Changes in protein biosynthesis during the differentiation of *Pisolithus* – *Eucalyptus grandis* ectomycorrhiza

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**Abstract**: Protein biosynthesis in *Pisolithus* – *Eucalyptus grandis* ectomycorrhiza was related to the stage of ectomycorrhizal development using two-dimensional polyacrylamide gel electrophoresis of proteins labelled by in vivo incorporation of $^{35}$S radiolabelled amino acids. Nineteen-day-old seedlings were radiolabelled and the primary root was divided into 1-cm segments. With increasing distance from the tip of the primary root, the lateral roots developed as follows: segment 1, no lateral tips; segment 2, three lateral tips, 1–4 days old; segment 3, five lateral tips, 3–8 days old; segment 4, five lateral tips, 7–12 days old. Six-day-old ectomycorrhizas were fully formed with a mantle and Hartig net. During ectomycorrhizal development, there was a decrease in all plant proteins and differential accumulation of fungal proteins. The apical segment of the primary root had a biosynthesis profile very similar to that of noninoculated roots. By contrast, the other segments of the primary root, with attached lateral roots, had biosynthesis profiles that were similar to those of the free-living hyphae. Thus, plant biosynthesis was shown to be predominantly associated with the primary root meristem. The domination of the fungal partner in the protein biosynthesis of developing ectomycorrhiza is probably a consequence of stimulated fungal growth and the corresponding decrease in plant meristematic activity. Ectomycorrhizal development was associated with a differential accumulation of fungal polypeptides and the appearance of a group of symbiosis-related acid fungal polypeptides between 27 and 37 kDa. As the polypeptides were present in a similar magnitude throughout ectomycorrhizal development (lateral tips 1–12 days old), it is suggested that they function as structural proteins associated with mantle formation.

**Key words**: ectomycorrhizal development, *Eucalyptus*, *Pisolithus*, protein biosynthesis, symbiosis-related polypeptides.

**Introduction**

Phenotypic changes in protein expression can be used to study alteration in gene expression during ectomycorrhizal development (Hilbert and Martin 1988; Hilbert et al. 1991; Simoneau et al. 1993; Martin and Tagu 1995; Burgess et al.)
1995). These types of studies depend upon a simple axenic system for the synthesis of ectomycorrhiza. Eucalypt species are ideal host species for in vitro studies, as they grow rapidly, germinating within 3 days and producing lateral root tips within 10 days (Malajczuk et al. 1990; Burgess et al. 1994, 1995, 1996). It is possible to obtain fully developed ectomycorrhiza in less than 2 weeks. Hilbert and Martin (1988) used the paper sandwich technique and collected mature *Pisolithus – Eucalyptus globulus* Labill. ectomycorrhiza. However, sufficient quantities of inoculated roots from earlier stages of development could not be easily collected. Hilbert et al. (1991) adopted the technique developed by Malajczuk et al. (1990), where pregerminated seedlings were transferred onto actively growing fungal colonies. By sampling whole roots, over a time series, from contact to 14 days, changes in protein biosynthesis during early stages of ectomycorrhizal development were observed. The main disadvantage with this technique is that the fungus rapidly colonized the primary root and appeared to restrict the emergence of lateral root tips. Burgess et al. (1995) used a modified Petri dish system (Malajczuk et al. 1990) to study *Eucalyptus grandis* W. Hill ex Maiden seedlings inoculated with three *Pisolithus* isolates of differing aggressiveness. The seeds were germinated in the same Petri dish as the actively growing fungal colonies. The presence of exudates from the fungus resulted in stimulation of root tip production and a greater proportion of ectomycorrhizal root tips. In addition, lateral tip initiation and ectomycorrhizal formation were concurrent.

Changes in protein expression during ectomycorrhizal formation have been observed regardless of the system used to synthesize ectomycorrhiza (Hilbert and Martin 1988; Hilbert et al. 1991; Guttenberger and Hampp 1992; Simonneau et al. 1993; Burgess et al. 1995). Commonly, there was a reduction in the expression of many proteins, increased expression of others, and the de novo synthesis of a few symbiosis-specific polypeptides (see Martin and Tagu 1995 for review). However, Guttenberger and Hampp (1992) sampled individual *Amanita – Pinus* ectomycorrhiza and demonstrated that protein profiles differed both quantitatively and qualitatively between tips, indicating stage specific differences in gene expression during ectomycorrhizal development. Thus, sampling whole roots with lateral tips at different stages of development (Hilbert et al. 1991; Simonneau et al. 1993; Burgess et al. 1995) would reduce the likelihood of observing polypeptide changes associated with a discrete stage of ectomycorrhizal development, only allowing general trends to be observed.

