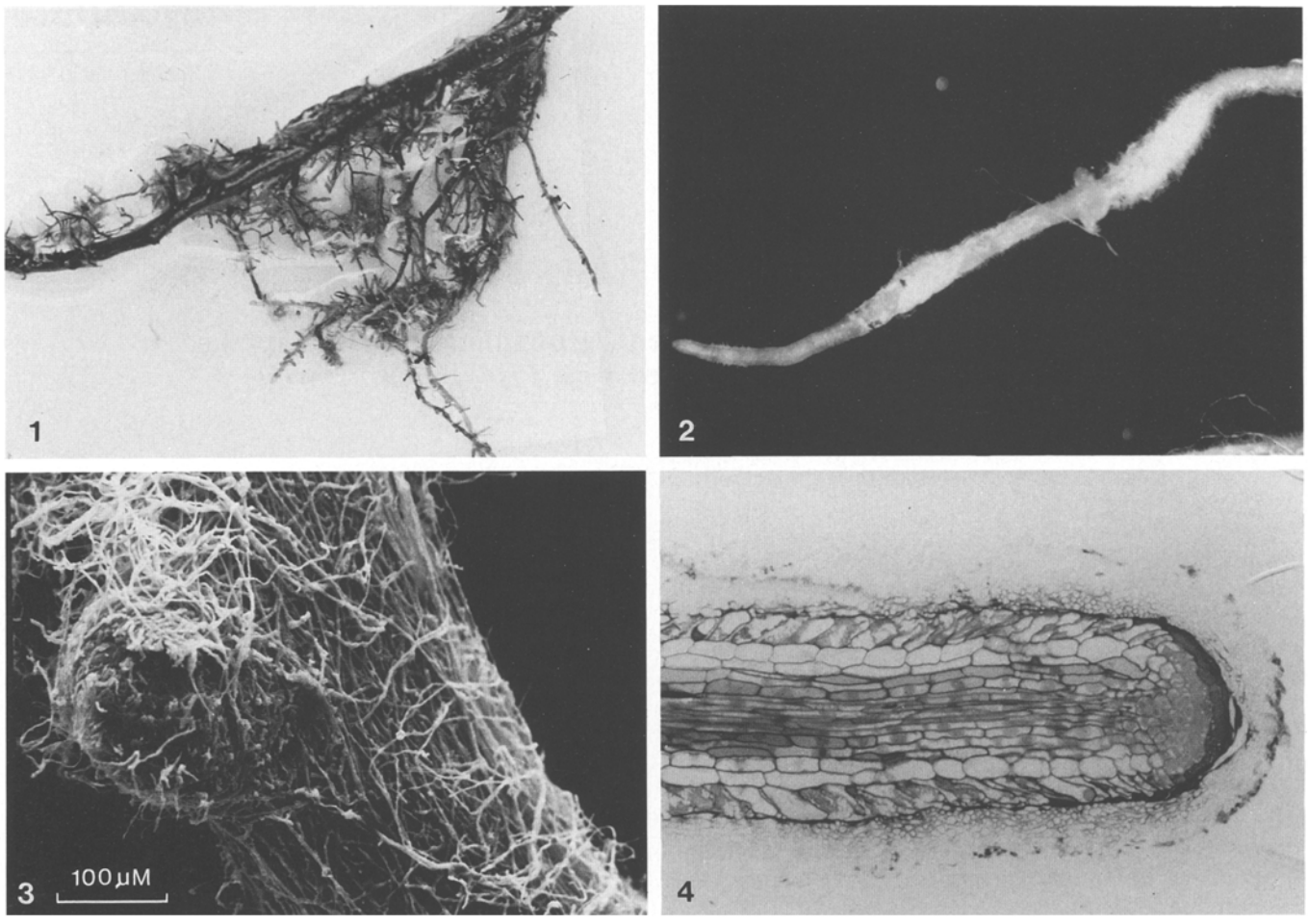

Fig. 1. External morphology of synthesized mycorrhizal clusters of *Quercus myrsinaefolia* seedlings infected by *Pisolithus tinctorius*. Note the hyphal strands. ×2

