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In vitro synthesis of *Pisolithus-Eucalyptus* ectomycorrhizae: synchronization of lateral tip emergence and ectomycorrhizal development

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Abstract A simple and reproducible in vitro system is described for the synthesis of *Pisolithus-Eucalyptus grandis* ectomycorrhizae. Hyphal discs from actively growing colonies were placed in large petri dishes containing minimum nutrient agar overlaid with cellophane and allowed to grow for 7 days. Seeds were then surface sterilized and placed above the expanding fungal colonies and the plates slanted. Seedlings that germinated and grew in the presence of fungal hyphae had twice as many lateral root tips as seedlings that germinated before they were transferred onto hyphal mats. In addition, the lateral root tips of inoculated seedlings had a faster maturation rate and emerged closer to the primary root apex than non-inoculated seedlings. All lateral tips emerged in contact with fungal hyphae and the differentiation of ectomycorrhizae was followed by examining lateral tips basipetally along a single primary root. Typical ectomycorrhizae had formed on 4-day-old lateral tips, i.e. a mantle, radially elongated epidermal cells and a Hartig net commencing about 0.3 mm behind the lateral root apex. Thereafter, the mantle continued to thicken and the apical meristem diminished. The Hartig net often surrounded the apex of 11- to 12-day-old lateral root tips. This model system will facilitate detailed studies on synchronized ectomycorrhizal development and associated molecular and biochemical changes.

Key words Ectomycorrhizal development · *Eucalyptus* · *Pisolithus* · Synthesis system

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Introduction

The examination of ectomycorrhizal development, or any physiological and biochemical changes associated with or controlling ectomycorrhizal development, relies on the use of simple, reproducible and easily manipulable model systems (Fortin et al. 1983; Ineichen and Wiemken 1992). Systems for slow-growing host species, such as *Pinus* and *Picea,* were reviewed by Ineichen and Wiemken (1992). Ectomycorrhizal synthesis with these species may take up to 60 days and, to provide suitable growth conditions, it is often necessary to have axenic roots and non axenic shoots (Duddridge 1986a,b; Wong and Fortin 1989). Many systems used previously for fast-growing seedlings have also been partially or totally non-axenic. With the non-axenic growth pouch technique (Fortin et al. 1980; Godbout and Fortin 1985; Grenville et al. 1986; Massicotte et al. 1987b,c), inoculum plugs were placed amongst a predeveloped root system. The paper sandwich technique (Chilvers et al. 1986; Horan et al. 1988) utilized a card covered with fungal inoculum which was placed over the root system, simultaneously inoculating the lateral root tips. However, fast-growing species such as *Eucalyptus* germinate within 3 days and produce lateral root tips within 10 days (Burgess et al. 1994; Malajczuk et al. 1990). An axenic petri dish technique was developed by Malajczuk et al. (1990) in which pregerminated seedlings of *Eucalyptus* were positioned onto actively growing colonies of *Pisolithus*. Brun et al. (1995) used a similar system to synthesize *Betula-Paxillus* ectomycorrhizae. However, in all these synthesis systems, the roots were developed before inoculation with the fungus. Consequently, the ectomycorrhizal root tips examined were a mixture of tips that had emerged prior to inoculation and later colonized and tips of different ages that had emerged after inoculation. Therefore, studying key developmental events associated with symbiosis formation in these systems is difficult.

Burgess et al. (1994, 1995a) described a system for the synthesis of *Pisolithus-Eucalyptus* ectomycorrhizae

whereby sterilized seeds were placed concentrically around an actively growing fungal colony. The seeds germinated in the presence of fungal hyphae, resulting in a stimulation of lateral tip production. However, the seedlings had to be repositioned after germination so that the roots could contact the fungal colony. In the present study, we describe an optimization of the petri dish system, allowing for the synchronization of lateral root tip emergence and ectomycorrhizal development. This technique was developed to facilitate temporal and spatial studies on molecular and biochemical changes during ectomycorrhizal formation.

