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Ectomycorrhiza formation in *Eucalyptus*

V. A tuberculate ectomycorrhiza of *Eucalyptus pilularis*

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SUMMARY

The structure of mature tubercles collected from a *Eucalyptus pilularis* forest in Queensland, Australia, is described. The smooth, pale yellow tubercles (5–20 mm diam.) consisted of a rind (200–250 μm thick) enclosing a dense coralloid mass of ectomycorrhizas (150–300 μm diam.) and rhizomorphs (200–300 μm diam.). The outer rind region was cemented together with an interhyphal matrix of carbohydrate containing embedded lipid deposits. Dolipores were common in the inner rind indicating the fungal component to be a basidiomycete. Mycorrhizas had thin mantles and well-formed Hartig nets. Protein and lipid reserves were present in mantle hyphae. Rhizomorphs formed around non-mycorrhizal roots inside the tubercles. The central zone of rhizomorph tissue contained thick-walled hyphae which stained positively for lignin-like material. The tubercles are similar to structures formed in associations between *Rhizopogon* and conifers in the northern hemisphere.

Key words: Tuberculate ectomycorrhiza, *Eucalyptus pilularis*, anatomy, rhizomorphs.

INTRODUCTION

Tuberculate ectomycorrhizas are tuber-like bodies, 1–2 cm in diameter, in which clusters of mycorrhizas are enclosed by a rind of fungal tissue. These associations have been observed with Douglas fir in Europe (Dominik, 1963; Dominik & Majchrowicz, 1967) and in North America (Trappe, 1965; Zak, 1971). In this paper we describe the structure of similar tubercules which are associated with the surface roots of *Eucalyptus pilularis* in natural forest stands in north eastern Australia. Previous studies on *E. pilularis* (Massicotte, Peterson & Ashford, 1987) have concentrated on the more typical eucalypt ectomycorrhizas which form in contact with soil and litter. The work reported here continues our investigations (Malajczuk, Dell & Bougher, 1987) into types of ectomycorrhizas in Australian eucalypt forests.

MATERIALS AND METHODS

Clusters of tubercles were collected from beneath a tall, open *Eucalyptus pilularis* regrowth forest (50- to 100-year-old), in the Mapleton State Forest, Blackall Range, Queensland, in May 1988. After washing with water the tubercles were dissected and fixed in 3% glutaraldehyde in 25 mM phosphate buffer, pH 7·0. Samples for TEM were postfixed in 1% aqueous osmium tetroxide, dehydrated in acetone and embedded in Spurr's epoxy resin. Sections for TEM were stained with uranyl acetate and lead citrate. Tissues for light microscopy were embedded in either the epoxy resin or methacrylate (LKB Historesin). Prior to some staining procedures, the epoxy resin was dissolved using potassium ethoxide (Imai, Sue & Yamaguchi, 1968). The following staining reactions were carried out on sections (0·5–2·0 μm): 1% toluidine blue in 1% borax (O'Brien & McCully, 1981) for general tissue components; 0·025% toluidine blue in benzoate buffer (O'Brien & McCully, 1981) for general tissue components; 0·5% azure II/0·5% methylene blue in 1% borax (Richardson, Jarrett & Finke, 1960) for general tissue components; 0·1% toluidine blue in 1% acetic acid, 1% acid fuchsin (Alexander & Bigg, 1981) for plant cell walls (blue/purple) and fungal cells (red); periodic acid Schiff's (PAS) (Feder & O'Brien, 1968) for starch, glycogen and some polysaccharides; PAS/diastase for glycogen (McManus, 1946) (glycogen is not stained after diastase treatment – rat liver tissue was used as a control); mercuric bromophenol blue (Pearse, 1968) for protein; coomassie brilliant blue (Fisher, 1968) for protein; alcian blue, pH 1·0, for sulphated