Fig. 2. A rapidly growing root apex having ruptured the enveloping hyphal sheath. ×10

Fig. 3. Scanning electron micrograph of an emerging lateral root pushing through the hyphal weft of the first-order lateral root

Fig. 4. Longitudinal section of a synthesized mycorrhiza of *Q. myrsinaefolia/P. tinctorius* showing typical features of the ectomycorrhizal association. *Scale bar*, 100 μm

Fixed tissue was also post-fixed in 1% osmium tetroxide in the same buffer for 1 h, washed in distilled water, dehydrated in a graded series of ethanol followed by critical point drying, shadowed with platinum-palladium, and then examined in a Cambridge Stereoscan 150 scanning electron microscope.

Growth analysis of seedlings

Twenty-four weeks after planting, three seedlings were randomly selected for analysis from pots containing (a) the artificially inoculated soil, (b) soil supplemented with natural soil and (c) from the uninoculated control. Roots were carefully washed under running tap water to remove adhering soil particles and examined for mycorrhizas.

Data were recorded from individual seedlings for shoot length, number of leaves, leaf surface area (plotted with a LI-COR portable area meter), the distribution of short roots, the total number of short roots, mycorrhizal roots (the percentage of infection per seedling calculated as the total number of infected roots divided by the total number of lateral roots), the dry weight of roots, the dry weight of shoots, and the root:shoot dry weight ratio.

Chemical analysis of seedlings

Seedlings were divided at the hypocotyl region into shoot and root regions and the total dried plant material for each portion was analysed for nutrients following its digestion in sulphuric acid/hydrogen peroxide (Grimshaw et al. 1989). Total nitrogen was determined by the free ammonia method following steam distillation in the presence of excess alkali. Phosphorus was determined colorimetrically by the molybdenum blue method. Potassium and calcium levels were determined using a flame photometer.

Results

Mycorrhizal development

Root development in the three treatments differed greatly:

a) In uninoculated artifical soil, the root system consisted of a very long main root with first- and second-order laterals. After 4 months, many of the older laterals had produced fourth-order rootlets.