Burgess et al. (1996) described an in vitro system for the synthesis of *Pisolithus – Eucalyptus* ectomycorrhiza in which the age and stage of development of individual lateral tips was clearly defined. In the present study, this synthesis system was used to synchronize changes in protein biosynthesis with discrete stages of ectomycorrhizal development.

**Materials and methods**

**Biological materials**

Ectomycorrhizas were synthesized between half-sib seeds of *Eucalyptus grandis* seed lot No. 17867 (Australian Seed Centre, Canberra, Australian Capital Territory), and *Pisolithus* isolate H2144 (CSIRO Herbarium, Division of Forestry, Perth, Western Australia). This isolate was selected based on the rapid development of ectomycorrhiza in vitro (Burgess et al. 1994, 1995) and because detailed protein biosynthesis profiles were available (Burgess et al. 1995). *Pisolithus* isolate H2144 was collected in 1988 beneath *Eucalyptus wandoo* Blakely at Moora, Western Australia. Cultures were maintained on modified Melin Norkan’s medium, 1% glucose (Marx 1969).

Ectomycorrhizas were synthesized as described by Burgess et al. (1996). Briefly, large round petri dishes (140 x 20 mm) were partially filled with 30 mL of complete nutrient agar (1.5 mM Ca, 0.7 mM K, 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 mM Mn, 0.2 mM Zn, 0.005 mM Co, 0.03 mM Mo, 0.03 mM Na, 0.003 mM 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, 5 mm in diameter, were cut from the edge of 14-day-old colonies and placed 1.5 cm apart in two rows (four or five plugs per row) in the centre of the Petri dish. The Petri dishes were sealed around the edges with 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 autoclaved water. Seeds were placed in a row 1 cm above the fungal hyphae and the Petri dishes resealed. Petri dishes were slanted at 70° and kept for 3 days in the dark at 25°C and then transferred to a controlled environment growth chamber with 24 h light (25°C, 200 μmol • m⁻² • s⁻¹). Petri dishes of noninoculated control seedlings were manipulated identically.

Earlier experiments had shown that there were no differences in the protein composition profile between hyphate grown on medium, overlaid with cellophane, and hyphate grown in liquid medium (Burgess 1995). This is important as colonies grown on liquid medium are superior for radiolabelling, as they are not as hydrophobic and have a higher rate of incorporation than colonies grown on solid medium. In addition, the colony is not damaged when transferred for radiolabelling. Consequently, fungal colonies were obtained as follows. Autoclaved pieces of polyester fabric (3 cm in diameter, with a 0.25-mm² mesh) were placed in Petri dishes (6.5 cm in diameter) containing 5 mL of complete nutrient medium (as above, 1 g glucose • L⁻¹). A hyphal plug (5 mm in diameter), cut from the edge of 14-day-old colonies, was placed in the centre of the Petri dish. After 10 days (colony diameter was 2.5 – 3 cm), the nutrient medium was removed and replaced with fresh medium (1 or 10 g glucose • L⁻¹). After a further 10 days, the colonies were harvested.

**Experimental design and sampling**

Ten days after seed sterilization and every subsequent 2 days, new primary 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 primary root on which they had emerged. On day 22, moderately sized seedlings with primary roots 4–5 cm in length (about 30% of the seedlings produced) were selected for radiolabelling. The experiment was replicated in triplicate on separate occasions.