Materials and methods

Aseptic synthesis of ectomycorrhizae

Ectomycorrhizae were synthesized between half-sib seeds of *Eucalyptus grandis* W. Hill ex Maiden, seed lot no. 17867 (Australian Seed Centre, Canberra, ACT), and *Pisolithus* isolate H2144 (CSIRO Herbarium, Division of Forestry, Perth, Western Australia). This isolate was selected for its rapid development of ectomycorrhizae in vitro (Burgess et al. 1994). The taxonomy of the genus *Pisolithus* is currently under review and species names have not yet been assigned to isolates (Burgess et al. 1995b). *Pisolithus* isolate H2144 was collected in 1988 beneath *E. wandoo* at Moora, Western Australia. Cultures were maintained on modified MMN, 1% glucose (Marx 1969).

Burgess et al. (1994) described an in vitro system in which the seeds were germinated in the presence of fungal exudates and lateral tip emergence was concurrent with ectomycorrhizal development. Various aspects of that system have been modified and improved for this experiment. Large round petri dishes (140 × 20 mm) were partially filled with 30 ml of complete nutrient agar (1.5 mM N, 0.7 mM Ca, 0.6 mM Cl, 0.5 mM Mg, 0.5 mM S, 0.46 mM K, 0.08 mM P, 0.02 mM Fe, 0.01 mM B, 2.28 μM Mn, 0.2 μM Zn, 0.005 μM Co, 0.03 μM Mo, 0.03 μM Na, 0.003 μM thiamine, 0.2 g glucose l^{-1} and 0.8% agar; adjusted to pH 5.8 using HCl). The agar was overlaid with washed, autoclaved cellophane discs. Hyphal plugs, 10 mm in diameter, were cut from the edge of 14-day-old colonies and placed 1.5 cm apart in 2 rows (4–5 plugs per row) in the centre of the petri dish (Fig. 1). The petri dishes were sealed around the edges with plastic tape. Fungal growth took place at 25 °C in the dark for 7 days (fungal colonies were 20 mm in diameter). Seeds of *E. grandis* (0.4–0.7 mm diameter) were pretreated for 1 min with 70% ethanol containing 0.1% Tween 20, surface sterilized with 5% NaOCl for 10 min and washed in three changes of sterile water. Seeds were placed in a row 1 cm above the fungal hyphae. The petri dishes were resealed and slanted at 70° for 3 days in the dark at 25 °C before being transferred to a controlled environment