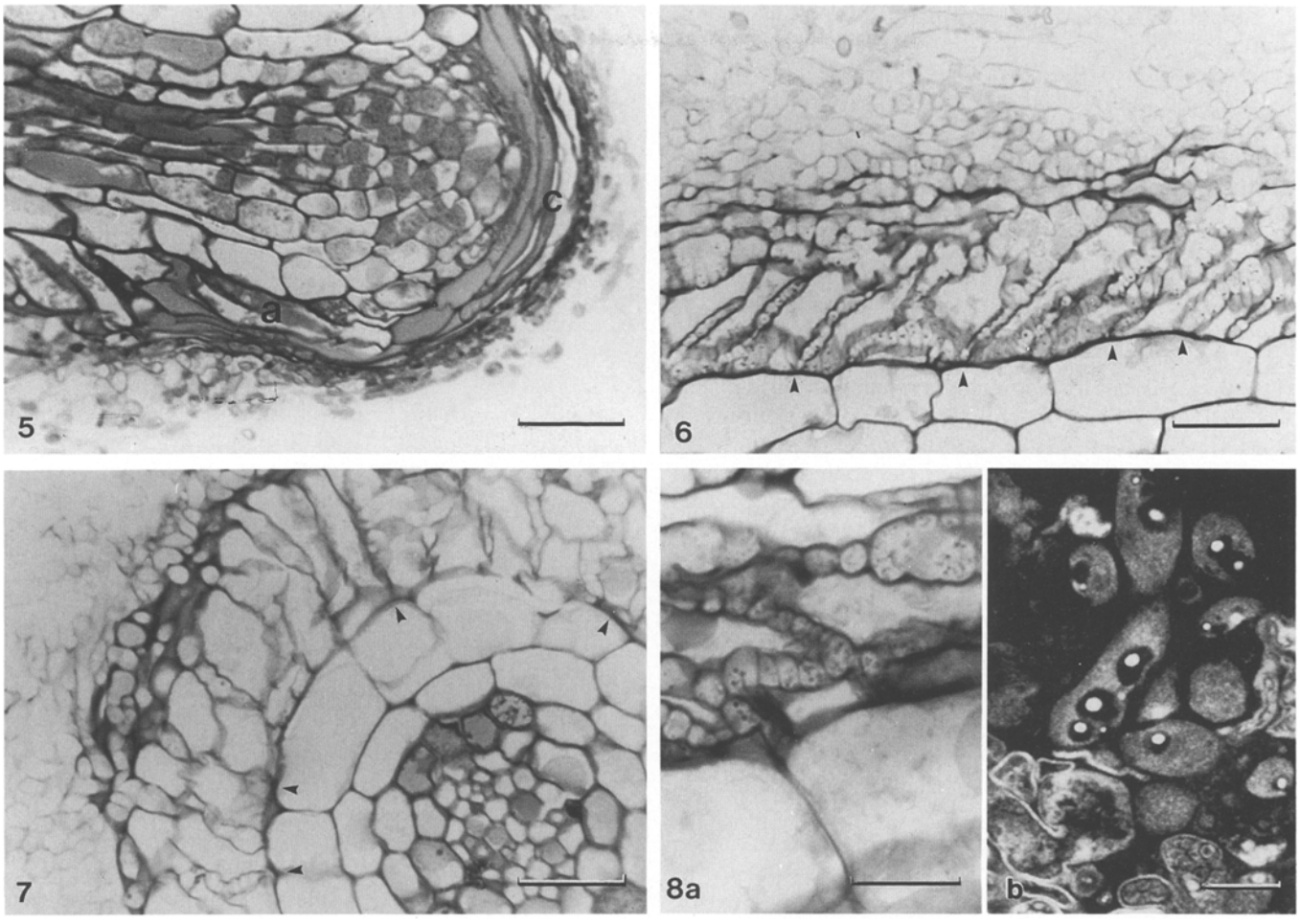

Fig. 5. Enlarged portion of the apposition zone (*a*) and root cap cells (*c*). Note darkly stained polyphenolic cell contents. *Scale bar*, 20 μm

Figs. 6, 7. The Hartig net zone of a synthesized mycorrhiza demonstrating the distribution of polyphenolic compounds. Note the highly vacuolated epidermal cells and the darkly stained polyphenol impregnated outer cortical walls (*arrows*). *Scale bar*, 20 μm

Fig. 8a, b. Enlarged portion of the Hartig net showing variable, discrete bodies (possibly polyphosphate granules) located in the hyphal vacuoles. **a** Light microscopy; **b** confocal microscopy. *Scale bar*, 10 μm

b) In artifical soil which had been supplemented with soil taken from under the mother tree, laterals were far less well-developed producing secondary and tertiary order roots only, some of which bore black mycorrhizal tips. After 4 months, roots bearing black sheaths had further divided to form small mycorrhizal clusters.

c) In soil inoculated with *P. tinctorius*, the main root remained short, lateral root development was pronounced and extensive root branching had occurred in the region of the root nearest to the soil surface, resulting in the formation of extensive mycorrhizal clusters (Fig. 1).

During the course of mycorrhizal development, it was observed that clusters developed 4 weeks after the inoculation; lateral roots deeper in the soil subsequently became infected following the development and growth of thick, yellow mycelial strands from the hyphal weft surrounding the mycorrhizal clusters. This means that hyphal wefts developing on younger laterals are always connected to, and are derived from, the colonizing mycelial strands. After a 4-month growth period, approximately 75% of all lateral roots bore hyphal mantles and could be considered mycorrhizal. Another conspicuous event was the gradual change from the bright yellow of newly formed mycorrhiza to the light brown of mature mycorrhizal roots. The developing hyphal weft derived from mycelial strands frequently engulfed the lateral roots including the root tip of first order laterals. However, these roots frequently grew through the enveloping weft to resume their uninterrupted growth (Fig. 2).