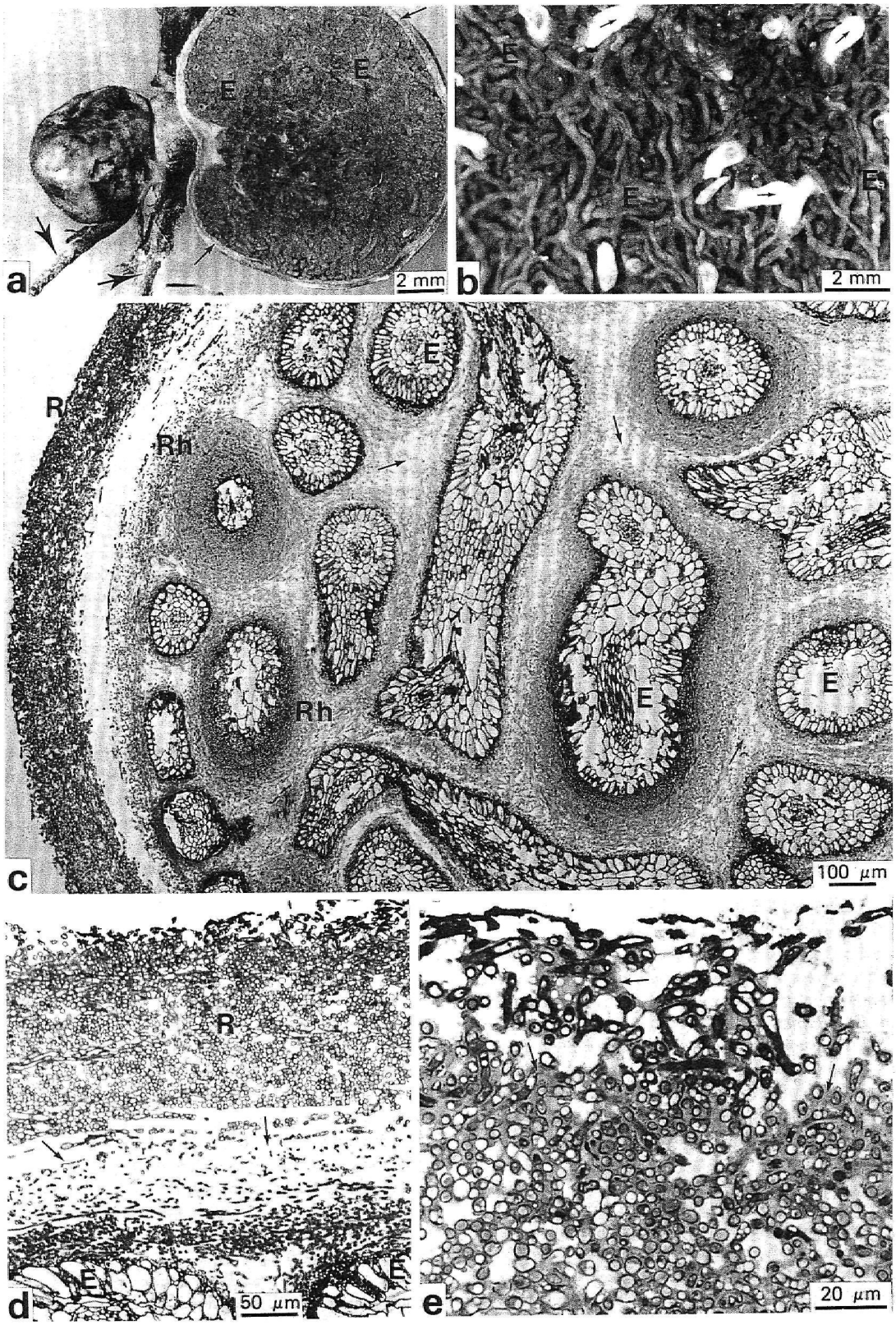


Figure 1. For legend see opposite

mucopolysaccharides; alcian blue, pH 2·5, for weakly acidic mucopolysaccharides (Pearse, 1968); Lugol's iodine (Peacock, 1973) for starch; phloroglucinol/HCl (Peacock, 1973) for lignin; lead sulphide (Ashford, Ling-Lee & Chilvers, 1975) for polyphosphate granules; Mayer's tannic acid/ferric chloride (Ling-Lee, Ashford & Chilvers, 1977) for mucopolysaccharides. Unstained sections were observed under ultra violet light (excitation filter 390–420 nm, barrier filter 450 nm) to determine the distribution of lignin and lignin-like cell walls.