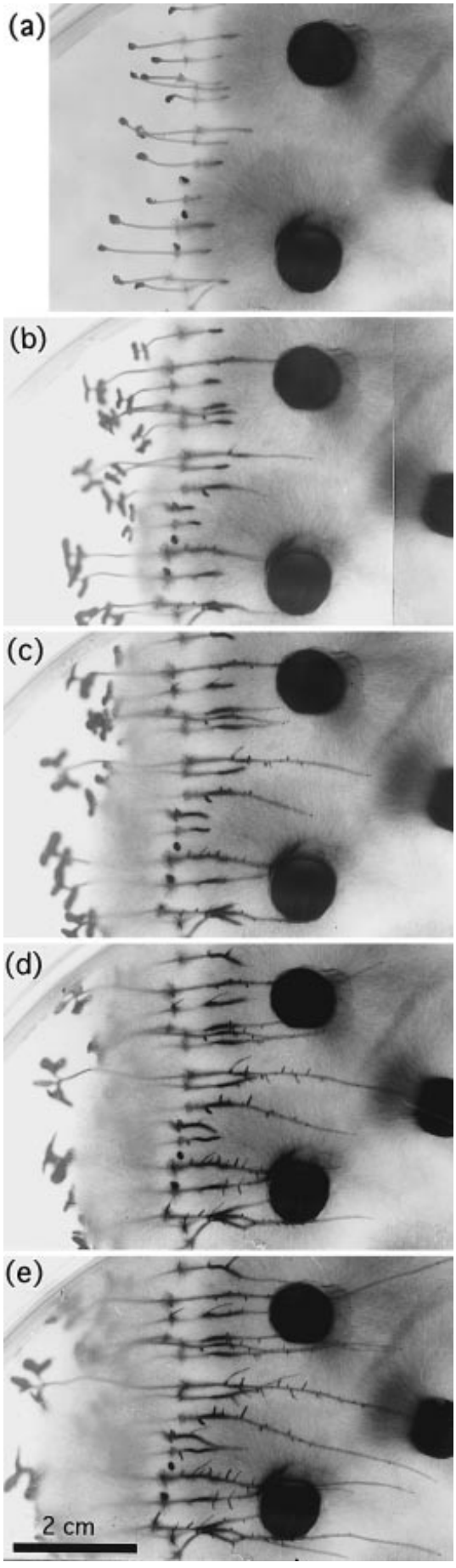


Fig. 1a–e The development of *Pisolithus-Eucalyptus* ectomycorrhizae is illustrated by a time sequence taken from the underside of a single plate. On day 0, seeds were surfaced sterilized and placed in a row 1 cm above the advancing hyphal front. The seeds germinated in the dark on day 2–3 and the plates were transferred to light on day 3. **a** Day 5 – the tap root was 0.5–1 cm long and in contact with the fungal colony. There were no lateral root tips and the cotyledons were still contained in the seed coat. **b** Day 10 – all of the tap root (1–2 cm) was in contact with the fungal colony and root tip initiation had commenced. **c** Day 14 – tap root 2–3 cm long with an average of 5 lateral tips per seedling. **d** Day 18 – tap root 3–4 cm long with an average of 9 lateral tips per seedling. **e** Day 22 – tap root 4–5 cm long with an average of 13 lateral tips per seedling

growth chamber with 24 h light (25 °C, 200 $\mu m^{-2} s^{-1}$). The slant on the petri dishes ensured that the tap root grew towards the fungus (Fig. 1). Petri dishes of non-inoculated control seedlings were manipulated identically. For comparison, seedlings were also inoculated by the method of Malajczuk et al. (1990). This involved germinating seeds on horizontal petri dishes. After 14 days, they were transferred onto actively growing fungal colonies.

Experimental design and sampling

Ten days after seed sterilization, and at 2-day intervals, new tap root growth and all lateral root tips were marked on the underside of the plates. This enabled the determination of the number of lateral tips that emerged over time and the age of the tap root on which they had emerged. In addition, lateral root tips that had emerged in contact with the fungal hyphae, 1–2, 3–4, 5–6, 7–8, 9–10, 11–12 and 13–14 days old, were sampled for light microscopy.

Data on the relationship between lateral tip emergence and tap root age were collected from three separate experiments, each involving a total of 52 petri dishes (488 seedlings). Data on the number of lateral root tips were collected from nine separate experiments involving 116 petri dishes (1206 seedlings). Data comparing lateral tip emergence in horizontal and vertical systems were from two separate experiments involving 10 petri dishes (103 seedlings). Treatment effects were assessed by the analysis of variance of raw data and treatment means compared by least significant difference ($P < 0.05$).

Light microscopy

Root tips were fixed in 0.01 M phosphate buffer (pH 7) containing 3% gluteraldehyde for at least 2 days and then postfixed for 2 h in 1% osmium tetroxide, washed in two changes of 0.01 M phosphate buffer (pH 7) and dehydrated with an acetone series. The samples were then infiltrated for at least 3 h in a series of solutions containing 5, 10, 20, 30, 50, 70, 90 and 100% Spurr's resin in acetone (Spurr 1969). Median longitudinal sections and 1-μm transverse sections, from approximately 0.5 mm behind the root apex, were cut with glass knives using a Sorvall microtome. The resin was removed using a saturated solution of KOH in 100% ethanol and the sections stained with 1% azure II and 1% methylene blue in 1% borax. Sections were photographed using a Zeiss Photomicroscope III.

Results

Root growth and lateral tip emergence

In general, 95–100% of seeds germinated, of which 30–40% exhibited a mean primary root extension of 2 mm day^{-1}. The data presented in Figs. 2 and 4 are for those seedlings. Seeds germinated in the dark 3 days after sterilization and came into contact with the fungal hyphae between days 3 and 5 (Fig. 1a). By day 10, the primary root was 1–2 cm long, in full contact with the fungal hyphae, and first order lateral tips had begun to emerge (Fig. 1b). Seedlings and hyphae continued to grow (Figs. 1c–d), and by day 22 (Fig. 1e) the primary root was 4–5 cm in length with an average of 13 lateral tips per seedling.