The development of mycorrhizal clusters occurred after a first-order lateral root became enveloped in a hyphal mantle which formed a loose network over the root surface. Growth of this lateral diminished and then ceased, and new laterals appeared which pushed their way through the hyphal weft (Fig. 3). Growth of this second-order root then continued until it too had become engulfed in the rapidly expanding hyphal weft. On cessation of growth, third-order laterals emerged through the mantle.

Longitudinal sections cut through developing and mature mycorrhizal roots illustrate the sequence of events leading to the close association between fungal hyphae and the internal root tissues; the following distinct zones could be observed: (1) a small number of root cap cells bearing dark-staining contents, (2) the apical meristem, which was somewhat reduced in area when compared to uninfected roots, (3) an apposition zone where hyphae were in close contact with the epidermal cells but where no fungal penetration occurred, (4) a zone where hyphae from the sheath had penetrated between the epidermal cells, and (5) a zone furthest away from the apex where hyphae had developed a distinct, typical Hartig net of colonizing hyphae. Epidermal cells were radially enlarged in all zones behind the meristem (Fig. 4).

The root cap cells and cells of the epidermal layer in the apposition zone (Fig. 5) had dark-staining contents characterized as polyphenolic compounds by the toluidine blue reaction. In the zone where the Hartig net was well developed, the epidermal cells had lost their dark-staining reaction but cell walls of the outer cortex gave a strong, positive polyphenol reaction (Fig. 6). Serial transverse sections clearly showed that as the root-colonizing hyphae became established the phenolic reaction slowly disappeared from the cells adjacent to the hyphae and appeared in cell walls newly in contact with and somewhat in advance of the intercellular hyphae (Fig. 7).

A distinct and invariable feature of the root region bearing a mature Hartig net was the appearance in the intercellular hyphae (Fig. 8a) and in the hyphae of the inner mantle of variable, discrete bodies stained red with toluidine blue. They were located in the vacuole of the hyphal cell (Fig. 8b).

Further back from the root apex, the stele was well developed and was bounded by a distinct endodermis and pericycle. These tissues were found nearer to the apex when compared with uninfected roots. Lateral roots developed from primordia arising in the pericycle and grew outwards, destroying cortical cells and epidermal cells of the Hartig net zone during their emergence. Colonization of the new laterals did not occur during passage through the cortex and required the growth of superficial hyphae to form a mantle after emergence. The sequence of events leading to the formation of a Hartig net in the new laterals followed the pattern described above.

Table 1. The distribution of infected and uninfected short root apices in the root system of *Quercus myrsinaefolia* seedlings inoculated with *Pisolithus tinctorius* after 4 months growth

Position in the root system	Total no. of root apices	No. of infected root apices	No. of uninfected root apices	Percentage of mycorrhizal infection
Upper	601	542	59	90.2
Middle	248	128	120	51.6
Lower	61	28	33	45.9

The distribution of mycorrhizas in the root system

The distribution of infected and uninfected root apices in the whole root system was recorded by dividing the root into three regions: "upper", i.e. the topmost third close to the soil surface; "middle", i.e. the central third; and "lower", i.e. the lowermost third close to the main apex. The overall numbers of infected and uninfected root apices were recorded and an estimation of the mycorrhizal infection was made in each region. The findings are summarized in Table 1. It can be observed that the total number of mycorrhizal apices and percentage infection decrease down the root system.