RESULTS

Field observations

Mature tubercles were observed in clusters beneath the litter layer and in the mineral soil to a depth of 10 cm. They ranged in diameter from 5 to 20 mm and were joined by one or more short woody roots (Fig. 1*a*), 2–4 mm in diameter, to the sub-surface perennial root system of *Eucalyptus pilularis*. The tubercles were mostly spherical, of a rubbery consistency, and when adhering soil particles were removed, were smooth-textured and pale yellow. Few rhizomorphs were seen running from the tubercles into the soil. Tubercles resembled, in size and appearance, some of the hypogeous sporocarps that are common in eucalypt forests. At the time of sampling, the common ectomycorrhizal fungi fruiting in the area were species of *Hydnangium*, *Hymenogaster*, *Hysterangium*, *Macowanites* and *Russula* (N. L. Bougher, personal communication).

Tubercle structure

All tubercles contained a dense coralloid mass of interwoven ectomycorrhizas (150–300 μm diameter) interspersed by rhizomorphs (200–300 μm diameter) (Fig. 1*a*). Typical details of tubercle structure are shown in Figure 1*c*. Space between the live roots and rhizomorphs was loosely filled with fungal hyphae. Rhizomorphs inside the tubercles typically developed in association with small nonmycorrhizal roots and the dense rhizomorph tissue was distinct from the thin mantle of the mycorrhizal roots. Old tubercle structures were also observed in which the rind had broken down revealing compacted dark brown roots and bleached rhizomorphs (Fig. 1*b*). Like the live tubercles, these were strongly attached to woody roots of the host tree. No spores were observed in the tubercles.

Rind

The rind consisted of two parts, an outer zone of closely packed hyphae forming a layer some 150–200 μm deep and a thinner inner zone with smaller diameter hyphae forming a loose mycelial mass (Fig. 1*d*). Under the light microscope, surface hyphae of the outer zone reacted more intensely with toluidine blue and PAS reagents than the subsurface hyphae (Fig. 1*e*). Some hyphal walls reacted weakly for sulphated mucopolysaccharides. The hyphae contained small PAS-positive granules but neither starch (Lugol's iodine) nor glycogen (PAS diastase) could be detected histochemically. The outer rind contained extensive deposits of extracellular material which was closely associated with hyphal walls (Fig. 1*e*). This matrix was PAS-positive and gave a positive reaction for mucopolysaccharides (tannic acid/ferric chloride). However, it reacted only weakly with alcian blue (pH 1·0). Protein was not detected (mercuric bromophenol blue, coomassie brilliant blue). The extracellular carbohydrate appeared fibrillar under the transmission electron microscope and contained electron-dense, amorphous, lipid-like droplets (Figs 2*a*, *b*). Hyphae of the inner rind had similar, though less extensive, deposits of extracellular carbohydrate and lipid (Fig. 2*c*). Dolipore septa were typical of hyphae in this region (Figs 2*d*, *e*).

Ectomycorrhizas

All fine roots within the tubercles were mycorrhizal; each had a loose fungal mantle some 20–30 μm thick and a well-developed Hartig net running between slightly expanded epidermal cells to the hypodermis (Fig. 3*a*). Nearly all ectomycorrhizas had deposits of phenolic material either adjacent to the epidermis (Fig. 3*b*) or between hyphae within the mantle (Fig. 3*c*). Within the root, deposits of lipid were isolated and the hypodermis was not lignified. Hyphae in the mantle and extending to nearby rhizomorphs contained abundant storage granules (Fig. 3*d*, *e*), the largest about 0·2 μm across. Histo-