Pisolithus isolate H2144 significantly increased the number of lateral root tips compared with non-inoculated seedlings (Fig. 2). Stimulation of lateral tip emergence was significant from day 12 and by day 26 the number of lateral tips emerged on inoculated seedlings was double that of non-inoculated seedlings. The effect of fungal hyphae on the emergence of lateral root tips was demonstrated by transferring non-inoculated seedlings onto fungal colonies at day 14 (method of Malajczuk et al. 1990). During the subsequent 4 days, these seedlings and the non-inoculated controls produced on average less than one lateral tip per seedling (Fig. 3). During the same time, seedlings germinated in the presence of fungal exudates produced over 3 lateral tips per seedling.

In addition to increasing the total number of lateral tips, the presence of fungal hyphae also reduced the age

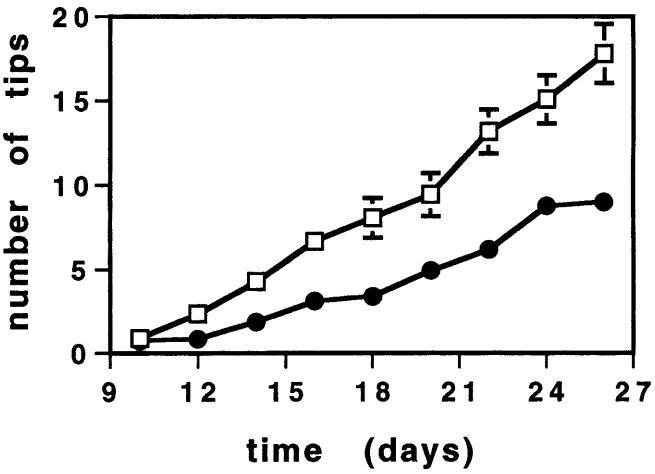


Fig. 2 Emergence of lateral root tips over a 27-day period in the presence of *Pisolithus* isolate H2144 (□) compared with non-inoculated control seedlings (●). *Bars* represent the standard errors of the mean (where absent, the error was within the symbol size)

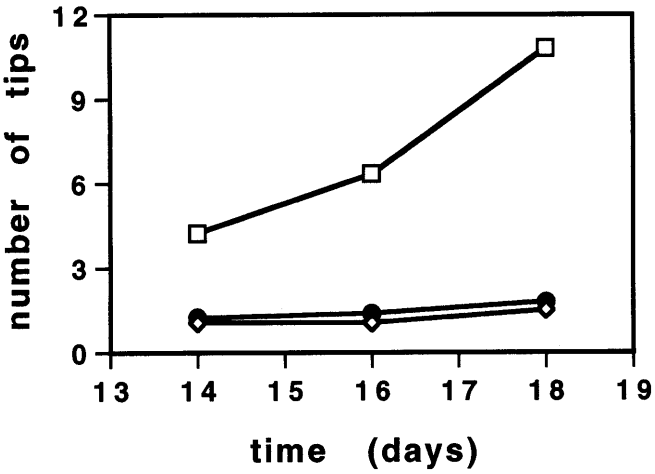


Fig. 3 Comparison of lateral root tip emergence of seedlings germinated in the presence (□) or absence (◇) [using method of Malajczuk et al. 1990)] of exudates from *Pisolithus* isolate H2144 and non-inoculated control seedlings (●). Standard errors of the mean were within the symbol size