Growth analysis

A summary of various growth parameters collected from uninoculated control, inoculated and natural soil seedlings is presented in Table 2. Compared to the control, the shoot height of the inoculated seedlings and natural soil seedings increased by 18% and 32%, respec-

Table 2. Growth parameters under three conditions of *Q. myrsinaefolia* seedlings after 24 weeks growth. The values are in every case the means of three samples

	Uninoculated control	Inoculated with *Pisolithus tinctorius*	Natural soil
Mycorrhizal infection (%)	0	65.6	13.3
Shoot height (cm)	6.2	7.3	8.2
Number of leaves	4.7	5.7	5.3
Leaf area (cm^2)	28.7	41.0	35.2
Leaf area ($cm^2\ g^{-1}$ total dry wt.)	64.2	95.1	86.5
Shoot dry wt. (mg)	262.0	302.0	269.0
Root dry wt. (mg)	185.0	129.0	137.0
Total dry wt. (mg)	447.0	431.0	407.0
Root:shoot ratio	0.71	0.43	0.49
No. of short roots	325.0	905.0	280.0
Short roots (mg^{-1} root dry wt.)	1.8	7.0	2.0

Table 3. Chemical analysis of *Q. myrsinaefolia* seedlings after 24 weeks growth. The values in every case are the means of three samples

			Uninoculated control	*Pisolithus tinctorius* inoculation	Natural soil
Mycorrhizal infection (%)			0	65.6	13.3
Nitrogen	N (% dry wt.)	Shoot	0.96	1.30	1.14
		Root	0.67	1.22	1.00
		Seedling	0.84	1.28	1.04
	Total N (mg)	Seedling	3.76	5.53	4.24
Phosphorus	P (% dry wt.)	Shoot	0.06	0.11	0.08
		Root	0.06	0.16	0.07
		Seedling	0.06	0.12	0.07
	Total P (mg)	Seedling	0.28	0.53	0.29
Potassium	K (% dry wt.)	Shoot	0.40	0.71	0.53
		Root	0.18	0.17	0.29
		Seedling	0.31	0.55	0.46
	Total K (mg)	Seedling	1.40	2.36	1.85
Calcium	Ca (% dry wt.)	Shoot	0.51	0.55	0.57
		Root	0.59	0.63	0.60
		Seedling	0.54	0.57	0.58
	Total Ca (mg)	Seedling	2.43	2.47	2.36

tively; the shoot dry weight of inoculated seedlings was about 15% higher, whereas that of the natural soil seedings was not markedly different. More significantly, the leaf area, particularly the area in cm^2 per gram dry weight, of both the inoculated and the natural soil seedlings increased by 48% and 35%, respectively, compared to the control. However, in both cases there was a significant reduction in root dry weight of 30% and 26%, respectively. A more dramatic decrease was found in root:shoot ratio, viz 39% and 31%, respectively, compared to the control. The overall dry weight of the seedlings in the three treatments did not vary greatly. However, a great difference was found in the number of short roots per milligram root dry weight between the inoculated seedlings and both the control and the natural soil seedlings.

Chemical analysis

As shown in Table 3, the inoculated seedlings showed a significant increase in uptake of N, P and K in terms of percentage dry weight and total dry weight per seedling, but the natural soil seedlings only showed a significant increase in N and K when compared to the uninoculated control. In general, nutrient uptake in the shoot and root samples of the three treatments was such that % N and % K in shoots was always greater than roots; the % P in shoots is more or less similar to that of roots except in inoculated seedlings, where a higher % P was found in roots. The % Ca in shoots was always lower than in roots. It is interesting that the levels of both P and Ca found in root samples were higher in inoculated seedlings than in uninoculated control.

Discussion

In this paper we have established that one of Hong Kong's native oak trees, *Q. myrsinaefolia*, can form mycorrhizal associations with *P. tinctorius* in artificially inoculated soil, and that the association partially enhances the growth of seedlings and dramatically increases nutrient uptake. We chose *P. tinctorius* as the fungal symbiont because it is a well-established mycorrhiza former in other species of *Quercus*, and despite the existence of local isolates in Hong Kong, we chose this particular isolate from the ATCC because of its rapid growth rate and its previous proven success as a mycorrhiza former in Hong Kong soils (Chan and Griffiths 1988, 1991).