**In vivo labelling of proteins**

For each replication, approximately 100 inoculated seedlings, 50 noninoculated seedlings, and 3 fungal colonies were labelled in vivo by immersion of roots in (whole root system, still attached to the shoots), or flotation of fungal colonies on, 3 mL of autoclaved complete nutrient solution containing 50 mg • L⁻¹ of the antibiotic streptomycin and 8500 GBq of ³⁵S cell labelling mix (Amersham, 70% methionine, 30% cysteine). Samples were incubated for 5 h with gentle agitation at a light intensity of 600 μmol • m⁻² • s⁻¹. After this labelling period, samples were washed (5 x 10 min) in fresh nutrient medium containing 5 mM unlabelled methionine, frozen in liquid N₂, and stored at −20°C before extraction. The roots were
Table 1. Summary of the stage of ectomycorrhizal development of lateral roots and the associated changes in expression of major polypeptides.

<table>
<thead>
<tr>
<th>Age of primary root segment (days)</th>
<th>Number of lateral roots per segment of primary roota</th>
<th>Age of lateral rootsb (days) from emergence</th>
<th>Stage of ectomycorrhizal development</th>
<th>Fungal polypeptides showing increased expression in colonized roots</th>
<th>Plant polypeptides present in colonized roots</th>
<th>Apparent symbiosis-specific polypeptides in colonized rootsb</th>
</tr>
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<tbody>
<tr>
<td>1–4</td>
<td>0.2±0.2</td>
<td>1±0</td>
<td>Hyphae attached to root surface</td>
<td>F31, F37, F36, F35, F34</td>
<td>F35, F34</td>
<td>F35, F34</td>
</tr>
</tbody>
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aMean ± SD of all seedlings in the three experiments.

bPolypeptides enclosed in circles in Fig. 2.

separated from the shoots after the first wash. The roots were pooled and divided into four segments, approximately 1 cm in length, corresponding to primary root age of 1–4, 5–8, 9–12, and 13–19 days. Primary roots of noninoculated seedlings were also divided after radiolabelling. However, as there were no discernible differences in quality or quantity of labelled polypeptides from the different root segments, data are presented for whole noninoculated roots. Hyphae for protein extraction were from the edge 5 mm of the fungal colony.

Protein extraction

A phenol extraction for soluble protein (Schuster and Davies 1983) was used. Briefly, proteins were extracted in 800 μL of extraction buffer (0.7 M sucrose, 0.5 M Tris HCl, 50 mM ethylene diaminetetraacetic acid, 0.1 M KCl, 2 mM phenylmethylsulfonyl fluoride and 1% β-mercaptoethanol added at the time of extraction). Samples were maintained at 0°C for at least 10 min before 800 μL of phenol was added. The solution was then mixed, left for 1 h at ambient temperature, and centrifuged for 5 min at 14 000 × g. The phenol layer was washed three times with 600 μL of extraction buffer. Protein was precipitated overnight at −20°C by the addition of 800 μL of 0.1 M ammonium acetate in methanol to 200 μL of the phenol layer. The sample was centrifuged for 10 min at 14 000 × g, and the precipitate washed twice with 400 μL of methanol and once with 200 μL of acetone. The pellet was solubilized in either Laemmli’s buffer (Laemmli 1970) for one-dimensional (1D) SDS polyacrylamide gel electrophoresis (PAGE) or O’Farrell’s buffer (O’Farrell 1975) for isoelectric focusing. The amount of radioactivity incorporated into the phenol soluble polypeptide fraction was determined by adding 2 μL of dissolved protein into 3 mL of cystosynt (Amersham) and measured by scintillation counting (Beckman).

Polyacrylamide gel electrophoresis

One-dimensional SDS PAGE gels were 200 × 160 × 0.75 mm in size and contained 15 wells. Monomer parameters of the resolution gel were T = 16%, C = 0.6% and for the stacking gel, T = 5.3%, C = 5.2%. Gels were run in a Biorad Protein II electrophoresis tank. After preelectrophoresis at 1 W per gel for 30 min, each well was loaded with 1.85 GBq of 35S labelled protein and run at 6W per gel for 4 h.

Two-dimensional (2D) PAGE of labelled proteins was undertaken as described by O’Farrell (1975) and modified by Hilbert et al. (1991) and Burgess et al. (1995). The ampholye concentration was modified to obtain a better distribution of the symbiosis-related acid fungal polypeptides (SPAPs). The ampholies were added to a final concentration of 4% (1.2% ampholies, pH 3.5–9 (Biorad); 0.8% ampholies, pH 5–8 (Pharmacia); and 2% ampholies, pH 3–5 (Biorad)). Five microlitres of O’Farrell’s buffer were loaded on the basic end of the gel before the pre-electrophoresis. Samples containing 18.5 GBq of labelled protein were then loaded and the gels ran in a Biorad isoelectric focusing cell at 1200 V for 17.5 h. The gels were extruded from the glass tube, equilibrated, and loaded onto the second dimension (gel composition as for 1D SDS PAGE) and run at 1W per gel for 1 h and 6W per gel for approximately 4 h.