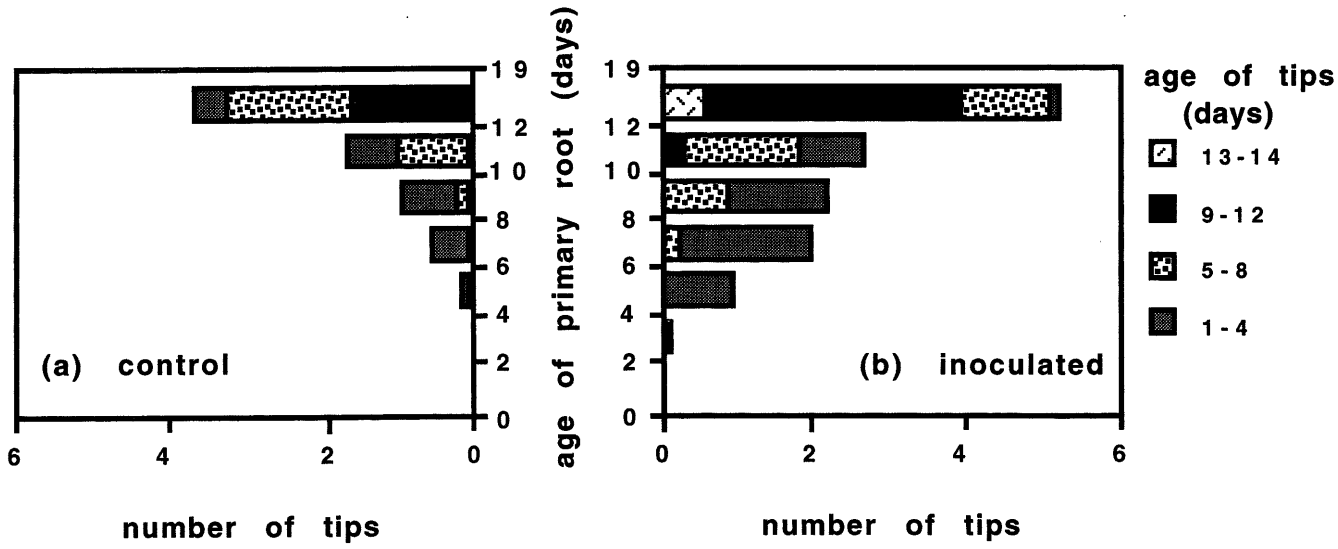


Fig. 4 Emergence of lateral root tips (number of tips per seedling) as a function of root tip age and tap root age. **a** Non-inoculated *E. grandis* seedlings. **b** Ectomycorrhizae synthesized with *Pisolithus* isolate H2144

of the primary root from which lateral tips emerged (Fig. 4ab). On average, lateral tips of inoculated seedlings emerged on segments of the primary root that were 2–6 days old, compared with segments of the primary root that were 5–8 days old for non-inoculated seedlings. This was a real increase in the maturation rate as the length of primary roots of inoculated (42 ± 6 mm) and non-inoculated (45 ± 10 mm) seedlings did not differ significantly.