Figure 1. Morphology and structure of tubercles associated with *Eucalyptus pilularis* roots. (*a*) External (left) and internal (right) appearance of tubercles connected to *E. pilularis* roots (large arrows). Each tubercle contains a dense interwoven mass of ectomycorrhizas (E) surrounded by a thin rind (small arrows). (*b*) Part of the contents of an old tubercle after decomposition of the rind. White rhizomorphs (arrows) are interspersed between the ectomycorrhizas (E). (*c*) Section through a tubercle showing the major structures: rind (R), ectmycorrhizas (E) and rhizomorphs (Rh). Loosely packed mycelium (arrows) lies between the roots. (*d*) Section of the rind adjacent to two ectomycorrhizas (E). The bulk of the rind consists of closely packed hyphae (R) which becomes more diffuse and of a smaller diameter on the inner margin (arrows) (e). Considerable extracellular material (arrows) occurs in the rind near the outer surface. Stains: (*c*) PAS/methylene blue/azure II; (*d*, *e*) 1 % toluidine blue in borax.

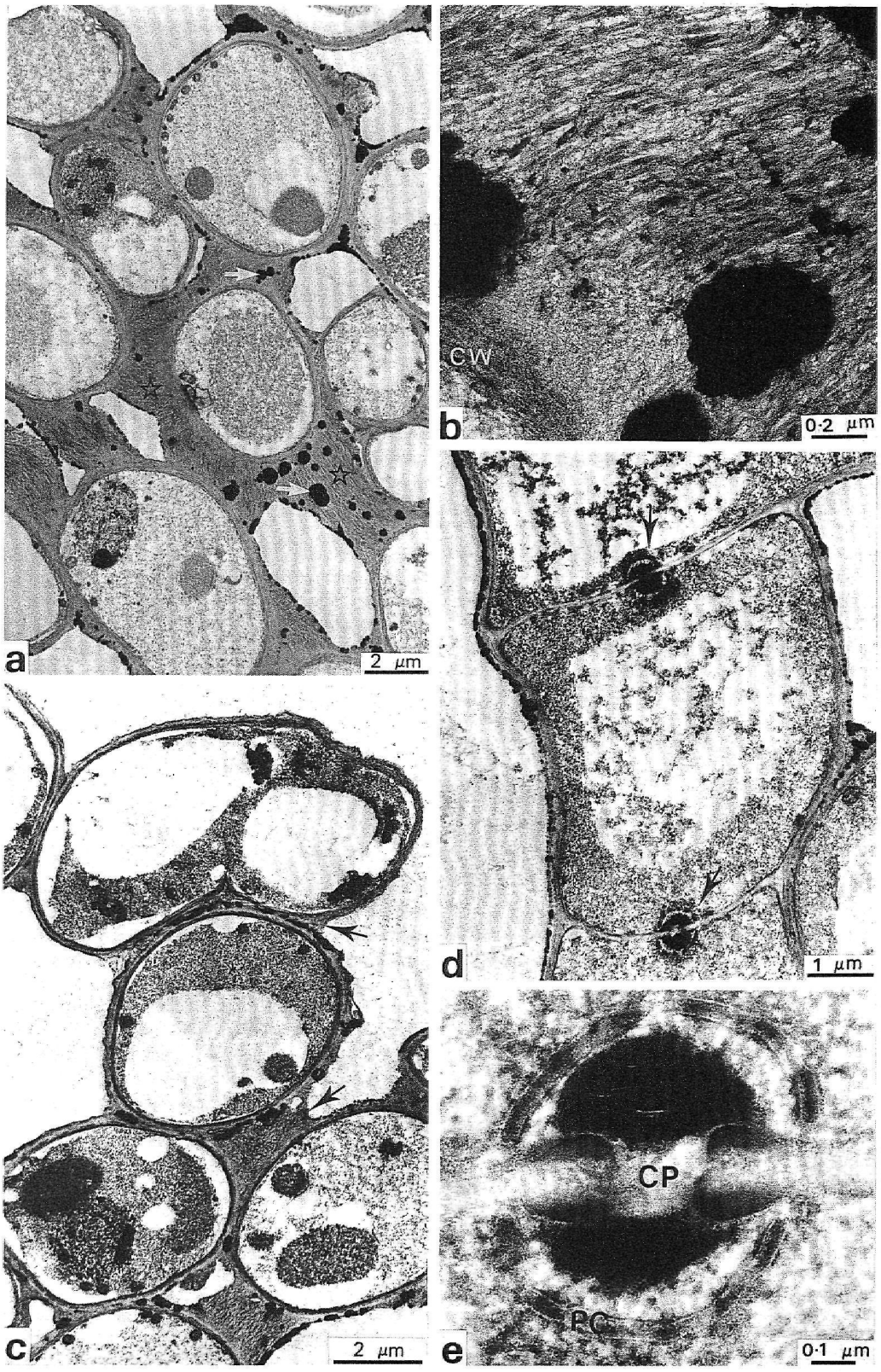
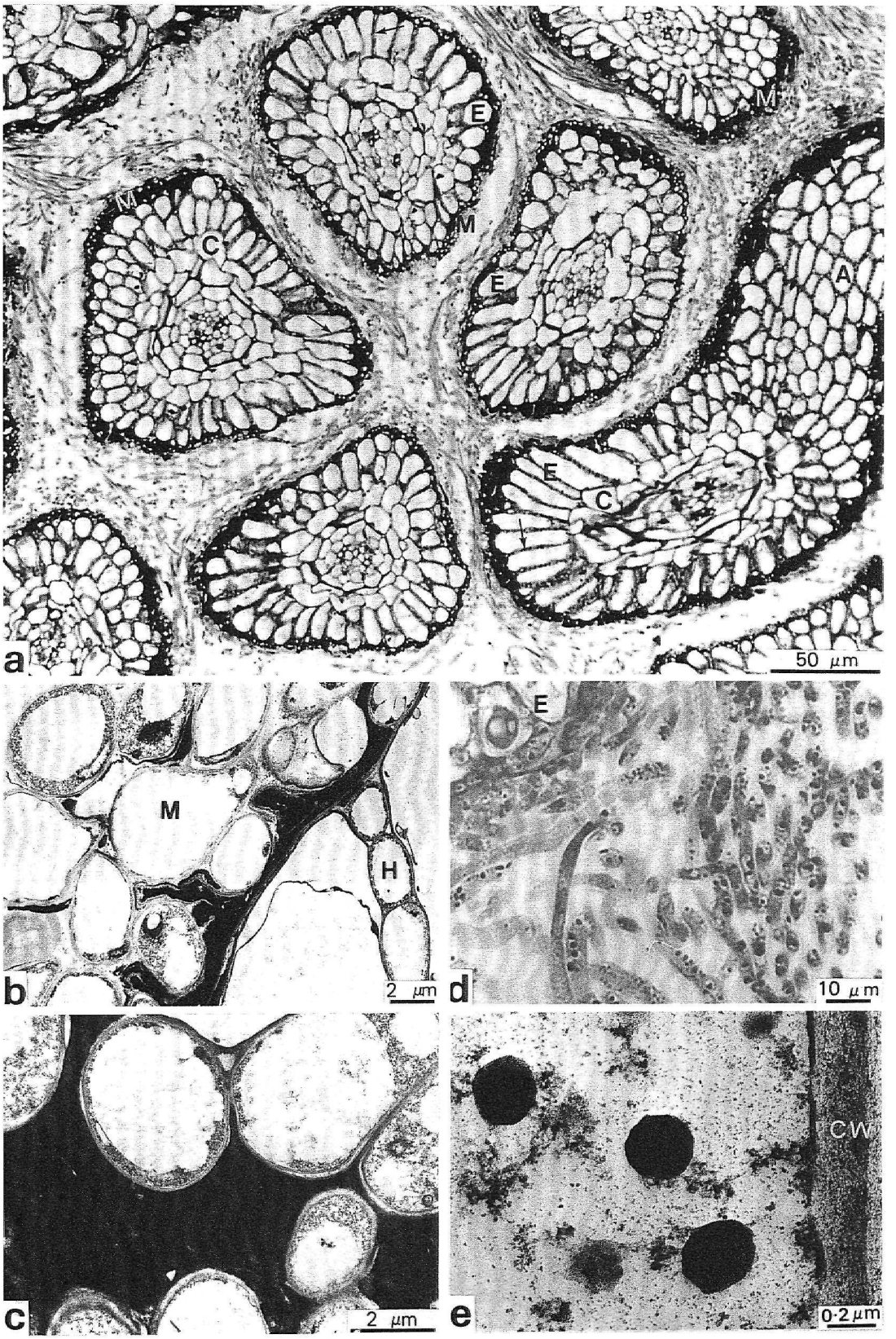