Evaluation of autoradiograms

Following electrophoresis, gels were silver stained (Blum et al. 1987), photographed, impregnated with Amplify (Amersham), dried onto a filter paper backing, and autoradiographed onto Hyperfilm β-max (Amersham) in Cronex enhancing screens at −70°C. Visual comparison of autoradiograms identifies changes in the relative accumulation of specific polypeptides. The apparent molecular weight (kilodaltons) and pl of polypeptides were estimated from their migration in the gel in relation to that of standard proteins with known molecular weight and pl (Biorad). In this experiment, attention was focussed on the gel region where the most dramatic alterations in synthesis occurred (27–37 kDa, pl 4–5.5). Major polypeptides were selected from the complete protein pattern to match gels. Photographs of autoradiograms were underexposed to eliminate the background and many of the less abundant polypeptides. Autoradiograms presented are from one of the replicate experiments.

Results

Characterization of ectomycorrhizae

Seedlings were harvested 22 days after seed sterilization. At this time, the primary root was 4–5 cm in length with an average of 12 lateral tips per seedling. Table 1 provides a
Fig. 1. One-dimensional PAGE of the changes in protein biosynthesis during the development of Pisolithus – E. grandis ectomycorrhiza compared with that of noninoculated seedlings and free-living hyphae. (i) noninoculated E. grandis; (ii–v) inoculated E. grandis with primary root (ii) 1–4 days old, (iii) 5–8 days old, (iv) 9–12 days old, (v) 13–19 days old; (vi) free-living hyphae of Pisolithus isolate H2144. A few major fungal (← F) and plant (P →) polypeptides are indicated to illustrate their loss or accumulation along the primary root. Subscripts denote the apparent molecular weight (kDa) of the polypeptides.

summary of the stage of ectomycorrhizal development of laterals associated with the different segments of the primary root. The youngest segment of the primary root was 1–4 days old, with less than 0.2 ± 0.2 (mean ± SD) lateral tips per seedling of 1 day old. There were hyphae attached to these tips. The next segment of primary root was 5–8 days old, with 3 ± 1.4 lateral tips, 1.8 ± 1.2 days old. Two-day-old inoculated tips were characterized by fungal attachment to the root surface and the commencement of epidermal cell elongation. The next segment of the primary root was 9–12 days old, with 5 ± 1.3 lateral tips of 5.5 ± 2.1 days old. Hartig net development commenced on 3-day-old inoculated tips. Eight-day-old inoculated tips were fully developed with a greatly reduced apical meristem. The oldest segment was 13–19 days old, with 5 ± 1.8 fully developed ectomycorrhizas, 9.7 ± 2.9 days old (see Burgess et al. 1996).

In vivo protein synthesis
Changes in protein biosynthesis during ectomycorrhizal development were examined by 1D SDS PAGE and 2D PAGE of labelled proteins from the free-living symbionts and inoculated roots. Inoculated roots and fungal hyphae incorporated 35S methionine at a rate of 401 ± 111 GBq · g−1 fresh weight (FW) and noninoculated roots at 1221 ± 333 GBq · g−1 FW over a 5-h labelling period. Autoradiograms of 1D gels revealed 50–70 polypeptide bands, whilst autoradiograms of 2D gels revealed 500–800 polypeptides. Of these, approximately 30 major fungal and plant polypeptides were selected to describe the effect of ectomycorrhizal development on protein biosynthesis.

The youngest segment of the primary root had a profile similar to that of the noninoculated root (Fig. 1). Profiles of the older primary root segments bore a greater resemblance to the fungal profile (Fig. 1). Some major fungal bands, F95, F50, F39, F33, and F13.5, were less dominant in the ectomycorrhizal profile, whilst other fungal bands, F71, F37, F55, F34, F19, F18.5, and F18 were stimulated. The dominance of the fungal bands, and the loss of plant bands as the primary root ages, can be seen clearly using 1D SDS PAGE (Fig. 1). However, due to the overlap of fungal and plant bands, the profiles were examined in more detail using 2D PAGE.