Ectomycorrhizal development

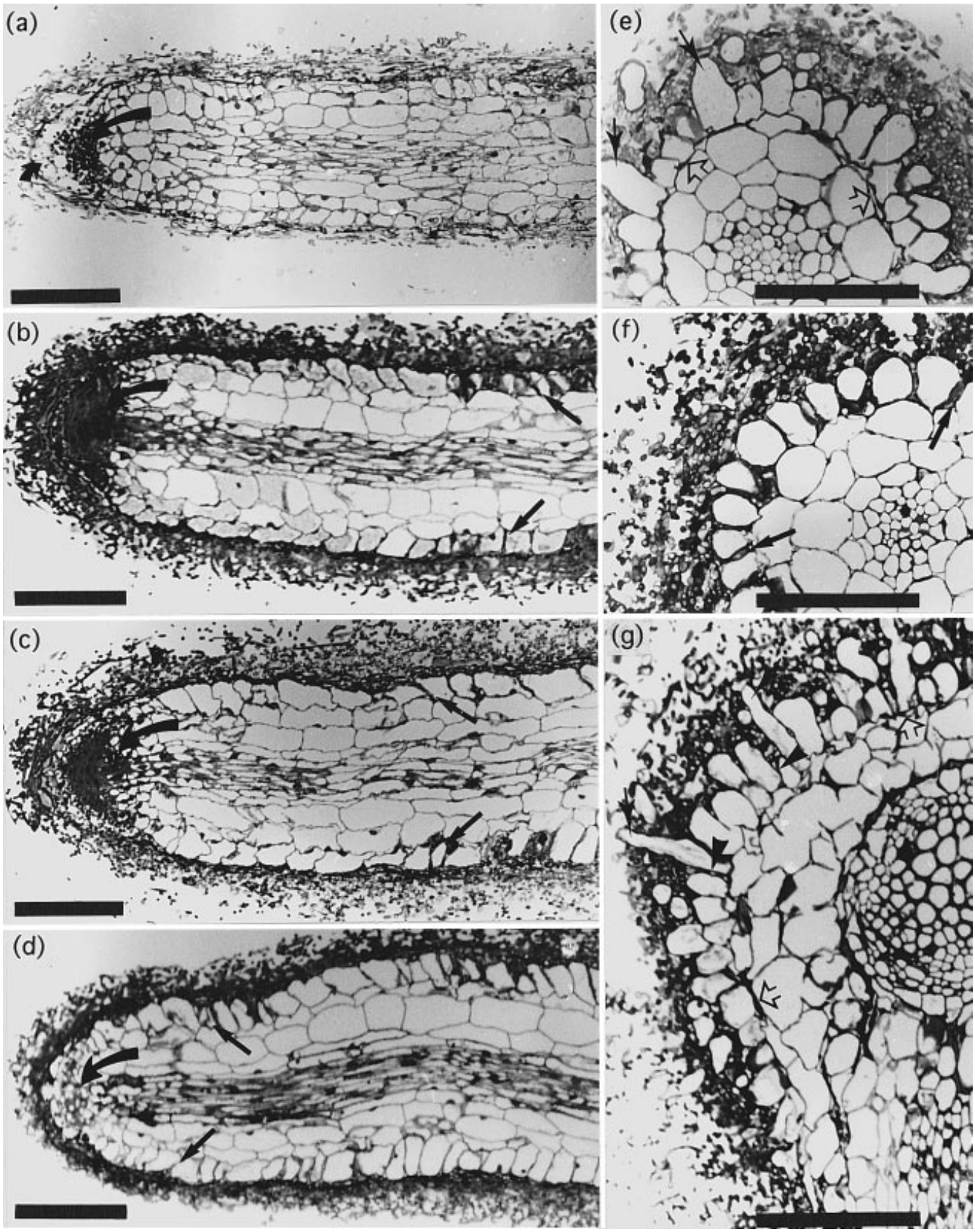
H2144 formed a compatible association with *E. grandis*. All lateral root tips emerged in contact with fungal hyphae. Ectomycorrhizal development of lateral roots was as follows. Day 1: fungal hyphae attached to the root surface, the apical meristem and root cap cells were clearly visible, but there were no root hairs (Fig. 5a); day 2: Hartig net began to develop and elongation of epidermal cells commenced (Fig. 5b); day 4: the mantle thickened and there were obvious layers of outer prosenchyma (mantle with air spaces) and inner synenchyma (mantle with no air spaces), the cortical cells expanded and the Hartig net commenced 0.3 mm behind the apex (Fig. 5c); day 6–12: further elongation of epidermal cells and thickening of the mantle occurred, the apical meristem was reduced and the Hartig net commenced closer to the apex (Fig. 5d).

Fungal hyphae surrounded the primary root, filling the spaces between root hairs and, after 10–14 days, occasionally forcing their way between epidermal cells (Fig. 5 g). By this time the hypodermis had collapsed. These features are very similar to those observed for lateral roots that contact hyphae post emergence (Fig. 5e). Alternatively, mycorrhizae that form concurrently with lateral tip emergence were free from root hairs, had a complete Hartig net between radially elongated epidermal cells, and the hypodermis did not collapse (Fig. 5f). We do not consider the structures formed on the primary root (Fig. 5 g) or on previously emerged lateral tips (Fig. 5e) to be true mycorrhizae (Fig. 5f).