Figure 2. Ultrastructure of the rind of tubercles associated with *Eucalyptus pilularis*. (*a*) Closely packed hyphae in the outer rind. Electron dense material (arrows) lies within the extensive deposits of extracellular carbohydrate (☆). (*b*) The extracellular material appears fibrillar at higher magnification. CW, fungal cell wall. (*c*) Loosely packed partially vacuolate hyphae in the inner rind. Only small deposits of extracellular material (arrows) occur in this region. (*d*), (*e*) Similar region to (*c*) showing the presence of dolipore septa (arrows) between adjoining cells. CP, central perforation; PC, pore cap. Stains: uranyl acetate/lead citrate.



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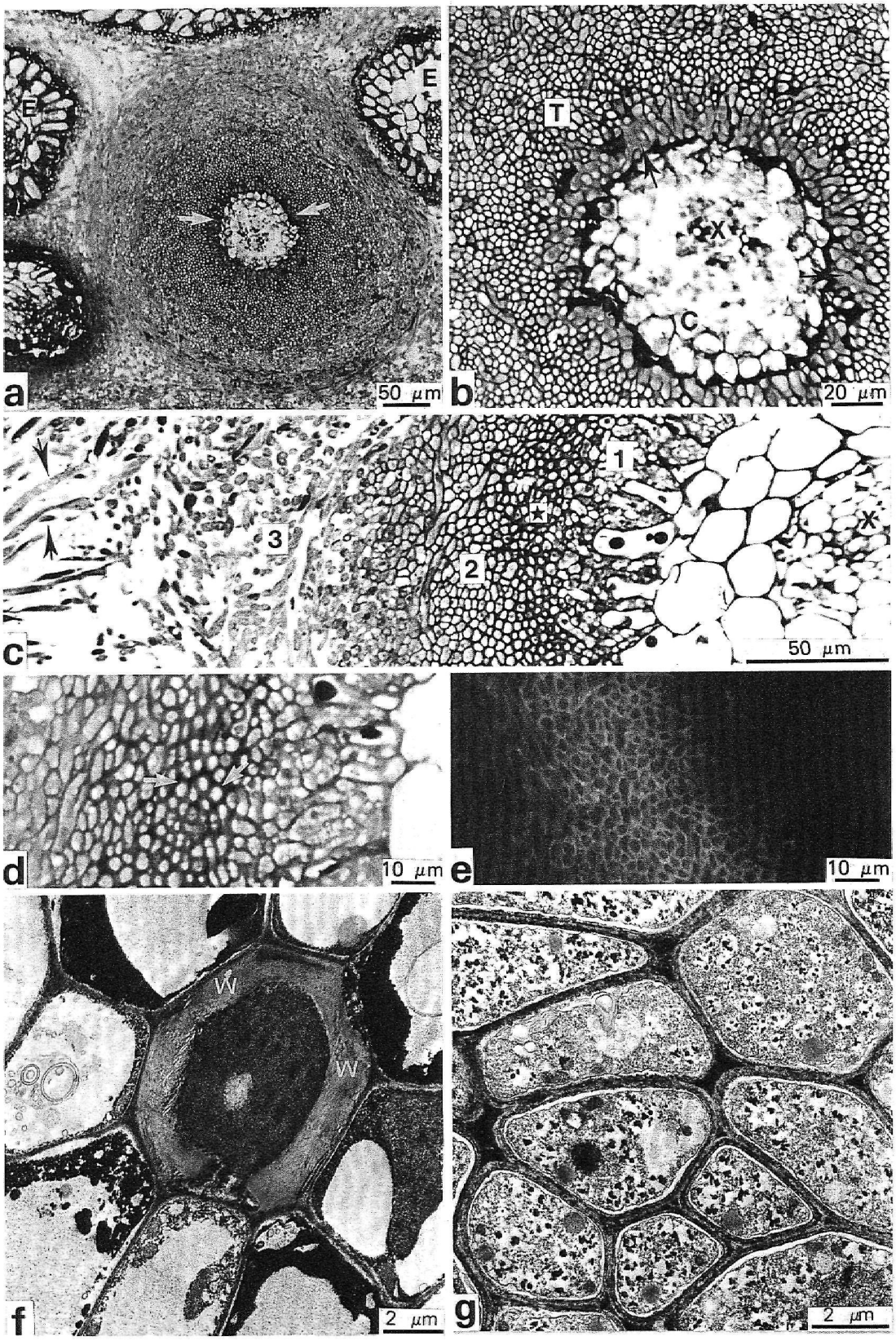