Segments of the inoculated primary roots were compared with fungal and noninoculated root patterns (Fig. 2, Table 1). The majority of plant polypeptides, and some fungal polypeptides, were present in the youngest segment (1–4 days old) of the primary root of inoculated seedlings (Fig. 2b). The fungal polypeptides present were acid polypeptides between 27 and 37 kDa. This group had been highlighted by Burgess et al. (1995) and referred to as SRAPs.

The intensity of the plant polypeptides decreased rapidly and they were barely visible in the 5- to 8-day-old segment (Fig. 2c). As the primary root aged (Figs. 2b–2d), the root polypeptides P26, P20, P17.5, and P13.5 were conserved, although their relative synthesis decreased. The rest of the plant polypeptides disappeared from the autoradiograms as their synthesis decreased below detectable levels. In the oldest segment of the primary root (Fig. 2e), synthesis of plant polypeptides was not discernible. There was no detectable increase in the synthesis of any root polypeptides in the ectomycorrhiza.

With the exception of the youngest segment of the primary root, protein biosynthesis in inoculated roots was predominantly fungal and this far outweighed the expected fungal contribution based upon a fungal biomass of 25% (as determined by Burgess et al. 1995). The profile of the youngest segment of the primary root (Fig. 2b) was very different from the profiles of the older segments (Figs. 2c–2e). The profiles of the older segments resembled the pattern of the free-living hyphae (Fig. 2f), but with differential biosynthesis of some polypeptides. There was increased synthesis of fungal polypeptides F71, F54, F37, F36, F35, F34, F31, F29, F28.5, F24, F33, F19, and F18.5 in inoculated roots and decreased synthesis of polypeptides F95, F79, F78, F39, F26, and F13.5.

As with previous experiments (Burgess et al. 1995), there appeared to be a few highly synthesized polypeptides specific to the symbiosis (Figs. 2b–2e, polypeptides enclosed...
Fig. 2. Two-dimensional PAGE of the changes in protein biosynthesis during the development of Pisolithus – E. grandis ectomycorrhiza compared with that of noninoculated seedlings and free-living hyphae. (a) Noninoculated E. grandis; (b–e) inoculated E. grandis with primary root (b) 1–4 days old, (c) 5–8 days old, (d) 9–12 days old, (e) 13–19 days old; (f) free-living hyphae of Pisolithus isolate H2144. Reference polypeptides of fungal (F) and plant (P) origin are indicated. Subscripts denote the molecular weight (kDa) of the polypeptides. Polypeptides enclosed in circles were present only in the profile of inoculated roots.
Fig. 3. Comparison of (a) protein biosynthesis in *Pisolithus* — *E. grandis* ectomycorrhiza (see Fig 2d); (b) protein biosynthesis of free-living hyphae of *Pisolithus* isolate H2144 grown at 1.0 g glucose·L⁻¹ (see Fig. 2f); (c) protein biosynthesis of free-living hyphae of *Pisolithus* isolate H2144 grown at 10 g glucose·L⁻¹; (d) protein composition of free-living hyphae of *Pisolithus* isolate H2144. Reference polypeptides of fungal (F) origin are indicated. Subscripts denote the molecular weight (kDa) of the polypeptides. Polypeptides enclosed in circles were present in the biosynthesis profile of ectomycorrhiza and in the composition profile of hyphae. Polypeptides enclosed in squares were present in the biosynthesis profile of ectomycorrhiza and hyphae grown at 10 g glucose·L⁻¹.

in circles, Table 1). These polypeptides were in the group of SRAPs and were present even in the youngest segment of the primary root. However, if protein biosynthesis of ectomycorrhiza (Fig. 3a) is compared with protein composition in free-living hyphae (Fig. 3d), it can be seen that, whilst protein biosynthesis is low (Fig. 3b), these polypeptides do accumulate in free-living hyphae (Fig. 3d). Also protein biosynthesis in hyphae grown at 10 g glucose·L⁻¹ (Fig. 3c) was very similar to that of the inoculated roots (Fig. 3a). Polypeptides F₃₇, F₃₆, F₃₅, F₃₄, F₃₁, F₂₉, and F₂₈₅ were highly synthesized in both developing ectomycorrhiza and in hyphae grown at high glucose levels.