Discussion

The petri dish system described here for the two-step in vitro synthesis of ectomycorrhizae proved to be rapid and reproducible. This technique is superior to earlier attempts with horizontal petri dishes (Burgess et al. 1994, 1995a) as it removed one manipulation step, that of repositioning the seedlings to confront the fungal colonies. Thus, there was less chance of contamination and of stressing or damaging the plants. In addition, slanting the plants resulted in straight tap roots which were easy to sample. Unlike other model systems

Fig. 5 Longitudinal sections of developing *Pisolithus-E. grandis* ▶ ectomycorrhizae synthesized in vitro. **a** Day 1 – fungal hyphae attached to the root surface, the apical meristem (▰) and root cap cells (◣) were clearly visible and there was no Hartig net development, no elongation of epidermal cells and no root hairs. **b** Day 2 – mantle developed and epidermal cells elongated. **c** Day 4 – the mantle thickened and there was obvious outer and inner layers, cortical cells expanded and the Hartig net (⟵) commenced 0.3 mm behind the apex. **d** Day 10 – Hartig net developed along whole root. **e** Transverse section of 10-day-old lateral root that contacted fungal hyphae 2 days post emergence. Features included the presence of root hairs (←) and a collapsed hypodermis (◈). **f** Transverse section of a 10-day-old lateral root that contacted fungal hyphae at emergence. **g** Transverse section of a 22-day-old primary root indicating similar features to **e**. Hyphae forced their way between some epidermal cells, but did not form a complete Hartig net (◅). *Bar* 50 μm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(Chilvers et al. 1986; Fortin et al. 1980; Malajczuk et al. 1990; Wong and Fortin 1989), all lateral root tips emerged in contact with the fungal hyphae. The exact age of ectomycorrhizal root tips, useful information for experiments following the differentiation of ectomycorrhizae, was easily determined. Ectomycorrhizal development could be time-sequenced by sampling lateral tips along an individual primary root. This method addressed a number of issues that are important to the development of ectomycorrhizae:

Concurrent development of ectomycorrhiza

In previous systems, it is unlikely that ectomycorrhizal development was concurrent on all lateral root tips. The fungal inoculum was generally introduced onto the predeveloped root system as inoculum plugs or with a paper card covered with a fungal mat. There is a time lag as hyphae grow out from the inoculum plug and consequently root tips were encountered by these hyphae at different times. Fungal mats consist of hyphae of different ages and growth rates and, as pre-developed root systems are composed of lateral tips in different planes, not all lateral tips contacted the hyphae at the time that the two symbionts were juxtaposed. In our system, the primary root also grows through hyphae of different ages; however, young hyphae tended to grow along the primary root as it extended and it is these hyphae that contacted the lateral roots as they emerged.

Production of physiologically similar lateral root tips

A predeveloped root system is composed of lateral root tips at many different physiological stages. When challenged with the symbiont, different processes are likely set in motion. In the present experimental system, however, lateral root tip emergence and ectomycorrhizal development were simultaneous. Ectomycorrhizae were characterized by their lack of epidermal root hairs, uniform thickness and the extension of the Hartig net from just behind the tip apex to the junction with the primary root. Alternatively, differentiated segments of roots are known to be unreceptive to colonization by ectomycorrhizal fungi (Chilvers and Gust 1982; Horan 1991; Massicotte et al. 1987a, 1989; Wong et al. 1990). For a wide range of ectomycorrhizal associations, these authors have shown that pre-emerged roots develop club-shaped ectomycorrhizae. The distal region of the root has expanded cortical cells, elongated epidermal cells and no root hairs, whilst the proximal region (emerged prior to contact with the hyphae) of the root tip becomes surrounded by the hyphae to form a mantle with entrapped root hairs, but a Hartig net does not develop and the root does not swell.

Ectomycorrhizal development on short apices

In the present study, ectomycorrhizae developed only on lateral root tips. Hyphae did proliferate and surround sections of the primary root, but a typical Hartig net did not develop. Some primary root sections appeared to have a Hartig net, but generally this was due to lysis of epidermal cells. Similarly, when fungal inoculum was introduced to a predeveloped root system, the first ectomycorrhizae were formed predominantly on short apices which had just emerged from the long root (Chilvers and Gust 1982; Wong et al. 1990). Alternatively, Martin and Tagu (1995) consider that ectomycorrhizae develop directly behind the primary root apex of young eucalypt seedlings. Dexheimer et al. (1994) demonstrated that the primary root apex can become mycorrhizal in horizontal in vitro systems but not in vertical systems. However, transverse sections of primary roots show the presence of entrapped root hairs, a collapsed hypodermis and a lack of elongation in the epidermal cells (Dexheimer et al. 1994; Martin and Tagu 1995). These are general characteristics of incompatible ectomycorrhizal associations (Duddridge et al. 1986a,b). Under natural conditions, the primary root tip would continue to grow. This is an important issue as the supposed penetration of mature epidermal cells would require enzymatic degradation of fully formed cell walls and the middle lamella (Massicotte et al. 1986). In comparison, it has been suggested that hyphal penetration between developing epidermal cells is probably mechanical (Horan 1991). Gea et al. (1994) suggest that fungal IAA facilitates Hartig net development by modifying the structure of the cell walls, leading to wall loosening, and thus facilitating the mechanical penetration of the Hartig net.