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chemical tests showed that these were mainly a mixture of protein and lipid. Polyphosphate granules could not be detected with lead sulphide.

Rhizomorphs

In transverse section, rhizomorphs typically contained three distinct regions (Fig. 4*a*, *b*, *c*). The inner zone, which was characterized by swollen hyphae in close contact with the epidermis or outer cortex of intact or partly decomposed roots, often had a dense cytoplasm which showed staining properties with the metachromatic dyes that were different from those shown by the outer regions. No Hartig nets were observed. The bulk of the central zone consisted of tightly-packed hyphae, aligned longitudinally forming a pseudo-parenchyma. Under the light microscope the thick hyphal walls of this zone reacted positively for the presence of lignin-like material (phloroglucinol/HCl, toluidine blue, PAS). In unstained sections the walls fluoresced with violet and ultra-violet excitation. The emission was similar to but weaker than that observed for lignified xylem vessels in the same sections. Detail of one of these thick-walled hyphae is given in Figure 4*f*. The thick-walled hyphae contained amorphous electron-dense material. Adjacent thin-walled hyphae were highly vacuolate but the cytoplasm appeared functional. Hyphae at the outer edge of the central zone were slightly thickened (Fig. 4*g*) but the walls did not contain lignin-like residues. The outer zone consisted of loosely interwoven hyphae. Fine structures of these cells were similar to those of the inner rind (Fig. 2*c*).

DISCUSSION

The mature tuberculate mycorrhiza of *Eucalyptus pilularis* resembles in general morphology a tuberculate mycorrhiza formed in an association between *Rhizopogon* and Douglas fir (Zak, 1971). There are some differences, however, in the arrangement of the roots and structure of the internal rhizomorphs. In the conifer system the ectomycorrhizas occur as deformed pyramidally pinnate fans. In the eucalypt system the single ectomycorrhizal short roots are interwoven into a tight ball. Remnants of old rinds observed by Zak were not seen inside sectioned eucalypt tubercles.

Since the eucalypt tubercles were not studied in detail in the field, detail of external rhizomorph density and structure is lacking. In Douglas fir, rhizomorphs extend from tubercles along roots and into the soil (Zak, 1971). The rhizomorphs inside the eucalypt tubercle are unusual in that development is associated with non-mycorrhizal roots and the root becomes an integral part of the rhizomorph. It remains unclear whether these structures have apical growth or are built up gradually around an existing root. It seems likely that the mantles of mycorrhizas are connected with the internal rhizomorphs and these extend through the rind of the tubercle into the soil. The few mycelial strands of ectomycorhizas which have been examined closely contain large diameter hyphae, lacking contents, known as vessel hyphae (Foster, 1981; Fox, 1987). These hyphae are believed to be pathways for assimilate and water transport (Duddridge, Malabari & Read, 1980; Brownlee *et al.*, 1983). However, vessel hyphae were not observed in the internal rhizomorphs in the current study. Apart from vessel hyphae, rhizomorph anatomy resembles the mycelial strands of non-mycorrhizal fungi such as *Serpula lacrimans* (Jennings & Watkinson, 1982) since both contain thick-walled 'fibre' hyphae. It has been suggested that these hyphae provide the structural rigidity