**Discussion**

Along the primary root, from youngest to oldest segments, there was a reduction in the expression of root proteins and differential expression of fungal proteins. Many fungal polypeptides, whose biosynthesis was low in the free-living hyphae, were enhanced in the ectomycorrhiza. In addition, there were at least five polypeptides that appeared to be symbiosis specific (i.e., they could not be detected in the biosynthesis profile of the free-living hyphae). However, these polypeptides were also present in the protein composition profile of free-living hyphae. Thus, they were fungal polypeptides with greatly enhanced expression in ectomycorrhiza. This enhanced expression of a number of fungal polypeptides is probably as physiologically important to the development of the symbiosis as symbiosis-specific polypeptides, especially as many of these symbiosis-related polypeptides are found in the same region (28–37 kDa) as the previously observed symbiosis-specific polypeptides (Hilbert et al. 1991; Simonneau et al. 1993; Burgess et al. 1995).

In earlier experiments (Burgess et al. 1995), protein biosynthesis in *Pisolithus*—*Eucalyptus* ectomycorrhiza was examined by sampling inoculated roots over a time series from precontact to 8 days after contact. A gradual reduction in the expression of root proteins was observed. By contrast, in the present experiment, very few plant polypeptides could be recognised in segments of primary roots older than 4 days. In fact, whilst the youngest segment of the primary root had a relatively high biosynthesis of plant proteins, the older segments had almost none. Downregulation of plant proteins or genes is not common with other plant—microbe interactions such as *Rhizobium* (Brewin 1991) or endomycorrhizal fungi (García-Garrido et al. 1993; Dumas-Gaudot et al. 1994). However, some nematode infections result in the downregulation of genes in feeder cells, which is vital in preventing the host cell from mounting a defence against the nematode (Sijmons et al. 1994).

The decreased synthesis of plant proteins during ectomycorrhizal development was probably a consequence of a decrease in root metabolic activity as the ectomycorrhizal structure matured. Cytological observations have indicated that ectomycorrhizal development reduces root meristematic activity and extension (Kottke and Oberwinkler 1987; Horan and Chilvers 1990). At the same time, the fungal hyphae were actively differentiating into the new tissue types of the mantle and Hartig net. Outer mantle hyphae were highly vacuolated like the extramatrical hyphae. The inner mantle and Hartig net hyphae had an active cytoplasm (Bonfante-Fasolo and Scannerini 1992) with a large amount of rough endoplasmic reticulum (Massicotte et al. 1986, 1990), indicating rapid protein biosynthesis. These cytological observations were supported by the biochemical observations of Timonen et al. (1993). They observed that the activity of δ-tubulin, a component of the cytoskeleton, was higher in the fungal component of ectomycorrhiza. The microtubule tracks formed by tubulins have been implicated in cell division and organelle movement and distribution (Timonen et al.
The higher the activity of the cytoskeleton, the more active a cell. Martin and Tagu (1995), in experiments on the localization of mRNA in the fungal component of ectomycorrhiza, indicated that \( \alpha \)-tubulin activity was greatest in the Hartig net and inner mantle, diminishing towards the outer regions of the mantle. The youngest segment of primary root contained the actively growing primary root apex, the site of rapid plant biosynthesis and growth (Barlow 1984). The other segments only contained the meristems of developing ectomycorrhizal tips. The more developed the ectomycorrhiza, the less the plant meristematic activity and the fewer plant proteins observed. Thus, there was not a gradual change from plant biosynthesis to fungal biosynthesis, as observed in the time series (Burgess et al. 1995), but rather a sharp change, with the biosynthesis of plant polypeptides predominately confined to the primary meristem and fungal biosynthesis dominating in the rest of the root. The method of sampling was superior to using a time series (Burgess et al. 1995) because only one incorporation was required, thus reducing the variability associated with each incorporation. In addition, each segment of the primary root had lateral root tips at similar stages of development. This increased the chance of observing stage-specific changes in protein biosynthesis.