The process of mycorrhizal initiation was very similar to that seen in other associations in *Eucalyptus* (Chilvers and Gust 1982; Massicotte and Peterson 1986), i.e. lateral root development was stimulated near the zone of inoculation and further development of the association was achieved by means of mycorrhizal strands, resulting in a high percentage of mycorrhizal roots per root sys-

tem. In contrast, seedlings growing in artificial soil supplemented with natural soil exhibited a much lower percentage of infected roots.

A frequently observed feature was the growth of root apices through the enveloping fungal mantle, indicating that the growth rates of mycobiont and phytobiont under the conditions we established for artifical inoculation were dissimilar, though both partners exhibited tissue compatability.

The histological features of our mycorrhizal roots were similar to those observed previously in eucalypt by Massicotte and Peterson (1986). In our associations, epidermal cells bore dark polyphenolic compounds when first in contact with hyphae forming the mycorrhizal weft. As hyphae penetrated between the epidermal cells, the polyphenols gradually disappeared but similar substances then occurred in the cell walls of the epidermis and more distinctly in those of outer cortex. These intracellular polyphenols may serve as a chemical barrier to penetration and colonization of roots by plant pathogens (Mace and Howell 1974). It is evident that the polyphenols we observed in the epidermal cells were mobilized during intercellular fungal penetration, and were resynthesized within the host cell walls in advance of further fungal invasion.

One feature of interest is the absence of starch grains from epidermal cells following fungal invasion and also from other regions of the mycorrhiza, although they were previously recorded in natural mycorrhizas of *Eucalyptus* (Ling-Lee et al. 1977). Their absence in our mycorrhizal roots may be explained by the continuous illumination to which seedlings were subjected. Previously, Bjorkman (1942, 1956) demonstrated a positive relationship between light intensity and soluble carbohydrate concentration in root systems, with high internal soluble carbohydrate supply favouring infection.

Our results indicate a moderate increase in shoot height and a significant increase in leaf area per unit g dry wt. in seedlings inoculated with *P. tinctorius* and in seedlings growing in natural soil, when compared to the controls. These results are similar to those obtained by Daughtridge et al. (1986), who suggested that mycorrhizal fungi have the fundamental potential to alter the relationship between leaf area and plant mass by increasing the relative investment of fixed carbon in the leaf area. However, in our results the overall dry weights of the three treatments are not significantly different, mainly due to the great reduction of root dry weight in the inoculated seedlings compared to the controls. The dry weight reduction in mycorrhizal seedlings seen in our experiments may be due to the altered root system physiology following early stages in infection and may be explained by the source - sink relationship described by France and Reid (1983), Cairney et al. (1989) and Dosskey et al. (1990). Carbohydrate flow from the host to the fungus contributes to the formation of hyphae of the mantle, Hartig net and extramatrical mycelial strands and also provides metabolic energy for nutrient absorption.

The root:shoot ratios of mycorrhizal plants were lower than the non-mycorrhizal plants, possibly because of a greater capacity of the former to absorb nutrients and to increased concentrations of nutrients in their tissues (Harley and Smith 1983). In our findings, both artifically and naturally inoculated seedlings demonstrated a significant increase in nutrient uptake when compared to the controls and this may be explained by the increased absorptive surface of the mycorrhizal roots (France and Reid 1983). Particularly significant was the increase of P in mycorrhizal roots. A visual indication of P accumulation was provided by the presence of large numbers of polyphosphate granules, which have been observed previously by Ling Lee et al. (1975), White and Brown (1979), Chilvers and Harley (1980), Cox et al. (1980), Strullu et al. (1982), Ashford et al. (1986). The actual composition of the granules is controversial. It has often been suggested that the balancing cation for the polyphosphate is Ca; others have suggested that Mg, K and Na are also involved. Our data show a moderate increase in Ca level in the mycorrhizal roots and offer circumstancial evidence for a linkage between P and Ca. This will be the subject of further investigation.

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