Figure 3. Structure of tuberculate ectomycorrhizas of *Eucalyptus pilularis*. (*a*) TS ectomycorrhizas with hyphal mantle (M) and well-developed Hartig net (arrows) between slightly expanded epidermal cells (E). At (A) the Hartig net is revealed in a paradermal slice of the epidermis. The inner mantle appears dark due to the accumulation of polyphenols. The outer cortical layers (C) are unlignified. (*b*) TEM of hyphal mantle (M) and Hartig net (H) between two epidermal cells. (*c*) Portion of the hyphal mantle close to the epidermis showing deposits of electron-dense material between vacuolated fungal hyphae. (*d*) Light micrograph of mantle hyphae with large and small granules. E, ectomycorrhiza. (*e*) TEM portion of a mantle hypha with electron-dense deposits in the cell. CW, cell wall. Stains: (*a*) methylene blue/azure II; (*d*) methylene blue/azure II/acid fuchsin; (*b*, *c*, *e*) uranyl acetate/lead citrate.

Figure 4. Structure of two types of rhizomorphs inside tubercles associated with *Eucalyptus pilularis* roots. (*a*), (*b*) T.S. of rhizomorphs with an densely cytoplasmic internal layer of expanded hyphae (arrows) surrounding partly decomposed roots (C, cortex; X, xylem). External to this region is a parenchyma-like tissue (T) in which the hyphae are mostly arranged parallel to the longitudinal axis of the root. E, ectomycorrhiza. (*c*–*g*) T.S. of rhizomorphs with a layer of thick-walled hyphae. (c) Section of rhizomorph from the vascular core (X) of the encased root to the sparse hyphae at the outer surface (arrows) which interconnect with the mantles of ectomycorrhizas. The following regions are visible, 1 – inner hyphae closely associated with epidermal cells of the root, 2 – central densely packed zone containing thick-walled hyphae (★), and 3 – outer zone of loosely interwoven hyphae. (*d*) Portion of zones 1 and 2 showing the thick-walled hyphae (arrows). (*e*) Similar region to (*d*) illuminated with violet radiation. The central hyphae exhibit lignin-like fluorescence. (*f*) TEM of zone 2. Note the heavily thickened hyphal wall (W). (*g*) Intermediate tissue between zones 2 and 3. Here the hyphae are uniform and the hyphal wall is only slightly thickened. These hyphae contain cytoplasm, unlike the thickened hypha in (*f*). Stains: (*a*, *b*) PAS/methylene blue/azure II; (*c*, *d*) toluidine blue in borax buffer; (*e*) unstained; (*f*, *g*) uranyl acetate/lead citrate.

necessary to withstand internal forces due to pressure-driven mass flow (Thompson, Eamus & Jennings, 1985; Eamus *et al.*, 1985). In *E. pilularis*, it is also possible that the internal dead root space inside the tubercle rhizomorphs could be a transport conduit.

It is now well established that eucalypt ectomycorrhizas can enhance the growth of young trees on nutrient-poor sites (Malajczuk, 1987). Growth stimulation due to enhanced phosphorus uptake by the tree is facilitated through contact of mycorrhizal roots and hyphae with soil phosphorus reserves. Enclosing the mycorrhizas within the rind of the tubercle may reduce such contact to rhizomorphs or mycelial cords which connect the tuberculate ectomycorrhizas with mycelial fans in the soil (Trappe, 1965). Zak (1971) has suggested that the tubercles may protect the mycorrhizas from attack by pathogens and sap-sucking insects.

As the Australian ectomycorrhizal fungal flora becomes better known it is likely that additional hypogeous structures will be described. The development of ectomycorrhizas is known to occur inside other fungal bodies, namely within the sporocarps of truffle-like Basidiomycetes. It remains to be established whether the tubercles play a significant role in nutrient cycling.

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