The most commonly used fungal control is hyphae from the edge of colonies grown under identical nutrient conditions to those used for the synthesis of ectomycorrhiza (Hilbert et al. 1991; Simoneau et al. 1993; Burgess et al. 1995). However, recent experiments (Burgess 1995) indicated that there was little difference in the profile of hyphae grown on liquid or solid medium. Colonies grown on liquid medium are much easier to handle during in vivo labelling and were used in this experiment as the fungal control. Associated experiments (Burgess 1995) demonstrated that the glucose concentration influenced protein biosynthesis in free-living colonies of *Pisolithus* isolate H2144, especially in the region between 27 and 37 kDa. Polypeptides in this region were highly expressed in ectomycorrhiza and present in the composition profile of free-living hyphae at all glucose levels and in the biosynthesis profile of free-living hyphae grown at high glucose levels. This suggests that the highly expressed proteins are structural and, as protein synthesis is coupled with hyphal growth (Wessels 1994), increased synthesis could be indicative of rapid growth rather than a symbiosis-related response. Hyphae growing and proliferating in the rhizosphere also have access to a high level of soluble carbohydrate. This soluble carbohydrate extends a few millimetres from a nonmycorrhizal root (Koske and Gemma 1992). Mantle formation in ectomycorrhiza would effectively arrest the movement of carbohydrate away from the root surface (Ashford et al. 1988), with the ectomycorrhizal fungi utilizing the trapped carbohydrate for growth. However, in natural situations (i.e., the rhizosphere) carbohydrate is limiting for the extramatrical hyphae and it is only by contact with the root that the fungi are likely to proliferate. This poses an important question. Is the correct fungal control one that is artificially grown at a high carbohydrate level, thus simulating the root surface, or is the correct fungal control one that is grown at a low carbohydrate level comparable with the greater soil volume? If the latter is true, then the increased growth caused by root carbohydrate is an ectomycorrhizal effect and the increased protein synthesis is symbiosis related if not symbiosis specific.

As in previous experiments (Hilbert et al. 1991; Burgess et al. 1995), a group of highly synthesized polypeptides (SRAPs) between 27 and 37 kDa has been highlighted. Symbiosis-related polypeptides were observed within the group of SRAPs. The 35- and 34-kDa polypeptides were present in the youngest segment of the primary root. This segment did not have any lateral root tips. The 32.5, 28, and 27 kDa polypeptides were present in the older segments. These segments all had lateral root tips, ranging in age from 1 to 14 days. Thus, some polypeptides were synthesized in response to the primary root, whilst others were synthesized specifically during ectomycorrhizal formation. The latter group could be involved in the attachment of the fungal hyphae to the root surface. However, the stage of ectomycorrhizal development of the root tips increased with the age of the primary root and yet there were no further detectable SRAPs. This suggests that the functions of the fungal SRAPs must be stable throughout the early stages of ectomycorrhizal development.

In this experiment, the intensity of the SRAPs was less than observed previously (Burgess et al. 1995). This could perhaps be explained by a loss of aggressiveness in *Pisolithus* isolate H2144. In earlier experiments (Burgess et al. 1994, 1995), fully developed ectomycorrhiza formed 4 days after contact, compared with 6–8 days in this experiment. Loss of aggressiveness by isolates maintained for long periods in pure culture has been documented by Marx (1981) and Thomson et al. (1993).

In conclusion, this experiment has provided information on the synchronization between the stage of ectomycorrhizal development and protein biosynthesis. The SRAPs appear in the protein profile of inoculated roots even prior to the emergence of lateral root tips. The continued presence of the SRAPs throughout mycorrhizal development suggests that their function is stable. It is highly likely that they are secreted polypeptides involved in the formation of the fungal mantle. Isomers of these SRAPs have been observed in the cell wall and secreted into the external medium by pure cultures of *Pisolithus* (de Carvalho 1994; Burgess 1995). The association between protein biosynthesis and ectomycorrhizal development and the existence of symbiosis-related polypeptides is now firmly established (Hilbert and Martin 1988; Hilbert et al. 1991; Simoneau et al. 1993; Burgess et al. 1995; this experiment). What needs to be determined is their physiological role during ectomycorrhizal formation. Future experiments will explore this topic.

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**References**