Exposing germinating seedlings to fungal hyphae

Roots that emerge in the soil encounter a diverse range of microorganisms including ectomycorrhizal fungi. Interactions with these organisms can influence the rate of tip initiation, tip emergence and tip development (Bolton et al. 1992; Piola et al. 1995; Torrey 1986). Allowing the seedlings to germinate and grow in the presence of fungal hyphae, as in this experiment, does not mimic the complexities of the rhizosphere. However, a direct consequence of the presence of fungal exudates was a stimulation in the production of lateral root tips and a reduction in the distance from the primary root apex on which the lateral roots emerged. Similar effects have been observed previously and ascribed to ectomycorrhizal fungi altering the normal host hormone balance either by supplying hormones (Gay et al. 1989, 1994; Gay and Debaud 1987; Gea et al. 1994; Gogala 1991; Ho 1987a,b; Wong et al. 1989) or by eliciting an alteration in endogenous hormone levels (Gogala 1991). IAA-overproducing mutants of *Hebeloma* devel-

op ectomycorrhizae more rapidly than the wild type (Gay et al. 1994; Gea et al. 1994), and recently an indole compound similar in structure to auxin and constitutively expressed in pure cultures of *Pisolithus* was found to be highly increased in the mantle of *Pisolithus-Eucalyptus* ectomycorrhizae (Beguiristain et al. 1994). Application of exogenous auxin can also result in the emergence of lateral root tips closer to the primary root apex (Torrey 1986). Thus, the alteration in the root emergence patterns of inoculated seedlings observed in this experiment may be due to exogenous auxins from the fungus overriding the normal internal controls of the root. This could also explain why primary root extension was less for inoculated seedlings.

Optimization of nutrient levels for ectomycorrhizal synthesis

This synthesis system, has a low level of carbohydrate and an optimal level of mineral nutrients. The nutrient medium was developed to provide optimal nutrient requirements for seedlings growing in vivo in sand culture (T. S. Grove, personal communication). Low glucose concentrations are essential, as high levels have been shown to result in artefacts such as phenolic deposition and irregular mantles (Duddridge 1986a,b; Duddridge and Read 1984; Gibson and Deacon 1990). In addition, carbohydrate in the medium may block receptor sites for recognition and attachment on hyphae or host cell walls (Anderson 1988, 1992). The system described by Malajczuk et al. (1990), used to synthesize *Pisolithus-Eucalyptus* (Hilbert et al. 1991; Lei et al. 1990) and *Paxillus-Betula* (Brun et al. 1995) ectomycorrhizae for a variety of studies, has a high exogenous sugar level which appears to imbalance the symbiotic relationship in favour of fungal growth. In these systems, the transfer of pregerminated seedlings onto fungal colonies would have resulted in much of the previously exuded carbohydrate remaining on the original plate and yet the primary root is rapidly colonized. Consequently, it is difficult to determine whether the fungus was attracted to the primary root or whether it was simply growing rapidly on the exogenous carbohydrate supply.

In conclusion, the in vitro system described here allows for the rapid production of physiologically similar *Pisolithus-Eucalyptus* ectomycorrhizae. All lateral tips emerge in contact with the fungus and ectomycorrhizal development can be followed by sampling tips in a basipetal sequence along a single primary root. Using this clearly defined system, it will be possible to align the morphological changes observed during ectomycorrhizal development with molecular, physiological and biochemical changes recorded.

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